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A Study of Cholesterol and Lecithin Blood Levels in Rh Sensitized Women Treated with Rh Hapten

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A STUDY OF CHOLESTEROL AND LECITHIN BLOOD LEVELS IN Rh SENSITIZED WOMEN TREATED WITH Rh HAPTEN

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This Thesis Is Dedicated
To
The Memory Of
Prof. (Dr.) Roy Fraser
To Whose Teaching And
Philosophy The Author
Is Deeply Indebted

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The Problem And Its Background

Introduction

Perhaps one of the most popular subjects, and a subject which has been discussed a great deal by the layman is that of Rh and Rh incompatabilities.

Landsteiner[1] discovered the Rh property of human blood by means of immune sera prepared with the blood of rhesus monkeys. He was assisted in this work by Wiener[2], who states that the Rh property was found to be present in about 85% of white individuals, and independent of the blood groups and M-N type. It is these individuals who are referred to as Rh positive, the remaining 15% being Rh negative.

Wright[3] in his textbook explains very simply what may happen when an Rh negative mother is pregnant with an Rh positive foetus. He says, "Cells containing D- pass across the placenta from the foetus to the mother; the latter responds by forming Anti-D which returns to the foetal circulation and tends

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1. Landsteiner, Karl, The Specificity of Serological Reactions. Cambridge: Harvard University Press, (1946), 88.
2. Wiener, Alexander S., Blood Groups and Transfusions. Springfield, Ill, Charles C. Thomas, (1943), 245.
3. Wright, Samson, Applied Physiology. London: Oxford University Press, 9th ed., (1953), 180.

to destroy the foetal red cells." The amount of damage to the foetus depends on the amount of anti-D formed by the mother and the ability of these agglutinins to cross the placenta. (D being an agglutinogen formed in the blood). Generally little harm, if any, is done during the first pregnancy, but in succeeding pregnancies difficulties may arise. Wright[1] goes on to say that if the woman had been immunized previously by Rh positive blood, a dangerously high response may occur during the first pregnancy. In contrast to so much 'bunk' current on this subject today, he makes the following sound statement, "It should be emphasized that in most cases agglutinins are not formed and the great majority of matings between an Rh negative woman and an Rh positive man result in normal offspring." The agglutinogens of the foetus can be considered as antigens to the mother. An antigen being defined by Potter[2] as "Any substance whose introduction into the tissues of an animal results in the appearance of antibodies in the blood serum after a suitable length of time." Just how the Rh antigen enters the circulation of the mother is not agreed upon by all

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1. Ibid.
2. Potter, Edith L., Rh . . . Its Relation to Congenital Hemolytic Disease and to Intra_rou_ Transfusions. Chicago: The Year Book Publishers, (1947), 22.

investigators in the field, e.g. Dippel[1] and Wiener[2] indicate that there is an actual break in the placental wall, while Kabat[3] says the question of how the antigen passes is unknown. The mother responds to the Rh antigen by forming antibodies which pass back through the placenta and into the blood of the baby, causing destruction of its red blood cells. Potter[4], Pickles[5] and others describe the stages that may be seen in erythroblastosis foetalis which are jaundice, anemia, an increase in the nucleated red cells, and, if severe enough, death.

For some time now, it has been believed that a transfusion is the only treatment for erythroblastosis. Wiener[6], along with others in the field, believed that the child suffering from the disease should be transfused with Rh negative blood of the same blood group as the infant.

Potter[7] makes reference in her book to the attitude, which at one time was current, that marriage of an Rh negative woman

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1. Dippel, A. Louis, "The Prevention of Erythroblastosis Foetalis by the Use of Rh Hapten." Reprint from Southern Medical Journal, XLV, (Oct. 1952).
2. Op. Cit., p. 369.
3. Kabat, Elvin A., Blood Group Substances. New York: Academic Press Inc., (1956), 12.
4. Op. Cit., p. 204
5. Pickles, M. M., Haemolytic Disease of the New Born. Springfield, Ill., Charles C. Thomas; (July 1949), 92-3
6. Op. Cit., p. 88
7. Op. Cit., p. 204

to a Rh positive man should be prohibited by law.

With the discovery of Rh Hapten, erythroblastosis (the disease resulting from Rh incompatabilities) can be done away with as long as treatment is begun early in pregnancy or in some cases even before pregnancy.

Because of the importance of Rh Hapten in the treatment of individuals prone to bearing children with erythroblastosis foetalis, much work of late has been done in this field. This thesis is concerned with Rh Hapten and its constituents.

The Purpose

It is the purpose of this paper to reveal the experimental findings of the research work done in the last year at Western Michigan University on Rh hapten, its constituents and its oral administration.

The Background of the Administration of Rh Hapten

I propose to give a brief synopsis of the reports on the use of Rh hapten as found in the literature.

Carter[1] demonstrated the isolation of a substance which

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1. Carter, Bettina B., "Preliminary Report on a Substance which Inhibits Anti-Rh Serum." Reprint from American Journal of Clinical Pathology, XVII, (August 1947), 646.

completely inhibited high titered human standard anti-Rh serum. At this time group O, Rh-positive red blood cells were used in producing the substance. The pooled, washed and packed cells were shaken at 4°C with an equal volume of distilled water in order to lake the cells. The laked cells were then mixed with five volumes of 95% ethyl alcohol in order to precipitate the protein. This mixture was then shaken for 30 minutes and allowed to stand overnight at 4°C. The red precipitate was separated from the alcohol by filtering and allowed to dry once again at 4°C. To the precipitate was added 5 volumes of anesthesia ether. The flask was rotated for 30 minutes and kept at 4°C. Each day for eight days this mixture was mixed. Then the red powder was separated, by means of filtration, from the ether. The filtrate was evaporated to dryness with an electric fan. The end product of the evaporation was a cream colored lipid material. In a later publication, Carter[1] reports that this crude lipid fraction is dissolved in absolute alcohol and then made ready for intramuscular injections by diluting with saline so that the hapten is present in the concentration of 1:500.

Various medical practitioners have accepted the Rh hapten

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1. Carter, Bettina B., "Rh Hapten: Its Preparation, Assay and Nature." Reprinted from The Journal of Immunology, LXI, (January 1949), 80.

as isolated by Carter.

Dr. Ruth Darrow[1] prepared and has used the hapten according to the methods of Carter with great success. She used Rh hapten in an immunological experiment devised to see whether passive anaphylaxis could be induced in guinea pigs with serum from Rh-negative mothers of children affected with erythroblastosis foetalis. Following erythrocytes of specific Rh subtype, a reversed passive anaphylaxis was demonstrable. Following Rh hapten, antiserum caused no reaction, nor did hapten elicit it following antiserum, even when both were given intravenously. Goldsmith[2], using the method of Carter with very slight modifications, has found that the Rh hapten has proven to be useful in preventing erythroblastosis foetalis in some women sensitized to the Rh factor. Carter, Williamson, Loughrey and Ingram[3] report that of 135 cases of women with a previous history of bearing children with hemolytic disease, 53% had normal infants after treatment with Rh hapten during pregnancy.

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1. Letter from Ruth Darrow, 4575 Oakenwald Avenue, Chicago, dated March 22, 1949.
2. Goldsmith, Joseph W., "Experiences with Rh Hapten." Reprinted from Wisconsin Medical Journal, (May 1950), 1.
3. Carter, Bettina B., Williamson, A. C., Loughrey, Joseph, and Ingram, C. H., "Evaluation of Rh Hapten." Reprint from American Journal of Obstetrics and Gynecology, LXXII, (Sept. 1956), 658.

Current Research

All the work to date on Rh hapten has involved its intramuscular administration. The study of the oral administration was undertaken with the attitude that if hapten could be administered with satisfactory results in this manner, it would alleviate much of the pain which accompanies intramuscular injections. Also there is the possibility that the oral administration would give more positive results than the intramuscular injections.

Because of the fairly well accepted theory that arteriosclerosis may be caused by faulty lipid metabolism, it seems necessary to study the cholesterol metabolism in the women under treatment since one of the constituents of the crude hapten is cholesterol or a similar steroid. The Merck Manual[1], designed especially for Doctors of Medicine, has the following to say concerning the etiology and incidence of arteriosclerosis, "The cause of arteriosclerosis is unknown. It has been variously attributed to abnormal metabolism of fats; exogenous toxins, such as lead; and endogenous toxins and infection. There is clear cut evidence that heredity plays a large part in its development. Long established hypertension appears to be a predisposing

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1. ———— "Arteriosclerosis." The Merck Manual, Rahway, N. J., Merck & Co., (1950), 183.

factor. The disease is most common in obese individuals and in males. The onset usually occurs during the 4th or 5th decade of life." The Rh hapten is a crude lipid fraction containing according to Carter[1] cholesterol and 2% nitrogen which is phospholipid in nature. This has necessitated the testing of the blood of the treated women to see what effect the oral administration of hapten has on the phosphorus levels in the blood.

