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The Apparent Effects Of RH Hapten Ingestion On The Circulating Blood Phosphorus

Barbara Lyke
Western Michigan University

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**THE APPARENT EFFECTS OF RH HAPTEN
INGESTION ON THE CIRCULATING BLOOD PHOSPHORUS**

**A Thesis Submitted to the Graduate Faculty
in Partial Fulfillment of the Requirements
for the Degree of Master of Arts in Biology**

Barbara Lyke

1959

Western Michigan University

Kalamazoo, Michigan

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Chapter 1

THE PURPOSE AND BACKGROUND

Purpose

The purpose of this work carried on at Western Michigan University during the past year is an attempt to elucidate further the action of Rh hapten within the body, or more specifically, the effect of its ingestion on the circulating blood phosphorus.

Rh hapten is a substance which upon administration to sensitized Rh negative women enables them to bear normal healthy infants. Extracted from Rh positive erythrocytes, the hapten appears to neutralize Rh antibodies in vivo and in vitro. The administration of Rh hapten has been demonstrated repeatedly to result in the elimination of Rh agglutinins from the circulation of immunized Rh negative individuals and sensitized laboratory animals. In addition, the hapten will inhibit saline agglutinating anti-Rho serum and will fix complement. Adsorption of antibody nitrogen has also been demonstrated. The substance appears to be lipid in nature containing neutral fat, unsaturated sterols, phosphorus and nitrogen.

Because of the nature of Rh hapten, its observed action in vitro and its postulated action in vivo, the effect of its administration on the lipid phosphorus values of the serum is of interest. Elevation of serum phospholipid levels has been observed experimentally after the consumption

of large quantities of both lecithin and neutral fat. The treated women, however, received only a small dosage of hapten daily (one hundred to four hundred milligrams). Increase in phospholipid values has also been noted in rabbits during active immunization. The ingestion of adrenocorticotropin will also raise phospholipid levels during the course of treatment. This hormone has been demonstrated to lower antibody titers as well and a relationship between these two observations is possible. Stimulation of some endogenous mechanism to release phospholipid into the bloodstream, therefore, might occur.

The approach to this investigation has been twofold. As in all research, the initial interest lay in a study of the literature pertaining to the history of erythroblastosis fetalis and attempts to counteract this malady. In addition, the metabolism of phospholipids and pertinent factors altering this process were also perused. The second attack has been of an experimental nature. Serum lipid phosphorus determinations were carried out on bi-weekly blood samples from the Rh hapten treated women over a period of seven months. Two control groups took part in this study. Identical tests were run on blood specimens collected from (1) a series of nontreated normal pregnant women and (2) from a group of nontreated nonpregnant individuals, since pregnancy is reputed to produce a rise over normal values

of serum phospholipides. The techniques utilized included the well standardized methods of Ficks and Subbarow and of Zilverman and Davis.

Hemolytic Disease of the Newborn

Erythroblastosis fetalis, or hemolytic disease of the newborn, has long been known to the medical world. Indeed, Pickles reports that a case described by Hippocrates was accepted by Ballantyne in 1892 as an account of hydrops fetalis.¹ The cause of this disease has been elucidated only recently and the possibility that erythroblastosis fetalis, hydrops fetalis, and icterus gravis could be varying degrees of the same disease passed unrecognized until 1932².

In 1905 Dianst proposed the concept of erythroblastosis fetalis as a consequence of immunization of the mother against a protein foreign to her but present in the fetal blood.³ As a result, many investigators attempted, with little success, to find some correlation between the syndrome and conflicting ABO blood groups.⁴ Conflicting blood groups are now recognized as the causative factors in a very small percentage of erythroblastotic infants along with the Kell factor, N antigen, the Lutheran factor, the Lewis factor⁵ and the Duffy antigen.⁶ In 1938, Darrow suggested that erythroblastosis fetalis might be the result of an antigen-antibody reaction involving an agglutinin other than the A and B substances.⁷

Landsteiner and Wiener, in 1937, observed that serum from rabbits injected with red cells from Macacus rhesus monkeys agglutinated human erythrocytes carrying the M antigen.⁸ In an attempt to find further agglutinins in human blood, these researchers produced a serum capable of clumping the erythrocytes of thirty-nine of forty-five human bloods tested.⁹ These results agreed with those of no other known blood group and the workers concluded that a new antibody had been demonstrated. Designated Rho, the new agglutinin became the first of the continually expanding Rh series.

About the same time, Levine and Stetson demonstrated in the serum of a woman who had delivered a stillborn child an agglutinin which clumped the cells of 80 percent of her own group.¹⁰ This observation paralleled those of Landsteiner and Wiener and led to the discovery of the Rh antigens. As a result, these investigators also suggested that the occurrence of erythroblastosis fetalis could be attributed to incompatibility of blood groups other than the ABO system. Shortly thereafter, Levine and his associates convincingly demonstrated that immunization of the mother against one or more of the Rh agglutinogens constituted the major cause of hemolytic disease of the newborn.¹¹ The disease itself appears when maternal antibodies, usually against an Rh antigen, pass through the placenta by osmosis and into the fetal bloodstream where they hemolyze the red cells present.

The severity of the disease is dependent upon the degree of erythrocyte destruction.

Following identification of the specific antigen-antibody mechanism involved in erythroblastosis fetalis, many workers turned to the study of the mode of maternal isoimmunization. This has yet to be fully explained. Transfusion of the mother at some time in her life with Rh positive blood has been known for several years to increase the possibility of a diseased first child. Levine observed that transfusion sensitized women gave birth to a higher percentage of firstborn erythroblastotic infants than the nontransfused.¹² Pickles indicated that incompatible transfusion increases the possibility of sensitization through pregnancy from less than 5 percent to more than 50 percent.¹³

Pregnancy is also known to sensitize some women. Gainey and his collaborators reported that obstetrical trauma resulting in introduction of Rh positive fetal blood into an Rh negative mother's bloodstream gave rise to a higher incidence of afflicted infants in subsequent pregnancies.¹⁴ In addition to this, an interchange of blood at the placenta during some pregnancies has been postulated and indeed the work of some investigators seems to substantiate this theory. Weiner cites the work of Dunsford who claimed that fetal cells appeared in the circulation of an Rh negative woman.¹⁵

