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Experimental investigation of the effect of chylomicronemia on blood coagulation

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AN EXPERIMENTAL INVESTIGATION OF THE EFFECT OF CHYLOMICRONEMIA ON BLOOD COAGULATION

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INTRODUCTION

The clotting of blood has been an intriguing and perplexing problem over the centuries and many investigations have been carried on to solve its mysteries. With the advent of modern medicine the coagulation mechanism has assumed ever increasing importance and at the present time much knowledge has been gained, although a complete understanding of the problem must await further investigation.

Interest in this phase of the subject was stimulated by the work of Waldron, Beidelman, and Duncan (1), who have recently reported a definite decrease in the clotting time of whole venous blood after the oral ingestion of cream. Doctor Herbert P. Jacobi considered the phenomenon worthy of further investigation and recommended that the project be undertaken with the following objectives in mind:

1. To repeat the experiments of Waldron et. al. and substantiate their findings if possible.

2. To investigate the degree of correlation between chylomicronemia and changes in coagulation time following ingestion of fat.
3. To investigate the effect of ingestion of fat on prothrombin time.
4. To investigate the effect of fat ingestion on circulating heparin-like substances by protamine titration.

REVIEW OF PREVIOUS WORK

A review of the literature reveals conflicting opinions and a comparative scarcity of experimental work relating to the effect of fat ingestion on blood coagulation. Mills and Necheleo(2) in 1928 reported that tests with man and dogs show that the coagulability of the blood increases after meals. They further state that the increase is not due to food in the digestive tract since carbohydrate and fat foods are without effect; nor can it be due to large lymphatic return to the blood during absorption, since the absorption of fat, as shown by the milky serum, produces no change. They found that, of the foodstuffs, protein alone appeared to be able to shorten the clotting time and suggested that blood changes are intimately associated with changes in body metabolism since

violent exercise was observed to have similar effects on the blood coagulability. They conclude that it is not the increase in the combustion processes of the body which effects the clotting time, but some as yet unknown factor, possibly connected with amino acids. This "unknown factor" might well be epinephrine released from the adrenal medulla during violent exercise or high metabolic activity. Cannon(3) demonstrated that epinephrine, when injected into the experimental animal in small amounts, accelerates blood coagulation.

The report of Mills and Necheleo(2) is in direct contradiction to the findings of Waldron et. al. (1) regarding the effect of fat on blood coagulation. These investigators conducted controlled experiments on 100 routine hospital patients by feeding them 60 c.c. of 20 per cent cream following a twelve hour fast and determining venous blood coagulation time at 0, ½, 1, 2, and 3 hour intervals using a modified Lee and White method. Their results show an average 33.7 per cent decrease in clotting time in the first 30 minutes and 43.7 per cent drop at the end of one hour. During the second and third hours the clotting time increased steadily toward the basal level. The

average response of 10 control patients drinking only water showed only minor fluctuations in clotting time over the three hour period, indicating that the increased coagulability after the ingestion of cream is not due to repeated venipunctures, the act of eating, or other numerous variables.

Dilution tests were also carried out to further test whether cream makes blood hypercoagulable. Normal blood was diluted with 0.85 per cent sodium chloride to 60 per cent and 30 per cent blood and the clotting times determined for the dilutions and compared with that of undiluted whole blood. Their results show a decrease for the 30 per cent dilution followed by an increase for the 60 per cent dilution. This is in keeping with the findings of Tocantins (4) who explains the phenomenon on the basis of first diluting out the inhibitors of coagulation in the 60 per cent blood and finally diluting out both inhibitors and accelerators in the 30 per cent blood. The same test was then run on blood made hypercoagulable by the ingestion of cream and it was found that dilution only prolonged the coagulation time without the diphasic result obtained with normal blood. This is explained by the inhibitors of coagulation being absent or completely blocked by the ingestion of cream.

Local effect of cream on blood coagulation was also investigated by Waldron et. al. (1). They reasoned that by doing dilution tests on normal fasting blood with a solution of clot accelerator (cream) instead of physiological saline, the clot accelerating properties of the dilutent should be evident by a greater drop in coagulation time due not only to the dilution of inhibitors but also to the addition of clot accelerators. This was found to be the case.

On the strength of these findings, along with other advantages of cream ingestion such as inhibition of gut motility and acid buffering capacity, these workers propose a physiological basis for the early feeding of cream in the medical management of gastro-intestinal bleeding.

Duncan and Waldron (5) have found that other orally ingested fats as well as cream have a profound effect on increasing the coagulability of blood. Experiments were performed by these workers on six dogs using three different fats--refined corn oil, olive oil, and 40 per cent cow's cream. Control experiments were performed to determine the normal fluctuations in the rate of coagulation of the blood

in each dog over a six hour period in order to evaluate the effect of such variables as multiple venipunctures and the constantly confined position of the animal for this period. While rather wide fluctuations in the coagulation time occurred in the control experiments with an initial rise reaching a peak in two hours followed by a significant shortening of the clotting time at the end of five hours; the shortening which followed the ingestion of fats was rapid and sustained during the five hour observation period. (ref.graph no.20)

In discussing the relationship of fat of various types to the prothrombin level in the blood, Doctor Wright (5) remarks that in recent studies normal subjects have been fed six eggs a day for a month and prothrombin levels taken daily with the result that the variation of mean averages was of very small degree--only 6/10 of a second in whole plasma and a proportionate change in 12½ per cent plasma tests. He then asked Doctor Duncan (5) if he had any information as to what factor in the coagulation mechanism is disturbed by the ingestion of fats and also whether he has taken into consideration the possible relationship of the prothrombin. Doctor Duncan replied, "So far, in very limited studies, any changes in the

prothrombin values following ingestion of fat are not considered to be significant. The manner in which the coagulation time is changed following the ingestion of certain fats is not clear. We assume that the effects of the fat on the coagulation time are related to changes in the activation of the prothrombin in some manner." Doctor Duncan further remarks that he has observed prompt reduction in coagulation time in diabetic patients who also had a hyperlipemia, and since diabetic patients are notoriously prone to develop coronary artery occlusion, the possibility is suggested that the feeding of fat may play a part in the increased coagulability of the blood superimposed on arterial disease.

A high incidence of coronary occulsion in patients on a high fat peptic ulcer diet in conjunction with a previous history of coronary artery disease has been reported by Plotz (6). In one group of 10 patients, all died within seven months after being placed on a high fat diet. All of these patients had definite coronary artery disease, nine had peptic ulcer, and one was on a high fat diet following a severe respiratory tract infection. Another similar group reported by Plotz (6) consisted of 17 patients, twelve of which

developed much worse symptoms of angina pectoris within a period of one to three months after starting an ulcer diet. Three of the twelve patients developed myocardial infarction. He attributed this to an acceleration of the arteriosclerotic process. Waldron (1) however suggests that the increased coagulability of the blood on an already damaged intima may have been the determing factor giving rise to a secondary thrombotic episode.

Sole (7) reported the use of human milk as a local hemostatic agent in 1935 and Kraszewski (8) attributed this activity to its thromboplastic properties. It has been shown by Pavlovski (9) that cow's milk contains thromboplastin which possibly accounts for the local hemostatic effect of cream.

Hsien Wu (10) in 1934 studied the clotting phenomenon in the entire absence of lipoid and concluded that lipoid as well as calcium is essential for the activation of thrombin. However, once the thrombin is formed the presence of lipoid was not found to be necessary. Plasma, from which lipoid had been removed, clotted readily on the addition of either natural serum, lipoid free serum, or calcium chloride and lipoid. However, no clot occurred when calcium chloride or lipoid alone was added.

Sanford (11) in 1932, reported that the withholding of fat and protein from the diet of newborn infants tends to decrease the fibrinogen content of the blood.

In studies of the relationship between anticoagulants and lipemia, Waldron and Friedman (12) in 1948 found that small doses of heparin administered intravenously cleared alimentary lipemia and also lipemia due to carbon tetrachloride poisoning. Other anticoagulants were found to have the same effect. In addition it was found that if corn oil is administered orally in an amount which in itself does not produce lipemia, the blood will become lipemic within five minutes after the intravenous injection of heparin or other anticoagulants. This lipemia potentiating effect of anticoagulants does not occur in vitro or without the prior administration of fat. The occurrence of both the lipemia-clearing and lipemia-potentiating effects of fat were correlated with blood coagulation time and the anticoagulant effect of heparin was found to be modified by ingestion of fat.

Lastly it is interesting to note that Collins et. al. (13), and Gorens et. al. (14), in their investigations of the use of intravenous fat emulsions for parenteral feeding, apparently have not noted

changes in blood clotting sufficiently striking for comment. In view of these discrepancies and conflicting opinions of the possible significance of dietary fat in blood coagulation, further study and investigation is clearly indicated.

THE BLOOD CLOTTING MECHANISM

While it is not an objective of this thesis to give a detailed account of the vast amount of research which has been done to bring us to our present state of knowledge regarding the mechanism of blood coagulation, it is felt that further advancement is contingent upon a basic understanding of the mechanism and that the thesis would not be complete without a brief history of this fascinating subject.

The philosophers of Greece were probably the first to record their observations on the clotting of blood. Plato (15), in his Tinaeus remarked that throughout the blood are scattered numerous fibers which give to the fluid certain measures of tenuity and density, and that "when let out quickly coagulates in consequence of the cold surrounding it". Also Aristotle considered that the blood contains a substance which is liquid as long as the blood is in the living

body, but which becomes solid and fiberous as the result of extraction from the body. Thus, as might be expected, probably the oldest hypothesis is that which attributes coagulation to loss of heat.

The fallacy of this hypothesis was shown by Hewson and Metcalf (15) who proved that actually an increase in temperature favors coagulation while blood will remain fluid much longer at low temperatures. Hewson (15) then proposed the equally incorrect theory that coagulation occurs as the result of exposure of blood to air. This theory was based on his observation that blood, retained or tied up in a vessel of an animal, coagulates much more slowly than when exposed to the atmosphere.

In the seventeenth century a number of authors believed that coagulation of the blood results simply from lack of motion. Brown (15) in 1684 wrote that blood clots are most commonly found in the right side of the heart; an effect due mainly to the slow motion of the returning blood through these parts of the circulation. Bostock (15) supported this view and wrote that "as fibrin is gradually added to the blood particle by particle while this fluid is in a state of agitation in the vessels it has no opportunity of concreting; but when it is suffered to be at rest,

either within or without the vessels, it is then able to exert its natural tendency". Collins (15) in 1685 also supported this theory and contended that polypous concretions in the heart are identical in structure with the buffy coat of coagulated blood and that they arise from a languid motion of the blood current.

Another interesting hypothesis was that of Thackrah (15) who concluded from a series of experiments that the blood's fluidity was maintained and controlled in the living organism by a nervous force generated in the medullary substance of the nerve centers and communicated by the nerve-cords and filaments to the blood in the lungs.

Hunter (16) thought that coagulation of blood was an act of life, and was analogous to the contraction of a muscle fiber. Hewson (16) noticed that blood could be kept fluid for months by addition of certain neutral salts and considered this as evidence against Hunter's theory since it was impossible to conceive that "vital power" could last for such a length of time.

Other early investigators thought that something, probably of a gaseous nature, was given off from the blood at the time of coagulation. Richardson (15) in

1858 considered that fibrin was held in solution by free ammonia which escaped from the blood on exposure from the vessels. This theory was exploded by Lester (16) who found that, on inserting a tube into the circulating system, fibrin separated from the blood in a short time and coagulum coated the inner surface of the tube even though the vessel was sealed.

Cooper and Thacknut (16) noted that the blood vessels exerted a specific influence in preventing coagulation. They found that blood of a turtle injected into an empty heart remained fluid for many hours; while some of the same blood exposed in an open vessel coagulated in a few minutes.

The advent of modern chemistry led to many new chemical hypothesis and the coagulation mechanism began to assume a more sound and scientific basis. Buchanan (17) in 1831 found that certain animal fluids, such as fluids from the pericardium, hydrocoele, hydrothorax, and ascites, which do not coagulate spontaneously, could be made to coagulate by the addition of fluid obtained by squeezing a blood clot in a linen cloth. Further investigation lead him to believe that the substance responsible for this coagulative power was derived from white corpuscles. He concluded that fibrin exists

in solution in serous fluids and that it has no tendency to coagulate until it is brought in contact with a substance derived from the white corpuscles. This substance he considered to be of the nature of a ferment and he compared its action to that of rennet upon milk.

The work of Buchanan (16) was almost entirely forgotten until it was re-discovered by Schmidt (18). In 1861 he endeavored to isolate the substance responsible for the coagulative power of blood and subsequently believed that he had identified it with serum-globulin which he called fibrinoplastic substance. By precipitating serum globulin from serum by diluting it with water and passing through it a stream of carbon dioxide, he demonstrated that serum from which the fibrinoplastic substance had been removed had lost its coagulating power. Schmidt considered that there is little or no serum globulin in the circulating blood, but that it is liberated from the white corpuscles, which, according to him, undergo rapid destruction when the blood is withdrawn from the vessels.

Schmidt also attempted to ascertain upon what the coagulating property of transudates depends, and subsequently identified it with a substance which also belonged to the class of globulins. To this substance

he gave the name fibrinogen. Transudates from which fibrinogen had been precipitated no longer coagulated on the addition of fibrinoplastic substance. However when these two substances were isolated and mixed in alkaline solution containing a certain proportion of salts, fibrin was formed. It was then supposed that coagulation was due to the interaction of fibrinogen and fibrinoplastin (serum-globulin) in saline solution. Further investigation however revealed that these two substances could be present together in such fluids as that of hydrocoele, which may contain considerable quantities of serum-globulin, without coagulating. Since this fluid formed a clot on the addition of blood or blood serum, it then appeared evident that a third factor was necessary for coagulation. Schmidt named this third factor fibrin ferment and found that it too was derived from the white corpuscles of the blood. He showed that blood received directly from a vessel into absolute alcohol, which prevented the destruction of white corpuscles, yielded no ferment whatever.

In summary then, Schmidt's theory of coagulation was that it is due to the union of two substances, fibrinogen and serum-globulin (fibrinoplastin), under the influence of a ferment; and that the fibrinogen

exists as such in the circulating blood, while serum-globulin and ferment are formed from the white corpuscles after the blood is withdrawn from the vessels.

Hammarsten (19) in 1878 agreed with Schmidt that fibrin is a precipitate caused by the union of soluble substances in the blood, but presented evidence which tended to prove that serum globulin is an unnecessary factor in the formation of fibrin. According to him fibrin is derived solely from fibrinogen under the influence of the ferment. He was able to prepare fibrinogen free from all traces of serum globulin, and caused it to coagulate by the addition of the ferment only.

Later observations by Norris (20) and Hayem (21) associated the formation of fibrin with changes in certain morphological elements of the blood. Norris termed these elements the "invisible corpuscles" or "advanced lymph discs" while Hayem called them "haemotoblasts" from their supposed share in the formation of the red blood cells. These morphological elements are identical with the "elementary corpuscles" of Zimmerman (22) and the "grains of sarcodiques" of Valpian (23). While various opinions were held as to the nature and origin of the blood platelets, it was

generally recognized that they were intimately associated with the clotting mechanism. Hayem (21) determined the number per cubic millimeter in healthy human blood and described the morphological changes which they undergo during clot formation. Rannier (24) regarded them as particles of fibrin which serve as centers of coagulation in much the same manner as a crystal of sodium sulfate serves as a center of crystillization when dropped into a solution of the same.

In 1887 it was observed by Halliburton (25) that the coagulation time of plasma was considerably reduced when muscle juice or fresh muscle was added to the plasma. Wright (26) in 1902 substantiated this observation by conducting experiments on the coagulation time of rabbit and dog blood in test tubes containing pieces of washed and unwashed muscle, and also sodium chloride extracts of muscle. These results were compared with controls run in clean test tubes and tubes containing a loose pledget of cotton-wool. The unwashed muscle and muscle extracts were found to be most effective in shortening the coagulation time and the hypothesis was set forth by Wright that the coagulative element is supplied by the lymph. To test this hypothesis lymph was obtained from heat

and chemical blisters raised on the skin and the blister fluid mixed with blood. It was found to be highly active in hastening coagulation time. Further investigation to determine the active element in the lymph was carried on by comparing the coagulative influence of freshly drawn lymph with that of lymph serum. It was found that while the influence of freshly drawn lymph was, as had already been shown, very striking, the influence of lymph serum was either slight or altogether absent. Wright concluded from these experiments that the effect of lymph is attributable to the coagulative albuminous element which is removed by clotting.

By the turn of the century much of our fundamental knowledge of blood coagulation had been established. The discoveries of thrombin and its precursor prothrombin as well as fibrinogen and fibrin had been made, and the role of calcium, thromboplastin and platelets demonstrated. The stage was now set for the assimilation of this data into a single theory. This accomplished by Morawitz (27, 28) in Germany in 1905 and probably marks the greatest single contribution to our understanding of the blood coagulation mechanism. This classical theory is the fundamental concept upon which most present day theories are based. Simply stated

the theory is that prothrombin, fibrinogen, and calcium are normally present in the plasma; and with the shedding of blood thromboplastin is released from tissue or from platelets, activating the whole mechanism. This can be diagramed as follows:

Prothrombin + Thromboplastin + Calcium ⟶ Thrombin

Fibrinogen + Thrombin ⟶ Fibrin

The fibrin forms as insoluble protein strands and serves as a structural framework for the clot. The blood is kept fluid in the vascular system due to the absence of free thromboplastin.

During the next thirty years, from 1905 to 1935, much investigation was carried out but little progress was actually made in further understanding the blood clotting phenomenon. The work of Howell (29) probably stands out as the most significant contribution in this period. In 1911 Howell formulated a theory based on the assumption that blood contained an anti-coagulant or an inhibitor of clotting. He thought that the presence of this anticoagulant substance rather than the lack of thromboplastin was responsible for maintaining the fluidity of the blood within the vascular system. In 1918 Howell (30) and his associates

succeeded in discovering such a substance in the liver which gave support to his theory. This substance, heparin, was subsequently isolated in pure form by Charles and Scott (31) in 1933. Howell's theory received a large number of supporters in this country while in Europe most investigators continued to hold to the classical theory. By 1935 so much conflicting evidence and unjustified speculation had appeared in the literature that the whole question was in a state of confusion and most workers had returned to the classical theory.

The year 1935 marks the beginning of the modern period and is characterized by great evolution in clinical interest in blood coagulation. This interest was stimulated by several new discoveries, the most important of which was the discovery of Vitamin K and its role in production of prothrombin. In the years between 1929 and 1933 a hemorrhagic disease in chickens was discovered independently by Dam (32), Holst (33) and McFarlane (34). These workers first thought that the hemorrhagic disease was due to scurvy but were later able to rule out this as well as other known vitamin deficiencies. It was first suggested by Dam in 1934 that this was a new dietary deficiency disease and he suggested that the anti-hemorrhagic factor be called Vitamin K. Both

McFarland and Schonheyder (35) observed that chickens with this hemorrhagic disease had a prolonged clotting time, and Schonheyder showed that the hemorrhagic tendency was due to hypoprothrombinemia. Thus the relationship between Vitamin K and blood clotting was firmly established.

From several different laboratories in the past decade there have appeared descriptions of substances in plasma which are concerned with the speed of formation of thrombin from prothrombin. Liggenhazer (36) in 1940 demonstrated that the kinetics of the clotting of blood could not be explained on the basis of the classical theory alone. He showed a latent period between the initiation of the reaction and the time that thrombin appeared and postulated that a preliminary reaction took place activating a plasma prothrombokinase to thrombokinase which then acted on prothrombin.

In 1943 Quick (37) observed that the prolonged prothrombin time of human plasma which developed due to storage could be restored to normal by the addition to it of fresh prothrombin free plasma. This was prepared by heating plasma of dogs and rabbits with aluminum hydroxide. Also plasma from dogs made markedly hypoprothrombinemic as a result of dicumerol

therapy had the same effect in restoring prothrombin time of stored plasma to normal. To explain these observations Quick postulated that there were two components which went to form prothrombin. One of these, which he called component B, was deficient in dicumerol therapy or in aluminum hydroxide absorbed plasma. The other, component A, was not affected by dicumerol therapy or absorbed on aluminum hydroxide but was lost when oxalated plasma was stored.

