MAITRA, Tushar Kanti, 1938-
A STUDY OF THE ALPHA-2 FRACTION OF SERUM PROTEIN IN VIRAL HEPATITIS.

The Ohio State University, Ph.D., 1970
Health Sciences, pathology

University Microfilms, A XEROX Company, Ann Arbor, Michigan
A STUDY OF THE ALPHA-2
FRACTION OF SERUM PROTEIN IN VIRAL HEPATITIS

DISSERTATION
Presented in Partial Fulfillment of the Requirement for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Tushar Kanti Maitra, M.B.B.S., M.Sc.

* * * * * *

The Ohio State University
1970

Approved by

[Signature]
Adviser
Department of Pathology
ACKNOWLEDGMENTS

I wish to express my sincere appreciation and indebtedness to my advisers, Dr. S.G. Murphy, for his guidance, criticism and active participation and to Dr. C.R. Macpherson for his criticism and encouragement throughout the present investigation.

I am grateful to Dr. A.S. Klainer and his laboratory personnel for helping me carry out various phases of the investigation.

My sincere thanks are also due to Drs. L. Ayers, K. Skitarelic and Mr. Richard Sellers and to other fellow graduate students and residents of the Department of Pathology and to the members of the Department of Pathology.
VITA

January 15, 1938  Born - Purnea (Bihar), India

1954  B.Sc. (Chemistry Honours), Calcutta University, Calcutta, India.

1960  M.B.B.S., Calcutta University, Calcutta, India.

1960 - 1963  Worked as house officer in Obstetrics and Gynecology, Calcutta Medical College Hospital. Also obtained diploma in Obstetrics and Gynecology from Calcutta University, India.

1963 - 1964  Rotating internship, De Paul Hospital, Norfolk, Virginia.

1964 - 1965  First year residency in Anatomical Pathology at Mount Sinai Hospital, Cleveland, Ohio.

1965 - 1969  Completed residency training in Anatomical and Clinical Pathology at the Ohio State University Hospital.

1968  Obtained M.Sc. in Pathology, Ohio State University, Columbus, Ohio.

FIELDS OF STUDY

Major Field: Pathology

Laboratory Medicine. Professor Colin R. Macpherson
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>81</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSION</td>
<td>91</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>93</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

SGOT = Serum glutamate-aspartate transaminase
SGPT = Serum glutamate-pyruvate transaminase
LDH = Lactic dehydrogenase
K_{AV} = \frac{Ve - Vo}{V_t - Vo}
Tris = Tris (hydroxymethyl) - aminoethane
DEAE = diethylaminoethyl
Ve = elution volume in ml
V_t = total volume in ml
IH = infectious hepatitis
SH = serum hepatitis

Alpha VH band = An alpha-2 glycoprotein noted in the sera of patients with viral hepatitis. The words "glycoprotein" and "mucoprotein" have been used interchangeably.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Chemistry of the flocculation tests</td>
<td>7</td>
</tr>
<tr>
<td>2 Total protein and percentage distribution of electrophoretic fractions</td>
<td>46</td>
</tr>
<tr>
<td>3 Percentage distribution of glycoprotein fractions in serum</td>
<td>47</td>
</tr>
<tr>
<td>4 Summary of screening experiments</td>
<td>57</td>
</tr>
<tr>
<td>5 Sephadex G-200 chromatography</td>
<td>66</td>
</tr>
<tr>
<td>6 Electrophoretic and immunoelectrophoretic patterns of various fractions of serum proteins</td>
<td>80</td>
</tr>
<tr>
<td>7 Comparison of Australia antigen and alpha VH band</td>
<td>89</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>68</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>11</td>
<td>73</td>
</tr>
<tr>
<td>12</td>
<td>79</td>
</tr>
</tbody>
</table>
INTRODUCTION

Viral hepatitis describes those forms of hepatitis that are caused by two or more hepatotropic filtrable infectious agents not yet fully identifiable by either specific antigenic or cultural methods. These agents produce systemic diseases with a characteristic type of liver injury (73). Two forms of the disease, infectious hepatitis (IH) and serum hepatitis (SH) have been known for the last 25 years. Knowledge of etiologic agents of these two forms has been derived largely from experimental human transmission studies. Though certain similarities have been observed in the clinical manifestations and in the liver morphology between IH and SH, they differ in their incubation period (27), and possibly in their immunologic properties (33), and mode of transmission (27).

In spite of the knowledge which has accumulated about IH and SH infections, no specific diagnostic tests for viral hepatitis are available. The diagnosis depends mainly on clinical and epidemiologic evidence and the pattern formed by certain nonspecific laboratory data.

In the differential diagnosis of viral hepatitis from hemolytic jaundice and obstructive jaundice, the most commonly
employed laboratory tests are the determination of serum bilirubin and its conjugates, and of bile derivatives in urine and feces. The dye excretion test using bromsulfophalein (BSP) is of particular value in identifying liver dysfunction in the absence of jaundice. Serum protein determination and serum flocculation tests, because of their lack of specificity, are of limited value. Recently the most promising results have been obtained from enzyme determinations of which the serum transaminases are considered the most important (108). Validation of these diagnostic procedures is dependant on comparison with the histologic features of liver obtained by biopsy. Though liver biopsy has provided much information, its clinical applicability is limited by occasional complications such as intra-abdominal hemorrhage, bile peritonitis and pneumothorax. Liver biopsy is contraindicated in patients with hypocoagulability of blood sometimes encountered in viral hepatitis (96). Moreover it is not always possible to arrive at correct etiologic diagnosis from the morphologic feature seen in liver biopsy material (5).

In cases of viral hepatitis having classical clinical manifestations the above mentioned laboratory procedures frequently help to establish the diagnosis. The problem however, is the diagnosis of anicteric viral hepatitis, which often
lacks general systemic manifestations. Recently attempts have been made to diagnose this variant of viral hepatitis by the use of serum transaminase determinations. As in mild clinical manifestations, the serum transaminase levels in anicteric hepatitis may show no, or only a transient, rise. The recent identification of a hepatitis-related antigen by Blumberg (11) and by Prince (82) may be a major step towards a specific method of diagnosis.

During the last 30 years the technique of electrophoretic separation of proteins in various body fluids has been incorporated as one of the routine diagnostic procedures of the clinical laboratory. Though alterations in the serum protein patterns have proved valuable in the differential diagnosis of many diseases, in viral hepatitis a consistent pattern has not been reported. The literature contains many contradictory findings with respect to both protein and glycoprotein changes in this disease.

Within the last decade, gel chromatography, in which the separation of protein is dependant on molecular size, has become a clinically applicable tool as well as an excellent method for the separation of proteins. If two parameters of separation are employed e.g., size and charge, minor changes not demonstrable with a single method may become manifest.

Utilizing the above approach, serum protein patterns from individuals with viral hepatitis were examined after applying
two parameters of separation. The overall objectives of this study were:

i) to determine and delineate the specificity of serum non-gamma globulin changes in viral hepatitis,

ii) to evaluate the diagnostic importance of such changes in this disease,

iii) to correlate these changes with other liver function tests in viral hepatitis.
REVIEW OF LITERATURE

In order to define the diagnostic problems in viral hepatitis various epidemiologic, clinical and pathophysiologic studies have been undertaken using spontaneous clinical cases and experimentally transmitted viral hepatitis in human volunteers (24, 40, 43, 73).

The majority of the pathophysiologic studies have dealt with the changes in various serum components. Consequently, many nonspecific laboratory diagnostic tests for viral hepatitis have evolved. The literature presented covers primarily those investigations which relate either directly or indirectly to alterations in one or more of the serum protein components.

A) Serum flocculation tests

These are empirical tests which differ only in the nature of the precipitating agent, the pH, and the ionic strength of the test system. More than 25 flocculation and turbidity tests have been reported (88). During the last 50 years, a few of them have been used consistently as part of the "liver function test battery." Table 1 is a summary of the most commonly used
flocculation and turbidity tests (61). Using electrophoretically separated protein fractions from normal and pathological sera, it was observed that an increase of gamma globulin and decrease of albumin were the significant factors which caused the flocculation and turbidity reactions (21, 44, 68). The reaction was not due to a simple alteration in albumin-globulin ratio. The addition of normal serum albumin inhibited the reaction to a greater extent than albumin from patients with viral hepatitis. This inhibitory effect of normal albumin was lost if it was stored at 4\(^\circ\). Deviations from normal in other serum components also influenced these tests. Increased alpha-1 globulin, seromucoid and mucoproteins were inhibitory, whereas decreased alpha-2 globulins and lipoproteins favoured these reactions (88).

In the earlier literature the flocculation tests were evaluated singly or in combinations for their diagnostic reliability. Often they were utilized in conjunction with enzyme assays, bilirubin determinations, and BSP retention among others, in order to obtain greater reliability (43, 60). This procedure of multiple test analysis was required since these tests were relatively nonspecific for viral hepatitis. The fact that flocculation tests are nonspecific has been observed in numerous studies on liver disease and its related laboratory findings. Studies of this type have been published regularly for the past fifty years (88).
<table>
<thead>
<tr>
<th>Test</th>
<th>Precipitating agents</th>
<th>Proteins fractions active</th>
<th>Results indicated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takata-Ara</td>
<td>Hgcl₂</td>
<td>alpha, beta &amp; gamma globulins</td>
<td>albumin</td>
</tr>
<tr>
<td>Cephalin-cholesterol</td>
<td>Emulsion of cephalin and cholesterol</td>
<td>(alpha, beta), gamma globulins</td>
<td>albumin</td>
</tr>
<tr>
<td>Colloidal Gold</td>
<td>Colloidal Gold</td>
<td>gamma globulins</td>
<td>albumin, alpha beta globulins</td>
</tr>
<tr>
<td>Thymol tests</td>
<td>Thymol solution</td>
<td>(beta), gamma globulins</td>
<td>albumin</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>ZnSO₄ soln.</td>
<td>gamma globulin</td>
<td>-----</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>(NH₄)₂SO₄ soln.</td>
<td>gamma globulin</td>
<td>-----</td>
</tr>
<tr>
<td>Colloidal Red</td>
<td>Colloidal susp. of Scarlet red dye</td>
<td>gamma globulin</td>
<td>Amount of flocculation in the tube</td>
</tr>
</tbody>
</table>

*Based on Maclagan's table (61).
The most widely used flocculation test has been the cephalin-cholesterol. It has in certain studies been found superior to the thymol and colloidal gold tests. The cephalin-cholesterol test has been reported to be the most sensitive indicator of hepatic parenchymal involvement (72). It is however nonspecific since there is little or no correlation between the flocculation test and histologic changes in the liver. In addition the test is positive in certain diseases which do not appear to affect the liver (88).

