Heparin - A Challenging Enigma

L. B. Jaques, D.Sc.[1]

Heparin was developed as a drug for clinical use sixty years ago by research groups in Toronto and Stockholm headed by Professors Charles H. Best and Erik Jorpes. These groups were involved in insulin manufacture and they applied this experience in the development of heparin (cf. W.G. Bigelow, *Mysterious Heparin*, McGraw-Hill Ryerson, Toronto, 1990). Bigelow (p. 36) describes how Best initially instructed the chemists, A. F. Charles and D. A. Scott in the Connaught Laboratories, University of Toronto to find a more readily available source for heparin than canine liver and then to purify the extract so that it was potent and suitable for safe use. They did this successfully and transferred the process to the commercial scale. This meant that considerable amounts of heparin became available for investigation. By arrangement the first to use the new material was to be a member of the Department of Surgery, D. W. Gordon Murray. I graduated with an Honours degree in Physiology and Biochemistry at the University of Toronto in 1933. I immediately embarked on a graduate programme in Biochemistry. I designed a research project - a comparison of the pH-activity curves on sucrose and maltose for the invertase and maltase of yeast *(Saccharomyces cereviseae).* In February 1934, my supervisor, Dr. A. M. Wynne, knowing that I was not receiving any financial support for my graduate work (I earned the money for my fees working weekends in a drugstore) told me that Dr. Best was looking for a student to work on heparin and that I should apply. I saw Dr. Best. He referred me to Dr. Murray. I had not done very well at my Physiology oral examination the previous year which could explain a certain lack of enthusiasm on Dr. Best's part. On going to Dr. Murray's office at the Medical Arts Building in Toronto, I received a warmer welcome, the first of many cups of tea and an indication of the programme. I was loath to give up my programme in Biochemistry. I certainly had no idea that I was embarking on a life-time vocation as a result of the switch.

The programme which I began with Dr. Murray was to determine in dogs if heparin could be used to prevent thrombosis. First it was necessary to determine the effect of the heparin we received in the dogs which were to be used for the thrombosis test. Increasing amounts were injected intravenously. Blood samples were taken at 5, 10, 15 minutes, etc. and the coagulation time determined. After three weeks of testing and as I was about to use a very large dose, I received a phone call from Dr. Best's office to drop whatever I was doing and come over immediately. When I arrived, Dr. Best asked what I had been doing. I showed him my record book. He said, "that's all very well Jaques, but do you realize that you have used up the world's supply of heparin?" I cancelled the scheduled extra large dose of heparin and proceeded to the next phase of the study. This was to produce thrombi in superficial veins. Veins were exposed, crushed with hemostats and removed six hours later. No obstruction to blood flow was found. The test was repeated with a linen thread inserted in the vessel before crushing. On microscopic examination it was evident that the resulting obstruction to blood flow was due to a mixed platelet thrombus. When heparin was injected before crushing the vein, no thrombus resulted showing that heparin could prevent thrombosis.

A second project for Dr. Murray was to test if heparin could be used for surgery of the blood vessels. Present day techniques and the principles involved were established by Carrel in 1911 and were used by physiologists for a generation. Such surgery was not attempted in humans since blood flow in the operated vessels failed in a few hours blocked by the formation of a thrombus and clot. A few hours was sufficient time for a physiological experiment which was completed in one working day but of no value for the treatment of patients. Dr. Murray taught me to transect accessible vessels (carotid and femoral arteries) and rejoin the cut ends with fine silk sutures. On removing the bull-dog clamps on the vessel, normal flow resulted. Blood flow

1. Dept. of Oral Biology, College of Dentistry, University of Saskatchewan, Saskatoon, Sask., Canada S7N 0W0.

quickly ceased in control animals but continued unimpeded in animals which were given heparin. Hence when supported by heparin, surgery of blood vessels, the heart, etc. for permanent repairs to the vascular system was possible.