However, as these cells were demonstrated in the maternal blood after delivery, Weiner admits to the possibility of introduction at that time. In addition, Weiner reported a case in which the blood of a pregnant Rh negative woman gave evidence of the presence of some Rh positive cells when routine typing was carried out.¹⁶ A recheck eight days later revealed only the typical reaction of negative cells. In 1954, Chown cited a case in which the blood of a mother who had delivered an infant with severe nonhemolytic anemia was demonstrated to contain 5 to 10 percent fetal cells.¹⁷ He then suggested that such infants have undergone severe hemorrhage prior to birth during which time some of the cells escape into the mother's bloodstream. However, this also occurred in blood collected subsequent to delivery. Chown then reported an additional case in which an Rh negative mother appeared to undergo a severe transfusion reaction prior to the birth of her baby.¹⁸ Upon delivery, the placenta was found to have a large hole through which Rh positive cells presumably escaped. In addition, Javert has found evidence of decidual bleeding during normal pregnancy.¹⁹ Mengert and his coworkers showed through the use of cells tagged with Fe⁵⁹ and cells carrying the sickling trait that erythrocytes cross the placental barrier under normal circumstances.^{20, 21} Both the tagged cells and those with the sickling tendency could be demonstrated in the cord

blood of 86 percent and 50 percent respectively of infants whose mothers had been transfused with the cells prior to delivery. Finally, Kling through careful examination of the placentas of both normal and erythroblastotic babies from all stages of pregnancy, demonstrated that numerous breaks in the vessels do occur.^{22, 23} This he indicated, begins toward the end of the third month and occurs more frequently involving more vessels as pregnancy progresses. This allows, of course, fetal blood to enter the uterine intervillous spaces thereby providing, in a small number of cases, the necessary antigenic stimulus.

Although about 15 percent of all Caucasians are Rh negative, the incidence of hemolytic disease of the newborn is much smaller than might be anticipated. In practice, about 10 percent of all pregnancies involve Rh negative mothers with Rh positive infants. Of these, about 3 percent produce diseased children. This anomaly has been attributed to relative permeability of the placenta,^{24, 25} the size and combining powers of the types of Rh antibodies produced,^{26, 27} the susceptibility of the individual to isoimmunization, i.e. the constitutional factor K²⁸, increased resistance of Rh negative women having Rh positive mothers,²⁹ and sensitization due to transfusion.³⁰ To further complicate the picture, Carter reported four instances of clinically normal babies from demonstrably immunized mothers.³¹ Several other

investigators have reported this phenomenon also^{32, 33, 34}. Lockery and Sachs have suggested this may be due to the passage of antibodies through the placenta only under certain conditions.³⁵ However, Carter demonstrated the antibodies in the blood of three of these infants. Relative immunity of certain babies to antibody damage has also been considered.³⁶ Such immunity, be it active or passive, has been ascribed to the secretion of Rh substances by the tissues of some infants³⁷, the stage of development of the serum proteins of the fetus when maternal immunization takes place³⁸, the duration of exposure to the agglutinins,^{39, 40} and resistance due to genetic factors.⁴¹

Despite the relatively small percentage of erythroblastotic births, many families have been limited to one or two children while others remain childless. Fortunately, an erythroblastotic first child is a rare occurrence providing the mother has not been sensitized prior to pregnancy. However, once a woman has borne a diseased child, the chances of reducing a less seriously affected infant are small. Often, the mildly diseased baby may be saved if treatment is undertaken quickly. Many obstetricians now make an immediate examination of the blood of an infant suspected of being potentially erythroblastotic for hemoglobin level, icterus index, red cell count, immature red cells and Coombs test. If erythroblastosis is suggested, simple or exchange transfusion of Rh negative blood may be employed with frequent

success.

These measures, however, do not help the stillborn infant. As a result, several lines of research aimed at prevention or alleviation of erythroblastosis fetalis have been undertaken. Four considerations have been the objective of most research to date. First, the prevention by chemical means of completion of the antigen-antibody reaction has been attempted. Kariber and his associates employed ethylene disulphonate, an antihistamine, with little success.⁴² In addition Scherer and his coworkers were unable to control the antibody levels of rabbits undergoing immunization against Rh antigens through the use of sodium salicylate, pyribenzamine, and ethylene disulphonate.⁴³

Second, inhibition of antibody formation by some immunological means offered another avenue of approach. Wiener, therefore, following his theory of competition of antigens, attempted to forestall erythroblastosis fetalis by administration of typhoid vaccine to pregnant Rh negative women with poor prognosis.⁴⁴ Theoretically, the production of antibodies against a strong antigen would inhibit the formation of agglutinins against the weaker agglutino-gen. This, too, soon proved fruitless.

Third, a few investigators have attempted to strengthen the placenta the point of attack Dippel deems weakest.⁴⁵ Jacobs unsuccessfully tried to use Vitamin C derivatives.⁴⁶

Vitamin K and progesterone therapy, employed by Hoffman⁴⁷ and his collaborators as well as by Glienon and his associates⁴⁸ has been abandoned also.

Fourth, Calvin and his coworkers, among many others, suggested that the possibility of obtaining a hapten fraction from the Rh antigens present in the stroma of red blood cells seemed worth investigating.⁴⁹ This, they felt, could be important in erythroblastosis fetalis. Accordingly, in 1946 they separated, through ultracentrifugation, a fraction which inhibited anti-Rh serum.⁵⁰ This fraction they termed elinin. In 1947, Carter was able to separate by chemical means a fraction from Rh positive cells which showed the same specificity.⁵¹ Later, this substance was designated Rh hapten in accord with the Landsteiner definition of a hapten. Rh hapten has been employed successfully to forestall erythroblastosis fetalis by Carter,^{52, 53, 54} and, repeating her work, by Goldsmith,⁵⁵ Barnard,⁵⁶ Ehrenberg⁵⁷ and Schubert and Granberg.⁵⁸