The thymol turbidity test has been used to differentiate hepatitis from obstructive jaundice. However cases of cholestatic jaundice due to drugs such as chlorpromazine have been found to cause marked thymol turbidity. Moreover, some cases of infectious mononucleosis gave positive thymol turbidity tests, but it must be remembered that hepatitis does occur in this disease. Compared to thymol turbidity, the thymol flocculation reaction was less frequently positive in acute parenchymatous liver disease (88).

Two examples of the multiple test approach are summarized to illustrate the type of literature often encountered.

A comparative evaluation of cephalin-cholesterol, colloidal gold thymol tests and determination of serum alkaline phosphatase was made on 489 cases of jaundice. In 137 cases of post-hepatic jaundice the different flocculation reactions and
the serum alkaline phosphatase levels showed a similar diagnostic value. When considered in pairs the greatest diagnostic reliability was found in a combination consisting of any of the flocculation tests with the phosphatase determination. In $85\%$ of the obstructive jaundice cases, all flocculation tests were negative whereas the phosphatase values were elevated. Out of 361 cases of hepatocellular jaundice, all flocculation tests were positive in $70\%$ of the cases. Thus in $30\%$ of the cases of hepatocellular jaundice the results of a single test were spurious (25).

In a study of 34 cases of experimental human hepatitis, the following liver function tests were compared for their diagnostic value:

1. BSP excretion at 45 minutes.
2. Total and direct serum bilirubin.
3. Urinary bilirubin by Harrison spot method and Methylene blue test.
4. Urinary urobilinogen.
5. Cephalin flocculation at 24 and 48 hours.
6. Thymol flocculation.
7. Thymol turbidity.
8. Colloidal gold reaction.

Certain differences were observed in the early and convalescent stages of the disease. The BSP retention and bilirubinuria were the first to indicate hepatic disturbance but the flocculation tests appeared superior in demonstrating persistent hepatic involvement in the convalescent stage (71).
The other flocculation test such as the Takata-Ara, Zinc sulfate, colloidal red, colloidal gold, and others have been utilized in comparative studies. None has appeared to be specific for hepatitis. Since all flocculation test depend upon serum protein alterations which are found in other primarily non-hepatic diseases such as malaria, infectious mononucleosis, Kala-azar, multiple myeloma and collagen diseases they are nonspecific (88). Sherlock has stated, "There is little to be said for performing more than two flocculation tests, preferably the thymol turbidity and the zinc sulfate method, and much to be gained from abandoning them" (100). This point of view is mandatory if the literature is even partially reviewed.

B) Serologic and immunologic tests

Eaton and Murphy reported the occurrence of an antibody in some viral hepatitis patients, which fixed complement in the presence of liver tissue. The antibody was found to be nonspecific because sera from patients with diseases other than viral hepatitis also fixed complement (26).

Further attempts were made to isolate a specific viral antigen or to elicit a specific antibody against the causative organism by injecting tissue homogenates from various organs of hepatitis patients. These attempts met with uniform failure in that cross-reactions occurred with normal
tissue extracts as well as those from other disease states. In addition such antibodies often cross-reacted with antigens from other animal species. These results have now been explained on the basis of tissue or organ specific antigens (75). Contradictory findings were reported on the occurrence of heterophile antibody in patients with viral hepatitis (39).

Red cells from several animal species have been shown to agglutinate when mixed with sera from patients with viral hepatitis. This assay was abandoned when it was shown that normal sera and sera from patients with diseases other than hepatitis also caused agglutination (42, 64, 69). The nature of these hemagglutinating factors has not been clarified (64).

O'Malley and co-workers isolated a virus from the NIH-6 icterogenic plasma pool in 1961, which they called A-l isolate. They used a plaque-reduction neutralization test to demonstrate antibodies against the A-l isolate in sera from patients with experimental viral hepatitis (76). Five isolates from human volunteers inoculated with the plasma pool and one isolate from the liver of a fatal case of infectious hepatitis were used to immunize rabbits. The antisera showed serological cross-reactivity between the isolates. In addition complement-fixing and neutralizing antibodies were shown to be present in the convalescent sera. The complement-fixation test was unsatisfactory since sera from these volunteers were anticomplementary (12). When latex particles were coated with the above viral
isolates antibodies were demonstrated in nearly all cases of viral hepatitis and in a varying percentage of the normal population. This test was designated as the HIM test (13). In a clinical evaluation, 150 control sera and sera from patients with various other diseases were assayed. The HIM antigen detected agglutinins in 90-100% of cases with viral hepatitis and infectious mononucleosis. However, 40% of the control sera as well as sera from patients with other diseases were positive (107).

Another antigenic component, an alpha protein was described by Blumberg and Allison in 1961 (11, 59). This antigen was later called "Australia antigen" because of its natural occurrence in the sera of some of the Australian aborigines. More recently Prince has described an antigen which he called SH-antigen. It was found in the serum during the incubation period of a classical post-transfusion hepatitis case. In 9 cases of post-transfusion hepatitis he noted the occurrence of this antigen in 7 (82). The antibody to both Australia and SH-antigens was detected in patients receiving multiple blood transfusions, particularly hemophiliacs. The Australia antigen of Blumberg and the SH-antigen of Prince are immunologically identical (83). Australia antigen has also been reported to occur in the sera of patients with lepromatous leprosy, leukemia, and Down's syndrome (8, 9, 10, 103).
C) **Serum enzyme determinations**

Many serum enzyme determinations (108) have been utilized in the evaluation of viral hepatitis and other liver diseases. Of these, serum alkaline phosphatase, the transaminases, and lactic dehydrogenase are most frequently evaluated.

i) **Serum alkaline phosphatase**

The role of serum alkaline phosphatase in the diagnosis of liver disease was recognized over 30 years ago. In 1933 Roberts attempted to differentiate several types of jaundice by blood phosphatase and Van den Bergh determination. All cases of obstructive jaundice showed a direct Van den Bergh reaction with very high blood phosphatase activity. Blood phosphatase activity was moderately increased in patients with toxic or catarrhal jaundice (90). A later study demonstrated that the majority of the obstructive jaundice cases had marked elevation of serum alkaline-phosphatase, while catarrhal jaundice cases showed a moderate elevation. Certain cases of liver cirrhosis and toxic jaundice gave results similar to catarrhal jaundice (37).

Maclagan observed that in some of his cases of viral hepatitis the serum alkaline phosphatase values were high but nondiagnostic because both hepatitis and biliary obstruction had elevated serum phosphatase values. The
diagnosis could be established by combining the determination of serum alkaline phosphatase with one of the three flocculation tests, colloidal gold, thymol turbidity or thymol flocculation (60). In some studies occasional higher levels of serum alkaline phosphatase in viral hepatitis have been reported (108). However, most investigators have concluded that marked elevation of serum alkaline phosphatase is diagnostic of both intra and extra hepatic biliary obstruction. Values obtained for viral hepatitis are quite variable (108, 116, 117).

Increased serum alkaline phosphatase activity is not a consistent indicator of acute or chronic parenchymatous liver damage.

ii) Transaminases

Karmen, Wroblewski, and Ladue in 1955, introduced the method for the determination of transaminases in serum and whole blood (46). Increased glutamate-aspartate transaminase (SGOT) activity appeared to be more specific than the previous enzyme for both icteric and anicteric viral hepatitis. On the basis of animal experiment, it was shown that the rise in SGOT was directly related to the degree of liver damage induced by carbon tetrachloride or virus infection (114).
In a series of cases with various liver diseases, 20 to 500-fold increase in SGOT levels were seen in patients with acute viral hepatitis and carbon tetrachloride poisoning. The initial elevated SGOT level fell after 5-10 days. In no case was there any correlation between the transaminase level and other liver function tests (113). During the asymptomatic stage of viral hepatitis when the other liver function tests were normal, the SGOT showed elevation. Subsequently the transaminase increased further as the patient became clinically ill (114). In a study of 200 cases of viral hepatitis, most patients had SGOT levels from 10 to 100 times the normal. With resolution a gradual decrease was characteristic with a normal level being reached at 4 to 8 weeks after the onset of the disease. Persistent elevation of the SGOT level reflected active disease while a secondary rise signified relapse. The changes in SGOT is not specific for viral hepatitis, since moderate to higher elevation of SGOT has been reported in various hepatic and nonhepatic diseases (6, 108).

Wroblewski and his co-workers introduced the determination of serum glutamate-pyruvate transaminase (SGPT), in the evaluation of hepatic diseases in 1956. They noted
that in acute liver cell injury, as seen in acute hepatitis, there was marked increase in the SGPT activity. Elevation of SGPT was more pronounced than SGOT (115). It was observed that both these enzymes showed elevation during the prodromal stage of viral hepatitis and reached their peaks at a time when the patients were seriously ill. With subjective and objective improvement of the patient, the transaminases tended to return to normal (116).

Chinsky and co-worker did a comparative evaluation of SGOT and SGPT changes in various diseases. In a variety of liver diseases both of these transaminases were elevated. In viral hepatitis the SGPT elevation was often, but not always higher than SGOT (19). As such, the determination of SGOT/SGPT ratio becomes of less diagnostic value, though some investigators have considered a drop below 1 of this ratio as diagnostic of viral hepatitis (22).

While discussing the diagnostic value of serum transaminase determination Batsakis and Briere concluded. . . . "the serum transaminases are of value in the recognition of acute hepatic injury; i.e., infectious hepatitis, damage from hepatotoxins, and neoplastic metastases to the liver. In the early stages of the disease, the transaminases
will aid in distinguishing the foregoing from obstructive jaundice or cirrhosis. With increasing chronicity of extrahepatic obstruction, secondary alterations in the hepatic parenchyma occur, and a distinction on the basis of transaminase determinations is not always possible. The relative degree of elevation of the levels may be of some diagnostic value" (5). Therefore neither single transaminase determinations nor evaluation of their ratio can be accepted as a reliable diagnostic procedure in viral hepatitis.

iii) **Lactic dehydrogenase (LDH)**

LDH was found to be elevated in the early stages of viral hepatitis but the rise was relatively small compared to that seen in SGOT and SGPT (6). No correlation was noted between LDH and transaminase values. There were cases with normal LDH and very high transaminase values, whereas some cases with moderate transaminase levels showed a marked rise in LDH activity (18). LDH activity was found to be markedly elevated in cases with hepatic cancer metastasis, and often was considered a sensitive index of neoplastic involvement of the liver (117). With respect to LDH isoenzymes, elevation of the slowest moving (cathodal) isoenzyme has been considered a specific and
sensitive indicator of active hepatocellular damage. It was observed that slight elevation of this enzyme in serum could be of importance in the diagnosis of the preicteric phase of viral hepatitis. However its elevation has also been reported in congestive cardiac failure with hepatic necrosis, and following injury of the skin (6).