The initial experiments on the relation of heparin dosage and coagulation times produced suggested to me conducting further studies on my own, of physiological and pharmacological factors affecting the response. Possibly as a defense against criticism, I determined the amount of heparin in the tissues of the animals receiving this very valuable material. I arranged with Dr. Charles for me to do this in his laboratory in the Connaught Laboratories evenings and weekends. At that time it was assumed that the liver was of chief significance for heparin in view of its name (hepar = liver). The injected heparin was found in the intestinal wall together with the considerable amount normally present.

In a little more than a twelve month the project for which I had been taken on, had been completed. Dr. Best had indicated that I could present the results I obtained for an M.A. thesis in Physiology. I therefore stayed home to write a fairly extensive thesis covering literature, physiology (distribution), prevention by heparin of venous thrombosis and embolism, value of heparin for blood vessel surgery. I suspect that Dr. Murray pointed out to Dr. Best that I had all the data books, codes, etc. so that if any credit for the year's work based on documented evidence was to be obtained I could not be just discarded. I received another summons to come to Dr. Best's office. He informed me that my funding from the grant would terminate the end of May as it would be needed for a surgeon who would take over the project so as to be able to introduce heparin clinically. Dr. Best then asked me what I wished to do. I stated that I wished to continue my own studies on heparin. He stated that he would arrange laboratory space in the Department of Physiology but could provide no salary. This arrangement continued for one year and then I received an appointment with pay as a demonstrator in the Department of Physiology.

There are a number of points that may be made from these personal anecdotes. Several are related to the economics of research. The amount of heparin which I used in the first three weeks (a large part of "the world's supply" in units was the amount which later was given routinely to one patient in 48 hours. Amounts of drug required increase exponentially as testing proceeds from in vitro to whole animal to patients. Costs increase proportionately. However, unit costs reflecting production and packaging costs decrease accordingly. The twenty animals used in the initial studies of heparin for thrombosis and vascular surgery provided the crucial evidence for the administration of heparin and other anticoagulants for the prevention of thrombosis and pulmonary embolism to millions of patients all over the world and for the initiation of vascular and cardiac surgery with even more dramatic surgical successes.

As emphasized by Bigelow, the overall project required the participation of four individual university departments, a most unusual event in the 1930s. Arranging this cooperation and the necessary funding was an important contribution by Dr. Best. The difficulty in producing a well recognized pathological condition in a normal living animal is common but not often recognized. "Animal models" are more evident today than they were sixty years ago but there is little appreciation how much careful observation and thought has been responsible for their development. Another example in our own work is the "spontaneous bleeding" model described later.

The story illustrates the difference in conditions for graduate students sixty years ago compared to today. There were very few possibilities for financial support other than from personal effort and family. To work on heparin, I received for the first fourteen months $50 per month from the Banting and Best Research Fund. This fund was established by the Provincial Government of Ontario. It was in recognition of the discovery of insulin. It was the first such fund in Canada for the medical sciences. Several private foundations such as the John & Mary R. Markle Foundation played a similar role in the U.S.A. Fortunately for us they did not consider the international boundary prevented them from making grants to projects in Canada. Of course accepting project research meant some sacrifice of independence. As a graduate student in Biochemistry I had devised my own research project. I only recall discussing it briefly with my supervi-

