BIOCHEMICAL PARAMETERS OF LIVER FUNCTION

by

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ABSTRACT

Following studies in animal models of hepatotoxicity four enzymes were selected for investigation as indices of hepatic function in human disease. Isocitrate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and glycyl prolyl-p-nitroanilidase activities were investigated in patients with hepatobiliary disease. Isocitrate dehydrogenase appeared to be a sensitive and specific index of hepatocellular damage. The serum activity of glycyl prolyl-p-nitroanilidase was markedly increased in all hepatobiliary diseases studied.

Although it has become customary practice to use plasma enzyme activities, such as aspartate aminotransferase and alkaline phosphatase, as the biochemical parameters of choice for assessment of liver damage, these enzymes are not true indicators of liver function. Indeed, increased plasma concentrations of the aminotransferases, \( \gamma \)-glutamyltransferase and other hepatocellular enzymes, may reflect only increased permeability of the hepatocyte plasma membranes, enzyme induction, or reversible hepatocellular damage.

In the safety evaluation of drugs there is a real need to be able to evaluate liver function with greater reliability. There have been many instances in recent years, when the use of plasma enzyme levels as liver function tests have failed to detect drug-induced hepatotoxicity or to the contrary have given false indications of potential liver damage.
Measurement of the serum concentration of prealbumin, a protein of short half life, synthesised and secreted by the liver into the blood, is a true test of hepatic function. Concentrations of prealbumin have been determined in the serum of patients with hepatobiliary disease and also in patients who have taken an overdosage of paracetamol. Concentrations of this protein have also been assessed in the serum of mice treated with hepatotoxins. Prealbumin appears to be a sensitive index of impaired liver function in human hepatobiliary disease and drug toxicity and also in animals treated with hepatotoxic chemicals.

This protein may ultimately be of great value in the safety evaluation of new drugs and also as a prognostic tool following the overdosage of hepatotoxic drugs.
CHAPTER ONE

INTRODUCTION

The liver is made up of sheets of hepatocytes (Figure 1.1) which intermingle in such a way that they form a labyrinth of lacunae made up by sinusoids.

The liver is supplied with blood by two routes: the hepatic artery and the portal vein which feed into the sinusoids. The hepatocytes are arranged in such a way that their walls form bile canaliculi, which progressively enlarge to form the biliary system.

Figure 1.1
Structure and subcellular components of a normal liver cell
(From Tietz: Fundamentals of Clinical Chemistry, W.B. Saunders Company, 1970)
The Functional Unit of the Liver

The functional unit of the liver is known as the liver acinus. This represents a microscopic parenchymal mass, irregular in size and shape, and arranged around an axis consisting of a terminal hepatic arteriole, a terminal portal venule, bile ductules, lymph vessels and nerves. (Figure 1.2)

The acinus lies between two or more terminal hepatic venules. The branches of central veins and biliary channels give rise to a vascular pattern simulating a hexagon.

The simple liver acinus can be divided into three circulatory zones. Zone one is the area of cells situated close to the supplying vessels, they are bathed by blood of a composition similar to that of the afferent vessels. Cells in zone three are situated most distant from the supplying vessels, at the microcirculatory periphery of the acinar unit and receives blood that has already exchanged gases and metabolites with cells in zones one and two. These cells are therefore most sensitive to damage due to ischaemia, anoxia, congestion and nutritional deficiency. (Rappaport, 1963) Zone two cells of the liver acinus are transitional cells between zones one and three.

Metabolism in the Liver Acini

From data collected in the literature on histochemistry and enzymology, the liver acinus was shown to be a site of metabolic organisation in close connection with the direction of blood flow.
Figure 1.2

The Liver Acinus: Zones and Enzyme Distribution.
The cells of zone one are geared to glycogen synthesis by virtue of high activity of the enzymes of glycogenesis (Katz, 1977). These cells contain numerous long mitochondria and the oxidative processes operate at a high level via the tricarboxylic acid cycle. Here too the activity of the respiratory enzymes are increased and the abundance of lysosomes rich in acid phosphatase allows a high rate of pinocytosis and the uptake of materials from nutrient laden portal blood.

Protein metabolism and synthesis of plasma proteins has also been shown to occur mainly in zone one of the liver acinus (Le Bouton, 1968).

Zone three is the site of NAD and NADH tetrazolium reductase activity as shown in Figure 1.2. Glycogen is stored in cells of this zone and fat and pigments are formed there. Cells of zone three are rich in microsomes involved with the metabolism of steroids, drugs, toxins and toxic substances. Enzymic specificity and metabolic heterogeneity of cells in different circulatory zones must be implicated in the selective toxic injury of cells in different parts of the liver acinus (Stoner, 1956).

The zonal distribution of drug induced toxic hepatic lesions is due to location of enzymes involved in the metabolism of the substance and its toxic metabolites. Glucuronidation of some drugs proceeds at a more efficient pace in zone one but microsomal transformation and detoxication of other drugs occurs in zone three.
**Production of Plasma Proteins**

The liver is the source of nearly all of the plasma proteins. It is estimated that one third of total proteins synthesised by the liver are released into the circulation as plasma proteins (Peters, 1968). Production of these proteins is maintained even in the case of severe protein depletion (Whipple, et al, 1947).

Plasma protein secretion is a one way process from the liver as little or no degradation occurs in the liver (Gordon, 1962). They are probably broken down to free amino acids elsewhere in the body.

Biosynthesis of albumin occurs at the ribosomes. The newly formed albumin is quickly transferred to the contents of the vesicles of the endoplasmic reticulum (Peters, 1962). In the liver, there is evidence for messenger RNA of both fast and slow turnover. Otsuka and Terayama (1964) suggested that the messenger RNA of albumin was among those metabolised less rapidly, thus explaining the slow response of albumin production to changing conditions.

**The Role of the Liver in Carbohydrate Metabolism**

The storage of glycogen occurs in most tissues but the liver is the only organ capable of releasing glucose from stored glycogen, thus initiating its transport with blood to other tissues. This means that the liver has a unique function, to serve as a central energy storage depot for all tissues (Sunderman, 1968).
Bilirubin Metabolism in the Liver

Bilirubin is formed each day from the break down of red blood cells by the reticuloendothelial system. Haemoglobin is liberated from the cells and is catabolised to haem and then bilirubin. Bilirubin is insoluble in water and is subsequently bound to albumin and transported in the plasma to the liver for excretion as a conjugate with glucuronide. Bilirubin conjugate does not pass through the glomeruli of the kidney and hence in haemolytic anaemia, when vast numbers of red cells are destroyed, no bilirubin appears in the urine although circulating bilirubin concentration does increase.

A scheme of bile pigment metabolism is shown in Figure 1.3.

The haemoglobin molecule is composed of a protein, globin, and an iron porphyrin, haeme. When haemoglobin undergoes degradation, iron and globin are liberated and reutilized but the porphyrin residue is not.

The pathway of haeme breakdown was recently studied in the rat (Brown, 1978). This study showed that although the great majority of haeme resides in haemoglobin, 15-20% of biliary bilirubin is derived from other sources. The other source may be from hepatic cytochrome P-450.

An NADPH-dependant enzyme, haeme oxygenase (E.C. 1.12.99.3) was found to be present in the microsomal fraction of rat liver cells (Tenhunen, 1969). This enzyme is involved in the initiation of haeme oxidation.
Figure 1.3
Pathway of haeme catabolism with some possible intermediates
The nature of the porphyrin cleavage step remains uncertain as is the role of possible intermediates between oxyhaeme and biliverdin (Figure 1.3). Studies on the haeme oxygenase system by $^{18}$O-labelling showed that both terminal oxygen atoms incorporated into bilirubin were derived from different molecular oxygen. Thus the formation of bilirubin by a hydrolytic step does not occur in the haeme oxygenase system.

A failure of the liver to excrete bilirubin in the bile gives rise to jaundice. In the past, the degree of jaundice has been used as an index of liver function. Jaundice is classified into three types (Sherlock, 1963, Billing, 1963):

a) Prehepatic Jaundice

This type of jaundice results from the over production of bilirubin due to excessive haemolysis. This in turn may be due to abnormalities in the red cell or to haemolysis of normal cells by abnormal antibodies or drugs and chemicals.

Phenylhydrazine, snake venom and occasionally sulphonamides have given rise to a haemolytic jaundice. The condition could easily be confused with jaundice due to hepatic damage in the case of drug toxicity. Other liver function tests should be considered, these being mainly normal in haemolytic jaundice.
b) **Hepatic Jaundice**

Hepatic jaundice may arise from three distinct causes. There may be a failure to conjugate bilirubin hence preventing its excretion. A common example of this type of jaundice is in the neonate. Virtual absence of conjugation during the first few days of life greatly increases jaundice due to physiological haemolysis.

Jaundice can also occur when liver function is impaired to some degree, although conjugation of bilirubin may be normal. This is the second type of hepatic jaundice. Centrilobular necrosis, due to drug or chemical toxicity, or infective hepatitis, may destroy the architecture of the liver cells or there may be swelling of the hepatocytes causing blockage of the sinusoids and thus preventing excretion of bilirubin. (Weinbren, 1952) Infective processes may destroy small bile capillaries and cause cholangiolytic hepatitis.

The third type of hepatic jaundice is that of normal conjugation but the conjugated bilirubin fails to reach the large bile ducts. This may be caused by impaired transport mechanisms within the liver, as in the case of the inborn error of metabolism, the Dubin–Johnson syndrome (Dubin, 1954). Primary biliary cirrhosis is another example of a disease causing this type of jaundice. It occurs insidiously usually in middle aged women. The disease is thought to be an autoimmune disease and no mechanical obstruction of bile flow is present.
c) Post Hepatic Jaundice

This type of jaundice is caused by mechanical obstruction. There is thought to be a regurgitation of bile into the bloodstream taking place through the hepatic lymphatics or directly from the distended bile capillaries.

Obstruction to the main bile passages may be caused by calculi, strictures and by a variety of malignant tumours originating in the bile ducts, pancreas or gall bladder.

Jaundice Due to Drugs

Drugs producing liver damage may or may not give rise to jaundice. Those drugs that do cause jaundice may do so by a variety of mechanisms (Sherlock, 1964).

As previously described prehepatic jaundice may be produced by phenylhydrazine. Para-aminosalicylate, phenacetin and quinine, cause jaundice and act by the formation of antibodies which destroy the erythrocyte. Hepatic jaundice may be caused by a generalized toxic action, as in the case with carbon tetrachloride. A hepatitis like jaundice, indistinguishable from infective hepatitis, is caused by iproniazid and its derivatives. A sensitivity reaction occurs with chlorpromazine giving rise to a cholestatic type of jaundice. Jaundice due to drugs may also be caused by competition with conjugation or transport mechanisms.
It is become evident that the mechanism of toxicity of many drugs and chemicals involves oxidation. Oxidants produce haemolysis, for example methaemoglobin formation, giving rise to a haemolytic jaundice.

Microsomal preparations undergoing lipid peroxidation show a loss of haem from cytochrome P-450. (De Matteis and Sparks, 1973). Peroxidation by carbon tetrachloride, for instance, may give rise to jaundice by this mechanism.

Drug Metabolism and Hepatotoxicity

The liver has numerous essential functions to life. Carbohydrate metabolism, fat metabolism, protein metabolism and formation and destruction of cells are all important liver functions.

The liver also plays an important part in the modification of the structure of a large number of foreign compounds including drugs, pesticides, industrial intermediates and solvents. Although this mechanism of modification is called detoxication it is now well known that metabolism of a foreign compound may also give rise to a more toxic metabolite than the original substance.

Williams (1959) divided the major metabolic conversions of drugs into two groups:

a) Phase One Reactions, involving oxidation, reduction and hydrolysis of drugs to produce both active and inactive metabolites.
b) Phase Two Reactions giving rise to conjugates of the drug generally yielding inactive metabolites that are readily excreted.

Although metabolism and conjugation reactions occur in all liver cells, it has been suggested in the previous section that drugs are metabolised more extensively by phase one reactions in the zone three cells of the liver acini and that conjugation of drugs occurs to a greater extent in the zone one cells of the acini (Stoner, 1956).

It is therefore reasonable to suppose that drugs and chemicals that are metabolised by Phase One reactions to yield a toxic metabolite are likely to lead to hepatotoxicity the site of which will be damage to the cells in zone three of the liver acinus. This is in fact the case with carbon tetrachloride and bromobenzene. Both substances are widely used as model compounds to induce hepatotoxicity.

Paracetamol is an analgesic drug which also gives rise to hepatotoxicity due to its metabolism to a toxic metabolite. This metabolite is rendered inactive by glucuronide and sulphate conjugation, which are phase two reactions, occurring in the zone one cells of the liver acine.

The Hepatotoxicity of Carbon tetrachloride

During the past decade the study of carbon tetrachloride induced liver injury has been dominated by two related ideas. Firstly that toxicity depends on the cleavage of the CCl₃-Cl bond and secondly that metabolism of carbon tetrachloride causes a peroxidation of structural lipids within the liver cell.
Formation of a Fatty Liver

Early investigation of carbon tetrachloride toxicity was centred around the problem of the fatty liver. It is now known that the key alteration which accounts for the fatty liver is blockade of the exit of hepatic triglycerides to the plasma as very low density lipoproteins (VLDL), (Recknagel R.O., 1967).

Cellular mechanisms responsible for transport of triglycerides from liver cells to their eventual appearance in the plasma as VLDL are complex. Initially the triglycerides that are synthesised in the hepatic endoplasmic reticulum are released into channels of the endoplasmic reticulum (Hamilton et al, 1967) in association with other lipids and protein. The intracellular lipoproteins move within the cell via processes involving Golgi apparatus. Minor carbohydrate components present in plasma lipoproteins may be acquired there. The intracellular lipoproteins eventually reach the cell surface and are discharged into the plasma where further complexing with plasma HDL and LDL occurs to form plasma VLDL. (Robinson, 1970).

A wide variety of toxic agents and conditions could be expected to give rise to a fatty liver through the same pathophysiological mode of action by interference with this hepatic triglyceride secretion. It is partly for this reason that carbon tetrachloride proved to be a good model for the study of liver cell injury in general (Recknagel et al, 1960).
Mechanism of Action

Soon after the administration of carbon tetrachloride to an experimental animal severe cell injury can be observed in the liver. Within 15 minutes there is initial blockade of hepatic triglyceride secretion which leads to the characteristic fatty liver (Recknagel, 1967, Schotz, 1960). Hepatic protein synthesis rapidly declines and the structure and enzyme activity of the endoplasmic reticulum are altered pathologically. Liver enzymes soon appear in the plasma. Hepatic water balance and electrolyte balance are disturbed. Gradually the mitochondrial elements of the cell become non-functional. Widespread hepatocellular necrosis is observed.

The mechanism of action is now widely accepted (Recknagel, 1973). The lethal cleavage of the CCl₃-Cl bond occurs, localized at the cytochrome P-450 site in the mixed function oxidase system of the endoplasmic reticulum. This homolytic cleavage gives rise to a large number of trichlormethyl free radicals in the vicinity of membrane phospholipids, with a high density of polyenoic fatty acids. The trichlormethyl free radical is able to bind to cytochrome P-450 causing uncoupling of flavoproteins and generation of other radicals such as the superoxide anion.

Lipid peroxidation can usually be curtailed by the scavenging of free radicals by antioxidants such as vitamin E and the dismutation of the superoxide anion free radical by superoxide dismutase (McCord et. al, 1969). Glutathione peroxidase can also protect against lipid peroxidation.
converting lipid hydroperoxides to corresponding alcohols.
(Christophersen, 1968).

In the case of carbon tetrachloride toxicity, however, the number of free radicals overwhelm the protective mechanisms with consequent disruption of normal membrane structure and function.

Membrane phospholipids have a high density of polyenoic fatty acids. These fatty acids are particularly susceptible to free radical attack and this occurs in carbon tetrachloride toxicity as shown in figure 1.4. It can be seen that for each radical formed by carbon tetrachloride metabolism, two organic radicals are formed. Thus the theory provides a mechanism of understanding the devastating effects of very low doses of carbon tetrachloride, since relatively few $\text{CCl}_3$-Cl bond cleavages would result in the appearance of many new free radicals. The process would be expected to proceed from sites relatively distant from the locus of the initial cleavage and provides a mechanism for the explosive character of the pathological changes set in motion by carbon tetrachloride poisoning.

Figure 1.5 shows a timetable of events occurring in carbon tetrachloride induced liver injury.

The Effect of Carbon Tetrachloride on Protein Synthesis

Marked depression of protein synthesis and dispersion of polyribosomes occur early in carbon tetrachloride liver cell injury (Alpers, 1968; Seakins, 1963; Smuckler, 1962). It is possible that sulphhydryl groups, vital to protein synthesis,
Figure 1.4 Lethal Cleavage and Mechanism of Action of Carbon Tetrachloride
### Timetable of Events in Carbon Tetrachloride Induced Liver Injury

<table>
<thead>
<tr>
<th>TIME AFTER DOSE (hours)</th>
<th>EVENT</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>Disturbance to lipid metabolism</td>
</tr>
<tr>
<td>1</td>
<td>Change in appearance of endoplasmic reticulum</td>
</tr>
<tr>
<td></td>
<td>Depression of microsomal enzyme activity</td>
</tr>
<tr>
<td></td>
<td>Depression of hepatic protein synthesis</td>
</tr>
<tr>
<td>2 - 4</td>
<td>Increasing hepatic calcium content in mitochondria</td>
</tr>
<tr>
<td></td>
<td>Electrolyte balance disturbances</td>
</tr>
<tr>
<td></td>
<td>Swelling of liver cells</td>
</tr>
<tr>
<td></td>
<td>Depletion of hepatic glycogen</td>
</tr>
<tr>
<td>5 - 10</td>
<td>Lysosomal disruption</td>
</tr>
<tr>
<td></td>
<td>Focal necrosis (midzonal)</td>
</tr>
<tr>
<td></td>
<td>Intracellular enzymes appear in plasma</td>
</tr>
<tr>
<td>10</td>
<td>Mitochondrial damage</td>
</tr>
<tr>
<td>12</td>
<td>Centrilobular cells exhibit prenecrotic changes</td>
</tr>
<tr>
<td></td>
<td>Balloon cells in midzonal region</td>
</tr>
<tr>
<td>24</td>
<td>Marked centrilobular necrosis (up to half of lobule)</td>
</tr>
</tbody>
</table>
may be attacked by trichlormethyl radicals generated from the metabolism of carbon tetrachloride. There is at present no evidence to support this view, however. Alternatively the free radicals could attack membrane proteins, especially the free sulphhydryl groups resulting in the membrane as a result of lipid peroxidation, thus causing a depression of protein synthesis (Ellman, 1959).

The Effect of Inducing Agents on Toxicity

A number of drugs and chemicals have been shown to cause an increase in the activity of the liver microsomal drug metabolising enzymes of treated animals (Conney, 1962; 1972). An example is the action of phenobarbitone.

Bromobenzene

It is well established that bromobenzene, a halogenated aromatic hydrocarbon, causes centrilobular necrosis of the liver. When administered to animals the first histological event to occur is the depletion of cytoplasmic glycogen after 12 to 16 hours. Centrilobular necrosis is observed after 24 hours (Reid et al, 1973).

Bromobenzene is thought to be metabolised initially to an epoxide, 4-bromobenzene oxide (Jollow et al, 1974; Azouz et al (1953); Daly et al, 1972). Epoxides are known to undergo non-enzymic rearrangement to phenols, hydration by an epoxide hydrase to yield dihydrodiols or conjugation with glutathione by a glutathione-S-epoxide transferase to yield mercapturic acids, (Boyland et al, 1969; Daly et al, 1972). The epoxide metabolite of bromobenzene can follow any of these courses.
Pathway of the Metabolism of Bromobenzene in the Rat
The 3,4-bromobenzene oxide can act like all other epoxides to be toxic. Epoxides have mutagenic properties, they can cause malignant transformation of tissue culture cells and they can be alkylating agents reacting with proteins and nucleic acids (Jollow et al, 1974; Grover et al, 1971; Huberman et al, 1971). The nature of the bromobenzene epoxide possesses all the required characteristics needed for toxicity. It is highly electrophilic leading to a rapid reaction with cellular nucleophiles. The epoxide also has sufficient stability to be transported from the site of formation to vital organelles in the cell.

Small doses of bromobenzene lead to little toxicity. This is due to the protective role of glutathione forming a mercapturic acid and removing the toxic epoxide metabolite of bromobenzene from circulation. However, in high dosage, glutathione stores become depleted and high concentrations of 3,4-bromobenzene oxide bind to macromolecules covalently causing necrosis of liver cells (Figure 1.6).

Much investigation has been done on the protection of the liver against bromobenzene toxicity. Phenobarbitone has been shown to enhance the toxicity of bromobenzene by inducing greater metabolism to the toxic epoxide. Conversely blocking the metabolism of bromobenzene to its epoxide with piperonylbutoxide or SKF 525A prevents the liver necrosis and decreased the covalent binding of the toxic metabolite (Zampaglione, 1973).
Paracetamol Mechanism of Toxicity

Paracetamol, or Acetaminophen as it is known in the United States, is safe at therapeutic doses, but it can produce a fulminating hepatic necrosis when taken in overdose.

When administered to rats and mice, paracetamol was found to produce centrilobular hepatic necrosis similar to that found in man, (Prescott et al, 1979; Boyer and Rouff, 1971; Boyd and Bereczky, 1966). Investigation of the mechanism of hepatic necrosis by Mitchell et al (1973) showed that phenobarbitone enhanced the toxicity of paracetamol and piperonyl butoxide, a metabolite inhibitor, prevented the necrosis due to paracetamol toxicity. These observations lead to the hypothesis that paracetamol is metabolised to an intermediate that causes the hepatotoxicity and necrosis.

A further study by Jollow et al (1973 a) showed that the degree of necrosis in the liver paralleled the extent of binding of radiolabelled paracetamol. Rees and Tarlow (1967) postulated that the intrahepatic localization of the enzymes that produce toxic metabolites may determine the distribution of necrosis. This hypothesis was predicted on the finding that periportal necrosis was caused by allylformate and allyl alcohol, presumably as a result of their conversion to the reactive aldehyde, acrolein, by an alcohol dehydrogenase localized in periportal hepatocytes.
Figure 1.7

Pathways of Paracetamol Metabolism
Histochemical studies reveal a centrilobular distribution for several drug metabolising enzymes of the type that metabolise paracetamol. (Koudstaal et al 1969, 1970) Thus the centrilobular location of the paracetamol induced lesion might be explained by the formation in the centrilobular cells of a highly reactive metabolite of paracetamol that alkylates nearby macromolecules thereby causing cellular damage.

Further work revealed that paracetamol caused the depletion of hepatic glutathione (Mitchell et al 1973c) when given in overdose to mice.

The proposed mechanism for metabolism and toxicity of paracetamol is shown in Figure 1.7.

A therapeutic dose of paracetamol does not cause liver damage because small amounts of active metabolite are rapidly inactivated by preferential conjugation with hepatic glutathione and subsequently excreted in the urine as cysteine and mercapturic acid conjugates (Mitchell, J.R., 1975). However, hepatic glutathione is rapidly depleted by toxic doses of paracetamol and when stores are reduced to less than about 30% of normal, the excess metabolite is free to alkylate vital cell constituents causing necrosis.

More recently lipid peroxidation following paracetamol intoxication has been studied by measuring exhaled alkanes (Wendel, 1979). It was shown that when liver glutathione levels are depleted by paracetamol, lipid peroxidation occurs.
Paracetamol Overdose

Self poisoning with paracetamol is very common. The physician is often confronted with the problem of determining the risk of liver toxicity in a patient who may have ingested an overdose of paracetamol. An ingested dose of 15g of paracetamol may be associated with liver toxicity. The normal half life of the drug is two hours, and concentrations in blood are usually assessed after four hours. If the plasma level is greater than 300 µg/ml after four hours the incidence of hepatotoxicity is 100%, if less than 120 µg/ml there is no danger (Ambre, 1977).

