An assessment of the diagnostic and prognostic value of serum vitamin B₁₂ levels in liver disease

C. D. HOLDSWORTH, MICHAEL ATKINSON, J. A. DOSSETT, AND R. HALL

From the Department of Medicine, University of Leeds, and St. James Hospital, Leeds

EDITORIAL SYNOPSIS In this study of serum B₁₂ levels in cases of liver disease normal levels were found in patients with extrahepatic biliary obstruction unless there was an associated cholangitis or intrahepatic metastases. Levels were also normal in chlorpromazine jaundice, but were consistently raised when necrosis of liver cells was present, whether necrosis was due to a virus infection or to drugs of the amine-oxidase inhibitor type.

Vitamin B₁₂ is stored in the liver and raised serum levels of the vitamin, as assayed biologically, are present in a variety of liver disorders, particularly those accompanied by acute necrosis of liver cells. Kristensen (1956) suggested the use of serum vitamin B₁₂ level as a liver function test and Rachmilewitz, Stein, Aronovitch, and Grossowicz (1958), finding raised levels in acute and chronic hepatitis and portal cirrhosis, state that this is a 'useful and

specific test for the presence and degree of hepatocellular damage'. However, others have reported less consistent changes in the serum vitamin B₁₂, particularly in chronic parenchymal liver disease (Table I) and raised levels have been found in extrahepatic biliary obstruction and when the liver is invaded by metastatic neoplasm.

The purpose of this investigation was to assess the diagnostic significance of raised serum vitamin B₁₂

TABLE I

CHANGES IN SERUM VITAMIN B₁₂ LEVELS IN LIVER DISEASE

Author and Assay Method	Acute Hepatitis		Chlorpromazine Jaundice		Active Chronic Hepatitis		Portal Cirrhosis		Obstructive Jaundice, Biliary Cirrhosis (B.C.) and Extrahepatic Obstruction (E.O.)		Hepatic Metastases		Chronic Congestive Cardiac Failure	
	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level	No. of Patients	No. with Raised B ₁₂ Level
Kristensen (1956)														
<i>Lactobacillus leichmannii</i>	10	9					3	3	14	3				
Jones <i>et al.</i> (1957)														
<i>Euglena gracilis</i>	6	6			10	10	11	Majority	B.C. 5	Minority				
Rachmilewitz <i>et al.</i> (1958 and 1959)														
Merzbach and Grossowicz (1961)	37	37	2	0	5	5	9	6	B.C. 6 E.O. 12	5 1	54	36	28	23
<i>Esch. coli</i>														
Cowling and Mackay (1959)														
<i>Euglena gracilis</i>	7	5	1	1	20	17	14	7	E.O. 7	5	6	5		
Nelson and Doctor (1960 and 1962)														
<i>Lactobacillus leichmannii</i>	6	5					19	3	E.O. 7	1	41	20		
Salera <i>et al.</i> (1961)														
<i>Euglena gracilis</i>	9	9					15	15					5	5
Present series														
<i>Euglena gracilis</i>	10	7	5	0	15	10	39	20	B.C. 4 E.O. 28	2 9	29	16		

levels in various types of liver disease, and to attempt, by means of serial observations, to evaluate the prognostic significance of this determination in both acute and chronic parenchymal liver disease.

METHODS

Vitamin B₁₂ was assayed by the method of Hutner, Bach, and Ross (1956), using the 'Z' strain of *Euglena gracilis* as a test organism. Total B₁₂ was measured after heating the serum dilutions in medium buffered to pH 3.6 to 100°C. for 15 minutes and free B₁₂ after heating to 56°C. for 30 minutes. The difference between these levels represented combined serum vitamin B₁₂. Our normal range was determined from estimations on 300 sera, selected from routine investigations after it had been verified that they did not come from patients suffering from anaemia or any condition known to affect the serum B₁₂ level. In all but three of 200 cases of pernicious anaemia the serum B₁₂ level was less than 110 µg./ml. We therefore regard the low values below 110 µg./ml. as indicative of B₁₂ deficiency of some severity. The range 110 to 180 µg./ml. we termed the 'intermediate' range and into it fall partially treated cases of pernicious anaemia, together with a small proportion of 'normals'. Interpretation of values falling within this range is, therefore, difficult. Although the normal values are scattered between 110 and 1,000 µg./ml., 95% of the results fell between 180 and 700 µg./ml., and this we regard as the normal range. We have designated the range between 700 and 1,000 µg./ml. the 'high' range. As with the intermediate range, values falling here are difficult to interpret and will include some disorders known to produce high values of serum vitamin B₁₂. Above 1,000 µg./ml., the 'very high' range, some related pathological abnormality is considered to be present. In sera from normal subjects we have not encountered free vitamin B₁₂; all the vitamin is present in the combined form.