Summary

It has been the author's intention in this chapter to give the reader an introduction into the history of the Rh factor, the effects of Rh incompatability and the use of Rh hapten. After giving the purpose of the thesis to be one of revealing the experimental findings with relation to the constituents of Rh hapten and its oral administration, the background of the Rh hapten administration was discussed and finally an introduction into the current research of the last year at Western Michigan University.

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1. Carter, Bettina B., "Rh Hapten: Its Preparation, Assay and Nature." Reprinted from The Journal of Immunology, XLI, (Jan. 1949), 83.

Chapter II

Current Method of Preparation of Rh Hapten in Comparison with Previous Preparations

It is the author's wish to show, in this chapter, that the method of preparation of Rh Hapten has been modified considerably from the time of its first isolation to the present.

The current procedure is as follows: The cells of Rh positive blood type, any group, are pooled and to these cells is added two volumes of 95% ethyl alcohol. The mixture is stirred by means of an electric stirrer and is allowed to stand overnight at room temperature. The alcohol causes a precipitation of the protein and also removes carbohydrate. The mixture is filtered by means of a Buchner funnel under suction. The filtrate is discarded and the precipitate is treated with two volumes of methylene chloride and mixed once again with the electric stirrer. The methylene chloride being a fat solvent removes the crude lipid fraction, the Rh hapten, from the precipitated blood. This mixture is filtered by means of a Buchner funnel. The filtrate containing methylene chloride and lipid is evaporated by means of an electric fan to dryness. The residue represents the crude lipid fraction. The residue is scraped from the evaporating dish and weighed. It is then made into pills of 100 mg. consistency very simply as will be described. The waxy like lipid of determined weight is cut into 100 mg. portions on a pill board and coated with

milk sugar. These portions are then picked up into a capsule and stored in the refrigerator until used.

Prior to this rather simple method of preparation, longer procedures were employed. Carter[1] first prepared the material shaking the pooled, packed, washed, group O, Rh + cells with an equal volume of distilled water in order to lake the cells. Then five volumes of 95% alcohol was added to the laked cells in order to precipitate the protein. The flask was rotated for 30 minutes and allowed to stand overnight at 4°C. The red precipitate was separated from the alcohol by filtration and allowed to dry at 4°C. Five volumes of anesthesia ether was added to this powder. The flask was rotated for 30 minutes and kept at 4°C. Extraction continued for eight days. At the end of this period, the ether was separated from the red powder by filtration. The ether filtrate was evaporated in an evaporating dish with the aid of an electric fan.

In a later publication, Carter[2] varied her procedure of Rh Hapten preparation. The cells were not washed because comparison of results with washed and unwashed cells revealed no detectable difference. This later publication also was different from the

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1. Carter, Bettina B., "Preliminary Report on a Substance Which Inhibits Anti-Rh Serum." Reprinted from American Journal of Clinical Pathology. XVII, (August 1947), p. 646
2. Carter, Bettina B., "Rh Hapten: Its Preparation, Assay and Nature." Reprinted from The Journal of Immunology. LXI, (January 1949), p. 79-80

preliminary report in that as well as treating the cells with 95% alcohol and allowing to stand overnight, the cells were treated with 50% and 25% alcohol, mixed for 10 minutes and filtered. The procedure following in this order: 95% - 50% - 25%. The value of the successive washings of the pooled cells with descending concentrations of alcohol was believed to be in the removal of extraneous materials, notably the group specific substances.

In an attempt to try to find the best procedure for the preparation of Rh hapten, other investigators began working. Very slight changes were made by Goldsmith[1]. He found that the rapid treatment of the protein precipitate with the dilute alcohols referred to previously did not completely remove substances which interfere with the activity of the final product and found that by keeping the precipitate in contact with each of these alcohols for at least twelve hours, a more active hapten was obtained. He also found that more active agitation of the precipitate in the ether gave increased yields and hence used a high speed electric stirrer. Carter, Williamson, Loughrey and Ingram[2] in 1956 report a preparation of Rh hapten

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1. Op. Cit., p. 1
2. Carter, Bettina B., Williamson, A. C., Loughrey, Joseph, and Ingram, C. H., "Evaluation of Rh Hapten." Reprinted from American Journal of Obstetrics and Gynecology, LXXII, (Sept. 1956), p. 655

which is exactly the same as the one used currently and described at the beginning of this chapter.

I have in this chapter shown the change over from the more complicated and time consuming method of preparation of Rh hapten to the more simplified method. I want to stress that these changes have been made with the realization that certain of the steps in the earlier procedures were nonessential and that these changes have not been done just for the mere sake of simplicity although simplicity in any scientific work is one of the Principles of the Scientist.

Chapter III

The First Approach to the Study of Cholesterol and Phospholipid

Knowing that the constituents of Rh hapten were cholesterol, or similar steroid, and phospholipid, and because of the belief by some medical authorities that these constituents, especially cholesterol, are the cause of arteriosclerosis, it seemed essential to examine the blood of the patients periodically to note any change in cholesterol and phospholipid levels from the beginning of treatment onward. With this in mind and because of the desire to administer Rh hapten orally, the first type of research to carry out was of a library nature where the digestion and absorption of cholesterol and phospholipid was studied by reviewing the literature. This provided a theoretical answer as to whether the oral administration of Rh hapten would be satisfactory and whether its constituents would reach the blood stream and hence could be experimentally studied.

It is the purpose of this chapter to express the findings obtained in the review of the literature.

The Theoretical Answer to the Digestion and Absorption of Cholesterol

The question of just what happens to cholesterol when ingested has long been a matter of controversy. Throughout the discussion

of the digestion and absorption of cholesterol and phospholipid, "digestion" will be considered to be the overall utilization of a foodstuff in the gastro-intestinal tract while "absorption" will be referred to as the process by which foodstuffs are removed from the small intestine. This breakdown of these two terms is in accord with Deuel[1], one of the authorities on the biochemistry of lipids.

Cholesterol is digested through the process of hydrolysis, the process believed to be one of esterification. All chemists are in general agreement as to how the esterification reaction works. Deuel[2] and White, Handler, Smith and Stetten[3] have very clearly discussed this reaction. The reaction is one affecting the carboxyl group wherein whether one starts with a mole of acid and alcohol on the one hand or ester and water on the other, one finds the four reagents present in the same relative amounts if one waits a sufficient length of time.

The esterification reaction is altered by a number of considerations. Deuel[4] mentions (1) the proportion of reactants as altering the equilibrium of the esterification reaction; (2) Prevention

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1. Deuel, Harry J., The Lipids. New York: Interscience Publishers, Inc., II, (1955), 195.
2. Deuel, Harry J., The Lipids. New York: Interscience Publishers, Inc., I, (1951), 113.
3. White, Abraham, Handler, Philip, Smith, Emil L., and Stetten, Dewitt, Principles of Biochemistry. New York: McGraw-Hill Book Company, Inc., (1954), 80.
4. Op. Cit., Vol., I, p. 115.

of accumulation of the reaction products, (3) temperature (4) the nature of the reacting components (5) the nature of the acid component which has an effect on the esterification reaction. All of these factors are important in the in vitro esterification. In terms of esterification in vivo, White, Handler, Smith and Stetten[1] cite the two most essential chemicals to be the H+ ion and specialized enzymes.

The esterases are enzymes which hydrolyze ester linkages of acids and alcohols. Some of these enzymes are broad in their action, while others are highly specific. The lipases are the most important esterases necessary for the digestion of cholesterol.

White, Handler, Smith and Stetten[2] state that some gastric digestion of cholesterol may take place in the stomach because of the presence of the H+ ion. These individuals do not mention that the gastric lipase may aid in the digestion. It is a question whether the hydrolysis of fat in the stomach is due to gastric lipase secreted by the mucosa or whether it is due to regurgitation of pancreatic lipase from the intestine. Deuel[3], citing Wilstatter and Memmen, has offered considerable evidence that gastric lipase exists in active form and occurs in greater concentration in the cardiac than in the fundic end of the stomach. The optimum pH is on the alkaline side of neutrality, as in the case of pancreatic lipase. The pH

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1. Op. Cit., p. 454.
2. Ibid.
3. Op. Cit., Vol., II, p. 7.

usually existing in the stomach is unfavorable not only for the emulsification of fat but also for the action of gastric lipase.

While the gastric digestion of fats is of only minor importance in itself, it does provide some free fatty acids which accelerate the rate of emulsification of the lipids, once the chyme is passed into the small intestine.