Since glutathione is depleted in paracetamol poisoning many sulphhydryl-containing amino acids have been tried as antidotes to paracetamol toxicity. However, these antidotes themselves are not totally innocuous. Cysteamine was found to be successful in treating severe paracetamol overdosage (Prestcott et al, 1974). Cysteamine however causes flushing, nausea, vomiting, drowsiness and a generally miserable feeling, as side effects for up to 36 hours after administration. It is thought that cysteamine protects against hepatic necrosis by combining with active paracetamol metabolite within the hepatocyte in the same way as glutathione thus preventing alkylation of liver-cell macromolecules (Mitchell et al, 1973, a, b, c).

As well as protecting the levels of hepatic glutathione, cysteamine may also inhibit the oxidation of paracetamol to the toxic metabolite (Leading Article, Lancet). Cysteamine is only effective if given within ten hours of ingestion of
paracetamol however and there is a risk of precipitating hepatic coma if it or any of the other S-amino acids are given more than ten hours after the overdose. This is due to the fact that the already damaged liver may be unable to metabolise them (Murphy, 1976).

Earlier treatments with corticosteroids, antihistamines, heparin haemodialysis and charcoal column haemoperfusion failed to prevent liver damage in severely poisoned patients. (Editorial, British Medical Journal, 1977). Administration of glutathione also proved unsatisfactory since cell penetration is low. Alternatives used in recent years have included cysteine, methionine and cysteamine. N-Acetyl cysteine as an antidote to paracetamol poisoning has been proposed. (Piperno, 1976).

Intravenous infusion of N-Acetyl cysteine is recommended as the treatment of choice in acute paracetamol poisoning. (Prestcott, 1979). Virtually complete protection against liver damage was achieved in 40 patients treated with N-Acetyl cysteine within eight hours after ingestion of paracetamol. In the same study N-Acetyl cysteine was shown to be more effective than cysteamine and methionine and free from adverse effects. Controversy exists however, with other workers suggesting that methionine is as effective as N-Acetyl cysteine and much cheaper to use (Widdop, 1979).

Paracetamol overdose is a real problem. Early clinical assessment is impossible, even in severe poisoning the initial features are mild with little other than pallor, nausea and occasional vomiting. Liver damage may not become apparent clinically for five or six days. Furthermore there is a considerable individual variation in susceptibility to
hepatic damage and in the presence of alcohol or barbiturates the liver toxicity may be enhanced.

The results of conventional liver function tests including enzyme tests are unreliable indices since several days may elapse before these indicate severe toxicity. (Leading Article, Lancet 1975, and Editorial, British Medical Journal, 1977). There is indeed a need for a liver function test that is able to monitor the effects of paracetamol overdose more efficiently.
The safety evaluation of new drugs and chemicals is difficult, expensive and complex. Trials and tests in animal species can only yield reliable information about the drug in that species and it may be dangerous to assume similar toxicity or metabolism in the human.

Recently there has been great concern with the role of vinyl chloride as a cause of angiosarcoma of the liver (Popper 1975) and the relationship of natural hepatotoxins to carcinogenesis in man.

Concern has focused on the hepatic injury caused by a number of medicinal agents (Zimmerman, 1978). The past few years have witnessed hepatocellular disease produced by reactions to isoniazid (Black et al, 1975), halothane (Bottinger, 1976), α-methyldopa (Rodman, 1976), oral contraceptives (Schmidt, 1977; British Medical Journal Editorial, August 1977; Fechner, 1977) and numerous other drugs all of which were available on prescription.

Acute hepatic injury produced by some drugs is characterised by damage to the hepatocytes. Other drugs lead to cholestatic injury manifested by arrested bile flow and jaundice. Some drugs lead to a mixture of both types of injury, that is they lead to cytotoxic and cholestatic injury.

The development of steatosis and fatty cirrhosis in some patients after prolonged administration of methotrexate has become a problem of major concern to physicians using this drug to treat psoriasis. Testing for the development of fatty liver and cirrhosis in recipients of methotrexate appears to
require periodic liver biopsy since biochemical testing is insufficiently sensitive. Clearly the approach to the diagnosis and prediction of drug induced hepatic injury requires considerable improvement (Zimmerman, 1978).

A large number of tests have been proposed to detect and monitor liver function in experimental animals and man. It is desirable to have cognizance of sensitivity, specificity and simplicity as well as reliability of the biochemical determination to evaluate hepatotoxicity. Tests may provide information on the functional status of one or more of the structural units of the liver; hepatocytes, biliary network, vasculature and mesenchymal cells.

Hepatotoxicity reactions which are often predictable and can be reproduced in animals, are dose dependant. Occasionally however they are unpredictable and do not produce characteristic untoward effects in animals. Animal studies are used to detect predictable reactions and these can provide guidance for future investigations of drug or chemical hepatotoxicity in Man. Sensitivity or idiosyncratic reactions, however, may not be detected.

A standard progression of liver function tests are used in animal studies, dependent on the practical limitations imposed by the size of the animal used, the ability to do tests without anaesthesia and the purpose or design of the experiment. Acute and chronic studies are employed with attention to toxicity, teratology, mutagenicity and carcinogenicity in the animal trials of new drugs and chemicals.
Studies in Man are designed to evaluate the relative risk of predictable hepatotoxicity of drugs and chemicals whose pharmacology indicates liver lesions may develop following administration or exposure to the drug. They are also used to detect predictable reactions in Man that do not occur in animals and to detect reactions that are not predictable.

There seems however no ideal liver function test that can be used to both monitor and detect hepatotoxicity of drugs and chemicals. Volumes of literature on the choice of liver function tests confesses to a failure of inadequate monitoring of true liver function.

Many drugs available on prescription are known to have a small incidence of hepatotoxic side effects. These effects need to be detected before damage is severe in the unlucky patient who develops hepatotoxicity.

**Hepatotoxicity of Perhexiline Maleate**

A drug currently prescribed for attacks of angina, PEXID, has also shown to have occasional hepatotoxic side effects. As an anti-anginal drug PEXID, perhexiline maleate, is second to none. Trials of the drug showed that a mean reduction of seventy percent in anginal attacks was observed. The drug can be used in patients with asthma or bronchitis as no significant alteration in ventilatory function occurs. Perhexiline improves the efficiency of the heart, thus reducing the number of anginal attacks. It does not act as a beta blocker or vasodilator. The effectiveness of the drug in patients with crippling anginal pain is proven beyond doubt (Armstrong, 1973).
However perhexiline administration is being increasingly associated with hepatotoxicity and neurotoxicity in a proportion of patients receiving the drug. There is a strong indication that wide differences in the rates of metabolic deactivation and excretion may be responsible for a genetic disposition to perhexiline toxicity.

The hepatic changes of centrilobular necrosis and hepatitis were common on a dose of 400 mg perhexiline daily. The damage was dose related and reversible (Pilcher et al., 1973). Histopathology studies indicate that the hepatotoxicity is associated with a sphingolipidosis. Larger than normal lysosomes are present with abnormal structure suggesting damage to the endoplasmic reticulum and impaired synthesis of lysosomal enzymes (Beaugrand et al., 1977). The damage to the endoplasmic reticulum causes a loss of glucose-6-phosphatase and hence an accumulation of hepatocellular glycogen and hypoglycaemia, observed in perhexiline toxicity.

Perhexiline undergoes N-methylation which may cause interference with choline metabolism resulting in a decrease of phosphatidyl choline synthesis, and abnormal functioning of the endoplasmic reticulum. This might also result in the degranulation of the endoplasmic reticulum, a process known to be associated with malignant cell transformation and carcinogenicity.

Perhexiline is also metabolised by hydroxylation of the two cyclohexane rings and presumably glucuronide conjugation. Metabolites are excreted in both the urine and faeces (Wright et al., 1973) (Figure 1.8).
Figure 1.8

Metabolic Pathways of Perhexiline
Population studies reveal a market genetic difference in the detoxication and excretion of perhexiline. Plasma half life varies from thirtysix hours to more than twenty days. There are fast and slow hydroxylators which results in overdosage in some twenty percent of patients. (Bousser, 1977). In order to prevent the hepatotoxicity it is clear that individual tailoring of the dose to the patient is required. Secondly liver function tests must be followed before and throughout the course of treatment in order to detect early changes in liver function.

Isoniazid

In a study of nearly two hundred patients receiving isoniazid, serum enzymes and bilirubin concentrations were determined monthly. About twenty percent showed abnormal values which subsided while the patient continued to take isoniazid (Mitchell, J.R., 1975). Thus isoniazid was reported to be hepatotoxic in a large proportion of individuals but most adapted to the insult and recovered rather than progressing to severe hepatic injury. No correlation of toxicity was found with slow acetylation of isoniazid, and no anti-isoniazid antibodies were found to explain an immunological sensitivity to the drug.

A major study in the United States on over ten thousand isoniazid patients discovered that isoniazid related liver injury could not be distinguished, biochemically or morphologically, from other causes of acute hepatocellular injury, such as viral hepatitis (Black, 1973). This study also showed no evidence of a hypersensitivity reaction to the drug.
A third study of isoniazid (Rao, 1970) discovered the fast and slow acetylation of isoniazid to have some association with toxicity. About thirty percent of the patients with hepatic reactions were residents of Honolulu and of Oriental origin. This group would be genetically expected to be fast acetylators of isoniazid.

Animal studies on the relative toxicity of the various metabolites (Figure 1.9) of isoniazid revealed the toxicity of acetylhydrazine. The hypothesis that fast acetylators would be more susceptible to isoniazid toxicity than slow acetylators arose. The fast acetylators would produce more acetyl hydrazine than the slow acetylators. The acetyl hydrazine was shown to produce centrilobular necrosis at low doses in the rat (Snodgrass, 1974). Other clinical observations do support this hypothesis. Rifampicin, an excellent inducer of drug metabolising enzymes increases the incidence of isoniazid toxicity. This may be explained by increasing activation of acetyl hydrazine to its toxic form. Para-aminosalicylic acid decreases the incidence of toxicity by inhibiting acetylation of isoniazid (Lees, 1971).

Here again is a drug that needed careful monitoring of liver function in the animal studies and then clinical trial stages of its safety evaluation. The inadequacy of such liver function tests make evaluation of drugs of this type very difficult.
Isoniazid

\[ \text{'N' ACETYLATION} \]

N-acetylisoniazid

\[ \text{Acetyl hydrazine.} \]

Hepatic Necrosis and Neoplasms

Covalent binding to cell macromolecules

Figure 1.9 Metabolism of Isoniazid (Mitchell, 1973)
1. Clearance Tests

An evaluation of uptake, storage and biliary excretion of dyes, drugs and endogenous metabolites have been widely used to detect subclinical liver injury. The test substance that has been most widely used to assess liver function in the past is bromosulphalein (BSP). More recently indocyanine green has been used for this purpose. Either may be used in animal and human studies in assessing hepatotoxicity.

The dyes are assumed to be treated in the liver in the same manner as naturally occurring organic anions. This is generally true with regard to uptake, intracellular binding and biliary excretion but certain differences in metabolic transformation, for example conjugation reactions, are recognized.

The molecular mechanisms of transport, binding and conjugation processes are largely unknown and elimination kinetics of these dyes have not been completely elucidated (Quittner, H., 1968).

The bromosulphalein test was introduced by Rosenthal and White in 1925 (Rosenthal, 1925) This was followed in 1931 by the less widely accepted Rose Bengal test (Delprat, 1931) and in 1959 by the use of indocyanine green (Leevy, 1959).

A number of substances have been employed as tolerance tests, among them benzoic acid and cinnamic acid. Conjugation of the substance was measured. Of this group
the benzoic-hippuric acid test was most widely used. Its major drawback has been that it requires an accurately timed four hour urine specimen.

2. Flocculation Tests

Since 1917 simple and inexpensive procedures have been proposed which consist of adding reagents to the serum in question with the production of a precipitate or flocculate which is easy to detect or measure. These type of tests are known as flocculation tests.

The tests are based on a concurrence of conditions that are usually present in the serum of patients with liver disorders. The precipitate obtained is made up of gammaglobulin and the reagent used for flocculation. The presence of gammaglobulin in other pathological conditions gives rise to spurious positive results whilst the globulin may not be present in some liver diseases giving rise to false negative results (Reinhold, 1960).

The cephalin flocculation and thymol turbidity tests are commonly and universally used. The cephalin-cholesterol test has many difficulties with the method since the working reagent is easily prepared improperly. The cephalin-cholesterol emulsion acts as an 'antigen' to which the gammaglobulin binds causing flocculation.

The thymol turbidity test (Mac Lagan, 1944) was developed as a result of an observation that barbital solutions containing phenols caused turbidity when added to the serum of patients with hepatitis but not with the serum of healthy persons. Here again the gammaglobulins produced in liver disease bind to the thymol causing
turbidity.

Although in liver disease elevation of serum gammaglobulins and decreased concentrations of albumin, alpha\textsubscript{1}-globulins and mucoproteins occur, this also happens in tuberculosis, myeloma and macroglobinaemia. Thus false positives are an important consideration in the interpretation of the flocculation tests.

3. **Plasma and Serum Enzyme Activity Changes**

The idea of the liver as a 'bag of enzymes' lead to the investigation of the release of enzymes from the bag when the liver was damaged.

Many factors governing the release of enzymes from the liver should be considered. Intracellular distribution, stability, changes in the permeability of the cell membrane and the presence of coenzymes, activators and inhibitors are all factors which affect the release of enzymes.

It is important to stress, from the beginning, that enzymes are not tests of liver function. The dye clearance tests rely on a function of the liver whereas enzymes are only indicators of hepatocellular damage. Enzyme studies, however, have been invaluable in aiding the diagnosis of liver disease. Current methodology has allowed screening of serum enzymes in hospitals to be routine. Enzymes that are determined for the investigation of liver function have been chosen for a number of reasons (Burke, 1978).
a) They are easily determined by automation.
b) They have activities in serum that are easily measured.
c) They show sensitive changes to disease conditions, not necessarily liver diseases.
d) They have reliable and consistent levels in normal serum.
e) They are stable in blood and not affected by haemolysis, high bilirubin concentrations, etc.
f) The method of determination is well established and reproducible.

To review every enzyme that has been used to study liver damage in the past would be impractical. The most commonly used and probably the best enzymes for this purpose will be reviewed here.

The hepatotoxicity of drugs and liver disease have in common an important parameter. Both require early diagnosis and then adequate monitoring. Enzymes have been used to do this but all have their disadvantages as well as advantages. No enzyme has been found to specifically detect and monitor liver damage or hepatotoxicity (Wilkinson, 1970).

**Serum Aminotransferases**

Aminotransferases, or transaminases as they have been known until recently, are intracellular enzymes found in nearly all tissues. They are most active within the cells of the liver, heart and skeletal muscle, adipose tissue, brain and kidney where they catalyse a wide variety of key metabolic reactions.
Plasma activity is believed to be due to the release of enzyme from tissue cells as a result of cell turnover or injury. Aminotransferases are constantly being removed from plasma and their half-lives depend on their particular structure.

The aminotransferases almost universally used for the study of liver damage are aspartate aminotransferase (AST, EC 2.6.1.1. formally glutamate-oxaloacetate transaminase) and alanine aminotransferase (ALT EC 2.6.1.2., formally glutamate-pyruvate transaminase). These enzymes catalyse the transfer of an amino group from the donor amino acid, either aspartate or alanine respectively, to a receptor keto-acid, in this case oxaloacetate. (Figures 1.10 and 1.11)

Aspartate aminotransferase is found in substantial quantities in heart muscle, kidney and skeletal muscle as well as the liver. Alanine aminotransferase however is located largely in the liver although a small quantity is found in heart muscle (Wroblewski, 1956). Aspartate aminotransferase occurs in the soluble fraction and the mitochondria (Eichel, 1961) but the two forms differ in electrophoretic mobility, pH optima and substrate affinity. Alanine aminotransferase occurs solely in the soluble fraction (Boyd, 1961; Fleisher, 1960).

A variety of acceptable methods of eliminating aminotransferases have been worked out depending on colorimetric or spectrophotometric methods. The changes undergone by these two enzymes in liver disease have been extensively investigated (Wroblewski, 1958; Wilkinson, 1961).
Reactions Catalysed by Aspartate Aminotransferase

Aspartate

\[ \text{CH}_2\text{COOH} \quad \text{CH}_2\text{CH}_2\text{COOH} \]

\[ \text{CH(NH}_2\text{)COOH} \quad \text{CO.COOH} \quad \alpha\text{-ketoglutarate} \]

Aspartate Aminotransferase

oxaloacetate

\[ \text{CH}_2\text{COOH} \quad \text{CH}_2\text{CH}_2\text{COOH} \]

\[ \text{CO.COOH} \quad \text{CH(NH}_2\text{)COOH} \quad \text{Glutamic Acid} \]
Figure 1.11

Reaction Catalysed by Alanine Aminotransferase

\[
\begin{align*}
\text{Alanine} & \quad \xrightarrow{\text{Alanine Aminotransferase}} \quad \alpha\text{-ketoglutarate} \\
\text{Pyruvate} & \quad \xrightarrow{\text{Alanine Aminotransferase}} \quad \text{Glutamic Acid}
\end{align*}
\]
The coupled reaction spectrophotometric method of determining the aminotransferases is used as an automated routine laboratory procedure. Aspartate aminotransferase catalyses the reaction shown in Figure 1.10. The oxaloacetate formed is detected by a second coupled reaction using added malate dehydrogenase and NADH, reading the decrease in optical density at 340 nm. as the NADH is oxidised. Alanine aminotransferase is determined by measuring the amount of pyruvate formed in the reaction (Figure 1.11).

This is also done by using a second coupled reaction. Pyruvate is reduced by NADH and lactate dehydrogenase, the decrease in optical density is read once again as the NADH is oxidised.

Automation of the aminotransferase assays has introduced methodological differences and interpretive difficulties. The Technicon technique for example uses an incubation at 37.5°C for 6.1 minutes for the AST reaction before initiating the second reaction by dialyzing the oxaloacetate formed into a second stream containing NADH and malate dehydrogenase.

The resulting units of activity cannot be called either International or Karmen Units, but machine derived values do have a rough correspondence that clinicians should interpret with caution. (Guidelines by F.D.A. after Fogarty International Conference, November, 1977.)
More Specific Liver Enzymes

A number of enzymes have been investigated and reported as being more specific to the liver than aminotransferases. Some of these enzymes will be outlined below.

a) Alkaline Phosphatase (E.C. 3.1.3.1)

Alkaline phosphatase is widely distributed in various organs of the body, including liver. Isoenzymes may be located histochemically in the sinusoidal and canalicular borders of the hepatocyte. The function of the enzymes is thought to be one of membrane mediated transport. Alkaline phosphatase is excreted in the bile, hence disturbance of the excretion of conjugated bile acids or mechanical obstruction of the biliary tree, causes increased amounts of hepatic alkaline phosphatase to enter plasma, resulting in elevated alkaline phosphatase activity.

The placenta, bone and intestine also contribute to alkaline phosphatase activity in plasma but it is possible to identify the tissue of origin of the alkaline phosphatase in a serum sample. This is done by electrophoretic fractionation. Paper, starch blocks, cellulose acetate and agar gels have been used to do this in the past. (Gutman, 1959; Stanton, 1962; Keiding, 1959; Korner, 1962; Warnock, 1966; Guilleux, 1979; Sakiyama, 1979)
In healthy individuals serum alkaline phosphatase is a composite derived from liver, bone and in some individuals intestine. In the non-jaundiced patient, alkaline phosphatase activity in serum is a useful detection and monitoring tool in drug-induced cholestasis, suspected obstruction of the common bile and hepatic ducts, suspected tumours, primary biliary cirrhosis and liver abscess and infections. An increase in the liver isoenzyme of alkaline phosphatase is indicative of a hepatic lesion, usually one that gives rise to cholestasis.

High activity of the liver isoenzyme is observed in the serum of patients jaundiced due to drug toxicity, for example, chlorpromazine (Nørredam, 1963; 1957; Russell, 1973; Zimmerman, 1978.) in which there is minimal hepatocellular damage but extensive intracanalicular obstruction.

Because of the specificity in the character of the liver type alkaline phosphatase the enzyme is valuable in the monitoring of patients with cholestasis.

b) 5' Nucleotidase (E.C. 3.1.3.5.)

This enzyme is found in the hepatocyte in a distribution similar to that of alkaline phosphatase and its role is also believed to be one of membrane mediated transport. Although the enzyme is found in organs other than the liver, such as placenta, bone and the pituitary gland, no serum elevation is seen in diseases of these organs.
For this reason, serum 5'-nucleotidase has been used in conjunction with alkaline phosphatase in distinguishing between hepatic and non hepatic causes of increased serum alkaline phosphatase activity.

Difficulties in the determination of 5'-nucleotidase activity occurs due to the presence of alkaline phosphatase which hydrolyzes the substrates used in the determination. A complex reaction procedure involving inhibition of 5'-nucleotidase activity with the Nickel ion has meant that automation of the 5'-nucleotidase assay is difficult. Manual estimation of the enzyme is tedious and time consuming.

In known hepatobiliary disease, elevations in alkaline phosphatase and 5'-nucleotidase activity tend to parallel each other. 5'-nucleotidase has no advantages over the determination of liver specific alkaline phosphatase isoenzyme and its use in the clinical laboratory is limited. (Dixon, 1954)

c) **Gamma-Glutamyl Transferase** (E.C.2.3.2.2.)

The enzyme gamma-glutamyl transferase (GGT) is now widely accepted as an enzyme of importance in the investigation of liver disease.

The enzyme catalyzes the transfer of gamma glutamyl groups from gamma-glutamyl peptides to other peptides, L-amino acids and water. It participates in peptide nitrogen storage, in protein synthesis and in the regulation of tissue glutathione levels. (Rosalki, 1975).
In human tissues GGT activity is prominent in kidney, pancreas, liver and prostate (Rosalki, 1974); the kidney shows the highest activity. Elevated activities of this enzyme have been shown to occur in hepatobiliary diseases especially in biliary obstruction and malignancy with hepatic involvement. In obstruction the incidence surpasses that of alkaline phosphatase, leucine aminopeptidase and 5'-'nucleotidase. Although of little use in acute hepatocellular disease, GGT activity in chronic hepatocellular disease exceeds that of the aminotransferases.

The specificity of GGT to liver disease is not absolute but the enzyme shows no elevation in bone disease (unlike alkaline phosphatase) and muscle disease (unlike aminotransferases) (Rosalki, 1975).

Activities greater than normal are found in myocardial disease and in diabetes (Goldbarg, 1963). The enzyme has also been shown to be sensitive to induction by drugs, including alcohol. The fact that GGT is elevated either due to induction or due to hepatocellular damage has given rise to its use as an enzyme of special sensitivity in the diagnosis of alcoholism (Hamlyn, 1979).

A recent study has suggested that GGT was more susceptible to spurious elevation in the absence of hepatobiliary disease than other tests. The same group suggested that GGT had little value other than for monitoring alcohol abuse and enzyme induction (Ellis, et al, 1979).
Other enzymes indicating hepatocellular damage

Other serum enzymes with a high specificity for liver disease have been reported. Ornithine Carbamyl Transferase (E.C.2.1.3.3.), isocitrate dehydrogenase (E.C.1.1.1.42) and glutamate dehydrogenase (E.C.1.4.1.2.) and sorbitol dehydrogenase (E.C.1.1.1.14) have all been studied to varying degrees but most reports claim that they are less sensitive indicators than the amino-transferases (Wilkinson, 1968).

Glutamate dehydrogenase is an NAD (or NADP) dependent enzyme which catalyzes the oxidative deamination of glutamate. Normally, only traces of this enzyme can be detected in the serum but significant elevation occurs in severe hepatocellular damage especially due to drugs in obstructive jaundice, alcoholic hepatitis and liver metastases (Cerdan, 1978; Leiber, 1978).