FOOTNOTES

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Chapter 2

THE HAPTEN

As indicated previously, the use of Rh hapten in prevention of hemolytic disease of the newborn appears to be the most promising approach to date. A hapten, according to Landsteiner, is a substance which, in itself, is nonantigenic but which, when combined with a suitable protein carrier, will stimulate antibody formation.¹ This hapten, then, has the ability to react specifically with the indicated antibody. Barnard describes Rh hapten as an antigen configured hapten which reacts directly and noncompetitively with Rh antibodies.²

That Rh hapten is truly hapten in nature is supported by the following evidence. Rh hapten does not stimulate antibody formation in vivo as demonstrated by injection into guinea pigs. Yet, when injected in combination with egg albumin, the specific Rh agglutinins can be demonstrated. On the other hand, the injection of the hapten into sensitized guinea pigs decreases the antibody titer.³ The administration of the hapten to immunized women has also been demonstrated to drop this titer.^{4, 5, 6, 7}

Rh hapten appears to be lipid in nature. It is soluble in the conventional fat solvents and insoluble in water. It has been demonstrated to contain at least 35 percent

unsaturated sterols and gives a positive Lieberman-Burchard test. The hapten also contains about 2 percent nitrogen and 0.5 to 1 percent phosphorus. The phosphorus is incorporated in lecithin and possibly other phosphorus containing lipid material. No carbohydrate or protein is demonstrable.⁸

Preparation of Rh hapten is accomplished at the present time in the following manner. The cells from six pints of outdated Rh (D) positive bank blood are pooled. Two volumes of alcohol are added slowly to this pool in a four gallon wide-mouth bottle and the whole is stirred thoroughly. The precipitated blood in alcohol is allowed to stand overnight at room temperature. The next day the precipitate is separated by filtration with suction and to this is added two volumes of dichloromethane. The precipitate is allowed to remain in the dichloromethane for at least six hours or as long as is convenient, with intermittent, thorough shaking. Then the solvent is separated from the precipitate by suction filtration and this liquid is distilled off or evaporated under a hood with an electric fan. The residue is the active material.⁹

The original mode of administration, intramuscular injection, while producing no local or systemic reactions, caused pain upon injection with soreness developing later. This encouraged experimental oral administration which is proving fully as effective as the intramuscular route¹⁰.

For oral administration, the fraction dried to the consistency of petroleum jelly, is first weighed and then mixed with powdered lactose to the point where it can be handled easily. It is then rolled on a graduated pill tile and cut into aliquots, each containing not less than one hundred milligrams of hapten. Each of the aliquots is taken up into a No. 2 gelatin capsule. Until they are dispensed, the capsules filled with hapten are kept refrigerated.¹¹

The mode of action of the hapten in vivo would appear to be a direct neutralization of Rh antibodies.^{12, 13} Three types of determinations have been conducted in vitro which tend to substantiate the specificity of Rh hapten. Fixation of complement has been demonstrated through the use of a modified Kolmer technique. In addition, specific inhibition of saline agglutinating anti-Rho serum by the hapten has been observed.^{14, 15, 16} These findings, employed in assay of the activity of the hapten have been confirmed by Barnard.¹⁷ Howe and Rustigian¹⁸ and Osborn¹⁹ reported unsuccessful attempts to confirm this due, apparently, to variations in technique. More recently, quantitative studies of antibody nitrogen adsorbed by Rh hapten revealed a consistently higher degree of adsorption by the hapten fraction obtained from Rh positive cells than by an analogous lipid fraction isolated from Rh negative cells. Carter and Harris feel that this indicates that the adsorption of nitrogen is a specific effect.²⁰

As indicated previously, a favorable outcome in cases of known isoimmunization would be expected if the antibody titer could be eliminated prior to pregnancy. Accordingly, Carter and her coworkers, in 1956, indicated the loss of only one baby in their series when treatment was begun prior to the initiation of a new pregnancy and continued with daily administration as soon as pregnancy was suspected until term.²¹ (The one failure was later demonstrated to be due to preeclampsia). Since that time, Carter has lost no infants under the conditions of the series reported in 1956.²² The failures reported by Wolf and his colleagues,²³ Harsters and his associates²⁴ and Spurling and his associates²⁵ appear to be due to beginning treatment too late and/or the administration of too little hapten.

FOOTNOTES

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Chapter 3

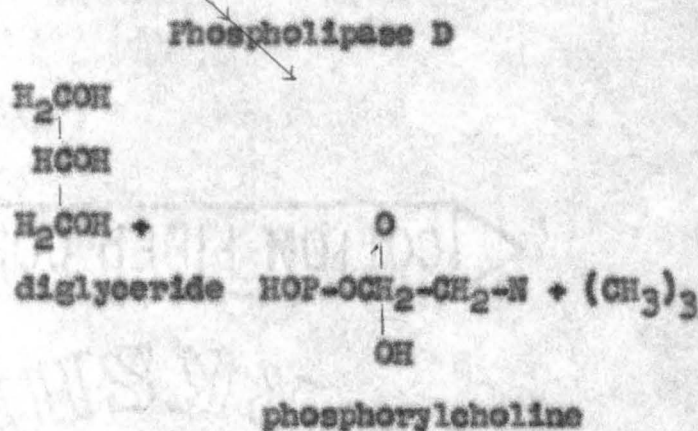
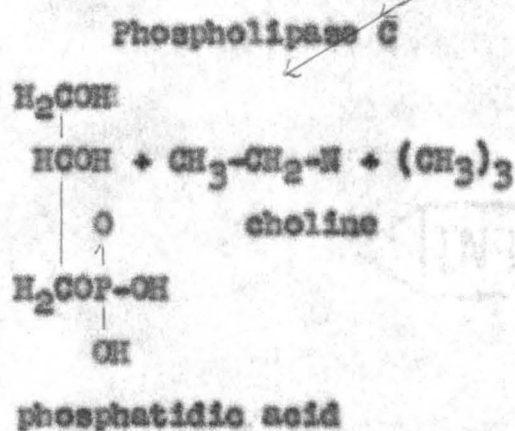
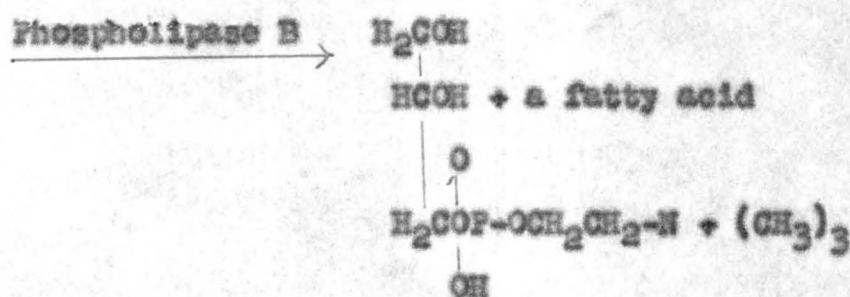
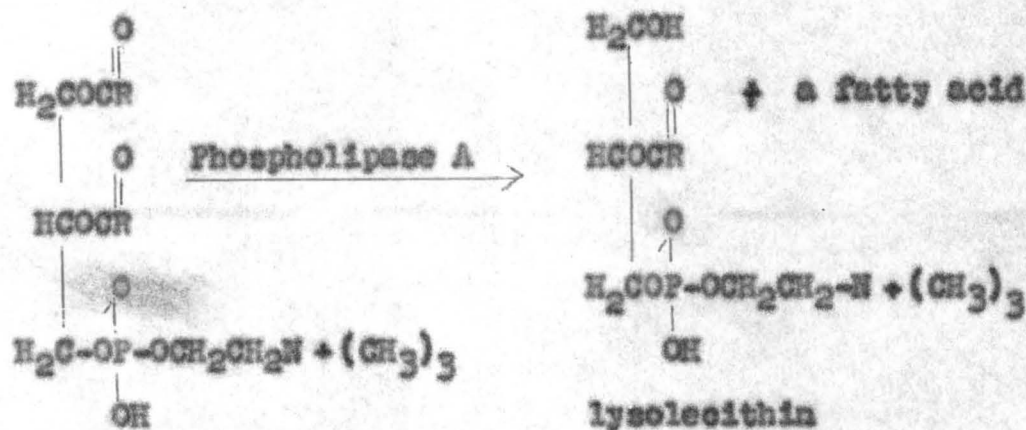
THE METABOLISM OF PHOSPHOLIPIDES AND SOME RELATED CONSIDERATIONS