The major site of digestion is in the small intestine. In the duodenum the cholesterol encounters two fluids of importance in its digestion, the bile and pancreatic juice, and in the lower small intestine the secretion of the intestinal mucosa also participates.

White, Handler, Smith and Stetten[1] and Cantarow and Trumper[2] both give the function of the bile in cholesterol digestion and absorption as being an emulsifier. In the presence of bile salts, fat globules are reduced in size, and the total surface area exposed to the lipolytic enzyme is thereby increased, digestion correspondingly facilitated.

Any textbook on physiology describes how the bile reaches the intestine. It is intermittently discharged from the gall bladder to the duodenum. The contraction of the gall bladder being under hormonal regulation, the hormone cholecystokinin arising in the

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1. Op. Cit., p. 454.
2. Cantarow, Abraham, and Trumper, Max, Clinical Biochemistry. Philadelphia: W. B. Saunders Company, (1945), 132.

upper segment of the small intestine and entering the circulation when cholesterol or fatty foods are introduced into the small intestine.

The churning effect of peristalsis, with the presence of the bile salts, results in a progressively finer and finer state of distribution of the cholesterol in the continuous aqueous phase.

Lipolysis has been wonderfully described by White, Handler, Smith and Stetten[1] as involving the participation of water and water soluble lipases. Since cholesterol is insoluble in water, the reaction of hydrolysis is biphasic and occurs only at the interface between the cholesterol droplet and the aqueous phase. The rate of reaction is determined by the area of this interface, and it is evident that the higher the degree of emulsification, the smaller the individual cholesterol droplet and the larger the area will be. The bile promotes contact between the water-soluble and water insoluble components of the reaction. Cantarow and Trumper[2] maintain that the emulsification process is aided by the fact that the bile salts dissolve fatty acid with a lowering of surface tension.

The flow of pancreatic lipase is under hormonal regulation. Prosecretin granules in the mucosa of the small intestine are activated by the acid of the chyme to yield secretin, which enters the

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1. Op. Cit., p. 450.
2. Op. Cit., p. 132.

circulation to stimulate the acinous tissues of the pancreas and promote secretion of pancreatic juice. Deuel[1] claims it was Claude Bernard in 1856 who first recognized that the function of the pancreatic juice was to cause emulsification and hydrolysis of fats.

Steapsin is the name given to the enzyme which is most active in the digestion of fat. When secreted from the pancreas it is partially inactive. It was believed at one time that this lipase was produced as an inert zymogen called "steapsinogen", which was converted to the active form in the small intestine by contact with bile salts. However, Willstatter and Bamann, according to Deuel[2], have shown that bile salts activate both the gastric and pancreatic lipase only after the enzyme is purified.

As has been said earlier in the chapter, just what happens to cholesterol when once ingested is a matter of controversy.

There are two probable processes to be considered in cholesterol absorption.

McDowall[3] states that Munk, and later Moore and Rockwood, showed that in the intestine fat may be broken down into glycerol and fatty acids. This is felt of McDowall[4] to be true of cholesterol also.

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1. Op. Cit., Vol., II, p. 6.
2. Ibid.
3. McDowall, R. J. S., Handbook of Physiology and Biochemistry. London: John Murray, 42nd ed., (1955), 333.
4. Loc. Cit., p. 335.

The fatty acid which is now in the cell combines with the glycerol which is absorbed at the same time, to resynthesize neutral fat. This synthesis passes through an intermediate stage of phosphatide formation. The glycerol is phosphorylated and combines with the fatty acids.

At this point, the importance of lecithin in cholesterol absorption will be discussed. In the mucosal epithelium the phosphatide is transformed again into neutral fat and appears in the lymph. Significant amounts, according to McDowall[1], escape this transformation and appear in the lymph as lecithin and so increase the lecithin content of the blood. This lecithin is found in combined form with cholesterol in the blood. McDowall[2], citing Verzár, says that there is no difficulty in explaining how the fat leaves the blood stream again, since the capillary walls have a permeability comparable with artificial membranes through which a great part of the serum fat can diffuse out.

All the cholesterol may not necessarily be broken down and absorbed as above, but may be absorbed in an unchanged form. According to McDowall[3], citing Mellanby, the emulsified cholesterol can pass freely into the columnar cells of the villi without previous

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1. Loc. Cit., p. 334.
2. Ibid.
3. Ibid.

hydrolysis. If adequate emulsification takes place, the direct, perhaps selective, absorption of cholesterol will occur.

Deuel[1] in his textbook believes that fat ingested at the same time as cholesterol has an accelerating effect on the digestion and absorption of cholesterol. He is the first individual who has considered the importance of fat in the absorption of cholesterol. It is he who refers to the demonstration of Dubach and Hill that cholesterol can be absorbed without an oil carrier. Apparently the requirement for fat to insure cholesterol absorption varies with the species. Deuel[2], citing Popják, noted that rabbits and guinea pigs were able to absorb small quantities of cholesterol on a low fat diet. On the other hand, he goes on to say that Cook found that if rats were fed on a low fat diet but containing cholesterol, the sterol can be recovered quantitatively from the feces.

Even if cholesterol is absorbed to a degree without fat, Deuel[3] says that it has been shown by Stamler and Katz as well as Peterson that the absorption is facilitated by the extra fat in the diet.

The role of the bile in cholesterol digestion has been discussed but little has been said about bile in relation to absorption. Bile salts, according to Deuel[4], are a more important factor in stimulating

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1. Op. Cit., Vol., II, p. 260.
2. Ibid.
3. Ibid.
4. Loc. Cit., p. 261.

the absorption of cholesterol than is the solvent action of fat. He goes on to say that although the normal bile secretion may be sufficient to result in a minimum absorption of cholesterol from the gastro-intestinal tract on a cholesterol-rich diet, a large excess of bile salts must be furnished if appreciable amounts of cholesterol are to be deposited in the liver.

According to Deuel[1], Siperstein, Chaikoff and Reinhart, employing C^{14} labeled cholesterol, conclude that bile plays an obligatory part in the passage of cholesterol from the intestinal tract to the lymph.

The mechanism by which bile salts facilitate the absorption of cholesterol is uncertain. Deuel[2] claims it was Wieland and Sarge who found that cholesterol is a substance which can be dissolved by the hydrotropic action of bile acids. The best explanation for the absorption of cholesterol is that it is rendered possible by the hydrotropic action of bile salts, and that it is facilitated when cholesterol is dissolved in fat.

Whether esterification in the intestinal mucosa is a prerequisite for the absorption of cholesterol is not known. It has been generally accepted that a cholesterol-esterase is present in the intestinal mucosa. Although it was once believed that this enzyme originated in the

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1. Ibid.
2. Ibid.

pancreas, according to Deuel[1], work by Stamler and Katz has shown the pancreas is not the origin of the enzyme. To date, its origin is unknown. Whereas no final picture can be given for the route of absorption of cholesterol, White, Handler, Smith and Stetten[2] report that the evidence suggests that after an as yet undetermined amount of hydrolysis in the lumen of the small intestine, the products of hydrolysis enter the lacteals almost quantitatively.

Of the dietary sterols only cholesterol seems to be absorbed to any appreciable extent. Neither the products of its reduction, cholestanol or coprosterol, nor its homologue in the vegetable world, the phytosterols, appear to be absorbed to any appreciable extent.

Although the evidence is not complete enough to give a clear cut picture of the absorption of cholesterol, we nevertheless have enough facts to know that cholesterol is absorbed and so from this first (theoretical) approach it can be said that cholesterol is digested and absorbed.

The Digestion and Absorption of Phospholipid

The same approach as was undertaken for the study of cholesterol digestion and absorption was followed for phospholipid.

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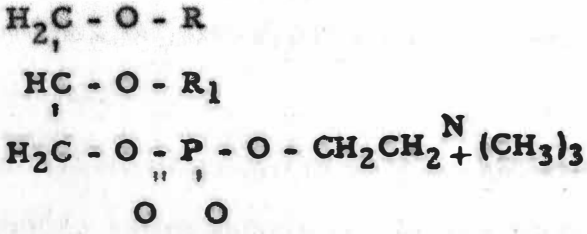
1. Loc. Cit., p. 264.
2. Op. Cit., p. 457.

Lecithin is the phospholipid whose digestion and absorption is described but there is no conclusive evidence that the phospholipid is a lecithin. Because the digestion and absorption of all the phospholipids is very much alike, it is justifiable to speak in terms of the particular one lecithin. Apart from this there is more evidence that lecithin is the phospholipid than any other of the phospholipids. Osborn[1] gives some indication that the phospholipid of Rh hapten is lecithin. Much more work has to be done in this field before this will be conclusive.