The enzymes of the urea cycle are usually specific to the liver mitochondria and have subsequently been investigated for use as potential markers of hepatobiliary disease (Maier, 1979).

Ornithine carbamyl transferase (OCT) is one such enzyme. The amount of this enzyme in the serum of a healthy individual is almost indetectable but significant increases are highly specific for hepatocellular injury. Although the method of determination of the enzyme is tedious (Vassef, 1978) and large quantities of serum sample are used, OCT
still has the advantage of being liver specific and a high serum level must be indicative of hepatocellular damage (Brown, 1959; Reichard, 1961).

Ornithine carbamyl transferase occurs in bile and could be eliminated by this route (Reichard, 1960). Reichard showed that sensitive elevation of activity of the enzyme occurred in the serum of patients with viral hepatitis, obstructive jaundice, biliary colic, acute cholecystitis and metastatic carcinoma of the liver.

OCT has also been reported to be a sensitive indicator of hepatocellular damage following chemical poisoning in the laboratory animal (Drotman, 1978).

NADP-dependent isocitrate dehydrogenase, an enzyme of the tricarboxylic acid cycle, has been investigated in numerous diseases of the liver. All diseases where there has been an active process of hepatocellular damage has given rise to high activities of serum isocitrate dehydrogenase. No elevated activity of the enzyme was recorded when hepatocellular damage was absent. (Cohen, 1961).

Increased activity of the enzyme has only been reported in liver disease and also in the presence of haemolysis (Bowers 1959; Sterkel, 1958; Okumura 1960). The aminotransferases have the disadvantage of increased activity during both myocardial and hepatic damage. Comparisons were made by Cohen (1961) and Bodansky (1960) of the sensitivity of isocitrate dehydrogenase
and aminotransferases in detecting liver damage.

Both studies showed that isocitrate dehydrogenase is a sensitive and specific indicator of hepatocellular damage but slightly less sensitive than alanine aminotransferase. The elevated activity, due to an outflow of enzyme from liver cells, was found to be at a maximum in patients with metastatic carcinoma of the liver.

Watts (1966) suggested that there may be an extrahepatocellular source of isocitrate dehydrogenase in order to explain high serum activities in acute inflammation of the biliary tract. Earlier Baron (1963) had stated that the enzyme was normal in obstructive jaundice. Variably raised values of serum enzyme activity have been reported by other workers in obstructive jaundice thus complicating suggestions that serum isocitrate dehydrogenase could be used to differentiate between intra- and extrahepatic jaundice (Sterkel, 1958).

Serum isocitrate dehydrogenase appears to be a good, sensitive index of hepatocellular damage. Although no more sensitive than the aminotransferases in detecting liver damage, its advantages in methodology of determination and better specificity make the enzyme worthy of further investigation in the realm of monitoring for hepatotoxicity. Furthermore unlike many other enzymes there is no evidence to suggest that isocitrate dehydrogenase is induced by drugs or chemicals.
Asada (1963) suggested that sorbitol dehydrogenase activity is elevated in serum in liver disease but is a less sensitive indicator of hepatocellular injury than the aminotransferase activities. Sorbitol dehydrogenase enzyme is, however, more specific for parenchymal injury than the other enzymes studied. In normal serum there is no detectable level of sorbitol dehydrogenase therefore any significant increase is a pathological sign. The determination of the enzyme is simple but employs the use of fructose as a viscous solution causing practical difficulties with spectrophotometry.

Another enzyme that has been extensively measured as an indicator of hepatic damage is lactate dehydrogenase. Isoenzyme studies revealed that the fifth isoenzyme of lactate dehydrogenase, on electrophoresis, was liver specific. An increase in the LDH₅ isoenzyme has been shown to be associated with hepatocellular damage in disease and chemical toxicity (Cohen, 1966).

A study of the hepatotoxicity of beryllium by Clary and Groth (1973) showed lactate dehydrogenase to be a poor detector of early changes of early hepatic subcellular changes. On the other hand the aminotransferases and isocitrate dehydrogenase were shown to be excellent serum indicators of early subcellular changes such as vacuolization but these enzymes are poorly correlated in the later stages of necrosis.
It must be stressed again that enzymes are not tests of liver function and many liberties have been taken when enzyme studies have been termed liver function tests. Some, however, are sensitive and reliable indicators of hepatocellular damage (Burke, 1978).

4. Plasma Proteins as Indexes of Liver Function

Proteins that are synthesised in the liver and 'secreted' into the plasma have long been considered to be an actual index of liver synthetic function. The advantage over enzyme determination is clear. A real function of the liver is monitored.

A damaged liver should synthesise less protein, hence a reduction in the plasma concentration of protein is observed. This gives rise to many of the clinical signs of chronic liver disease. Blood clotting factors become in short supply leading to increased clotting times, and ascites results from a direct fluid loss from plasma into the tissues.

A reduced plasma protein concentration could arise in a number of ways. There may be impaired protein synthesis in the liver, or there may be impaired 'secretion' into the plasma. Accelerated protein turnover or altered interactions of the protein could also account for a reduction in blood concentration. Protein is also lost in burns, renal failure and enteropathies (Kohn, 1978).

With the birth of immunological techniques for the determination of protein, methodology is specific and sensitive. Immunodiffusion, immunoelectrophoresis and radioimmunoassay are now frequently used to measure the
concentration of plasma proteins.

Protein tests, therefore, have the advantage of being true indicators of liver function, synthesis, and are easily, specifically and sensitively determined.

Many serum proteins have been reviewed as indexes of liver function. Albumin has been extensively studied as have prothrombin, caeruloplasmin, transferrin and $\alpha$-fetoprotein in hepatobiliary disease (Noren, 1972; Hoe, 1973; Purves, 1970)

**Albumin**

The mechanisms for regulating the rates of synthesis and degradation of albumin are not completely elucidated. The long half life of albumin, however, about 20 days, gives rise to serum levels that are maintained until the chronic stages of hepatic damage. Clinical signs have become evident by this time and thus albumin offers no assistance in the detection of hepatic damage either due to disease or toxicity.

Albumin has been used as an index of the chronicity of liver disease and hypoalbuminaemia in liver damage gives rise to many of the clinical signs of liver disease.

The lack of correlation between albumin synthesis and serum albumin, especially at low concentrations diminishes its utility (Burke, 1978).
Prothrombin time is commonly used as an index of liver function in hepatobiliary disease. The test is, however, relatively insensitive. There are at the present time no entirely satisfactory reference methods available for any coagulation test or factor level (Burke, 1978; Noren, 1972).

At one time the prothrombin time was thought to be a specific measure of plasma prothrombin (factor II) but it is now known to depend on levels of factors V, VII, X and fibrinogen.

**Acute Phase Reactants**

The term acute phase proteins or reactants is customarily used to describe plasma proteins whose concentration increases significantly as a response to the acute phase of inflammatory processes, trauma, tissue breakdown, infection and so on (Kohn, 1978).

The main acute phase reactants are alpha\textsubscript{1}-acid glycoprotein, C-reactive protein, haptaglobin, caeruloplasmin, transferrin and fibrinogen. They are glycoproteins and synthesised in the liver and for this reason it is expected that serum concentrations must fall in severe liver damage (Koj, 1974).

Caeruloplasmin has been found, however, to have increased serum concentration in primary biliary cirrhosis and disorders of the biliary tract. Occasionally elevated concentration is seen in patients with chronic active hepatitis and cirrhosis of the liver. In these cases caeruloplasmin is acting as an acute phase reactant, as an indicator of inflammation. The use of this protein as an index of liver function is severely handicapped
(Ritland, 1977).

Low plasma caeruloplasmin concentration is observed in hepatolenticular degeneration (Wilson's disease) which is an inborn error of copper metabolism.
The clinical value of bile acid determinations is undergoing thorough evaluation at the present time (Billing, 1979). With the exception of bacterial overgrowth, the bile acids found in serum are in the conjugated form.

Bile acids are synthesised from cholesterol in the liver and are conjugated to taurine or glycine before excretion. The determination of primary bile salts in serum is by gas liquid chromatography or radioimmunoassay giving specific, sensitive results (Baquir, 1979; Pennington, 1979).

Serum bile acids are thought to be of use in the discrimination of non-icteric patients with liver disease and for monitoring of drug treatments. A recent report showed bile acids to be elevated in serum before jaundice following paracetamol intoxication (Collins, 1979).
Many drugs and foreign compounds that are metabolised by enzymes located within the liver have been shown to have altered metabolic rates in liver disease (Shand, 1979; Vessell, 1979). 

Many factors can vary the rate or way a drug is metabolised in the body (Farrell, 1979). Genetic constitution, various disease states, other drugs, age, sex and environment have all been shown to affect the metabolism of drugs (Lang, 1976).

The rate of hepatic metabolism of aminopyrine (dimethyl aminoantipyrine) has been used to assess liver function by a number of investigators (Hepner, 1974; Hepner, 1975; Hepner, 1976; Sultatos, 1978).

Aminopyrine is readily absorbed, evenly distributed in total body water and primarily metabolised in the liver. Metabolism occurs mainly through N-demethylation (Brodie, 1950). In liver disease there is a reduction in the clearance rate of the drug as shown by using a $^{14}$C radioactive label on the drug. The metabolic clearance is determined by measuring the breath output of labelled carbon dioxide.

The technique has the advantage that it is non-invasive and a sensitive reflection of overall liver dysfunction. The ethics of the test however are questionable. In the patient with hepatotoxicity the liver may be under severe stress, an additional dose of another drug can only add to that stress. When low doses of aminopyrine are given to animals dosed with paracetamol, the paracetamol has been shown to interfere with the N-demethylation of aminopyrine causing an effect similar
to that expected in liver damage (Sultatos, 1978). A study has recently suggested that the aminopyrine breath test is a good way of monitoring liver function after paracetamol overdose (Saunders, 1980).

Misleading results may also be observed in patients taking enzyme inducing drugs. High alcohol intake or phenobarbitone therapy both give rise to increased drug metabolising enzymes. The aminopyrine test in these people would show an accelerated clearance of labelled drug (Leiber, 1979).
The present study aims to investigate the following:

1. To investigate existing and new serum enzymes to monitor liver diseases, to evaluate their sensitivity and specificity to hepatobiliary disease and drug toxicity in the human, especially in the acute situation.

2. To evaluate the use of serum proteins as real indexes of liver function and to determine the sensitivity of C-reactive protein and prealbumin to detect disease and drug toxicity.

3. To apply any successful test of liver function to the animal model and investigate its effectiveness as an index of liver function in drug toxicity.
CHAPTER TWO
CHAPTER TWO

Studies of Experimental Hepatotoxicity
by Standard Plasma Enzyme Determinations

Introduction

It is now known what enzymes show elevated activities most sensitively in liver damage. It is now important to discover how quickly and how sensitively each enzyme responds to hepatotoxic drugs.

By dosing animals with carbon-tetrachloride, bromobenzene or paracetamol and measuring enzyme activities at various times after dosing, it should be possible to identify which enzymes leak from the cells at an early stage and hence deduce which enzymes are likely to be sensitive indices of hepatocellular damage.

Carbon tetrachloride is one of the most potent of the known hepatotoxins. Causing hepatocellular necrosis and fatty infiltration of the liver its hepatotoxicity results from the formation of an active intermediate which binds to cell macromolecules and initiate autoxidation (Chapter One).

In the following studies carbon tetrachloride was used to experimentally produce damage in the livers of treated animals so that the most suitable enzymes could be selected as indicators of hepatocellular damage and "liver function" for further investigation.

Measurement of the activities of enzymes in plasma or serum present special difficulties in clinical biochemistry.
Variables affecting activity include choice of substrate, substrate concentration, pH, choice of buffer and its concentration, temperature and a wide variety of other factors special to particular assays.

Considerable efforts have been made to standardize the various methods of measurement. Certain conditions have been specified by the German Society for Clinical Chemistry, the Scandinavian Society for Clinical Chemistry and Clinical Physiology and the Societe Francaise de Biologie Clinique. More recently the International Federation of Clinical Chemistry (IFCC) has set up an Expert Panel on Enzymes which has published a series of Provisional Recommendations with particular reference to aspartate aminotransferase. All of the above organisations were in agreement that enzyme activities should be measured at a specific temperature but the Germans recommended 25°C, the IFCC and the French 30°C and the Scandinavians 37°C. In Britain most laboratories work at 37°C but some use 30 or 25°C (Duggan, 1979).

The methods employed to determine enzyme activities in the present study are those recommended, and optimised, by Bergmeyer (1974).

The reliability of enzyme determinations in clinical biochemistry is at present in question. Drug interference of enzyme determination is now well known (McNeely, 1978). Enzyme determinations are highly sensitive to changes in pH, temperature and equipment and great care is needed to obtain reproducible results.
Enzyme activities often have to be measured in unstored samples which are free from haemolysis. Isocitrate dehydrogenase activity is high in haemolysed serum from a healthy individual.

Chemically induced liver damage in the rat was investigated recently by Drotman (1978). Rats were dosed with carbon tetrachloride to induce liver injury and the time and magnitude of peak liver injury assessed by histopathological examination of liver specimens taken at intervals after dosing. Ornithine carbamyl transferase was shown to be more sensitive in indicating hepatocellular damage than isocitrate dehydrogenase, alanine and aspartate aminotransferase, and sorbitol dehydrogenase. Good correlation between the peak ornithine carbamyl transferase activity and maximal histological damage was found.

This report contradicted the findings of other groups who suggested both ornithine carbamyl transferase and isocitrate dehydrogenase to be less sensitive indicators of hepatocellular damage than histopathology for detecting liver damage (Korsrud, 1972; Korsrud, 1973).

Sorbitol dehydrogenase is another enzyme found predominantly in the liver (Gerlach, 1959). Work by Asada and Galambos in 1963 showed that aminotransferase and sorbitol dehydrogenase activities decreased in the liver and increased in the serum of animals treated with carbon tetrachloride. This was shown to be a dose related effect. Rees (1960) was unable to show a dose related effect with isocitrate dehydrogenase activity following carbon tetrachloride intoxication but Asada suggested the dosage regimen used by Rees was too high.
Methods of Enzyme Determination

1. Isocitrate Dehydrogenase (E.C.1.1.1.42, ICD)

- Bernt, E. and Bergmeyer, H.U.

Principle:

\[
\alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \\
\text{Mn}^{2+} \rightleftharpoons \text{ICDH} \\
\text{Isocitrate} + \text{NADP}^+
\]

At pH 7.5 the equilibrium is completely on the side of the reduced cofactor, NADPH. The determination uses isocitrate as substrate and NADP as coenzyme. The amount of isocitrate oxidised is determined by the increase in extinction due to the formation of NADPH, which in turn is a measure of ICD activity.

Method

An assay mixture consisted of triethanolamine (80 mM) DL-isocitrate (3.7 mM) and sodium chloride (42 mM). The sample (0.1 ml) was added to this mixture in a quartz cuvette and, after mixing, incubated for five minutes at 25°C.

Cofactor solution, consisting of NADP (0.32 mM) and manganese sulphate (3.9 mM, Mn\(^{2+}\)), was then added to start the reaction.
The change in extinction with time at 340 nm was measured continuously, using a Perkin Elmer SP1800 spectrophotometer with a paper recorder, against an air blank. Temperature was maintained at 25°C throughout the determination by a constant temperature cuvette holder.

Activity of ICD was determined in International Units per litre by using a calculation formula.

\[
\text{Volume Activity} = 997 \times \Delta E/\text{min} \quad \text{IU/l}
\]

**Precision**

The coefficient of variation is given to be 10% with values around 20 IU/l a standard deviation of 2 IU/l is recorded.

**Specificity**

The activity of all NADP-dependant isocitrate dehydrogenase is recorded. The sources of this enzyme are the liver, the heart and red blood cells. In serum, however, the liver enzyme is predominant with little contribution of heart enzyme and that from red blood cells in non haemolysed serum samples.

2. **Alanine Aminotransferase (E.C. 2.6.1.2.)**

**Principle:**

The method described here was first published by Henley et al (1955) and later modified by Wroblewski and La Due (1956).
\[
\begin{align*}
\text{(i) } &\text{ L-Alanine} + \alpha-\text{keto glutarate } \xrightarrow{\text{ALT}} \text{ Pyruvate} + \text{L-Glutamate} \\
\text{(ii) } &\text{ Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+
\end{align*}
\]

The equilibrium in reaction (ii) lies far to the right, as shown. The pyruvate formed by the action of alanine aminotransferase in (i) is therefore immediately converted to L-Lactate. The activity of alanine aminotransferase is determined from the rate of NADH oxidation in reaction (ii).

**Method**

The sample (0.1 ml) was added to a preincubation mixture of phosphate buffer (pH 7.4, 80 mM) L-alanine (0.8 M), NADH solution (0.18 mM) and lactate dehydrogenase (6.8 g/ml; 3.7 IU/ml) in a quartz cuvette. After mixing thoroughly the pyruvate, oxaloacetate and other substrates in the serum were allowed to react and oxidise the NADH for three minutes, before addition of \(\alpha\)-ketoglutarate (18 mM) to start the alanine aminotransferase reaction.

The mean change in extinction at 340 nm was measured for three minutes following addition of \(\alpha\)-ketoglutarate, using a Perkin Elmer SP1800 spectrophotometer at a constant temperature of 25°C. Extinction was read against an air blank.

**Activity** was determined using a calculation formula,

\[
\text{Volume Activity} = 1190 \times \Delta \text{E/min} \quad \text{IU/l}
\]

The coefficient of variation for the method is 5.5%, becoming less with sera of high enzyme activity.
3. **Gamma-Glutamyl Transferase (E.C.2.3.2.2.)**

**Principle**

\[
\text{HOOC-CHNH}_2-\text{CH}_2-\text{CH}_2-\text{CO} + \text{H}_2\text{N-CH}_2-\text{CONH-CH}_2\text{ COOH} \rightarrow \gamma\text{-glutamyl-p-nitroanilide} + \text{glycylglycine}
\]

\[
\text{HOOC-CHNH}_2-\text{CH}_2-\text{CO} \rightarrow \gamma\text{-glutamyl glycylglycine} + \text{p-nitroaniline}
\]

The gammaglutamyl transferase activity is directly proportional to the amount of p-nitroaniline liberated per unit time (Bergmeyer, 1974). p-Nitroaniline at pH 8.2 has an absorption maximum around 400 nm while the substrate does not absorb at this wavelength.

**Method**

The GGT in human serum reacts most rapidly with L-\(\gamma\)-glutamyl-p-nitroanilide at pH 8.2 (Szasz, 1969; Goldbarg, 1978), the same activity being found in ammonium, diethanolamine, triethanolamine and tris buffers. The enzyme is saturated at a substrate concentration of 4.0 mM.
L-γ-glutamyl-p-nitroanilide (4.0mM), glycylglycine (50 mM), magnesium chloride (10 mM) and ammediol buffer (48 mM) were mixed at 25°C (constant) in a quartz cuvette before addition of the serum sample (0.1 ml).

After mixing the change in extinction was determined for three minutes at 405 nm by continuous measurement in a Perkin Elmer SP 1800 spectrophotometer with a chart recorder attached.

The activity of GGT was calculated in International Units per litre of serum.

i.e. Activity (IU/l) = 2120 x Δ E/min

The coefficient of variation for this method is reported to be 3.8 to 5.5% (Szasz, 1969).

4. 5' Nucleotidase (E.C. 3.1.3.5.)

**Principle:**

\[
\text{Adenosine - 5' monophosphate} + \text{H}_2\text{O} \rightarrow \begin{array}{c}
\text{5' nucleotidase} \\
\downarrow \\
\text{Adenosine + H}_3\text{PO}_4
\end{array}
\]

The dephosphorylation of the C₅ position of the ribose ring by isodynamic phosphomonoesterases must be taken into account in the determination of 5' nucleotidase. By selectively inhibiting the activity of 5' nucleotidase with nickel ions, (Ahmed, 1958) the activity of the non-specific phosphatases can be found.
In the presence of nickel ions the activity of non-specific phosphatase is determined, while in their absence the sum of the activities of non-specific phosphatases and 5'-nucleotidase is measured. The difference in the amount of phosphate ion liberated per unit time in the two assays as determined by the Fiske and Subbarrow method (1925) is a measure of 5'-nucleotidase activity. Campbell (1962) modified Fiske's original method.

**Reagents**

i. Manganese sulphate (20 mM)

ii. Veronal buffer (40 mM; pH 7.5)

iii. Nickel chloride (0.1 M)

iv. Adenosine-5'-monophosphate, AMP (10 mM)

v. Trichloroacetic acid (0.68 M)

vi. Ammonium molybdate (40 mM)

vii. Reducing solution: potassium bisulphate (0.02 M) and methylaminophenol sulphate (0.01 M)

viii. Sodium acetate (2.5 M)

**Method**

Three test tubes were required.

A : without nickel ions

B : with nickel ions and

C : phosphate blank of the sample

The scheme laid out below was followed. Tubes were read against air in a Cecil Spectrophotometer using glass cuvettes.
### Scheme of Nucleotidase Determination

<table>
<thead>
<tr>
<th>Volumes in millilitres</th>
<th>TUBE A</th>
<th>TUBE B</th>
<th>TUBE C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veronal buffer</td>
<td>1.5</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Manganese sulphate solution</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Nickel chloride solution</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Sample (serum)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>AMP solution</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

**MIX AND INCUBATE FOR 30 MINUTES AT 37°C**

<table>
<thead>
<tr>
<th>ADD TRICHLOROACETIC ACID</th>
<th>2.0</th>
<th>2.0</th>
<th>2.0</th>
</tr>
</thead>
</table>

The enzymic reaction is stopped. Centrifuge for 5 minutes at 3000g. Discard the precipitate. Pipette successively into separate tubes:

<table>
<thead>
<tr>
<th>Supernatant fluid</th>
<th>2.5</th>
<th>2.5</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Molybdate solution vi</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Reducing solution vii</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**MIX AND ALLOW TO STAND FOR 10 MINUTES**

<table>
<thead>
<tr>
<th>THEN ADD ACETATE SOLUTION</th>
<th>4.0</th>
<th>4.0</th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DISTILLED WATER</strong></td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**MIX AND ALLOW TO STAND FOR 5 MINUTES**

**MEASURE EXTINCTIONS AT 578 nm**
Calculations

The difference TubeA - TubeB is found. The extinction of Tube C is then subtracted from this result, representing the amount of phosphate ion liberated by $5^{1}$-nucleotidase. (The Lambert-Beer law is obeyed up to a concentration of 0.04 mg phosphorus per reaction mixture.)

Conversion of the activity to International Units per litre is done by multiplication of the extinction difference by 441.

The coefficient of variation of the method is 2.86% (Gerlach, U. and Hiba, W., 1959).

5. Ornithine Carbamyl Transferase (E.C.2.1.3.3., OCT)

Principle

This enzyme was found to catalyse the reaction between carbamyl phosphate and ornithine in the urea cycle (Reichard and Reichard, 1958).

$$\text{Carbamyl phosphate} + \text{ornithine} \xrightarrow{\text{OCT}} \text{citrulline} + \text{phosphate}$$

OCT occurs mainly in the liver, in Man, (Reichard, 1960) the intestine being the second major source of the enzyme.

Elevated OCT activities in serum were observed in patients with most diseases of the liver (Reichard, 1961) but the study also stressed that disease processes in the small intestine must be ruled out. The same study suggested that OCT was a specific indicator of liver cell damage.
The enzyme is determined by colorimetric determination of citrulline. The amount of citrulline formed per unit time is a measure of OCT activity. Serum urea, which is also chromogenic is removed by adding urease to the incubation mixture. (Vassef, 1978).

