A STUDY OF LIVER FUNCTION TESTS IN RABBITS

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A STUDY OF LIVER FUNCTION TESTS IN RABBITS

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GENERAL INTRODUCTION

The term "liver function test" was originated in the days when investigators envisioned discovering a single procedure which would measure accurately the status of all the functions of the liver. Over a period of about fifty years many tests have been developed, some on a sound physiological basis, others quite empirically; few have proved to be of value.

Within the last few years research in this field has followed a different trend. There has been an increasing interest in investigation into the physiological principles underlying these tests, with the result that great improvements have been made in some of the procedures, and there has been a sharper delineation of the normal ranges of many tests. Nevertheless, today the view is widely accepted that no single test is adequate to measure all hepatic functions, and the repeated performance of different tests is now advocated in the estimation of hepatic function (1).

In recent years there has also been a renewed and widespread interest in the study of liver damage in animals. Investigators have employed various criteria in estimating the condition of the liver: many have made post mortem histological examinations or liver lipid determinations; biopsies have occasionally been used; liver function tests have been

performed infrequently, and have been confined almost entirely to the dog. In view of the fact that much of the more recent work on liver damage is being performed on small animals whose use permits of better control and is more practical in many ways, it seemed that liver function tests might be performed more frequently in these animals, so that the degree of damage present might be assessed without sacrificing the animals.

The present investigation is essentially a study of liver function tests on small animals. Tests were chosen which seemed to represent a wide range of different functions, and which could be rather easily applied to small animals. Although the rabbit was used in this study, because of the ease with which repeated injections may be made and repeated blood samples taken, the analytical methods have been modified to require very small quantities of blood, so that the tests might be applicable to even smaller animals. For the purposes of the investigation it seemed desirable to produce in the test animals a slowly progressing type of liver damage, over a period of weeks. This was achieved in some of the animals, and the results of the tests were followed during both the period of damage and the recovery period.

It was found that liver function tests can be performed repeatedly on rabbits with little difficulty, and that if a combination of two or three tests is used, valuable information can be obtained concerning the state of the liver. The tests studied can be modified rather easily for use on the rat.

HISTORICAL INTRODUCTION

1. Physiology of the Liver

The liver has been most appropriately described by Cowdry (2), as "the jack-of-all-trades of the body". Situated between the heart and the great area of intestinal absorption, the liver receives a dual blood supply. A small quantity of arterial blood enters through the hepatic artery, but a much larger amount of venous blood is carried to the liver from the digestive tract, pancreas, spleen, and gall-bladder by the portal vein. The situation of the liver and the origin of the portal blood supply suggest that the liver serves as a clearing-house for foodstuffs; indeed, the liver is essential for the intermediary metabolism of carbohydrates and proteins, and plays a large part in fat metabolism. The liver performs other tasks, as well; cholesterol, bile acids, glycogen, and plasma proteins are synthesized, and many compounds are detoxified by combination with substances formed in the liver. This organ removes from the blood and excretes into the bile several physiological compounds such as bilirubin, bile salts, cholesterol, and urobilinogen, as well as certain foreign dyestuffs. Glycogen, fat, protein, and many vitamins are stored in the liver, and sufficient glycogen is broken down to maintain the normal level of glucose in the

blood. The liver is also concerned in maintaining the water content of the blood, and in the storage of a compound essential to the formation of red blood cells. Thus the liver is probably the most versatile organ in the body; not only does it serve as a storehouse and an excretory organ, but it plays a vital role in the catabolism and anabolism of a wide variety of compounds.

In spite of the wide range of the liver's activities, the hepatic parenchymal cells are all apparently exactly the same, and it has never been proved that different functions are performed by different sections of the liver lobules. Therefore it is probable that a variety of enzyme systems within each cell is responsible for the wide diversity of reactions carried out by the hepatic parenchyma. The large number of "Kupffer cells" found in the liver form part of the reticulo-endothelial system ("R.E.S."). Beyond the formation of bilirubin and the removal of many colloidal substances (e.g. injected dyes, plasma substitutes, and so forth) from the blood, it is doubtful whether the Kupffer cells play any part in the activities of the liver.

2. Liver Function Tests: Their Uses and Limitations

In general, liver function tests may be divided into two groups. One type of test involves merely the determination of some substance in the blood, such as prothrombin, bilirubin, or alkaline phosphatase, or the measurement of

urinary bile salts or urobilinogen, the level of which has been found to depend chiefly on the condition of the liver. The other group includes the "tolerance" or "clearance" tests in which a substance is administered and the rate of its excretion or removal from the blood is estimated. The latter type of test, although more difficult to perform, has generally been found to be more sensitive than the former. Delprat and Whipple (3) in 1921 proposed that the ideal liver function test should consist of the intravenous administration of some non-toxic substance which would "test by a synthetic demand the functional reserve of the liver"; the product of the synthesis should be obtainable from the blood rather than from the urine, in order to eliminate the effect of variations in renal function. Such a test would not be affected by the rate of intestinal absorption, and being independent of the bile flow, would be applicable in all cases of jaundice. (See p. 64).

It is claimed by many, however, that there is a "dissociation of the functions of the liver". Not all the functions are influenced to the same degree by stress or damage; some functions may be seriously impaired, while at the same time others appear to be undisturbed. This circumstance imposes a serious limitation upon the usefulness and interpretation of any liver function test. As Boyce and McFetridge (1) assert, "No test is universally applicable, none infallible, and none should be accepted as correct unless it is repeated

or made serially".

Another important consideration lies in the fact that the capacity of the normal liver to carry on its many functions greatly exceeds the normal needs of the organism; even a very small amount of hepatic tissue may carry on the normal functions of the liver. Furthermore, the tremendous capacity of liver tissue for regeneration (see p. 12) may compensate for the presence of mild chronic liver injury. To quote Elton (4):

> "The reliability of tests depends on the existence of diffuse involvement of the liver or of sufficiently widespread focal involvement to exhaust the reserve power of the organ. Localised lesions, completely walled off, may involve a large part of the liver without causing any functional impairment whatever, provided the remaining parenchyma is normal."

In the light of these firmly established observations, it is obvious that in many cases of non-diffuse liver damage the results of liver function tests will be normal.

Ivy (5) has drawn attention to the fact that since we do not yet know the real cause of death after hepatectomy, we have not been able to develop a test for the "vital" function or functions of the liver. It is entirely within the realm of speculation whether such a test, if found, would be extremely sensitive or whether the function would be affected only in severe liver injury.

Liver function tests are employed naturally in the hope of detecting damage to the liver when it is present. However present-day tests cover a wide range of sensitivity.

Not only is there a difference in the susceptibility of the different functions to derangement, but different modifications of the same type of test vary in sensitivity. Liver function tests have been vaguely classified into a "more sensitive" and a "less sensitive" group, and each group serves an important purpose. The "more sensitive" tests are used when it is desired to detect liver damage which may not be severe. Functional impairment is expected in diseases of the liver and biliary tract, but it has been found in many other diseases as well; frequent repetition of liver function tests allows one to follow roughly the progress of the hepatic lesion and the effectiveness of therapeutic measures. Again, when a patient is about to undergo surgical treatment, it is important to know the condition of the liver so that the degree of operational risk and the need of pre-or post-operative therapy may be anticipated. Chemotherapy frequently causes some liver damage; the early detection of functional impairment is most important in patients receiving arsenicals, salicylates, and certain other drugs. In all these cases it is necessary to use several tests; one or more of the "less sensitive" ones may furnish additional knowledge concerning the severity of the hepatic lesion.

Liver function tests find an important application in the differential diagnosis of jaundice (see p. 64). Here an extremely sensitive test is undesirable, for it would detect the slight amount of liver injury present in the early stages

of biliary obstruction. A positive result with a less sensitive test within the first few weeks after the appearance of icterus affords stronger evidence that the jaundice is not caused by obstruction to the bile outflow but is the result of damage to the hepatic parenchymal tissue.

Liver function tests will not, of course, distinguish among the different pathological conditions of the liver parenchyma; they merely detect parenchymal cell damage as such. There is, in fact, evidence that under certain conditions there may be an impairment of hepatic function in the absence of any demonstrable damage to the liver cells (6, 7).

3. Order of Presentation of Material

Our present extensive knowledge of the many functions of the liver has been gained partly through studies of liver disease in humans, but largely through experimentation with animals. By partially or entirely removing the liver, or by producing lesions through the administration of one or more of the many drugs which attack the liver, investigators have made great progress in the study of liver function both in health and in disease. A tremendous amount of work in this field has been done within the past fifty years particularly from the clinical aspect. It must suffice in this introduction, therefore, to describe what has been accomplished through animal experimentation, with only brief mention of clinical studies. It has been necessary to omit many references

concerning theoretical topics which, although very interesting, are either not directly concerned with the problem, or are still highly controversial.

Because of the close relation (both historical and theoretical) between studies on liver damage and liver function, it is impossible to discuss either of these topics adequately without reference to the other. In the present discussion, the methods used in the experimental production of hepatic insufficiency will be described first, and the similarities between the pathological conditions produced in animals and the types of liver disease found in humans will be pointed out. Then will follow a survey of the present knowledge of the many functions of the liver, with emphasis on those functions for which tests have been proposed. The section will be concluded with a description of the results of liver function tests in liver disease and in experimental liver damage, as well as the more reliable analytical methods in current use.

4. The Experimental Production of Hepatic Insufficiency

Bollman and Mann (8) of the Mayo Clinic, two of the foremost investigators in the field of liver damage and liver function, state that "it is possible to produce experimentally most of the abnormal conditions usually associated with hepatic disease in humans". Several methods have been used to produce pathological changes in the liver and to study their effects on hepatic function:

a. Partial or total removal of the liver

b. Shunting of portal blood away from the organ (e.g. "Eck fistula")

c. Obstruction to the biliary outflow

d. Administration of one or more chemical substances which poison the liver cells

e. Maintenance on a diet leading to changes in the liver such as fatty infiltration, necrosis, and cirrhosis. Whereas the first four methods have been employed by investigators for many decades, the last has been extensively developed only within the past ten years.

a. Partial and complete hepatectomy.