D) Serum glycoproteins

Winzler described a method for the isolation of carbohydrate rich protein fractions from human plasma in 1948. These compounds were called mucoproteins (109).

Mucoprotein patterns of serum from patients with liver disease were evaluated by Greenspan and co-workers. They observed that in the majority of viral hepatitis patients the serum mucoprotein content as determined by the biuret method was below normal or in the lower range of normal. However, the polysaccharide content of the mucoprotein as determined against a galactose-mannose standard remained within the normal range. This disproportionate increase of polysaccharide in the mucoprotein was noted in many of the patients with viral hepatitis. Patients who showed normal mucoprotein values initially, exhibited decreased or subnormal values on serial mucoprotein determination during the course of the disease. Cases of portal cirrhosis showed a similar pattern. The change in the mucoprotein level was independent of the abnormalities revealed by the serum
the serum bilirubin, thymol turbidity, zinc sulfate turbidity, alkaline phosphatase determination, prothrombin time or the BSP test. It was proposed that the mucoprotein alterations reflected changes in the alpha globulins (36). A similar observation has also been made by other authors (14, 63).

Contrary to the above findings, chemically-fractionated serum mucoprotein failed to exhibit a consistent decrease in uncomplicated cases of viral hepatitis, when assayed by the biuret method. With superimposed extra hepatic complications slightly elevated mucoprotein as obtained by chemical fractionation of serum did not represent a single chemical or biological entity. Any increase or decrease of certain of its constituents such as protein, hexosamine, hexose or polysaccharide would not necessarily reflect equivalent elevations of the whole complex (62).

In patients with viral hepatitis, determinations of serum electrophoretic glycoprotein patterns revealed no diagnostic change in the alpha and beta-globulins nor in the carbohydrate associated with them. Albumin and also albumin-bound carbohydrate were decreased during the acute phase of the disease. Conversely, elevation of the gamma-globulin was associated with an increase in carbohydrate. In addition, splitting of the gamma fraction into gamma-1 (IgM or IgA) and gamma-2 (IgG) was noted. Of these two fractions, gamma-1 (IgM or IgA) had the
higher carbohydrate content (93).

In a study of the electrophoretic analysis of serum proteins, glycoproteins and lipoproteins of children with viral hepatitis, it was noted that albumin as well as the carbohydrate bound to it was markedly decreased. The alpha-1 globulin was within the normal range, while the alpha-1 glycoprotein was slightly to moderately depressed. The beta globulin was slightly elevated in 50% of cases with a concomitant increase in beta-glycoprotein. In one-half of the cases the alpha-2 globulin was slightly increased whereas the alpha-2 glycoprotein was decreased. The gamma globulin and gamma glycoproteins were markedly increased in most cases (3).

In contrast to these observations, Klainer et. al. employing an improved glycoprotein staining technique for cellulose acetate strips, noted in viral hepatitis a relative decrease of alpha-1 glycoprotein with an increase of beta and gamma glycoglobulins. This was considered to be a characteristic finding (49).

The variable results of glycoprotein electrophoretic studies could explain the lack of any consistent pattern of change in chemically determined serum mucoproteins in viral hepatitis.

E) Studies of serum protein by chemical and electrophoretic fractionation:

Chemical methods for the determination of total protein and the albumin/globulin ratio antedate electrophoretic analysis.
When electrophoretic analysis became available, it was observed in a number of laboratories that the agreement between the electrophoretically determined albumin and the albumin obtained by salt fractionation was poor. In normal subjects and in many diseases, the albumin obtained by the chemical method was in fact the sum of albumin and the alpha globulins (80, 86).

No significant difference in total serum protein between viral hepatitis patients and normal subjects has been noted (35). In some studies, however, a slight decrease in the total serum protein during the acute phase of the disease was reported (43). The albumin/globulin ratio determined by chemical fractionation showed a relative increase of globulin and decrease of albumin in sera from patients with viral hepatitis (66, 81).

a) Serum albumin

Gray and Barron, the first to study the electrophoretic pattern of serum from patients with viral hepatitis, noted a decrease in albumin (35).

A serial study was made of the electrophoretic patterns of serum from patients with viral hepatitis up to 64 days following the onset of the disease. Maximal depression of the albumin fraction was noted during the first 10 days of the disease (66). In another study, cases having the most severe alterations during the acute phase, had persistently lower values of serum albumin for over a year. These patients were asymptomatic with essentially normal liver function tests.
In a 10 to 36 month follow-up of post viral hepatitis patients with abnormalities in one or more of the liver function tests, decreased serum albumin values were observed (89).

Popper and co-workers (1951) studied the electrophoretic serum protein pattern in hepatobiliary diseases and concluded "... serum albumin was markedly decreased in all hepatobiliary diseases (especially in cirrhosis) including obstructive jaundice, in which the decrease parallels the drop in the cholesterol/ester ratio. The decrease has limited diagnostic significance, since it occurs to a frequently similar degree in many non-hepato-biliary diseases, although usually not as marked as in cirrhosis" (81). This opinion appears to be generally accepted (3, 7, 77).

b) Alpha globulins

A slight increase of alpha globulin in the serum (15, 35) and plasma (29) of patients with viral hepatitis has been reported. Plasma values were higher than the observed serum values. A marked elevation of alpha globulin was characteristic of obstructive jaundice (29).

An equal number of increased and decreased serum alpha globulin values were reported in cases of viral hepatitis. Generally the total value in severe cases was lower than in mild or recovering cases. In obstructive
jaundice, alpha globulin levels exceeded the normal range in 70% of the cases. Analysis of the individual alpha-1 and alpha-2 values gave no additional information. There was no relationship between the alpha globulin values and the results of the biochemical and flocculation tests (81).

In a serial electrophoretic serum protein study of viral hepatitis patients for two months, Martin noted an increase in the alpha-2 globulin between the 14th and the 30th days (66). In a group of children with viral hepatitis, a slight elevation of the alpha-2 globulin with noted in half of the cases, with no alteration in the alpha-1 region (3). In a follow-up of 12 post viral hepatitis cases, having residual symptoms referable to their original attack, three had a persistent increase of the alpha-2 globulin (86). Others have also reported variable alpha-2 globulin changes in viral hepatitis (89).

Probably the inconsistent patterns of alpha globulin changes as noted above were related to the severity and clinical stage of the disease. It was observed that in mild cases of hepatitis, both alpha-1 and alpha-2 globulin values were at the lower limit of normal, but a pronounced decrease of both these components was seen in severely affected cases (104).

In fact, Knedel observed that the alpha globulin changes in viral hepatitis were related to the stage of
the disease. An initial increase in the alpha-2 globulin was followed by a decrease (51). Approximately half the adult cases of infectious hepatitis showed a slight initial increase of alpha-1 and alpha-2 globulins, whereas in serum hepatitis somewhat lower values were observed (7).

From the foregoing review, it appears that the majority of the investigators did not observe any consistent alteration of the alpha-1 globulins, whereas both increase and decrease of the alpha-2 globulins depending on the clinical severity and the stage of viral hepatitis, were observed by most authors.

Alterations of alpha lipoprotein and several specific alpha-2 proteins, namely haptoglobin, ceruloplasmin, cholinesterase, and c-reactive protein, have also been reported.

Normal or subnormal haptoglobin levels during viral hepatitis have been observed. Subnormal levels of haptoglobin were seen mainly in patients with long preicteric and icteric phases of the disease (74). An elevation of serum haptoglobin was seen in cases with obstructive jaundice (45) as well as in many non-hepatic diseases.

Isolated reports of increased serum ceruloplasmin in viral hepatitis have been published. An increase of serum ceruloplasmin was observed during the first week of the disease in cases of uncomplicated viral hepatitis. Cases
of viral hepatitis, complicated by other diseases like diabetes, showed a much higher rise of serum ceruloplasmin which remained high for a longer period of time. However, when there was advanced hepatic parenchymal damage due to viral hepatitis, a considerable decrease in serum ceruloplasmin was noted (38).

C-reactive protein has been reported in the sera of patients with viral hepatitis (7), but Havens and co-worker in their study failed to find c-reactive protein in any of their 90 cases of viral hepatitis regardless of the phase of the disease (41).

Impaired synthesis of alpha lipoprotein has been observed in viral hepatitis (56). This is manifested by a decrease in high density lipoproteins and cholesterol (65, 99). Serum lipoprotein electrophoresis of sera from children with viral hepatitis demonstrated a marked diminution in the alpha lipoprotein and elevation of the beta lipoproteins (3).

c) Beta globulins

Moderate elevation of serum beta globulin has been consistently noted in hepatitis, whereas marked elevation was characteristic of obstructive jaundice and tumor metastasis to the liver (15, 29, 35, 104).

In viral hepatitis elevation of the beta globulin was noted between the 14th and 30th days of illness (66)
No relationship could be demonstrated between elevation of beta globulin and the clinical severity of hepatitis (89). Several investigators have failed to observe any consistent increase of beta globulin or of the carbohydrate associated with it (77, 93).

Popper et al., reported increased beta globulin in 62% of the cases of viral hepatitis. The mean beta globulin level was higher in severe than in mild cases of viral hepatitis. Recovering cases in general had lower values (81).

Alterations in serum beta lipoproteins in viral hepatitis are probably related to changes in the beta globulin fraction as described above. The concentrations of beta and low density lipoproteins and free cholesterol were frequently high in viral hepatitis (3, 56). Flotation rates of these lipoproteins have been known to alter, probably because of the association between free cholesterol and the lipoprotein components (99). This increase in beta lipoprotein was used by Kellen as a criterion for the diagnosis of acute viral hepatitis and the post-viral hepatitis state. Increased beta lipoproteins were noted in 50% of cases, 5 years, and in 34% of the cases 10 years after the attack of hepatitis (47, 48). This elevation of beta lipoproteins might not have been reflected in the serum electrophoretic pattern of hepatitis.
patients in some of the studies, because paper electro-
phoresis did not often record the beta lipoprotein
increase. In contrast the moving boundary method of
electrophoresis did reflect the beta lipoprotein ele-
vation in the beta globulin region (79).