sor. Of course it came within his own field of interest which is why I selected him in the first place. Certainly I did not submit a written proposal to a Graduate Study committee as is required generally today. Public funding for research through grants for specific projects did not exist to any degree before the 1950s. It is interesting that the practice developed earlier in both countries with Agriculture. In the U.S.A., research was a program limited chiefly to the larger universities. In Canada constitutional reasons inhibited the federal government from directly subsidizing education. In the U.S.A., the Land Grant colleges and their equivalent in Canada were active in Agriculture Research using funds made available by federal and state (provincial) departments of Agriculture through contracts and grants. In the period between the two World Wars, considerable experience was obtained and by this means a meaningful relationship developed between officials of public departments and universities. After World War II during which the importance of research had been demonstrated, there occurred a tremendous increase in student numbers and increasing costs to universities. As a result, the public in both countries accepted in the 1950s the need to make available large sums of money to universities. In both countries grants for research projects provided a known mechanism for distributing funds. With the money assigned to departmental accounts and to be assigned through competition with evaluation of the relative merits of projects proposed, both legislators and civil servants avoided being involved in hassling for funds. While considerable sums reached universities by grants for capital expenditures on specific buildings and equipment, much grant money was assigned to salaries of assistants. These were particularly graduate students since they were temporary and their personal interests were tied to the project. The university professors were happy as they had control of making applications and of spending the money; with the principle of "peer evaluation" there was a reasonable expectation of receiving funding. University business administrators while initially happy with the resulting kudos for the university, eventually became unhappy because of the increasing demands and costs for processing the research accounts. This is a source of increasing tension. Some university professors resented the need to explain in the grant applications what they proposed to do. This did not bother me since I had served an apprenticeship under Dr. Best who was a skilful practitioner of "grantsmanship" many years before the term was invented. The importance of science (particularly engineering, physics, medicine) came to the fore during World War II and shortly thereafter. Hence granting bodies to distribute public funds for research were established in these areas - the National Science Foundation, Atomic Energy (started in the Manhattan Project in 1943), the National Institutes of Health - U.S. Public Health Service, in the U.S.A.; in Canada, the National Research Council established earlier but now with a greatly expanded programme, and the Department of National Health and Welfare. The medical division of the National Research Council was later established as the separate Medical Research Council. In addition in both countries semi-public bodies such as the Red Cross, the Cancer Societies, etc. organized their own programmes of research grants.

By 1937 many people believed that the development and resulting knowledge of heparin was complete. Charles and Scott had crystallized heparin as the barium salt and Jorpes had identified sulphate as an important component. Chemists thought that synthesis would be achieved shortly. The known anticoagulant properties were assumed to explain the clinical effectiveness of the drug. These simplistic assumptions were not accepted by the initial investigators. The chemists in both groups were very much aware of the complexity of the material they were producing and for some years there was a controversy between them as to whether heparin was a single substance or a mixture. There was even greater resistance to the idea that a substance known as an anticoagulant could prevent thrombosis. This was due to a controversy among pathologists as to whether a thrombus was a fibrin clot or a mass of platelets. The latter was the new fashionable view in the 1930s. Hence an anticoagulant ipso facto could not prevent thrombosis. Hence every vein examined in the first study was removed, fixed and sectioned for microscopic examination. Bigelow indicates that this was still the practice for investigators in Toronto forty years later. The identification of the experimental thrombus

as a mixed platelet thrombus was reinforced by Best, Cowan and MacLean who demonstrated that heparin prevented the build-up of platelets in a glass shunt connecting artery and vein. They recorded the blood flow through the shunt by cinematography, an early use of this technique. Bigelow reports the impact of this movie on viewers at that time. However, these initial studies included, as frequently happens, other observations which differed even more from accepted beliefs.

These were reported but were ignored by readers. Thus in Best, Cowan and MacLean's animals while the blood was anticoagulated within five minutes, platelet deposition did not diminish until fifteen minutes and likewise platelet deposition did not resume immediately once the coagulation time was normal. These observations showed a dissociation of the heparin anticoagulant effect from other biological actions. More important from the standpoint of heparin use was the observation that in the large number of animals which received heparin, bleeding did not occur if pressure was applied on puncture points until bleeding ceased. The question of bleeding was asked frequently after early presentations of our data and firmly answered - None occurred. The answer was not stressed in our publication. Emphasis was placed on the results obtained judged by other indicators, the benefits of heparinization, and we purposely avoided being sidetracked by a specious alarm.