Studies of completely and partially hepatectomised animals have contributed greatly to our knowledge of the functions of the liver. Hepatectomy involves much more than the mere removal of the liver; it is necessary to make provision for an unimpeded flow of blood to the heart from the abdominal viscera and from the hind quarters. Markowitz (9), in his textbook on experimental surgery, describes the technique for such an operation.

The symptoms which follow hepatectomy have been described many times, and are well established (10) (11) (12). Among the early changes is a rapid fall in the blood sugar, even in depancreatised animals. The normal fasting blood sugar level may, however, be maintained in the partially hepatectomised dog by only 20 per cent

of the liver tissue, although evidence indicates that in certain other aspects hepatic insufficiency exists at this time. Mann and Magath (13) performed an interesting experiment in which the blood of a moribund hepatectomised dog, A, was allowed to circulate through the liver of a well-fed, normal dog, B. Within half an hour the blood sugar of A was restored almost to the normal level, and the animal's condition appeared to be normal. The glycogen of B's liver was depleted. Removal of the perfused liver (B) caused an immediate decrease in the blood sugar of A, and the development of the characteristic symptoms of hepatectomy. The blood sugar was not elevated when the glycogen-free liver of a fasted animal was used in a similar experiment.

Following hepatectomy, jaundice gradually develops, and bile pigments are found in the plasma and urine. There is a progressive fall in the level of blood fat. Amino-acids accumulate in blood and tissues, and leucine and tyrosine, amino-acids of very low solubility, crystallize out in the urine. Urea formation stops, and if urea excretion continues through the kidneys, the blood and tissues become almost free of urea. The destruction of uric acid ceases, and the compound accumulates in the blood, tissues, and urine; if injected intravenously into a hepatectomised animal, uric acid may be recovered almost quantitatively in the urine. Because of a lack of prothrombin and fibrinogen, the liverless animals show a very pronounced tendency to bleed from

abraded and traumatised surfaces, and at autopsy bloody fluid is always found in the abdomen.

Death follows within a few hours after hepatectomy, but administration of sufficient glucose to maintain the normal blood sugar will prolong life temporarily. After 24 to 48 hours, however, a number of characteristic symptoms appear, including loss of hearing, vision, and the normal reflexes, development of muscular rigidity, coma and convulsions, followed by death. Even when substitutional therapy is undertaken through glucose injections, blood transfusions, and administration of bile salts and substances normally stored in the liver, it is impossible to keep hepatectomised animals alive. The real cause of death in these animals has never been determined.

Markowitz (9) likens the completely hepatectomised animal to a patient with yellow fever or acute yellow atrophy; most of the pathologic liver conditions, such as portal cirrhosis and prolonged complete obstruction of the common bile duct, represent chronic hepatic insufficiency.

When an animal is subjected to repeated partial hepatectomy, within six to eight weeks the normal weight of the liver is restored through hyperplasia and enlargement of the remaining lobes. Intense and rapid multiplication of hepatic cells by mitosis, and formation of hepatic cells from proliferating bile ducts, assist in maintaining a roughly normal histological "architecture". The unextirpated part

of the liver responds as rapidly and completely to successive partial hepatectomies as to the first one. Mann (14) states that "many of the changes noted in patholcgical conditions of the liver, and the persistence of functioning hepatic tissue in opposition to exceedingly adverse conditions, are due to the almost irresistible urge of the hepatic tissue to be restored."

That the cause of the hepatic restoration is evidently the relatively increased blood supply, is indicated by two facts; first, that tying branches of the portal vein results in a complete atrophy of the corresponding parenchyma, with an increase in the size of the remaining organ, and second, that regeneration after partial hepatectomy is prevented or greatly reduced when the portal blood supply to the liver is reduced, as in an Eck fistula (see p. 14). Other causes of poor regeneration of liver tissue are the presence of jaundice, extensive cicatrisation of the liver, and the continued administration of toxic agents (15) (16). Thus, if proper dietary precautions are taken, one may maintain an animal with less than twenty per cent of the normal amount of hepatic tissue. Fatal liver insufficiency develops in rabbits, however, when they are deprived of ninety per cent of the liver (17). Regeneration of removed liver tissue is much more rapid on a high carbohydrate diet than on a mixed diet, and is depressed by a high proportion of fat in the blood.

b. Eck fistula

The "Eck fistula" diverts the flow of portal blood into the vena cava, rather than through the liver; the hepatic blood supply becomes entirely arterial, and has been estimated to be 25 to 35 per cent of normal (19). Within a few weeks the size of the organ is decreased by one-half. There is no significant fibrosis, and the cells appear to be normal, except for the presence of a few fat droplets.

Such livers show few indications of hepatic insufficiency; phenol conjugation, however, is reduced (20), there may be a slight diminution in bile salt production, and in the anaemic animal there is a decreased utilization of standard diet factors and iron for the production of haemoglobin and plasma proteins (19). Instead of an animal with an Eck fistula, Mann (21) prefers for studies of hepatic insufficiency an animal in which the fistula is employed to prevent regeneration after partial hepatectomy. In such an animal with 70 per cent of the liver removed, there is a gradual loss of weight, strength, and appetite, with resulting emaciation. Although jaundice does not occur, uric acid appears in the blood and increases in amount as the condition becomes progressively worse. Occasionally, in an animal, the general condition improves and uric acid disappears from the blood. In these cases, necropsy reveals that a certain amount of regeneration of the remaining liver tissue has taken place.

c. Obstruction to the biliary outflow

A condition resembling complete biliary obstruction in humans has been produced in dogs by ligation of the common bile duct. Because of distention of the gall bladder with bile, icterus is delayed for several days, but if the gall-bladder is removed, bilirubinemia and bilirubinuria promptly develop. Although there is a decreased production of bile salts, there is little interference with other functions, and the hepatic parenchyma is not greatly injured unless obstruction is maintained for several months (21) (22). After about three months of complete obstruction of the biliary outflow, the survival time of dogs largely depends upon the nature of the diet. The low level of plasma proteins is scarcely sufficient to prevent oedema, and the administration of an unidentified substance present in meat extract will precipitate a condition of ascites, followed by death within a week. If the original meat diet is withdrawn before death, the ascites disappears within a few days (22), and the animal will live several months on a diet of milk and syrup. Dogs with complete biliary obstruction gradually decline, however; they develop abdominal and oesophageal varices, have frequent haemorrhages into the gastro-intestinal tract, and manifest ascites spontaneously in later periods of obstructive jaundice. Such animals usually fail to survive more than a year, in spite of careful dietary treatment. Mann (21) emphasizes that after biliary obstruction

of several months' duration, when the hepatic parenchyma shows damage, the effect of obstruction itself on the other body tissues is so great that to interpret the results of functional studies in terms of hepatic injury, is hazardous.

Studies of animals with biliary fistulae, however, have shown that if adequate care and a suitable diet are maintained, complete loss of bile is compatible with life indefinitely (22).

d. Drugs that produce liver damage. i. General considerations.

The damage caused to the cells of the liver by hepatotoxic drugs is transitory; when administration of the toxic agent ceases, regeneration takes place, even after severe injury, to such a degree that the liver may appear normal (8) (21) (23). To produce a condition of chronic liver insufficiency similar to portal cirrhosis or subacute yellow atrophy in man, it is necessary to administer a hepatotoxin repeatedly, in doses large enough to cause acute necrosis, and frequently enough to prevent adequate regeneration after each dose. Eventually such treatment results in extensive cicatrisation which mechanically hinders restoration of healthy liver tissue. Jaundice and a reduced blood supply to the liver, both of which prevent regeneration, are also usually present in cirrhosis.

Crandall and Ivy (18) have criticised the use of toxic agents for the production and study of liver insufficiency,

on the grounds that the damage produced is not uniform, and that regeneration is prompt. These workers recommend instead an Eck fistula, or the obstruction of biliary outflow.

Hepatic poisons rarely produce complete hepatic insufficiency (24). Other organs are undoubtedly affected, and in many instances when death occurs it is not caused primarily by functional damage to the liver. Bollman and Mann (8) report that many of their animals died from haemorrhage (presumably from prothrombin deficiency, although this was not realized at the time); other animals developed chronic or subacute duodenal ulcers, with subsequent perforation and peritonitis. In this connection Crandall and Ivy (18) state,

> "It is perhaps significant that every procedure which has been successful in the production of chronic experimental ulcer either involves outright liver damage, or can be suspected of bringing functional liver changes in its wake."

Because of the wide variety of drugs which cause damage to the liver, and because many of these drugs have either been used frequently in the experimental production of hepatic insufficiency or are commonly employed therapeutically, a rather detailed discussion of these hepatotoxins is warranted.

ii. Liver glycogen

There is much evidence to show that one of the most important factors affecting the resistance of the liver to damage is the level of glycogen in the hepatic parenchyma

(8) (14) (25).

A rapid depletion of liver glycogen such as occurs during fasting has been shown to render the liver much more susceptible to injury by hepatotoxic drugs (3) (8). Graham (26) found that the unusual resistance shown by pups against liver damage by chloroform was presumably the result of the high glycogen level in young dogs, and could be lowered considerably by starvation.

The length of the fasting period required for this depletion depends somewhat on the size of the animal. In rats, mice, and small rabbits, the liver glycogen level may be reduced to less than one per cent in eighteen to twenty-four hours (27) (28) (29) (30) (31) (32) (33) (34) (35); larger rabbits (2 kg. or more) retain their glycogen for a period of twenty-four to forty-eight hours (36) (37). The liver glycogen in dogs, on the other hand, usually is not depleted before the third or fourth day (38) (39). In rats and rabbits, glycogen synthesis begins again on the second or third day, but the level remains very low throughout the fasting period (35) (40) (41) (42).

The level of liver glycogen in different animals, however, is not simply a function of the length of the fast. Many workers have observed that in rabbits and rats marked variations in the liver glycogen content occur even when every precaution is taken to feed and maintain all the animals in a uniform manner (42) (43). Even fasted animals show

variation in their liver glycogen content. Evans and co-workers (44) found that the liver glycogen in cats fasted 46 hours might vary from 2.5 to less than one per cent, and a similar variation was noted by Donhoffer and MacLeod (37) in rabbits fasted for 16 hours. Cori (29) has reported that although rabbits usually have less than one per cent of liver glycogen after a forty-eight hour fast, the level in some animals may be as high as two to three per cent.