Quick (38) modified his view somewhat in 1947 after studying two cases of idiopathic hypoprothrombinemia in two unrelated families. By mixing the pathological plasmas with equal parts of dicumerol plasma, or stored plasma, or absorbed plasma and then determining the prothrombin time of the mixtures he showed the following results: 1. Both patients' plasmas if fresh will restore stored plasma to near normal. 2. One patient's plasma is similar in its defect to the plasma of a dicumerol treated patient while the other patient's plasma is not. 3. A mixture of equal parts of both patients' plasmas resulted in a plasma with a normal prothrombin time. Since neither patient appeared to have the deficiency found in stored plasma and each patient appeared to

have a deficiency different from the other, Quick postulated that there are three different factors involved rather than just two as he first thought in 1943 (37). He also changed his terminology. His original component A he now calls the labile factor and has given the name component A to the new factor apparently lacking in Vitamin K deficiency.

In summary then Quick (38) has three components which he says form a prothrombin complex; a labile factor which is lost on storage and is not absorbed on the usual absorbants of prothrombin; a component A in the synthesis of which Vitamin K is essential; and a component B the formation of which is suppressed by dicumerol therapy.

In 1946 Fantl and Nance (39) reported the presence of an accelerator of coagulation that was present in prothrombin free plasma. Their investigations were done to determiné the cause of discrepancies noted between the undiluted and diluted one stage prothrombin method of Quick. (40) By plotting dilution curves using saline and prothrombin free plasma respectively they showed the presence of a much greater activity in the plasmas diluted with prothrombin free plasmas. On the basis of this observation they reported the presence of an accelerator of coagulation which was not prothrombin.

Owren (36, 41) in 1947 put the concept of this accelerator factor in blood coagulation on a more firm basis. His conclusions were based primarily on his observations of a 29 year old female patient under his care. His patient gave a history of numerous episodes of severe bleeding since the age of 3½ years. These episodes were in the form of severe epistaxis, spontaneous hemorrhages into her vitreous humor, many massive subcutaneous hemorrhages as a result of minor trauma, and severe hemorrhage during her menstrual periods requiring blood transfusions. The transfusions were noted to have a rapid hemostatic effect. There was no history of bleeding in her family. Laboratory studies showed a prolonged clotting time of 25 minutes compared with a control of 6 minutes. The platelet count was 400,000. Quick prothrombin times were 70 to 80 seconds compared with controls of 15 to 20 seconds. Clot retraction, plasma fibrinogen, and serum calcium were all within normal limits. Liver function studies failed to reveal any evidence of disfunction and massive doses of Vitamin K had no effect on the prothrombin time or bleeding tendency. No cause for the prolonged prothrombin time could

be found and the presence of an anti-coagulant could not be demonstrated.

Owren prepared prothrombin free plasma from the patient's plasma and also from normal human, guinea pig, and ox plasma. This was accomplished by the use of prothrombin absorbents such as magnesium hydroxide, aluminum hydroxide, and barium sulfate. He then made dilutions for the patient's plasma with these prothrombin free plasmas and found that the prothrombin time was markedly reduced by all these preparations except the patient's own prothrombin free plasma which had almost no effect. In other words, by adding small amounts of something in plasma other than prothrombin the prolonged prothrombin time was reduced to normal. He concluded that this "something" was lacking in the patient's plasma and that it could not be prothrombin, thromboplastin, calcium or fibrinogen. Owren (41) termed this missing substance "factor V" and the disease caused by its absence parahemophilia.

Owren (41) studied his factor V very thoroughly and described it in considerable detail. It is protein and exists in plasma in an inactive form. It must be activated by calcium and thromboplastin to and active form which Owren calls factor VI.

Seegers (42, 43, 44, 45, 46, 47) and his co-workers have described a similar new factor in the coagulation process. In 1945 Seegers' attention was focused on prothrombin and he succeeded in preparing a highly concentrated preparation. His method was based on absorption of the prothrombin on to magnesium hydroxide with subsequent elution by carbon dioxide under pressure. This preparation was found to be a glycoprotein with a molecular weight of about 140,000. Its activity was about 1500 units per milligram of dry weight which is about 500 times the concentration normally present in plasma protein. However when Seegers compared this concentrated preparation with plasma prothrombin in its native state in plasma, he found it to be much less active. By retracing the steps in his isolation procedure and analyzing the by-products, he was able to demonstrate a protein that greatly accelerated the conversion of his pure preparations. Small amounts of this protein increased the activity to equal that of a similar amount of prothrombin in plasma. This factor was first reported in 1947 (45) and was named accelerator globulin and abbreviated to Ac.--globulin.

Further investigation of Ac-globulin by Seegers (47) and his group revealed that when Ac-globulin is isolated from plasma, it has considerable less activity than when it is isolated from serum. By adding minute amounts of thrombin to Ac-globulin from plasma its activity is stepped up to equal that of Ac-globulin isolated from serum. These were called plasma Ac-globulin and serum Ac-globulin respectively and are distinguishable on the basis of their activity.

There are other reports in the literature which describe somewhat similar accelerator factors in the blood and will be mentioned briefly. Honorato (48) working with Quick in 1947, described what he termed the "plasmatic co-factor of thromboplastin". While studying the absorption of prothrombin by tri-calcium phosphate and aluminum hydroxide gels, a factor was isolated which is not prothrombin but is necessary for the action of thromboplastin in conversion of prothrombin to thrombin.

Milstone (49) at Yale University in 1948 described a three stage method of blood coagulation based on the theory of Leggenhager (36). In this method he was able to determine the presence and amount of a plasma prothrombin accelerator which he called

prothrombokinase. According to Milstone the first stage of blood coagulation is an activation of the prothrombokinase which then initiates and accelerates the conversion of prothrombin to thrombin.

de Vries (50) and his associates described a "serum prothrombin conversion accelerator" (S. P. C. A.) in 1949. They found that the addition of a small amount of serum to plasma resulted in a much reduced prothrombin time but were unwilling to identify this phenomenon as being the same as that described by Seegers (47) or Owren (36).

Thus it appears firmly established that prothrombin accelerators exist and it is likely that the factor V of Owren, the plasma Ac--globulin of Seegers, the labile factor of Quick, the plasmatic co-factor of thromboplastin of Honorato, and the prothrombokinase of Milstone all represent the same substance. Also it may be that the factor VI of Owren, the serum Ac--globulin of Seegers, and the S. P. C. A. of de Vries are terms designating the same substance. Conclusive evidence of this is lacking at the present time.

With the recent advances in our knowledge of circulating accelerator factors there has also been evidence presented to show the presence of circulating inhibitor factors in the blood. Since Howell (29, 30)

succeeded in isolating heparin from the liver in 1911 there have been many attempts to show its presence in normal blood. These attempts were unsuccessful until 1938 when Wilander (51) isolated heparin from the blood of dogs which were in peptone shock. Three years later Jacques and Waters (52) isolated heparin from dogs in anaphylactic shock and also showed that the heparin came from the liver.

In 1948 Allen (53) and his associates at the University of Chicago reported on studies on animals exposed to ionizing radiation. It had been previously supposed that the hemorrhagic tendency which develops following long exposure to X-rays was due to the thrombocytopenia which also occurs. These workers observed however that their animals developed a markedly prolonged clotting time before the platelet count fell or before the platelets showed any other changes. They were then successful in demonstrating the presence of an anticoagulant in the blood which they suspected of being heparin. Jorpes (54) in 1939 had shown heparin to be an acid compound and a heterogenous group of sulfuric acid esters of mucopoly saccharides, and that its activity could be neutralized by certain basic dyes such as toluidinic blue or basic

compounds such as protamine sulfate. The addition of these compounds to the blood of these animals in vitro reduced the clotting time to normal, and injection of comparable amounts intravenously had a similar effect. This gave further evidence that the bleeding resulting from X-ray irradiation is probably due to a heparin-like anti-coagulant. Similar evidence was presented by Smith (55) and his co-workers in 1948 to show the presence of a heparin-like anti-coagulant in the blood of patients treated with nitrogen mustard.

Jacques (56) in 1949 described a much improved method for the isolation of heparin. He has isolated a heparin-like compound from normal human blood and he estimates that the normal level of this substance is of the order of 0.009 milligrams per milliliter.

Since 1940 several investigators have reported anti-coagulants in the blood which do not appear to be heparin-like materials. Lozner (57) and his associates in 1940 described a patient in whom a hemorrhagic diathesis was associated with a prolonged coagulation time and the proven existence of an anticoagulant in the blood plasma. In this 61 year old male negro there was no deficiency of calcium, fibrinogen, prothrombin or platelets, and

titration with protamine produced no effect on coagulation time. This showed that the prolongation was not due to the presence of heparin. When small amounts of the patient's plasma were added to normal human blood the coagulation time was increased from 7 to 28 minutes. This anticoagulant effect of the patient's plasma was striking even in high dilution. There was no past or family history to suggest the diagnosis of true hereditary hemophilia.

A similar case was reported by Soulier (58) in 1948. This patient, a 21 year old male, was clinically diagnosed as a hemophiliac based on a prolonged clotting time, normal bleeding time, and the occurrence of hemorrhagic episodes in joint hemorrhages. However, like Lozner's case, there was no family history of the disease and blood transfusions had no beneficial effect. Also the patient's blood or plasma had a definite anti-clotting effect which is contrary to what occurs in true hemophilia. Protamine and toluidine blue did not neutralize the anticoagulant.

Still other cases very similar to the two cited above have been reported by Munro (59) in 1946; Lawrence and Johnson (60) in 1942; Dieter, Spooner and Pohle (61) in 1949; and Conley, Rathbun, and

Morse (62) in 1948. The circulating anticoagulants reported by all these workers appear to have the following common characteristics: 1. They prolong the coagulation time of normal blood. 2. They were thermostable. 3. They showed no antithrombic activity. 4. They were not neutralized by protamine. 5. They did not pass through semipermeable membranes. 6. They were not extracted by ether. These anti-coagulants generally reveal themselves by precipitating atypical hemorrhagic diseases with laboratory findings resembling those in true hemophilia. However they have been reported in both men and women and many of the cases have definitely not been hemophiliac. The exact nature of these anti-coagulants is not known but they do not appear to be heparin-like materials.

With the advances made in our knowledge of blood coagulation since Morawitz (27) presented his classical theory in 1905, it now becomes necessary to fit this knowledge into the framework of that theory to form a modern working hypothesis. This has been done by Frommeyer and Epstein (63) by using a combination of theories of Quick (64) and Seegers (47). The prothrombin accelerators of plasma and prothrombin accelerators of serum reported by Owren (36, 41),

Seegers (42, 44), and others have already been discussed. Seegers, calling his plasma and serum prothrombin accelerators plasma Ac$_1$--globulin and serum Ac$_2$--globulin respectively, believed that they played the following part in blood coagulation:

Prothrombin + Thromboplastin + Calcium $\longrightarrow$ Thrombin

Plasma Ac$_1$--Globulin + Thrombin $\longrightarrow$ Serum Ac$_2$--Globulin

Prothrombin + Thromboplastin + Calcium + Serum Ac$_2$--Globulin $\longrightarrow$ Thrombin

Fibrinogen + Thrombin $\longrightarrow$ Fibrin

Since it was shown by Seegers (47) that plasma Ac$_1$--globulin has essentially no effect on the yield of thrombin produced from a mixture of purified prothrombin, thromboplastin, and calcium; and that serum Ac$_2$--globulin produced a large yield of thrombin in such a mixture; it therefore became necessary to show graphically the conversion of plasma Ac$_1$--globulin to serum Ac$_2$--globulin. To accomplish this it was suggested by Seegers (47) that thrombin played a part in this conversion as shown in the above diagram. The mechanism for this conversion has not been clearly shown however and it leaves one weak point in the foregoing theory, namely, that purified prothrombin, tissue thromboplastin, and calcium form very little or no thrombin as shown by both Owren (36) and Seegers (47).

It is possible to eliminate this weak point in Seegers' theory by also incorporating the theory of Quick (64). Quick's hypothesis, substantiated by experimental findings, is that the platelets liberate an enzyme on disintegration which is essential for the conversion of thromboplastinogen to active thromboplastin. This theory is diagramed as follows:

Thromboplastinogen + Platelet Enzyme ⟶ Thromboplastin

Prothrombin + Thromboplastin + Calcium ⟶ Thrombin

Fibrinogen + Thrombin ⟶ Fibrin

With the platelet enzyme of Quick augmenting the thrombin formation from thromboplastinogen, the weakness of Seegers' theory is eliminated and the two theories can be combined into a five step modern hypothesis as follows:

Platelet Enzyme + Thromboplastinogen ⟶ Thromboplastin

Thromboplastin + Prothrombin + Calcium ⟶ Thrombin

Thrombin + Plasma Prothrombin Accelerators ⟶ Serum Prothrombin Accelerators

Prothrombin + Thromboplastin + Serum Prothrombin Accelerators + Calcium ⟶ Thrombin

Fibrinogen + Thrombin ⟶ Fibrin

THE CHYLOMICRON

Having reviewed the development of our knowledge of the blood clotting mechanism to its present state, attention will now be turned to the chylomicron and its relationship to blood coagulation.

All the food materials in the blood except the fats are in solution, and can be followed after being absorbed from the gastro-intestinal tract only by chemical means. The major part of the fat however, after passing through the wall of the intestine, appears in the lacteal vessels and blood stream as a fine emulsion which can be seen clearly by the aid of a high power dark field microscope.

As early as 1622 it was observed by Aselius (65) that food absorbed from the small intestine caused the mesenteric lymphatics to become visible by their milky appearance. He thought that the chyle was carried to the liver by the lacteals but Pecquet (65) in 1650 traced the lacteal vessels to the jugular and subclavian veins to show that the chyle was carried directly to the blood stream. Boyle (65) in 1665 noted that the blood serum itself could be made milky by an abundance of food and Hewson (65) in 1774 showed that the milky appearance was due to

the presence of fat since it left a greasy spot when dried upon paper. Hewson also was the first to observe the fat particles under the microscope. These fat particles were more fully described by Gulliver (65,66) in 1840 and he showed that the particles in the blood stream were the same as those in the lacteal fluid. Edmunds (67) in 1877 gave us our first account of the appearance of blood when studied under the dark field microscope. He described it as appearing like a wholly new substance with multitudes of glancing particles which look like "motes in a sunbeam".

As more and more interest was directed to the constantly moving particles in blood serum a diversity of opinions were expressed by various investigators as to their biochemical composition. Kahne (68) in 1895 considered them nothing more than particles of protein contamination from the skin while Raehlmann (68) in 1905 thought that they represented granules of disintegrating leukocytes. Munk and Rosenstein (67) were the first to suggest that the particles were fat and they gave them the name "fat dust". Knudson and Grigg (69) in 1923 confirmed this view by noting that the lipid content of the blood increased as the number of particles increased following a fat meal.

Gage and Fish (65, 70, 71, 72, 73) from 1920 to 1925 probably made the greatest single contribution to our knowledge of the particles in blood serum (which they termed chylomicrons) through a series of experiments in which selected healthy individuals were fed moderate amounts of fat, carbohydrate, and protein and their blood lipid content studied both chemically and by dark field microscope. From these experiments they were able to show the following:

1. Neither protein nor carbohydrates, nor any combination of them give rise to chylomicrons in the blood.
2. Fatty food is the only food that gives rise to emulsion of chylomicrons in the chyle and blood.
3. The emulsion in the chyle and blood is known to be fat because:
   a. The particles rise and form a cream on top as with milk.
   b. If in sufficient amount the emulsion when dried on paper gives a greasy spot.
   c. The emulsion when dried and extracted with ether or other fat solvents and the solvent evaporated leaves a solid fatty substance.

d. This fatty substance stains black with osmic acid and red with sudan III and scarlet red.

e. It has the iodine absorption of fat.

f. It has the refractive index and dispersion of fat.

4. The time after eating fatty food for the fat particles to appear in the blood is from one-half to one and one-half hours.

5. The time required for a given kind and quantity of fat to be digested, absorbed, and to disappear from the blood with any given individual or animal varies from about six to ten hours.

6. There is considerable variation in digestibility, time of absorption and assimilation of different kinds of fat by the same man or animal.

7. There is considerable variation in digestibility of the same fat by different individuals and animals.

8. Mental conditions influence digestion. Cessation of digestion was observed during severe mental effort.

9. The presence of a wholly indigestible oil does not seem to interfere with the normal digestion of a digestible fat.
10. The remobilized fat after prolonged fasting appears in the blood as an emulsion of fine particles of the same size and character as those appearing after a meal of fatty food.
11. The size of the chylomicron ranges from one-half to one micron in diameter.

McDonagh (74) in 1927 investigated chemically the fine particles in active Brownian movement in serum and came to the conclusion that they were protein rather than fat as reported by Gage and Fish (65). He called attention to their behavior in an electric current and the modification in their number during various diseases. These observations were confirmed by Peters (75) in 1936 but he disagreed with McDonagh's concept that they were protein on the ground that normal serum protein, when fully hydrated, should have a refractive index so near to that of water that the particles would not be visible under the ultra-microscope. He also showed that dehydration and percipitation of the serum proteins actually caused an increase in the number of particles visible.

In 1937 Frazer and Stewart (76) published the results of a series of investigations on the effect of feeding on the particle content of the serum of human subjects. Their findings confirm those of Gage and Fish (65) in that the serum exhibits a considerable increase in particles following ingestion of fat, while ingestion of carbohydrate or protein has no effect. They conclude that the particles are lipid in nature and suggest that they are probably enclosed in an absorbed protein film. These workers further observed that there are two distinct types of particles in the serum; one appearing brightly illuminated and the other dull. Cunningham and Peters (77) in 1938 extended this observation to include three distinct types, namely, large bright, medium dull, and small dull. By a series of physico-chemical investigations they came to the conclusion that the large bright particles are neutral fat but that the smaller particles are protein. These conclusions were based on their observations that extraction with ether removed the large bright particles but not the smaller ones; the large bright particles were removed by pancreatin, probably through the action of lipase; and the large bright particles rose

to the top on centrifuging the serum while the smaller particles were drawn to the bottom.

The controversy as to whether the particles in blood serum were composed of fat or protein was settled to the satisfaction of most investigators by Eckles, Frazer, and Stewart (68) in 1939. These workers studied the action of various fat solvents and other substances on the serum as seen under dark-ground illumination and concluded that the main bulk of the particles in normal blood is fatty, and that there may be a layer of absorbed globulin at the oil-water interface.

The suggestion by Eckles, Frazer, and Stewart (68, 76) that the chylomicron is enclosed in an absorbed protein film is strongly supported by the work of Ludlum, Taft, and Nugent (78, 79). These workers in 1929 recognized the chylomicron suspension in serum as a colloidal system and made a study of the fat emulsion to determine the nature of the emulsifying agent. Working on the basis that the surface characteristics of the dispersed emulsion are necessarily those of the emulsifying agent, they added dilute hydrochloric acid to serum in increasing concentration and noted the change in dispersion of the chylomicrons

by dark field microscopic examination. Flocculation of the chylomicrons was found to occur at a pH of 4.7--5.1 which was assumed to be the isoelectric point of the emulsifying agent. This pH is in the range of the isoelectric points of serum proteins, thus supporting the prediction that chylomicrons are stabilized by protein films. Frazer and Stewart (80,81) in 1939 studied this phenomena more closely by making comparisons of the behavior of chylomicrons with that of protein protected soap stabilized oil in water emulsions. They concluded that maximum flocculation occurs at pH 5.3 which indicates that the protein film consists of globulin.