Alteration in the serum concentration of transferrin, another beta globulin, have been suggested, but the
results have been contradictory (16, 20, 87, 94, 102).

d) Gamma globulins

Electrophoretic fractionation of sera from patients
with viral hepatitis demonstrated a marked increase in
the gamma globulin region (35). Gamma globulin reached
its peak as the albumin level fell. With gradual increase
later in the albumin level, the gamma globulin level
decreased (66). In an eight month follow-up study of 20
cases of viral hepatitis with no residual symptoms, no
alteration of gamma globulin was noted. With residual
symptoms elevated gamma globulin levels were often noted
(86).

Among all cases of liver disease, elevation of
gamma globulin was most marked in cirrhosis of the
liver, less so in hepatitis and least in obstructive
jaundice. Cirrhosis and hepatitis cases showed a
splitting of the gamma peak into a "gamma-2" and a fas-
ter moving "gamma-1." The gamma-1 globulin was found to
have an electrophoretic mobility similar to fibrinogen and was therefore buried under the fibrinogen peak in the plasma electrophoretic pattern. Thus the elevated gamma-1 accounted for the apparent increase in fibrinogen in the plasma electrophoretic pattern (29). The heterogeneity of the increased gamma globulin was elucidated by other investigators. Immunoelectrophoretic study of infectious hepatitis sera showed all the gamma globulin components, namely gamma-2 (IgG), gamma-1A (IgA), and gamma-1M (IgM) to be increased (77).

A reasonable summary of this mass of data on electrophoretic components of sera was made by Wall, "If isolated electrophoretic analyses are made, they must be interpreted in view of the clinical stage of the disease" (105). It therefore appears that this method of analysis is nonspecific with respect to viral hepatitis.

In serial quantitative immunoglobulin determinations, in children up to 7 months after the onset of viral hepatitis, no alteration of the serum IgA level was noted. The IgM values were highest during the acute stage and persisted in certain cases. The IgG was noted to be lowest during the acute stage of the disease and gradually increased over the next 6 months. In the majority of the cases IgG increased as the IgM levels declined (57). Slightly different results were noted in a study of experimental viral hepatitis in children. Children with MS-1
type infections (a disease similar to classical infectious hepatitis) showed more pronounced changes in serum immunoglobulin levels compared to those with the MS-2 type of infection (similar to serum hepatitis). Elevation of IgM was first noted 3 to 4 days after SGOT activity increased. The high IgM level persisted for an average of 17 days during the acute phase of the disease. In certain cases IgA was elevated in similar fashion to the IgG (33).

The patterns of change of the immunoglobulins in adult hepatitis patients were similar to those seen in children. The serum immunoglobulin values determined by an immunochemical method did not show any correlation with total serum protein, SGOT, and serum bilirubin values. There was no correlation between the gamma zone electrophoretic patterns and IgG levels, nor was there any indication in the beta zone that IgA or IgM were abnormally high (58). Almost all cases with infectious hepatitis showed alterations in immunoglobulin levels, whereas only half of the patients with serum hepatitis showed such changes. After noting the pattern of immunoglobulin changes in viral hepatitis, Wollheim concluded, "... the striking feature being a gamma-M increase out of proportion to the relative increment of gamma-G, which in turn is greater than the gamma-A reaction. There is no indication that the magnitude of the gamma-M response has any prognostic significance" (111).
Summary of the literature review

Most of the flocculation tests reflect alterations of the serum albumin/globulin ratio due to hepatic parenchymal damage caused by various etiologic agents. Also, certain tests gave positive reactions because of alterations in the serum proteins due to non-hepatic diseases.

The determination of serum alkaline phosphatase was found to be more helpful in establishing the diagnosis of obstructive jaundice. The alteration of other enzymes was noted to be a sequela of acute injury to liver cells, rather than to viral hepatitis specifically.

The study of glycoproteins and of the various electrophoretic fractions of serum protein in viral hepatitis revealed a consistent decrease in albumin and increase in the gamma globulin fractions. The pattern of alterations of the glycoproteins, alpha and beta globulins was not consistent and even the results within a single study sometimes appeared contradictory. Such nondiagnostic patterns could be inherent in the methodology used, in the sense that proper delineation of the component or components responsible was not achieved.

Of the immunologic and serologic studies, the Australia antigen appears most promising. However this is not
a straightforward method because it involves a multi-stage operation of antigen isolation and antibody preparation.
MATERIALS AND METHODS

Collection of Serum and plasma

Ten to fifty ml. of serum was collected by routine venepuncture. Larger samples were collected by plasmaphoresis with subsequent recalcification of the plasma. Serum was stored at 4°C until utilized. In certain specific experiments fresh frozen plasma was utilized.

Analytical electrophoresis

Analytical zone electrophoresis was performed using cellulose acetate as the support matrix. The electrophoresis chamber, cellulose acetate strips and high resolution buffer were obtained from the Gelman Instrument Company, Ann Arbor, Michigan, a 10 lambda applicator was used to apply samples. Electrophoresis was carried out for 120 minutes at 1.5 ma per strip.

Strips were stained for protein according to the method of Briere and Mull (17). The glycoprotein staining method of Klainer (50) was used to demonstrate glycoproteins. Stained strips were cleared prior to densitometric scanning according to the method of Klainer et. al. (50). Cleared strips were scanned on an Analytrol, (Beckman Instruments) according to manufacturer's directions.

-32-
Gel chromatography

Sephadex and Blue Dextran, a void volume marker, were obtained from Pharmacia Fine Chemicals Incorporated, Piscataway, New Jersey. Upward flow adaptors for 2.5 and 5.0 cm columns as well as 0.9 cm columns were obtained from the same company. Glass pipe was purchased from Mooney Brothers Incorporated, Little Falls, New Jersey.

Sephadex G-200 was dry-sieved prior to use on a Tyler portable shaker (Fisher Scientific Company). U.S. standard sieves were used to size the chromatographic matrix. In general, two size cuts were taken, 44-74 μ for analytical columns and 74-120 μ for preparative columns. Sephadex other than G-200 was utilized as supplied by the manufacturer.

Unless otherwise indicated the gel chromatographic buffer was 0.05M tris-HCl, pH 8.3, + 0.1M NaCl. A stock buffer of 1.0M tris HCl was prepared and diluted 20-fold prior to addition of NaCl. Tris base was purchased from Sigma Chemical Company, St. Louis, Missouri as "Trizma 121." All pH determinations were made on a Radiometer, PHM 26 (London Company, West Lake, Ohio).

Chromatographic design

Small columns were purchased as previously stated. The 2.5 and 5.0 cm columns were constructed by using Sephadex upward flow adaptors as bottom end pieces and silicone rubber
stoppers for tops. The columns were Corning Double Tough glass pipe cut to the desired length on a glass saw. Stainless steel 20 gauge blunt needles were used in the tops and PE 100 tubing (Clay Adams) was used to connect the buffer reservoir with the column and the effluent tubing to the fraction collector. The preparative columns, 7.5 cm, were constructed with glass pipe and end piece constructed with glass pipe and end piece construction, similar to that described by Gelotte and co-workers (31).

Sephadex was swollen in 0.14M NaCl for 24-48 hours prior to equilibration with the running buffer. Since the Sephadex G-200 was sieved, defining of the gel was unnecessary. Unsieved Sephadex was defined as suggested by the supplier.

Before packing each column, buffer was poured into the glass tube to check for leaks prior to filling the column with a slurry of gel. The ratio of media to buffer in the slurry was 50:50. The outlet was opened slightly when a packed bed of 3-4 cms appeared at the bottom. The outlet was opened completely allowing a maximum flow rate as soon as a 10 to 15 cms packed bed was obtained at the bottom of the column.

When the packed zone reached the desired level a porous polyethylene disc was inserted to protect the even surface of the gel. At least two column volumes of running buffer were collected prior to the application of sample on either a new
Sample application was accomplished using a disposable pipette. The sample was applied on the polyethylene disc which rested directly on top of the gel. The flow rate of all columns was controlled by a Microbilateral roller pump (Extracorporeal Company, Mount Laurel, New Jersey) which was connected to the effluent side. It functioned solely as a metering pump. Chromatographic fractions were collected on a time basis using a Gilson Medical Electronics Company fraction collector. Aliquots of fractions were monitored spectrophotometrically at 280 m\(\mu\) on a Bausch and Lomb Spectronic 600.

After continued use, the flow rate of Sephadex decreased. When this became limiting the gel was removed from the column and washed in saline in order to remove absorbed protein and fine particles which limited the flow rate.

**Calibration of Sephadex G-200 column for molecular weight determination**

A Sephadex G-200, 44-74\(\mu\)(2.5 x 50 cm) column was calibrated with a reference protein kit (Mann Chemical Company, New York, New York). Blue Dextrane was used as a Vo marker. All elutions points were determined at the elution volume showing the peak concentration of the protein.

**Concentration methods**

Volumes less than 7 ml were concentrated by ultrafiltration in the Centro-flow ultrafiltration system, (Amicon
Corporation Lexington, Massachusetts). Force was applied by centrifugation in an International Refrigerated Centrifuge model PR2 at 1000 \( x \) \( g \) for approximately 1 hour. The actual time varied with the concentration of the sample. Volumes larger than 7 ml were concentrated by dialysis at 4°C in Visking tubing, usually 20/32, against 25 to 30% polyethylene glycol in either Ringer's solution or 0.14M NaCl solution. Samples remained in the polyethylene glycol solution until sufficient concentration was achieved.

**Ion exchange chromatography**

DEAE cellulose (Whatman DE 52, Reeve Angel Incorporated, Clifton, New Jersey) was cycled in 0.5 Normal NCl and 0.5 Normal NaOH. Each equilibration step was followed by five to six changes of distilled water prior to equilibration in 0.5 M Tris-HCl buffer, pH 8.3. Columns were gravity-packed and washed with running buffer until the pH of the buffer influent equalled that of the effluent. No attempt was made to measure the conductivity of either. Serum protein fractions were eluted by either step-wise elution or gradient elution utilizing a linear gradient. In both cases, the method of elution was an increase in NaCl concentration.