Reagents

i. Phosphate buffer, (67 mM pH 7.0) was made with disodium hydrogen phosphate and potassium dihydrogen phosphate.

ii. Urease 0.5 mg/ml (Activity 5IU/mg)

iii. Urease/substrate solution consisted of carbamylphosphate (12.5 mM), ornithine (25 mM) and urease (0.5 mg/ml).

iv. Citrulline standard (10 mM)

v. Trichloroacetic acid (10% w/v)

vi. Antipyrine/Ferric sulphate solution (4g/l) (50mg/l)

vii. Acetic acid (5% v/v)

viii. Diacetyl monoxime (0.5% w/v)

ix. Colour Reagent

Two volumes of solution vi were mixed with one volume of solution viii immediately before use.

Method

A flow diagram for the determination is shown in table 2.2. All incubations were carried out at 37°C. The citrulline content of the sample was determined separately (tube "citrulline").
Table 2.2

Procedure for the Determination of Ornithine Carbamyl in Serum.

<table>
<thead>
<tr>
<th>Pipette into tubes:</th>
<th>OCT</th>
<th>Serum citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Urease/substrate (iii)</td>
<td>0.8ml</td>
<td>-</td>
</tr>
<tr>
<td>Urease solution (ii)</td>
<td>-</td>
<td>0.8ml</td>
</tr>
</tbody>
</table>

Mix and incubate for 15 minutes

| Trichloroacetic acid (v)     | 1.1ml     | 1.1ml            |

Mix and centrifuge for 10 minutes at 3000 rpm. Use the supernatant fluid

<table>
<thead>
<tr>
<th>Pipette into test tubes:</th>
<th>OCT</th>
<th>Serum citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fluid</td>
<td>1.5ml</td>
<td>1.5ml</td>
</tr>
<tr>
<td>Colour Reagent (ix)</td>
<td>1.5ml</td>
<td>1.5ml</td>
</tr>
</tbody>
</table>

Mix and place in a boiling water bath for 25 minutes

| Add Ethylene glycol          | 0.2ml     | 0.2ml            |

Allow to cool. Read extinctions at 460 nm.

\[ E = E_{OCT} - E_{citrulline} \]
The extinction of a reference standard is measured using citrulline (50 nmoles) instead of the serum sample.

All extinctions (E), measured at 460 nm, are read against a blank containing buffer (i) instead of sample.

**Calculation of Activity**

The OCT activity was calculated by measuring the concentration of citrulline (nmole) liberated per minute at the conditions stated (37°C, 15 minutes incubation, standard solution of citrulline, 500nmol/ml).

The following relationship was used:

\[
\text{Activity (IU/l)} = \frac{\Delta E}{15 \times E_{\text{standard}}} \times 500
\]

\[
= \frac{\Delta E}{E_{\text{standard}}} \times 33.5
\]

The coefficient of variation for the method is 5% (Ceriotti, 1974).

6. **Sorbitol Dehydrogenase (E.C.1.1.1.14)**

**Principle**

\[
\text{D-SORBITOL} + \text{NAD}^+ \xrightarrow{SDH} \text{D-FRUCTOSE} + \text{NADH} + \text{H}^+
\]

Sorbitol dehydrogenase activity can be measured in either direction of the reaction above. For measurements in serum the use of D(-) fructose has been preferred.
The decrease in extinction as the NADH is oxidised to NAD is followed spectrophotometrically, this being a measure of SDH activity.

**Method**

Triethanolamine buffer (1.07M, pH 7.4), NADH solution (0.4 mM) and the serum sample (1.0ml) were incubated in a quartz cuvette for thirty minutes at 25°C until extinction was constant.

D(-)-Fructose solution (0.4M) was then added to the cuvette and the change in extinction measured per minute for five minutes in a Perkin Elmer SP1800 spectrophotometer at 340 nm.

The enzyme activity was calculated according to the calculation formula:

\[
\text{Activity (IU/l) = } 482 \times \Delta E/\text{min}
\]

(Bergmeyer, 1974)

The coefficient of variation for this method is 1.14% (Gerlach and Hiby, 1959; Bergmeyer, 1974). The long preincubation before addition of fructose in the determination is to allow the sorbitol dehydrogenase to catalyse numerous other reactions in the presence of NAD or NADH (i.e. ribitol ribulose, xylitol xylulose, etc.) Only after sufficiently long preincubation and stabilization of the extinction is SDH activity measured specifically after the start of the reaction with fructose.
7. **Alkaline Phosphatase (ALP, E.C.3.1.3.1.)**

**Principle**

The determination of phosphatase by continuous measurements has the advantage that only one pipetting is necessary and the measurements can be carried out rapidly. (Walter and Schutt, 1974).

p-Nitrophenylphosphate is commonly used as substrate in the determination of alkaline phosphatase. p-Nitrophenol is liberated from the substrate proportional to the activity of the enzyme.

\[
O_2N-\text{-OPO}_3\text{H}_2 + \text{H}_2\text{O} \rightarrow \text{p-nitrophenylphosphate}
\]

\[
\text{ALP} \rightarrow \text{O}_2\text{N-\text{OH} + H}_3\text{PO}_4 \quad \text{p-nitrophenol}
\]

**Method**

Diethanolamine (0.99M) and nitrophenylphosphate (14.85mM) were mixed and added to a quartz cuvette at 25°C. The serum sample (0.02ml) was then added to the cuvette and the rate of change of extinction measured at 405nm in a Perkin Elmer SP1800 spectrophotometer.
Calculation

The change in extinction per minute was determined. The calculation formula below was then applied to find the volume activity in International Units per litre at 25°C.

\[ \text{Activity (IU/l)} = 5460 \times \Delta E/\text{min}. \]

The coefficient of variation reported by Walter was 3.9%.
Investigation of Serum Enzyme Activities of Rats Treated with Carbon Tetrachloride

Method

Twenty male Wistar albino rats (200 - 250g) bred by the University of Surrey Animal Unit were randomised into two equal groups. Animals were allowed food and water ad libitum and maintained in conditions usual for the University Animal Unit.

After a period of "settling in" Group 1, the control animals, were dosed intraperitoneally with corn oil and Group 2, the test group, were dosed intraperitoneally with carbon tetrachloride in corn oil (50% v/v) at a dose of 0.4g/kg body weight.

Eighteen hours after dosing the rats were bled by cardiac puncture and killed by cervical dislocation. Blood was allowed to clot for twenty minutes at room temperature before serum was collected. Livers were removed from the animals and washed before preservation in formyl-saline solution (10%) for histology.

Isocitrate dehydrogenase, alamine aminotransferase 5'-nucleotidase, sorbitol dehydrogenase, alkaline phosphatase gammaglutamyl transferase ornithine carbamyl transferase were determined in serum by the methods previously described.

Lactate dehydrogenase was determined by the method of Bergmeyer and Bernt (1974). Preserved livers were sectioned and stained, with haematoxylin and eosin. Histology was done to confirm hepatocellular damage was present (see Appendix).
The activities of several enzymes following administration of carbon tetrachloride

Isocitrate dehydrogenase shows the greatest elevation above control values (table 2.4). Another enzyme located in the cytosol, alanine aminotransferase, also shows significant elevation in enzyme activity. $5^1$-Nucleotidase, located in the nuclear membrane of the cell, and ornithine carbamyl transferase, the urea cycle enzyme, both showed elevated activity following injury to the liver. Alkaline phosphatase, gammaglutamyl transferase and lactate dehydrogenase showed only small elevations in serum activity following an acute dose of hepatotoxin. It is known that these enzymes are better indicators of hepatic damage in a more chronic situation.

The relative merits of each enzyme as an index of acute damage can be considered in view of these results but consideration should also be given to the methods of their determination.

$5^1$-Nucleotidase and ornithine carbamyl transferase have complex methods of determination with a final stage involving colorimetry. Long tedious methods prevent a large number of samples being determined quickly and these methods also use large quantities of serum.

Despite this fact ornithine carbamyl transferase, a mitochondrial enzyme, showed sensitive elevation in activity following hepatic abuse and is worthy of further investigation. Sorbitol dehydrogenase is determined by a method involving fructose, forming a viscous solution and hence creating
Table 2.4

Enzyme activity in rat serum following administration of carbon tetrachloride

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>ACTIVITY (IU/l)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GROUP 1</td>
<td>GROUP 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Means ± Standard Deviation</td>
<td></td>
</tr>
<tr>
<td>Isocitrate Dehydrogenase</td>
<td>5.2 ± 2.1</td>
<td>65.2 ± 18.9 **</td>
<td></td>
</tr>
<tr>
<td>Alanine Aminotransferase</td>
<td>22.4 ± 6.3</td>
<td>63.4 ± 26.9 **</td>
<td></td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>&lt;1.0</td>
<td>8.2 ± 1.6 **</td>
<td></td>
</tr>
<tr>
<td>Sorbitol Dehydrogenase</td>
<td>&lt;1.0</td>
<td>1.2 ± 0.7 **</td>
<td></td>
</tr>
<tr>
<td>Gamma-Glutamyl</td>
<td>18.4 ± 1.7</td>
<td>31.2 ± 7.4 **</td>
<td></td>
</tr>
<tr>
<td>transpeptidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ornithine Carbamyl</td>
<td>4.8 ± 3.5</td>
<td>44.3 ± 4.2 **</td>
<td></td>
</tr>
<tr>
<td>transferase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>1782.0 ± 322.0</td>
<td>2059.0 ± 395.0 *</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>58.4 ± 7.2</td>
<td>68.6 ± 9.3 +</td>
<td></td>
</tr>
</tbody>
</table>

** p < 0.001
* p < 0.5
+ p < 0.02
problems in spectrophotometry due to density effects. This plus the fact that no real elevation in serum activity occurred after carbon tetrachloride treatment, leads sorbitol dehydrogenase to be omitted from further investigation.

From the enzymes studied, therefore, isocitrate dehydrogenase, alanine aminotransferase and ornithine carbamyl transferase were chosen for further investigation of their activities in acute hepatotoxicity.
Enzyme activity at various times following dosage with hepatotoxin

Male Wistar albino rats (103) with weights in the range 150 to 250g were randomised into the following groups:

a) **Carbon tetrachloride**

Fifteen animals (controls) were dosed with a solution of corn oil. Twenty-eight experimental animals were dosed intraperitoneally with carbon tetrachloride; 1.5g/kg in corn oil (50% v/v).

Four rats in the experimental group were killed by cervical dislocation following cardiac puncture to obtain blood for the collection of serum, at 1.0, 2.0, 4.0, 8.0, 18.0, 24.0 and 36.0 hours post dose.

b) **Bromobenzene**

Twenty animals (controls) were dosed with peanut oil intraperitoneally and twenty experimental animals were dosed with bromobenzene, 1.0 g/kg body weight (50% v/v in peanut oil), by the same route.

Four animals in the experimental group were bled by cardiac puncture and killed by cervical dislocation 1.0, 4.0, 8.0, 24.0 and 48.0 hours after dosing.

c) **Paracetamol**

Twelve rats in the control group were dosed orally with a solution of gum tragacanth (1% w/v). Experimental animals (12) were dosed orally with a suspension of paracetamol (20% w/v) in tragacanth (1%), 2g/kg body weight.
Two experimental animals were bled by cardiac puncture 1.0, 4.0, 8.0, 15.0, 24.0 and 48.0 hours after dosing.

Control animals were bled and killed at the same time as the experimental animals.

All animals were allowed food and water ad libitum throughout the experiment.

After killing the liver of each animal was removed, washed in sodium chloride solution (0.1M) and preserved in formyl-saline solution (10%) for histology. Serum was prepared from clotted blood by centrifugation.

All enzymes were determined by the methods described earlier in this chapter.

Results and Discussion

The results of the experiment are shown in the following tables.
Enzyme activity at various times following administration of hepatotoxin

The results of this experiment are shown in tables 2.5, 2.6 and 2.7.

a) Carbon tetrachloride

Significant elevation in the activity of isocitrate dehydrogenase, alanine aminotransferase and ornithine carbamyl transferase occurred after only one hour following administration of carbon tetrachloride. The rapid absorption following intraperitoneal injection of hepatotoxin in corn oil gives rise to high blood levels soon after dosing and the rapid effect on the liver is shown by these high enzyme activities.

Following the high initial activities a further elevation was seen to occur as the time after dosing increased until peak activities of isocitrate dehydrogenase and alanine amino transferase were observed, 24 hours after dosing. A peak in the activity of alanine aminotransferase was observed 4 hours after dosing but this was accompanied by a high standard deviation.

Ornithine carbamyl transferase activity was greatly increased one hour after dosing but a steady fall in serum activity occurred following this time. The activity in serum after thirty six hours however was still elevated by seven times the mean control value. It appears that isocitrate dehydrogenase and
<table>
<thead>
<tr>
<th>Time after dose (hours)</th>
<th>Enzyme Activity (IU)</th>
<th>Alanine Amino Transferase</th>
<th>Ornithine Carbamyl Transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (15)</td>
<td>3.2 ± 1.9</td>
<td>18.6 ± 6.0</td>
<td>4.8 ± 3.5</td>
</tr>
<tr>
<td>1.0 (4)</td>
<td>29.1 ± 4.5</td>
<td>45.9 ± 19.4</td>
<td>50.3 ± 3.3</td>
</tr>
<tr>
<td>2.0 (4)</td>
<td>33.5 ± 4.7</td>
<td>61.3 ± 12.6</td>
<td>50.2 ± 5.2</td>
</tr>
<tr>
<td>4.0 (4)</td>
<td>38.3 ± 8.0</td>
<td>87.4 ± 17.0</td>
<td>42.2 ± 8.8</td>
</tr>
<tr>
<td>8.0 (4)</td>
<td>45.4 ± 5.1</td>
<td>84.3 ± 8.4</td>
<td>43.6 ± 7.4</td>
</tr>
<tr>
<td>18.0 (4)</td>
<td>87.7 ± 3.2</td>
<td>81.0 ± 6.3</td>
<td>44.3 ± 4.2</td>
</tr>
<tr>
<td>24.0 (4)</td>
<td>111.2 ± 69.6</td>
<td>87.8 ± 5.5</td>
<td>40.4 ± 9.8</td>
</tr>
<tr>
<td>36.0 (4)</td>
<td>76.4 ± 12.1</td>
<td>72.6 ± 4.4</td>
<td>33.2 ± 7.8</td>
</tr>
</tbody>
</table>
alanine aminotransferase are rapidly released into the blood stream following abuse to the liver by the hepatotoxin, carbon tetrachloride. The location of these enzymes in the cytosol allows rapid leakage through the damaged cell membrane.

The mitochondrial enzyme ornithine carbamyl transcrase seems to be released rapidly by the damaged mitochondrial membrane but at a more controlled rate, giving rise to serum levels of enzyme that are elevated, but steadily falling as the mitochondrial membrane restabilizes.

b) Bromobenzene

In the animals dosed with bromobenzene a similar picture was seen. Isocitrate dehydrogenase and alanine aminotransferase showed rapid elevation in serum activity following dosage and high activities after one hour increased to a maximum twenty four hours after dosing. Ornithine carbamyl transferase activity in serum was also increased one hour following dose and a maximum activity was observed four hours post dose before a steady fall in activity with time.

After 48 hours, alanine aminotransferase showed an elevation of only 1.5 times the mean activity of the controls whereas isocitrate dehydrogenase and ornithine carbamyl transferase were more than 4 times greater than the mean control activity.
Table 2.6

Enzyme Activities at various times following an intraperitoneal dose of 1.0g/kg bromobenzene in the rat

<table>
<thead>
<tr>
<th>Time after dose (hours)</th>
<th>Enzyme Activity (IU)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isocitrate Dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (20)</td>
<td>4.1 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 (4)</td>
<td>45.2 ± 22.8</td>
<td>22.0 ± 4.4</td>
<td>5.3 ± 2.8</td>
</tr>
<tr>
<td>4.0 (4)</td>
<td>25.7 ± 10.8</td>
<td>48.5 ± 26.3</td>
<td>35.2 ± 14.4</td>
</tr>
<tr>
<td>8.0 (4)</td>
<td>58.6 ± 2.0</td>
<td>52.6 ± 27.5</td>
<td>12.7 ± 2.4</td>
</tr>
<tr>
<td>24.0 (4)</td>
<td>110.8 ± 42.7</td>
<td>94.8 ± 29.9</td>
<td>28.4 ± 13.3</td>
</tr>
<tr>
<td>48.0 (4)</td>
<td>18.2 ± 3.2</td>
<td>37.0 ± 8.2</td>
<td>24.8 ± 14.3</td>
</tr>
</tbody>
</table>

Means ± standard deviation
After four hours the activities of ICD and ALT were seen to be less than the activity of these enzymes after one hour and at eight hours post dose. As bromobenzene concentration in the serum is initially high, damage will be done to the liver. The membrane could then restabilize as the bromobenzene metabolite becomes conjugated to glutathione. This gives rise to lower activities of enzymes that have leaked from damaged cells into the blood. As the glutathione levels become depleted the bromobenzene metabolite causes further damage and further enzyme leakage giving rise to increasing serum enzyme activities.

c) **Paracetamol**

By varying the route of administration to oral dosing a delay of the increase in enzyme activity was observed following dosage with paracetamol.

A drug given orally takes longer to reach high blood levels than one administered intraperitoneally. Therefore it was no surprise that no significant increase in the activity of isocitrate dehydrogenase was observed until four hours following dosage. It was at this time that elevated serum alanine aminotransferase activity was also observed. Ornithine carbamyl transferase activity on the other hand showed no real increase until 15 hours after the dose when the damage to the liver was at a maximum as shown histologically. This suggests that ICD and ALT are more sensitive indicators of hepatocellular damage than OCT.
Table 2.7

Enzyme Activities at various times following an oral dose of 2g/kg paracetamol suspension in tragacanth, in the rat.

<table>
<thead>
<tr>
<th>Time after dose (hours)</th>
<th>Enzyme Activity (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isocitrate Dehydrogenase</td>
</tr>
<tr>
<td>Controls (12)</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>1.0 (2)</td>
<td>6.4</td>
</tr>
<tr>
<td>4.0 (2)</td>
<td>14.4</td>
</tr>
<tr>
<td>8.0 (2)</td>
<td>13.2</td>
</tr>
<tr>
<td>15.0 (2)</td>
<td>176.5</td>
</tr>
<tr>
<td>24.0 (2)</td>
<td>133.7</td>
</tr>
<tr>
<td>48.0 (2)</td>
<td>48.2</td>
</tr>
</tbody>
</table>
After twenty four hours the maximum enzyme activity was observed. The values of ICD and ALT were far greater than those observed following dosage with carbon tetrachloride and bromobenzene. The histology of rat livers treated with paracetamol showed haemorrhage around the portal tracts. This haemorrhage may have given rise to high activities of isocitrate dehydrogenase from the red blood corpuscles plus the enzyme leaking from damaged cells.

Histology

Photographs taken from sections stained with haematoxylin and eosin are shown in Figures 2.1 to 2.4.
**Figure 2.1**

H & E section of normal rat liver (x 100).

---

**Figure 2.2**

H & E section (x 40) from the liver of a rat dosed with carbon tetrachloride, (1.5g/kg) killed 24 hours after dosing. Low grade hepatocellular degeneration can be seen with vacuolation peripheral to the lesion suggestive of fatty change (confirmed by a positive Oil Red O reaction).
Figure 2.3
H & E section (x 40) from the liver of a rat dosed with bromobenzene (1g/kg) and killed 48 hours after dosing. Massive diffuse hepatocellular degeneration and necrosis with a few localised areas of hydropic change at the periphery of the lesion can be seen with necrosis of bile ducts and complete destruction of hepatocytes in the areas surrounding the portal veins.

Figure 2.4
H & E section (x 40) from the liver of a rat dosed with paracetamol (2g/kg) and killed 24 hours after dosing. Massive and confluent areas of necrosis can be seen. The hepatocellular degeneration is mainly but not exclusively associated with the central veins. Hepatocytes in the vicinity of the portal tracts are unaffected.
CHAPTER THREE
CHAPTER THREE

Glycyl-Prolyl-p-Nitroanilidase as an Indicator of Hepatocellular Damage

Introduction

In 1966, an aminopeptidase enzyme which cleaved \( N \)-terminal-X-proline from peptides was discovered (Hopsu-Havu and Glenner, 1966). By using a newly synthesised chromogenic substrate, glycyl-prolyl-\( \beta \)-napthylamide, activity was found in porcine liver and kidney (Hopsu-Havu, et al, 1968) and in the sera and tissues of various animals and Man, (Nagatsu et al, 1968 (a); Nagatsu et al, 1968 (b)). The serum enzyme is thought to be derived from liver (Hino et al, 1976 a).

The enzyme has also been shown to be present in human saliva, (Nagatsu et al, 1968) and salivary glands (Oya et al, 1972, 1974).

Because the substrate used in the determination of the enzyme, a napthylamide, produced \( \beta \)-napthylamine, a potent carcinogen, other substrates for the enzyme were tried (Nagatsu et al, 1976). Glycyl-prolyl-p-nitroanilide was found to be the best substrate compared to other p-nitroanilides (Hino et al, 1976). This substrate was considered safe and efficient for routine use.
This and the other chromogenic substrates were used to investigate the rate of hydrolysis by the enzyme and to examine optimum pH and Km values using homogenous enzyme from human submaxillary gland and crude enzyme from human serum.

The N-terminal amino acid of these substrates was found to affect the Vmax and Km values (Nagatsu, 1976). Glycyl-prolyl-p-nitroanilide was the best substrate at the optimum pH, 8.7. This substrate forms crystals as tosylate which are fairly stable in the dark and easily solubilized in water containing a non ionic detergent, the aqueous solution being stable below pH 8.0.

The Japanese workers showed that serum glycyl-prolyl-p-nitroanilidase (GPN) was abnormally high in patients with hepatobiliary diseases and was decreased in patients with gastric cancer (Hino et al, 1975).

Following carbon tetrachloride intoxication in rats, the activity of the enzyme was shown to be increased in serum and decreased in liver suggesting that the enzyme is released from the liver in hepatic injury (Hino et al, 1976).
More recently the activity of GPN in serum from patients with hypertension was found to be significantly higher than from normotensive healthy subjects. (Fuyamada, Hino et al, 1977).

A study of the enzyme in patients with hepatic cancer (Kojima, 1979) showed that significant elevation of activity occurred in these patients but no significant elevation occurred in patients with other hepatobiliary disease.

In view of the results obtained with patients with hepatobiliary disease and in cases of drug toxicity it was decided to investigate the use of glycyl-prolyl-p-nitroanilidase as an indicator of hepatocellular damage and liver disease more thoroughly than the Japanese had done.

Methods of Determination in Serum

(a) Materials

Glycylprolyl-p-nitroanilide tosylate was synthesised in the laboratories of the Protein Research Foundation, Osaka, Japan. The white crystalline solid was dissolved in 1% aq. Tritron x-100 to give a concentration of 3mM, Glycine buffer, pH 8.7, 0.2M, was made and its pH adjusted with IM NaOH. This buffer was diluted 1:1, (v/v) before use.
(b) **Method**

Glycylprolyl-p-nitroanilidase substrate solution (0.2ml) and diluted glycine buffer (0.2ml) were added to a quartz cuvette, volume 1.0ml. Small quantities of substrate were used due to its difficult availability and high cost. Reagents and cuvette were maintained at 25°C. Serum (20μl) was added to the cuvette in order to start the reaction (outlined in Figure 3.1) and the extinction change was measured against a reagent blank for three minutes at 380 nm using a Perkin Elmer, SP1800, spectrophotometer on its most sensitive range.