Two hypotheses exist for the absorption of fat from the intestine. The Lipolytic Hypothesis of Verzar and McDougall (82) which has been generally accepted in the past, states that the neutral fats of the food are emulsified and digested completely to their constituent fatty acids and glycerol in the small intestine. The presence of bile in the duodenum aids digestion and absorption by activating pancreatic lipase and providing a favorable hydrogen ion concentration for its activity. The bile salts also lower surface tension and thus promote emulsification which offers a greater surface for enzymatic

action. Verzar and his colleagues believe that fatty acids form a complex with the bile salts which is water soluable and readily diffusible into the epithelial cells. After transference across the epithelial boundary it breaks down into its components and the fatty acid recombines with glycerine which has also diffused into the cell from the intestinal lumen. There is evidence that an intermediate stage in the resynthesis of the neutral fat is the formation of a specific phospholipid. Sinclair (83) found that during fat absorption a change occurred in the composition of phospholipid in the intestinal mucosa. After fat with a high iodine number has been fed, the phospholipid of the mucosa has a high iodine value also. Verzar (82) supports this belief that phosphorylation occurs as a step in the synthesis of neutral fat.

A more recent hypothesis has been presented by Frazer (84, 85) for the origin of the chylomicron. According to this partition hypothesis triglycerides are digested only to di-and monoglycerides and fatty acids. This is based on the fact that Frazer and his colleagues were unable to isolate glycerol from digestions of fat with lipase either in vitro or vivo.

They believe that following the initial digestion of a small amount of fat to mono-and diglycerides, these compounds, together with biles salts, start to emulsify the rest of the fat with a great deal of it remaining as emulsified triglyceride. The mono-di-and triglycerides are then absorbed directly through tiny canals in the membranes of the intestinal mucosal cells and from there to the systemic circulation by way of the lacteals and thoracic duct. The fatty acids which have been liberated (representing only about 30 per cent of the total contained fatty acids) are believed to be absorbed by the portal vein and carried to the liver.

Various physiological and pathological conditions have been shown to effect the chylomicron level in the blood. Frazer (84) found that stout individuals have higher and more prolonged levels than those of thin people. He explained this as being due to the fact that more fat goes directly to the fat stores via the general circulation in the stout individual while a greater percentage is hydrolized and taken to the liver via the portal vein in the thin individual. Gage and Fish (65) observed that physical exercise increases the chylomicron level while severe mental

effort produces a prolonged low level. Frazer (76) found that anxiety gave the same effect and it was thought to be due to decreased bowel motility and digestion. Frazer (76) also has described a "fat crisis" occurring about every two hours in a normal fasting subject in which a shower of chylomicrons from the fat stores raised the blood level above that of fasting.

Setala (86) has reported that X-ray irridiation lowers the chylomicron level of the blood. He explains this by the escape of the chylomicrons from the blood stream through altered capillary membranes but it is interesting to note that Allen (53), as has already been mentioned, found the presence of a heparin-like anti-coagulant in the blood following X-ray irridiation, and that Waldron and Friedman (12) found that small doses of heparin administered intravenously cleared the blood of chylomicrons. Thus the hypochylomicronemia following X-ray irridiation may be due to the presence of heparin-like substance rather than to altered capillary membranes as supposed by Setala (86).

Bloor and Gillette (87) studied fat metabolism in diabetes mellitus and found that the basal chylomicron level of the blood was elevated in the uncontrolled state of this disease. These observations

were confirmed by Zon and Shields (88) in 1935. Gage and Fish (72) observed that the basallevel was also elevated in nephrosis and hyperthyroidism while Fourman (89) found that diarrheas and sprue produced low chylomicron levels; presumably because of poor fat absorption from the intestine. Cooper and Lusk(90) report that the chylomicron level never rises above that of fasting in obstructive jaundice. Peters (75) observed a tendency for chylomicrons to clump and settle out of the serum of patients with chronic infections, and to rapidly disappear from the serum of patients with acute infections. Gage and Fish (72) have reported that non-infectuous toxic conditions such as chronic alcoholism, prolonged morphine narcosis, and phosphorus poisoning tend to elevate the chylomicron level of the blood; while Day (91) found that ether anesthesia lowers the level in some cases.

EXPERIMENTAL WORK

FIRST OBJECTIVE

The first experimental objective of this thesis was to substantiate, if possible, the findings of Waldron et. al. that the ingestion of cream has a definite effect in decreasing the clotting time of whole venous blood. To accomplish this, coagulation time tests were conducted on a total of eighteen subjects selected from University Hospital patients. In their selection attention was given to diagnosis and general physical and mental condition. Patients with diabetes mellitus and other metabolic or hemorrhagic disorders were not used.

Each subject was fed a test meal following a twelve hour over-night fast. Fifteen of the subjects received fat in the form of 1 cc. of 20 per cent cow's cream per kilogram of body weight. The other three received carbohydrate in the form of glucose in water to serve as controls. The amount of glucose given was equivalent to the caloric value of the fat test meal based on 1 cc. of cream per kilogram of body weight.

The blood coagulation time was determined at the bedside of the patient immediately before, and

at hourly intervals after the test meal for a period of three hours. In the early tests a one-half hour determination was also made, and in one test a five hour determination was made. Uncontrollable circumstances made it necessary to discontinue after two hours in five of the tests.

A modified Lee-White method (63) was used for the coagulation time determinations. Blood was drawn from the median basilic vein after thorough cleaning with 1:1000 tincture of zepherin solution. A sterile 5 mL. syringe and 20 gauge 1½ inch needle was rinsed twice with sterile physiologic saline solution and all solution expelled just prior to its use in drawing the blood. A turniquet was applied just before the venipuncture and removed immediately after to avoid stasis. The venipuncture was made with the least possible trauma and every effort was made to avoid contamination of the blood sample with tissue juice or air bubbles. If a clean venipuncture was not accomplished in the first attempt, the needle and syringe were discarded and another sterile needle and syringe used after rinsing with sterile saline. If traction on the plunger was required in filling the syringe, it was applied very gently to avoid agitation

or foaming. A stop watch was started the instant the syringe began to fill. After a 5 ml. sample of blood was obtained, the needle was removed from the syringe and 2 ml. allowed to run down the sides into each of two chemically clean test tubes (size 10x75 mm.) which had been rinsed twice with physiologic saline solution and all excess expelled just before drawing the blood. The remaining 1 ml. of blood was not used in the coagulation time determination because it was assumed to contain unavoidable traces of tissue juice which might introduce serious error. (This blood was used for the chylomicron counts which will be described later.) The two test tubes were kept in a constant temperature water bath at 37.5° C. One tube was examined for coagulation at one minute intervals by gently tilting until it could be inverted without spilling. The other tube was then examined in like manner at one-half minute intervals and the end point taken as the time required for the second tube to coagulate sufficiently to allow inversion without loss of contents. The experimental error using this method was found to be within one-half minute as determined by repeated coagulation time determinations at approximately ten minute intervals on three healthy normal subjects. (ref. chart no. 1)

Other methods of determining coagulation time were investigated and discarded in favor of the method just described. The method of Wright (92) using capillary blood drawn into capillary tubes was found to be completely unreliable and useless. Collodian lined test tubes were used in tests no. 4 and 5, and paraffin lined test tubes were used in test no. 6 instead of plain glass tubes rinsed with saline. These modifications gave relatively longer coagulation times but were less satisfactory because of greater experimental error induced by a less sharply defined end point.

The coagulation time of blood was found to be an extremely sensitive and highly arbitrary biologic procedure and its determination is fraught with many sources of error. Solis (92) has made a thorough study of factors affecting the clotting time of shed blood and has discussed the major sources of error. They are contamination with tissue juice, contact with foreign material, dirty equipment, mechanical disturbance of the blood sample, evaporation, temperature, dilution, end point adopted, and the personal equation. Extreme care was used to reduce these sources of error to a minimum. The glassware was

made chemically clean by first washing in alconox solution followed by rinsing in distilled water, boiling for several minutes in chromic acid cleaning solution, rinsing again in distilled water, and drying in an electric oven. Wetting the syringe, needle, and test tubes in physiologic saline solution reduced the effect of contact with foreign material. The efforts to avoid tissue juice, mechanical disturbance, and use of a constant temperature water bath have already been discussed. All experimental work was done by the same person which held the personal equation error to a minimum.

The data obtained in the first experimental objective is presented in graphic form (ref. graphs 1 to 18). The ordinate represents the coagulation time (in minutes) and the abscessa represents the time (in hours) after the test meal. Of the fifteen subjects tested for coagulation time following a fat meal, eleven showed a definite increase in blood coagulability while three showed only slight change (ref. graph 7, 8, 9) and one showed a definite decrease (ref. graph 10). The average per cent decrease in clotting time for the eleven subjects (excluding tests 7, 8, 9, and 10) is 44 per cent at

the end of one hour, 26 per cent at the end of two hours, and 22 per cent at the end of three hours. This compares favorably with the results reported by Waldron et. al.(ref. graph 19).

No explanation can be offered for the failure of tests 7, 8, 9 and 10 to give similar results and it can only be assumed that unknown factors were involved which neutralized or counteracted the effect of chylomicronemia on the clotting time. The failure cannot be explained by poor assimulation and digestion of fat since all four tests gave the characteristic rise in chylomicron count following the fat meal.

The three controls in which glucose was used for the test meal did not show an increased blood coagulability which indicates that the change noted after fat ingestion is due to the chylomicronemia per se rather than to any dynamic metabolic action resulting from food ingestion in general; or any of the other variable factors of the test. (ref. graphs 16, 17, 18)

SECOND OBJECTIVE

The second objective of the experimental work was to determine the degree of correlation between changes in coagulation time and changes in chylomicron content of the blood. This phase of the work was

carried out in conjunction with the first objective. Samples of blood were obtained for the chylomicron count from the same sample drawn for the coagulation time determination. This was accomplished by allowing the blood to flow from a drop on a glass slide into small glass capillary tubes by capillary action. These capillary tubes were made by heating a section of 1/4 inch glass tubing over an oxygen enriched gas flame to the plastic state and then drawing it out to an inside diameter of approximately 1/4 mm. This tubing was then cut into 5 inch length, cleansed with alconox and boiling chromic acid, rinsed with distilled water, dried in the electric oven, and stored in a stoppered test tube.

After the capillary tubes were filled to within 1 inch of the end of the tube, the blood was allowed to clot and the unfilled ends were sealed over a gas flame. The tubes were then centrifuged for 10 minutes at 2500 R. P. M. to extract the serum. The serum was then placed on a glass slide by breaking the tube at the serum-cell interface and forcing the serum out of the tube by means of a small bulb syringe. A cover slip was placed over the drop of serum, tapped gently to obtain an even distribution, and the edges

sealed with castor oil in preparation for microscopic examination. The slides and cover slips were cleansed in the same manner described for test tubes and capillary tubes and stored in 95 per cent alcohol prior to use. Their surfaces were polished with lens paper just before applying the drop of serum to the slide.

The method used in making the chylomicron counts is essentially the same as that developed by Gage and Fish (65) in 1925. The microscope used in this work was fitted with a paraboloid condenser and built in pointolite illuminator for dark field illumination. The oil emersion objective contained a funnel stop which reduced the aperature to less than 0.8 mm.; and the 10X occular was fitted with a grid micrometer consisting of 100 squares. Each square was .075 mm. by .075 mm. in size. Immursion oil was used between cover slip and objective, and between condenser and slide.

In adjusting the microscope the light from the dark field illuminator was first brought to a fine point on the slide and then the objective was carefully lowered and focused until the brilliant reflections from the chylomicrons in active brownian movement came into view between the two planes formed by the inner surfaces of the slide and cover slip. After making final

adjustment on the focus it was not changed again until the count was finished. Five different microscopic fields were counted and the average value taken as the final count. The count in each field consisted of counting the chylomicrons in 10 small squares of the micrometer grid and multiplying this value by 10 for the total count. Two small squares were counted in each corner of the grid and two in the center. All particles, both bright and dull, in brownian motion were counted.

Probably the greated source of error in the chylomicron determination is the difficulty in accurately counting the particles as they dance in and out of the micrometer gride squares in rapid brownian movement. However, with practice, and one person making all counts, it was found that results could be repeated with a reasonable degree of accuracy. All contamination by foreign particles was reduced to a minimum by cleaning and storing the glassware in the manner already described. Error due to different depths of focus was eliminated by focusing only once for all five microscopic fields.

The data obtained in the second experimental objective is also presented in graphic form and is plotted

in juxtaposition to coagulation time of each experimental subject for ease of comparison (ref. graphs 1 to 18). The ordinate represents the chylomicron count (total chylomicrons per micrometer grid) and the abscissa represents the time (in hours) after the test meal.

In nine individuals the maximum decrease in coagulation time occurred at the peak of chylomicronemia following the test meal (ref. graphs 1,2,3,4, 5,6,11,12,14). Two individuals showed the maximum decrease in coagulation time to precede the maximum chylomicron count by one hour (ref. graphs 13 and 15). There is, of course, no correlation between changes in coagulation time and chylomicron count in the three individuals which failed to show a coagulation time change (ref. graphs 7, 8, 9). One individual showed a progressive decrease in coagulation time as the chylomicron count increased following the test meal.

The test results indicate that there is a direct time relationship between rise in chylomicron count and decrease in blood coagulation. This relationship does not appear to be a quantitative one however, since there is no consistent correlation between degree of rise in

chylomicron count and degree of change in coagulation time of the various individuals tested.

Gage and Fish (65) have pointed out that several factors must be considered in evaluating the results of chylomicron studies. They found a wide variation in the speed of digestion, absorption, movement of fat from the intestine to the blood stream, and metabolism of fat after it reaches the blood. Also the ingestion of excess fat is known to decrease the emptying time of the stomach to different degrees in different individuals. This is amply confirmed in the experimental results. The chylomicron curves show a high sharp peak and rapid return to the basal level in some individuals (ref. graphs 1, 3, 8, 13) while others show a more gradual rise and fall in the chylomicron count (ref. graphs 4, 5, 6, 15). Still others show relatively low peaks (ref. graphs 11, 12, 14). Another factor which must be kept in mind is the "fat crisis" which Frazer (76) has described as occurring about every two hours in the fasting subject (ref. p. 45). The results shown in graph no. 14 suggest that the initial blood sample was taken at the time of such a crisis. Lastly, it has been pointed out by Gage and Fish (65) and emphasized by Frazer and Stewart (93)

that the chylomicron count actually represents the difference between the amount of fat added to the blood stream and the amount removed. The relative efficiency of these two factors in the metabolism of fat therefore has a profound influence on the shape of the chylomicrograph.

THIRD OBJECTIVE

The third objective of the experimental work was to investigate the effect of fat ingestion on prothrombin activity. This was accomplished by making prothrombin activity determinations on a healthy human subject before, and at hourly intervals after the ingestion of a test meal, for a period of four hours. The test meal consisted of 1 cc. of 20 per cent cow's cream per kilogram of body weight. The experimental subject had had no food for twelve hours prior to the test.

The one stage method of Quick (94) was used in the determination of prothrombin activity. The principle of this method rests on the fact that when optimal amounts of thromboplastin and of calcium are added to citrated plasma, prothrombin is converted to thrombin allowing the coagulation mechanism to proceed to completion. The measurement of the speed of this

conversion, as determined by the clotting time of the plasma, is therefore a measurement of the prothrombin activity. The technique used in this determination will be described in detail.

Each blood sample was taken from the median basilic vein using a dry sterile 10 m. syringe and 20 gauge needle. All precautions to avoid bubbling, stasis, tissue juice contamination, and excessive mechanical disturbance, as described in the first objective were strictly observed. Exactly 9.0 ml. of the blood sample was quickly transferred to a chemically clean test tube containing 1.0 ml. of 0.1 molar sodium citrate solution. The tube was stoppered and inverted several times to insure thorough mixing. Plasma was obtained by centrifugation of the citrated whole blood at 1000 revolutions per minute for five minutes. 0.1 ml. of citrated plasma was then accurately pipetted into a chemically clean test tube (10x75mm.). To this was added 0.1 ml. of thromboplastin solution and the tube gently shaken to insure mixing. With stop watch in hand, exactly 0.1 ml. of 0.025 molar calcium chloride solution was added and at the same instant the stop watch was started. The tube and reagents were maintained at 37.5° C. in a water bath. For 10

seconds the tube was gently shaken in the water bath and then removed and slowly rocked back and forth in the horizontal position. The stop watch was stopped at the instant the plasma was no longer completely fluid and this was taken as the end point.

The thromboplastin solution was prepared from lyophilized rabbit brain obtained from the University Hospital laboratory. To 0.15 gm. of this preparation were added 4 ml. of sterile sodium chloride solution (0.85 gm./100 ml.), and 0.05 ml. of sodium oxalate solution (1.34 gm./ 100 ml.). This mixture was incubated at 48° C. for 10 minutes with agitation every 3 minutes. It was then filtered through cotton and kept frozen until time for use.

A total of five prothrombin time determinations were made on each hourly sample of blood and the average of these taken as the final value. It was found that results could be repeated within 1 second for each sample of blood.

Test results are presented in tabular form showing both prothrombin time in seconds and per cent change for each hourly determination (ref. chart 2). Chylomicron counts were also made on each blood sample to correlate the degree of change in prothrombin activity

with the chylomicron blood level. These results are presented in graphic form (ref. graph 21). No significant change in prothrombin activity was observed following ingestion of cream.

FOURTH OBJECTIVE

The fourth objective of the experimental work was to investigate the effect of fat ingestion on circulating heparin-like substances of the blood. This was accomplished by titrating the blood with protamine using the method developed by Allen (95) and his associates. The experimental subject, test meal, fasting state, method and intervals of obtaining blood samples and precautionary measures to eliminate sources of error were identical with those described in the third objective. The experimental work of the third objective preceded that of the fourth objective by several days.

The principle of the protamine titration test is based on the fact that when a blood sample is made incoagulable by the addition of a standard amount of heparin and then titrated back to a clotting end point by the addition of a standard amount of antiheparin (protamine sulfate), the amount of protamine sulfate required to re-establish coagulation is theoretically

proportional to the concentrations of native "heparins" and "antiheparins" in the blood. Thus by comparing the end points of a series of blood samples taken before and after the ingestion of fat by a healthy human subject, any alteration in the concentration of native heparin-like substances in the blood due to ingesting fat should be apparent.

The technique used in the protamine titration was as follows: Protamine sulfate solution was placed into each of ten dry and chemically clean test tubes (10x75 mm.) from a micropipette in 0.02 ml. increments, beginning with 0.02 ml. (0.02 mg.) in the first tube and ending with 0.20 ml. (0.20 mg.) in the tenth tube. 0.10 ml. (1.0 mg.) of liquid commercial heparin was then micropipetted into a conical centrifuge tube graduated to 15 ml. After drawing the blood sample the centrifuge tube containing the heparin was quickly but gently filled with blood to exactly the 11 ml. mark, stoppered, and inverted several times to insure mixing. A 10 ml. pipette was then filled with the heparinized blood and 1 ml. delivered to each of the ten test tubes containing protamine sulfate. These tubes were stoppered and each inverted ten times to insure mixing. The entire series of tubes was then allowed to stand undisturbed for one hour before reading. The end point was

taken as the tube containing the least amount of protamine sulfate in which a solid clot had formed after one hour.

The test results are presented in graphic form in terms of protamine content of the end point tube. Chylomicron counts were also made on each blood sample and are plotted in juxtaposition to the protamine titration curve for ease of comparison (ref. graph 22).

These results show a definite increase in coagulability in the blood sample taken two hours after ingestion of cream. In this sample coagulation occurred with 0.12 mg. of protamine sulfate while all other samples taken before and after the two hour sample did not coagulate with less than 0.14 mg. of protamine sulfate. Chylomicronemia also reached its highest level at two hours following ingestion of cream. This strongly indicates that the heparin-like circulating anticoagulants of the blood are in less concentration or in less active form due to chylomicronemia following fat ingestion.

Coagulation at an end point of 0.14 mg. of protamine sulfate agrees perfectly with the normal value reported by Allen (95). The end points were very definite and unmistakeable for all blood samples. The blood in the end point tube and all tubes beyond formed a firm clot while all tubes of lower protamine content remained fluid.