**Preparative zone electrophoresis**

Pevikon-C 870 (Mercer Chemical Company, New York) was utilized as the solid phase support. Separation of whole
sera or serum fractions was performed utilizing standard methods for this procedure (78). The electrophoresis buffer was 0.05M Barbital buffer, pH 8.3. Electrophoresis was carried out for 16 hours at 10 volts per centimeter. Since the migration rate was variable it was monitored by adding one to two drops of 3% aqueous Bromphenol blue solution at the edge of the sample trough. The Bound Bromphenol blue migrates with the albumin to which it is bound and is an indicator of the length of migration. Pevikon blocks were cut into sections with a spatula and eluted in a Buchner funnel with physiologic saline. Prior to further purification procedures, these fractions were concentrated as previously described.

**Precipitation methods**

The perchloric acid method of Winzler was utilized to prepare glycoprotein-rich fractions (109). This method depends upon the differential precipitation of glycoprotein in perchloric and phosphotungstic acid. Serum protein fractions were concentrated by several precipitating techniques. Eighty percent ethanol at 0°C was used to precipitate material, as were 50% and 60% saturation with ammonium sulfate. The latter was achieved by either the addition of saturated ammonium sulfate in small volumes, or by the addition of the salt.

**Enzymatic digestion**

Pronase, a proteolytic enzyme, was obtained from Cal. Bio. Chem. Digestions were carried out at room temperature, pH
range of 8 to 8.5. The protein to enzyme ratio was 20:1 by weight, pH was maintained at approximately 8 by the addition of 0.5 normal NaOH.

**Lipoprotein-free serum**

This material was prepared by ultracentrifugation of sera according to the method of Gidez (32). The density of the serum was adjusted to 1.21 gm/ml by the addition of sodium bromide solution. The final density of the mixture was checked at room temperature by determining the mass of a known volume of the mixture. Duplicate samples were loaded into cellulose nitrate centrifuge cups, (11.5 ml) and centrifuged in a Model L preparative ultracentrifuge (Division of Beckman Spinco) at 35,000 RPM (100,000 x g for 24 hours). The yellowish lipoprotein floating at the top of the tube was removed with a 20 gauge needle attached to a syringe. The lipoprotein-free serum with sodium bromide mixture was aspirated from the bottom of each centrifuge tube and dialyzed against 0.05M tris HCL containing 0.1M sodium chloride, pH 8.3, for 24 hours.

**Immunoochemical methods**

Immunoelectrophoresis was performed according to the method of Scheideger (95). The antisera (Goat antihuman serum, goat antihuman haptoglobin, and goat antihuman IgG) used were obtained from Hyland Division Travenol Laboratories, Incorporated.

Several sera from patients with viral hepatitis were evaluated for SH (Australia) antigen, by the standard radial
immunodiffusion method, (83). The SH antibody and control SH antigen were kindly supplied by Dr. Alfred M. Prince of the New York Blood Center.

Operational schemes

The standard screening procedure developed during the course of these experiments was gel chromatography of serum on a Sephadex G-200 column (44-74 microns), which was 2.5 x 50 cm in length. To this column was applied 5 ml of serum and fractions of 5 ml were collected. These were concentrated approximately ten-fold and analyzed by cellulose acetate electrophoresis. This is summarized in Figure 1. Over 100 sera were analyzed in this manner. In all separated material cellulose acetate electrophoresis was performed regardless of the previous parameters of separation. Initially duplicate strips were run with one stained for protein and the other for glycoprotein. This was abandoned, and for better comparative evaluation each strip was split into two longitudinal halves, one half being stained for protein while the other half for glycoprotein. In order to obtain readable patterns it was often necessary to make as many as six to eight applications to the cellulose acetate strip. It was therefore necessary to mark the strip with a pencil or a ball point pen to ensure the same point of application. Serial fractions were compared in the same electrophoretic chamber. If this is not done, individual variation precluded any evaluation of migration.
Diagramatic presentation of the operational scheme.
SEPARATE SERUM

CONCENTRATE 10X WITH CENTRIFLOW FILTER

SEPARATION OF SERUM ON SEPHADEX G-200

ELECTROPHORESIS ON CELLULOSE ACETATE

HALF STAINED Ponceau S DYE (PROTEIN)

HALF STAINED PAS (GLYCOPROTEIN)

STRIp CUT INTO EQUAL PARTS
Serum chemistries and liver biopsy

The determinations of serum bilirubin, alkaline phosphatase, SGOT, SGPT and LDH on hospitalized patients were performed by the Clinical Chemistry Laboratory of the Ohio State University Hospital.

The liver biopsy specimens were processed and interpreted by the Surgical Pathology Division of the Ohio State University Hospital.
RESULTS

Serum protein and glycoprotein electrophoretic patterns:

The electrophoretic patterns from sera of 25 patients were analyzed. Their distribution as to disease was:

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral hepatitis</td>
<td>7</td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td>4</td>
</tr>
<tr>
<td>Portal cirrhosis</td>
<td>1</td>
</tr>
<tr>
<td>Normal (blood donors)</td>
<td>13</td>
</tr>
</tbody>
</table>

Patients with viral hepatitis and infectious mononucleosis were in the first week of their illness. Table 2 is a summary of the serum protein electrophoretic analysis obtained on these patients. The only markedly abnormal pattern was found in a patient with portal cirrhosis. Otherwise values were normal, slightly decreased, or slightly increased over the normal range. The serum glycoprotein values are listed in Table 3. Analysis of these data demonstrates that the beta and gamma regions were slightly increased with the most marked increased in gamma fraction is found in the patient with portal cirrhosis. No consistent alteration in either the protein or glycoprotein values of the alpha 1, alpha 2 or beta region was noted. These values were obtained from densitometric tracings of the electrophoresis patterns. An example of the glycoprotein tracing is shown in Figure 2 and the protein pattern in Figure 3.
FIGURE 2

Diagram of the densitometric tracing of the normal electrophoretic serum glyco-protein pattern.
Alb 8.9%
\alpha_1 21.4%
\alpha_2 36.8%
\beta 19.6%
\gamma 14.3%
Table 2
Total Protein and Percentage Distribution of Electrophoretic Fractions

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total Protein gm/100 ml</th>
<th>Albumin</th>
<th>Percent of Total Proteins</th>
<th>Alpha-1</th>
<th>Alpha-2</th>
<th>Beta</th>
<th>Gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious Hepatitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.N.</td>
<td>6.1</td>
<td>53.8</td>
<td>4.3</td>
<td>7.7</td>
<td>11.1</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>M.K.</td>
<td>7.2</td>
<td>44.2</td>
<td>5.0</td>
<td>8.6</td>
<td>14.3</td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td>S.H.</td>
<td>7.2</td>
<td>54.7</td>
<td>4.7</td>
<td>6.1</td>
<td>12.2</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>B.S.</td>
<td>8.0</td>
<td>61.7</td>
<td>4.8</td>
<td>6.8</td>
<td>15.0</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>E.H.</td>
<td>8.1</td>
<td>64.7</td>
<td>1.9</td>
<td>9.3</td>
<td>7.4</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>R.G.</td>
<td>7.05</td>
<td>66.0</td>
<td>1.8</td>
<td>7.0</td>
<td>10.5</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>L.W.</td>
<td>7.4</td>
<td>60.0</td>
<td>5.0</td>
<td>5.0</td>
<td>13.3</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Infectious Mononucleosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W.F.</td>
<td>8.5</td>
<td>52.0</td>
<td>1.6</td>
<td>9.4</td>
<td>11.8</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>R.N.</td>
<td>7.2</td>
<td>55.8</td>
<td>4.1</td>
<td>9.0</td>
<td>13.1</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>Portal Cirrhosis</td>
<td>7.2</td>
<td>22.1</td>
<td>1.8</td>
<td>6.2</td>
<td>5.3</td>
<td>64.6</td>
<td></td>
</tr>
<tr>
<td>Normal Range*</td>
<td>6.7-8.7</td>
<td>50.0-63.0</td>
<td>2.4-5.1</td>
<td>8.8-12.4</td>
<td>9.8-12.7</td>
<td>11.5-25.5</td>
<td></td>
</tr>
</tbody>
</table>

*Normal range is average of 13 individual analysis of serum obtained from blood donors.
Table 3

Percentage Distribution of Glycoprotein Fractions in Serum

<table>
<thead>
<tr>
<th>Electrophoretic Region</th>
<th>Percentage of total PAS stained material in association with each electrophoretic fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>Infectious Hepatitis</td>
<td></td>
</tr>
<tr>
<td>1. J.N.</td>
<td>2.5</td>
</tr>
<tr>
<td>2. M.K.</td>
<td>6.0</td>
</tr>
<tr>
<td>3. S.H.</td>
<td>4.6</td>
</tr>
<tr>
<td>4. E.S.</td>
<td>3.9</td>
</tr>
<tr>
<td>5. E.H.</td>
<td>5.0</td>
</tr>
<tr>
<td>6. R.G.</td>
<td>2.2</td>
</tr>
<tr>
<td>7. N.W.</td>
<td>0.0</td>
</tr>
<tr>
<td>Infectious Mononucleosis</td>
<td></td>
</tr>
<tr>
<td>8. W.F.</td>
<td>0.0</td>
</tr>
<tr>
<td>9. R.N.</td>
<td>2.9</td>
</tr>
<tr>
<td>10. S.M.</td>
<td>2.4</td>
</tr>
<tr>
<td>11. H.T.</td>
<td>2.9</td>
</tr>
<tr>
<td>Portal Cirrhosis</td>
<td></td>
</tr>
<tr>
<td>12. W.C.</td>
<td>2.0</td>
</tr>
<tr>
<td>Normal Range*</td>
<td>4.8-10.8</td>
</tr>
</tbody>
</table>

*Normal range is average of 13 individual analysis of serum obtained from blood donors.
FIGURE 3

Diagram of the densitometric tracing of the normal electrophoretic serum protein pattern.
Alb 61.7%
α1 4.5%
α2 12.1%
β 10.2%
γ 11.5%
Serum protein and glycoprotein patterns in serum fractions

Serum fractions obtained from gel chromatography of patient's sera were analyzed by electrophoresis. In a preliminary experiment 7 individual sera were fractionated and the fractions analyzed after concentration. The diagnosis of these individuals was:

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral hepatitis</td>
<td>2</td>
</tr>
<tr>
<td>Contact with viral hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>Past history of hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>Normal (blood donors)</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 4 is an example of a typical chromatogram obtained by fractionation of 10 ml of serum on a Sephadex G-200 column. As indicated in Figure 4, aliquots from individual fractions were pooled and concentrated prior to electrophoretic analysis. An obvious difference was noted when comparing protein and glycoprotein strips from normal sera and sera obtained from patients with viral hepatitis. This difference was restricted to the alpha region of the second pool. In normal sera there was an alpha-2 protein and an alpha-2 glycoprotein band. However, in sera obtained from viral hepatitis it was noted that there was an extremely strong glycoprotein band but corresponding to this glycoprotein band was a sharp, well delineated clear area in the strip stained for protein, Figure 5. Sera from two individuals with a past history of viral hepatitis or a history of prolonged contact with hepatitis demonstrated a
FIGURE 4

Gel filtration of serum on Sephadex G-200, 44.74 (2.5 x 110 cm). Sample volume, 10 ml. Flow rate, 3 ml/cm²/hour. Fraction volume, 5.0 ml pools numbered I, II, III, IV, and V were made out of the fractions.
similar clear area in the alpha-2 region. In order to further evaluate this finding, 32 sera were fractionated on Sephadex G-200 and the concentrated fractions analyzed by electrophoresis. Eight samples were obtained from normal blood donors. The remainder were from patients with liver disease or other related infections. The size of the Sephadex G-200 column was reduced from a height of 110 cm to 50 cm permitting the fractionation to be accomplished within a 24 hour period. Even with a reduction in sample size, from 10 to 5 ml, the fractionation was inferior to that obtained on the longer column. Sacrifice of precision was made in order to permit a more reasonable time sequence in the analysis. Although the three major peaks were not as well separated, the clear alpha-2 pattern was readily discernible if alpha-2 macroglobulin was not in the fraction. The presence or absence of this protein was determined by immunoelectrophoresis. In addition the electrophoresis strips were split with one-half stained for protein and the other for carbohydrate. This permitted a much more accurate comparison of the stained and unstained alpha region. An example of a chromatographic pattern obtained on these columns is shown in Figure 6. Three pools were made as indicated and concentrated prior to electrophoresis. All 7 cases of viral hepatitis demonstrated the clear band in the alpha region. In addition the 3 individuals with a past history of hepatitis also showed an abnormal pattern. Three of 8 samples obtained from rejected donor plasma also evidenced a clear region. Normal donors and 6 individuals with other hepatobiliary diseases
FIGURE 5

Diagram of cellulose acetate electrophoretic patterns of Pools I, II, III, IV and IV (Fig. 4)
P = Protein stain,
G = Glycoprotein stain
did not show the clear alpha-2 pattern. Prior to fractionation no consistent difference could be demonstrated in the electrophoretic pattern of normal and abnormal sera in the alpha region. A definite difference was associated with the abnormal sera following chromatography and concentration of the fractions, Figures 7 and 8.

A total of 103 individual sera were screened for the presence or absence of the characteristic clear area in protein stained strips. The electrophoretic migration of this serum component can best be described as interalpha or possibly an alpha-2. Table 4 is a summary of the screening experiments. Positive findings were noted in 29 of 30 patients with viral hepatitis. The only other disease which exhibited a high percentage of positive results was infectious mononucleosis.

**Time sequence evaluation**

In eight cases of viral hepatitis, serum collected at weekly intervals during hospitalization demonstrated the characteristic alpha-2 pattern. The intensity of the pattern decreased with time. Samples from the third and fourth week were not as strong as those in the first and second week. Long term follow-up, 8-12 weeks, demonstrated that in the three patients observed, there was a progressive alteration which eventually became normal. This was in contrast to those persons with a past history of hepatitis who demonstrated a
## Table 4

### Summary of Screening Experiments

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total No. of Cases</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver Diseases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral Hepatitis</td>
<td>30</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>Sub-acute hepatitis and cirrhosis</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Past history of viral hepatitis</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Obstructive jaundice</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Acute yellow atrophy</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Alcoholic hepatitis</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Drug induced hepatitis</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Rejected blood donor (icteric plasma)</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>History of contact with viral hepatitis</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Infectious Diseases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Mucormycosis</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Rocky mountain spotted fever</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Bacterial pharyngitis</td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Viral pneumonia</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Bacterial meningitis</td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Sub-acute bacterial endocarditis</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety state</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Heart disease with liver disease</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Extensive surgery</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>ABO incompatible blood</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Accepted blood donors</td>
<td>24</td>
<td>2</td>
<td>22</td>
</tr>
</tbody>
</table>
(Top) Gel filtration of serum on Sephadex G-200 44-74Å, (2.5 x 50 cm). Sample volume, 5 ml. Flow rate, 3 ml/cm²/hour. Fraction volume, 5.0 ml. Pools numbered I, II, and III were made out of the fractions.

(Bottom) Diagram of "split strip" cellulose acetate electrophoretic pattern of Pool II
P = Protein stain
G = Glycoprotein stain
Optical density at 280 nm

Elution Volume (Vₑ) in ml
FIGURE 7

"Split strip" cellulose acetate electrophoretic patterns of Pool II (Fig. 6). Normal vs. viral hepatitis.
P = Protein stain
G = Glycoprotein stain
Pool II Normal.
Pool II Viral Hepatitis.
"Split strip" cellulose acetate electrophoretic patterns of Pool II (Fig. 6, Top). (Normal vs. viral hepatitis).
P = Protein stain
G = Glycoprotein stain
Pool II Normal
Pool II Viral Hepatitis
characteristic alpha-2 positive pattern.

The positive pattern was subsequently called the "alpha VH band" for descriptive purposes.

Correlation of alpha VH band and liver function data

All hospitalized cases of viral hepatitis had elevated SGOT, SGPT, serum alkaline phosphatase and serum total bilirubin. In 10 of these cases the diagnosis was confirmed by biopsy. Of the 30 cases of viral hepatitis, 29 showed a clear alpha VH band in their sera after fractionation on Sephadex G-200 columns. There was no correlation between other liver function tests and the alpha VH band. As described in Table 4, 15 cases of other hepatic and non-hepatic diseases demonstrated abnormal values for enzymes and bilirubin, comparable to the cases of viral hepatitis, but were negative for the alpha VH band. Thus it was concluded, that the alpha VH band was not related to alterations in SGOT, SGPT, alkaline phosphatase and serum bilirubin levels.

Characterization of the alpha VH band

Preparative gel chromatography of serum from a patient with viral hepatitis was performed on Sephadex G-200. As in the analytical columns the fractions eluting at the leading edge of the second peak demonstrated the alpha VH band. Positive fractions were pooled and concentrated ten-fold. An aliquot of the concentrated pool was refractionated on an analytical
Sephadex G-200 column. A single peak eluted from the column. Positive fractions were found at the leading edge of this peak. The principle protein components as determined by immunoelectrophoresis were haptoglobin and IgG. Sera from 6 patients were rechromatographed as described. In each case the alpha VH band eluted at the same position as described above. Since the elution position of the alpha VH band did not change, it seemed appropriate to analyze certain molecular parameters of the material utilizing gel chromatography. The $K_a V$ was 0.14, calculations being made from the most intensely stained strip of the calibrated elution volume.

When the data (Table 5) were plotted in an Andrews plot, (2) Figure 9, the material has an approximate molecular weight of 180,000. This assumes a spherical molecule. During these experiments it was noted that the alpha VH band is not recovered quantitatively in concentrating operations. It is possible that some of the material was lost by adsorption on the column and on the membrane during the concentration procedure. This fact greatly increased the difficulty of procedures which are normally used for the separation of classical glycoproteins or proteins.

**Ion exchange chromatography**

When serum from a patient with viral hepatitis was subjected to ion exchange chromatography on DEAE cellulose, the chromatogram shown in Figure 10 was obtained. Ten pools, as indicated
## Table 5
### Sephadex G-200 Chromatography

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol. Weight</th>
<th>Ve</th>
<th>KAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
<td>169 ml</td>
<td>0.51</td>
</tr>
<tr>
<td>BSA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>67,000</td>
<td>146 ml</td>
<td>0.38</td>
</tr>
<tr>
<td>IgG</td>
<td>160,000</td>
<td>113 ml</td>
<td>0.19</td>
</tr>
<tr>
<td>Horse Apoferritin</td>
<td>480,000</td>
<td>83 ml</td>
<td>0.005</td>
</tr>
<tr>
<td>Alpha VH band</td>
<td>1.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>107 ml</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Ve = Elution Volume;

\[ KAV = \frac{Ve - Vo}{V_t - Vo} \]

\[ V_t = (\text{total column volume}) = 250 \text{ ml} \]

\[ Vo = (\text{void volume}) = 82 \text{ ml} \]

<sup>1</sup>Bovine Serum Albumin
FIGURE 9

Plot of $V_e$ versus log of the molecular weight. Values other than alpha VH band are literature values.
- Ovalbumin (45,000)
- BSA (67,000)
- IgG (160,000)
- Alpha VH Band
- Horse Apoferritin (480,000)

Log Molecular Weight
in Figure 10, were made and concentrated prior to analysis by zone electrophoresis. Pool X, which eluted at approximately 0.15 Molar NaCl, demonstrated an alpha VH band. Since it eluted at the end of the chromatogram, the flat gradient was replaced by one utilizing a higher concentration of sodium chloride. Due to the intense absorption of the alpha VH band to DE 52 and the dilution obtained in eluting the material, it was decided not to utilize ion exchange chromatography for the first step isolation procedure but rather to utilize the Sephadex G-200 and follow this with ion exchange chromatography. When the pooled alpha VH was chromatographed on DE 52, it was eluted at 0.2 M sodium chloride, (Figure 11). The results of these experiments were disappointing in that recovery approximated less than 10%. As the material is further purified it tends to become more strongly absorbed to any surface, at least at the pH which was utilized in these fractionation procedures.