Parallel to the studies on heparin in Toronto and Stockholm were those of Albert Fischer and Tage Astrup in Copenhagen. These demonstrated that heparin reacted with many proteins, basic substances and dyes. Some enzymes were activated and more were inhibited. Protamine was a very effective binder of heparin. From this we developed the protamine titration for determining heparin in blood and plasma. Dr. Waters and Dr. Markowitz in the Department of Physiology, University of Toronto were initiating a study of the relationship of the liver to canine anaphylaxis. I joined them to do protamine titrations. This work showed that release of heparin from the liver was a marked feature of canine anaphylaxis. Disintegration of liver mast cells was also shown. The accompanying fall in blood pressure (hence the shock label) was shortly shown to be due to histamine. This study with Dr. Waters and Dr. Markowitz resulted in my lifetime interest with heparin, for histamine, mast cells, liver, sensitivity and antibody-antigen reactions.

K. P. Link and associates at the University of Wisconsin isolated the toxic agent in spoiled sweet clover - dicumarol. They found that this substance was an indirect anticoagulant -one which did not affect blood clotting when added direct to blood but reduced the ability of the blood to clot when fed to animals. They proposed dicumarol as a substitute for heparin but were unable to demonstrate inhibition of thrombosis. Dicumarol was synthesized by a chemist in our group and Miss Dale and I proceeded to repeat with it the experiments that I had carried out ten years earlier with heparin. These experiments showed that dicumarol could be an effective anti-throm-botic agent. In 1946 I moved to Saskatoon to the University of Saskatchewan. In Saskatoon Dr. John Spinks was setting up a programme in the Chemistry Department for the use of radioactive tracers. He and I launched a programme studying dicumarol using a C^{14}-label. This was one of the earliest drug studies using this technique. We extended this to a study of the K-vitamins in view of the reversal of the prothrombinopenic effect of dicumarol by the K-vitamins. These studies showed that the pharmacodynamics of dicumarol and K-vitamins reflected the drug concentration in the liver. With the onset of the war in Korea, frostbite was considered to be a serious problem and thrombosis a factor in its development. We therefore tested the influence of dicumarol administration. As was the case with other investigators we found that the anticoagulant delayed the onset of gangrene without preventing it. However, we considered a side observation was much more valuable. This was the considerable mortality from internal bleeding. Others assumed that this was the normal consequence of anticoagulant treatment. In some cases they did not report it. Since we had given anticoagulants to many animals without bleeding, we concluded that it must be the consequence of the frostbite procedure. This suggested stress was the contributing factor. The bleeding was very typical. It was always internal and occurred in all animals receiving both treatments. Death or survival depended on the location of the bleeding and volume of blood lost from the circulation. We thus had a new concept for an age-

old clinical problem - spontaneous bleeding. Rather than being an erratic uncontrollable accident, it was the result of the combination of two interferences with two of the normal protective mechanisms of hemostasis. In the first experiment these were frostbite (stress) and dicumarol (anticoagulant). Extending this principle in a large series of investigations, it was established that internal bleeding (spontaneous hemorrhage) resulted when treatments simultaneously interfered with blood coagulation and platelets or blood coagulation and vessel wall or platelets and vascular wall. All types of stress (physical agents, chemical agents, drugs, psychic such as restraint, etc.) when combined with treatments which interfered with fibrin formation or with platelets produced spontaneous hemorrhage. Stress interferes with the vascular component of hemostasis. This principle provides a very objective measure for stress. These experiments demonstrated the multifactorial nature of hemostasis and the value of conducting experiments designed on this principle.

In 1962 when visiting Utrecht I learned that the new procedure of gel electrophoresis for proteins could be applied successfully to heparin using toluidine blue for identification. This introduced new possibilities for studying heparin. First the multiple bands seen in the gel showed that all heparin preparations contained many components differing in molecular weight and charge density. At this time the arrival of n.m.r. (nuclear magnetic resonance) equipment at the N.R.C. Prairie Regional Laboratory in Saskatoon resulted in a very valuable collaboration with Dr. Arthur Perlin since this made possible identification of sugar components of heparin without disintegration of the sample. These approaches clarified the confusion regarding the chemical nature of heparin. Heparin preparations were shown to consist of over a hundred closely similar sulfated polysaccharides. Anticoagulant tests showed that these measured only a small fraction of the heparin present in a sample and the fraction measured was different with different coagulation tests. All could be measured with toluidine blue.