DISCUSSION

Any attempt to explain the mechanism whereby chylomicronemia increases the coagulability of the blood must naturally be based on the factors involved in the biochemical processes of blood clotting. Of these factors it is apparent from our present knowledge of the clotting mechanism that prothrombin occupies a central and dominant position (ref. p. 34). It is reasonable to suppose therefore that the increased coagulability phenomenon might be explained by an influence of chylomicronemia on the activation of prothrombin. It has been established in the review of the development of our knowledge of the clotting mechanism that a number of circulating accelerator factors are essential to the activation of prothrombin. Calcium is essential for the physiologic activation of prothrombin and must be present in optimum concentration, but it is untenable that chylomicronemia could produce its effect through this factor since the calcium level of the blood is many times that required to activate prothrombin. Thromboplastin is also essential for prothrombin activation and it is a possibility that platelet enzyme is in some way liberated more rapidly after fat ingestion and thus

accelerating the formation of thromboplastin from its precursor thromboplastinogen. Serum and plasma prothrombin accelerators (accelerator globulins) are proteins which have been shown to be essential for the rapid activation of prothrombin and it might be postulated that their activity is in some way enhanced by ingestion of fat. These hypotheses are purely speculative however, and not supported by experimental results since no significant change in prothrombin activity was observed following the ingestion of fat.

In addition to circulating accelerator factors it has also been shown that there are circulating inhibitors of coagulation in the blood (ref. p. 28). The best known of these is heparin or substances quite similar to heparin. Experimental results indicate that the heparin like substances in the blood are rendered less active or reduced in concentration due to chylomicronemia as shown by protamine titration. It has previously been pointed out that there is evidence of a direct relationship between heparin and lipemia (ref. p. 9). Waldron and Friedman (12) found that heparin could produce both a lipemia-clearing and lipemia potentiating effect in vivo under certain conditions of fat ingestion. These effects were correlated

with blood coagulation time and the anticoagulant effect of heparin was found to be modified by ingestion of fat. These findings, along with the protamine titration results obtained in this thesis, strongly support the hypothesis that the chylomicrons in some way bind the heparin-like substance of the blood or buffer its anticoagulant activity.

Quick (96) has made a critical study of the mode of action of heparin as an anticoagulant and has concluded that it acts in three distinct ways: first, it prevents the liberation of thromboplastin from platelets; second, it prevents, with the aid of a plasma co-factor, the conversion of prothrombin to thrombin; and third, it forms with serum albumin a strong antithrombin. On first consideration these modes of action seemingly introduce a weakness in the above hypothesis in view of the fact that no significant change in prothrombin activity was observed following ingestion of fat. However, since the normal level of heparin-like material in human blood has been estimated by Jacques (56) to be only 0.009 mg. per ml., and since the conversion of prothrombin to thrombin in the Quick prothrombin time determination is greatly accelerated by the addition of thromboplastin,

it is believed that the amount of thrombin formed excedes and completely obscures the variation in antithrombic and antiprothrombic activity of the heparin-like substances of the blood.

Lastly, it is interesting to note that Seegers(97) has found a variety of artificial colloidal substances, such as acacia and gelatin, to have a favorable influence on the activity of thrombin in converting fibrinogen to fibrin. This raises the intriguing question as to whether chylomicrons, since they also are colloidal substances, exert a similar effect.

SUMMARY

Previous work relating to the effect of fat ingestion on blood coagulation has been reviewed and a brief history of the development of our knowledge of the blood coagulation mechanism has been presented, including a modern working hypothesis of the blood coagulation mechanism. Also a brief history of the development of our knowledge of the chylomicron has been presented and its origin, transportation, biochemistry, physical chemistry, colloid chemistry, and method of study by dark field microscope have been discussed. The effect of various physiological and pathological states on chylomicronemia was considered.

The experimental work of the thesis consisted of four objectives; first to investigate the effect of

fat ingestion on whole blood coagulation time and to compare the test results with those reported by other investigators; second, to investigate the degree of correlation between changes in whole blood coagulation time and chylomicronemia; third to investigate the effect of fat ingestion on prothrombin activity; and fourth, to investigate the effect of fat ingestion on heparin-like substances of the blood by means of protamine titration. The method and technique used in each experimental objective is described in detail and the experimental accuracy, sources of error, and test results are discussed.

A final discussion of possible explanations for the phenomenon of increased blood coagulability following fat ingestion is presented in view of the experimental results and our present knowledge of the coagulation mechanism. This includes an original hypothesis which the author believes merits further investigation.

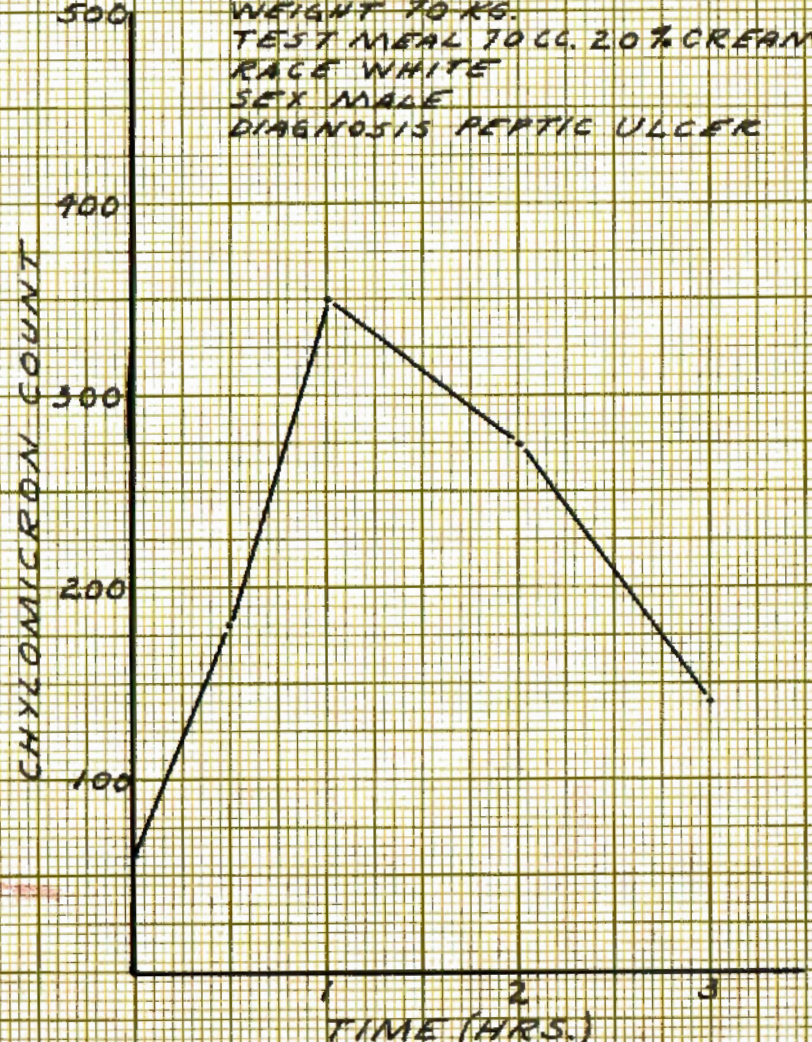
CONCLUSIONS

While it would be pushing the experimental data of this thesis beyond its just limits to draw definite conclusions from it, it is believed that the following statements are justified and constitute strong indications for further work on this subject.

1. The ingestion of cream causes whole venous blood to become hypercoagulable.
2. The average maximum decrease in coagulation time of whole venous blood after ingestion of 1 cc. of 20 per cent cream per kg. body weight is approximately forty per cent.
3. The time of maximum decrease in coagulation time of whole venous blood appears to occur at the time of maximum chylomicronemia following ingestion of cream.
4. There appears to be no direct correlation between degree of chylomicronemia and degree of change in coagulation time following ingestion of cream.
5. The ingestion of glucose produces no consistent change in coagulation time of whole venous blood.
6. No significant change in prothrombin time of venous blood can be demonstrated by the Quick one stage method following ingestion of cream.
7. Protamine titration tests on whole venous blood following the ingestion of cream indicate that the chylomicrons in some way buffer the anticoagulant activity of the heparin-like substances of the blood.

TEST NO. 1

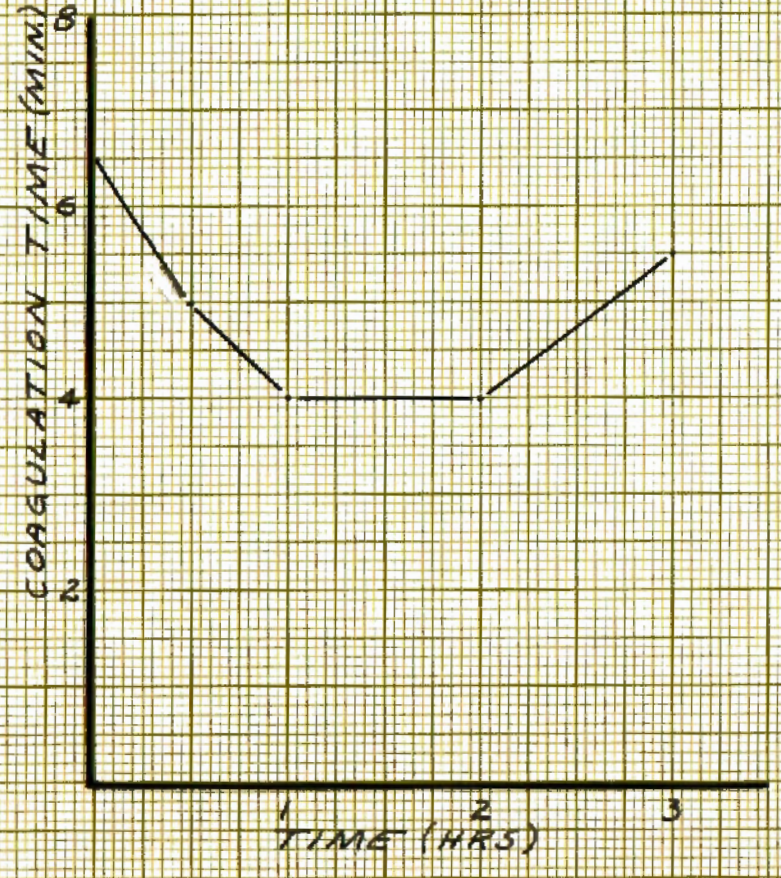
DATA:
AGE 29
WEIGHT 70 KG.
TEST MEAL 70 CC. 20% CREAM
RACE WHITE
SEX MALE
DIAGNOSIS PEPTIC ULCER

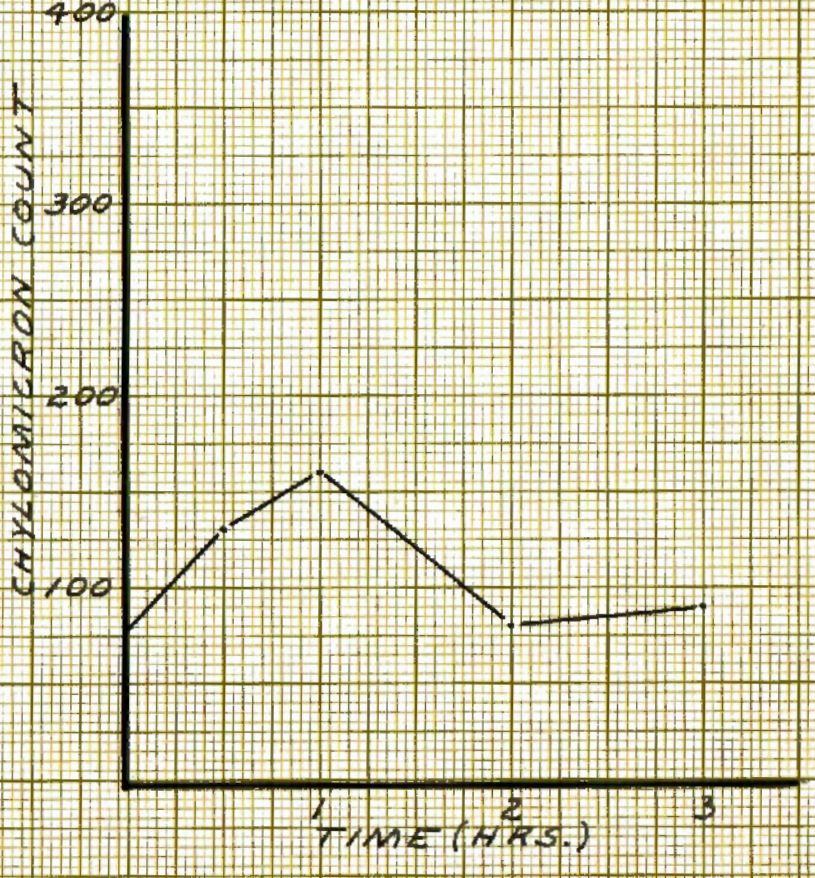


GRAPH NO. 1

TEST NO. 2

DATA:
AGE 63
SEX MALE
WEIGHT 80 KG
TEST MEAL 8 CC. 20% CREAM
RACE WHITE
DIAGNOSIS CIRRHOSIS OF LIVER

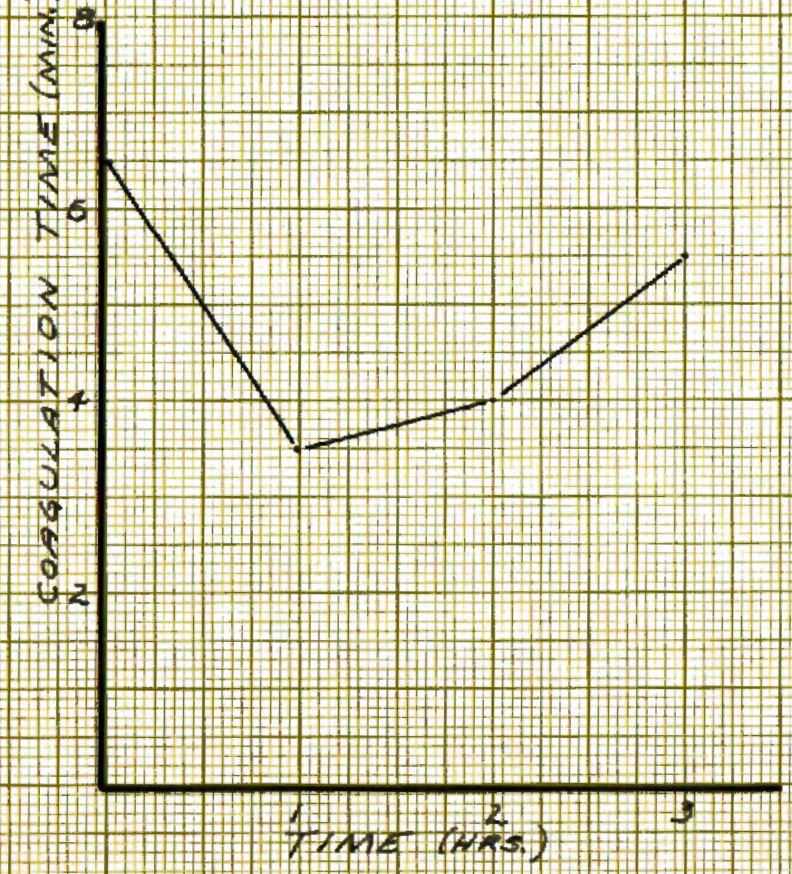


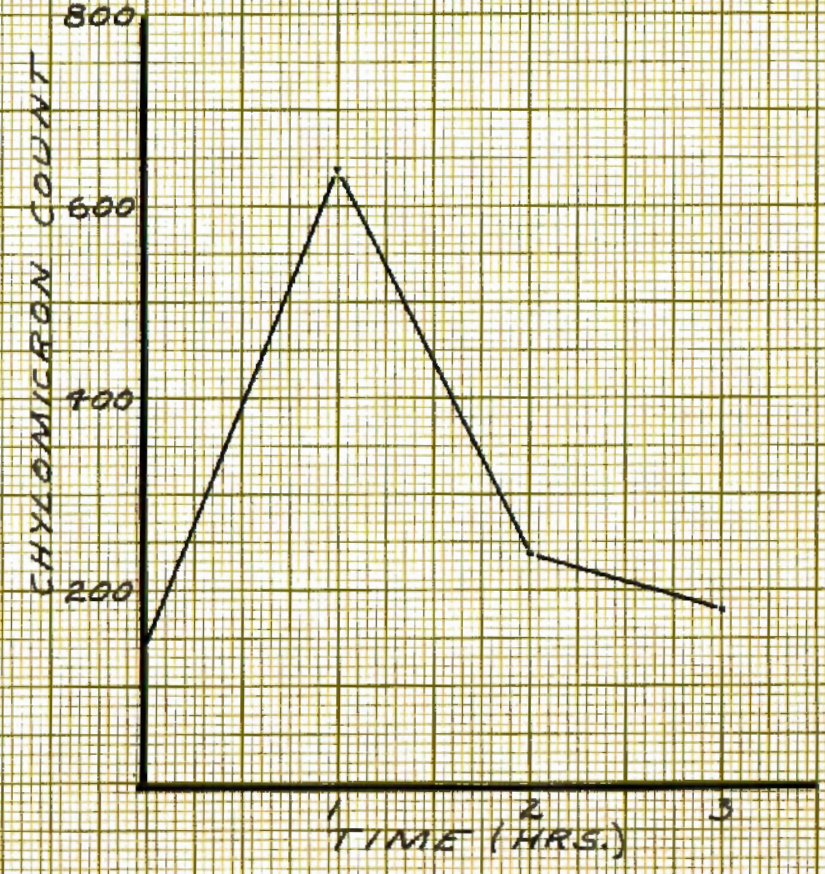


GRAPH NO. 2

TEST NO. 3

DATA:
AGE 69
WEIGHT 54 KG.
TEST MEAL 54 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS PEPTIC ULCER



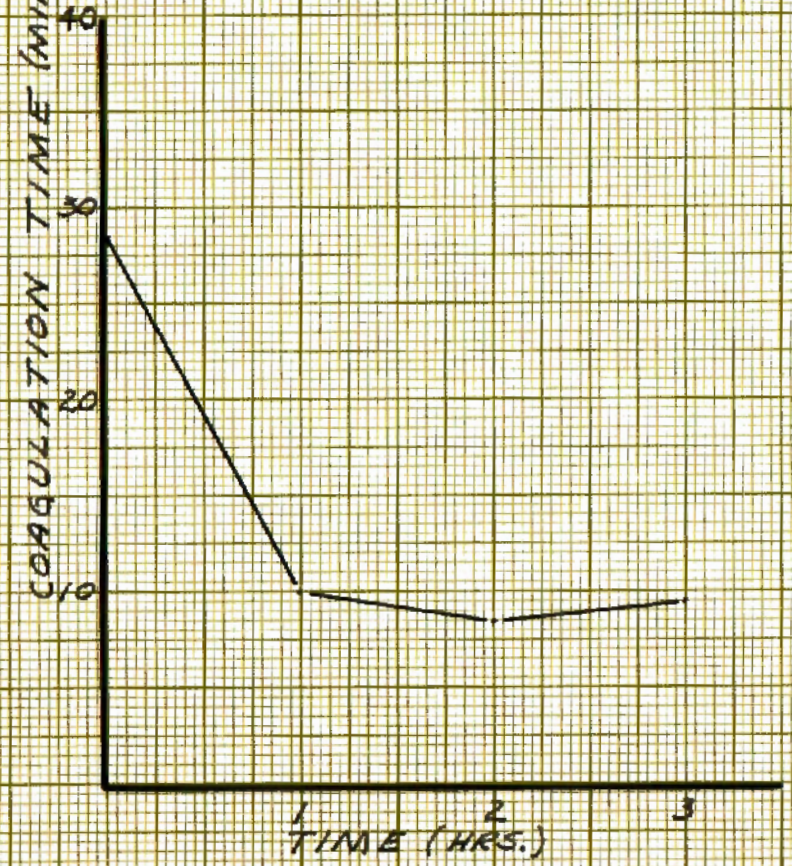


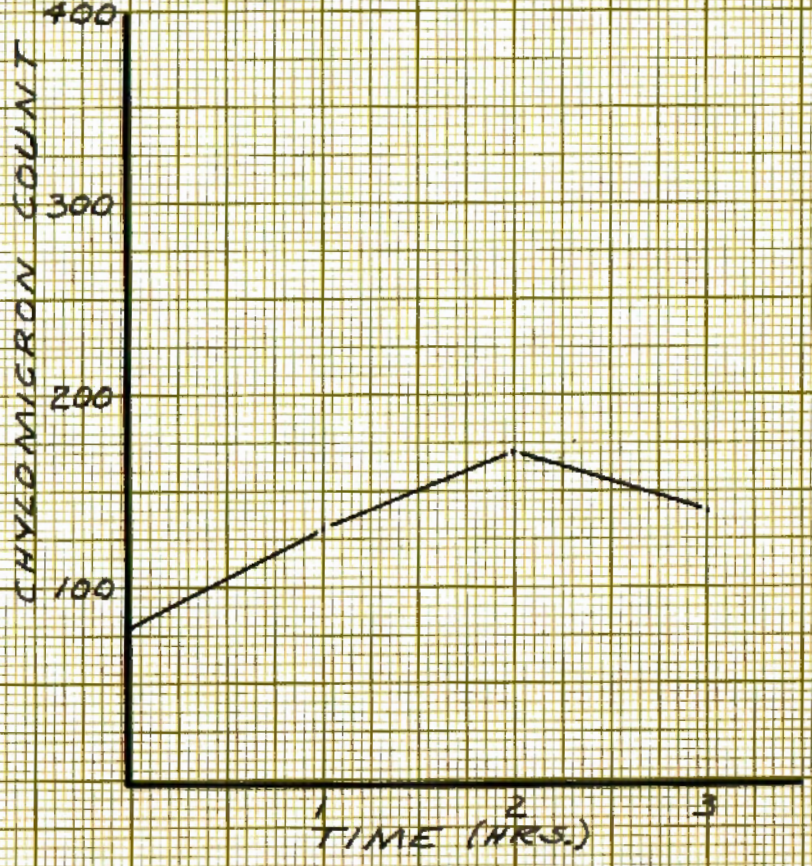
GRAPH NO. 3

TEST NO. 4

DATA:
AGE 21
WEIGHT 60 KG.
TEST MEAL 60 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS BRONCHITIS