Aside from the individual variability in carbohydrate metabolism in different animals, there is considerable evidence that external conditions may contribute to the variations in liver glycogen in normal animals. Thus, it has been observed that liver glycogen is higher in winter than in summer (36) (45). Althausen and Stockholm (46) claim that in spring this level tends to be still higher than in winter. Evans et al (44), too, have suggested that seasonal variations may be of importance. Meyer (41) observed considerable variations in the liver glycogen of different groups of rats, and attributed them to differences in season and external conditions. Barbour and co-workers (42) have reported that on certain days the majority of liver glycogen values in rats deviated considerably from the mean, and were sometimes very low. Meyer (41) found seasonal variations in the rate of fructose absorption in rats. Deuel (47) discovered that male rats had a higher liver glycogen content than female rats, while in guinea pigs this difference was not observed.

It is apparent, then, that the liver glycogen level is affected by a number of known factors, many of which are difficult to control, and doubtless by some as yet undiscovered factors. Not only does the presence of glycogen in the liver have a protective action against hepatotoxins, but the glycogen level can be depleted by many substances which cause hepatic damage.

It has been known for nearly one hundred years that chloroform steadily reduces the concentration of liver glycogen (26) (48), and causes a rise in the blood sugar. During the first two hours of anesthesia one-third of the glycogen is lost; at the same time the fatty acid concentration of the liver is increased. Evans and co-workers (44) found that ether causes a loss of fifty per cent of liver glycogen in one hour; Lauber and Bersin (49) report that vitamin B_1 (thiamine), if given three days in advance, checked the loss of liver glycogen with resulting hyperglycemia and glycosuria, although the vitamin was ineffective if given immediately before the anesthetic. Newburger and Brown (50) found that the amount of glycogen lost as a result of ether anesthesia, or starvation, was not affected by previous diet. Amytal slowly reduces the liver glycogen level (44). Hafkesbring et al (51) have reported that sodium sulfathiazole has no effect on the blood sugar or liver glycogen. After single or repeated doses of sulfapyridine, however, the blood sugar remains high and the liver glycogen

low for twenty-four hours or longer after the drug is discontinued.

The liver glycogen content is markedly reduced by biliary obstruction (52) (53) as well as by liver damage. Ravdin (53) found the liver glycogen level to be only about one per cent in dogs suffering from obstruction of the common bile duct for two to six weeks, as compared with the normal value of about five per cent. In a case of chronic post-operative hypoglycemia in a child, autopsy showed a liver free of glycogen but with extensive fatty infiltration (54). The decrease in liver glycogen in the damaged liver may be due to a reduced capacity for the formation of glycogen from glucose, insufficient gluconeogenesis, excessive glycogenolysis, or a combination of these factors (46).

Hyperthyroidism lowers the level of liver glycogen (48) and Buell and Strauss (55) have found that the conversion of lactic acid to glycogen is impaired in hyperthyroid individuals. The data of McIver (27), however, do not support the view that the increased susceptibility to chloroform poisoning in the hyperthyroid rat is chiefly due to the low level of liver glycogen found in these animals.

As long ago as 1908 Wells (56) and Beddard (57) advocated glucose administration in the treatment and prevention of chloroform poisoning, and since then glucose has been found to be of value in every type of liver disease (58). It decreases the post-operative drop in liver function, and

is of definite value following surgical procedures in animals with extensively injured livers (59). A semi-hepatectomised dog must be maintained on glucose injections for several days following operation; animals with Eck fistulae or with obstructive jaundice are benefited by the addition of large amounts of glucose to the diet (58). It should be noted here that Best and Huntsman (60) found moderately fatty livers in rats after the administration of sucrose alone; choline was necessary to prevent accumulation of the fat.

Mann, in his many studies, found that the impaired liver functions best on a high carbohydrate diet, with the addition of simple foods to provide the protein necessary to maintain body requirements and to synthesize some of the special nitrogen containing compounds required by the body (14).

Although many observations indicate the great importance of glycogen in the normal metabolic functions of the liver (61), there are few data to elucidate the role of the polysaccharide. Doljanski's work (62) showed that in the embryonic chick liver glycogen disappears in tissues undergoing active proliferation and appears in tissues in which proliferation is hindered. Forsgren (63) found that the liver glycogen was low when there were large amounts of bile in the parenchymal cells, and vice versa. From these results one might infer that glycogen is used up in the process of tissue proliferation and bile formation, although

the experiments did not establish that this was so.

Gassman (64) apparently considers this to be the case, for in answering the question as to whether the decreased glycogen causes liver cell damage or is the result of such damage, he concludes that the process becomes a vicious circle, in which liver damage reduces gluconeogenesis and glycogenesis, so that the supply of liver glycogen, needed for regeneration, is not adequate. Administration of glucose is beneficial because it interrupts this circle. Rosenfeld (65) has postulated that in chloroform poisoning, after the glycogen is used up, the liver cells are starved because their metabolism is so altered by the poisoning that proteins, and especially fats, are imperfectly used. Under these conditions glucose administration tends to obviate the need for fat transport and in this way prevents the cells from dying from acute inanition. Ravdin et al (66), however, found a simultaneous increase in glycogen and liver fat during the recovery period following chloroform anesthesia, which was not in accord with Rosenfeld's hypothesis of a reciprocal relationship between these two substances.

Davis, Hall, and Whipple (67) studied the sudden rise in urinary nitrogen caused by chloroform poisoning. More nitrogen was eliminated in fasted animals than in sugar-fed ones, and if fasting was continued, the curve of excretion often remained well above the ordinary fasting level. Administration of glucose caused a rapid drop in the nitrogen

excretion. Repair was much more rapid and complete on a high carbohydrate diet than during starvation, and Davis and Whipple (68) conclude that this indicates a true conservation of protein split products in the liver, and that liver glycogen spares protein by taking an active part in the reconstruction of new protein substance.

In a recent review Ivy (5) suggests a number of possible roles for liver glycogen. He proposes that it may prevent the accumulation of fat in the liver, spare the protein, and reduce the tendency to acidosis by preventing formation of acetone bodies. Glucose (and therefore glycogen upon breaking down) may place at rest or facilitate other functional processes such as detoxification. It has been shown that glucose decreases cholic acid synthesis, although, as Ivy points out, this synthesis is so much reduced in the damaged liver that it would not be a strain upon the synthetic capacity of the organ.

iii. Arsenicals

The widespread use of arsphenamine and neoarsphenamine in the treatment of syphilis has resulted so frequently in damage to the liver (69) (70) (71) (72) (73) (74) (75) (76) that publications on the so-called "post-arsphenamine jaundice" have appeared repeatedly in the clinical literature of the past few years (77) (78) (79). The typical liver lesion is a mild form of acute hepatic parenchymal degeneration (77) resembling that found in epidemic hepatitis (80), although

in some cases damage is so severe that death results (81) (71). Occasionally jaundice results from intrahepatic biliary obstruction caused by concentration of the bile within the bile canaliculi (82) (83). Residual damage has been demonstrated by the bilirubin excretion test in many cases following recovery from postarsphenamine jaundice (84) (85).

In animals the arsenicals are capable likewise of producing extensive necrosis of the liver, with fatty degeneration (86) (87) (88) (89). Clinical evidence indicates that neoarsphenamine is more toxic than arsphenamine (90). However, Kolmer and Lucke (91) found that the tissue changes produced in rabbits by neoarsphenamine were less severe than those following arsphenamine. Liver lesions from administration of these drugs have seldom been observed in rats (92) (93); in these animals, the liver appears to be rather resistant to the action of arsenic. Renal changes have been observed after large doses of neoarsphenamine (88) (92) (94), and arsphenamine (91).

Messinger and Hawkins (89) studied the influence of diet on the liver injury produced in dogs by arsphenamine. Carbohydrate or protein diets were highly protective, but when the diet was high in fats, there resulted marked progressive jaundice from severe and often fatal liver injury. It was noted that some hepatic damage occurred in all the dogs, regardless of the diet.

McJunkin (95) found that therapeutic doses of arsphenamine did not increase the liver injury caused by acute or

chronic chloroform poisoning in rabbits.

VonGlahn et al (96) have reported that lead, copper, and sodium arsenates cause necrosis of the liver in rabbits but are less toxic to the rat and ferret. Scott (97) has described a case of jaundice apparently caused by ingestion of fruits and vegetables sprayed with an insecticide containing an inorganic compound.

In this investigation, neoarsphenamine was one of the drugs employed in the production of liver damage in rabbits (see p.).

iv. Anaesthesia and hypoxia

Almost every anaesthetic known, especially the inhalant anaesthetics, seems to exhibit a depressing effect upon the liver. Although Bourne (98) excepts cyclopropane, Boyce (59) claims that operation under any anaesthetic causes a fall in liver function. Ether (98) (99), divinyl ether (100), vinethene (44) (142), propethylene, nitrous oxide and ethylene (99) (101), morphine, avertin (101) and the barbiturates (102) have been studied by many workers and all have been incriminated from this standpoint. The effects of the most noxious of all anaesthetics, chloroform, and the many related chlorine compounds have been so extensively investigated that a separate discussion will be devoted to this subject.

Liver function during and after anaesthesia has been studied in both humans and animals (99) (101). The fact that

prolonged retention of bromsulfalein in the blood was always found before any abnormalities in bile pigment metabolism were noted, might be construed to indicate that the R:E cells are injured by anaesthesia before the parenchymal cells of the liver are affected.

As is true in the case of other toxic agents, damage from anaesthetics is lessened by a high level of glycogen in the liver. While the liability of liver injury is, in general, proportional to the duration of anaesthesia and the age of the patient (101), the most important factor in determining the amount of liver damage produced is probably the concentration of oxygen with which the anaesthetic is administered (99) (103). Goldschmidt et al (100) found that the protective action of oxygen against necrosis from chloroform and from divinyl ether compared favourably with that of a high carbohydrate diet prior to the period of anaesthesia. It was shown by Ravdin et al (66) that volatilisation of chloroform with oxygen led to a more rapid restoration of the normal liver glycogen concentration following the anaesthesia.