Urinary estimation of vitamin B₁₂ was not always satisfactory, because if contaminating organisms grew in the urine during the period of collection, it was not certain whether the B₁₂ had been excreted by the patient or manufactured by the organisms. However, up to 50 mµg. per 24 hours was found in the urine of normal subjects.

Specimens obtained by needle biopsy of the liver or at necropsy were examined histologically to verify the clinical diagnosis and to relate pathological changes to serum B₁₂ levels from sera collected within two days of obtaining the liver sample. An attempt was made to assess histologically the degree of cellular damage, but since we found no feature that could be equated with the B₁₂ levels, the full details of this side of the investigation will not be set out in this paper.

RESULTS

ACUTE HEPATITIS Ten episodes of jaundice were studied in nine patients. Acute viral hepatitis accounted for four of these episodes while the remaining six, although following a similar clinical course,

FIG. 1. Serum vitamin B₁₂ levels in 132 patients with liver disease.

Each dot represents the initial level in a single subject.

had resulted from the administration of amine oxidase-inhibiting drugs (Holdsworth, Atkinson, and Goldie, 1961). The serum vitamin B₁₂ level was initially in the very high range in six of the 10 episodes of jaundice and in the high range in one. Thus 70% of the cases gave values above the normal range (Fig. 1). It returned to normal with recovery in five patients and rose in the other two, both of whom died of hepatic necrosis. The initial serum B₁₂ level was normal in the remaining three patients. In two of these and in the patient whose B₁₂ was in the high range, the earliest estimation was made at a time when the jaundice was already receding and it is likely that earlier in the course of the disease very high levels would have obtained. In the final patient a normal level of 550 µg./ml. fell to a low value on recovery from the jaundice (75 µg./ml.) and a Schilling test one year later showed a reduced uptake, suggesting an inherent defect in B₁₂ absorption.

The degree of jaundice in patients with acute hepatitis was closely related to the B₁₂ value, the correlation coefficient between serum vitamin B₁₂ and serum bilirubin being 0.81. Changes in the serum B₁₂ level usually paralleled those in the serum bilirubin during recovery from acute hepatitis (Fig. 2).

In several patients free and combined B₁₂ levels were measured and the increase in acute hepatitis appeared chiefly in the free fraction. Urinary excretion of vitamin B₁₂ was followed in a few cases and large increases were found to accompany high serum levels (Fig. 2). Liver cell necrosis was present in all of these patients in varying degree, but the B₁₂

FIG. 2. Serum levels of free and combined vitamin B₁₂, serum bilirubin, and urine excretion of vitamin B₁₂, during the course of a typical attack of acute viral hepatitis in a 56-year-old woman. The total serum B₁₂ level and serum bilirubin showed parallel changes during recovery. Initially the major part of the B₁₂ was in the free form and this fell during recovery, the combined fractions remaining constant.

level could not be predicted from the amount of necrosis present.

PROGRESSIVE HEPATITIS Fifteen patients with progressive hepatitis characterized by persistent jaundice of several months' duration were studied. Needle liver biopsy was obtained in 14 and showed variable degrees of liver cell necrosis, regeneration, fibrosis, and infiltration of the portal tracts by inflammatory tissue.

In progressive hepatitis the initial serum B₁₂ level was less consistently raised than it was in acute hepatitis and, indeed, active liver cell necrosis as assessed histologically and biochemically, sometimes occurred in the presence of a normal serum B₁₂ level, particularly in those patients with the longest histories of jaundice. In this group there were seven with very high levels, three with high levels, three with normal levels and one in each of the intermediate and low ranges. Thus 10 (66.6%) had values above the normal range and two (13.3%) had values below the normal range (Fig. 1).

Serial measurements of serum vitamin B₁₂ were made during the course of the illness in 12 patients. The majority were treated with prednisone which usually caused a reduction or disappearance of jaundice. Serum vitamin B₁₂ usually paralleled the changes in serum bilirubin levels (Fig. 3) and a falling serum B₁₂ level was never encountered in the absence of clinical improvement. The only patient whose serum B₁₂ remained high in spite of a falling bilirubin relapsed three months later and his disease

has continued to run a very active course. On the other hand, in two patients whose liver function was deteriorating, serum vitamin B₁₂ levels remained normal throughout the period of study (Fig. 4), and hence the trend seems of more prognostic importance than the absolute level of serum B₁₂ in progressive hepatitis.