Lecithin is a phospholipid or phosphatide. Its structural formula is as follows:



From this formula, it can be seen that lecithin is made up of phosphoric acid, glycerol, choline and long chain fatty acids. The fatty acids may be either saturated or non-saturated.

Just what happens in the digestion and absorption of lecithin is not agreed upon by all authorities. The majority of the evidence suggests in the course of digestion of lecithin in the gastro-intestinal tract the two fatty acid residues are removed through the enzymatic reaction of

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1. Osborn, D. A., "The Question of the Rh Hapten." Journal of Clinical Pathology, IV, (1951), 470.

pancreatic juice. There is likely a step wise removal of the two fatty acid residues. The removal of only one fatty acid, probably an unsaturated acid such as oleic acid, results in the formation of a product known as lysolecithin which is strongly hemolytic; this hydrolytic reaction is attributed to the enzyme lecithinase A, which is found not only in animal tissues but also in snake venom, bee sting and scorpion venom. The hydrolytic removal of the fatty acid residue of lysolecithin destroys its hemolytic power; this hydrolysis is attributed to the enzyme lecithinase B, found in many animal tissues. Various opinions exist as to how the other parts of the lecithin molecule are hydrolyzed. Cantarow and Trumper[1] credit lipase for the hydrolysis of the whole molecule. These individuals seem to stand alone on this matter. Other chemists, namely West and Todd[2], say that Lecithinase C removes the choline from the lecithin, while Lecithinase D hydrolyzes the lecithin molecule at the glycerol-phosphoric acid linkage. Najjar[3] claims that the splitting of lecithin to yield choline and phosphatidic acid has been observed only in plants and that splitting to yield phosphorylcholine and a diglyceride has not been described in higher animals but has been

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1. Op. Cit., p. 139
2. West, Edward Staunton, and Todd, Wilbert R., Textbook of Biochemistry. New York: The MacMillan Company, 2nd ed., (1955), 171.
3. Najjar, Victor A., Fat Metabolism. Baltimore: The Johns Hopkins Press, (1954), 154.

described in snake venom.

This hydrolysis of lecithin was considered to be the complete picture for a long time. It was assumed that the lecithin was absorbed in the form of its hydrolytic products. Deuel[1] gives a somewhat different picture of the hydrolysis of lecithin. He says that Bokay in 1877 proposed that lecithin was broken down into fatty acids, choline and glycerophosphate; the latter ester was believed to be split into glycerol and phosphoric acid in the intestine by phosphatase. Deuel[2], citing Abderhalden and Paffrath, demonstrated that choline originated when lecithin was allowed to remain in intestinal segments of the rabbit in vitro, as well as when lecithin solutions were incubated with intestinal juice. He also says that Kahane and Levy proved that Lecithinase B is present in rat intestine. This enzyme splits the two fatty acid residues from the lecithin molecule, leaving glycerylphosphoryl-choline. In earlier work, Deuel[3] says, Schmidt, Hershman and Thannhauser reported that duodenal juice was without effect on lecithin but that the intestinal mucosa was able to hydrolyze lecithin.

These same individuals have reported that alkaline phosphatase has the ability to convert glyceryl phosphoryl choline into

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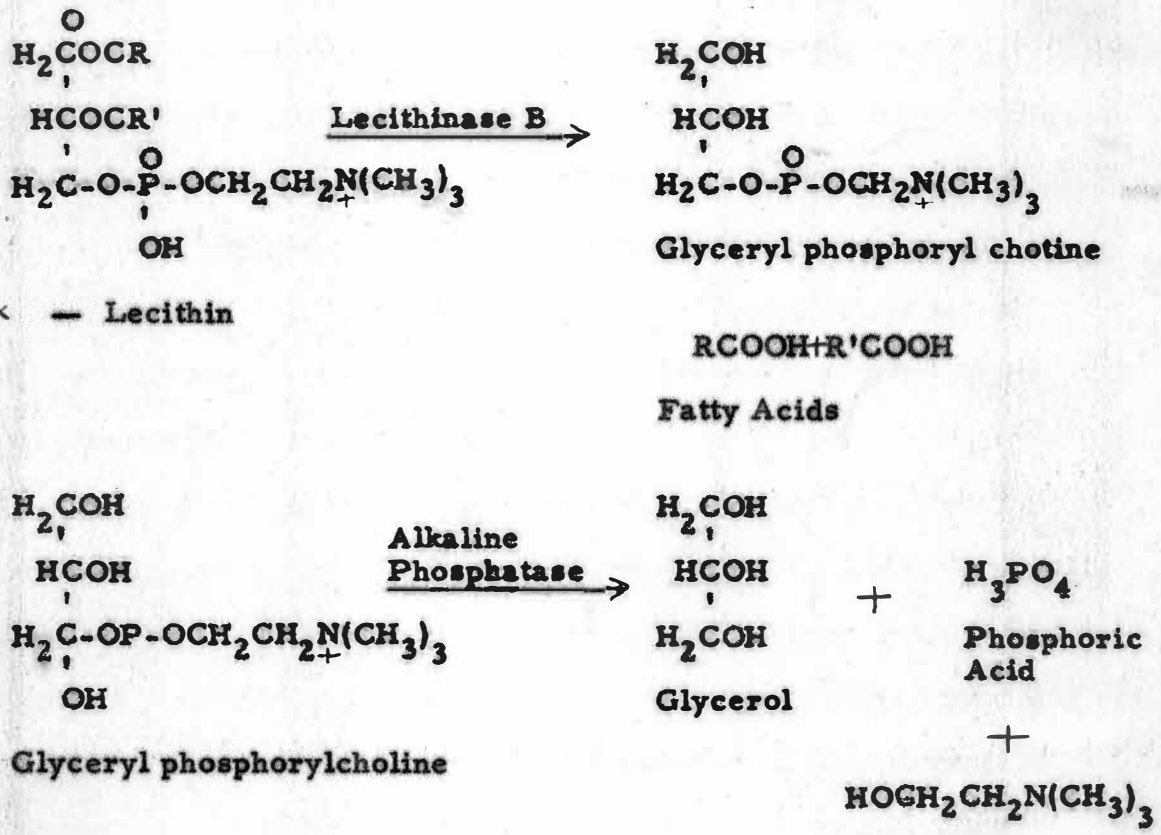
1. Op. Cit., Vol., II, p. 248.
2. Ibid.
3. Ibid.

glycerol, phosphoric acid and choline. Deuel[1] gives the mechanism for the hydrolysis of the lecithin molecule as follows:



∝ — Lecithin

1. Loc. Cit., p. 249.

Deuel[1] claims significant increases of lecithin in the lymph following administration of this phospholipid have been reported by Slowtzoff and Eckstein, while Eichholtz has demonstrated this pholipid in the blood following its administration. The tissue phospholipid content was shown to be increased after continual feeding of the phospholipid. (Deuel[2], citing Rewald, Serejski and Heinlein.) Heinlein further demonstrated that this tissue increase did not result when bile is excluded from the intestine, a condition known to result in the impairment of phospholipid absorption. Whether lecithin can be absorbed as such is an open question.

Artom and Swanson, says Deuel[3], demonstrated that part of the phospholipid is absorbed without hydrolysis. This conclusion was based upon the demonstration that the content of phospholipid, labeled with P^{32}, was considerably greater in the blood and liver after the feeding of phospholipid so labeled than resulted in control rats receiving non-labeled phospholipid and P^{32} as glycerophosphate in an equivalent amount. However, these same men state that phospholipids may be absorbed at various stages of hydrolysis. There appears to be definite evidence that part of the phospholipid is hydrolyzed in the gastro-intestinal tract, so that inorganic phosphate

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1. Ibid.
2. Ibid.
3. Loc. Cit., p. 250.

or glycerophosphate is set free.

The absorption of lecithin is indistinguishable from that for neutral fat. There are two theories with respect to the changes taking place in the intestine prior to the absorption of fat. Referred to by Deuel[1], one is the lipolytic theory of Verzár, the other is the partition theory of Frazer.

These theories of absorption will be briefly discussed. There will be some overlapping with the description of the absorption of cholesterol, but it is the author's contention that separate descriptions of these theories will clarify the attitudes current on the absorption of fat.

The lipolytic theory is one in which it is believed that fat is hydrolyzed, in the intestine, to fatty acids and glycerin. The bile salts act as hydrotropic agents to bring the water-insoluble fatty acids into solution. It is presumed that the bile acids combine with fatty acids and convey them through the intestinal wall. Whereupon the bile salt fatty acid combination breaks up and the bile salt is set free. Bile salts are supposedly absorbed to the surface of the epithelial cells where they can dissolve more fatty acid molecules and convey them into the cells.