NOTE:
COLLOIDIN LINED TUBES



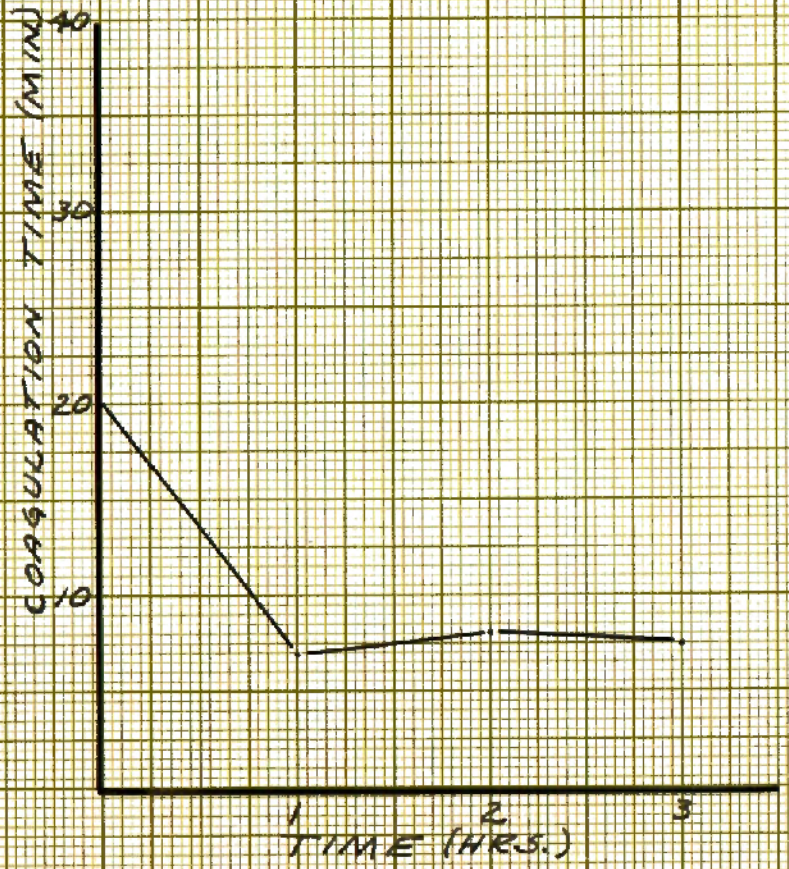


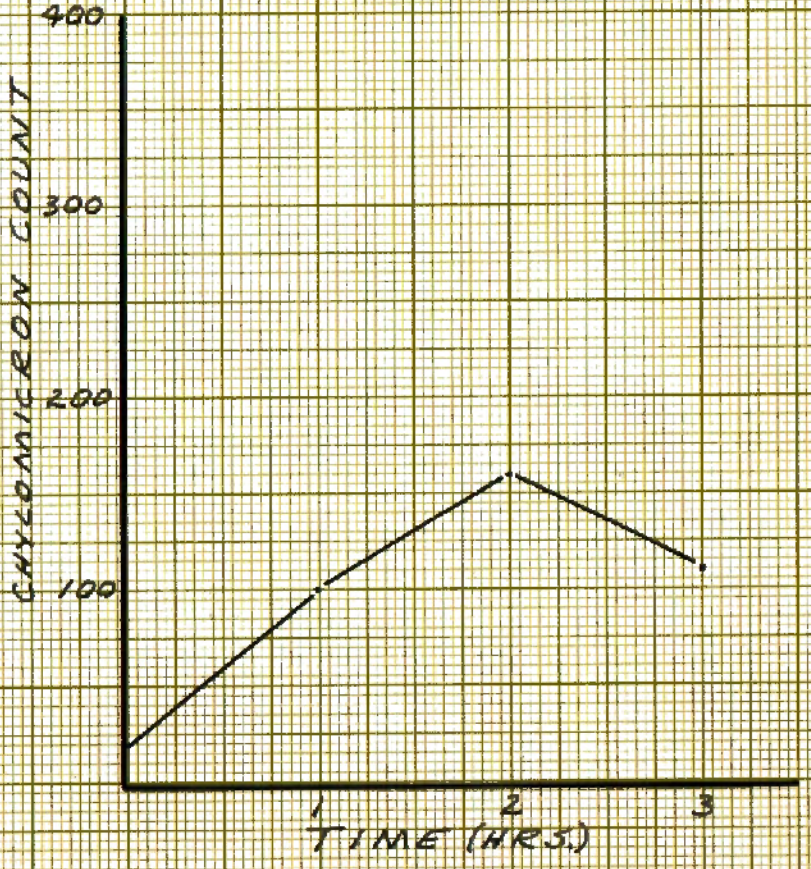
GRAPH NO. 4

TEST NO. 5

NOTE:
COLLOIDIN LINED TUBES

DATA:
AGE 70
WEIGHT 60 KG.
TEST MEAL 60 CC 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS BRONCHIECTASIS

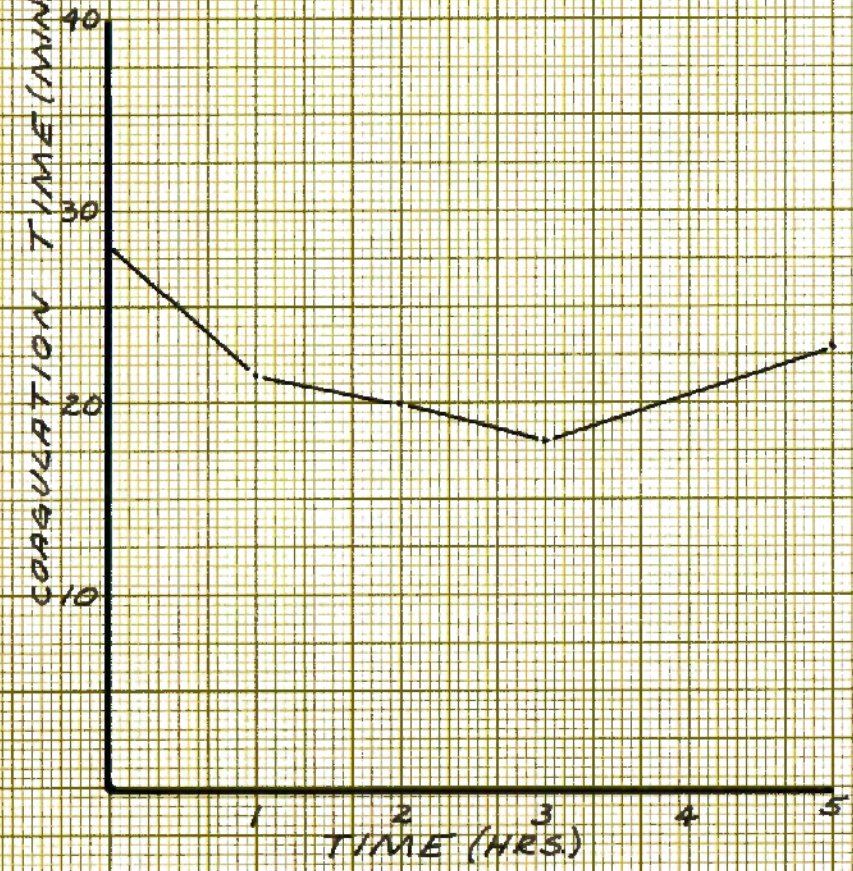


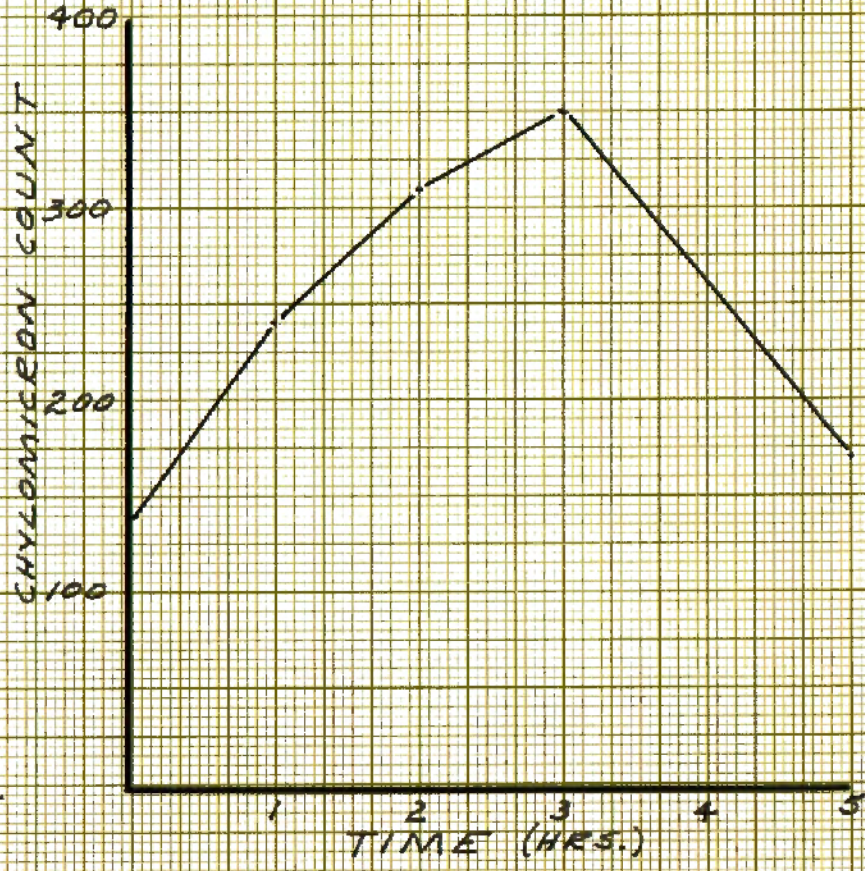


GRAPH NO. 5

TEST NO. 6

DATA:
AGE 69
WEIGHT 122 KG.
TEST MEAL 122 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS CARCINOMA OF BOWEL

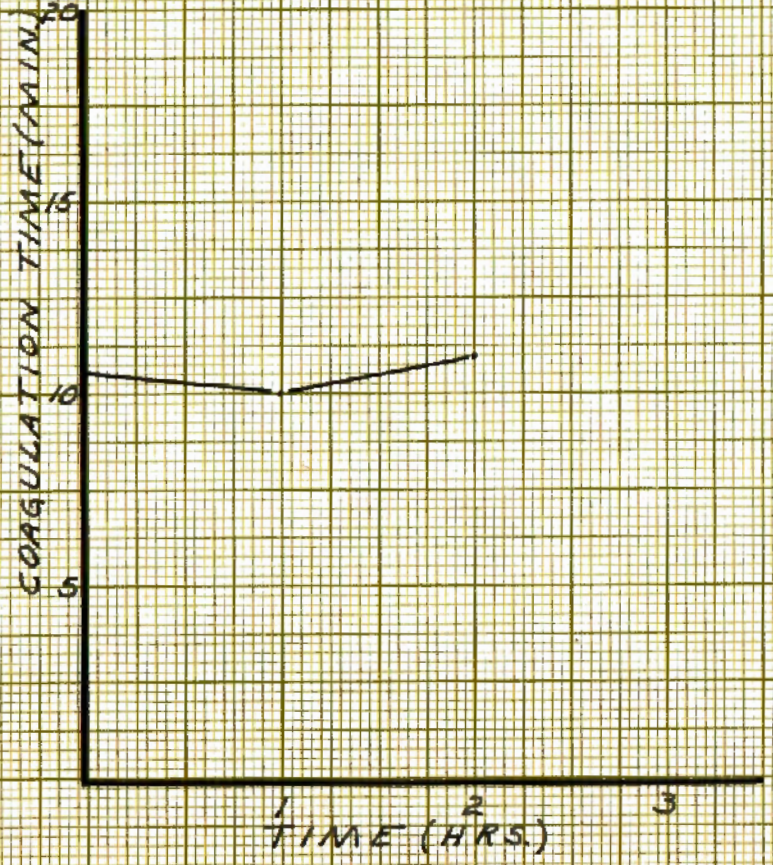


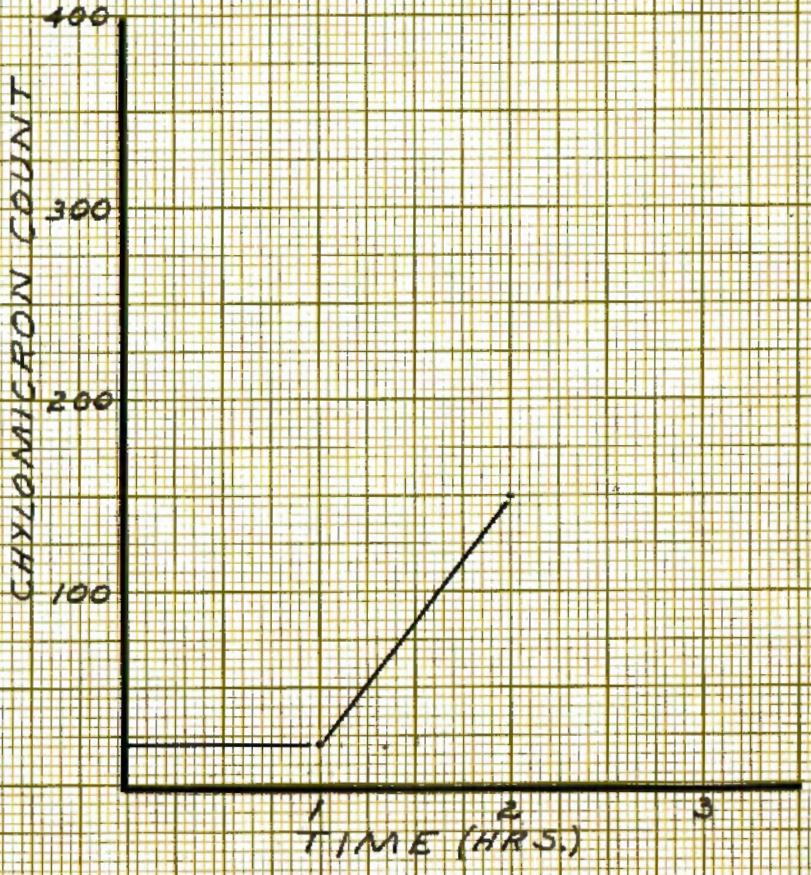


GRAPH NO. 6

TEST NO. 7

DATA:
AGE 60
WEIGHT 76 KG.
TEST MEAL 76 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS ESOPHAGEAL DIVERTIC.

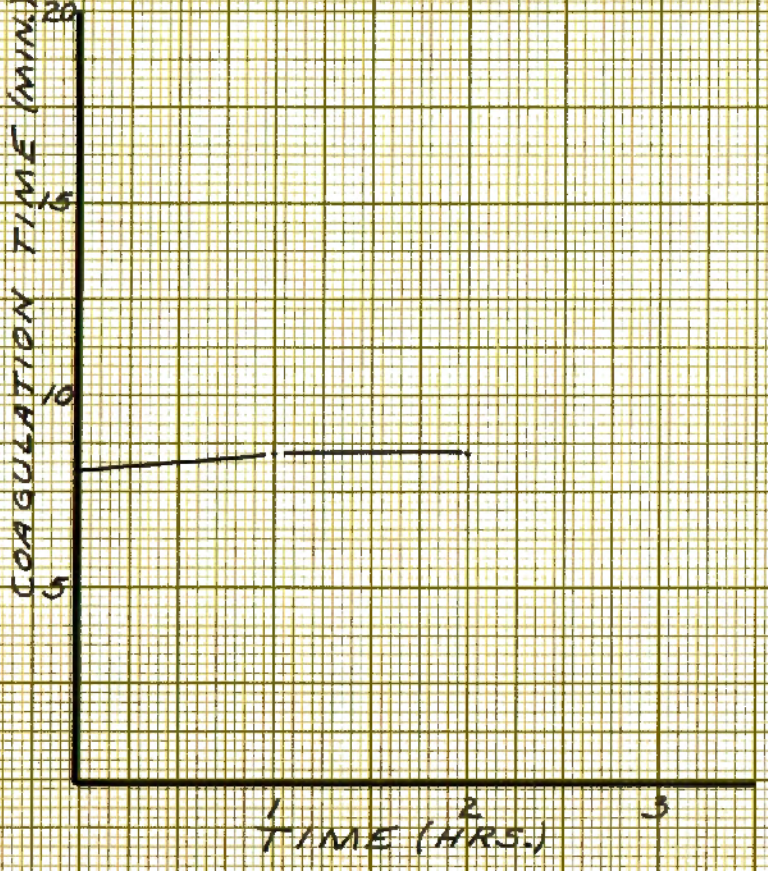


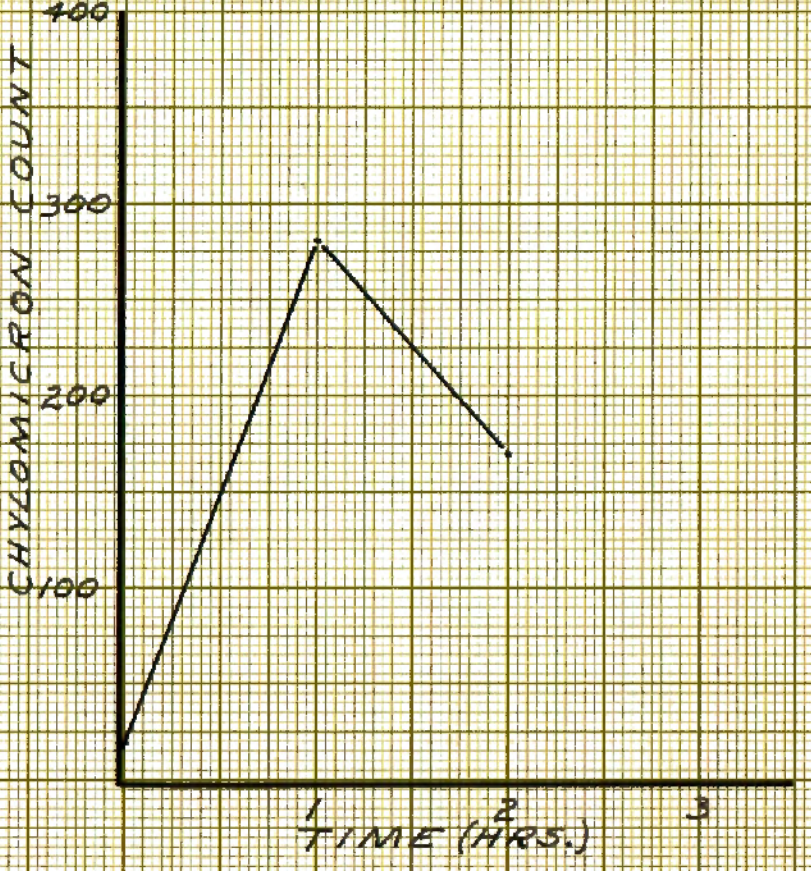


GRAPH NO. 7

TEST NO. 8

DATA:
AGE 16
WEIGHT 54 KG.
TEST MEAL 54 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS RHEUMATIC HEART DISE.