FIG. 3. Serum vitamin B₁₂ and bilirubin concentration in a man of 48 with progressive hepatitis. Parallel changes occurred during response to treatment with prednisone.

FIG. 4. Serial observations of total serum B₁₂ levels, plotted logarithmically, in 11 patients admitted with progressive hepatitis. In seven patients (indicated by a continuous line) jaundice improved considerably during this period, serum bilirubin falling from initial values of between 4.5 mg./100 ml. and 19 mg./100 ml. to levels on discharge of between 0.9 mg./100 ml. and 2.1 mg./100 ml. Four patients (indicated by an interrupted line) failed to respond in any way to treatment with prednisone; the two patients with very high values throughout died while under observation.

PORTAL CIRRHOSIS In the 39 patients with portal cirrhosis studied, the aetiology was unknown in all except two; one of these was an alcoholic and the other had haemochromatosis. The dominant clinical

feature on the first admission was ascites in nine cases, and coma, hypersplenism, jaundice, and bleeding from oesophageal varices each in three cases. The remainder presented with abdominal pain, *Esch. coli* septicaemia, or unrelated conditions.

The serum B_{12} values were very high in seven patients, high in 12, normal in 16, and intermediate in four (above normal in 19 (49%) and below normal in four (10%). Thus high, or very high values were obtained in just under half the patients, compared with in two-thirds of the patients with progressive hepatitis. There was little correlation with the clinical manifestations or with the results of biochemical tests of liver cell function in portal cirrhosis. In six patients admitted in hepatic coma, the highest level was 880 $\mu\text{g./ml.}$ and the mean 431 $\mu\text{g./ml.}$ One of these died and necropsy revealed extensive recent necrosis of liver cells in a shrunken cirrhotic liver, yet the serum B_{12} level shortly before death was only 232 $\mu\text{g./ml.}$ As in acute hepatitis a steadily rising level of serum B_{12} is of ominous prognostic significance and in this series invariably indicated progression of liver disease. On the other hand, in portal cirrhosis a fixed level of serum B_{12} does not necessarily indicate a poor immediate prognosis and it may remain high for periods of weeks or months without obvious deterioration in hepatic function.

In a small number of our patients with chronic liver disease we estimated the free and combined fraction of serum vitamin B_{12} . In contrast to our findings in acute hepatitis, most of the vitamin is present in the combined form. Jones, Mills, and Capps (1957) demonstrated an increased serum- B_{12} -binding capacity in chronic liver disease and suggested that a responsible binding globulin was present in increased amounts. We have been unable to find any constant increase in a protein fraction correlating with a raised B_{12} level as judged by electrophoresis, total proteins, and albumin and globulin fractions, but these methods may be too crude for this purpose.

CHOLESTATIC HEPATITIS In the six patients in this group, jaundice had followed administration of chlorpromazine and in each the serum vitamin B_{12} level was normal.

PRIMARY BILIARY CIRRHOSIS In the four patients in this group, the serum B_{12} level was high in two and intermediate in two. The highest level (940 $\mu\text{g./ml.}$) was found in a patient who died in hepatic coma.

EXTRAHEPATIC BILIARY OBSTRUCTION Twenty-eight patients with obstruction of the larger bile passages were included in this series. Twenty-one were caused

by neoplasm which originated in the pancreas (10), bile ducts (8), ampulla of Vater (1), or involved glands at the porta hepatis (2). In the majority inspection of the liver was possible either at operation or at necropsy within a week or two of the serum B_{12} assay. Six of eight patients with hepatic metastases (75%) showed a raised serum B_{12} level (three high and three very high), but in only one of the 13 in whom no hepatic metastases were found was the serum B_{12} level elevated. Hence a raised serum B_{12} level in a patient with obstructive jaundice due to neoplasm strongly suggests that intrahepatic metastases are present. In the remaining seven patients, jaundice was caused by gall-stone obstruction and the serum B_{12} level was raised in two, both of whom had a severe associated cholangitis. Two of the other patients in the group developed cholangitis while under observation and in each the serum B_{12} level became elevated. At necropsy two further patients were also shown to have cholangitis, yet the serum B_{12} level a week before death had been within normal limits, suggesting that the cholangitis developed as a terminal event.