The protection afforded by oxygen against liver damage caused by anaesthesia is probably related to the fact that the hepatic parenchyma is easily damaged by a low concentration of oxygen in the inspired air (100) (103). Martin et al (104) kept rabbits in an atmosphere of low oxygen concentration for a week. At the end of this period, liver,

kidneys, and heart were found to be damaged; the liver cells most remote from the blood supply showed the most pronounced lesions. Ravdin and co-workers (66) showed that hypoxia causes a rise in blood sugar and a fall in liver glycogen.

Rich and Resnick (105) report that in pernicious anaemia, as well as in experimentally produced anaemia, the liver cells about the efferent veins of each lobule were damaged; it is suggested that this might be the result of a lack of oxygen.

The data of Holman (106) indicate that repeated bleeding causes cirrhosis of the liver in dogs fed a high fat diet, whereas neither diet nor repeated bleedings alone produced such changes in the liver. Holman suggests that the lesions might be due to hypoxia, to the relatively high lipemia, or to the loss of some substance necessary for the continued integrity of the liver. Impairment of the circulation in the liver increases the toxicity of the damaging agents (8). The name "cardiac cirrhosis" has been given to the condition resulting from the prolonged failure of the circulatory system in humans (107). Engel and colleagues (108) suggest that the rise of blood amino nitrogen in shock is caused by an accumulation of the products of protein breakdown, because of liver damage resulting from continued hypoxia.

In the light of the above observations, the findings of Wakim and Mann (109) are of interest. In a study of the

intrahepatic circulation of blood in the intact animal during the inhalation of carbontetrachloride, it was found that inhalation of the drug caused an immediate vasoconstrictor effect; when inhalation was continued for half an hour, pathological changes were produced which became apparent in four to eight hours and maximal in twenty-four hours. Hepatic enlargement with fatty degeneration, hydropic changes, and haemorrhages in the liver were noted. Recovery was complete in two to four weeks. The vascular changes during the development of cirrhosis of the liver following repeated inhalations of the drug were studied in a similar manner.

Doljanski and Rosin (110) administered urethane to rats, and although the evidence obtained was insufficient to exclude a direct action of urethane on the liver cells, it was strongly suggestive that the parenchymal lesions were caused by vascular injury.

Thus the available evidence shows strongly the deleterious effects of a lack of oxygen upon the liver cells, whether the results of poor circulation, anemia, or a low concentration of oxygen in the inspired air. On the other hand, it is well known that the breathing of pure oxygen for several hours is dangerously toxic. Hawkins (111) has observed that the clotting of the blood is greatly accelerated in dogs and humans after breathing almost pure oxygen; the actual cause of this acceleration is unknown.

v. Alcohol

The frequency with which cirrhosis occurs in chronic alcoholics (112) has led to extensive studies on the effects of alcohol on the liver. The results of these studies are conflicting and contradictory.

MacNider (115) in a study of kidney function in dogs using the phenolsulphonphthalein test, administered 10 cc. of forty per cent ethanol per kilogram of body weight daily to his animals for more than six months. The drug was found to be only slightly nephrotoxic (114); some of the animals, however, showed an increased elimination of dye in the urine, and this increase was found only in those animals in which liver injury was demonstrable (115). The increased elimination was attributed either to a decreased capacity of the liver to destroy the dye, or to a decreased excretion of the dye by the liver into the intestine. When alcohol was withheld for forty-eight to seventy-two hours, the elimination of the dye in the urine was reduced.

A further study of the effects of ethanol on the liver was then made. After a fast of two days, dogs were given 10 to 15 cc. of forty per cent alcohol per kilogram by stomach tube. Such animals were intoxicated for twenty-four to forty-eight hours, during which time functional and histological studies were carried out. There was noted both increased elimination of phenolsulphonphthalein by the kidneys, and abnormal retention of phenoltetrachlorphthalein in

the blood. The liver cells developed severe oedema, and other pathological changes were noted. After an intoxication of twelve hours, recovery was almost complete within three days; twenty-four hours' intoxication required twelve days for functional and histological recovery. During the intoxication, biochemical studies showed a decrease in the reserve alkali of the blood; with histological and functional improvement, the acid-base equilibrium returned to normal (117).

Rosenthal (118) using doses only half as large as those of MacNider, studied liver function in dogs before and after the administration of ethanol. Although there was no increase in urinary urobilinogen or blood bilirubin in two to six hours after alcohol was given, delayed removal of bromsulfalein from the blood, returning to normal within twenty-four hours, was demonstrable. A similar observation was made in humans by Cates (7) (119), who found that some alcoholics, after prolonged and continuous drinking, showed abnormal retention of bromsulfalein. Cates has attributed this retention to a physiological deficiency of the R.E. cells, rather than the parenchymal cells of the liver. Biopsy made in two cases showed no pathological disturbance in one alcoholic; in the second there was evidence of mild toxic hepatitis, with fatty infiltration. Beazell (120) was not able to demonstrate functional impairment of the liver in ten humans during acute alcoholic intoxication, even

though several tests, including the bromsulfalein test, were used. Four out of nine dogs, however, showed abnormal retention of the dye during intoxication. That the results of other tests were normal would lend support to Cates' view that the R.E. cells, rather than the parenchymal cells, were damaged.

Lillie et al (121) produced dietary cirrhosis in rats, and found some evidence that the pathological changes were more severe when about twenty per cent alcohol was substituted for drinking water.

Hanzlik (122) fed pigeons two to four per cent alcohol for thirteen to twenty-four months; in spite of the fact that the birds were frequently intoxicated, no pathological changes were noted in the heart, kidney, or liver. Machella and Higgins (123) found no evidence of liver damage in rats, except for an occasional fat droplet at the periphery of the lobules, after administering 6 cc. of twenty per cent alcohol daily for four weeks.

Bollman (124) reports that no gross or microscopic hepatic abnormalities were observed in dogs intoxicated with alcohol twice daily for more than two years, provided the animals were maintained on a well-balanced diet. Alcohol when given to a fasted animal, or to one fed a high fat diet, produced a fatty liver much more rapidly than could be produced by a high fat diet alone. Connor and Chaikoff (125) produced fatty livers in dogs by feeding them a high fat

diet for a month. Then four to seven day periods on a meat and alcohol diet were alternated with equal periods on a high fat diet. This regime resulted in excessively fatty livers, resembling those found in chronic severe alcoholism in man. In addition, four of the sixteen dogs showed cirrhosis, moderate in degree, but resembling in every way that found in early fatty cirrhosis of the liver in man.

Joliffe and Jelinek (112) in a review of the subject of liver cirrhosis in alcoholism, point out that fatty livers are extremely common in chronic alcoholics, and consider the investigations of Connor and Chaikoff to be one of the most important advances in the knowledge of the pathogenesis of cirrhosis of the liver in chronic alcoholism. Gyorgy and Goldblatt, on the other hand, feel that it is not necessary to assume a specific injurious effect for alcohol, since the diet of alcoholics is usually one deficient in B vitamins and protein, similar to the type of diet frequently used to produce cirrhosis in rats.

Alcohol has been shown to increase strikingly the toxicity of chloroform and carbon tetrachloride (118) (124) (127) (128); Jetter and McLean (129) report that phenobarbital and ethanol exhibit a synergistic poisonous effect. That the metabolism of alcohol is modified by the presence of liver damage has been shown by several workers (130) (131) (132). The last cited workers found, in studies on excised tissues, that the liver is the only tissue able to

oxidise significant amounts of alcohol. Bollman reports that in the presence of fatty liver in dogs, intoxication may be produced with one-half the usual amount of alcohol (124).

Ethanol, like many other hepatotoxic drugs, caused acidosis, depletion of liver glycogen (133) (134) and a rise in blood sugar in fed animals; there is also a rise in the blood vitamin A content, presumably through a decrease in the liver stores of the vitamin (135).

Although more work is necessary for the elucidation of the role of alcohol in the production of chronic liver injury, it is apparent that under certain conditions alcohol can cause damage to the hepatic parenchyma, and that the drug must be classed among those which are harmful to the liver.

vi. Halogenated compounds

The toxic action of chloroform upon the liver cells has been recognised for many decades, and this drug has been employed frequently in the experimental production of liver damage. Chloroform poisoning causes a central hyaline necrosis of the liver, with fatty degeneration which may be slight or extreme. The blood supply of the lobule has been found to bear no relation to the character and distribution of the liver necrosis (23). DeZalka (136) has made detailed histological studies of the livers of rabbits after subcutaneous injections of chloroform; Iwata et al (137)

report the production of experimental cirrhosis of the liver in rabbits by repeated subcutaneous injections of chloroform mixed with paraffin oil. Wells (56), in 1908, noting that chloroform seemed especially toxic to livers which had undergone fatty degeneration, suggested that this might result from a solvent action on the part of the fat, increasing the concentration and the duration of action of the anaesthetic upon the degenerated liver cells.

Davis and Whipple (138) have reported that they can produce a "unit chloroform necrosis" in dogs by measured periods of anaesthesia, but Williamson and Mann (24) found a wide difference in the reaction of animals to hepatotoxins, and were unable to produce a definite result by giving a definite dosage.

Carbon tetrachloride acts similarly to chloroform, in producing a central necrosis of the liver, (128) (139) (140) (141) (143) (144), regardless of the method of administration. Repeated doses of the drug over a period of months result in a condition comparable to cirrhosis of the liver as found in man (15) (16) (128) (139) (145) (146) (147) (148). Edwards (148) also found a high percentage of hepatomas in mice receiving such treatment.

Histological evidence of liver necrosis from chloroform or carbon tetrachloride is not seen until six to ten hours after the damaging treatment (23) (136); maximal damage to

the liver cells is not observed until twenty-four hours after administration of the drugs (136). Mantinengo (147) has observed a progressive decrease in the size of the liver cell nuclei during chloroform narcosis.