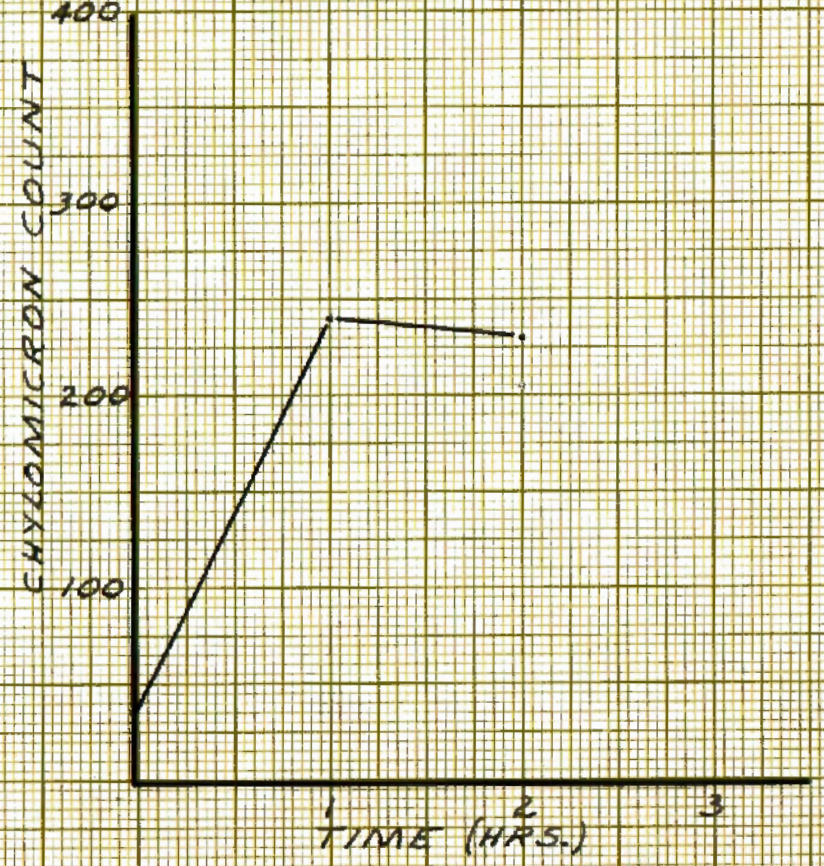




GRAPH NO. 8

TEST NO. 9

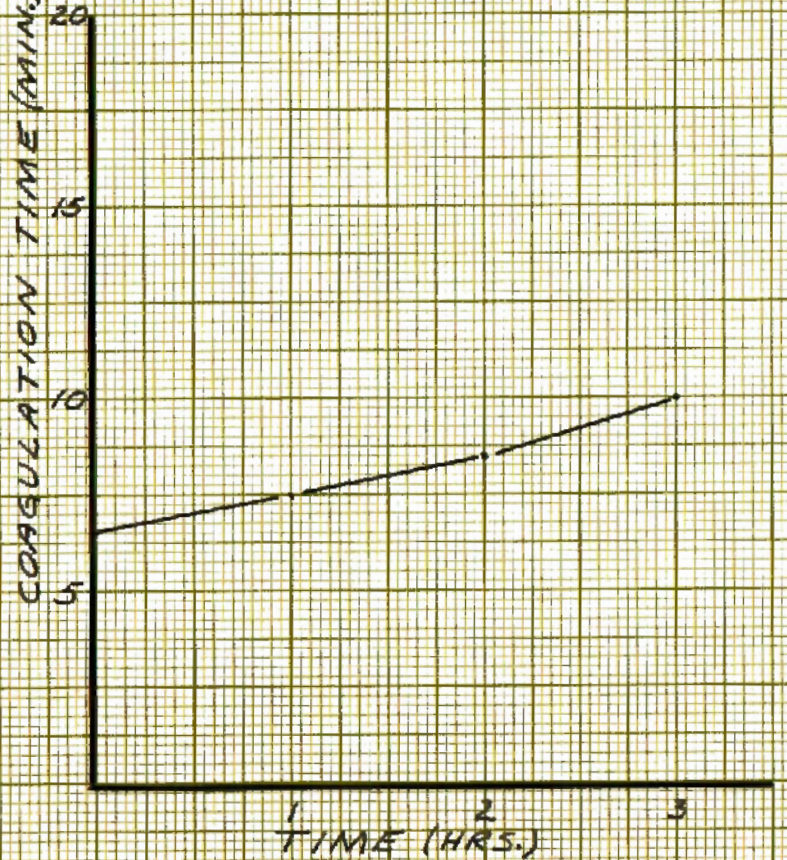
DATA:
AGE 32
WEIGHT 64 KG.
TEST MEAL 64 cc. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS BRONCHIECTASIS

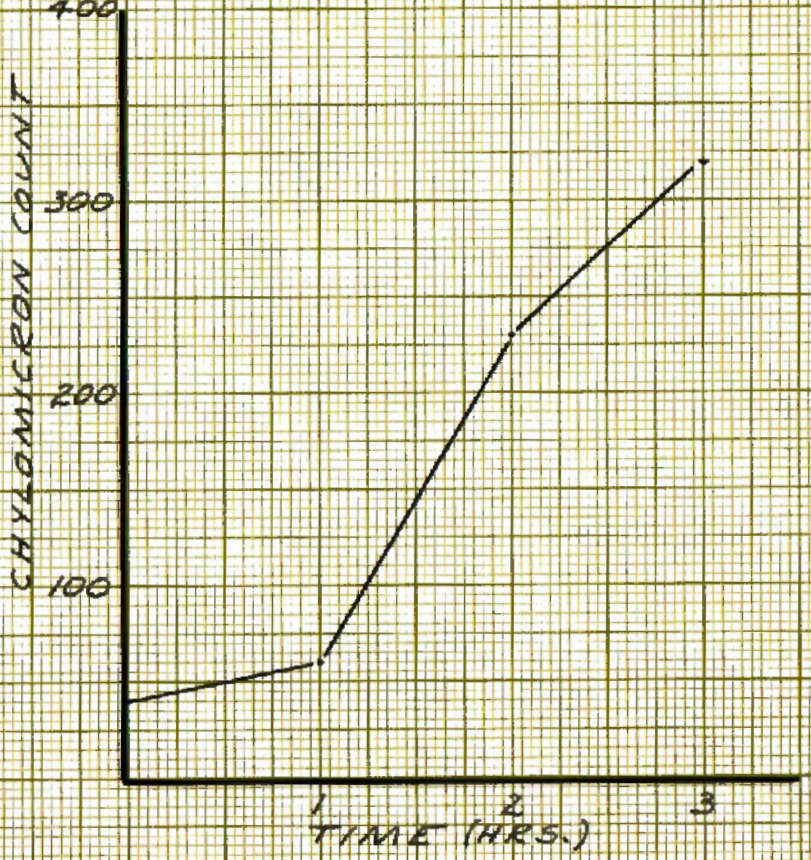


GRAPH NO. 9

TEST NO. 10

DATA
AGE 72
WEIGHT 82 KG.
TEST MEAL 82 GM. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS CARCINOMA OF SIGMOID

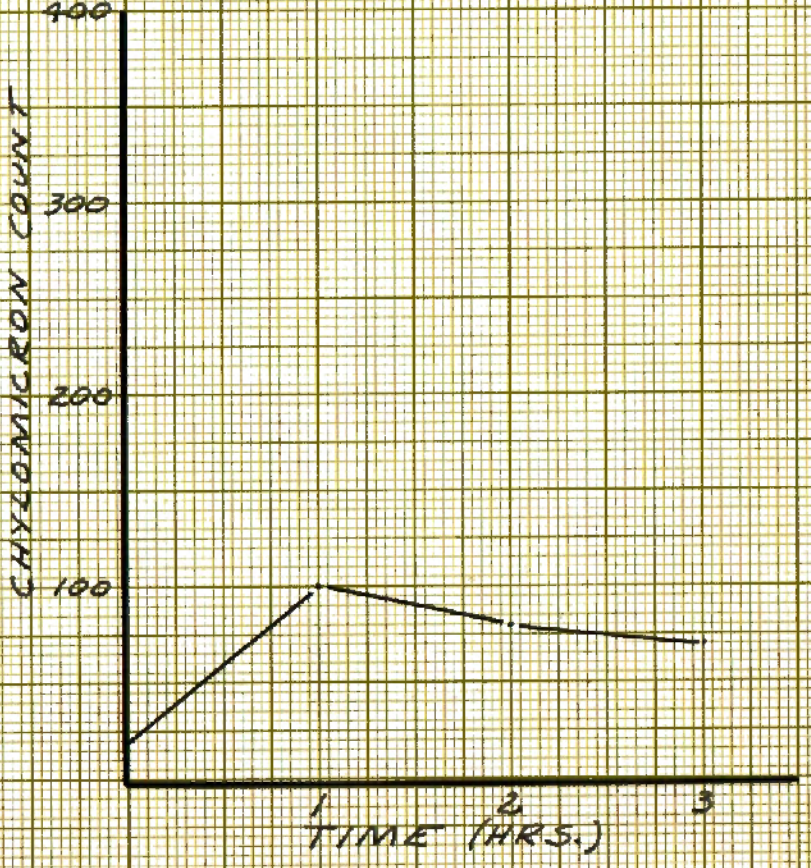




GRAPH NO. 10

TEST NO. 11

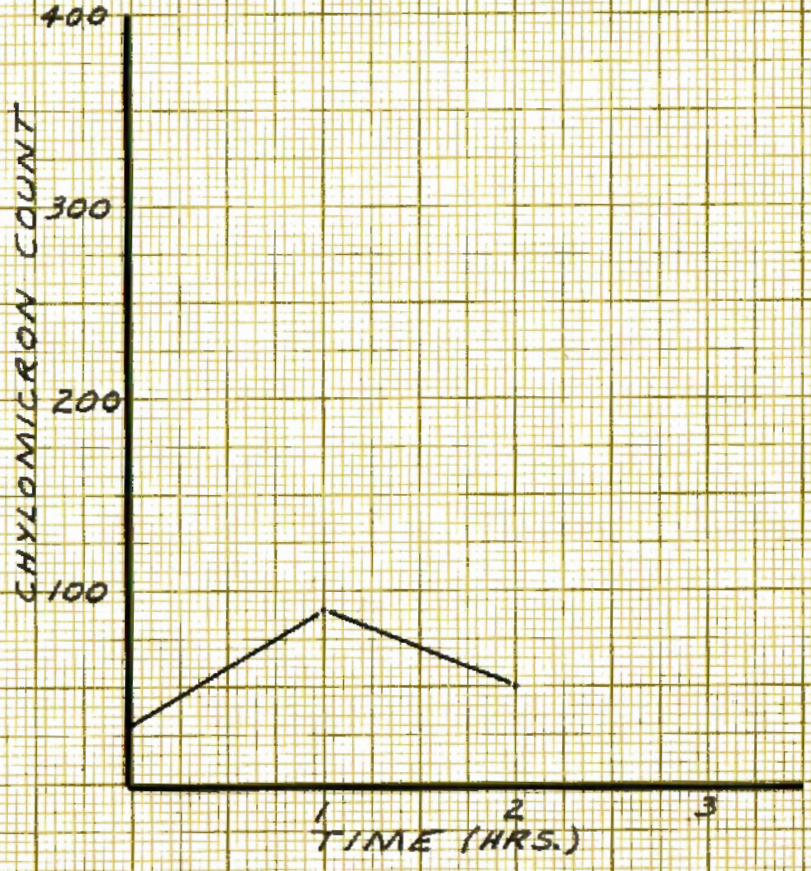
DATA:
AGE 37
WEIGHT 83 KG.
TEST MEAL 83 CC. CREAM
SEX FEMALE
RACE WHITE
DIAGNOSIS VAGINAL BLEEDING OF UNKNOWN ETIOLOGY



GRAPH NO. 11

TEST NO. 12

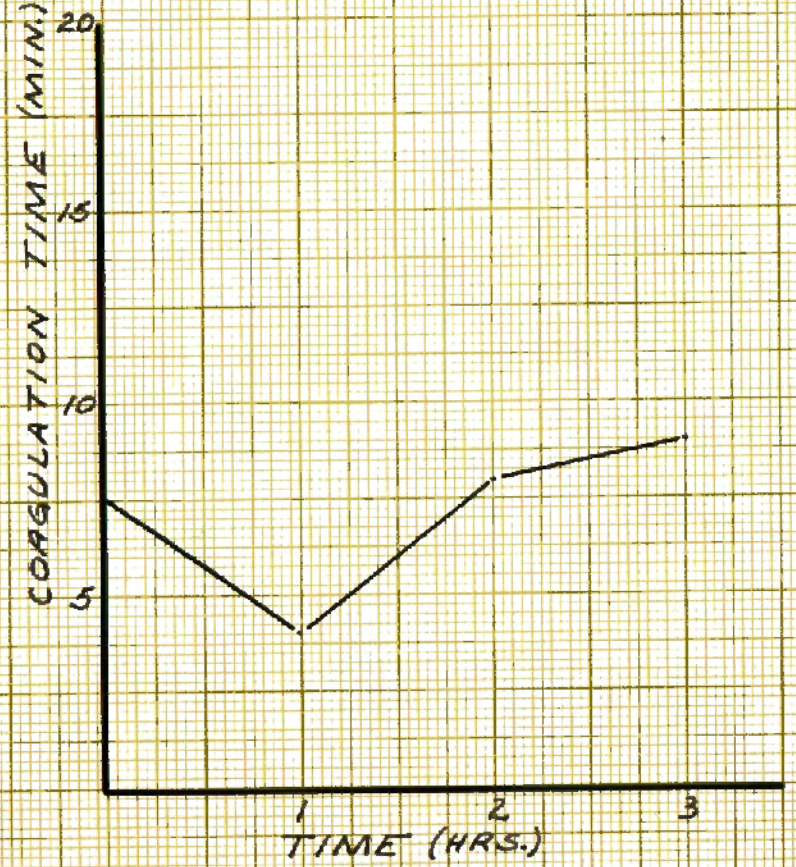
DATA:
AGE 73
WEIGHT 63 KG.
TEST MEAL 63 CC. 20% CREAM
SEX FEMALE
RACE WHITE
DIAGNOSIS VAGINAL CARCINOMA