NEOPLASM OF THE LIVER Twenty-nine patients with proven hepatic metastases were included in the series. Eight had obstructive jaundice and have been discussed in the preceding paragraph. The serum B_{12} reached very high levels in 12 and high levels in four (55% above normal values), normal levels were found in 12, and an intermediate level in one patient. The presence of an elevated serum B_{12} level bore no relation to the presence or absence of jaundice, nor to the source of the primary growth. A raised serum B_{12} level was never found unless the serum alkaline phosphatase level was raised, yet the latter was often elevated in those patients with normal serum B_{12} levels and, in the absence of jaundice, it would, therefore, seem to be of greater value in the diagnosis of hepatic metastases than is the serum B_{12} .

The serum B_{12} level bore no relation to the extent of the carcinomatous involvement of the liver. In one patient a serum B_{12} assay gave a level of 1,800 $\mu\text{g./ml.}$ He was thought to have a simple gastric ulcer and operation appeared to confirm this. The liver palpated at this time appeared normal. However, on histology the ulcer proved malignant and the patient died a few days later. At necropsy a single secondary deposit the size of a thumb nail was found in the left lobe of the liver.

Of the four patients with hepatoma, two had raised B_{12} levels in the very high range, and the other two had normal values. Histologically, all the tumours showed varying degrees of necrosis and large bizarre cells of parenchymal origin forming tumour masses. In the case with the highest serum B_{12} level, wide-

spread necrosis of liver cells had resulted from occlusion of the portal vein by growth.

MISCELLANEOUS DISEASES A serum B₁₂ level of 6,500 µg./ml. was recorded in a patient with extensive amyloid infiltration of the liver. There was no liver cell necrosis detectable histologically.

Very high serum B₁₂ levels were recorded in three patients with subphrenic abscess and in two with an intrahepatic abscess, one of which was pyogenic and one amoebic. These ranged from 1,360 to 3,250 µg./ml. These patients all had a very high polymorphonuclear leucocytosis. Whether these high values resulted from hepatic damage, which was minimal in the patients with subphrenic abscess, or whether they were caused by an associated leukaemoid reaction, it is not possible to say, for very high levels with no free B₁₂ are found in leukaemoid reactions, as well as in chronic myeloid leukaemia.

DISCUSSION

The liver in man is the main storage organ for vitamin B₁₂, containing an estimated 1 mg. of the material. The total serum content based on an average serum concentration of 300 µg./ml. is of the same order as the total requirements, estimated at about 1 µg. a day. This is the minimum dosage necessary to maintain a patient with pernicious anaemia in haematological remission (Darby, Bridgforth, Le Brocqy, Clark, De Oliveria, Kevany, McGanity, and Perez 1958). It is thought that most of the vitamin B₁₂ in the serum is bound to globulins, but it is probable that other serum protein fractions can also bind B₁₂. In a normal serum approximately half of the total binding capacity is utilized. In this laboratory we have found the average total binding capacity of normal sera to be 800 µg./ml. with variations from 550 µg./ml. to 1,200 µg./ml. Presumably it is because of this binding that very little is excreted in the urine, 30 mµg. being the average amount found by Register and Sarett (1951). There is a larger excretion in the bile, which contains vitamin B₁₂ in a concentration approximately 10 times greater than that of serum (Reizenstein, 1959), but is not entirely lost from the body since an entero-hepatic circulation occurs (Gräsbeck, Nyberg, and Reizenstein, 1958). We have found (Dossett and Leese, unpublished data) that when vitamin B₁₂ is given in dosage vastly in excess of the body requirements the binding capacity of the serum is swamped, and the excess free B₁₂ is usually excreted in 48 hours, though on rare occasions urinary excretion may continue for a week. Gräsbeck, Ignatius, Järnefelt, Lindén, Mali, and Nyberg (1961), using radioactive vitamin B₁₂, also found that equilibration is virtually complete within

three days; in addition radioactivity, presumably indicating retained B₁₂, remained in the liver for many months.

In acute hepatitis, whether this is viral or induced by drugs, the serum vitamin B₁₂ level is consistently raised during the early phases, but falls rapidly with remission of the disease as the jaundice begins to recede. The mechanism of this elevation appears to be a direct release of the vitamin from damaged or dying liver cells. In animals previously primed with radioactive B₁₂, acute liver necrosis produced by carbon tetrachloride results in a rapid rise of labelled serum vitamin B₁₂ and a concomitant fall in radioactive content of the liver (Auzépy, Bourdon, and Fauvert, 1959). Nelson and Doctor (1960) found a fall in vitamin B₁₂ content in serial liver needle biopsy specimens from a patient with acute hepatitis using *Lactobacillus leichmannii* as the test organism. Our findings accord with this view, since the B₁₂ levels were high in acute liver disease and the increase consisted largely of the free vitamin. This is at variance with the studies of Kato and Murakami (1959), who found that after carbon tetrachloride induced liver necrosis in the rabbit the increase in the serum level consisted mostly in the bound vitamin, and although some increase in the free form occurred, this was transient.