A standard curve was constructed using nitroaniline solutions and the change in extinction converted to concentration (μmol) of nitroaniline formed per minute per litre of serum. (International Units)

**Investigation of the Subcellular location of glycylprolyl p-nitroanilidase**

When chopped liver is homogenised and the cell membrane is ruptured, the cell sap disperses to become a simple solution and loses structure. Secondly the endoplasmic reticulum and to a considerable extent the cell membrane and the membranes of the golgi apparatus fragment to form microsomes. Thirdly, if the homogenisation procedure has been gentle, the nuclei, mitochondria and lysosomes are released intact.
Figure 3.1 The direct photometric assay of Glycylprolyl-p-nitroanilidase (Hino, 1976)
By differential centrifugation the particles can be separated into the five fractions, as below:

1. Nuclear Fraction
2. Mitochondrial Fraction
3. Lysosomal Fraction
4. Microsomal Fraction and
5. Cell Sap or Cytosol.

There is, however, always an overlap of fractions such that the cell sap will contain microsomes and the mitochondria will be contaminated with nuclei and so on.

Fractions are identified by marker enzymes characteristic to the fraction required. Succinate dehydrogenase is the mitochondrial marker.

Glycylprolyl p-nitroanilidase activity can be determined in a sample of each fractions to roughly detect the location of the enzyme in the rat liver cell.

Method

Nuclear, Mitochondrial, Lysosomal and Microsomal pellets were prepared by the method of Bandhuin and De Duve, (1964).

Acid phosphatase was determined in order to characterise the lysosomes, succinate dehydrogenase to locate the mitochondria, glucose 6-phosphatase to characterise the microsomes and glucose 6-phosphate dehydrogenase activity was determined to confirm one fraction was the cytosol (Bergmeyer, H.U., 1974).

Glycyl prolyl p-nitroanilidase was determined in each fraction by the method described previously using an aliquot of
resuspended fraction instead of serum.

Results

Glycyl prolyl p-nitroanilidase was found to be located primarily in the cytosol but some activity was also detected in the mitochondrial fraction.

Conclusions

This brief study of the location of glycyl prolyl p-nitroanilidase in rat liver cells has suggested that the enzyme may be present in cytosol and mitochondria. The majority of the activity was found in the cytosol and only a small activity in the mitochondrial fraction.

Investigation of the activity of serum GPN in rats treated with carbon tetrachloride and bromobenzene

Fortyfive (45) male Wistar albino rats (180-200g) were randomised into nine groups, each of five rats. Animals were allowed food and water ad libitum and maintained under standard animal house conditions. Control animals were given a single intraperitoneal dose of corn oil. Groups 2 - 5 were dosed with carbon tetrachloride in corn oil (50% v/v) as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>0.04</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.08</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.16</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Groups 6 - 9 were dosed intraperitoneally with bromobenzene in corn oil (50% v/v) as follows:

- Group 6: 50 mg/kg
- Group 7: 100 mg/kg
- Group 8: 250 mg/kg
- Group 9: 750 mg/kg

Eighteen hours following administration of the dose the animals were anaesthetised in ether and bled by cardiac puncture. After bleeding, rats were killed by cervical dislocation and livers removed for histology. Tissue was preserved in formyl-saline solution (10%). Blood collected was allowed to clot at room temperature, before centrifugation to collect the serum.

Isocitrate dehydrogenase (ICD), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and glycylylprolyl p-nitroanilidase (GPN) enzymes were measured in the serum.

**Results**

The results of the investigation are shown in tables 3.1 and 3.2.

**Discussion**

It was seen that a significant dose related increase in activity of isocitrate dehydrogenase and alanine aminotransferase occurred in rats dosed with both carbon tetrachloride and bromobenzene. This confirmed the results of previous experiments.

Glycylylprolyl-p-nitroanilidase on the other hand did not show this increase in activity to the same extent and only a slight significant increase was seen following bromobenzene intoxication. This enzyme appeared to mirror gamma-glutamyl transferase shown to be a good indicator of chronic liver damage.
Table 3.1  The activity of several serum enzymes following acute i.p. administration of carbon tetrachloride in the rat.

<table>
<thead>
<tr>
<th>Dose (g/kg)</th>
<th>Enzyme Activities (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICD</td>
</tr>
<tr>
<td>Controls (5)</td>
<td>5.2 ± 2.1</td>
</tr>
<tr>
<td>0.04 g/kg (5)</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>0.08 (5)</td>
<td>28.6 ± 13.9</td>
</tr>
<tr>
<td>0.16 (5)</td>
<td>47.3 ± 4.4</td>
</tr>
<tr>
<td>0.40 (5)</td>
<td>65.2 ± 18.9</td>
</tr>
</tbody>
</table>

(Means ± standard deviations)
Table 3.2 The activity of several serum enzymes following acute i.p. administration of bromobenzene in the rat.

<table>
<thead>
<tr>
<th>Dose mg/kg</th>
<th>Enzyme Activities (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICD</td>
</tr>
<tr>
<td>Control</td>
<td>5.2 ± 2.1</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>21.3 ± 19.3</td>
</tr>
<tr>
<td>100</td>
<td>36.5 ± 13.9</td>
</tr>
<tr>
<td>250</td>
<td>38.4 ± 2.7</td>
</tr>
<tr>
<td>750</td>
<td>54.2 ± 6.3</td>
</tr>
</tbody>
</table>

(Means ± standard deviations)
It may be suggested therefore that glycylprolyl-p-nitroanilidase did not indicate acute changes of necrosis within the liver as well as isocitrate dehydrogenase. This finding conflicts with those of Hino, et al. (1976) whose group found elevation of the serum activity of the enzyme following carbon tetrachloride administration.

It has been suggested that GPN activity in serum increases in diseases and damage of collagen and its metabolism in liver. Cirrhosis and hypertension are two causes of impaired collagen metabolism both of which give rise to high serum GPN activities, (Hino, 1976).

The investigation of GPN and other serum enzymes following chronic administration of cadmium chloride to the rat

Introduction

Cadmium is a metal resembling zinc. It occurs as an ecotoxin especially around zinc processing plants of the Bristol Channel. The toxicity of cadmium is being closely investigated. In biological systems cadmium may replace zinc and thus cause toxicity, the exact mechanism is still, however, largely unknown. The liver is severely affected in cadmium toxicity but the kidney is the major target organ.

Method

Eight (8) male Wistar albino rats (150 - 200 g) were randomised into two groups, each of four rats. Animals in the control group were dosed subcutaneously with saline, each day for 40 days. The four rats in the test group were dosed in a similar way with cadmium chloride solution 1.5mg/kg/day for 40 days.
Both groups were allowed food and water, ad libitum, under standard animal house conditions.

At the end of the 40 day period, the rats of both groups were anaesthetised in ether, bled by cardiac puncture, killed, liver preserved in formyl saline (10%) for histology. Blood was allowed to clot for collection of serum. Activities of several enzymes were determined in serum.

**Results**

The results are shown in table 3.3.

It can be seen that in this long term treatment with cadmium salts toxic effects are demonstrated by elevated enzyme activities corresponding to changes in liver histology. Gamma-glutamyl transferase (which may be of kidney origin), isocitrate dehydrogenase, ornithine transcarbamylase and alanine aminotransferase all show significant elevation, but Leucine aminopeptidase and alkaline phosphatase show insignificant changes in activity. Glycyl prolyl p-nitroanilidase was significantly elevated in the sera of the cadmium treated animals.

**Histology**

Despite poorly prepared histology, hepatocellular damage with necrosis was confirmed in test animals.

**Conclusions**

From the enzyme activities studied it was seen that isocitrate dehydrogenase (x 8) alanine aminotransferase (x 2) ornithine carbamyl-transferase (x 0.5) and γ-glutamyltranspeptidase (x 0.6)
<table>
<thead>
<tr>
<th>Group</th>
<th>Alkaline phosphatase</th>
<th>Gamma-glutamyl transferase</th>
<th>Isocitrate dehydrogenase</th>
<th>Alanine aminotransferase</th>
<th>Ornithine carbamyl transferase</th>
<th>Leucine aminopeptidase</th>
<th>GPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (4)</td>
<td>22.3 ± 2.9</td>
<td>14.3 ± 2.7</td>
<td>2.5 ± 1.0</td>
<td>10.9 ± 3.8</td>
<td>10.8 ± 2.5</td>
<td>32.0 ± 4.4</td>
<td>18.0 ± 2</td>
</tr>
<tr>
<td>Tests (4)</td>
<td>22.3 ± 5.8</td>
<td>25.5 ± 5.3</td>
<td>20.9 ± 11.5</td>
<td>28.2 ± 6.6</td>
<td>16.5 ± 1.8</td>
<td>36.1 ± 7.0</td>
<td>69.5 ± 2</td>
</tr>
</tbody>
</table>

Enzyme Activity (IU/l)

p<0.01  p<0.02  p<0.01  p<0.01  p<0.5  p<0.02
all showed significantly elevated serum activities in the group treated with cadmium chloride. Alkaline phosphatase and leucine aminopeptidase showed no activity changed. Isocitrate dehydrogenase appeared to be the most sensitive indicator of hepatic damage.

Ornithine carbamyl transferase is also regarded as being liver specific. Hence the increased activity of this enzyme was probably indicative of hepatocellular damage. The low alkaline phosphatase activity probably indicated the absence of cholestasis and severe damage to the bile duct epithelium.

Although the $\gamma$-glutamyltransferase may have been released from damaged kidney tissue, it was unlikely that the isocitrate dehydrogenase was indicating anything other than hepatocellular damage.

Glycyl prolyl p-nitroanilidase activity in the cadmium treated animals was significantly elevated, suggesting that it may indicate hepatocellular damage following chronic dosing with liver damaging chemical.
CHAPTER FOUR

Serum Enzyme Activities

in

Human Hepatobiliary Disease

Introduction

From the studies in the animal model (see Chapter Two) it was concluded that isocitrate dehydrogenase (NADP dependant) was a more sensitive index of hepatocellular damage than other workers had proposed. Isocitrate dehydrogenase activity should therefore be determined in human hepatobiliary disease to assess the sensitivity and specificity of the enzyme for diagnosing liver disease. A high concentration of the enzyme in the heart leads investigators to determine ICD in patients with myocardial infarction.

Glycylylproyl p-nitroanilidase (GPN) was also chosen for determination in human hepatobiliary diseases, despite the investigations which showed that insignificant increase in serum activity of this enzyme occur in acute hepatotoxicity in the rat. The reason for continuing with the determination of GPN was to investigate reports in the literature that cirrhosis gave rise to high activities of this enzyme. (HINO et al, 1976) The sensitivity and specificity of this enzyme would also be compared with those of the more traditional enzymes that are currently determined as indexes of liver function.
For comparison with the two enzymes, ICD and GPN, the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Alanine aminotransferase has similar properties to aspartate aminotransferase but its tissue distribution favours it as an indicator of hepatocellular damage. For whereas AST has high concentrations in heart, liver and kidney, the major location of ALT is the liver, with only minor activity in the heart.

The aims of the present study were to investigate the findings of Sterkel et al, (1958), and Cohen et al, (1961), that isocitrate dehydrogenase is a sensitive and specific indicator of hepatocellular damage in human patients, and also to ascertain whether or not serum glycylprolyl p-nitroanilidase activity is a good indicator of liver damage in hepatobiliary disease, as suggested by Hino et al, (1976).

Methods

Patients were selected and allocated to one of the following groups:

1. Secondary carcinoma of the liver
2. Alcoholic hepatitis
3. Chronic active hepatitis
4. Cryptogenic cirrhosis of the liver, including three patients with primary biliary cirrhosis
5. Obstructive jaundice
6. Myocardial infarction
7. Chronic inflammatory bowel disease
Diagnosis of patients in groups 1 to 5 were confirmed by biopsy or laparotomy. Patients with myocardial infarction were diagnosed clinically by E.C.G. changes, and those with chronic inflammatory bowel disease were diagnosed as having Crohn's disease or ulcerative colitis by radiography and rectal biopsy.

Patients were bled between 0830 and 1200 by venepuncture. Blood was allowed to clot for one hour at room temperature before centrifugation to collect serum.

Isocitrate dehydrogenase, alanine aminotransferase and glycerylprolyl p-nitroanilidase were determined in serum by the methods previously described.

Aspartate aminotransferase was determined by Vickers automatic analysis procedure.

The same enzyme activities were also measured in the control sera of 40 healthy patients. Quality control serum was used at all times to check the reproducibility of the enzyme determinations.

Results

The enzyme activities of patients in each group are shown in figure 4.1 and tables 4.1-7 (Appendix 4). A summary of these enzyme activities compared with controls from normal subjects is shown in table 4.8.
Figure 1.1  Enzyme Activities in Hepatic and Non Hepatic Disease

Enzyme Activity

x 10^{-10} Katals/l

- GPN
- ICD
- ALT
- AST
- GGT

Norm  Ca  AlcH  CAH  Cirr  Obs  CBD  MI
### TABLE 4.8

Summary of Serum Enzyme Activities in Hepatobiliary Disease

<table>
<thead>
<tr>
<th></th>
<th>Enzyme Activity (IU/litre)</th>
<th>Isocitrate Dehydrogenase (ICD)</th>
<th>Glycylprolyl p-nitroanilidase (GPN)</th>
<th>Alanine aminotransferase (ALT)</th>
<th>Aspartate aminotransferase (AST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Individuals</td>
<td></td>
<td>1.5 ± 1.1 *</td>
<td>38 ± 8 *</td>
<td>10 ± 3 *</td>
<td>23 ± 8 *</td>
</tr>
<tr>
<td>Secondary Carcinoma of the Liver</td>
<td></td>
<td>13.6 ± 3.0 *</td>
<td>123 ± 17 *</td>
<td>52 ± 7 *</td>
<td>75 ± 12 *</td>
</tr>
<tr>
<td>Alcoholic Hepatitis</td>
<td></td>
<td>6.2 ± 1.3 *</td>
<td>149 ± 24 *</td>
<td>43 ± 8 *</td>
<td>99 ± 30 *</td>
</tr>
<tr>
<td>Chronic Active Hepatitis</td>
<td></td>
<td>8.4 ± 2.3 *</td>
<td>86 ± 19 *</td>
<td>34 ± 9 *</td>
<td>128 ± 39 *</td>
</tr>
<tr>
<td>Cryptogenic Cirrhosis</td>
<td></td>
<td>4.6 ± 1.4 *</td>
<td>102 ± 18 *</td>
<td>40 ± 6 *</td>
<td>68 ± 13 *</td>
</tr>
<tr>
<td>Obstructive Jaundice</td>
<td></td>
<td>2.6 ± 0.6 +</td>
<td>83 ± 8 *</td>
<td>36 ± 9 *</td>
<td>97 ± 24 *</td>
</tr>
<tr>
<td>Chronic Inflammatory Bowel Disease</td>
<td></td>
<td>1.5 ± 0.2</td>
<td>43 ± 7</td>
<td>11 ± 2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td></td>
<td>2.5 ± 0.3</td>
<td>52 ± 4 *</td>
<td>22 ± 4 *</td>
<td>143 ± 40 *</td>
</tr>
</tbody>
</table>

Values given are the means ± S.E.M. taken from Tables 4.1-4.7. Values outside the normal range are underlined.  * p<0.001    + p<0.01
Most patients with secondary metastases in the liver had elevated serum isocitrate dehydrogenase activity (see table 4.1). Twelve of sixteen studied had an activity double the upper normal limit. Of the four people that had normal ICD activity (P.E., A.B., E.H., E.D.), two had abnormal aminotransferases (E.H., E.D.). The aspartate aminotransferase activity was abnormal in less than half of the cases studied (7/16) whereas the alanine aminotransferase activity was elevated in all but two of the cases studied (14/16, A.B. + M.H.). When the aspartate aminotransferase was within the accepted normal range (up to 45 IU/l) or only slightly elevated, the isocitrate dehydrogenase or alanine aminotransferase activities or both were elevated in all but one case (P.E.). Here, the patient had normal or slightly elevated enzyme activity, including a normal alkaline phosphatase activity, despite biopsy of the liver at laparotomy showing metastatic, but poorly differentiated, malignant melanoma tissue.

The activity of serum glycylprolyl p-nitroanilidase was elevated in all but two cases measured. One of the cases was P.E. (32 M) as described above but the other (T.G. 78 M) had abnormal isocitrate dehydrogenase activity and slightly abnormal aminotransferase activities. It can be seen that the highest activities of glycylprolyl p-nitroanilidase generally occur when the isocitrate dehydrogenase activity is grossly elevated.
In the patient, M.H. (67, F) the alanine and aspartate aminotransferase activities were within normal limits but the isocitrate dehydrogenase and GPN activities were markedly abnormal.

Patients with alcoholic hepatitis had grossly abnormal GPN activities in 9/10 of the cases measured (see Table 4.2). In the case where GPN activity was not elevated (P.H.) the isocitrate dehydrogenase and aminotransferase activities were also within the normal range. Only 5/10 cases had an elevated isocitrate dehydrogenase activity and only six had an elevated aspartate aminotransferase activity. Alanine aminotransferase was elevated in all but one (9/10) of the cases. In this patient (B.P.) aspartate aminotransferase was elevated, as was GPN but isocitrate dehydrogenase activity was well within the normal range.

In the patients with chronic active hepatitis (Table 4.3) it can be seen that high activities of serum ICD and AST are accompanied by the highest serum activities of GPN. However, only two of the seven cases studied had grossly abnormal GPN activities and four showed moderate elevation in serum activity. In one case, M.B., the GPN activity was normal whereas all other enzymes showed abnormality. In this patient the liver biopsy gave evidence of acute inflammation around multiple granulomas in the portal tracts without a fibrinoid necrosis.
Enzyme activities apart from GPN were also within the normal ranges in patient E.P. (56 F). Here the chronic active hepatitis was long standing and early portal cirrhosis was described in the liver biopsy. All but this one patient, showed enzyme activities above the normal range. Isocitrate dehydrogenase showed abnormal elevation in 6/7 cases of chronic active hepatitis as did alanine aminotransferase. Serum aspartate aminotransferase activity was abnormally high in 5/7 cases.

In cirrhosis of the liver, of unknown cause, (Table 4.4) isocitrate dehydrogenase activities were normal in all but one case. In this patient D.C. (70, M) the process of portal cirrhosis was highly active, as shown by biopsy. As expected the alanine and aspartate aminotransferase activities were elevated in this patient as was the activity of glycylprolyl p-nitroanilidase. In the other cases of cryptogenic cirrhosis the activities of ICD, ALT and AST were normal or only slightly elevated due to an inactive cirrhotic process as shown by liver biopsy. GPN activities however were significantly elevated in all cases studied. In the three cases of primary biliary cirrhosis included in this group the activities of the aminotransferases were high. Little or no elevation in the activities of ICD was observed but GPN activity was elevated in 2/3 cases.
Aspartate aminotransferase was elevated in 6/12 of the patients investigated with obstructive jaundice (see Table 4.5). The highest activities of this enzyme was associated with the greatest serum bilirubin concentrations. Serum glycylprolyl p-nitroanilidase activities were also above the normal range in most instances of hepatic obstruction (10/11). Isocitrate dehydrogenase activity was normal in all but three cases investigated. All three had raised aminotransferase and GPN activity and the serum bilirubin concentration was grossly elevated in each instance.

In the non liver disease states studied, namely, chronic inflammatory bowel disease and myocardial infarction, isocitrate dehydrogenase activity was normal in all instances. Alanine aminotransferase, aspartate aminotransferase and GPN activities were also normal in inflammatory bowel disease.

In myocardial infarction aspartate aminotransferase activities were usually grossly elevated, as expected. Similarly, ALT was increased in two cases and GPN activity was elevated in 6 cases of myocardial infarction suggesting either hepatic involvement or a distribution of GPN in heart tissue. The elevation of GPN in myocardial infarction was not as great as in the cases of hepatobiliary disease.
The use of GPN as an index of hepatocellular damage does not appear to offer substantial advantage over the aminotransferases. It does however seem to be better than isocitrate dehydrogenase as an index of liver cell damage.
CHAPTER FIVE

SERUM PROTEINS AS AN INDEX OF LIVER FUNCTION

IN HUMAN HEPATOBILIARY DISEASE

Introduction

Of the biochemical tests of hepatic function used to detect disease processes within the liver and to evaluate the degree and type of impairment of liver function, none is specific or affords direct evaluation of the integrity of the liver. Serum aminotransferases, especially aspartate aminotransferase (E.C. 2.6.1.1. AST), are usually determined for this purpose, as are alkaline phosphatase (E.C. 3.1.3.1. ALP) and gamma-glutamyl transferase (E.C. 2.3.2.2. GGT). Aspartate aminotransferase is not specific to the liver and increased serum activities of this enzyme are observed in myocardial infarction. Moreover, drug interference with the measurement of this enzyme may give rise to misleading serum activities (McNeely, 1978). Alkaline phosphatase and gamma-glutamyl transferase are sensitive indices of cholestasis, and both are elevated in more than 90% of patients with liver disease (Burke, 1978). Again, however, both tests lack specificity for liver disease, and elevated activities have been found in various circumstances including anticonvulsant therapy, heavy alcohol intake, enzyme induction, the healing stage of acute myocardial infarction, and infection (Burke, 1975).

Enzyme determinations are not true tests of liver function in that they indicate the degree of hepatocellular damage, which is not necessarily the same as loss of liver function.
Measurement of the serum concentration of proteins synthesised and secreted by the liver are true tests of hepatic function. One such protein is albumin. However, serum concentrations of albumin, because of the relatively long half-life of this protein, are usually within the normal range in acute hepatic disease. In chronic liver disease the serum albumin concentration is usually low and is considered a good index of the chronicity and severity of liver disease.

Prealbumin is a protein, synthesised in the liver, with a short half-life, 1.9 days (Oppenheimer, et al., 1965). For this reason, the serum concentration of prealbumin would be expected to be a sensitive indicator of any changes affecting its synthesis and catabolism, as in the case of liver disease. Prealbumin has a molecular weight of 60,000 (Harris and Kohn, 1974) and its name derives from its electrophoretic mobility relative to albumin, i.e. it migrates faster than albumin on electrophoresis at pH 8.6. It is a thyroxine binding protein (Harris and Kohn, 1974) with similar properties to those of albumin, and aids the transport of vitamin A by forming a complex with retinol binding protein, preventing its loss from the circulation (Peterson, 1971).

Concentrations of prealbumin in hepatobiliary diseases have been determined, but most workers have used only semi-quantitative methods such as inspection of cellulose acetate strips and polyacrylamide disc gels. Now, with a commercially available antiserum to human prealbumin it is possible to determine the protein quantitatively by specific, sensitive, immunological methods. Prealbumin concentration,
determined by polyacrylamide disc gel electrophoresis, was decreased in infective hepatitis and cirrhosis but was normal in patients with pulmonary tuberculosis and myxoedema (Helen, et al., 1975). In other studies serum concentrations of prealbumin were found to be decreased in liver disorders, burns, and ulcerative colitis, although in the latter two conditions, other serum protein concentrations were low due to direct loss of plasma proteins (Huezek-Glebocki, 1969; Prandota, 1975; Groza, 1976; Agostini, 1968; Skrede, 1975; Savoyi, 1977; Burdea, 1978).

The aim of the present study was to investigate, more rigorously, the effects of hepatobiliary disease and other disease states, on serum prealbumin concentration, using a sensitive immunological method and thus to assess whether prealbumin may be taken as an index of liver function.

The Normal Range

The normal range may be defined in terms of a range in which 'healthy' persons are expected to be found with a certain chosen probability, usually 95%. This means that the normal range is equivalent to the mean concentration plus or minus 1.96 standard deviations.

Methods

Immunological determination of serum proteins is both sensitive and specific. Commercially prepared antisera specific to particular proteins, are available. Methods of determination are based on the antigen-antibody reaction (Mancini, et al, 1965).

Single radial immunodiffusion is a simple, sensitive specific and relatively inexpensive technique that requires little apparatus. The antibody (from antisera) is
antigen is introduced into a well cut in the gel and is allowed to diffuse and react with the surrounding antibodies.