Due to the nature of Rh hapten and its apparent action within the body, an investigation of the possible effects of administration on the circulating blood phosphorus was attempted. Five considerations seemed pertinent to this study including the exogenous effect of lipid feeding, the increase of lipid phosphorus associated with pregnancy, the known effect of antibody production and attempts to depress this reaction on the lipid phosphorus and a possible stimulation of some unrelated endogenous mechanism to release phospholipide. This chapter will, therefore, review first the metabolic pathway of phospholipide within the body and secondly, the pertinent material relating to the five considerations of this study.

Metabolism of Phospholipides

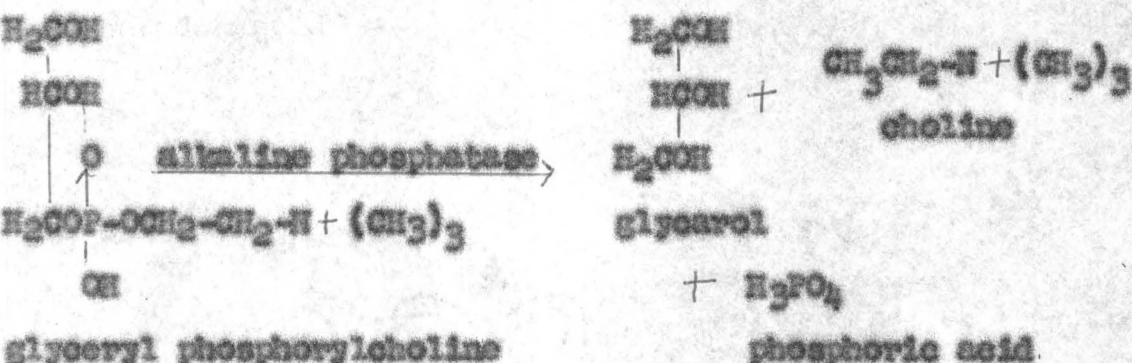
In this discussion, the metabolism of lecithin will be considered as representative of the phospholipides in general. Most of the work to date involving phospholipide metabolism has centered about this particular compound. In addition, some studies of the crude hapten seem to indicate that lecithin may very well be, in part at least, the phospholipide present.

The mode of metabolism of lipides is not, in general, clear at this time. The primary change in all fats in the gastrointestinal tract appears to be hydrolysis. This is, at best, minor in comparison to that which occurs in much of the protein and carbohydrate for in the case of the lipides, only the ester linkages may be attacked enzymatically. Within the phospholipide molecule, four ester linkages may be hydrolyzed and enzymes which apparently act upon these bonds have been isolated. The hydrolytic scheme for lecithin as proposed by Arton, is as follows:¹



Both Phospholipase A and B have been demonstrated in the gastrointestinal tract of many animals.² Specifically, Phospholipase A has been demonstrated in pancreatic extracts while Phospholipase B has been found in preparations of rat and rabbit intestine as well as in beef pancreas. Duodenal juice appears to have no hydrolytic effect on the phospholipides.³ The distribution of Phospholipases C and D does not appear to be as universal. Hanahan and Chaikoff demonstrated in steamed carrots and in steamed cabbage leaves a Phospholipase C which will form phosphatidic acids.^{4,5,6} The presence of Phospholipase D has been demonstrated by MacFarlane^{7,8} and by MacFarlane and Knight⁹ in the toxins of Clostridium oswaldense and Clostridium welchii. Furthermore, Phospholipases C and D appear to vary in their ability to attack the various phospholipide species.¹⁰

Deuel, reporting an alternate scheme for lecithin breakdown, indicates that the glycerylphosphorylcholine is split directly into glycerol, phosphoric acid, and choline by alkaline phosphatase.¹¹



Considerable controversy revolves about the extent to which lipides in general are hydrolyzed prior to absorption. Two theories have been put forth which, because of their applicability to the phospholipides, will be considered here. Frazer contends that complete hydrolysis of the fat molecule is essential for absorption to take place postulating the following mechanism for uptake in his Lipolytic Theory.¹² Fatty acids pass into the epithelial cells from the lumen of the intestine as fatty acid-bile salt complexes. (The bile salts Frazer feels bring the water insoluble fatty acids into solution.) These complexes are split within the epithelial cells into the free fatty acids which combine with the glycerol present to form new molecules of fat.