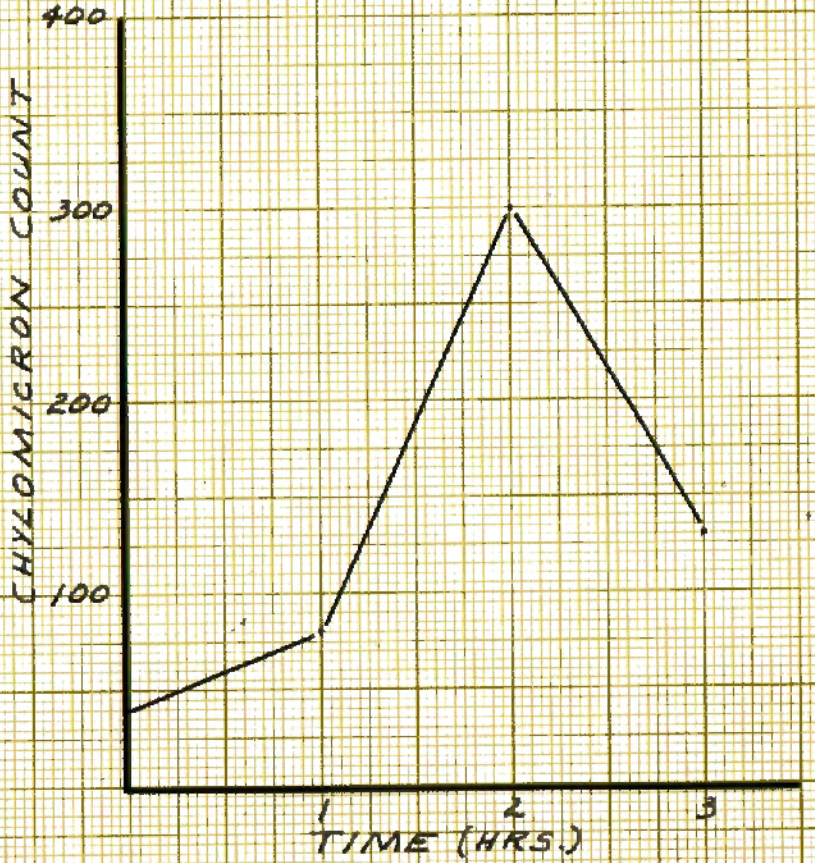


GRAPH NO. 12

TEST NO. 13

DATA:
AGE 65
WEIGHT 90 KG
TEST MEAL 90 CC. 20% CREAM
SEX MALE
RACE WHITE
DIAGNOSIS NO ORGANIC DISEASE

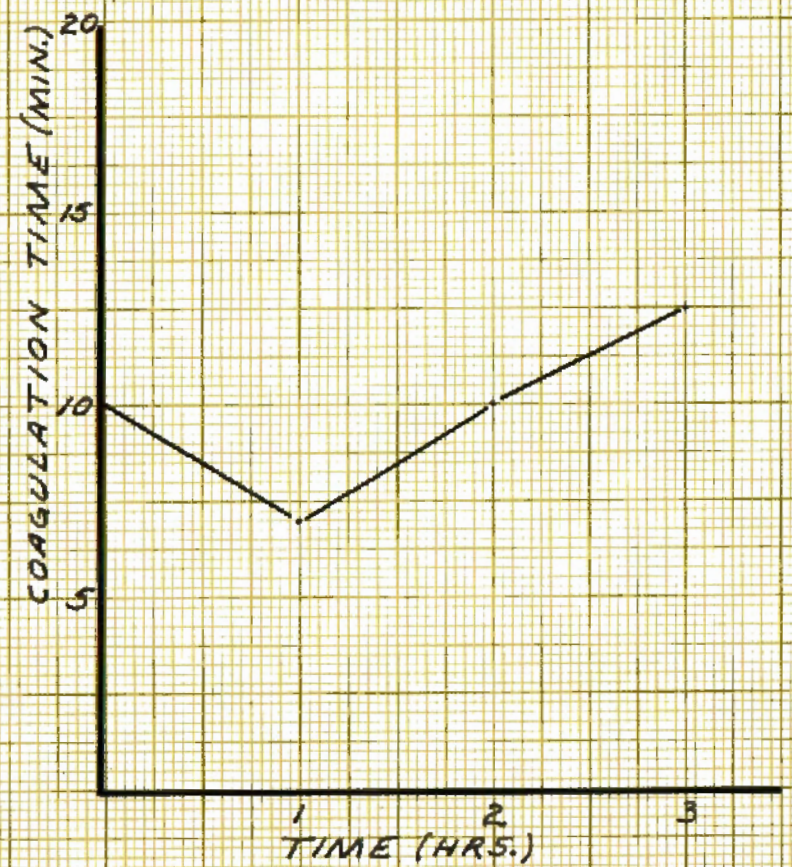


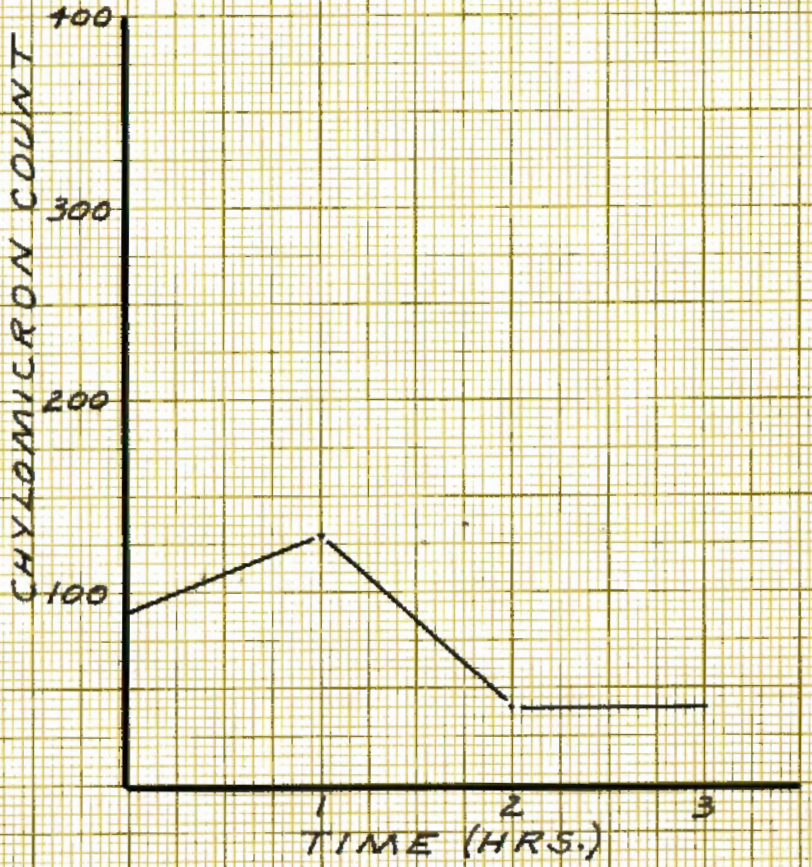


GRAPH NO. 13

TEST NO. 14

AGE 44
WEIGHT 70 KG.
TEST MEAL 70 c.c. 20 % CREAM
SEX MALE
RACE WHITE
DIAGNOSIS PNEUMONIA WITH PLEURAL EFFUSION

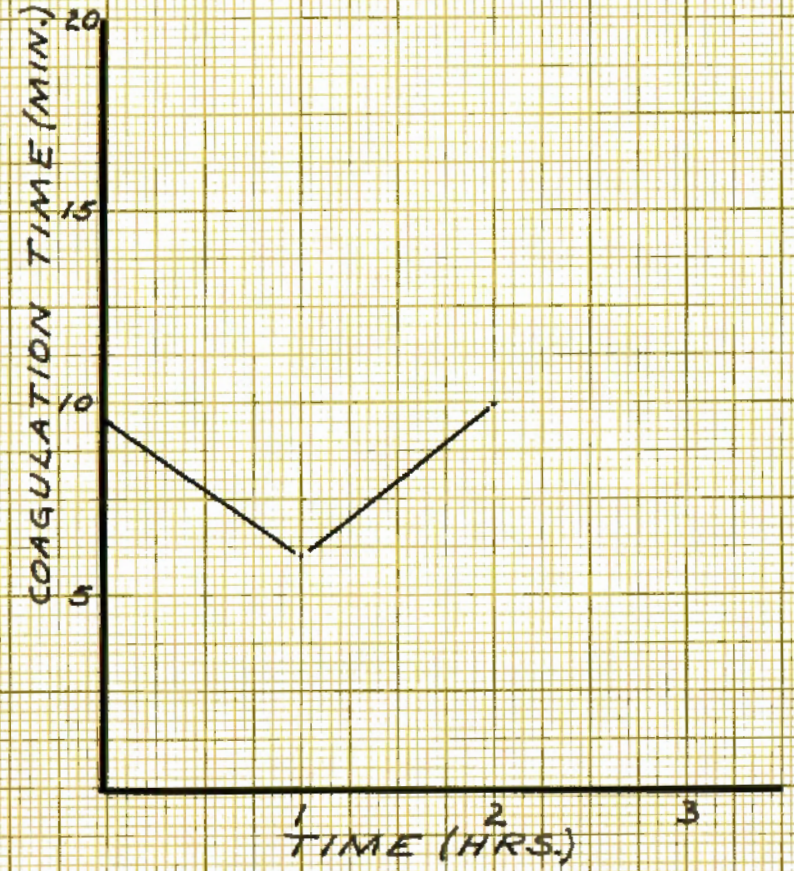


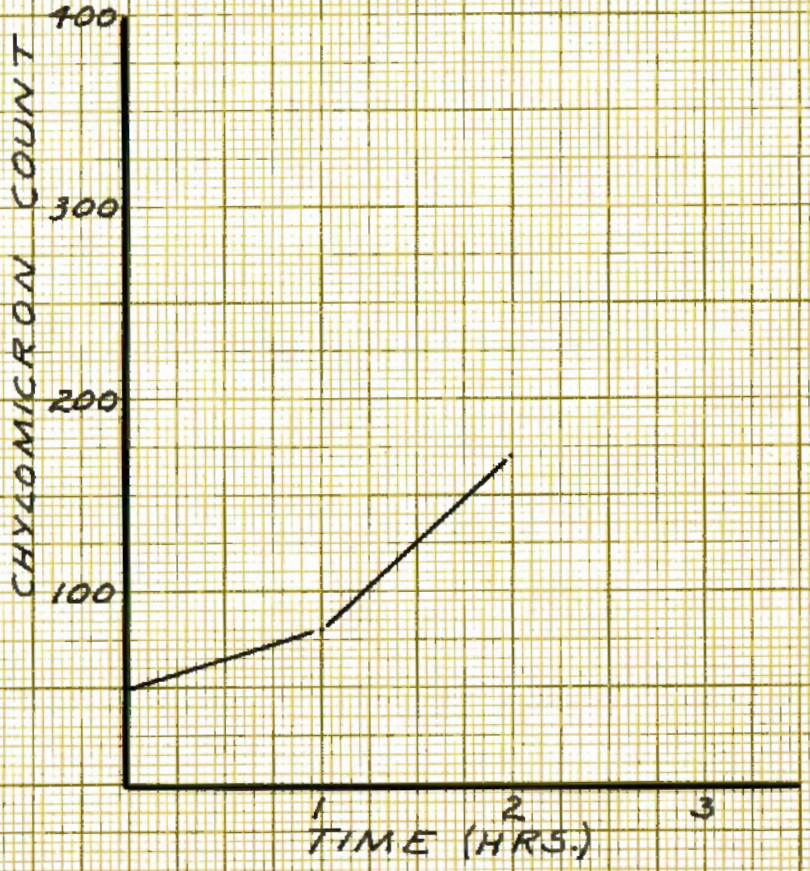


GRAPH NO. 14

TEST NO. 15

DATA:
AGE 33
WEIGHT 100 KG.
TEST MEAL 100 CC. 20 %. CREAM
SEX FEMALE
RACE NEGRO
DIAGNOSIS SALPINGO-OOPHORITIS



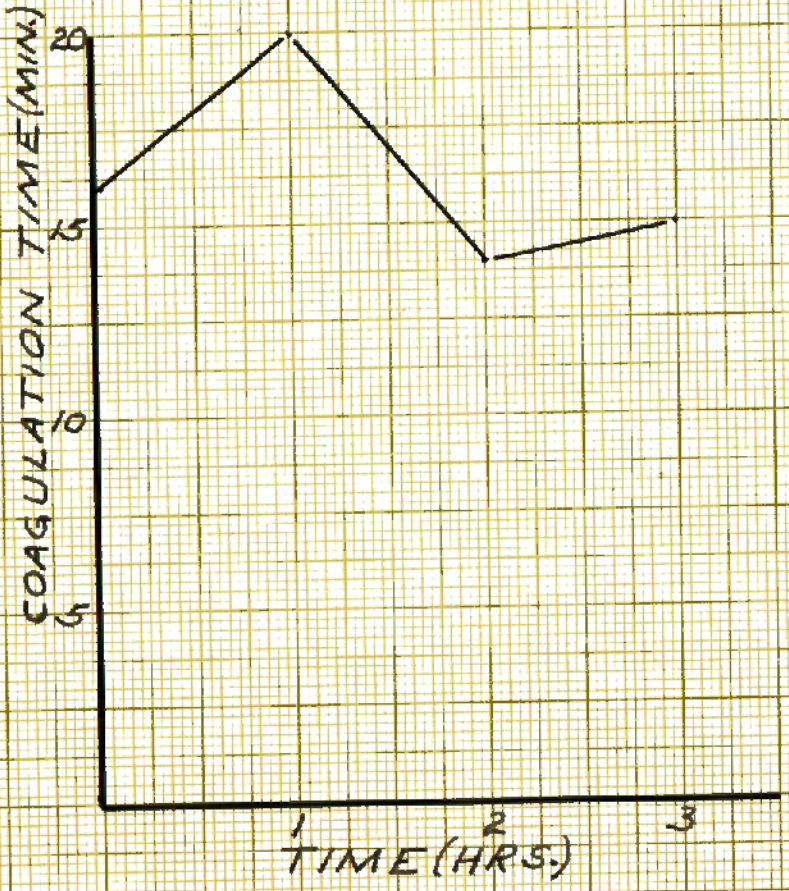


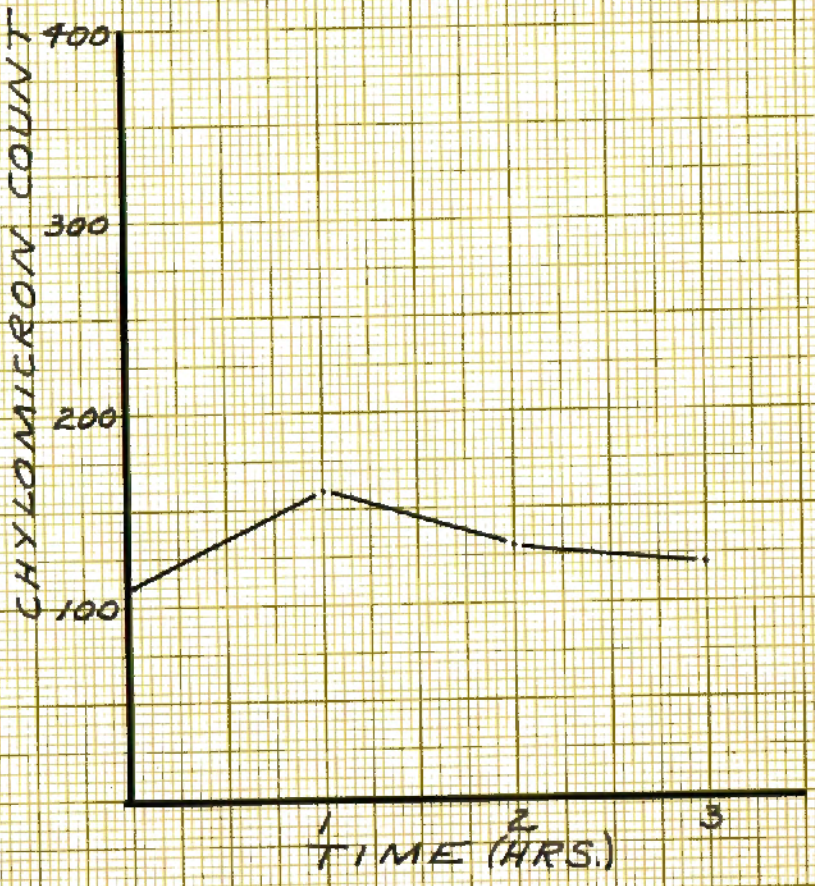
GRAPH NO. 15

TEST NO. 16

NOTE: CONTROL TEST

DATA:
AGE 17
WEIGHT 70 KG.
TEST MEAL 32 GM. GLUCOSE
SEX MALE
RACE WHITE
DIAGNOSIS FRACTURE OF MANDIBLE



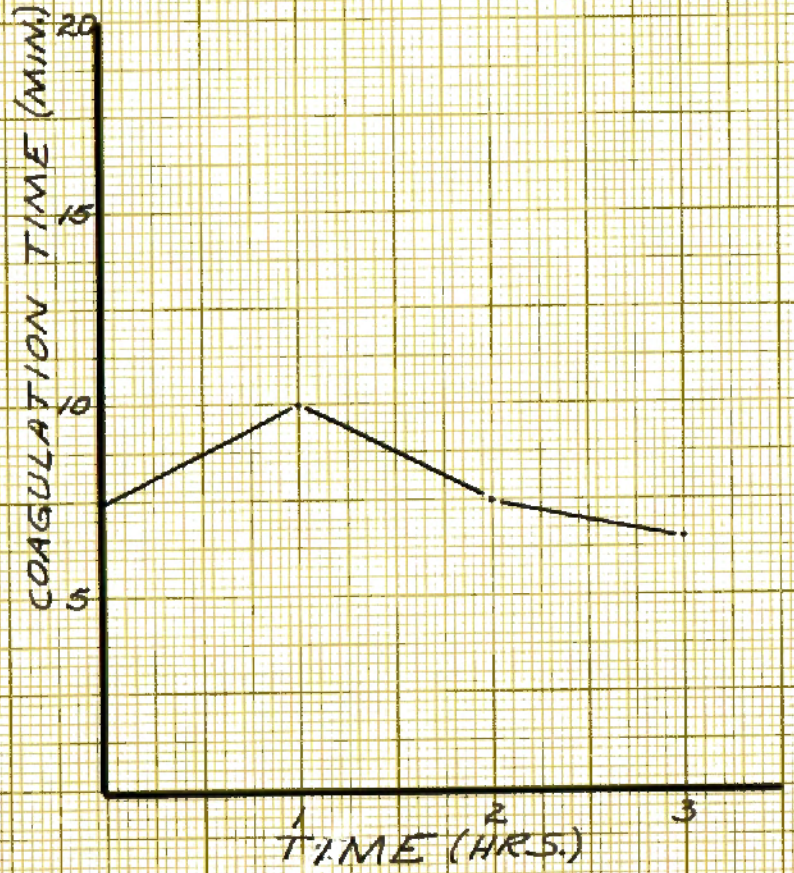


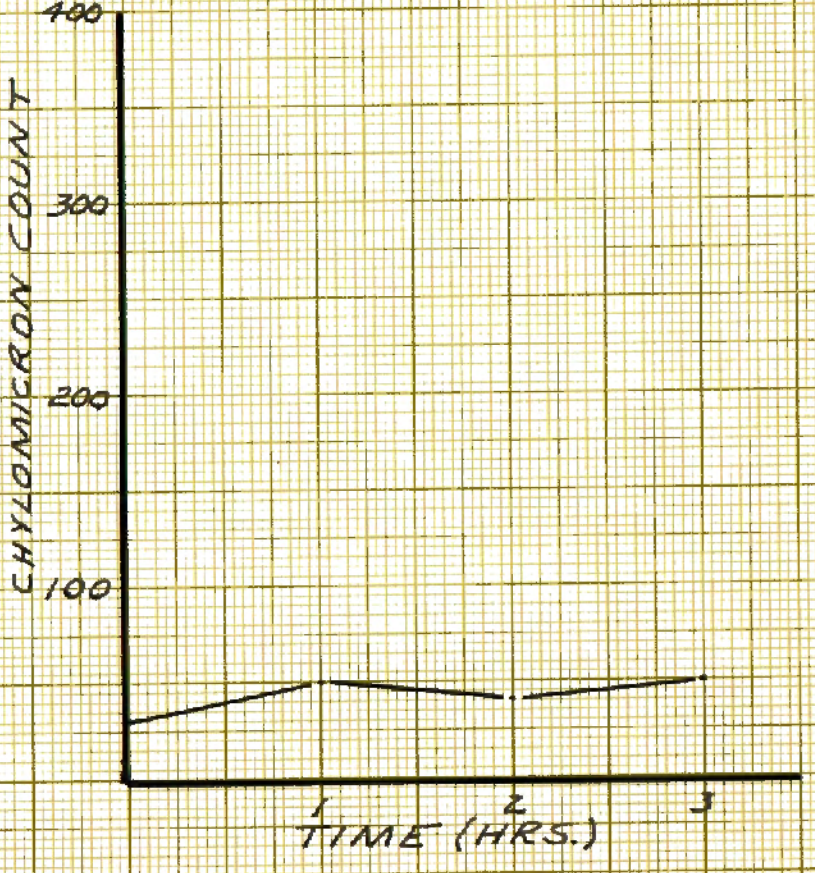
GRAPH NO. 16

TEST NO. 17

NOTE:
CONTROL TEST

DATA:
AGE 69
WEIGHT 66 KG.
TEST MEAL 30 GM. GLUCOSE
SEX MALE
RACE WHITE
DIAGNOSIS INGUINAL HERNIA



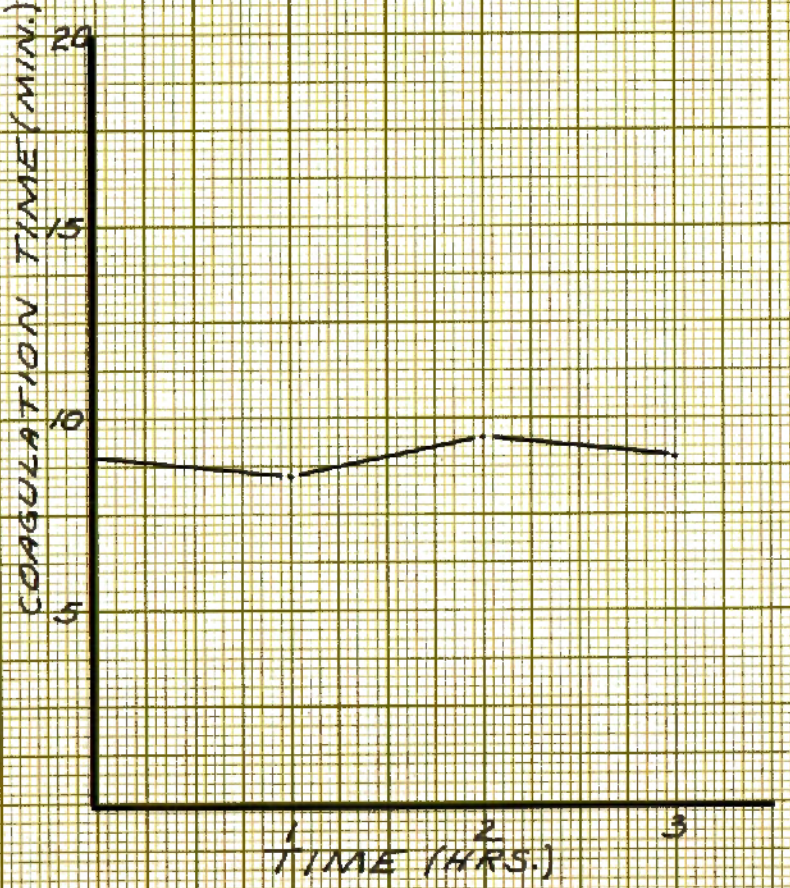


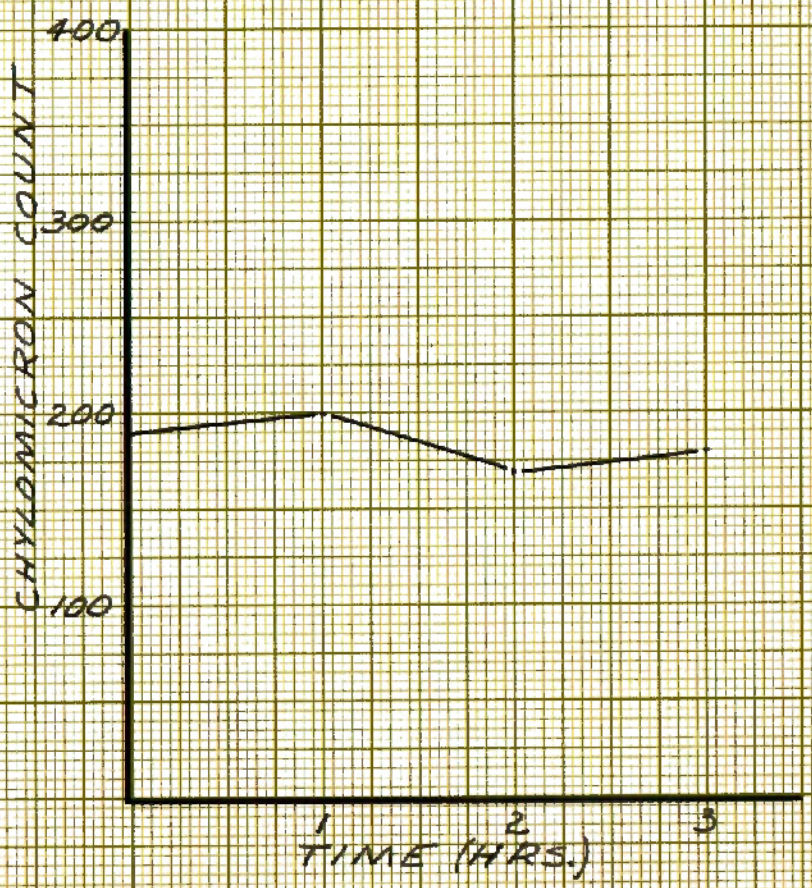
GRAPH NO. 17

TEST NO. 18

NOTE:
CONTROL TEST

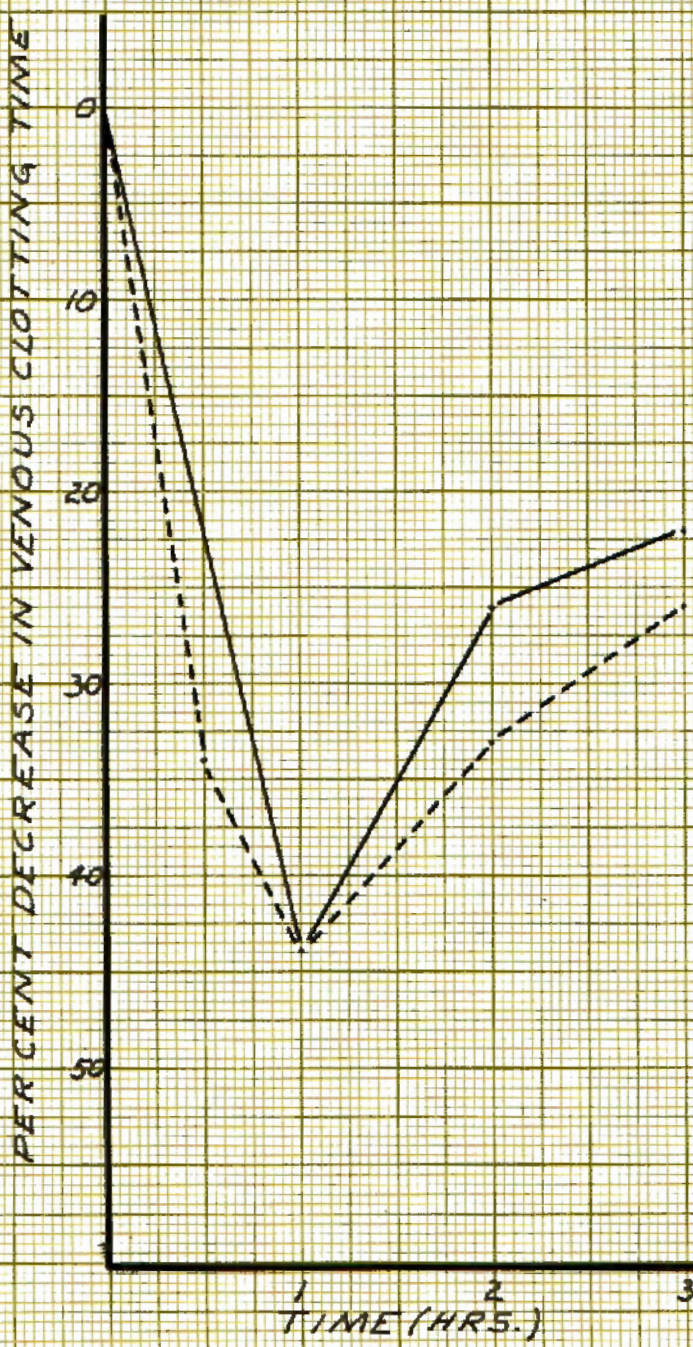
DATA:
AGE 38
WEIGHT 64 KG.
TEST MEAL 29 G M. GLUCOSE
SEX MALE
RACE WHITE
DIAGNOSIS - UNDIAGNOSED



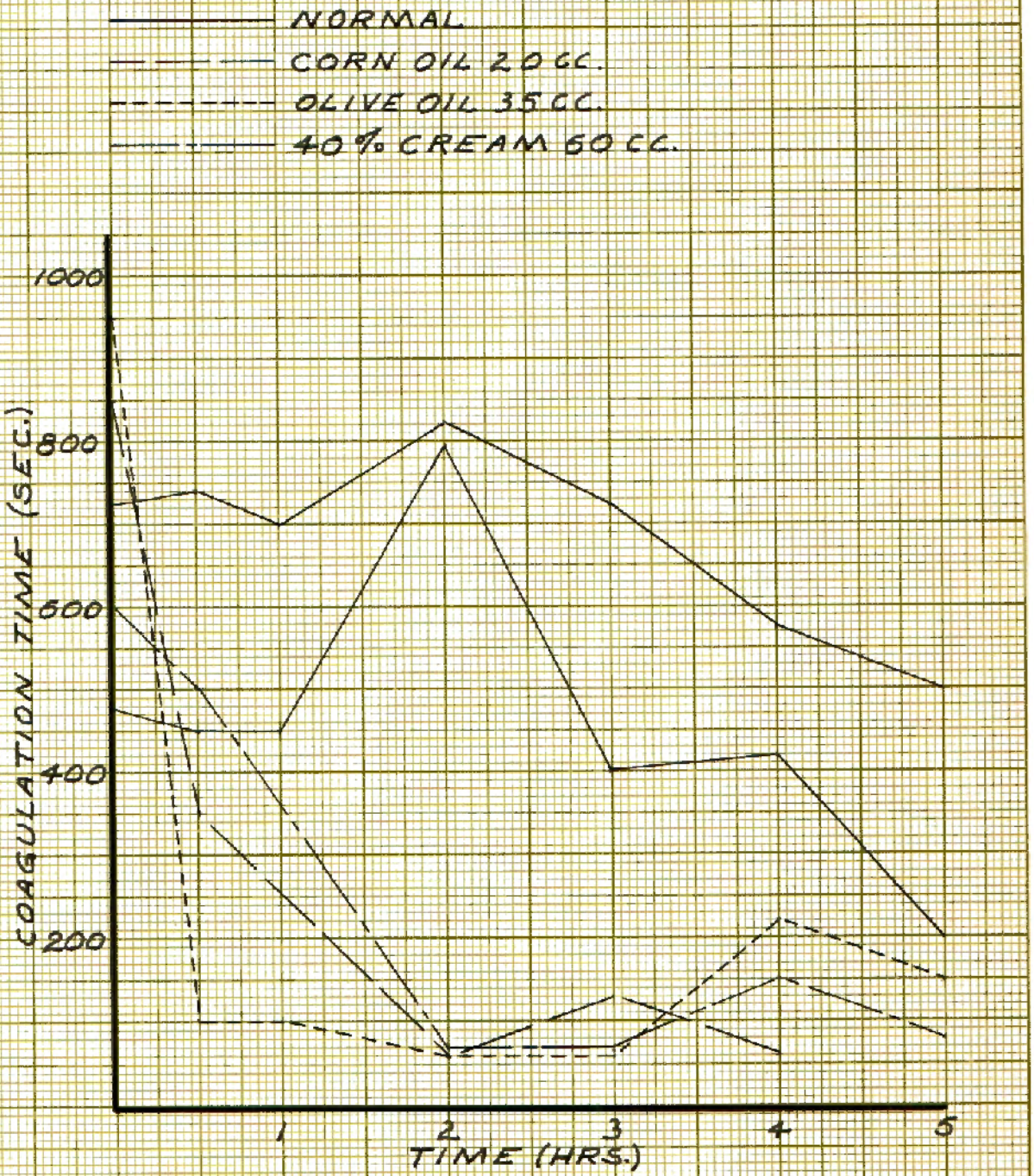


GRAPH NO. 18

AVERAGE PER CENT DECREASE IN VENOUS CLOTTING TIME AFTER INGESTION OF CREAM ~ COMPARATIVE TEST RESULTS WITH THOSE REPORTED BY WALDRON