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1. Loc. Cit., Vol., II, p. 146.

In the partition theory a part of the ingested fat is absorbed as highly emulsified, neutral fat particles. These enter the lacteals and are carried by the lymphatic circulation to the systemic blood. The other fraction of food fat is enzymatically hydrolyzed and the products in the case of long chain triglycerides are fatty acids and mono and di-glycerides rather than fatty acids and glycerol. The fatty acids through the hydrotropic action of bile salts and possibly by other means are transported through the intestinal wall, resynthesized into neutral fat molecules and enter the portal blood stream. After arrival at the liver, sufficient metabolic alterations supposedly intervene so that little of this fat finds its way into the systemic circulation. It can be realized from the foregoing that although the mechanism is not clearly understood, nevertheless absorption of phospholipid is known to take place.

The Practical Application of the Findings of Cholesterol and Phospholipid Digestion and Absorption.

Having determined from the literature that both of the constituents of Rh hapten, namely, cholesterol and phospholipid, are readily digested and absorbed, it was felt that the oral administration of Rh hapten could be undertaken with a good deal of assurance of its being absorbed and reaching the blood stream. The only sure answer to the question would be in the actual giving of the pill orally. This was done, and it was shown that the hapten was reaching the

blood stream, since in those treated women, who possessed high antibody, the concentration of antibody fell -- a reaction expected on the successful administration of hapten. In all of the treated women the antibody titre falls to zero. This means that the hapten has caused a neutralization of the antibody and there is every reason to believe that the children born will be well and free from hemolytic disease. Does the hapten administration cause a change in the blood levels of cholesterol and lecithin? This will be discussed in the next two chapters.

It is inconceivable to think that the amounts of cholesterol and phospholipid, which are present and ingested daily, in the Rh hapten capsule would cause any increase in the blood levels of these constituents. However, it is interesting to ponder on the possible catalytic effect of ingested hapten on endogenous formation of cholesterol. The body synthesizes cholesterol in the body from acetic acid accounting for the finding of blood cholesterol even in cholesterol free diets and this level remains within a certain range. Could the hapten act as a trigger mechanism to spark the formation of excess cholesterol formation?

Summary

It has been the author's desire, in this chapter, to present evidence for the decision that the constituents of Rh hapten are

absorbed and hence that its oral administration is feasible. Because lipid physiology and biochemistry represent a very challenging field, all theories of the digestion and absorption have been mentioned. The true answers lie in the future.

Chapter IV

Methods and Findings

Purpose

In this chapter, the author proposes to discuss the relationship of cholesterol and phospholipid to arteriosclerosis and then to describe the methods employed in the experimental work on cholesterol blood levels. Having done this, the findings will be interpreted both in table and graph form.

The Relation of Cholesterol and Phospholipid To Arteriosclerosis

Arteriosclerosis is defined by the Merck Manual[1] as being "A chronic vascular disease, characterized by thickening, hardening, and loss of elasticity of the arterial walls, with inflammatory, degenerative, or hyperplastic changes." This condition usually occurs in hypertension, although increased blood pressure may originate from a variety of other causes as well.

Cantarow and Trumper[2] as well as Deuel[3] discuss the fact

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1. Op. Cit., p. 183.
2. Op. Cit., p. 156.
3. Op. Cit., Vol. II, p. 472.

that hypercholesterolemia experimentally induced will cause the condition of atherosclerosis in rabbits. Various individuals have wondered how, if at all, the development of arteriosclerosis in man could be attributed to hypercholesterolemia. White, Handler, Smith and Stetten[1] note that in both experimentally induced atheromatosis and in arteriosclerosis the characteristic lesion is the deposition of cholesterol plaques in the aorta in atheromatosis and in the lesser vessels in arteriosclerosis. The lesion in atherosclerosis is not as confined to a particular area as one finds in arteriosclerosis, the coronary vessels representing the site of the greatest laying down of plaques. According to Deuel[2], citing Peters and Van Slyke, the experimentally induced atherosclerosis of rabbits differs from the arteriosclerosis in man by the speed of development and by the unusual susceptibility of the former to iodine and to the activity of the thyroid gland.

Whether hypercholesterolemia is a concomitant of hypertension is not generally agreed upon. Many investigators, says Deuel[3], including Fahring and Wacker, Koch and Westphall, Medvei, Wacker and Rahrig, and Harris, report that cholesterol is increased in hypertension. He goes on to say that Wacker and Rahrig report

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1. Op. Cit., p. 496.
2. Op. Cit., p. 472-3.
3. Loc. Cit., p. 473.

that all the lipids are increased and not only cholesterol. It has been found that in the coronary disease group, both cholesterol and phospholipids are increased in the serum, and also the cholesterol: phospholipid ratio is likewise increased, indicating that a rise in phospholipid has not kept pace with that of cholesterol. It is suggested that the factors favoring the deposition of cholesterol in the intima are enhanced because of the lack of sufficient phospholipid to act as a colloid stabilizer. Both Deuel[1] and White, Handler, Smith and Stetten[2] agree on this issue while many other writers do not have anything to say of this ratio being altered, but write only on increased concentration of cholesterol and its effects on arteriosclerosis.

From what has been said regarding the relation of cholesterol and phospholipid to arteriosclerosis, it is apparent that a study of the blood levels of these two constituents of hapten is essential in order to know the changes, if any, caused by hapten with respect to blood levels.

Laboratory Methodology in the Study of Blood Cholesterol Levels

The procedure followed for the determination of cholesterol in the blood of the treated women was that described by Hawk, Oser

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1. Ibid.
2. Op. Cit., p. 496.

and Summerson[1]. Approximately 10 ml. of acetone-alcohol mixture is placed in a 25-ml. glass-stoppered volumetric flask and to it is added 1 ml. of serum down the side of the flask. After withdrawing the pipet, the flask is swirled to produce a finely divided precipitate, the flask is then immersed in boiling water, with swirling to prevent bumping, until the solvent boils. Remove, cool to room temperature and make up to volume with alcohol-acetone mixture. Stopper, mix, and filter. The filtrate is collected in a dry test tube. The funnel is covered during filtration to minimize evaporation of the solvent. Three drops of potassium hydroxide solution is added to a 15-ml. graduated centrifuge tube. 3 c. c. of acetone-alcohol filtrate is then added to the tube, a glass rod inserted and the mixture is stirred vigorously until no droplets of alkali can be seen in solution. The rod is left in the tube and the tube and rod are placed in a preserving jar containing a layer of sand about 3 cm. deep which has been previously heated in a water bath until the temperature of the sand reached 45°C. The jar is closed tightly and placed in an incubator at 40°C. for 30 minutes. The tube is removed to the rack, allowed to cool to room temperature. The rod is then

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1. Hawk, Philip B., Oser, Bernard L., and Summerson, William H., Practical Physiological Chemistry. Philadelphia: The Blakiston Company, 12th edition, (1949), 532.

raised and acetone-alcohol mixture is added to the 6 c.c. mark. One drop of phenolphthalein solution is added, followed by 10% acetic acid, drop by drop with stirring, until the red color disappears. One drop is added in excess followed by 3 ml. of digitonin solution. The contents are thoroughly stirred, the tube and rod placed in a preserving jar, covered tightly, and allowed to stand at room temperature overnight. In the morning, the tube is transferred to a test tube rack and stirred gently to free particles of precipitate that may have adhered to the walls of the tube. The rod is removed from the tube, being careful not to rub off any of the adhering precipitate. It is preferable to transfer the rod to a wire numbered rack so that it will be replaced to the correct tube at a later time. The tube is centrifuged for 15 minutes at 2800 r.p.m. The supernatant is separated by decanting and the tube is drained by inverting for a few minutes. Any particles that float near the top of the supernatant are discarded with the supernatant. The stirring rod is replaced in the tube and the walls of the tube and the rod itself is washed down with 2 c.c. of acetone-ether mixture slowly by means of a pipette. The precipitate is stirred thoroughly, the rod replaced in the rack and the tube centrifuged for five minutes, after which the supernatant is decanted off. The rod is replaced in the tube and the precipitate is washed with ether in a manner similar to the above. The rod is replaced in the rack and centrifugation is done again for five minutes, discarding

the supernatant. The rod is then replaced in the tube. A layer of sand about 3 cms. deep is placed in a shallow pan and heated to 110-115°C. in an oven. The tubes, containing the precipitated and washed cholesterol digitonide are placed in the pan and it is returned to the oven. After 30 minutes, the pan is removed and while the tubes are still in the hot sand, 2 ml. of glacial acetic acid from a burette is added to them in such a way that the acid rolls down the wall of the tube and rod. The mixture is stirred well and allowed to remain in the hot sand for two minutes, but no longer. The tube and rod are then replaced in a rack and allowed to cool to room temperature. Now the tubes are ready for color development. They are placed in a water bath at 25°C. from which light is excluded and allowed to come to temperature equilibrium. Noting the time, 4 mls. of cold acetic anhydride-sulfuric acid reagent is added to the tube. The mixture is mixed well with the rod, and returned to the bath for 27 minutes. At the end of this time, the mixture is transferred to a colorimeter tube and read in the colorimeter immediately. The unknowns are read against a standard which is prepared as follows. 2 mls. of a standard solution of cholesterol in acetic acid, containing 0.2 mg. of cholesterol are transferred to a tube equipped with a stirring rod. The tube is placed in a water bath at 25°C. and allowed to come to temperature equilibrium. When ready, 4 mls. of acetic anhydride-sulfuric acid reagent is added, mixed well with the rod,

returned to the bath and allowed to stand for 27 minutes at the end of which time the color change is read in a colorimeter.