Phospholipides are believed by a number of investigators to play a major role as intermediates in this re-synthesis. Much evidence has accumulated which indicates that phospholipides are readily synthesized in the intestinal mucosa. Frazer and his associates found the cells of the small intestine to be the most active gastrointestinal site in phosphorylation.¹³ The proposed mechanism for fat re-synthesis involving the phospholipide intermediate consists of two steps. First, a fatty acid and a phospholipide would react to yield a base complex, phosphoric acid and glycerol. Then, the base complex, a new fatty acid and glycerol would combine to produce a new phospholipide. This obviously would result in

little turnover of phospholipide. Bollman and his colleagues, however, reported the passage of large quantities of this substance into thoracic and intestinal lymph during fat absorption.¹⁴ Contesting this observation, Arton indicated that the techniques employed in analyses of the phospholipides in lymph after fat feeding frequently pick up many non-phospholipide materials.¹⁵ In addition, Bloom and his colleagues demonstrated that 96 percent of the labeled fatty acids found in the lymph during absorption were not contained in phospholipide.¹⁶ Finally, Fraser pointed out that lipolysis could be forced in both directions in vitro in the absence of phospholipide.¹⁷

Arton maintains "there is at present, no acceptable evidence that phospholipides are obligatory intermediates in fat absorption. . . certain findings are at least suggestive however of a quantitatively less important role of phospholipide . . . several recent reports strongly support the hypothesis that complete hydrolysis of fats is not a prerequisite for their absorption."¹⁸

Directly opposed to the Lipolytic Theory, then, is the Partition Theory proposed by Fraser.¹⁹ This theory is based on the premise that unhydrolyzed fat is absorbed in a highly emulsified state. These observations have been supported by a number of recent investigations. Arton suggests that due to their greater affinity for water, monoglycerides and diglycerides can be absorbed as such.²⁰ Reiser and his

coworkers in a study of the thoracic duct lymph of rats after the feeding of labeled triglycerides, found 25 to 45 percent of the ingested triglycerides completely hydrolyzed and 55 to 75 percent split into the monoglycerides.²¹ The phospholipides in the lymph had been derived equally from the absorbed monoglycerides and fatty acids in this investigation.²² In addition, Arton and Swanson indicated that a portion of ingested phospholipide undergoes absorption without complete hydrolysis.²³ Rats fed labeled phospholipid materials showed a greater amount of p^{32} containing phospholipides in the blood than those having been fed equivalent amounts of tagged trisodium phosphate or glycerophosphate after a given period of time.

At any rate, the absorption of fats by the intestine has been demonstrated to consist of two phases. In the first phase, the products are transferred from the lumen of the intestine into the intestinal cells. Then the products, intact or otherwise, pass into the lymphatics or into the portal blood. Here again a division of opinion occurs. Fraser contended that fed free fatty acids were taken up by the portal blood while the fatty acids split from fed triglycerides passed into the lymphatics.²⁴ Reiser then demonstrated that in the rat, at least, the route of absorption is identical regardless of the form in which the fatty acid is ingested.²⁵ The fatty acids, he observed,

passed primarily into the thoracic and intestinal lymph. Bloom and his associates found this to be true also.²⁶ From the lymphatic system, the absorbed lipid material is presumed to pass into the systemic circulation from which it is actively removed by the liver.

Synthesis of phospholipid occurs in all cells, those of the liver, kidney and small intestine being the most active in this process.²⁷ Fairly conclusive evidence exists that the liver is the chief source of plasma phospholipid formation and degradation. Consequently, this discussion will be focused on activity in that organ.

Kedziora and his collaborators, Sinclair citing, found a 23 percent increase in the phospholipids of the blood leaving the liver of dogs.²⁸ Hovey and his associates and Fischler, Chalkoff citing, independently reported that tracers indicated the incorporation of inorganic phosphorus into plasma phospholipids occurs primarily in the liver. In addition, Fischler and his colleagues²⁹ and Goldman and his associates³¹ found that in the absence of the liver, p^{32} is not built into plasma phospholipids. Furthermore, Antman and his associates demonstrated that when the arterial supply to the liver remains intact, complete removal of the digestive tract has no effect on the rate of disappearance of labeled plasma phospholipids.³² Silverman and his associates reported that one-third of intravenously injected

labeled phospholipide could be found in the liver whereas none could be recovered in the thoracic lymph.³³ Reinhardt and his collaborators confirmed this and, in addition, showed that the thoracic duct lymph apparently returns to the plasma a significant portion of the phospholipides lost from it.³⁴

The mode of phospholipides synthesis in the liver is, at present, undergoing intensive investigation. Aronson indicates that through experiments with labeled molecules, all evidence points to direct synthesis from their components.³⁵ These compounds fairly well established as precursors include phosphate,³⁶ glycerol,³⁷ unsaturated fatty acids³⁸ and choline.^{39,40,41,42,43} Other compounds found to increase phospholipide turnover in the liver upon administration include betaine^{44,45} ethanolamine⁴⁶ glycine,⁴⁷ methionine⁴⁸ and cystine and cysteine⁴⁹. These, of course, represent precursors of choline and ethanolamine.⁵⁰

Aronson has postulated that because hydrolytic enzymes specific for the phospholipid linkages have been demonstrated, new phospholipid molecules may be built from the products of partial hydrolysis.⁵¹ He bases this premise on his own research in which he has observed the rapidity with which phospholipides are formed in the liver following single doses of choline, monoethanolamine, certain precursors and nitrogenous analogs. He feels that this system, in addition, would allow interconversion of the different types of

phospholipides and thereby explain changes in the relative amounts of liver phospholipides.⁵³

The manner in which the phospholipides are assembled in the liver has not been completely explained to date. However, cell free preparations of liver mitochondria have been demonstrated to synthesize phospholipides from inorganic phosphate in the presence of malate and adenosinetriphosphate.^{53,54,55}

Effect of Lipide Feeding on Plasma Phospholipide Levels

Bollman and his coworkers⁵⁶, Sinclair, citing Wendt,⁵⁷ McClure and his colleagues,⁵⁸ Flock and his associates,⁵⁹ and Deuel (quoting Eichholts)⁶⁰ have demonstrated that the feeding of neutral fat in large quantities results in a definite rise in lymph and plasma phospholipides. Flock found that phospholipide ingestion resulted in a rise in phospholipide, neutral fat and cholesterol in the plasma.⁶¹ Sinclair states that in the blood one can observe most readily a consistent and definite response to change in diet.⁶² One might conclude, therefore that the feeding of Rh hapten could be expected to result in a rise in plasma phospholipide levels. However, the women under treatment received merely one hundred to four hundred ug. of hapten daily for a period of fifteen to twenty months. There is no reason to believe that this small quantity of lipide would, in any way, alter the phospholipide level of the plasma.