Single radial immunodiffusion was first employed by Petrie (1932) in his studies on the growth of bacterial colonies. Ouchterlony (1949) was the first to exploit the technique for semiquantitative purposes in his work with strains of the diptheria bacterium.

Mancini (1964) showed that a linear relation existed between the area of the precipitate and the concentration of the antigen, provided the diffusion was allowed to proceed until all antigen had been combined. The single radial diffusion method is, therefore, suitable for very accurate quantitative determinations, without any resort to end-point methods and the interpolations they require.

**Preparation of the Agar**

Barbitone buffer (0.024M, pH 8.6) was prepared by dissolving sodium barbitone (4.1g) barbitone (0.8g) and preservative sodium azide (1.0g) in deionized water (1.01), and adjusting the pH accordingly.

Litex agarose (1.0g) was added to warmed buffer (100ml) and the mixture was then placed in a boiling water bath and stirred until all the agarose had dissolved. The hot agarose solution was then dispensed into aliquots (10ml) and allowed to solidify for further use. The stock supply of agar was stored in well stoppered tubes at 4°C.

**Preparation of the plates**

1. An aliquot of the agarose, prepared above, (10ml) was melted by placing the tube in a boiling water bath.
2. The molten agarose was then allowed to cool to 55°C in a constant temperature water bath.

3. A glass slide (8cm x 8cm) was cleaned with absolute alcohol and allowed to dry. The slide was then placed on a level surface.

4. When the agarose had cooled (55°C) antiserum, specific to the protein (Behring Ltd) to be measured, was added to the agarose. The gel was then mixed thoroughly taking care to avoid bubbles of air (Table 5.1).

5. The antibody-containing gel was then poured on to the glass slide, spread evenly and allowed to set.

6. Wells were then cut into the gel with a commercially prepared cutter (2.0 mm diameter) using a template to ensure even spacing.

7. Serum samples (4 μl) were then applied to the wells.

8. A few wet tissues were placed at the bottom of an airtight plastic box. The glass slide was then put into the moist box and incubated at room temperature until diffusion was complete (Table 5.1).

9. Serum samples of standard protein concentration(diluted 1 in 2, 1 in 5, 1 in 10, 1 in 20, 1 in 50, 1 in 100) were also placed in wells so that a calibration curve could be constructed.

10. Quality control serum samples were added to each plate.
Table 5.1

Antiserum concentration and time necessary for complete diffusion of antigen (4 μl) in radial immunodiffusion.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antiserum Concentration % of Agarose Volume</th>
<th>Minimum Diffusion Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>8</td>
<td>18</td>
</tr>
</tbody>
</table>
Washing and Staining Procedure

a) When diffusion was complete, the wells were filled with water and the gel was covered with a piece of wet filter paper. A wad of dry tissues was then placed over the gel surface and pressure (10g/cm²) applied and maintained for ten to fifteen minutes. This procedure ensured gel was squeezed in a very effective manner and the liquid phase of the gel containing non-precipitating proteins was eliminated.

b) The filter paper and tissues were then carefully removed from the gel before washing for ten minutes in saline (0.1M). The gel was then squeezed a second time.

c) The gel was then dried under a current of warm air.

d) The slide was then ready for staining in Coomassic blue solution (Coomassic Brilliant Blue R, 5g, ethanol 450ml, 96%, glacial acetic acid 100ml, and deionized water 450 ml) for five minutes.

e) Washing in destainer (ethanol 96%, acetic acid and deionized water 3:2:9 by volume) clears the background stain before drying the slide in warm air.

Construction of the Calibration Curve

The diameters of the precipitation rings were measured using a magnified graticule, calibrated in 0.1 mm units (Gallenkamp Ltd.) The squared diameter of the standard precipitation rings were then plotted against the amount of antigen applied to the wells.
When complete diffusion of the standards had been reached a linear curve with an intercept on the ordinate was obtained.

By interpolation on the curve, samples of unknown protein concentration were quantified.

A standard curve for prealbumin was calculated by linear regression using a series of points taken over ten slides.

Storage of Serum for Prealbumin Determination

Stabalini et al (1968) showed that prealbumin could be stored frozen in serum for many years without loss of concentration. The investigation to follow was designed to check this information and to ensure that samples collected for enzyme determination (Chapter Four) and stored frozen for up to one year, were suitable for measurement of prealbumin concentration.

Method

Five blood samples taken from healthy volunteers were allowed to clot and serum prepared in the usual way. Each sample was separated into five aliquots. Four aliquots were frozen and maintained at \(-40^\circ\text{C}\).

Prealbumin concentration was determined in duplicate in the serum in the unfrozen aliquot and then in aliquots one month, six months, twelve months and fifteen months after freezing.

Results

The mean concentrations of prealbumin in each aliquot are shown in Table 5.2.
Table 5.2

Prealbumin concentration in stored serum samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Prealbumin concentration (mg/100 ml) at the specified time (months stored at -40°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>20.5</td>
</tr>
<tr>
<td>B</td>
<td>18.5</td>
</tr>
<tr>
<td>C</td>
<td>20.0</td>
</tr>
<tr>
<td>D</td>
<td>31.5</td>
</tr>
<tr>
<td>E</td>
<td>28.0</td>
</tr>
</tbody>
</table>
Conclusion

The results suggest that no loss in concentration of prealbumin occurs following storage of serum at -40°C for a period of up to fifteen months.

It is reasonable to assume, therefore, that prealbumin determinations in patient serum stored for up to fifteen months will give valid results.

Determination of the Normal Range

Serum from 89 healthy volunteers taking part in a community survey on tuberculosis, was obtained from the Midhurst Research Centre. Approximately equal numbers of male and female volunteers were used. Most volunteers (65/89) were between the ages of 50 and 80 years. Eighteen were aged below 50 years and the remaining 6 volunteers were over 80 years old. No apparent correlation of prealbumin concentration with age or sex was observed.

The distribution of the prealbumin values determined is shown in Figure 5.1.

The normal range (mean ± 1.96 standard deviations) was found to be 8 - 30 mg/100ml. This normal range is slightly lower than ranges previously reported (Simmons et al, Med. J. Aust. (1969), 2, 494-506). None of the volunteers studied, however, had a serum prealbumin concentration of less than 12.5 mg/100ml and 95% of the values lie within the range 13.5 - 31.0 mg/100ml.
SERUM PREALBUMIN AS AN INDEX OF LIVER FUNCTION IN HEPATOBILIARY DISEASE

Methods

Blood was collected from selected patients between 0830 h and noon by brachial venepuncture. Serum was prepared for determination of prealbumin, by centrifugation of clotted blood for 15 min at 2000 r.p.m. Aspartate aminotransferase, alkaline phosphatase, bilirubin and albumin were determined by standard methods.

Stored serum (-40°C), (Stabalini, et al., 1968), was diluted (1:5) with barbitone buffer (0.024 M, pH 8.6) and the prealbumin concentration was determined by immunodiffusion (Mancini, et al., 1965). Litex Agarose (1% w/v in barbitone buffer, pH 8.6, 0.024M) containing monospecific antiserum to human prealbumin (1% v/v) was used as gel medium. Diluted serum samples (2 μl) were applied to individual wells (2 mm diam.) cut into the gel, and diffusion allowed to take place overnight. The gels were washed for two hours in 0.1M-NaCl, then pressed with absorbant paper under 12 g/cm² pressure. The gels were stained in Coomassie Brilliant Blue R (0.5% w/v in alcohol-acetic acid-water (9:2:9 v/v), washed with alcohol-acetic acid-water (5:2:9 v/v) and dried. The diameters of the diffusion rings formed by the antigen-antibody complex were measured to 0.1 mm using a magnified graticule (Gallenkamp Ltd.), and the prealbumin concentration calculated from a standard curve. The reproducibility from gel to gel was checked with quality control sera.
Patients with one of the following disease states were selected: a) secondary carcinoma of the liver, confirmed by biopsy or laparotomy; b) alcoholic liver disease; alcoholic hepatitis confirmed by biopsy, and in most cases with superimposed cirrhosis; c) cirrhosis of the liver of unknown origin, including primary biliary cirrhosis, confirmed by laparoscopy or biopsy; d) obstructive jaundice, with high serum bilirubin levels, of non-neoplastic origin; e) myocardial infarction confirmed by E.C.G. changes; f) chronic inflammatory bowel disease, including ulcerative colitis and Crohn's disease.

Prealbumin concentration was also determined in the sera of a group of healthy volunteers of similar ages to patients in the study. Except for patients with myocardial infarction, all blood samples were taken, where possible, during the most acute phase of the illness. In the case of patients with myocardial infarction, blood was taken three days after the infarct.

Results and Discussion

The mean serum prealbumin concentration in 89 healthy volunteers was 19.4 ± 5.6 mg/100ml. Activities of aspartate aminotransferase greater than 40 IU and alkaline phosphatase greater than 300 IU were considered to be greater than normal. The normal range of plasma bilirubin was taken as 2 to 19 µmol/l, and the normal range of plasma albumin as 38 to 54 g/l. Parameters measured are shown in scatter diagrams (Figures 5.2 to 5.6). Tables of values are shown in Appendix 5.

Nine of fifteen cases of secondary carcinoma of the liver studied (Table 5.3) the prealbumin concentration was decreased. In most cases the aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum bilirubin were elevated also.
in one case the prealbumin level was decreased but none of the routine parameters of blood biochemistry showed abnormality.

Similarly, 8/12 cases of alcoholic liver disease studied (Table 5.4), prealbumin was below normal. One of this group of patients had normal values for alkaline phosphatase, aspartate aminotransferase and bilirubin, and the prealbumin was only marginally decreased. The other patients had normal aspartate aminotransferase and bilirubin, but prealbumin and serum albumin were below normal, and alkaline phosphatase was increased. Another patient had normal alkaline phosphatase and bilirubin values, but prealbumin, albumin and aspartate aminotransferase were outside the normal ranges. Only 6/12 cases had notably elevated alkaline phosphatase activity and 7/12 elevated bilirubin levels. Albumin concentration was only marginally decreased in all the cases of alcoholic liver disease studied.

Two patients with chronic active hepatitis (Table 5.5) of long standing were within the accepted normal range for prealbumin, and all other blood biochemistry parameters, which seems to indicate that the liver was functioning normally in these patients. In the remaining five patients in whom the disease was active, high aspartate aminotransferase activities and greatly reduced prealbumin levels were found, yet in two of these alkaline phosphatase was normal, and in one bilirubin was also normal.

The two patients with primary biliary cirrhosis (Table 5.6) both had normal prealbumin levels, and abnormal aspartate aminotransferase and alkaline phosphatase activities. Of the five cases of cryptogenic cirrhosis (Table 5.6) studied, three had low serum prealbumin levels but the aspartate aminotransferase
(3/5 were raised) and alkaline phosphatase (2/5 were raised) had a wide range of values with the group.

All patients with obstructive jaundice (Table 5.7) had bilirubin concentrations greatly in excess of the upper normal range and aspartate aminotransferase activities were also increased. All but three of these patients had decreased prealbumin levels. In these exceptions aspartate aminotransferase and alkaline phosphatase activities were both abnormal, although aspartate aminotransferase and bilirubin in one of these patients were not greatly increased. In this case, presumably, the obstruction had resulted in little or no effect on hepatocellular function.

All cases of inflammatory bowel disease studied (Table 5.8) had normal prealbumin concentrations. The serum enzyme activities and albumin concentrations were also in the normal range, which is in contrast to the findings that liver function tests in some instances of inflammatory bowel disease are abnormal (Weeke and Jarnum, 1971; Perrett et al, 1971; Leading Article, BMJ, 1979).

In patients with myocardial infarction the serum concentrations of prealbumin (Table 5.8) were within the normal range in all of the cases studied. As expected, high aspartate aminotransferase activities were observed in 7/9 patients with myocardial infarction. Blood samples were taken from the myocardial infarction patients three days after the infarct. In some cases, therefore, the aspartate aminotransferase activity was past its peak value and was returning to normal levels.
Figure 5.1
The Distribution of Serum Prealbumin Levels in Healthy Volunteers

Prealbumin Concentration
mg/100ml

Scatter
Mean ±
2 SD
Figure 5.3  Albumin Concentration in Hepatic and Non-hepatic Disease

Albumin g/1

Norm  Ca  AlcH  CAH  Cirr  Obs  BD  MI
Figure 5.4  Aspartate Aminotransferase Activity in Hepatic and Non-Hepatic Disease
Figure 5.5  Alkaline Phosphatase Activity in Hepatic and Non-Hepatic Disease
Figure 5.6  Bilirubin Concentration in Hepatic and Non-Hepatic Disease
Table 5.9
Summary of Serum Prealbumin Concentrations in Hepatobiliary Disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>Concentration (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Volunteers</td>
<td>* 19.4 ± 5.6 (89)</td>
</tr>
<tr>
<td>Secondary Carcinoma</td>
<td>* 7.2 ± 2.6 (15)</td>
</tr>
<tr>
<td>Alcoholic Hepatitis</td>
<td>* 8.5 ± 4.3 (12)</td>
</tr>
<tr>
<td>Chronic Active Hepatitis</td>
<td>* 8.5 ± 7.3 (7)</td>
</tr>
<tr>
<td>Cryptogenic Cirrhosis</td>
<td>* 10.0 ± 5.0 (7)</td>
</tr>
<tr>
<td>Obstructive Jaundice</td>
<td>11.1 ± 6.3 (9)</td>
</tr>
<tr>
<td>Inflammatory Bowel Disease</td>
<td>24.7 ± 11.6 (6)</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>18.7 ± 4.0 (9)</td>
</tr>
</tbody>
</table>

Mean values ± S.D. are given, with the numbers of patients studied shown in parenthesis.

* p 0.001 (Duncans Multiple Range Test)
<table>
<thead>
<tr>
<th>GROUPING</th>
<th>MEAN</th>
<th>N</th>
<th>GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24.666667</td>
<td>6</td>
<td>IRD</td>
</tr>
<tr>
<td>A</td>
<td>19.42213</td>
<td>89</td>
<td>NOR</td>
</tr>
<tr>
<td>A</td>
<td>18.666667</td>
<td>9</td>
<td>MYI</td>
</tr>
<tr>
<td>B</td>
<td>11.11111</td>
<td>9</td>
<td>OBJ</td>
</tr>
<tr>
<td>B</td>
<td>10.00000</td>
<td>7</td>
<td>CTR</td>
</tr>
<tr>
<td>B</td>
<td>8.520909</td>
<td>11</td>
<td>ALD</td>
</tr>
<tr>
<td>B</td>
<td>8.500000</td>
<td>7</td>
<td>CAH</td>
</tr>
<tr>
<td>B</td>
<td>7.222222</td>
<td>15</td>
<td>SCL</td>
</tr>
</tbody>
</table>
If the serum prealbumin concentration were to change as a consequence of myocardial infarction, however, its half life of two days would mean that little change would be observed until this time.

Conclusion

In this study, the serum concentration of prealbumin has been shown in to be decreased in diseases affecting the function of the liver. Because raised plasma enzyme activities may indicate impaired biliary excretion (alkaline phosphatase) and enzyme induction (gamma-glutamyl transferase), as well as hepatocellular damage, prealbumin which reflects the ability of the liver to synthesise protein is consequently a more reliable parameter of liver function. Other workers have shown that the serum prealbumin may be decreased in patients with hepatobiliary disease. However, these workers used only semi-quantitative methods for estimation of prealbumin, whereas the quantitative immunodiffusion methods of determining prealbumin are specific, reproducible and more sensitive to low concentrations. Furthermore, because of its short half-life, prealbumin is able to show changes in liver function more sensitively than plasma albumin changes.

In addition to the diagnostic role, serial determination of serum prealbumin concentration with time would be especially valuable in ascertaining whether liver function was improving or deteriorating. In some of the patients with secondary carcinoma of the liver serial determination of prealbumin showed that the serum concentration of the protein decreased as the illness progressed.
Furthermore, a patient with alcoholic hepatitis had low prealbumin levels in the acute phase of the illness but as the patient recovered the serum prealbumin concentration increased.

The results of the study are summarized in Table 5.9 and 5.10 which show that prealbumin concentrations are significantly decreased in hepatobiliary diseases but not in non-hepatobiliary disease states such as inflammatory bowel disease and myocardial infarction. This confirms that serum prealbumin concentration is dependant on liver function. However, it is desirable also to determine the plasma albumin concentration to ensure that no general protein loss, from the kidney or from enteropathies, has occurred to give rise to artefactual low serum concentrations of prealbumin.
Introduction

C-Reactive Protein was first described in 1930 by Tillett and Francis, who noticed a precipitation reaction between the C-polysaccharide of the pneumococcal cell wall and "acute phase serum".

Work by many groups has shown C-Reactive Protein (CRP) to have low serum levels in the healthy individual, not usually exceeding 1.0 mg per 100 ml serum. In disease, particularly following trauma, inflammation or tissue breakdown, the level of CRP in the blood stream shows significant elevation (Yocum, 1957).

The molecular weight of CRP is 138,000 and, synthesised in the liver (Hurlimann, 1966), has a half life of days. It probably exists in a pentameric form. As an acute phase reactant, CRP by definition is a protein whose concentration increases significantly as a response to the acute phase of inflammatory processes, trauma tissue breakdown, infection, necrosis, etc. In liver diseases involving any of these processes, the CRP level in serum should be seen to rise. This will be investigated.

It is also thought that CRP levels correlate with the progress of a disease. Sequential studies of the serum levels show that CRP levels return to normal as the disease process subsides.
Method of Determination

C-Reactive Protein was determined by radial immunodiffusion by the method of Mancini (Mancini, 1965). Monospecific antiserum to human CRP (Behring Ltd.) was added to molten agarose prior to pouring on glass plates and allowing to gel.

Serum samples (2 dil 1 diluted 1 in 5) were pipetted into wells cut into the gel. Diffusion was then allowed to take place overnight before washing the plates in aqueous sodium chloride (0.1 M).

After staining, the diameters of the diffusion rings formed were measured by a magnified graticule (Gallenkamp Ltd.). The CRP concentration determined with reference to a standard curve.

Determination in Human Serum

Human pathological sera that had been stored at -40°C for up to six months was thawed. Dilutions (1 in 5) of each sample were made and CRP determined as described.

The serum samples had been obtained from patients in the following categories; secondary carcinoma of the liver, hepatitis (4 alcoholic, 4 chronic active, 1 granulomatous and 1 hyperplasia), cirrhosis (all long-term confirmed). Obstructive jaundice (2 cholecystitis, 1 methyltestosterone induced). Myocardial infarction, chronic inflammatory bowel disease and drug overdose (1 paracetamol, remainder tranquillisers and hypnotics).
All blood samples (except drug overdose) were taken between 9.00 a.m. and 12 noon. The blood was allowed to clot and then centrifuged (10 mins, 2500 rpm) to collect serum which was separated and stored frozen until required.

Results

The results are shown in Table 5.10. The upper normal concentration is marked.

Conclusions

The following conclusions emerge from this small study.

a) All cases of secondary carcinoma of the liver elevated serum CRP levels.

b) Some but not all cases of hepatitis studied had elevated serum CRP levels. In the cases where no elevation occurred (equally divided among the types of hepatitis considered) it was probable that the hepatitis was not active.

c) Long standing cirrhosis did not appear to cause an increase in serum CRP level.

d) Obstructive jaundice caused a slight elevation in serum CRP level, not correlating with the severity of jaundice.

e) Elevated serum CRP level was observed in myocardial infraction, in most cases.

f) Chronic inflammatory bowel disease seemed to increase serum CRP levels.

g) None of the cases of drug overdose studies caused an immediate rise in serum CRP levels.
Table 5.10

Serum C-Reactive Protein Levels in Disease

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serum C-Reactive Protein (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary Carcinoma of Liver</td>
<td>*</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>*</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>*</td>
</tr>
<tr>
<td>Obstructive Jaundice</td>
<td>*</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>*</td>
</tr>
<tr>
<td>Chronic Inflammatory Bowel Disease</td>
<td>*</td>
</tr>
<tr>
<td>Drug Overdose (Paracetamol)</td>
<td>*</td>
</tr>
</tbody>
</table>
h) In two cases of cardiac arrest, one with right ventricular failure, serum CRP levels rose to 20 x greater than the normal value.

This brief study confirms previous work that CRP may be a good index of inflammatory change but also shows the non-specificity of CRP elevation to hepatobiliary disease. The protein is therefore of little value as a liver function test.
CHAPTER SIX
CHAPTER SIX

Prealbumin as an Index of Liver Function
Following Acute Paracetamol Poisoning

Introduction

Although it has become customary practice to use plasma enzyme activities, such as aspartate aminotransferase and alkaline phosphatase, as the biochemical parameters of choice for assessment of liver damage after drug overdose, these enzymes are neither true nor reliable indicators of liver function (Chapter Three). Indeed, increased plasma concentrations of the aminotransferases, \( \gamma \)-glutamyltransferase and other hepatocellular enzymes, may reflect only increased permeability of the hepatocyte plasma membranes, enzyme induction, or reversible hepatocellular damage (Burke, 1975). Furthermore, it is now recognised that certain drugs may produce such effects without concomitant liver injury, and that other drugs may interfere with the determination of plasma enzyme activities giving rise to erroneous results (McNeely, 1978). In the safety evaluation of drugs there is a real need to be able to evaluate liver function with greater efficiency and reliability. For there have been many instances in recent years, when the use of plasma enzyme levels as liver function tests have failed to detect drug-induced hepatotoxicity or to the contrary have given false indications of potential liver damage.

Prealbumin is a tetrameric protein, with a molecular weight of 60,000 (Harris, 1974), which is synthesised in the liver and is secreted into the blood plasma as a functional
protein. Its name reflects its electrophoretic migration relative to albumin, i.e. it migrates faster than albumin at pH 8.6. It has been described (Harris, 1974) as a thyroxine-binding protein with similar properties to those of albumin, but is especially active in the binding of tri- and tetra-iodothyronine (Goodman, 1976). The short half-life of prealbumin, 1.9 days, makes it a sensitive indicator of any changes affecting its synthesis and catabolism suggested in Chapter Five, and for this reason serum prealbumin concentration is a true index of liver function.

The assessment of liver function is particularly important in patients who have ingested an overdose of hepatotoxic drugs, such as paracetamol or Distalgesic. Paracetamol, when taken in overdose, produces hepatic necrosis by a direct action on the liver (Potter, 1973; McJunkin, 1976). This damage is dose related (Chapter One). The drug is metabolised to reactive intermediates which may initiate autoxidation and progressive hepatocellular damage or may be detoxicated by conjugation with glutathione. It is at the occurrence of progressive hepatocellular injury that liver enzymes are released into the circulating blood, but when hepatocellular damage has become so extensive that the enzymes are no longer being synthesised by the liver cells, the serum enzyme levels begin to fall, even though the liver may not be functioning normally. In contrast, blood concentrations of prealbumin appear to be a good index of liver function both at the early and late stages of hepatic necrosis, and the serum prealbumin levels fall as the damaged liver fails to synthesise the protein in amounts sufficient to maintain the normal blood concentration.
In the Guildford area severe overdosing with paracetamol was infrequent. Numerous patients were admitted with suspected paracetamol overdose but most had undetectable or minimal blood levels of the drug.

Most patients investigated in the present study survived, suffering only minimal and reversible liver damage, as shown by routine blood biochemistry.

**Experimental**

**Case One**

The subject of the study was a female (G.A.), 50 years old, who presented, having been found unconscious at home. There were venepuncture marks in both arms. She is known to have taken overdoses in the past, there was no history of the misuse of drugs.

On examination, the patient was deeply unconscious with a blood pressure of 80/0. There were some indurated and blistered areas on the left ankle and right thigh suggestive of barbiturate blisters. Reflexes were absent, apart from both knee jerks. Pupils were small and fixed. The fundi were probably normal, although there were early cataracts.