The colorimeter used in this research laboratory was a Bausch and Lomb. The calculations for the colorimetric measurements were as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 \times \frac{100}{0.12} = \text{mg. of total cholesterol per 100 mls.}$$

Findings From Experimentation

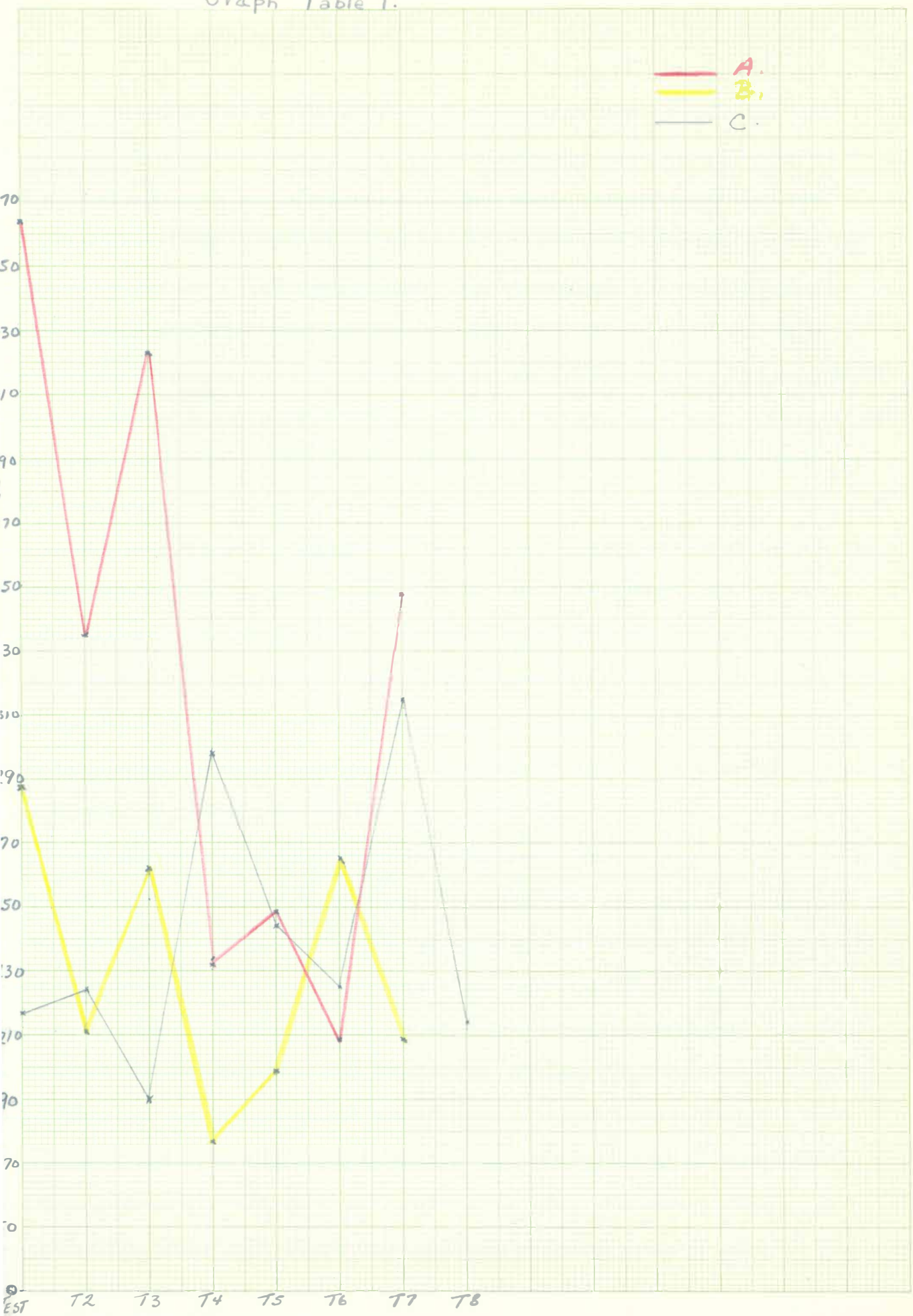
The findings from experimentation are found in tabular form which follows:

		Cholesterol Blood Levels. Mg./100 c.c. Blood								
Woman	T. P. *	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	Avg.
A	X	466.0	335.0	423.0	232.4	249.0	209.1	348.6		324.3
B	X	288.0	213.0	262.3	177.6	199.2	265.6	209.2		230.7
C	X	217.0	224.1	190.9	298.8	244.0	225.7	315.4	214.4	241.3
D	X	199.2	170.9	199.2	174.3					190.9
E	X	136.6	215.8	156.0	172.6	166.0	170.9	178.7		170.9
F+	X	215.8								215.8
G	X	166.0	194.2	180.9						180.3
H+	X	177.6								177.6

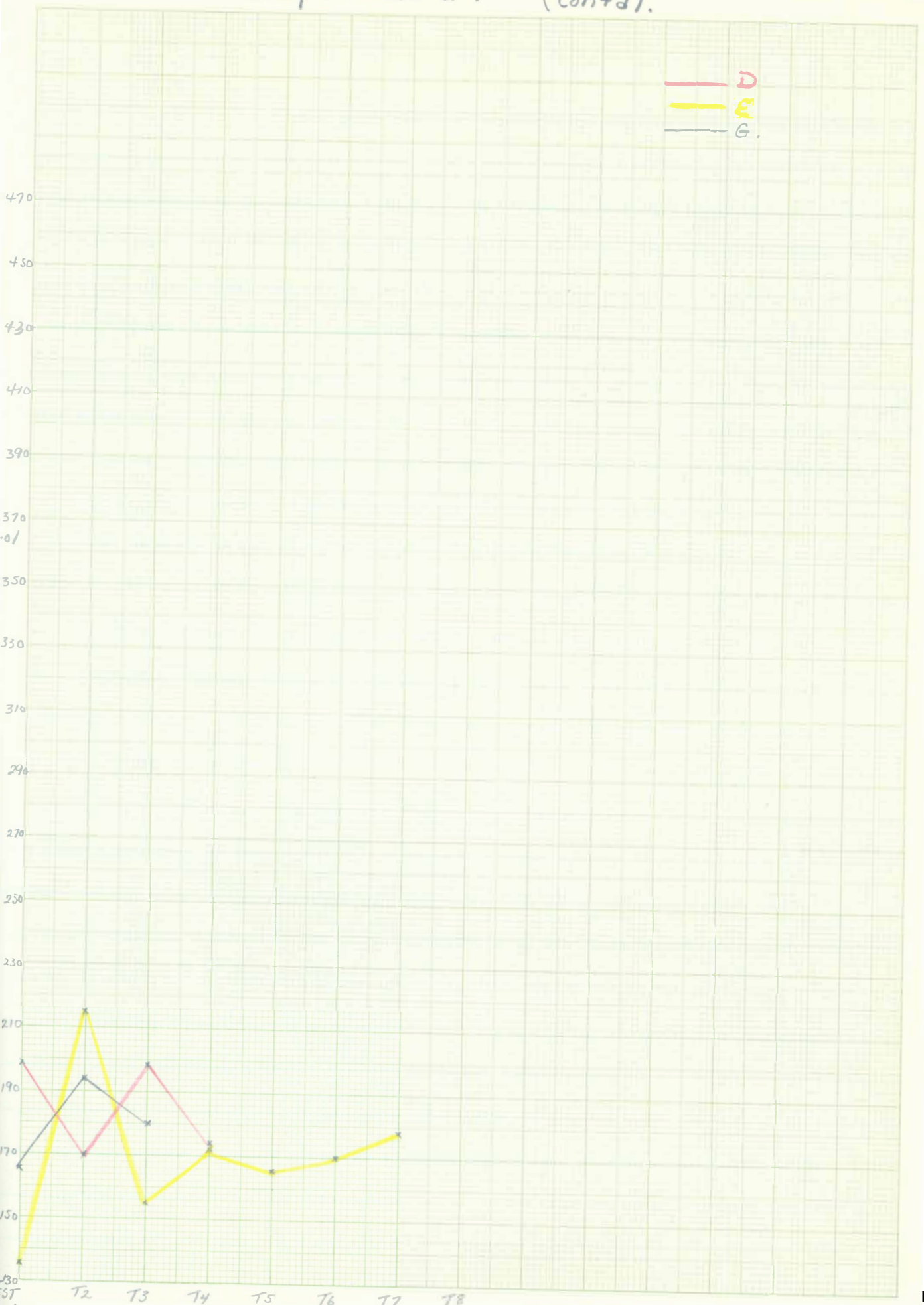
*T.P. - Treated Pregnant
These two cases are new and hence only one test has been done thus far.