The reason for the rise in plasma phospholipides following fat feeding is not clear. However, several roles have been ascribed to these phospholipides, two of which seem pertinent here. The Leathes Metabolic Theory, according to Sinclair, postulates that neutral fat is taken up by the liver, desaturated, transformed into phospholipides, passed into the blood and carried to the tissues where fatty acids are metabolized.⁶³ An increase in liver and blood phospholipide indeed occurs during fat absorption, during increased catabolism and transport of fatty acids and during increased heat production. Consequently, the increase of plasma phospholipides following fat feeding has been attributed to increased fat transport.⁶⁴ Indeed, Deuel states "since the original work of Leathes and Raper, it has generally been assumed the phospholipide function in fat transport."⁶⁵ However Chalkoff indicated that while a free interchange of plasma phospholipides occurs between the liver and plasma, no evidence can be found to indicate a similar interchange between the plasma and other tissues.⁶⁶ Entenman and his collaborators also challenge the transport theory by interpreting their experimental evidence on the rate of phospholipide turnover in hepatectomized dogs to indicate that the liver is far more important in the utilization of phospholipide than any other tissue.⁶⁷ Arton does believe, however, that some sort of transport of fat and cholesterol via

phospholipide is possible because of the polar groups within the phospholipide molecule.⁶⁸

Fraser attributes the major role of phospholipides in the bloodstream to the stabilization of the film on circulating fat droplets.⁶⁹ Indeed, fat droplets lacking this film have been demonstrated to flocculate in the presence of plasma proteins and a 1 percent sodium chloride solution at pH 5. Just how the phospholipide sheath is added to the particle is not clear, but may occur in the intestine.⁷⁰ An increase in plasma phospholipides could, therefore, be due to an increase in fat particles in the plasma.

Pregnancy and Phospholipide Levels

During pregnancy and lactation, some workers have found a mild increase in phospholipids of the blood. Boyd observed that the lipemia of pregnancy appears to be restricted almost entirely to the plasma.⁷¹ He reported that neutral fat begins to increase during the first trimester whereas phospholipide and cholesterol begin to rise during the second trimester. At term neutral fat increased about 100 percent of the value for non-pregnant women in his study whereas the phospholipide and free cholesterol showed approximately a 25 percent rise.⁷² The lecithin-cholesterol ratio remained fairly constant.

Tyler and Underhill found an increase of 33 percent in lecithin at term and were able to demonstrate an increase by the third month of pregnancy.⁷³ They also found that the

lecithin-cholesterol ratio appeared to be altered only slightly.

A slight increase in plasma phospholipid levels during pregnancy has also been observed by Oser and his coworkers.⁷⁴ In addition, Peters and associates reported the occurrence of a drop in phospholipid levels during the first twelve weeks after which a progressive rise could be observed until term.⁷⁵ After delivery, the value soon dropped to normal.

Immunization and Phospholipide Levels

The immunization of rabbits has been demonstrated to result in a rise in the phospholipid content of the immune serum⁷⁶ (Deuel citing Chino). This effect, however, may have been a result of the introduction of an antigenic substance rather than a result of antibody formation.

Adrenocorticotropin Administration and Phospholipide Levels

Recently, increasing evidence has accumulated indicating that lipid metabolism, along with that of the proteins and carbohydrates, may be regulated through an endocrine mechanism.⁷⁷ Indeed the adenohypophysis and the thyroid are already known to be involved. Aldersberg and colleagues in a further investigation of this concept, undertook a study of the level of plasma phospholipides during the administration of the adrenocorticotropic hormone (ACTH).⁷⁸ During the

time daily doses of seventy-five to one hundred mg. of ACTH were administered, a slight increase in plasma phospholipides could be observed. In addition, ACTH is known to decrease antibody formation. Since Mh hapten also decreases antibody production, it is not unreasonable to suspect that the administration of this substance could result in a similar release of phospholipide and thereby increase plasma phospholipide levels somewhat.

Summary

In summary, Arton, in 1952, stated, with foresight, "It seems likely that the differences in the rate of phospholipide formation observed in vivo are not due to differences in the amounts of specific enzymes present in each tissue but rather to regulatory mechanisms (perhaps of a hormonal nature) which maintain the rate of the process at a level characteristic of each tissue. . . in a given tissue changes will presumably occur in the amounts of phospholipides or in the rate of their synthesis when the metabolic needs of the tissue are increased or decreased, or when the supply of materials needed for the synthesis of phospholipides becomes insufficient, or when the efficiency of the regulatory mechanism is impaired."⁷⁹

FOOTNOTES

Chapter 3

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Chapter 4

THE METHODS AND FINDINGS

Description of the laboratory methods employed, presentation of experimental data and interpretation of this data will constitute the threefold objective of this chapter.

Methods

Three groups, two serving as controls, took part in this investigation of the circulating lipid phosphorus. The first group consisted of five pregnant Rh hapten treated women. Private physicians collected biweekly blood samples from these women and shipped the specimens to the laboratory by air mail. Upon receipt, the blood samples were refrigerated and the cells allowed to settle. Lipid phosphorus determinations were carried out on the specimens within twenty-four hours after arrival. The second group, a control group, was made up of eleven nontreated pregnant women for as long as previously, pregnancy has been demonstrated by some investigators to result in a rise in the lipid phosphorus levels over those observed for non-pregnant women. All except two of these blood samples were drawn by private physicians and shipped airmail to the laboratory. The third group, also a control group, consisted of seven nontreated nonpregnant individuals and served to insure the accuracy of the determinations as well as for comparison purposes. These blood specimens were taken in the laboratory and centrifuged thirty

minutes after drawing. The test for lipid phosphorus was carried out immediately thereafter.