Precipitation experiments

Since ion exchange and gel chromatography or a combination of both failed to yield a reasonably pure material as judged by immunoelectrophoretic monitoring, the precipitation methods described by Winzler (109) were utilized on material first separated by Sephadex chromatography. In the course of six separate experiments, it was observed that the VH band was highly soluble in perchloric acid and could be precipitated by phosphotungstic acid. However this approach was found to
FIGURE 10

Chromatography of serum on DEAE cellulose (2.5 x 40 cm). Serum (40 ml) was adsorbed and eluted with 1.2-liter linear gradient of 0.02 M Tris-HCl (pH 8.3) + 0.05 M NaCl to 0.02 M Tris-HCl (pH 8.3) + 0.15 M NaCl. Fraction volume, 16.0 ml. Pools numbered I through X were made out of the fractions.
Chromatography on DEAE cellulose (2.5 x 20 cm) of a pooled and concentrated alpha VH band material obtained by preparative Sephadex G-200 gel filtration of serum from a patient with viral hepatitis. The pooled alpha VH material (20 ml) was adsorbed and eluted with step-wise elution at 0.1 M, 0.2 M, and 0.4 M NaCl concentrations in 0.02 M Tris - Hcl (pH 8.3) buffer. Fraction volume, 10 ml. Pools numbered I through VIII were made out of the fractions. Fraction V showed alpha VH band.
be inoperable for the following reasons.

i) From the same starting material, similar protein preparations were never obtained as judged by the immunoelectrophoretic and electrophoretic protein and glycoprotein pattern.

ii) In some experiments, it was almost impossible to obtain a clear perchloric acid filtrate.

iii) Because of very highly acid (pH 2) working pH, the possibilities of irreversible molecular change existed.

It was found that the alpha VH band was precipitable by ethyl alcohol (80% concentration) or ammonium sulfate (60% saturation). In either case the precipitates could be resolubilized in buffer. Therefore, pooled fractions from a preparative Sephadex G-200 column were precipitated at 60% saturation with ammonium sulfate. The precipitate was redissolved and desalted on a Sephadex G-50 column using the standard chromatographic buffer (0.02 M Tris-HCl, pH 8.3). The desalted alpha-2 fraction was then precipitated with 80% ethanol at 4°C. The redissolved precipitate exhibited a strong alpha VH band. Experimental losses were comparable to those found in chromatography. Six preparations of pooled alpha-2 protein obtained by preparative Sephadex G-200 fractionation of 6 sera from patients with viral hepatitis were treated by the above precipitation methods and the results were similar.

Preparative zone electrophoresis

Although zone electrophoresis of concentrated pooled alpha VH band fractions from a preparative Sephadex G-200 column
produced a single, well-stained, PAS-positive alpha-2 band, the dilution of the material obtained when eluting it from the Pevikon required extensive concentration. This resulted in loss of the material on the concentrating membrane.

Lipoprotein studies

Concentrated aliquots from gel chromatography were subjected to electrophoresis and stained for lipoprotein by the method of the Gelman Instrument Company (30). Lipoproteins present in fraction 2 did not coincide with the clear area observed in the protein-stained fraction. Most lipoprotein staining material was noted in the alpha-1 region. In order to rule out the presence of lipoprotein as a cause for the clear area, sera from a normal individual as well as sera from a patient with viral hepatitis and sera from patients with a history of hepatitis were subjected to density ultracentrifugation. Lipoprotein-free, and untreated sera were then fractionated on identical Sephadex G-200 columns. The first peak eluted from the column was smaller in lipoprotein-free samples as compared to untreated sera. The clear alpha-2 area was observed in both the lipoprotein-free and in untreated sera from the patient with viral hepatitis. The normal pattern was also obtained in individuals with normal sera both lipoprotein-free and untreated.

Enzymatic digestion of alpha-2 material

Pooled fraction 2 was subjected to pronase digestion. There was a marked reduction in the protein-staining material.
This obliterated the sharp edges of the clear region in the protein stain. In addition the glycoprotein material which normally migrated in the alpha-2 region showed increased migration as well as insoluble material which no longer moved in the electric field.

**Glycoprotein nature of the alpha VH band**

Since quantitative recovery of the alpha-2 material appeared to be impossible by the techniques utilized, the following procedure was devised, based upon minimum loss, (Figure 12). Two sera from patients with viral hepatitis and one sample of pooled normal sera were fractionated. The results of immunoelectrophoretic and electrophoretic protein and glycoprotein patterns of the various fractions are shown in Table 6. In this experiment, separate electrophoretic strips of fractions of serum from patients with viral hepatitis showing the alpha VH band were also stained by the method of Langley and Weiss (52). It was observed that strips stained with a) Amido Schwartz, and b) pretreated with periodic acid and then stained with regular Ponceau SS or Amido Schwartz did not show any "washed-out pattern." All the strips showed a stained band corresponding to the glycoprotein band in the alpha-2 region.

**Immunochemical studies**

In the serum protein fractions obtained by the fractionation procedures described in the preceding sections, haptoglobin
was the only alpha-2 protein easily identifiable by immunoelectrophoresis using goat antihuman serum antiserum. Haptoglobin was consistently noted in all fractions of sera from patients with viral hepatitis, showing the alpha-2 glycoprotein pattern. It was also noted in the corresponding serum fractions from normal individuals.

In the comparative evaluation (Table 6) of the protein fractions between normal serum and serum from patients with viral hepatitis, immunoelectrophoretic determination of haptoglobin was important as a marker. An obvious difference was observed in the comparison of cellulose acetate strips stained for protein and glycoprotein following electrophoresis of fraction five. However, the immunoelectrophoretic patterns were similar. In contrast, fraction six demonstrated an obvious difference in the stained cellulose acetate strips but neither sample reacted with antiserum. Therefore it appeared that the alpha-2 glycoprotein, (alpha VH band), did not react with the commercial antiserum and that upon pronase digestion haptoglobin was removed but that an altered alpha VH band remained in the soluble fraction.

Results of SH antigen study of sera showing the alpha VH band

When 8 strongly-positive sera for alpha VH band were assayed by immunodiffusion for the presence of SH antigen negative results were obtained. Therefore the alpha VH band is not immunochemically nor physico-chemically related to SH antigen.
Flow diagram for the purification of alpha VH band for electrophoretic and immunoelectrophoretic evaluation. These stepwise procedures were employed on 2 sera from patients with viral hepatitis and on one control sample of pooled normal sera.
FIGURE 12

90 ml serum fractionated on preparative Sephadex G-200 column.

Pooled alpha-2 protein (1) containing fraction.

Concentrated 40% ammonium sulfate saturation.

4 ppt.

(2) Supernate ammonium sulfate saturation increased to 60%

Supernate (discarded)

(3) ppt. (Redissolved in buffer)

Desalted.

Fractionated in a DE 52 column

Material eluting at 0.2 ml NaCl was pooled and concentrated. (4)

Ethanol added up to 80% concentration at 0°C.

ppt. (Redissolved in minimum buffer) (5) Supernate (discarded)

Treated with Pronase

Insoluble precipitate → Soluble component (6)

Numbers in parenthesis indicate fraction numbers.
Table 6

**Electrophoretic and Immunoelectrophoretic Patterns of Various Fractions of Serum Protein**

<table>
<thead>
<tr>
<th>Fraction No. as of Fig. 10</th>
<th>Pooled Normal Serum</th>
<th>Serum from Viral Hepatitis (2 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Principle protein components by IEP</strong></td>
<td><strong>Electrophoretic protein/glycoprotein</strong></td>
</tr>
<tr>
<td>(1)</td>
<td>IgG</td>
<td>Well matched alpha-2 protein and glycoprotein band</td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haptoglobin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace albumin</td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>Trace IgG</td>
<td>As above</td>
</tr>
<tr>
<td></td>
<td>Haptoglobin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace albumin</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>Haptoglobin</td>
<td>As above</td>
</tr>
<tr>
<td></td>
<td>Trace albumin</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>Trace Haptoglobin</td>
<td>Poorly stained but well matched alpha-2 protein and glycoprotein bands</td>
</tr>
<tr>
<td></td>
<td>Trace albumin</td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td>No reaction</td>
<td>No stained band was noted</td>
</tr>
</tbody>
</table>

IEP: Immunoelectrophoresis
DISCUSSION

Electrophoretic analysis of unfractionated serum was not diagnostic for viral hepatitis in this study. After fractionating the serum in a Sephadex G-200 column, in more than 90% of the cases of viral hepatitis (including infectious mononucleosis) a specific alpha-2 glycoprotein was noted.

The constant elution pattern of the alpha VH band in Sephadex gel chromatography precludes the possibility of it being an artifact produced by gel filtration operation. Had it been so, the elution pattern would have changed during the course of repeated gel chromatography. Several investigators have reported changes in the elution pattern of protein (23) and glycoprotein (106) due to alteration in the molecule, denaturation or depolymerization of the molecule caused by the gel filtration procedure.

Based on the constant elution pattern of the alpha VH band in the Sephadex G-200 column, the molecular weight of this protein was found to be in the range $1.8 \times 10^5$. This molecular weight represents the order of magnitude only, since

1) The elution volume or $K_{av}$ of any protein is dependant on molecular size rather than molecular weight (53, 101).
ii) Glycoproteins are not compact molecules and therefore do not yield precise molecular weight values when compared with a series of globular proteins as standards (28, 34).

In spite of all these problems it would appear that the alpha VH band is produced by a single protein or a very closely related group of proteins. This conclusion can be made on the basis of the results of chromatographic experiments, precipitation experiments, electrophoretic properties and staining characteristics of the alpha VH band.

The alpha VH band is not a lipoprotein, nor related to the alterations in the serum lipoproteins. The staining characteristics of this protein are similar to those described by Langley and Weiss (52) for alpha-1 acid glycoprotein. The solubility in perchloric acid and precipitability by phosphotungstic acid of the alpha VH band also fulfills Winzler's (110) criteria for a mucoprotein. The persistence of PAS-staining material even after pronase digestion of the alpha VH band-containing fraction also supports the hypothesis that the presently described alpha protein is primarily a glycoprotein with a very high carbohydrate content. The loss of this protein during purification due to surface adsorption is probably related to its highly acidic nature. In general the acidity characteristic of any glycoprotein is related to its sialic acid content. Therefore one can probably conclude that this alpha-2 glycoprotein is also rich in sialic acid content. A similar loss in mucoproteins due to adsorption to filter paper has been observed.
Several authors have reported changes in the serum alpha-2 globulin (1, 3, 36, 81) and total serum mucoprotein levels in various diseases. Most of the investigators have used either Winzler's method (1, 36) or ion exchange chromatography (4, 28, 67) for the isolation of such components, and electrophoresis and immunoelectrophoresis for their identification. In the present work extensive use of gel chromatography was made for the isolation of the alpha-2 glycoprotein, and its identification depended on the electrophoretic pattern. Immunoelectrophoretic examination was not helpful since no antibody against this protein was noted in available antisera. Because of the varying experimental conditions in this study and in those reported by other authors (4, 67, 98) no definite conclusion can be made as to the similarity of this alpha-2 glycoprotein and those described by other authors (98). However, the existence of this carbohydrate-rich protein of the alpha-2 region probably explains the findings of several investigators (14, 36, 63). All these investigators consistently noted an increase in the proportion of carbohydrate in the mucoprotein, or seromucoid, components of serum from patients with viral hepatitis, isolated by Winzler's method (109). The alpha VH band glycoprotein was responsible for the proportionate increase of carbohydrate in the mucoprotein preparation noted by these authors, since using Winzler's method, it is precipitated in the
mucoprotein fraction.