Blood biochemistry showed that plasma electrolyte levels were normal but the serum paracetamol concentration was 1.9 mmol/l, well above the range predictive of severe liver damage.
She was intubated but did not require ventilation, and was treated with intravenous fluids, including 50 grams of glucose, as the blood glucose concentration was undetectable. Several hours after presentation, the patient was responding to pin prick and to tracheal suction, but was still hypotensive and was treated with dopamine infusion which maintained her systolic blood pressure at around 100. Blood biochemistry was performed on subsequent days by Vickers automatic analysis. Prealbumin concentrations in serum were determined by Mancini radial immunodiffusion using monospecific antisera to human prealbumin (Chapter Five).

On day 1 after presentation, her electrolytes were normal but plasma enzyme concentrations indicated the rapid onset of liver damage, and became progressively abnormal. The patient went into renal failure, with a urine output of less than 10 ml per day. An electroencephalogram at this stage showed diffuse encephalopathy, probably due to liver damage or due to prolonged hypoglycaemia before admission. On day 5 the patient improved such that she would open her eyes on command, and blood pressure and pulse were stable. The following day (day 6) she became more unconscious (hepatic coma) and severe peripheral oedema developed. Full supportive therapy was continued. Her condition remained more or less unchanged until her death 15 days after presentation.
Other Cases

Several other cases of acute paracetamol overdose were studied. All of the following patients presented to the Accident Department of Guys Hospital, London. Serum and plasma was collected daily by hospital staff and routine analysis performed at Guys Hospital Poisons Unit. Prealbumin was determined on stored samples (-40°C) sent from London to Guildford, by the methods described in Chapter Five.

In total, nine other patients were studied. All patients had plasma paracetamol concentrations accepted to cause hepatotoxicity, i.e. greater than 1.2 mmol/l four hours after the overdose.

Results and Discussion

Case One

The prealbumin concentrations and other blood biochemistry are shown graphically in Figures 6.1 and 6.2. Prealbumin concentrations were in the normal range (0.15 - 0.45 g/l) on the first day after presentation. By the second day the level of prealbumin had fallen, and by the third day after presentation, the level was 50% decreased. As the liver function deteriorated the serum prealbumin was further decreased, and no serum prealbumin could be detected shortly before the patient died.

Aspartate aminotransferase (AST) activity rose sharply after the overdose, and 24 hours after presentation, was 50 times greater than the upper normal level. At 48 hours the activity had risen to 200 times greater than the normal
Figure 6.1

Blood protein levels following acute paracetamol poisoning

Serum prealbumin concentration (g/l) (●●), and plasma albumin concentration (g/l) (■■) are shown following overdosage with paracetamol.
Plasma enzyme levels following acute paracetamol poisoning

Plasma aspartate aminotransferase activity (IU/l) (○○○), and plasma alkaline phosphatase activity (IU/l) (□□□) are shown following overdosage with paracetamol.
level. However, after this time AST activity started to fall and by day 4 after presentation, its activity in serum was only 5 times the normal level. The plasma activity of this enzyme continued to decrease until the patient died. The activity of aspartate aminotransferase clearly was not a reliable index of liver function in this case, but did indicate the degree of hepatocellular damage in the initial phase.

Rising plasma levels of bilirubin and alkaline phosphatase (ALP) supported the clinically observed deterioration in the patient's condition. The bilirubin level was slow to increase, at first, but rose to greater than 15 times the normal level before death. Alkaline phosphatase was not significantly raised until 4 days after presentation. Before death, activity was double the normal level in plasma.

The concentration of plasma albumin decreased slightly but the level was not greatly abnormal even before death. This indicates that little protein was lost from the circulation due to abnormal renal failure. The long half-life of albumin causes this protein to be an insensitive index of liver function compared with prealbumin.

Prothrombin time was determined routinely and serial measurements showed that the liver was severely damaged in the initial phase. However, prothrombin time began to return to normal on subsequent days following vitamin K therapy, thereby giving a false impression that the damaged liver was recovering.
In conclusion, the serum prealbumin concentration appeared to be a more reliable index of liver function than were plasma enzymes, plasma albumin concentration or prothrombin times. Now that this indicator of liver damage can readily be quantitatively determined it would seem desirable to include it in the routine biochemical assessment of liver function that is made in cases of drug overdosage, or that is employed to monitor safety during clinical trials with new drugs.

Other Cases

The results of two other cases where other blood parameters were available for comparison with prealbumin are shown in Figure 6.3 (BL) and Figure 6.4 (JW). Patients also presented with overdose of paracetamol, where serum was sent to Guildford from London, but inadequate or no other blood parameters were measured. The results of prealbumin concentrations are shown in Figure 6.5,

In Figure 6.3 the patient had high activities of aspartate aminotransferase and prolonged prothrombin time as expected during a period of hepatocellular damage. The AST activity began to fall towards normal levels within the first few days after presentation of the patient. Prothrombin time showed most abnormality two to four days after the overdose and then returned to normal after six days.
Figure 6.3

Blood Parameters following Paracetamol Overdose
in Patient B L

Protein Concentration (g/l) or Enzyme Activity (IU/l) vs. Days after overdose.

- Plasma aspartate aminotransferase activity (IU/l) (○○),
- serum prealbumin concentration (g/l) (●●), and
- prothrombin time (patient/control ratio) (■■) are shown following paracetamol overdosage.
Figure 6.4

Prealbumin Concentration, Aspartate Aminotransferase Activity and Prothrombin Time Following Paracetamol Overdose (Patient J.W.)

Plasma aspartate aminotransferase activity (IU/l) (○--○), serum prealbumin concentration (g/l) (●--●) and prothrombin time (patient/control ratio) (■--■) are shown following paracetamol overdosage.
Figure 6.5

Preactamin Concentration in Several Patients following Paracetamol Overdose

Days after overdose
0 2 4 6

Prealbumin Concentration (g/l)

0.1

0.2
Prealbumin concentration was in the normal range on presentation but a rapid and significant reduction in concentration had occurred within 36 hours of presentation. As the damaged liver recovered, prealbumin concentration returned to the normal range.

The results of patient JW (Figure 6.4) show a similar trend to those of the patient BL (Figure 6.3). Again AST became grossly elevated before returning to normal. Prealbumin concentration fell after the overdose before returning to the normal range upon recovery. Plasma albumin concentration remained within the normal range in this patient.

Prealbumin concentration was observed to fall in the serum of a further five patients a short time after paracetamol overdose (Figure 6.5).

**Summary and Conclusion**

In a female presenting with an overdose of paracetamol and dextropropoxyphene, serum prealbumin levels were compared with routine plasma enzyme determinations in the assessment of the patient's condition. The plasma aspartate aminotransferase returned to normal levels after 3 days; alkaline phosphatase was slow to show increases in activity, and serum albumin concentration was in the normal range throughout. In contrast serum prealbumin concentration decreased significantly after 36 hours and continued to decrease showing the course of failing liver function until the patient's death, 15 days after presentation.
Prealbumin concentration was shown to fall after overdose of paracetamol in seven other patients. In these patients, damage due to paracetamol was not severe enough to cause death. Prealbumin levels fell after the overdose and then returned to normal concentrations as the patient recovered.

The plasma concentrations of enzymes of hepatic origin are essentially indications of hepatocellular leakage and enzyme induction, and do not always act as reliable indices of liver function. In comparison, prealbumin is a functional plasma protein synthesised in the liver, and, having short half-life, is thus a true and reliable index of liver function. It would appear to be a most reliable biochemical parameter to determine as an indicator of liver function in drug overdose.
CHAPTER SEVEN
CHAPTER SEVEN

ENZYME ACTIVITY - TIME RELATIONSHIPS

IN FOUR CASES OF HUMAN DISEASE

Introduction

The development of a disease process cannot be assessed by a single enzyme or protein determination. For this reason a few patients, selected from the previous study of enzyme activities in hepatobiliary diseases (Chapter Four) were assessed throughout the duration of their illness. Enzyme activities and prealbumin concentrations were determined at various occasions throughout that period.

Two patients with multiple secondary metastases in the liver were selected along with a patient presenting with an acute attack of alcohol poisoning. A fourth patient reported here presented with crescendo angina which lead to a cardiac arrest.

Methods

Patients were bled by venesection at various intervals during the course of illness. Enzyme activities and prealbumin concentrations were determined according to the methods previously described (Chapter Two, Chapter Five).

Case 1

This 67 year old lady presented with a six week history of malaise anorexia, upper abdominal distention and dyspepsia. On examination she was found to have an enlarged liver, 11 cm below the costal margin. There were no other abnormal physical signs.
A liver scan revealed the grossly enlarged liver to have multiple filling defects compatible with the presence of metastatic disease. A liver biopsy was performed. Histology showed metastatic adenocarcinoma with a primary site of the gastrointestinal tract. She was started on a palliative course of radiotherapy but deteriorated considerably and treatment was stopped.

The patient died four months after presentation. A graph of enzyme activity and prealbumin concentration at various times during the illness is shown in figure 7.1.

Case 2

This 56 year old lady presented with a two month history of weight loss, anorexia and abdominal pain. On examination she was found to have an enlarged liver, 6 cm below the costal margin. A liver scan showed the liver to have filling defects compatible with metastatic disease. A liver biopsy was performed, the histology of which showed largely necrotic poorly differentiated adenocarcinoma in the liver.

Enzyme activities and prealbumin concentrations are shown in Figure 7.2.

Case 3

This 38 year old alcoholic man had been admitted to hospital on previous occasions with conditions associated with his high alcohol intake.
On this occasion he was admitted to hospital having noticed increasing icterus. He had not felt well for a few months prior to admission but admitted to a large alcohol intake (bottle of whisky/gin per day).

During his first four days on the ward his condition markedly deteriorated. He became confused and disorientated, his jaundice deepened and he became comatosed. Treatment, with vitamin supplements, diuretics and intravenous foodstuffs, antibiotics and vitamin K when necessary, was continued through the period of unconsciousness.

Six days later the patient's level of consciousness improved and his jaundice lessened. The patient's condition improved until his discharge, 39 days after admission.

Four months after discharge the patient represented in hepatic failure due to continued alcohol intake and subsequently died. (Figure 7.3).

Case 4

This 52 year old man was admitted to hospital following a three week history of chest pain. On examination his pulse was regular, blood pressure was normal and there were no signs of cardiac failure. Crescendo angina was diagnosed and the patient treated with bed rest and a beta blocker.

Twentyfour hours after presentation the patient collapsed with cardiac arrest and was found to be in ventricular fibrillation. He was resuscitated with success. An electrocardiogram showed an anterior myocardial infarct. He was now hypotensive and in cardiac failure.
Following the attack the patient was rested and mobilized, in
due course, to complete recovery. Enzyme activities and
prealbumin concentrations in the plasma of this patient are shown
in Figure 7.4.

Results and Discussion

Normal values for each parameter measured are shown in Table 7.1.

Case 1

In figure 7.1 the serum activity of all enzymes determined
increased as the illness progressed. This can be explained by
the tissue in the liver becoming more and more necrotic as normal
tissue was replaced with carcinoma tissue.

Glycylprolyl-p-nitroanilidase (GPN) activity was greatly elevated
in the sample taken soon after presentation. A subsequent fall in
activity of this enzyme over the following few days occurred. This
may be due to massive release of this enzyme followed by a period
of resynthesis in the liver. Further leakage occurs as the
tissue becomes necrotic.

Aspartate aminotransferase (AST) activities, although slightly
abnormal, do not increase greatly over the course of the illness
although alanine aminotransferase (ALT) rises from the normal
activity to become abnormally high a short time after presentation.

Alkaline phosphatase activity rises consistently as the illness
progresses, as expected, and this high activity supports the
view that this enzyme is a good indicator of hepatic carcinoma.
High activities of alkaline phosphatase also indicate that there
may be increasing cholestasis.
### Table 7.1

Normal values of some biochemical parameters in human blood

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Range (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>0 – 4</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>5 – 27</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase</td>
<td>3 – 25</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>0 – 8.5</td>
</tr>
<tr>
<td>Ornithine carbamyl transferase</td>
<td>0 – 3.5</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>66 – 300</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>6 – 40</td>
</tr>
</tbody>
</table>

The values quoted apply to the enzymes determined by the methods described in Chapter Two.
Enzyme Activities and Serum Prealbumin Concentration in Early Secondary Carcinoma of the Liver (Case 1)

Prealbumin concentration (mg/100 ml) and enzyme activities (IU/l)

Days after presentation

- Glycylprolyl p-nitroanilidase
- Aspartate aminotransferase
- Alkaline phosphatase (x 0.1)
- Alanine aminotransferase
- Isocitrate dehydrogenase
- Prealbumin (x 5)
Enzyme Activities and Serum Prealbumin Concentration in Carcinoma of the Liver (Case 2)

Prealbumin concentration (mg/100 ml) and Enzyme Activity (IU/l) vs Days after presentation

Key:
- □ Aspartate aminotransferase
- ● Glycylprolyl p-nitroanilidase (x 0.25)
- ○ Alanine aminotransferase
- ■ Isocitrate dehydrogenase
- ▼ Prealbumin
Figure 7.3
Enzyme Activities and Serum Prealbumin Concentration in Alcohol Overdose (Case 3)

Prealbumin concentration (mg/100 ml) and Enzyme Activity 250 (IU/l)

Key:
- Glycylprolyl p-nitroanilidase
- Aspartate aminotransferase
- Alkaline phosphatase (x 0.25)
- Isocitrate dehydrogenase
- Prealbumin (x 4)

Days after presentation [Graph showing changes in concentration and activity over days.]
Figure 7.4

Enzyme Activities and Serum Prealbumin Concentration
following Myocardial Infarction and Cardiac Arrest (Case 4)

Prealbumin concentration (mg/100ml) and Enzyme Activity (IU/l)

Arrest

Days after Presentation

□ Aspartate aminotransferase (x 0.3)
▼ Alkaline phosphatase (x 0.25)
● Glycylprolyl p-nitroanilidase
○ Alanine aminotransferase
■ Isocitrate dehydrogenase
▼ Prealbumin
The serum activity of isocitrate dehydrogenase was initially in the normal range but a steady elevation in activity was observed as the disease progressed. Activity was more than six times greater than the upper normal limit, sixty days after presentation, whereas aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were less than double the upper normal activity at this time. This suggests that ICD activity is a sensitive indicator of cellular damage in hepatic carcinoma.

Prealbumin concentrations in serum were slightly below the normal range on presentation but within three days the prealbumin level had reduced to a quarter of its initial concentration. This low level was then maintained until the final stages of the illness.

**Case 2**

In this second case of secondary carcinoma of the liver the disease process was at a more advanced stage than in the case just reported (case 1). (Figure 7.2) Massive elevation in GPN activity was observed and this continued to rise in serum until the patients death.

On the other hand, aspartate aminotransferase activity became less with progression of the illness. This was probably due to a complete release of the enzyme from the damaged liver cells which were unable to synthesise sufficient quantities of the enzyme to maintain further high blood levels. Alanine aminotransferase activity remained above the normal range.
Serum isocitrate dehydrogenase (ICD) activity was greatly elevated and continued to rise until the patient's death, when activity was greater than five times the upper normal limit. This supports the observation described in Case 1.

The low prealbumin concentration was even more marked than in Case 1. In all determinations prealbumin level was greatly reduced and could hardly be detected in serum.

**Case 3 (Figure 7.3)**

When the patient presented, his blood biochemistry was markedly abnormal. This was due to hepatic damage caused by a high alcohol intake. Aspartate aminotransferase (AST) activity was greatly elevated, suggesting a large degree of hepatic necrosis and cell damage. Alkaline phosphatase (ALP) activity was also greatly elevated and the patient was jaundiced suggestive of cholestasis.

Isocitrate dehydrogenase (ICD) activity was more than 10 times above the upper normal range.

The leakage of the aminotransferase and isocitrate dehydrogenase enzymes into the blood was thought to be caused by hepatocellular damage. As the patient abstained from alcohol the necrotic cells were replaced allowing the damaged liver to recover. The enzyme activities began to fall and the three enzymes AST, ALP and ICD returned to the normal range at almost the same time after abstinence.

Serum GPN activity was also greatly elevated when the patient presented and the activity of this enzyme continued to rise for a further few days as his condition deteriorated. As the patient then began to recover the GPN activity subsided but
still remained above the normal range when all other enzymes investigated had returned to normal.

GPN activity was elevated in alcoholic patients (Chapter Five). This may be due to microsomal induction of the enzyme by alcohol or it may be due to hepatocellular damage. It was suggested that the enzyme resembles gamma-glutamyl transferase in this respect.

Prealbumin levels in the serum of this patient correlated well with the clinical severity of the disease. On presentation serum prealbumin concentration was just below the normal range. Over the course of the next few days its concentration fell dramatically as liver failure developed. Levels finally rose and returned to normal as the clinical condition of the patient improved.

Case 4 (Figure 7.4)

In myocardial infarction aspartate aminotransferase activity is known to increase to very high levels above the normal range. This is due to a major distribution of the enzyme in heart muscle. The enzyme leaks from damaged cells after infarction. It was no surprise therefore to observe high activities of aspartate aminotransferase following the cardiac arrest suffered by this patient. As the damaged tissue repaired there was a decline in blood activity.

Alanine aminotransferase has a small distribution in heart muscle, and this enzyme also showed elevation in serum activity following the cardiac arrest. Values returned to normal more slowly than those of aspartate aminotransferase.
Alkaline phosphatase activity in blood increased considerably after the arrest. A lactate dehydrogenase isoenzyme pattern showed increases in LDH one and two isoenzymes typical of myocardial infarction but also in LDH five isoenzyme suggesting some hepatic involvement.

The serum activities of glycylprolyl-p-nitroanilidase and isocitrate dehydrogenase stayed within the normal range during the illness. This suggested that the concentrations of these enzymes in the heart is quite low. This is known for isocitrate dehydrogenase but not for glycylprolyl-p-nitroanilidase. A small increase in the activity of these enzymes after the arrest can be explained by effects on the liver. A certain degree of hypoxia in the liver results from cardiac arrest. This gives rise to necrosis of liver cells and a release of enzymes, the degree of which depends on the severity of the resulting damage.

Prealbumin concentration remained within the normal range throughout the illness.

Conclusions

Emphasis has been placed in earlier Chapters that enzymes are not true indicators of liver function. This study has confirmed that isocitrate dehydrogenase and alanine aminotransferase were good indicators of hepatocellular damage, with greater specificity than aspartate aminotransferase. This fact is well known. It has also been shown that ICD activity may well correlate with the degree of hepatic cell damage.
Prealbumin concentration appears to be a good indicator of liver function (Chapter Five and Chapter Six). The present study has also investigated this finding and showed that the use of prealbumin levels as a prognostic tool, could be valuable. Results given in this Chapter suggest that prealbumin concentrations, measuring the ability of the liver to function normally, correlates well with the degree of hepatocellular damage shown by elevated enzyme activities. Prealbumin thus appears to be an objective measurement of the clinical state of the liver and its functions.
CHAPTER EIGHT
CHAPTER EIGHT

INVESTIGATION OF SERUM PREALBUMIN CONCENTRATION FOLLOWING ACUTE DRUG HEPATOXICITY IN THE MOUSE

Introduction

The electrophoresis of mouse serum on cellulose acetate at pH 8.6 enables visual detection of mouse prealbumin (Reuter, 1966). Personal experience has proved that mouse prealbumin does not cross react with human antiprealbumin antiserum at a level sufficient to be a useful determination of the protein. Commerically prepared antisera to animal prealbumin are not available. Only quantitative evaluation of the protein in mouse serum is possible with the semi-quantitive scanning of the cellulose acetate strip assisting the interpretation.

Method

Twenty four male albino mice, weight 30 to 40 g, were randomised into six groups, each of four mice. All mice were maintained under normal animal house conditions and allowed food and water ad libitum.

The five animals in group one were intraperitoneally dosed with corn oil. Animals in group two were given a single intra-peritoneal dose of carbon tetrachloride in corn oil, (350 mg/kg). Animals in group three were also dosed intraperitoneally with carbon tetrachloride in corn oil (50 mg/kg).
Animals in groups four and five were dosed orally with paracetamol in gum tragacanth (1% w/v) at doses of 600 mg/kg and 300 mg/kg respectively on a single occasion. Group six mice were control animals, orally dosed with gum tragacanth (1% v/v).

Forty eight hours after dosing, all animals were bled under light ether anaesthesia by cardiac puncture and killed by cervical dislocation. Livers were then removed and preserved in formyl saline solution (10%) for histology. Blood was allowed to clot at room temperature before centrifugation to collect serum.

Prealbumin was identified quantitatively by inspection of a protein electrophoretic strip run on cellulose acetate (Smith, 1968; Sargent, 1975).

**Results**

In both groups of control animals, group one and group six, a prealbumin band could be identified on the electrophoretic strip. In animals treated with carbon tetrachloride at a dose of 50 mg/kg the band was still visible but in those animals dosed with 350 mg/kg no prealbumin band could be detected in any animal.

In the case of mice treated with paracetamol, all control animals (group six) and animals dosed with 300 mg/kg had clearly visible prealbumin bands. In only one of the four animals dosed with 600 mg/kg paracetamol was a prealbumin band visible after electrophoresis.
Histological examination of the livers of animals in the group treated with 350 mg/kg carbon tetrachloride showed severe centrilobular necrosis.

**Conclusion**

Liver damage, induced by paracetamol and carbon tetrachloride, was detected by histological examination of the liver (see appendix). Centrilobular necrosis and fatty deposition was observed in the livers of mice treated with carbon tetrachloride. The livers of mice treated with paracetamol showed centrilobular necrosis and some haemorrhage from the portal veins into surrounding tissue.

By visual inspection, the prealbumin band as seen by electrophoresis of serum on cellulose acetate, was not evident in the serum of mice treated with high doses of carbon tetrachloride and paracetamol. These results suggest that serum prealbumin determination in the mouse is likely to be a good indicator of liver dysfunction in drug toxicity. Ultimately, an antiserum to mouse serum prealbumin should be prepared (Reuter, 1968) and a more quantitative investigation undertaken in drug and chemical hepatotoxicity.

**Prealbumin Concentration at Various Times in the Serum of Mice Treated with Carbon Tetrachloride**

**Introduction**

It has been shown in the previous experiment that carbon tetrachloride, a potent hepatotoxin, causes histological damage to the livers of treated mice.
Prealbumin concentration has also been shown to be reduced in mice treated with carbon tetrachloride. The purpose of this investigation is to determine how quickly the prealbumin concentration is noticeably reduced in the serum of mice treated with carbon tetrachloride.

Method

Sixteen albino mice (30 - 40 g) were randomised into eight groups. Test groups (T1 - T4) each consisted of three animals; control groups (C1 - C4) each consisted of one animal. All animals were maintained in standard animal house conditions and allowed food and water ad libitum. All animals in the test groups (T1 - T4) were given a single intraperitoneal dose of a solution containing carbon tetrachloride in corn oil (35) mg/kg body weight). The control animals (groups C1 - C4) were given an equivalent volume of corn oil by intraperitoneal injection.

At 12, 24, 36 and 48 hours following dosing one group of test animals and one group of control animals were bled by cardiac puncture and killed by cervical dislocation before livers were removed for preservation in formyl-saline (10%). Blood collected by cardiac puncture was allowed to clot before centrifugation to collect serum.

Cellulose acetate electrophoresis at pH 8.6 was carried out on each serum sample and the resulting strip, stained with nigrosine to visualise the prealbumin band, on the anode side of the albumin band.
Glycylprolyl-p-nitroanilidase, alanine aminotransferase and isocitrate dehydrogenase enzymes were also determined in each serum sample.

Enzyme activities and prealbumin concentration by visual inspection were compared with routine histology performed on each liver.

Results

a) Cellulose Acetate Electrophoresis

In the table below each animal in the group is represented by either + or -. The plus sign indicates a prealbumin band to be visible in the serum of that animal. A minus sign indicates no prealbumin band was visible.