Each test reported represents a different blood sample run in duplicate and occasionally in triplicate. A normal control serum was run simultaneously with each group of blood samples.

The lipemia of pregnancy, Boyd observed, appears to be restricted primarily to the plasma.¹ Consequently, this study involved an investigation of the serum lipid phosphorus values only.

The initial method employed in determining the lipid phosphorus values of the serum followed that of Fiske and Subbarow as outlined by Hawk, Oser and Summerson.²

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.

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 percent 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 percent 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 photometer 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 2 ml. of aminonaphtholsulfonic acid reagent. Dilute with water to the 100 ml. mark, mix,

allow to stand five minutes. Compare the standard against itself in the photometer before reading the unknown. If the color of the unknown is particularly strong, repeat the reading a few minutes later to be sure that maximal color development has taken place. A reagent blank was also prepared in a manner analogous to that of the standard using a 25 ml. volumetric flask and the following quantities of reagents: 12 ml. of water, 2.5 ml. of molybdate I, 1 ml. of aminonaphtholsulfonic acid reagent, and dilution with water to the 25 ml. mark. Calculation of the quantity of lipid phosphorus present is based on a colorimetric determination and is made as follows:

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

1 readings were taken on a Bausch and Lomb photometer.

Frequently, difficulty in obtaining the desired color changes were encountered with this method. As this phenomenon could not be attributed to the reagents or to cleanliness of the glassware, a change to the method of Silverman and Davis was undertaken late in February.³ No further trouble occurred. This method will now be described.

Deliver 0.2 ml. of plasma into a centrifuge tube containing 3 ml. of water. Add 3 ml. of 10 percent trichloroacetic acid while shaking constantly, the first 1/2 ml. drop by drop and the remainder more rapidly. Allow

the tube to stand for 1 to 2 minutes, then centrifuge for several minutes until the precipitate is tightly packed. Decant the supernatant and invert the tube until practically all the supernatant has been removed. Add 1 ml. of 60 percent perchloric acid and a boiling chip. After 20-30 minutes heating, the solutions are clear and colorless. When cool, transfer the digest to a 10 ml. volumetric flask or to a calibrated test tube. However, to avoid loss of phosphorus by transfer, reagents may be added directly to the digestion flask in the following amounts: 7 ml. of water, 1 ml. of 4 percent ammonium molybdate solution, and 1 ml. of aminonaphtholsulfonic acid reagent. A reagent blank is prepared by mixing 0.8 ml. of 60 percent perchloric acid, 7 ml. water, 1 ml. of 4 percent ammonium molybdate solution, and 1 ml. of aminonaphtholsulfonic acid reagent. The standards and unknowns are arranged in the following order: 2 standards, the unknowns, and 1 standard. In adding the 4 percent ammonium molybdate solution, allow the reagent sufficient time to mix before adding the aminonaphtholsulfonic acid reagent. The latter reagent must be added at approximately the same rate the samples are expected to be read. Read the samples after approximately 20 minutes, setting the spectrophotometer at 0 absorbance with the reagent blank. The wavelength is 660 mμ. The computation may then be made in the following manner:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} = \frac{0.02}{0.2} \times \frac{1}{1} \times 100 = \text{mg. of lipid phosphorus per 100 ml. of serum or plasma}$$

FINDINGS

Table 1

Mg. of Lipide Phosphorus/100 cc. serum

Woman (Treated Pregnant)	Fiske and Subbarow						
	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7
A	10.6	10.3	10.2	14.25	16.3*	10.9*	14.2
B	9.55	9.4	11.4				
C	9.45	10.8 ⁺	N.C.C.	12.6	11.2*	12.5	
D	10.76 ⁺	9.8 ⁺	9.9				
E	N.C.C.	12.2*					
	Zilvermit and Davis						
	Test 8	Test 9	Test 10	Test 11	Test 12	Test 13	
A	16.3	18.65	17.8 ⁺	18.6	18.6	18.3	
B							
C	11.9	13.8	11.7 ⁺	11.5	14.7	15.6	14.3
D	12.4	13.8	13.4 ⁺	14.7	13.4	12.0	
E	11.6	11.9	16.1	8.9	12.3	11.7	

- * - Reagent believed bad
 - Test run before hapten begun
 - Hemolyzed sample
 N.C.C. - No color change

Level of Lipide Phosphorus in Biweekly Serum Samples of
 Pregnant Treated Women as Determined by the Methods of
 Fiske and Subbarow and Zilvermit and Davis

Table 2

Silvermit and Davis

Woman (Nontreated Pregnant)	Mg. Lipide P/100 cc. serum
F	14.0
G	15.0
H	18.3
I	12.9
J	10.1
K	11.1
L	14.8
M	13.2
N	11.5
O	11.2
P	12.1

(Several more samples were available but because these showed hemolysis, serious interference with color development occurred. These results do not appear in the table.)

Level of Lipide Phosphorus in the Serum of Nontreated Pregnant Women in the Second and Third Trimesters of Pregnancy as Determined by the Method of Silvermit and Davis

Table 3

Mg. of Lipide Phosphorus/100 cc. serumFiske and Subbarow

<u>Individual</u>	<u>Test 1</u>	<u>Test 2</u>	<u>Test 3</u>	<u>Average</u>
Q (female)	9.17	9.07	8.5*	
R (male)	10.5			
S (male)	9.45			
T (male)	10.8			
U (female)	12.3			
V (male)	11.7			
W (female)				

Zilversmit and Davis

	<u>Test 1</u>	<u>Test 2</u>	<u>Test 3</u>	<u>Average</u>
Q (female)	9.2	8.6		
R (male)				
S (male)	8.8	9.5		
T (male)	9.9			
U (female)				
V (male)				
W (female)	8.2			

* - Reagent believed bad

Level of Lipide Phosphorus in the Serum of Normal
Individuals as Determined by the Methods of
Fiske and Subbarow and Zilversmit and Davis