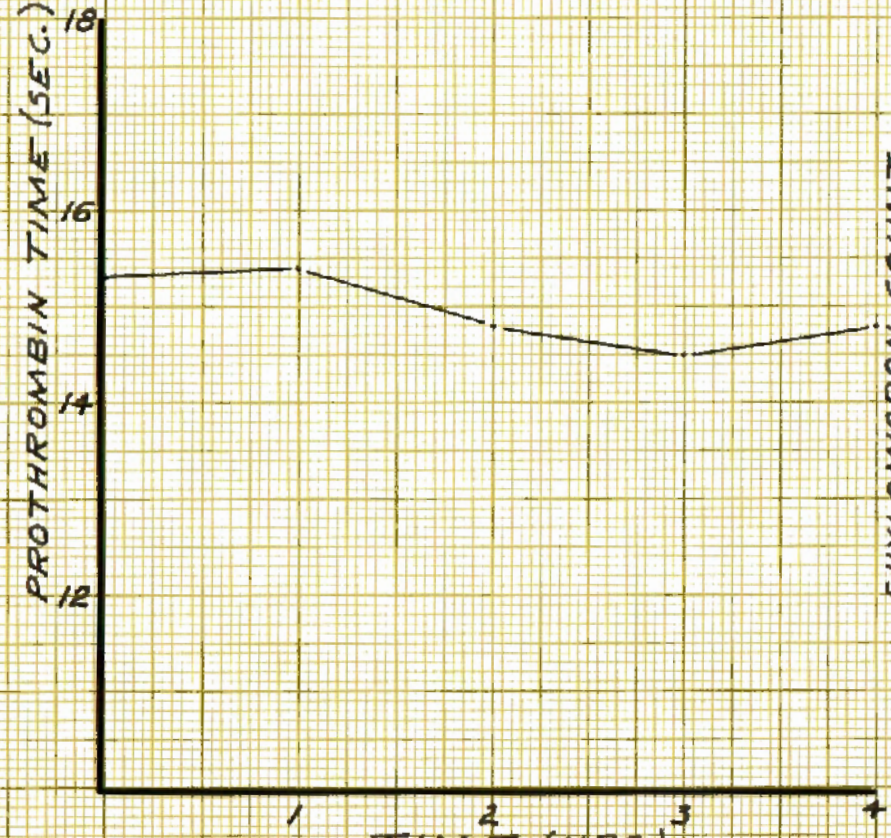


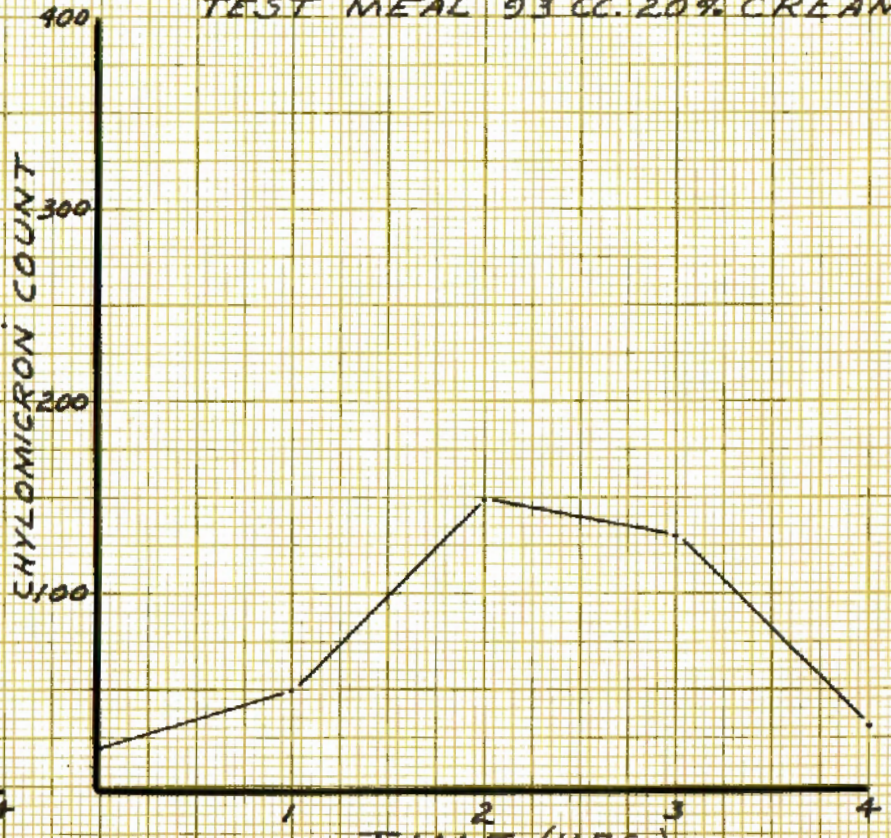
THE EFFECT OF THE INGESTION OF THREE DIFFERENT FATS IN FACILITATING THE COAGULATION OF BLOOD – CONTRASTED WITH CONTROL STUDIES USING THE SAME ANIMAL (REPORTED BY DUNCAN AND WALDRON 5)



CORRELATION OF PROTHROMBIN TIME AND CHYLOMICRONEMIA IN A NORMAL HUMAN

DATA:
WEIGHT 93 KG.
AGE 32
SEX MALE
RACE WHITE
TEST MEAL 93 CC. 20% CREAM

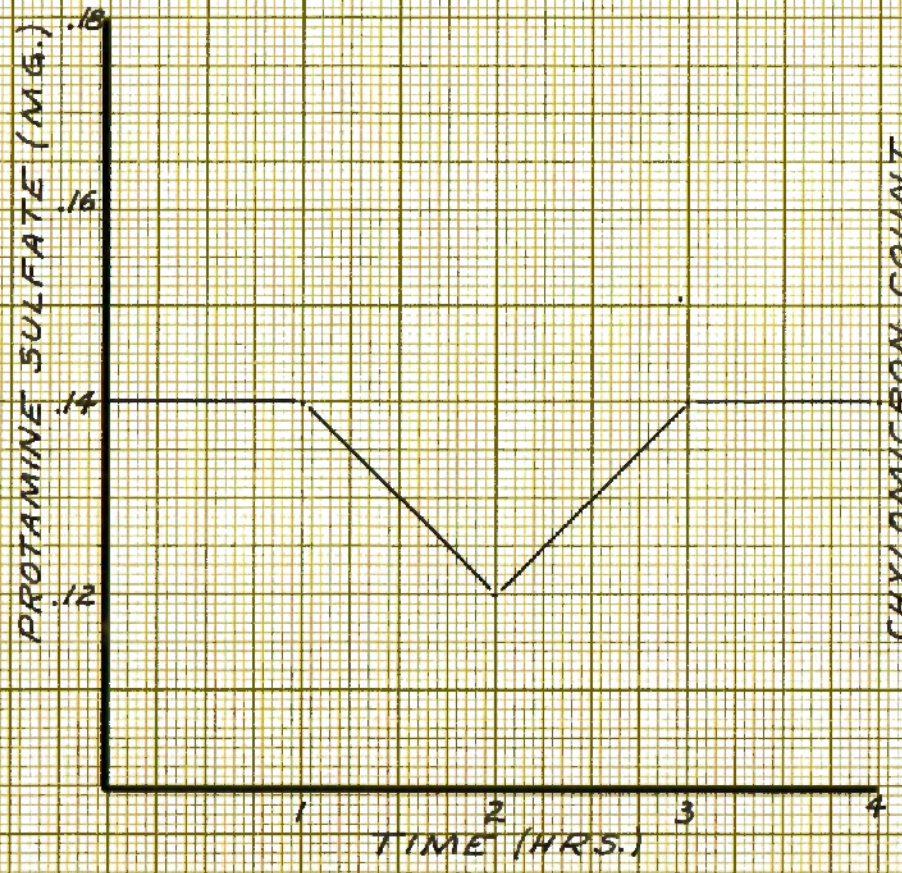


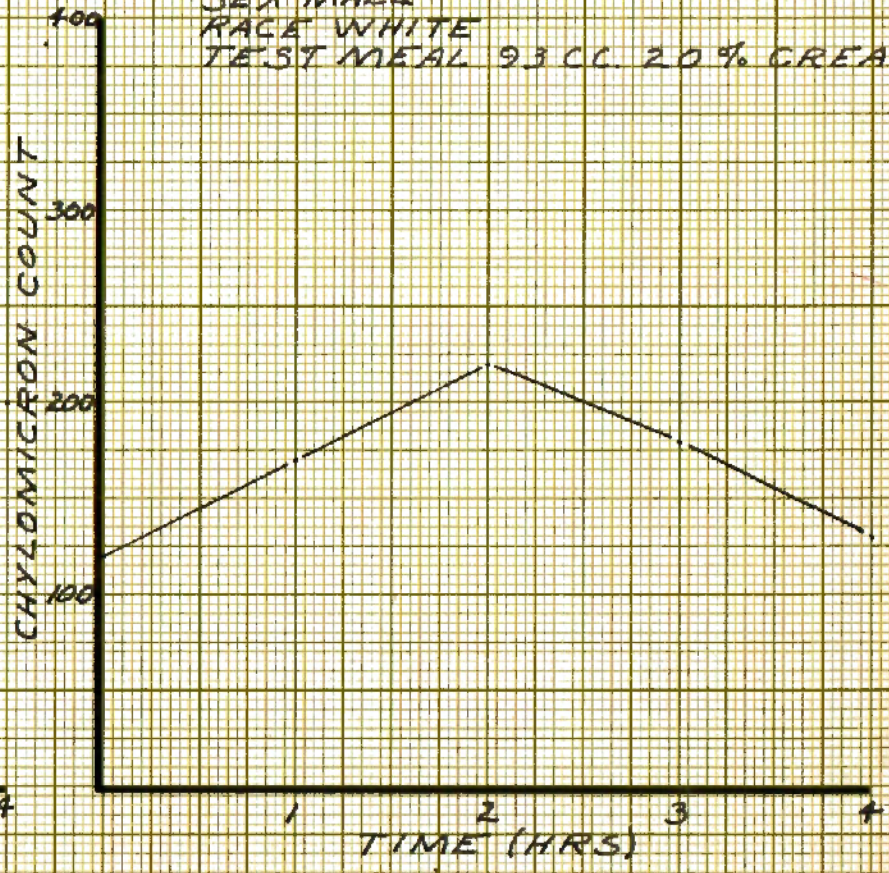


GRAPH NO. 21

CORRELATION OF PROTAMINE TITRATION OF WHOLE VENOUS BLOOD AND CHYLOMICRONEMIA IN A NORMAL HUMAN

DATA:
WEIGHT 93 KG.
AGE 32
SEX MALE
RACE WHITE
TEST MEAL 93 CC. 20% CREAM





GRAPH NO. 22

EXPERIMENTAL ACCURACY OF COAGULATION TIME DETERMINATION USING MODIFIED LEE-WHITE METHOD ON THREE NORMAL HUMAN SUBJECTS

Subject No.	Coagulation Time (min.)			
	Trial No. 1		Trial No. 2	
	First Tube	Second Tube	First Tube	Second Tube
1	5	9	5.5	9
2	6	8.5	6	8
3	6	8.5	6	8

EFFECT OF CREAM INGESTION ON PROTHROMBIN TIME OF NORMAL HUMAN SUBJECT USING QUICK ONE STAGE METHOD OF DETERMINATION

Hours After Test Meal	Prothrombine Time (seconds)					Aver. Value	% Change
	Trial No. 1	Trial No. 2	Trial No. 3	Trial No. 4	Trial No. 5		
0	15.6	15.1	14.9	15.5	15.3	15.3	
1	15.5	15.7	15.1	14.5	15.4	15.4	-0.5
2	15.2	15.5	14.5	14.5	14.5	14.8	3
3	14.3	14.8	14.4	14.6	14.3	14.5	5
4	14.6	15.0	14.5	14.8	15.3	14.8	3

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