Cholestatic hepatitis causes little necrosis of liver cells and the serum B₁₂ level remains within normal limits. This is in agreement with the finding of Moran, Eliakim, Suchowolski, and Ungar (1961) that in rats fed with α -naphthylisothiocyanate, which produces acute obstructive cholangiolitis but no liver cell necrosis, serum B₁₂ did not rise in spite of raised levels of serum bilirubin, alkaline phosphatase, and aspartate transaminase.

In chronic liver disease elevation of serum vitamin B₁₂ correlated less well with derangement of hepatic function and normal values were often found, particularly in long-standing portal cirrhosis. This may be explained by the fact that the actual rate of cellular damage and necrosis is much slower than in acute hepatitis and the smaller quantities of vitamin B₁₂ liberated could be adequately dealt with by the body. An alternative explanation is that the vitamin B₁₂ stores of the cirrhotic liver are already severely depleted and hence even rapid cellular necrosis would liberate less of the vitamin into the blood, presupposing that B₁₂ has previously been lost from cells in the liver before they ultimately succumb. Swendseid, Hvolboll Schick, and Halsted (1957) assayed tissue obtained at necropsy and found an average of 0.26 µg./g. in cirrhotic livers compared with 0.72 µg./g. in normal liver and 0.1 µg./g. in untreated pernicious anaemia. Nelson and Doctor (1960) confirmed this in needle biopsy specimens from

cirrhotic patients. That levels of B₁₂ become depleted in the chronically diseased liver is supported by our findings: in 54 patients with progressive hepatitis or portal cirrhosis six (11%) had serum B₁₂ levels below the normal range, which is greatly in excess of the percentage of values falling in the intermediate range in normal subjects.

The mechanism of B₁₂ elevation in patients with tumours involving the liver is also uncertain and appears to be unrelated to the source of the metastases or to their size and number. Liver cell necrosis is usually not a prominent feature, and tumour tissue itself contains very little B₁₂ (Nelson and Doctor, 1962). The explanation may lie in the circulation of abnormal amounts of binding protein.

From the clinical point of view our observations suggest that serum vitamin B₁₂ estimations are of definite, though limited, value in the diagnosis and assessment of prognosis in liver disease. The delay of seven to 14 days necessitated by the method of estimation detracts from the practical value of the test in acute liver disease, in which a clinical recovery is usually obvious before it is confirmed by a falling serum B₁₂ level. In progressive hepatitis a persistently high serum B₁₂ level, even in the face of a declining serum bilirubin, strongly suggests continued activity of the disease process and as such is of definite value in prognosis and the regulation of treatment. The converse does not apply, and in chronic liver disease rapid clinical deterioration may occur without elevation of B₁₂ levels. In patients with obstructive jaundice a high serum B₁₂ level suggests either that neoplasm with intrahepatic metastases is the cause or that cholangitis is also present. Alternatively, it may indicate a mistaken diagnosis suggesting acute hepatitis.

SUMMARY

The significance and diagnostic value of the serum B₁₂ level has been determined in 132 patients with hepato-biliary disease, in the light of clinical and histological findings.

In acute liver disease elevation of the serum level was a consistent finding when necrosis of liver cells was present, whether this was due to virus infection or drugs of the amine-oxidase inhibitor type. In chronic parenchymal liver disease high values were encountered less constantly, even when necrosis of liver cells was evident, and in a significant number of cases the serum level was lower than normal. While a rising level invariably indicated progression of the disease, a raised level could be present for years with no apparent deterioration in liver cell function. The serum B₁₂ level in extrahepatic biliary obstruction

was usually normal unless there was an associated cholangitis or intrahepatic metastases were present. It was also normal in chlorpromazine jaundice.

In patients with proven hepatic metastases the serum vitamin B₁₂ level was raised in a little over half, and hence was less useful in diagnosis than the alkaline phosphatase.

The serum B₁₂ level was high in three patients with subphrenic abscess and in two with an intrahepatic abscess.

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