Normal lipid phosphorus values of the serum range from 8 to 10 mg. per 100 cc. of serum for the method of Fiske and Subbarow according to Hawk and his colleagues⁴. Zilverman and Davis, however, found a range of 6.1 to 14.5 mg. of lipid phosphorus per 100 cc. of serum for normal individuals with a mean value of 9.2 mg.⁵ In spite of this apparent variation, the latter authors reported a very high correlation between the values obtained using their trichloroacetic acid method and those found for the ethanol-ether extraction.⁶

A fairly progressive rise in the lipid phosphorus values of the biweekly serum samples collected from haptan treated women can be observed. However, according to Peters and associates this is to be expected from the twelfth week of pregnancy to term.⁷ In comparing these values against those obtained for pregnant nontreated women, only three samples from one woman receiving the haptan exceeded the highest control value. The three samples, however, contained merely 0.3 to 0.35 mg. of lipid phosphorus more than that of the highest nontreated woman. The deviation on the samples, all run in duplicate or triplicate, never varied more than 0.2 mg. in any of the three groups.

A statistical comparison of the three groups revealed the following information. When the values for the treated pregnant women were compared against those of the nontreated pregnant women, $T = .21$. This is not significant. On the

other hand, comparing the treated pregnant women against normal nontreated nonpregnant individuals, $T = 4.26$. In addition, in a comparison of nontreated pregnant women against nontreated nonpregnant individuals, $T = 4.90$. The latter two values are highly significant at the 1 percent level of confidence. Therefore, no significant difference has been demonstrated in the serum phospholipide levels of pregnant women to whom K_n hapten has been administered and those receiving no hapten. The increase observed in these values may be attributed to pregnancy alone.

An additional interesting observation may be made here. The first test on treated pregnant woman D was run on a blood sample taken one week prior to the initiation of hapten treatment for the present pregnancy. The next two determinations, made on blood samples collected approximately one and three weeks after this time, showed somewhat depressed values which may have been due to the drop observed during the first weeks of pregnancy by Peters and coworkers.⁸

FOOTNOTES

Chapter 4

¹Boyd, op. cit., p. 347.

²P. B. Hawk, B. L. Oser, and W. H. Summerson, Practical Physiological Chemistry, New York: The Macmillan Co., Inc., 1954 (revised), 589.

³D. B. Zilvermit and A. K. Davis, "Phosphatides in Plasma," pp. 133-135 in Standard Methods of Clinical Chemistry, II. New York: Academic Press, Inc., 1953.

⁴Hawk and others, op. cit., p. 590.

⁵Zilvermit and Davis, op. cit., p. 135.

⁶D. B. Zilvermit and A. K. Davis, "Microdetermination of Plasma Phospholipids by Trichloroacetic Acid Precipitation," Journal of Laboratory and Clinical Medicine, XXXV (January 1950), 153 et seq.

⁷Peters, and others, op. cit., p. 388.

⁸Ibid., ad. loc.

Chapter 5

SUMMARY

Although the incidence of erythroblastosis fetalis is very small, this disease has been a source of grief to many families throughout history. Since the discovery of the first Rh agglutinin in 1940 and the identification of the specific mechanism involved in hemolytic disease of the newborn shortly thereafter, intensive work has been carried on to find some way to relieve or prevent the condition. At present, by far the most successful approach to this problem is through the use of Rh hapten. This material, extracted from Rh positive erythrocytes, appears to neutralize the antibodies in the mother which are so harmful to the unborn child. Providing the antibody titer is eliminated prior to pregnancy and treatment is again initiated as soon as pregnancy is suspected, Rh hapten allows the sensitized mother to bear normal infants.

The research carried on at Western Michigan University for the past year has been an attempt to evaluate further the effect of Rh hapten ingestion on the body, or, more specifically, its possible effect on the circulating blood phosphorus. The purpose of this thesis has been to describe this work.

Rh hapten, as indicated previously, appears to be a lipid in nature and contains nitrogen, phosphorus and

unsaturated sterols. Therefore, the ingestion of Rh hapten, due to its nature and its apparent action within the body, might be expected to alter the lipid phosphorus values of the serum. The reasons for this are several. First, the feeding of large quantities of fat has been demonstrated by numerous researchers to elevate the serum phospholipid level. However, in this study, dietary effects probably represent the least important consideration for the treated women received merely one hundred to four hundred mg. of hapten daily for a period of twelve to eighteen months. Secondly, during a time of active antibody formation, an increase in serum lipid phosphorus values has been noted. Third, administration of ACTH is known to lower antibody titers if prolonged as well as to increase the serum lipid phosphorus. Should the two observations be related, Rh hapten, which also lowers antibody levels, could conceivably raise the lipid phosphorus. Fourth, the stimulation of some unrelated endogenous mechanism to release phospholipids into the serum must be considered. An additional factor, unrelated to hapten administration also is of importance. Pregnancy has been demonstrated by a number of investigators to elevate the serum phospholipid level over that observed in non-pregnant women. Therefore, the demonstration of a significant increase in serum lipid phosphorus values over those shown in normal nontreated pregnant women would be a specific effect

of Rh haptan administration.

The approach to the problem involved the two avenues followed in all research. These include the extensive search of the literature for pertinent information as well as experimental observations.

The library investigation covered several areas related to this study. These included the background of erythroblastosis fetalis, the developmental and clinical work on Rh haptan to the present, the metabolism of phospholipides and the five factors just outlined.

The laboratory techniques for the determination of serum lipid phosphorus employed in this study involved, initially, the well standardized Fiske and Subbarow method. Later the method of Zilvermit and Davis which has been successfully utilized in experimental work with dogs prior to adaptation to clinical use was employed. Serum lipid phosphorus determinations were made not only on samples obtained from five pregnant treated women but on a group of nonpregnant nontreated individuals and on a series of nontreated pregnant women as well. The latter two groups served as controls.

Only three samples from one haptan treated woman exceeded the highest value obtained on nontreated pregnant women. This amounted to a maximum of merely 0.35 milligrams of lipid phosphorus over the control. In addition, a

statistical comparison between pregnant treated women and pregnant nontreated women showed no significant difference in the two groups. Therefore, no significant difference has been demonstrated between the lipid phosphorus levels of treated and nontreated pregnant women, due to Fh hapten ingestion.

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