Beattie et al (148) (149) have studied the sulphur balance in a man with acute carbon tetrachloride poisoning and have suggested that the real cause of the liver disturbance was an abnormal metabolism of methionine and related compounds.

Several other organic chlorine compounds are powerful hepatotoxins. Dichloromethane is less poisonous than either chloroform or carbon tetrachloride (150); tetrachlorethane has been labelled the most toxic of the chlorinated hydrocarbons (22) (151) (152). Abreu and colleagues (153), in a study of saturated and unsaturated halogenated hydrocarbons, found that several of these compounds containing either bromine or chlorine caused significant hepatic injury in rats and mice. Chlorinated naphthalenes produced in rabbits and guinea pigs lesions resembling those of the early stages of acute yellow atrophy of the liver (154). Studies of the pharmacological action of DDT, a highly chlorinated insecticide, have revealed that it is capable of producing liver damage as well as neurological symptoms in animals (155) (156). The literature contains numerous references to accidental poisoning by methyl chloride used as a commercial refrigerant (157) (158).

High fat and low protein diets cause an increased susceptibility in animals to the effects of chloroform or carbon tetrachloride; on high carbohydrate diets, liver necrosis is least, and regeneration after injury most marked (159) (160). High protein diets are also protective against the hepatotoxins; the degree of protection afforded is indicated by recent studies of Miller and Whipple (161), who found that twelve minutes' anaesthesia in a protein-depleted dog caused severe liver injury, whereas normal dogs tolerated ninety minutes of anaesthesia with little hepatic injury. Methionine seems to be the component of proteins responsible for the protective effect (162), and has been used successfully in the treatment of acute carbon tetrachloride poisoning in man (148).

Because of the widespread use of carbon tetrachloride in the treatment of hookworm, extensive studies of the toxicity of the drug were made in animals (127) (128) (163) (145). It was observed that the oral administration of very large doses of carbon tetrachloride produced no outward signs of intoxication in dogs on a well-balanced diet, although liver function tests showed the presence of hepatic damage. On the other hand, dogs receiving a diet low in calcium died in coma or tetanic convulsions within two to three days after ingestion of the drug; repeated intravenous injections of calcium salts before death were highly beneficial in relieving the symptoms of intoxication (164) (165) (166).

Function tests indicated, however, that the liver disturbance in the calcium-deficient dogs was about the same as in the dogs on a well-balanced diet, and it was found that even in dogs protected against the acute symptoms of carbon tetrachloride intoxication, repeated administration of the drug caused typical cirrhosis of the liver. An intoxication very similar to that of carbon tetrachloride poisoning was produced in dogs by the subcutaneous administration of a lethal dose of guanidine-hydrochloride (165). Because of the gradual rise in the level of blood guanidine after carbon tetrachloride is given, and because the increased toxicity of carbon tetrachloride when given with alcohol is accompanied by a much higher and more rapid rise in blood guanidine, Minot and Cutler (165) postulated that there was a causal relationship between the retained guanidine and the symptoms noted. Calcium was found to be highly beneficial in guanidine poisoning.

Wokes (167) studied the effect of calcium on carbon tetrachloride toxicity in mice, and found no significant difference in susceptibility when the calcium content of the diet was varied from 0.2 to 2.0 per cent of calcium. Oral administration of large doses of calcium lactate produced a decrease in susceptibility to the drug but did not provide complete protection.

Cantarow et al (168) have recently studied the problem in cats, making extensive functional and histological studies

of the livers of the animals used. The group of cats receiving intravenous injections of calcium gluconogalactogluconate following carbon tetrachloride ingestion manifested a greatly increased tolerance to the drug; hepatic functional impairment was less marked, and the mortality rate was lower than in untreated animals. Histological studies of the liver showed that in the group receiving calcium, there was consistently less hepatic cell disintegration, and a much better preservation of the hepatic cell cord arrangement. These results are of interest in view of the fact that Minot had found that calcium administration relieved the symptoms of carbon tetrachloride intoxication, but did not lessen the degree of liver damage (164).

A liver preparation of Forbes et al (169) (170) when given to animals prior to administration of chloroform or carbon tetrachloride, was found to provide protection against the damaging effects of the drugs; the preparation also accelerated the healing of liver tissue after damage; the liver fat of the treated animals was invariably less than that of the controls (171). The active material in the liver preparation was isolated in crystalline form and found to be identical with the sodium salt of xanthine (172) (173). Other purine compounds had a similar protective action, but purine oxidation products, allantoin and alloxan, increased the liver damage caused by the chlorine-containing drugs.

Vars et al (174) confirmed the protective action of a suspension of sodium xanthine against liver injury, but found that subcutaneous injections of a solution of sodium ricinoleate conferred a similar protection, and injections of colloidal carbon were partially effective. The property common to these three substances is the ability to produce an inflammatory reaction in the body. Vars et al suggest that they act by producing "split-products" for theuse of the liver, a theory first suggested by Daft and colleagues (175), and conforming with the findings that high protein diets are very protective against chloroform injury to the liver.

A similar mechanism might explain the protective action of a cholesterol ester colloid ("liposol") when injected intraperitoneally previous to carbon tetrachloride administration in rats (176).

MacNider (177) has reported the presence in the livers of fifteen out of twenty-one senile dogs, of an atypical flattened type of cell with large, deeply staining nuclei, replacing the normal polyhedral-shaped cells of the parenchyma. Although animals with such cells in their liver showed retention of the dye phenoltetrachlorphthalein (see p.81), they showed also a remarkable resistance to liver injury from alcohol or chloroform.

When uranium nitrate was given to normal young dogs, diffuse liver injury resulted (178). Four weeks later, dye

excretion tests yielded normal results in some animals, but in others there was abnormal retention of the dye. The livers of these animals showed the abnormal, flattened type of cell previously found only in senile dogs, and exhibited the same resistance to a second poisoning with uranium nitrate or with chloroform. Cantarow et al (168) found that repair following carbon tetrachloride intoxication in cats resulted in an atypical flattened type of epithelium, identical with that described by MacNider.

The sulfonamide drugs have been found to protect the livers of animals from the toxic effects of carbon tetrachloride; the mechanism of this phenomenon is at present not known (179) (180).

Graham (150) has proposed a theory that the toxicity of carbon tetrachloride, chloroform, and dichloromethane, lies in the release of hydrochloric acid in the body when these compounds are metabolised. He claims that hydrochloric acid in suitable concentrations produces a condition of necrosis, fatty changes, haemorrhages, and oedema, similar to delayed chloroform poisoning, and that alkali given in suitable concentrations simultaneously with chloroform markedly inhibits the production of liver lesions. Iodoform, bromoform, and the halogenated substitution products of ethane all yield halogen acids on breaking down in the body, and all produce similar hepatic lesions. On the other hand, chloral hydrate, which does not cause liver damage, is

excreted as trichloroethylglucuronic acid.

Acidosis is frequently found in cases of liver disease (57) (117) but whether it is the cause or the effect has never been determined. Only a few odd observations are found concerning this subject. Stehle and Bourne (181) have shown that ether anaesthesia causes acidosis, which they attribute in large part to the liberation of phosphoric acid from organic phosphate compounds in muscle. Atabrine, when given to rats, caused a severe irritation of the gastro-intestinal tract resulting in a flow of fluid into the stomach and intestines, followed by diarrhea. The animals became severely dehydrated, and a marked acidosis was produced. Atabrine administration also produced liver necrosis (182). MacNider (117) found a disturbance in the acid-base equilibrium of the blood in dogs intoxicated with alcohol, in which severe liver damage resulted. Acute liver injury in dogs receiving arsphenamine was accompanied by acidosis with very high levels of lactic acid in the blood (87). An acid-producing diet has been shown to be associated with lowered levels of liver glycogen in animals (183), which would in turn make the liver more vulnerable to damage, or perhaps even cause injury to the liver.

Graham's statement that the simultaneous administration of alkali largely inhibits the liver lesions produced by chloroform, has been challenged, however, by Davis and Whipple (184). In their experiments neither sodium carbonate in

hypertonic salt solution, nor phosphate solutions high in buffer content, were protective against the hepatotoxic effects of chloroform when given intravenously during the anaesthesia.

vii. Sulfonamides

The sulfonamide drugs, valuable in chemotherapy, have manifested varying degrees of toxicity, leading at times even to death with kidney and liver lesions (185). In less extreme cases, hyperbilirubinemia (186), lowered prothrombin levels (187) (188), and positive colloidal gold tests have been observed (189) (190).

The various sulfonamide drugs differ in their toxicity. Sulfanilamide has been reported to have caused toxic hepatitis (191) (192) (193) (194), jaundice (195), fat necrosis (196), and acute yellow atrophy (197). The drug increases the destruction of red blood cells and the formation of urobilinogen (192) (195) (196) (198) (199), and occasionally causes damage to the kidneys (196) (200).

Although Johannsen (201) found large doses of sulfapyridine to be relatively non-toxic to mice, Watson (195) has reported a case of jaundice, and Singh (202) a death following sulfapyridine therapy. In the latter case degeneration and necrosis of the liver cells were the only abnormalities found.

Sulfaguanidine, when fed for two months to rats in an amount equal to one per cent of the diet, was fatal and caused

extensive lesions throughout the body, including degeneration and necrosis of the liver cells (216). Addition of folic acid and biotin to the diet reduced the mortality from ninety to fourteen per cent, and the liver and splenic lesions tended to heal. Black et al (203) found that the addition of sulfaguanidine to a synthetic diet reduced both the growth rate and the blood prothrombin concentration in rats. Liver extract or p-aminobenzoic acid counteracted both effects; vitamin K was active only against the hypoprothrombinemia. The hypothesis that sulfaguanidine inhibits the bacterial synthesis of nutritional factors in the intestine was only partially satisfactory; a toxic action of the drug on the body tissues was also indicated.