+The findings from a study of cholesterol blood levels in pregnant individuals being treated with Rh Hapten because of having a history of bearing children suffering from hemolytic disease.

Graph Table 1.



Graph Table #1 (cont'd).

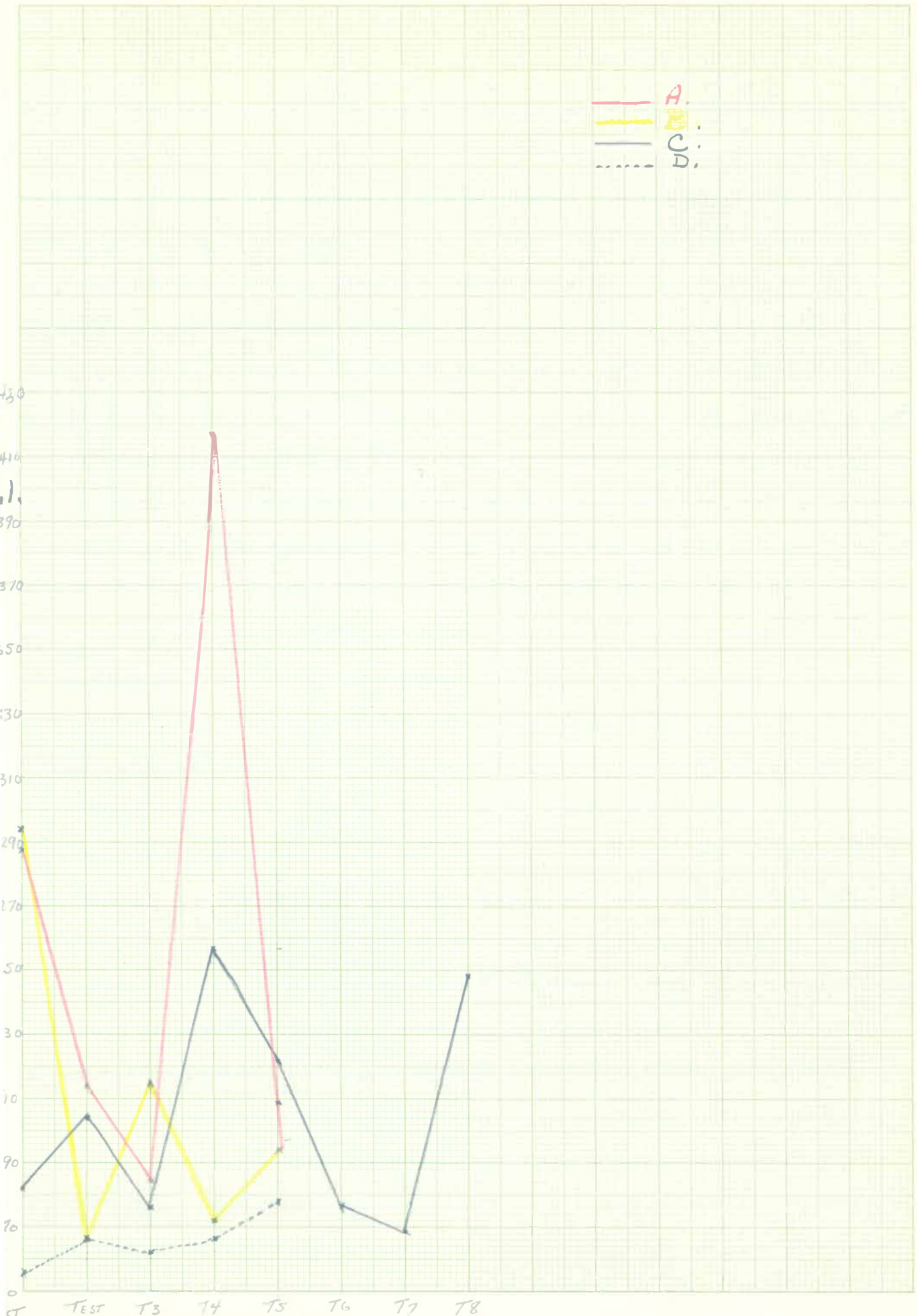


		Cholesterol Blood Levels. Mg. /100 c. c. Blood								
Woman	T. N. P. *	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	Avg.
A	X	288.0	214.2	184.3	415.0	194.2				259.1
B	X	294.5	166.0	215.8	172.6	209.2				201.6
C	X	182.6	204.2	176.6	257.0	222.4	177.6	169.3	249.0	207.3
D	X	156.0	166.0	162.7	166.0	178.7				165.7

*T. N. P. - Treated Not Pregnant

Table represents cholesterol blood levels of individuals who possessed a high titre of antibody and were advised to get the titre reduced before becoming pregnant. This is common practice with women who possess a high titre. If this is done, it makes better the chances of a normal birth.

Graph Table #2



Woman	P. N. T. *	Test 1	Average
A	X	227.4	227.4
B	X	332.0	332.0
C	X	194.2	194.2
D	X	170.9	170.9

*P. N. T. - Pregnant Not Treated

This table represents cholesterol blood levels of a few pregnant untreated women. Tests made for comparison with pregnant treated women.

Woman	S. N. P. *	Cholesterol Blood Levels. Mg. /100 c. c. Blood	
		Test	Average
A	X	262.3	262.3
B	X	294.5	294.5

*S. N. P. - Sensitized Not Pregnant

Because these individuals were not pregnant, they were untreated. Cholesterol levels done for comparison with pregnant women.

Individual	Cholesterol Blood Levels. Mg. /100 ml. Blood	
	Normal	Test
A (Man)	X	144.4
B (Man)	X	156.1
C (Man)	X	162.7
D (Woman)	X	184.3
E (Woman)	X	184.3
F (Man)	X	179.3
G (Man)	X	154.4

Tests done on normal individuals, some women and some men, both for comparison with pregnant individuals and as check on testing technique to see whether results approximated considered normal values.

The normal blood cholesterol levels range from 150 - 300 mg./100 c.c. blood. Nearly every textbook reports that with an advanced pregnancy it is not uncommon to find that the cholesterol level rises beyond this normal range. From all the tables it can be seen that in only two women do we find an overall (average) cholesterol level which is greater than 300. One of these is a Treated Pregnant woman and the other is an Untreated Pregnant woman. In the case of the Treated Pregnant woman her cholesterol level was not consistently high but showed fluctuation from 466.0 mg. to 209.1 mg.

An interesting finding is that in the Sensitized Not Pregnant woman tested the values of cholesterol, although not exceeding the maximum value, were better than 250 mg.

The majority of the normal runs were done on males and hence the readings from 144.4 - 184.3 represent normal values since the cholesterol level of males is considered to be somewhat lower than for women.

The graphs were drawn to show that in both Treated Pregnant and Treated Non-Pregnant women, the cholesterol levels fluctuated and that not one case shows a constant increase of cholesterol concentration with continuous administration of hapten. Test 1 in every case represented the first test made and all succeeding tests were done during the continuous use of hapten.

Summary

After discussion of the relation of cholesterol and lecithin to arteriosclerosis, a description of the laboratory methodology in the study of cholesterol blood levels was described. The findings, as shown in tabular and graph form, indicated that the cholesterol level did not rise above the normal range on continuous hapten administration nor was there a continuous rising of the level but great fluctuation from one test to another was the picture.

Chapter V

Methods and Findings

Purpose

It is proposed in this chapter to report on the methodology used and findings obtained from a study of phospholipid in blood.