When applied to extracts from plasma and tissues, gel electrophoresis measured all the heparin present. Linda Hiebert proposed studying the uptake of heparin by endothelium. She showed in a series of studies that she could find heparin in endothelium in concentrations hundreds to thousands greater than the concentration in plasma. This in conjunction with the observations of vascular surgeons provided a sound basis for understanding the action of heparin in preventing thrombosis. Vascular surgeons had found that when plastic tubing was used to replace a portion of a blood vessel, it was necessary to treat the plastic with heparin, preferable by incorporation. P. N. Sawyer showed that any condition that would result in a thrombus in a vessel was accompanied by a decrease in the electronegativity of the vessel wall. Administration of heparin restored the electronegativity of the vessel wall to normal levels. Thus the effectiveness of heparin in preventing thrombosis was due to the incorporation of the administered heparin in the vessel wall providing and maintaining the normal electronegativity in spite of adverse flow and damage. Thus the anticoagulant action of heparin in the general circulation is not important for the prevention of thrombosis. In fact when it occurs with other interference with hemostasis (e.g. by stress) it is a toxic effect of heparin as it causes bleeding.

Textbooks for over fifty years have stated that heparin is not effective by mouth and is not absorbed. This statement is presumably based on a publication by Fischer and Astrup in 1937. In 1952 when visiting me in Saskatoon, Astrup told me that this was not what they had observed. Rabbits were given heparin orally. No change was observed in the blood coagulation of the animals. The statements in the textbooks were inventions of their writers. Dr. Hiebert placed heparin in the stomach of rats and in five minutes was able to recover amounts of heparin from the endothelium of the vena cava and aorta equivalent to all the heparin administered when calculated for the total endothelial bed. Thus textbook authors can draw conclusions from published work that are not supported by the data and the original authors may have rejected these very conclusions. Again investigators can find new techniques may provide new insights and clues indicating time scales and other parameters should be changed profitably. The observation that heparin is effective orally will be a boon to nurses and patients by eliminating the need for needles. It also makes feasible many animal studies on

biological actions of heparin. These were not practical when heparin was injected every six or eight hours around the clock.

Dextran sulphate was produced in 1962 as a semi-synthetic substitute for heparin. We have found that the same procedures can be used for this drug with very similar results. It has been known since its development to have antiviral and other biological activities previously known for heparin and that it was much less expensive to produce with unlimited sources of raw material (dextrans). In 1991 with the appearance of AIDS (acquired immune deficiency syndrome) as a significant clinical problem, dextran sulfate was tested and shown to be effective in blocking the virus HIV-1 in vitro. However, the U.S. Food & Drug Directorate blocked the clinical use of dextran sulfate for the treatment of AIDS stating as the dextran sulfate was given orally and was not absorbed, so this was not an acceptable treatment. This is a flagrant example of the damage that textbook writers cause by making statements not substantiated by evidence. In Ottawa the Food & Drug division of the Department of National Health & Welfare slavishly followed the inhibition imposed by Washington although they had in their files a report from Saskatoon that dextran sulfate like heparin was rapidly absorbed from the stomach to give effective drug concentrations in the endothelium.

This personal account of sixty years of research presents the results of successful investigations conducted with colleagues, associates and students. Each step indicated another challenge. I was fortunate to come into this challenging programme even almost accidentally. I was fortunate that my initiation was with experienced investigators who emphasized the necessity of making and recording accurate observations and then firmly accepting what the observations meant even if contrary to current beliefs. These same standards continued with succeeding colleagues, associates and students. The pleasures of the mutual collaboration have been equalled by the challenges presented by this enigmatic group of drugs and by their continually increasing importance clinically. The observations, conclusions and reservations made in 1934 to 1937 have been fully confirmed and clarified using new procedures and methods. *Vita brevis ars longa.* I have been very fortunate to have lived long enough to see the contributions of my colleagues and myself become part of the Art.

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