The chief toxic action of sulfathiazole appears to be a slight impairment of renal function, caused by a physical blocking of the renal tubules by crystals of the acetylated compound formed by protective synthesis (204) (205) (206). Injury to the liver and spleen from sulfathiazole also has been reported. Waugh (207) found sulfathiazole to be less toxic to the liver of the thirteen-day old chick embryo than sulfanilamide or sulfapyridine.

Since in humans it has been impossible to correlate the degree of hepatic dysfunction with the amount of sulfonamide or duration of therapy, and since toxic manifestations are actually rather infrequent (Machella and Higgins (123) report that less than one per cent of patients receiving

sulfanilamide show liver damage), the theory that hepatic damage depends upon an idiosyncrasy to the drugs appears to be well-founded (189) (193) (208). Peterson (209) does not consider the presence of liver damage to be a contra-indication to therapy with sulfonamides in patients with infections against which the drugs are effective.

Machella and Higgins (123) (210) have studied the effect of sulfanilamide on rats with experimentally damaged livers, and found that the drug did not increase the injury to the liver caused by carbon tetrachloride hepatitis, or by the presence of obstructive jaundice. The sulfonamide did not impede the regeneration of the liver after carbon tetrachloride administration had been discontinued, nor did the simultaneous administration of alcohol and sulfanilamide result in hepatic damage. In fact, it was noted that the livers of those animals that received only carbon tetrachloride were more damaged than the livers of animals receiving carbon tetrachloride plus sulfanilamide. Leach and Forbes (180) have studied this strange protective action of the sulfonamide drugs against carbon tetrachloride poisoning, and found that neither sulfanilic acid nor any one of a number of vitamins and amino-acids tested increased the animals' tolerance to carbon tetrachloride.

viii. Atabrine

The toxicity of the now widely used antimalarial compound, atabrine, has been extensively studied in animals. A single

dose of the drug in rats (equivalent to fifty per cent of the L.D. 50) was found to produce a severe necrosis of the liver, abnormal retention of bromsulfalein and prolongation of the prothrombin time. Liver damage as indicated by function tests and by mortality was markedly increased by fasting the animals for varying periods before giving the drug (211). In rats a high-protein low-fat diet offered much greater protection against daily administration of the drug than did either a low-protein, or a high-protein and fat diet (212) (213). In dogs, too, the beneficial effects of a high-protein diet were noticeable. Thiamine deficiency had no effect upon the hepatic changes produced by atabrine (212). Increases in the plasma fibrinogen levels appeared to run parallel with the toxic action of the drug upon the liver, being slight in animals not showing grossly necrotic livers, and much more strikingly elevated in the presence of gross liver necrosis (213) (214).

These high levels of fibrinogen in the blood are probably the result of generalised tissue injury; severe damage to the liver parenchyma results in a decreased production of fibrinogen by that organ.

Gottfried and Levine (215) have studied liver function in soldiers who had received 0.1 gm. of atabrine daily for eighteen months. In spite of the wide variety of tests performed, only the hippuric acid test showed evidence of hepatic dysfunction, and with this test, abnormal results were

found only in three of the soldiers studied. Presumably the small doses of atabrine administered caused no subclinical liver damage.

ix. Selenium

Wheat grown on seleniferous soil produces in rats a well-marked nodular cirrhosis of the liver, when combined with a low-protein, high-carbohydrate diet (217). Although high-fat, low-protein diets, and high-protein diets do not offer complete protection against the toxic effects of organic selenium compounds, they greatly delay and decrease their frequency.

Selenium is poisonous in minute doses; as little as fifteen parts per million of selenium in the diet of the rat cause toxic effects (218). Higher animals appear to be even more susceptible to the poison. Liver tumors developed in many rats which survived for eighteen to twenty-four months on diets containing from five to ten parts per million of selenium (219).

The liver damage produced by selenium has several unique features (see p.57). Lillie et al (220) report that cirrhosis caused by selenium poisoning does not resemble that of cincophen, copper, silicic acid, or phosphorus poisthat of cincophen, copper, silicic acid, or phosphorus poisoning. It is obvious that the protective action of a high-fat, low-protein diet, and the deleterious effect of a high-carbohydrate, low-protein diet, are most unusual. Lastly, it has been shown that the addition of five parts per million

of arsenic as sodium arsenite to the drinking water of dogs, provided full protection against the liver damage caused by either sodium selenite, or seleniferous wheat (221) (232). The theory has been proposed that arsenic combines with selenium in the gastro-intestinal tract, decreasing its absorption. Moxon et al, however, found that arsenic effectively detoxified selenium regardless of the route of administration (oral or subcutaneous) of either metal (222).

x. Acacia and other colloidal materials

Gum acacia was once used as a blood substitute because of its capacity for retaining water in the blood stream, and maintaining the blood volume for a time. The polysaccharide is largely deposited in the liver, and is removed from this organ very slowly (223) (224) (225). Decreased excretion of bile acids, lowered levels of serum proteins and cholesterol, diminished glucose and galactose tolerance, and delayed clotting of the blood, all indicative of diminished liver function, have been reported following intravenous administration of acacia to animals (223) (224) (226) (227) (228) (229). The polygonal cells are swollen with deposits of acacia, and Yuile and Knutti (230) have proposed that the hepatic insufficiency might be caused by a physical "clogging" of the cells by the gum. Gum arabic is similarly retained for a long time in the liver cells and the R.E. cells of the viscera (231).

Many other colloidal substances that in small amounts

are innocuous are taken up by the R.E. cells and slowly eliminated through the bile. If injected in large amounts, they may cause hepatic damage. Cirrhosis of the liver has been produced in cats (233), dogs (234), and rabbits (235) by the repeated injection of colloidal silica. The lesions are rapidly recovered from, and even large doses are ineffective in the production of chronic damage unless the intervals between the injections are relatively short. The liver changes are accompanied by enlargement of the spleen and kidney lesions.

xi. Carcinogens and diseases affecting the liver

The development of liver tumors in rats and mice is induced by many organic chemical compounds, including p-dimethylaminoazobenzene (236) (237) and certain of the azonaphthalene compounds (238). Polson (239) found no parallel between carcinogenicity and toxicity to the liver, however, for although dibenzanthracene caused liver necrosis, 1,2-benzpyrene had no such effect, and the hepatotoxic action of tar did not seem to lie in its carcinogenic fraction.

Dibenzanthracene administered to normal rats was not found by Abels et al (240) to produce functional deficiency, according to the results of several tests; the concentration of vitamin A in the liver was depleted, however, and the theory was propounded that there is a competition between vitamin A and dibenzanthracene for some substance, possibly a protein, to which vitamin A may be bound in the liver.

Clayton and Baumann (241) found, similarly, that vitamin A is stored in the liver and released therefrom by a mechanism comparatively independent of other processes taking place in the liver; the powerful carcinogen p-dimethylaminoazobenzene did not alter the hepatic storage of the vitamin, nor was it affected by dicumarol or vitamin K, which greatly influence the prothrombin level of the blood, nor by the removal of large amounts of fat from the liver.

The susceptibility of the liver in rats to the carcinogenic action of p-dimethylaminoazobenzene is increased by the presence of cirrhosis of the liver, or by feeding a diet high in fat or low in the vitamin B complex (242) (243) (244) (245).

Patients with cancer of the gastro-intestinal tract show a high incidence of hepatic insufficiency, although the liver shows little or no morphological change, other than deposits of fat. Removal of the cancer causes disappearance of the hepatic dysfunction in many cases (246) (247).

Although hepatic dysfunction is frequently found in cases of hyperthyroidism (248) (249) (250) (251), no direct causal relationship between the two has yet been proved (252) (253) (254). The recent researches of Drill and his associates indicate that the problem may be elucidated in the near future. Hyperthyroid rats suffered a depletion of liver glycogen unless fed large doses of B-vitamins (255). Presumably the same is true in thyroid-fed dogs in which

removal of yeast greatly hastened, and a diet high in the B-vitamins delayed, the development of abnormal liver function as indicated by the bromsulfalein test. Serum phosphatase levels were found to be increased only when the dye retention was abnormal (256) (257) (258). McIver and Winter (27) report that a high protein diet does not afford protection against chloroform poisoning in animals receiving thyroid hormone.

xii. Miscellaneous damaging agents

Phosphorus has been used for many years as a hepatotoxin; it is usually administered subcutaneously dissolved in olive oil. Chronic and acute liver damage has been produced with phosphorus in rabbits (130) (259) (260), guinea pigs (261), dogs (262) and rats (263).

Elemental phosphorus is not a substance to which humans are commonly exposed, nor is it used therapeutically. Whether it produces the same type of damage and biochemical derangement as other commonly used hepatotoxic drugs is a question that has given rise to criticism of the use of phosphorus in experimental studies of liver function.

Many alkaloids cause extensive liver necrosis, with sinusoidal congestion and haemorrhage into the cell cords (264) (265) (266) (267).

Feeding a diet containing two milligrams of copper acetate per gram of food (0.2%) to rabbits resulted in pigmentation of the liver, and necrosis, developing in some

of the animals into cirrhosis (268).

Liver damage has also been reported to have followed the ingestion of cincophen, tolysin, sodium salicylate (269), hydrazine (270) (271) (272), and the seed of Amsinckia intermedia (273); the injection of toluylenediamine (141) (151) (22); and shale oil (285) (286); and prolonged contact with TNT (274) (275). The injurious effect of certain glycols was demonstrated in the perfused cat liver by Newman and colleagues (276).

Boldyreff and Humphrey (277) (278) have produced acute yellow atrophy of the liver in dogs by injecting pancreatic juice directly into the portal vein. The same lesion developed more slowly in dogs in which large amounts of pancreatic juice were introduced into the intestine through an intestinal fistula. These workers suggest that a markedly increased pancreatic secretion might be a factor in the development of acute yellow atrophy in humans.