In an attempt to explain the occurrence of the unique alpha-2 glycoprotein finding in more than 90% of the cases of viral hepatitis, one is confronted with the following 3 postulates:

a) Increase in the serum glycoproteins due to liberation and release of ground substance secondary to necrosis of liver cells.

b) Virus-induced increased synthesis of glycoprotein by the liver cells, as has been noted in Herpes Simplex infected human amnion cell cultures (91).

c) Reflection of abnormal synthesis of glycoprotein in the injured liver cells.

If these postulates are examined in the light of the present experimental data, it appears that the first postulate is not acceptable, because cases with massive active liver necrosis e.g., acute yellow atrophy of the liver, sub-acute hepatitis and Rocky Mountain spotted fever with very high SGPT values, failed to show the alpha VH band. Musil (70) has also noted that in viral hepatitis the increase in serum glycoprotein was not a result of tissue necrosis; rather it reflected reparation and proliferation of tissues. However in the present experiments, in 4 out of 7 cases (Table 4) of sub-acute hepatitis with (liver biopsy proven) regenerating nodules in the liver, no alpha VH band was noted. It thus seems that the appearance of alpha VH glycoprotein in cases of viral hepatitis is neither related to necrosis, nor reparative proliferation of liver cells.
The first postulate also would not explain the occurrence of this pattern in individuals who had viral hepatitis many years ago, and who were clinically normal at the time of examination.

The second postulate is also not acceptable for the following reasons:

i) The polysaccharide component of glycoprotein synthesis is not directly dependant on the DNA and mRNA-ribosome sequence (97), and as such it is difficult to visualize how a virus, be it DNA or RNA can enhance directly the synthesis of a specific glycoprotein.

ii) It does not explain the presence of the alpha VH band in cases with infectious mononucleosis, or in individuals in post viral hepatitis state.

The third postulate appears to be more acceptable, i.e., secondary to injury or inflammation of liver cells, either temporary or permanent derangement of glycoprotein synthesis occurs. The derangement may be in the peptide synthesis or in the conjugation of peptide to the polysaccharide in the formation of glycoprotein molecules (36). A similar example is noted in a reported case (92) of biliary cirrhosis showing altered forms of beta lipoprotein containing proportionately lesser amount of peptide. Apart from the failure of peptide synthesis, it is also possible that the alpha VH band represents a glycoprotein having abnormalities in the carbohydrate moieties, since the normal mechanism of synthesis of glycoproteins is not as precisely specified as the primary DNA directed protein biosynthesis. As a consequence a considerable degree of polydispersity is noted in blood group substance and in
preparations of all epithelial glycoproteins (34, 97). Therefore it is not unlikely that secondary to injury and inflammation, liver cells can very easily produce glycoproteins having abnormalities either in the peptide part or in the carbohydrate part or in both. A similar example is noted in the occurrence of various types of glycoproteins in patients suffering from malignant diseases (4). The persistence of this alpha-2 glycoprotein in some of the individuals, many years after the attack of the disease, is comparable to the persistent elevation of beta lipoprotein in some individuals many years after an attack of viral hepatitis (47, 48).

Though the exact chemical nature of the alpha VH glycoproteins could not be established because of its extremely labile nature in the experimental conditions of the present work, its usefulness in the diagnosis of viral hepatitis is evident from the data presented in Table 4. Apart from viral hepatitis this pattern was noted in 13 out of 30 cases with "other forms of liver disease." The other forms of liver diseases included sub-acute hepatitis, cholangiolytic hepatitis associated with obstructive jaundice, drug induced hepatitis, acute yellow atrophy, alcoholic hepatitis, hepatitis of infectious mononucleosis, and some blood donors. Among the blood donors the majority had been rejected because of hyperbilirubinemia. With the exception of the infectious mononucleosis cases, the possibility of exposure to viral hepatitis, or the
presence of active viral hepatitis could not be ruled out in other cases of hepatitis. In the case of rejected blood donors, since they were all apparently healthy individuals with no history of exposure to drug or toxic substance, it could be postulated that most of them were either suffering from subclinical viral hepatitis or infectious mononucleosis associated hepatitis.

The washed-out alpha-2 protein band was not related to the alterations in serum bilirubin, SGOT, SGPT or serum alkaline phosphatase levels. Since the presence of the alpha VH band does not depend solely on the necrosis of liver cells, as has been suggested earlier, its appearance and disappearance in the serum does not follow the pattern of SGOT and SGPT changes in the serum. Similarly the mechanism of hyperbilirubinemia and elevation of serum alkaline phosphatase in viral hepatitis and other forms of liver diseases is probably not the same, nor related to the alpha VH band.

Since the appearance of the alpha VH band is probably related to a specific type of injury to the hepatocyte, its occurrence in the serum can be considered specific and diagnostic of such a type of injury. In more than 90% of the cases of viral hepatitis and infectious mononucleosis, such a specific type of injury is produced to the liver cells as is evident from Table 4. Therefore it can be postulated that the occurrence of this alpha VH glycoprotein is much more specific and
diagnostic of viral hepatitis (infectious, serum and infectious mononucleosis associated) than the available methods of enzyme determination.

Australia antigen (9, 10) is also an alpha migrating protein and as such, in the earlier part of this investigation, it was thought that the alpha-2 protein probably represented Australia antigen. However, differences in characteristics between these two entities were evident (Table 7).

Australia antigen, when present in the serum is suggestive of the carrier state of viral hepatitis or active viral hepatitis, however its diagnostic value is limited by the fact that it can be demonstrated in only 30-40% of the cases of viral hepatitis in the acute phase of the disease (10, 11). In the present study in 8 sera from cases of viral hepatitis, no SH antigen was noted in any of them. SH antigen and Australia antigen are immunologically identical (83, 85). In contrast to these results of the SH antigen study, all 8 cases showed the strongest alpha VH band during the first 3-4 weeks of their illness. Similar findings of an alpha VH band were noted in all other cases of viral hepatitis and infectious mononucleosis. It thus seems that the alpha VH band glycoprotein is not related to SH (Australia) antigen, either immunologically or physicochemically.

Based on these findings it can be concluded that the determination of the alpha VH band in all cases of viral hepatitis
Table 7
Comparison of Australia Antigen and the Alpha VH Band

<table>
<thead>
<tr>
<th>Alpha VH glycoprotein</th>
<th>SH or, Australia Antigen (9, 10, 11, 55, 112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Chemically a glycoprotein having staining characteristics of a glycoprotein.</td>
<td>(1) Chemically a lipoprotein, stained by Sudan stains.</td>
</tr>
<tr>
<td>(2) Molecular weight around $1.8 \times 10^5$ and elutes in the IIInd peak after Sephadex G-200 chromatography.</td>
<td>(2) Described as a macroglobulin eluting at the 1st peak after Sephadex G-200 chromatography.</td>
</tr>
<tr>
<td>(3) On preparative ultracentrifugation at 1.21 gm/ml density alpha-2 glycoprotein is present in the infranate.</td>
<td>(3) On preparative ultracentrifugation at 1.21 gm/ml density, SH antigen is present in the supernate.</td>
</tr>
<tr>
<td>(4) Presence is detected in more than 90% of the cases of viral hepatitis.</td>
<td>(4) Presence is detected in 30-40% of the cases of viral hepatitis during the acute stage of the disease.</td>
</tr>
<tr>
<td>(5) Present in cases with infectious mononucleosis.</td>
<td>(5) Not demonstrable in cases with infectious mononucleosis.</td>
</tr>
</tbody>
</table>
could complement the results of Australia antigen determination. Moreover it has been suggested that Australia antigen is present only in cases of serum hepatitis (84, 85), and not in cases of infectious hepatitis. The alpha VH band appears uniformly in all cases of viral hepatitis and infectious mononucleosis associated hepatitis. In this respect its determination would be more effective than the determination of Australia antigen in the diagnosis of viral hepatitis.
SUMMARY AND CONCLUSIONS

A specific change in the alpha-2 fraction of serum protein, described as an "alpha VH band" was noted in patients with viral hepatitis and infectious mononucleosis associated hepatitis. The change was apparent as soon as the macroglobulins were separated from the serum by Sephadex G-200 gel chromatography. Based on precipitation reactions, chromatographic behavior, and staining characteristics, it appeared that the alpha VH band was caused by a mucoprotein or a group of similar mucoproteins containing a high percentage of carbohydrate. Because of loss of the alpha VH band material during various attempts at purification in the present experimental study, a clear-cut chemical characterization of the alpha VH band could not be achieved. This glycoprotein was immunologically nonreactive with commercially available goat antihuman serum antiserum. It was not related to the Australia antigen. The Australia antigen is not apparent in all cases of viral hepatitis, in all stages of the disease. But this alpha VH glycoprotein was found in more than 90% of cases of viral hepatitis, during the first 3 or 4 weeks of illness. It thus appears that in the diagnosis of viral hepatitis, detection of the alpha VH band could be used as a diagnostic procedure, complementary to detection of the Australia antigen. However, the
complexity of manipulation, time and expense involved in presently available methods of isolation of the alpha VH band limit its applicability as a routine diagnostic procedure in the clinical laboratory. It is hoped that the present experimental data will aid future attempts at isolation and complete chemical and immunologic characterization of the alpha VH band. Once it is thus characterized a prospective study involving a large number of cases of viral hepatitis and other diseases will provide a final answer to the question -- whether the alpha VH band is pathognomonic of virus infection of the liver.