Table 8.1

Existence of a prealbumin band on electrophoresis of serum at a given time after dosing

<table>
<thead>
<tr>
<th>Time After Dose (hours)</th>
<th>Existence of prealbumin band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test Animals (350 mg/kg) CCl₄</td>
</tr>
<tr>
<td>12</td>
<td>+++</td>
</tr>
<tr>
<td>24</td>
<td>+++</td>
</tr>
<tr>
<td>36</td>
<td>+--</td>
</tr>
<tr>
<td>48</td>
<td>---</td>
</tr>
</tbody>
</table>
b) **Table 8.2**

Enzyme activities at a given time, in the serum of mice treated with carbon tetrachloride in corn oil

<table>
<thead>
<tr>
<th>Time after dose (hours)</th>
<th>Mean Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Number of times greater than mean control value)</td>
</tr>
<tr>
<td></td>
<td>Isocitrate Dehydrogenase</td>
</tr>
<tr>
<td>12</td>
<td>2.5</td>
</tr>
<tr>
<td>24</td>
<td>12.5</td>
</tr>
<tr>
<td>36</td>
<td>5.0</td>
</tr>
<tr>
<td>48</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Table 8.3**

Histological assessment of liver damage at various times in mice treated with carbon tetrachloride

<table>
<thead>
<tr>
<th>Time after dose (hours)</th>
<th>Extent of Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test Animals</td>
</tr>
<tr>
<td>12</td>
<td>±</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>+++</td>
</tr>
<tr>
<td>48</td>
<td>+++</td>
</tr>
</tbody>
</table>
Evidence of centrilobular necrosis is indicated by +, the number of plus signs indicating the severity of the lesion.

Conclusions

The prealbumin band observed on cellulose acecate electrophoresis of mouse serum disappeared some thirtysix to forty eight hours after dosing the animal with carbon tetrachloride. This correlated with a high degree of centrilobular necrosis and elevated enzyme activities which indicate hepatocellular damage.
CONCLUSIONS

A review of the battery of currently used "liver function tests" indicates the valuable, although at times conflicting, information obtained from their results. Not only are a majority of these tests non-specific as diagnostic tools for liver disease, but their values frequently vary at different stages of a given hepatic disease. Results also may not reflect the severity of a hepatic problem.

The Requirements of a Liver Function Test

There are three main indications for the performance of liver function tests. Firstly the test should establish the presence of liver damage, due to a disease process or toxicity due to xenobiotics. Secondly the test should attempt to define the type of hepatobiliary dysfunction, and thirdly it should assess the extent and prognosis of the lesion. In choosing and interpreting liver function tests, it is of value to consider the specificity, sensitivity and selectivity of each test.

A test is specific if an abnormal result can only be due to liver dysfunction, sensitive if it is capable of detecting early, minor or subtle disturbances of liver function, and selective if it is capable of differentiating one liver disease from another.
Clearly no biochemical parameter currently used to assess liver function is completely specific, sensitive and selective. Although the aminotransferases are sensitive they are not specific. Ornithine carbamyl transferase activity changes are more specific to hepatic dysfunction but the enzyme is less sensitive compared with the aminotransferases. Alkaline phosphatase is quite sensitive, has good specificity for liver diseases by virtue of specific isoenzyme determinations, and is selective in that elevated alkaline phosphatase activity is usually indicative of hepatocellular damage due to bile stasis.

The ease and reliability of measuring a particular enzyme activity is also important when assessing its usefulness as a liver function test.

**Problems Involved with Enzyme Determinations**

There is evidence that drugs and chemicals may interfere with the determination of enzymes, particularly aminotransferases, in serum (McNeely, 1978; Hendriks, 1979).

An important but often unsuspected cause of aminotransferase elevation is drug interference. Therapeutic agents may derange serum aminotransferase activity by interfering with the chemical reaction used for analysis. Paracetamol, isoniazid, methyldopa are hepatotoxins and they have also been shown to interfere with aminotransferase determination. This could lead to errors in interpretation.
In certain pathological sera, such as those from advanced or chronic liver disease, an interfering reaction between α-ketoglutarate and ammonium ions forming glutamate, may be catalysed by serum glutamate dehydrogenase, also present in elevated amounts from injured liver cells. This may cause additional NADH oxidation which is read mistakenly for aminotransferase activity.

It is clear that efficiency of the determination of aminotransferases is in doubt and must hinder, in future, the use of these enzymes as liver damage markers.

Measurements of isocitrate dehydrogenase has several advantages over that of aminotransferase determination. Firstly isocitrate dehydrogenase is determined by a single step reaction, the kinetics of which can be followed spectrophotometrically in a single step. In contrast, the aminotransferases are determined by a coupled reaction. Secondly in the isocitrate dehydrogenase determination an increase in absorbency is measured whereas a decrease is measured in the aminotransferase determination. The readings for the citrate reaction are therefore made in a more sensitive range of the spectrophotometer (Sterkel, 1958). Thirdly, pyruvate in the serum makes necessary a preliminary preincubation in the aminotransferase determination which is not necessary in the measurement of serum isocitrate dehydrogenase activity. Finally, the product of isocitrate dehydrogenase action (NADPH) is stable in the presence of serum at neutral pH unlike the NADH product of the aminotransferases.
Another advantage of using isocitrate dehydrogenase as a marker of liver cell damage is that it is not induced by drugs and does not appear to be increased by chemicals which lead to an increase in plasma membrane permeability.

Selection of Enzymes to Indicate Hepatocellular Damage

This study has aimed to investigate the ability of existing and new serum enzymes to detect and monitor liver damage. In the animal model a number, but by no means a comprehensive list, of serum enzyme activities were investigated following administration of hepatotoxins (Chapter Two).

Carbon tetrachloride, paracetamol and bromobenzene were chosen as hepatotoxins because each acts by a different mechanism. Carbon tetrachloride is metabolised to a free radical, causing lipid peroxidation and cell necrosis. Bromobenzene is also metabolised to a reactive intermediate, this time an epoxide. Finally paracetamol is metabolised to an intermediate, probably a quinone-imine, which exerts its toxin effects by initiating cellular autoxidation and depleting intracellular glutathione.

From preliminary studies three enzymes were selected as being good indices of hepatocellular damage. Isocitrate dehydrogenase, alanine aminotransferase and ornithine carbamyl transferase all showed sensitive elevation in activity following administration of hepatotoxin.
Investigations were also made on a new enzymic method, first published by Hopsu-Havu and Glenner in 1966 (Chapter Three). Glycyl prolyl p-nitroanilidase showed great similarity to the enzyme gamma-glutamyl transferase, now well established as an indicator of chronic hepatic damage, particularly that to alcoholism. Although GPN showed minimal changes in activity following acute administration of carbon tetrachloride, significant serum elevation was observed after long term dosing with cadmium chloride. This contradicts the finding of Hino in Japan (1976) whose group suggested that the enzyme showed elevation following acute CCl₄ toxicity.

**Enzyme Studies in Human Hepatobiliary Disease**

Four enzymes, GPN, ICD, ALT and AST were measured in a number of patients with hepatobiliary disease (Chapter Four). Although most enzymes do not show complete specificity to hepatic damage, isocitrate dehydrogenase showed elevated serum activities in all cases of hepatic disease where an active process of cell death was indicated. No abnormality in the activity of ICD was observed in conditions not associated with liver damage.

Patients with hepatic cancer have been shown to have high serum GPN activities following a recent study in Japan (Kojima et al, 1979). This confirms the finding described in Chapter Four. GPN was also shown to have elevated activity in the serum of patients with other hepatobiliary disease but not in patients with disease not involving the liver.
Prealbumin as a Liver Function Test

Although it has become customary practice to use plasma enzyme activities, such as aspartate aminotransferase and alkaline phosphatase, as the biochemical parameters of choice for assessment of liver damage, these enzymes are neither true nor reliable indicators of liver function. Indeed, increased plasma concentrations of the aminotransferases, \( \delta \)-glutamyltransferase and other hepatocellular enzymes, may reflect only increased permeability of the hepatocyte plasma membranes, enzyme induction, or reversible hepatocellular damage (Burke, 1978).

Furthermore, it is now recognised that certain drugs may produce such effects without concomitant liver injury, and that other drugs may interfere with the determination of plasma enzyme activities giving rise to erroneous results (Hendricks, 1979). In the safety evaluation of drugs there is a real need to be able to evaluate liver function with greater efficiency and reliability. For there have been many instances in recent years, when the use of plasma enzyme levels as liver function tests have failed to detect drug-induced hepatotoxicity or to the contrary have given false indications of potential liver damage.

Prealbumin is a tetrameric protein, with a molecular weight of 60,000, which is synthesised in the liver and is secreted into the blood plasma as a functional protein (Chapter Five). Its name reflects its electrophoretic migration relative to albumin, i.e. it migrates faster than albumin at pH 8.6. It is a glycoprotein and has been described as a thyroxine-binding protein with similar properties to those of albumin, but is especially active in the binding of tri- and tetra-iodothyronine.
The short half-life of prealbumin, 1.9 days, makes it a sensitive indicator of any changes affecting its synthesis and catabolism, and for this reason serum prealbumin concentration is a true index of liver function. Blood levels of prealbumin were found to be low in conditions associated with liver disorders, congestive heart failure and burns (Skrede, 1975; Helen, 1975) and in healthy volunteers only 0.3% of 4,486 sera investigated had low concentrations of prealbumin as judged by cellulose acetate electrophoresis.

Prealbumin Levels after Paracetamol Overdose

The assessment of liver function is particularly important in patients who have ingested an overdose of hepatotoxic drugs, such as paracetamol of Distalgesic. It is at the occurrence of progressive hepatocellular injury due to drugs that liver enzymes are released into the circulating blood, but when hepatocellular damage has become so extensive that the enzymes are no longer being synthesised by the liver cells, the serum enzyme levels begin to fall, even though the liver may not be functioning normally.

In a female presenting with an overdose of paracetamol and dextropropoxyphene serum prealbumin levels were compared with routine plasma enzyme determinations in the assessment of the patient's condition. The plasma aspartate aminotransferase returned to normal levels after 3 days; alkaline phosphatase was slow to show increases in activity, and serum albumin concentration was in the normal range throughout.
In contrast serum prealbumin concentration decreased significantly after 36 hours and continued to decrease showing the course of failing liver function until the patient's death, 15 days after presentation. This observation was confirmed in other cases of overdose (Chapter Six).

The plasma concentrations of enzymes of hepatic origin are essentially indications of hepatocellular leakage and enzyme induction, and do not always act as reliable indices of liver function. In comparison, prealbumin is a functional plasma protein synthesised in the liver, and, having short half-life, is thus a true and reliable index of liver function. It would appear to be a most reliable biochemical parameter to determine as an indicator of liver function in drug overdose.

The reduction in serum prealbumin levels one expects to see in liver damage could arise in a number of ways. Impaired protein synthesis in the liver, impaired 'secretion' into the blood accelerated protein turnover altered interactions of the protein and direct loss from serum through burns, enteropathies and so on, would all give rise to reduced serum levels of prealbumin.

The most likely cause of lowered levels in hepatic disease is impaired synthesis of prealbumin in the liver or impaired secretion of the protein into the blood.

Mouse Serum Prealbumin and Hepatotoxicity

Investigation of prealbumin levels in an animal model of hepatotoxicity required the preparation of a monospecific antiserum to the protein, to enable its sensitive measurement.
A similar procedure to that used for the isolation of human prealbumin (Alper, 1969) was used in an attempt to prepare the protein from rat serum. Great difficulties were encountered and it became apparent that perhaps the properties of rat prealbumin (Navab, 1977) were different to those of the protein in human serum.

An antiserum containing antibodies to a minor rat protein was prepared in the rabbit. Unfortunately antibodies to rat albumin were also present and these hindered the ability to identify the minor protein antibodies.

Because of the large amount of time needed to advance the preparation of a suitable antiserum, it was decided to investigate prealbumin levels qualitatively by cellulose acetate electrophoresis.

No classical prealbumin band is seen when rat serum is separated by cellulose acetate electrophoresis. Mouse prealbumin is however readily observed on electrophoretic strips of serum.

Studies of acute toxicity in the mouse were therefore undertaken. These showed that following carbon tetrachloride and paracetamol toxicity, prealbumin levels were rapidly decreased. (Chapter Eight). These findings suggest that development of a quantitative estimation of animal prealbumin is desirable.

Prealbumin appears to be a sensitive index of impaired liver function in human hepatobiliary disease and drug toxicity and also in the animals treated with hepatotoxic chemicals.
This protein of short half-life may ultimately be of great value in the safety evaluation of new drugs, particularly those which are expected to have an effect on liver function. Monitoring serum prealbumin levels should also be of more reliable prognostic value in patients taking overdose of hepatotoxic drugs, notably paracetamol.
References


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Leading Article (1979) 'Liver Dysfunction in Inflammatory Bowel Disease', BMJ, 22nd September 1979, pp.688-689.


Slater, T.F. (1972) in Free Radical Mechanisms in Tissue Injury, Pion Ltd., Ch. 11, pp. 171-197; Ch. 9, pp. 91-170.


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In addition I should like to thank Mrs. Joan Pearne, SRN, SCM, for her dedication in the typing of this thesis, aided in part by Miss Toni Fransèes.

I am grateful for the generous support of the Science Research Council and Merrell (International) Limited, without which the work could not have been done.

Finally I would like to extend my special thanks to Dr. Martin Smith and to Professor Dennis V. Parke whose endless help, advice and patience have been very much appreciated.

Thank you.
APPENDICES
The following papers have been prepared:

1. "Prealbumin as an Index of Liver Function in Human Hepatobiliary Disease."
   by D.R. Hutchinson, R.P. Halliwell, M.G. Smith and D.V. Parke.

2. "Prealbumin as an Index of Liver Function Following Acute Paracetamol Poisoning."
   by D.R. Hutchinson, M.G. Smith, and D.V. Parke.
   (submitted to The Lancet)

3. "Glycyl prolyl-p-nitroanilidase in Hepatobiliary Disease"
   by D.R. Hutchinson, R.P. Halliwell, J.D.F. Lockhart and D.V. Parke
   (submitted to Clinica Chimica Acta)

4. "Prealbumin Levels in Paracetamol Overdose"
   Read at The Liver Club Meeting in Sheffield (July 1979)

5. "Prealbumin Levels following Drug Overdose"
   Poster presentation, Postgraduate School on Toxicological Testing Methods, Chelsea College, April 1980.
ROUTINE HISTOLOGY

PARAFFIN WAX EMBEDDED SECTIONS STAINED WITH HAEMATOXYLIN AND EOSIN

FIXATION

Pieces of tissue were fixed in 10% neutral buffered formalin for several days.

Formula for 10% neutral buffered formalin

Formalin (40% formaldehyde) 100 ml
Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O) 4.5 g
Anhydrous disodium hydrogen orthophosphate (Na₂HPO₄) 6.5 g
Distilled water to 1 litre

PROCESSING

The tissue was embedded in paraffin wax by the following method:
A piece of tissue 2-3 mm in thickness was cut from each sample and placed in a metal processing container together with the appropriate reference number. The containers were placed in a tissue basket which was then fitted to the Histokinette automatic tissue processor.

Tissues were transferred automatically from one beaker of fluid to the next and the processes of dehydration, clearing and impregnation with wax were carried out using the following processing schedule.
Dehydration

70% alcohol 1 hour
85% alcohol 1 hour
95% alcohol 1 hour
100% alcohol I 1 hour
100% alcohol II 1 hour
100% alcohol III 1 hour

Clearing

Toluene I 1 hour
Toluene II 1 hour

Impregnation

Paraffin wax I at 58°C 1 hour
Paraffin wax II 1 hour

The melting point of the wax was 56°C. After this process the tissue containers were transferred to a vacuum embedding oven containing paraffin wax at a temperature of 58°C for ½ hour. This acted as a third wax bath and the reduced pressure aided impregnation by ensuring that any remaining air bubbles and clearing agent were removed.

The tissue was blocked out by removing it from the container using a pair of electrically heated forceps and placing it in a plastic mound filled with molten wax. The tissue was orientated so that the surface to be cut rested on the base of the mould. A plastic block was placed in the mould with the reference number label.
Section Cutting

Sections were cut from the blocks at a thickness of 7μ using an American Optical Spencer 820 Rotary microtome. The sections were floated on distilled water at a temperature of 50°C until the creases disappeared.

The sections were mounted on slides by half submerging a clean slide into the water near the section and withdrawing it, so bringing the flattened section with it. The slides were placed initially on a drying hotplate and then left in an incubator at a temperature of 37°C overnight to dry.

STAINING

Sections were stained with Ehrlich's acid haematoxylin (obtained from R.A. Lamb Ltd.) and eosin (c.I. No. 45380 obtained from R.A. Lamb Ltd.).

Slides were placed in:

1. XYLENE to remove the wax 2 minutes
2. ABSOLUTE ALCOHOL 1 minute
3. 70% ALCOHOL 1 minute
4. 50% ALCOHOL 1 minute
5. DISTILLED WATER Rinse
6. EHRLICH'S ACID HAEMATOXYLIN 15 minutes
7. Blue-ed in TAP WATER 5 minutes
8. Differentiated in ACID ALCOHOL (1% Hydrochloric acid in 70% alcohol) by agitating for about 5 seconds
9. Returned to TAP WATER 1-2 minutes
10. Examined under low power microscope to ensure the sections were sufficiently differentiated

11. Blue-ed in TAP WATER 15 minutes

12. Counterstained in 1% AQUEOUS EOSIN 2 minutes

13. Rinsed in TAP WATER ¼ minute

14. Dehydrated in 85% alcohol "

15. " " 100% "

16. " " " "

17. Cleared in xylene

18. Cleared in xylene

The sections were mounted in D.P.X. (B.D.H. Ltd.) by placing a drop of the mountant on a clean coverslip, taking the slide direct from the xylene, inverting it over the coverslip and pressing gently so that the mountant spread under the coverslip.

Slides were examined at magnifications of x14, x100 and x400 using a Vickers M15c microscope.
APPENDIX 3

CHEMICALS

All of the chemicals used in these studies, unless otherwise stated, were of analar quality obtained from Sigma Chemical Company Ltd., Poole, Dorset, or BDH Limited, Poole, Dorset.
TABLE 4.1

Serum Enzyme Activities in Secondary Carcinoma of the Liver

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<thead>
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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Isocitrate Dehydrogenase (ICD)</th>
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<th>Alanine aminotransferase (ALT)</th>
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### TABLE 4.2

**Serum Enzyme Activities in Alcoholic Hepatitis**

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### TABLE 4.3

**Serum Enzyme Activities in Chronic Active Hepatitis**

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### TABLE 4.4

**Serum Enzyme Activities in Cryptogenic Cirrhosis**

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* primary biliary cirrhosis
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<td>Serum Enzyme Activities in Obstructive Jaundice</td>
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<td>M</td>
<td>105</td>
</tr>
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<td>M</td>
<td>43</td>
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<td>M</td>
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<tr>
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TABLE 4.6

Serum Enzyme Activities in Chronic Inflammatory Bowel Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Isocitrate Dehydrogenase (ICD)</th>
<th>Glycylprolyl p-nitroanilidase (GPN)</th>
<th>Alanine aminotransferase (ALT)</th>
<th>Aspartate aminotransferase (AST)</th>
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<td>M</td>
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<td>9</td>
<td>13</td>
</tr>
<tr>
<td>AW</td>
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<td>M</td>
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<td>54</td>
<td>9</td>
<td>-</td>
</tr>
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<td>M</td>
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<td>38</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>JS</td>
<td>37</td>
<td>M</td>
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<td>42</td>
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<td>F</td>
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<td>34</td>
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<td>14</td>
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<tr>
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<td>F</td>
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<td>37</td>
<td>7</td>
<td>-</td>
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TABLE 4.7

Serum Enzyme Activities in Myocardial Infarction

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<th>Enzyme Activity (IU/litre)</th>
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<td>VB</td>
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<td>M</td>
<td>5.0</td>
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<td>PC</td>
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</tr>
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<td>M</td>
<td>2.1</td>
</tr>
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<td>M</td>
<td>3.4</td>
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<td>RD</td>
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<td>M</td>
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<td>M</td>
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<td>TA</td>
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<td>M</td>
<td>3.0</td>
</tr>
<tr>
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<td>EA</td>
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<td>F</td>
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APPENDIX 5

TABLES TO CHAPTER 5
Table 5.3
Serum concentration of prealbumin and parameters of blood biochemistry in secondary carcinoma of the liver

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Prealbumin (mg/100 ml)</th>
<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (mol/l)</th>
<th>Albumin (g/l)</th>
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</thead>
<tbody>
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<td>116</td>
<td>49</td>
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<td>-</td>
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<td>38</td>
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<td>38</td>
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<td>-</td>
</tr>
<tr>
<td>P.L.</td>
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Table 5.4

Serum concentration of prealbumin and parameters of blood biochemistry in alcoholic liver disease

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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Prealbumin (mg/100 ml)</th>
<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (µmol/l)</th>
<th>Albumin (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>R.H.</td>
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<tr>
<td>B.P.</td>
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<td>F</td>
<td>7.5</td>
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<td>M</td>
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Table 5.5

Serum concentration of prealbumin and parameters of blood biochemistry in chronic active hepatitis

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<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (µmol/l)</th>
<th>Albumin (g/l)</th>
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<tr>
<td>E.P.</td>
<td>56</td>
<td>F</td>
<td>17.0</td>
<td>24</td>
<td>214</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>G.A.</td>
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<td>F</td>
<td>7.5</td>
<td>113</td>
<td>369</td>
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<td>34</td>
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<td>M.S.</td>
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<td>164</td>
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<td>F</td>
<td>3.0</td>
<td>314</td>
<td>279</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>D.F.</td>
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<td>F</td>
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<td>280</td>
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Table 5.6
Serum concentration of prealbumin and parameters of blood biochemistry in cryptogenic cirrhosis

<table>
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<th>Sex</th>
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<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (μmol/l)</th>
<th>Albumin (g/l)</th>
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<td>163</td>
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* Primary biliary cirrhosis.
<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Prealbumin (mg/100 ml)</th>
<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (µmol/l)</th>
<th>Albumin (g/l)</th>
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<td>26</td>
<td>40</td>
</tr>
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Table 5.8

Serum concentration of prealbumin and parameters of blood biochemistry in non-hepatobiliary disease

<table>
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<th>Age</th>
<th>Sex</th>
<th>Prealbumin (mg/100 ml)</th>
<th>Aspartate Aminotransferase (IU/l)</th>
<th>Alkaline Phosphatase (IU/l)</th>
<th>Bilirubin (μmol/l)</th>
<th>Albumin (g/l)</th>
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<td>9</td>
<td>39</td>
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<td>M</td>
<td>24.0</td>
<td>229</td>
<td>191</td>
<td>34</td>
<td>55</td>
</tr>
<tr>
<td>P.C.</td>
<td>55</td>
<td>M</td>
<td>23.5</td>
<td>32</td>
<td>135</td>
<td>19</td>
<td>45</td>
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<tr>
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<td>116</td>
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<td>R.G.</td>
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<td>M</td>
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<td>191</td>
<td>12</td>
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</tr>
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<td>R.D.</td>
<td>68</td>
<td>M</td>
<td>18.0</td>
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<td>1024</td>
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<td>38</td>
</tr>
<tr>
<td>P.L.</td>
<td>60</td>
<td>M</td>
<td>17.5</td>
<td>52</td>
<td>295</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.B.</td>
<td>61</td>
<td>F</td>
<td>17.0</td>
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<tr>
<td>E.G.</td>
<td>56</td>
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<tr>
<td>L.M.#</td>
<td>59</td>
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<td>10.5</td>
<td>254</td>
<td>213</td>
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<td>39</td>
</tr>
</tbody>
</table>

* Gluten-free diet.

# Increased LDH$_5$ isoenzyme in this patient.