Liver damage has been shown to follow severe burns (279) (280). Jaundice frequently develops as early as the third day, and functional studies indicate the presence of hepatic damage especially during the period from the third to the tenth day following the injury. The etiology of the liver damage has not been discovered, but it is suggested that infection, a circulating toxin, or hypoxia from capillary stasis might be the cause. Large doses of x-rays have been noted to result in increased amounts of fat in

the liver (281).

e. Diet

For many years investigators have noted a relationship between the diet fed to animals and the resistance of the liver to the action of hepatotoxic drugs. The protective action of liver glycogen and the beneficial effects of glucose administration in experimental hepatic insufficiency have already been described, and the generally deleterious results of high-fat diets prior to the administration of alcohol, atabrine, arsphenamine, chloroform, and carbon tetrachloride (282) have been mentioned. Workers have found high-protein or high-carbohydrate diets to afford a high degree of protection against these hepatotoxins. A curious exception is the metal selenium, which was shown to be much more harmful when given with a low-protein, high-carbohydrate diet than with a low-protein, high-fat diet.

Recently it has been shown that the condition of the liver itself can be profoundly affected by varying the diet with respect to the content of carbohydrate, protein, fat, amino-acids, and dietary factors such as choline and the B-vitamins (283) (284).

Because of the important part played by the liver in carbohydrate metabolism, in which several B-vitamins serve as co-enzymes, it would be anticipated that the liver requires an adequate supply of these vitamins. There are a few experimental observations which indicate that the

members of the B-complex are, indeed, necessary for the preservation of the normal state of the liver.

Gyorgy and Goldblatt (287) occasionally found degenerative and necrotic changes in the livers of rats on a diet deficient in vitamin B-complex, but supplemented with B_1, B_6, and riboflavin. The lesions could be regularly prevented by the addition of yeast to the diet. The liver injury could not be produced at will, and did not occur regularly even under identical experimental conditions.

Fouts (288) fed dogs a low-protein diet supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid; a deficiency state resulted, in which fatty cirrhotic livers were found. Choline, inositol, and p-aminobenzoic acid were not effective in curing or preventing the deficiency state, but the combined administration of choline and powdered liver extract caused the rapid disappearance of the signs of deficiency. Fouts concluded that the animals required a supplement other than the well-recognized members of the B-complex.

Street and Cowgill (289) irregularly observed an increased fat content of the liver cells in riboflavin-deficient dogs. However, a similar change was also seen in the inanition control dogs, so that apparently not the riboflavin deficiency but the accompanying inanition was the cause.

The lipotropic action of choline in fatty livers produced both by feeding diets high in fat or cholesterol, or by

the administration of a hepatotoxin such as phosphorus or carbon tetrachloride, has been extensively studied during the past few years (60) (290) (291) (292) (293) (294) (295). McHenry (296) has recently reviewed this subject and has suggested that choline should be considered not as a vitamin but merely as an essential dietary constituent.

The attribution of the lipotropic activity of casein (292) to its methionine content led to an investigation of the sulfur containing amino-acids. It was soon found in aminals on a high-fat diet that whereas methionine supplements reduced the hepatic fat content, large amounts of cystine greatly increased the amount of fat in the liver (297).

Prolonged feeding of various high-fat, low-protein, low-choline diets resulted not only in fatty infiltration, but eventually in cirrhosis of the liver in rats (126) (298) (299), dogs (300) (301), rabbits and guinea pigs (302) (303) (304). Choline and methionine, when administered, were partially or wholly effective in preventing the cirrhotic condition. It was felt that the extensive fibrosis in the livers of choline-deficient animals was stimulated by the presence of fat in the liver cells over a period of several months.

The production of cirrhosis of the liver in rats fed a low-fat, low-protein diet has been reported by Daft and his colleagues (121) (305) (306) (311); the lesions could be prevented by the addition of choline, methionine, or casein

to the diet. Under certain conditions the parenchymal cells of the cirrhotic liver contained a hyaline substance which has been named "ceroid" (307) (308); this compound was found also in the R.E. cells of the liver, spleen, and lungs. It has been noted that the deposition of ceroid is greatly influenced by the type of fat in the low-protein, low-choline diets; in addition, it has been found that 5% of l-cystine in the diet favours pigment deposition, while 1% choline and alpha-tocopherol inhibit it (309) (310).

The liver lesions produced by the addition of l-cystine to the diet have been studied by Earle and Victor (312) (313) (314). In rats fed diets containing ten per cent cystine the manifestations were fatty infiltration, portal haemorrhagic necrosis, and cirrhosis in animals surviving more than two weeks. The pathogenesis of the liver lesions could be modified by varying the diet. Fatty infiltration was found only with low-protein, high-fat diets; choline prevented the accumulation of fat, but did not protect the liver against the other effects of cystine. There was no correlation between the amount of fat in the liver and the incidence or degree of cirrhosis.

Earle and Victor have pointed out several ways in which the damage produced by cystine differs from that caused by low-protein, high-fat diets (313). The lack of response to choline is one important difference; in addition, the lesion develops more rapidly, is less diffuse, and is characterised

by more extensive bile duct proliferation than the liver damage produced on low-protein, high-fat diets.

It is interesting also to note that the cirrhosis caused by excess dietary cystine is unlike that produced by most hepatotoxins, with the exception of selenium. Both cystine and selenium produce damage in which the acute lesion consists of a periportal haemorrhagic necrosis; cirrhosis develops later. Another curious link between the two is the fact that certain fats, when fed at a high level in the diet, manifest a protective action against liver damage caused by excess selenium; the reduced toxicity of selenium when given with a high fat diet has already been mentioned.

Methionine and to a less extent cystine, given to protein-depleted dogs several hours before chloroform anaesthesia, has been found to exert a remarkable protective action against injury to the liver. Large cystine supplements frequently caused jaundice in the protein-depleted dogs, yet still protected the animals against a lethal anaesthesia (315). Methionine also protects protein-depleted dogs against liver injury caused by arsphenamine (316).

The lipotropic action of mathionine has been employed by several investigators in the treatment of cirrhosis in humans. Rimmerman and colleagues (317) fed their patients a diet low in fat and rich in carbohydrate and protein, with added choline and B-vitamins. Repeated liver function tests showed definite improvement in a number of cases. Fagin

et al (318) (319) administered amino acids parenterally or intravenously, and noted improvement in the condition of the patients after four weeks. In some cases liver specimens were analysed, and it was found that the specimens from the patients receiving amino-acids contained a higher percentage of protein and a lower percentage of fat than did the samples from patients who were not given amino-acids.

Beattie et al (148) successfully treated a case of acute chloroform poisoning with dl-methionine, and succeeded in delaying the onset of liver damage and in moderating its severity in patients receiving arsenicals, by the administration of methionine (79) during the period of treatment when the expected incidence of liver disease was maximal.

Very few liver function tests have been performed on animals with liver lesions of dietary origin, but the results available indicate that the tests should be of great value in following the course of the lesions.

Hough and Freeman (320) (321) found that the removal of protein from the diet of dogs soon resulted in an increase in serum phosphatase and a progressive impairment in the clearance of rose bengal from the serum. Fatty infiltration was noted after about sixteen weeks on the protein-deficient diet. Addition of protein to the diet in the form of casein or cooked egg white reversed these findings; choline was of temporary benefit, but could not prevent the extensive changes in the body resulting from protein deficiency.

McKibbin and his colleagues (322) (323) have studied the effects of choline deficiency in dogs on the results of several liver function tests. They, too, found an increased serum phosphatase activity, related to the degree of fatty infiltration of the liver; the clearance of bromsulfalein was markedly delayed, but these results correlated less well with the fatty infiltration. The prothrombin level of the blood was lowered only in the presence of an extremely high hepatic fat content; the colloidal gold test was of no value in their experiments. Unpublished experiments on pantothenic acid-deficient pups, however, revealed little functional abnormality of the liver even though fifty per cent of its dry weight was lipid material. Dutra and McKibbon (323) suggest that the mere presence of fat is not responsible for the diminution in liver function in choline-deficient dogs, and that the fatty metamorphosis may be only coincidental, rather than an anatomic manifestation of hepatic cell injury. The work of Li and Freeman (793) (794) (795), however, indicates that the reduced clearance of rose bengal occurring in dogs fed a protein-deficient diet with added cholesterol is due largely to an accumulation of lipids in the liver.

The elucidation of the role of dietary factors in the development of liver lesions is by no means complete, but in view of the extensive investigations being carried on in this field, the next few years should bring forth much knowledge of wide theoretical and practical interest.

5. Bile Pigment Metabolism a. Bilirubin formation and excretion

The principal bile pigments are bilirubin ($C_{33}H_{36}N_4O_6$),

an orange-red compound, and its oxidation product, the bluish-green biliverdin ($C_{33}H_{36}N_4O_8$). These compounds are formed when the haemoglobin molecule, liberated in red cell destruction, undergoes an opening of the ring system of the prosthetic group, with the loss of its four iron atoms and the protein component. In clinical and experimental investigations of the relation of the bile pigments to the liver, bilirubin is the only pigment which has been extensively studied.

Hawkins and Johnson (324) have inquired into the quantitative relationships in the formation of bilirubin from laked red cells, injected into anaemic dogs. Assuming, on the basis of their molecular weights, that the destruction of one gram of haemoglobin yields 34.9 grams of bilirubin, the investigators found that new haemoglobin was formed in an equivalent amount (90 to 100 per cent), and that concurrently there was a return (90 to 100 per cent) of the injected haemoglobin in the form of bile pigment. They suggest that under normal conditions muscle haemoglobin may be the source of from one-third to one-quarter of the daily excreted bilirubin, and that the remainder comes from red-cell haemoglobin.

The formation of the bile pigments takes place in the spleen, bone marrow, lymph glands, and liver. Mann and coworkers (325) studying totally hepatectomised dogs, showed spectroscopically that the yellow pigment in the urine,

plasma, and fat of these animals was bilirubin, proving that there was an extrahepatic source of the bile pigments. In 1926 (326) they showed that the liver also was concerned in this process, when they found that the bilirubin content of the blood leaving the liver was measurably greater than that of the blood entering the organ, after the excretion of bile pigment through the biliary tract had been prevented. Later in the same year Mann and his colleagues (327) developed an index of the rate of bilirubin formation by measuring the rate of development of bilirubinemia following removal of the gall bladder and obstruction of the common bile duct. Using this index they determined the effect of the loss of organs such as the liver and spleen on the rate at which bilirubinemia was developing, and obtained the normal amount of bilirubin in the absence of both these organs. They concluded that, in the dog, all but a small amount of bilirubin is formed outside the abdominal cavity, probably in the bone marrow.