Laboratory Methodology in the Study of Lipid Phosphorus in Blood

The method employed in the study of phospholipid was in accord with the procedure described by Hawk, Oser and Summerson[1]. A description of this procedure follows.

Transfer 18 ml. of alcohol-ether mixture to a wide-mouthed test tube (best 150 x 20 mm.) graduated at 20 ml., and drop in slowly, while shaking, 1 ml. of plasma or serum. Mix, place in a boiling water bath, and heat the contents of the tube to boiling. Remove and allow to cool to room temperature. Make up to the 20-ml. mark with alcohol-ether mixture, mix, and filter.

Transfer 8 ml. of filtrate to a 200 x 25 mm. pyrex test tube, add a silica pebble (from broken silica ware), place in a wire rack containing a wire bottom, over an electric hot plate, and evaporate to dryness.

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1. Op. Cit., p. 541, 580-1

Add 2.5 ml. of 5N sulfuric acid and a quartz chip to minimize bumping. Place in a slanting position over a micro burner, with the burner tip about 2 cm. below the bottom of the tube, or suspend in a wire basket about 1.5 inches above an electric hot plate. After evaporation is complete and the mixture turns brown or black with no further change, remove the tube, cool slightly, and add 1 drop of 30% hydrogen peroxide, allowing the drop to fall directly into the digestion mixture. Replace the tube and continue heating. The contents of the tube should become colorless; if not, repeat the addition of hydrogen peroxide and heating. When colorless, cool the tube, add a few ml. of water, and heat to boiling momentarily. Cool again and transfer the contents of the tube to a 25 ml. volumetric flask, with washings until the flask is about half full. Add 2.5 ml. of 2.5 per cent ammonium molybdate solution, followed by 1 ml. of aminonaphtholsulfonic acid reagent. Dilute with water to the 25-ml. mark and mix. Allow to stand five minutes, then compare in the colorimeter against a standard prepared at the same time, as follows: Transfer 5 ml. of standard phosphate solution, containing 0.4 mg. of phosphorus, to a 100-ml. volumetric flask, and add from a graduate 50 ml. of water. Add 10 ml. of molybdate I, mix, and add 4 ml. of aminonaphthalsulfonic acid reagent. Dilute with water to the 100 ml. mark, mix, and allow to stand five minutes. Compare the standard against itself in the colorimeter before reading the unknown.

If the color of the unknown is particularly strong, repeat the reading of the unknown a few minutes later, to be sure that maximal color development has taken place.

The calculations for the colorimetric measurements are done as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{0.4}{4} \times \frac{1}{0.4} \times 100 = \text{mg. of total acid-soluble phosphorus per 100 ml. of blood plasma or serum.}$$

Findings From Experimentation

The experimental work on the phospholipid analysis of blood has not been carried on a sufficient length of time for definite conclusions to be drawn. The study of phospholipid in blood was not undertaken until the second semester and then it was late in the semester before conditions were favorable for making analysis.

Toward the end of the analysis the writer ran into some difficulty. The color changes which normally occurred were no longer occurring. Careful study of the technique indicated that the fault lay in the reagents. Those reagents which were believed to be responsible were made up anew and runs made on three samples of serum just prior to the writing of this chapter showed much better color change, and results similar to those experienced at the time when the tests were first begun.

The author wants just to give the facts on the findings and asks the reader to consider this as a preliminary report and not an answer in any way, shape or form to the question of whether the hapten causes a phospholipid or phospholipid:cholesterol ratio change in the blood after its administration.

Woman	State of Treatment and Pregnancy	Test 1	Test 2	Test 3	Test 4
A	T. P.	10.8 mg.	10.4 mg.	14.0 mg.	* 6.3 mg.
B	T. P.	10.8 mg.	12.5 mg.	10.3 mg.	✳ 11.0 mg.
C	T. P.	*17.0 mg.	* 8.5 mg.		
D	T. N. P.	*16.6 mg.	* 5.4 mg.		
E	N. T. P.	* 5.5 mg.			
F	T. N. P.	* 7.5 mg.			
G	N. T. P.	* 6.1 mg.			
H	T. P.	✳ 11.2 mg.			
I	T. P.	* 5.4 mg.			
Control	N. P. N. T.	10.8 mg.	✳ 10.0 mg.		

* - Indicates abnormal color change.
✳ - Indicates tests done after new reagents made up. All other tests done before difficulties arose.

T - Treated
P - Pregnant
N - Not

Woman	State of Treatment or Pregnancy	Phospholipid:Cholesterol Ratio			
		Test 1	Test 2	Test 3	Test 4
A	T.P.	$\frac{10.8}{166.0} = \frac{1}{15.3}$	$\frac{10.4}{194.2} = \frac{1}{18.6}$	$\frac{14.0}{180.9} = \frac{1}{12.8}$	
B	T.P.	$\frac{10.8}{172.6} = \frac{1}{16.1}$	$\frac{12.5}{166.0} = \frac{1}{13.2}$	$\frac{10.3}{170.9} = \frac{1}{16.6}$	$\frac{11.0}{178.7} = \frac{1}{16.2}$
H	T.P.	$\frac{11.2}{174.3} = \frac{1}{15.5}$			

*Ratio only for tests which were unaffected by bad reagents.

Table of phospholipid-cholesterol ratios for three treated pregnant women.

Table indicating actual number of mgs.

More investigation needs to be carried on before any conclusion can be made regarding blood levels of phospholipid in Rh negative treated sensitized women.

Summary

It has been intended in this chapter to give a description of the methods employed in a study of blood phospholipids in treated pregnant women. Although the author has put in tabular form the results of the experimental investigation, it is hoped that the reader will not form a decision on the effect of hapten on blood phospholipid level but will consider this only a report preliminary to further investigation; especially with the realization that the author himself does not feel qualified at this time, because of difficulties encountered and too few samples, to form any conclusions.

Chapter VI

Summary of Paper

This paper has been written with the intention of revealing the nature of the research carried on during the past year at Western Michigan University on "The Study of Cholesterol and Phospholipid Blood Levels in Treated Rh Negative Sensitized Women."

Cholesterol and phospholipid appear to be the two major constituents of Rh hapten. Rh hapten is the substance contained in the capsule administered orally to sensitized Rh negative women, so that they are able to bear normal children, hapten acting as a neutralizer of the antibody which causes the red cell destruction of the baby.

The importance of this study is that there is a belief on behalf of some individuals that high cholesterol blood levels are the cause of arteriosclerosis. Another school of thought deems the cholesterol-phospholipid ratio the cause or, if not the cause, one of the symptoms of the disease. Because the treated individuals receive both cholesterol and phospholipid in the hapten pill daily it presented the problem of whether its administration may cause an alteration in the levels of these blood constituents and hence have a clinical importance.

Techniques Used in the Study

The techniques used in the investigation were two-fold, the first being of a library research nature where a review of the literature indicated that Rh hapten would be readily absorbed if administered orally. When this was realized and the hapten was administered in this manner, the next problem was to investigate experimentally the levels of the constituents of hapten in the blood to learn of any changes that may take place. The procedures for the experimental work have been given in detail. The methods used are ones that have been considered to be reliable through the years.

Conclusions

The writer believes that only one part of the findings can be considered conclusive, that being that with the oral administration of Rh hapten, there is not any rise in cholesterol level beyond normal range and hence if increased levels cause arteriosclerosis there is no indication, in the cases studied, that it (the cholesterol level) would do any damage to the women under treatment.

Not enough tests were done on the phospholipid blood levels and hence no conclusions can be drawn with respect to blood phospholipid levels in the treated women. However, the findings as reported in Chapter 5 might well serve as a preliminary report and be compared with findings done at a later time on the same women using same or similar experimental techniques.

The Future

The future in this field of Rh hapten administration is vast. As indicated above, more study needs to be done on phospholipid levels in the women and the ratio of cholesterol to phospholipid examined further.

Besides this work, as an outcome of the past year's research, there are brand new fields of investigation. One of these might well be to investigate the exact chemical structure of the hapten constituents. There has not been to date any electrophoretic patterns of the protein or lipoprotein fractions of the sera in the treated cases. There has been a tremendous lot of work done in the past few years using electrophoretic techniques in study of proteins and lipids, enzymes, antibody complexes and so on. Electrophoretic studies in this field of investigation might well answer questions that as yet are unanswered.

Isotopic studies are a challenging technique for labeling compounds and watching kinds of activity and ways of activity of certain chemicals. There is no reason to deny the use of isotopes in the investigations with respect to the administrations of Rh hapten.

Tremendous strides have been made since Rh hapten was first isolated in 1947. This is not, however, any reason to believe that all is known because much has yet to be learned, not only about Rh hapten and its neutralizing power but about the blood in general.

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