However, the cells of the reticulo-endothelial system ("R.E.S.") are generally believed to be responsible for the formation of the bile pigments; many people believe, in spite of Mann's results with eviscerated dogs, that the Kupffer cells of the liver are of major importance in this conversion. Gottlieb (328) (329) (330) in a study of the Kupffer cells, blockaded the cells with thorium dioxide and found that in the rat this blockade prevented the usual

accumulation of bilirubin in the blood after the administration of toluylenediamine. In dogs with biliary fistulas, blockade of the R.E. cells caused complete disappearance of bilirubin from the bile within forty-eight hours after the second injection of thorotrast.

The epithelial cells of the liver play no part in the formation of the bile pigments; they are involved only in the excretion of the pigments into the bile canaliculi. Harrop and Barron (331) found that bilirubin injected into the blood stream was not excreted by the kidneys, or retained by the R.E. system; it was totally excreted by the liver.

Normally, only a small portion of the liver is necessary to excrete all the bilirubin formed in the body. This remarkable factor of safety of the liver with respect to the removal of bilirubin was shown by Dragstedt and Mills (332) who ligated the hepatic ducts draining an estimated two-thirds of the liver in a dog; twenty-four hours later the clearance of intravenously injected bilirubin was normal.

These investigators studied the mechanism of the removal of injected bilirubin from the blood, and found that the rate of removal was retarded by anaesthesia, and immediately arrested by biliary obstruction. However, they reported an initial rapid removal of bilirubin which is apparently not accounted for by its excretion in the bile. That this rapid disappearance might be due to the R.E. system is suggested

by their finding that an artificially induced bilirubinemia causes retention of the dye, bromsulfalein, which has been shown by several investigators to be removed from the blood chiefly by the R.E. cells (333). Blockade of the R.E. cells did not retard the rate of removal of injected bilirubin as much as it did bromsulfalein, but the possibility that some of these cells may be involved was not excluded. Further treatment of this subject will be found in a later discussion on dye excretion (see p. 85).

b. Jaundice

Under certain pathological conditions, the concentration of bilirubin in the blood may increase to such an extent that the skin and the sclera of the eyes become yellow or greenish-yellow in colour, and jaundice or icterus results.

Maintenance of a low level of bilirubin in the blood is dependent upon the ability of the hepatic cells to remove bilirubin from the blood, and damage to the liver frequently causes an elevation of blood bilirubin. The amount of bilirubin in the blood depends also, however, on the amount of haemoglobin available, which in turn is influenced by diet, and by loss or destruction of blood. Herlitz (334) found that in diseases in which streptococcal infection is present, the blood bilirubin level is often increased because of breakdown of the red cells although other diseases, even when prolonged and severe, seldom increase the bilirubin level.

That the amount of bilirubin in the blood depends on many factors other than hepatic injury is illustrated strikingly by Mann (21) who found that an animal with obstruction to the biliary outflow will have a large amount of bilirubin in the blood a few days after the beginning of an obstruction, when the liver appears almost normal, and will have only a small amount of bilirubin in the blood several months after the obstruction, when the liver is very severely damaged. Similarly, in the blood of a dog with only a single cirrhotic lobe of liver remaining, there may be an almost undetectable amount of bilirubin, while in a normal animal, the administration of toluylenediamine will cause intense jaundice, with very little hepatic injury. Jaundice, therefore, may be the result of several different factors, and the causes are usually classified in the following manner:

a. Excessive haemolysis of red cells because of some abnormality of the cells, or because of some toxic substance capable of destroying red cells and resulting in the production of bilirubin in excess of the liver's normal excretory capacity (haemolytic jaundice).

b. Decreased excretion of bilirubin because of toxic or infectious damage to the liver cells and smaller bile passages (hepatogenous or catarrhal jaundice).

c. Obstruction of the larger bile passages, causing a reflux of bilirubin into the blood (obstructive jaundice).

However, if pure forms of jaundice exist, they do so only in the very early stages, for a high level of blood bilirubin from either haemolysis, or obstruction of the bile flow, soon injures the hepatic parenchyma, and conversely, pure hepatogenous jaundice causes some regurgitation of bile because of occlusion of some of the smaller bile passages.

Mann and Bollman (22) have reported jaundice in dogs following continuous injection of dextrose for many hours at the maximum rate of tolerance. The glycogen concentration was increased up to twenty percent of the liver weight, and the jaundice was ascribed to the blocking of the bile canaliculi by the swollen hepatic cells.

c. Icteric index

One of the simplest methods of following the changes in the concentration of bilirubin in the blood consists in determining the "icteric index", i.e. in comparing the intensity of the yellow colour of the blood serum with that of a standard solution of potassium dichromate (1:10,000). The icteric index in the normal person lies within the range of 4 to 6; an icteric index of more than 6 indicates latent jaundice, while with an index above 15 jaundice is usually clinically visible. Other yellow pigments, such as carotene, may be present in the blood, however, and must be removed by first precipitating the serum with acetone before the comparison is made. Of the index, Boyce and McFetridge (1) state that

it is

> "a test for jaundice, a simple, reasonably accurate test, the chief value of which is in the revelation of latent or subclinical jaundice, and in the demonstration of fluctuations in the icterus which cannot be detected by simple clinical observations".

Harrop and Barron (331), however, found no definite correlation between the serum bilirubin level and the icteric index, although both presumably serve the same purpose as a measure of bilirubinemia.

Today the icteric index is seldom used as a general test of liver function, because of the many more sensitive tests available.

d. Van den Bergh reaction

When bilirubin is treated with Ehrlich's diazo reagent (a mixture of sulfanilic acid, hydrochloric acid, and sodium nitrite), a reddish or reddish-violet compound, azobilirubin, is formed. Usually the colour develops promptly, reaching a maximum intensity within thirty seconds. On the other hand, the violet colour may appear only after preliminary precipitation of the serum protein with alcohol. The first is known as the "direct reaction", the second as the "indirect reaction". An intermediate type, in which the colour appears at once but the attainment of maximum intensity is gradual, is termed "biphasic". Why there should be such differences in the reaction between Ehrlich's reagent and bilirubin is not yet understood, but several theories have been advanced.

Barron (335) believes that the bilirubin normally circulating in the blood is indirect-reacting; some constituent of plasma, presumably albumin, combines with the pigment to yield a complex which does not react directly with Ehrlich's reagent. Only after the complex is dissociated by alcohol can the reaction take place. Sodium bilirubinate in solution gives a direct reaction. Barron added to serum increasing amounts of this pigment in a solution buffered to pH 8.43. The reaction was indirect until the concentration of bilirubin reached 12 mg. per 100 ml. of blood; in the concentration range from 12 to 16 mg. per 100 ml. of blood, the reaction was biphasic, and above a concentration of 16 mg. per 100 ml. of blood a "direct" reaction was obtained. Harrop and Barron (331) found that as soon as a solution of bilirubin (direct-reacting) was injected intravenously, the reaction became indirect, and believed that this was due to the adsorption of bilirubin by the protein of the blood.

Other investigators believe that the two types of reactions are given by two different compounds. Griffiths (336) has isolated from gall-bladder bile a substance distinct from bilirubin, yet giving the direct reaction. He called this compound cholebilirubin. Harrison (337) used this term to designate the pigment after it had passed through the hepatic cells; the pigment as it occurs in the blood stream normally was called haemobilirubin.

Greene (338) has used the term cholebilirubin, without

reference to chemical constitution, to represent the compound which is present in the blood giving the direct reaction; and the term haemobilirubin for that compound in the blood giving the indirect reaction. The direct-reacting form of bilirubin is water-soluble, the indirect-reacting form chloroform-soluble.

van den Bergh believed that indirect-reacting bilirubin was the product of haemolysis leading to haemolytic jaundice and that the direct-reacting form was produced by passage of the former compound through the hepatic cells, so that it could appear in the blood only through regurgitation of bile into the blood stream, in obstructive or hepatogenous jaundice in which there had been rupture of the bile capillaries.

Obstruction of the common bile duct in animals, leads within a few hours to the appearance of direct-reacting bilirubin in the blood (339). If now the animal is hepatectomised, the bilirubin which accumulates after this operation is indirect, and is not altered by the presence of direct-acting bilirubin, the reaction is a true biphasic one: therefore it seems that indirect bilirubin may be considered to be of extra-hepatic origin and as not having passed through hepatic cells, and that the direct reaction of bile pigment in the blood is evidence of the reabsorption of bilirubin from the hepatic cells, after they have converted indirect bilirubin to the direct-reacting compound.

That the interpretation of the van den Bergh reaction is not as simple and clear-cut as the above experiments would suggest, is indicated by the work of several other investigators. Herlitz (334) claims that if serum is allowed to stand for a moderate length of time exposed to light, a direct reaction may change to an indirect one, and that a nitrite surplus may elicit a direct reaction in haemolytic jaundice. At the onset of obstructive jaundice and during convalescence from it, the reaction is often indirect. In the haemorrhages of haemolytic jaundice or after splenectomy, there may be a direct reaction. He suggests that bile acids and salts liberate the bilirubin from its combination with albumin, to give a direct reaction, and that while an indirect reaction does not necessarily mean extra-hepatic jaundice the direct reaction probably proves injury of liver cells.

Recent studies of the chloroform-soluble and insoluble fractions of the blood bilirubin have shown that in hepatogenous and obstructive jaundice both types of bilirubin are present. In haemolytic jaundice the greater proportion of the total bilirubin was chloroform-soluble, but if the total pigment concentration was high, a prompt colour development was noted, not reaching a maximum, however, for fifteen to thirty minutes (340) (341).

Dameshek and Singer have reported two cases of familial non-haemolytic jaundice in which the indirect reaction was given (342).

Cantarow (343) (344), in a recent investigation of the van den Bergh reaction, mixed samples of serum from obstructive jaundice and from haemolytic jaundice in varying proportions with one another or with normal serum, and found that the actual proportion of direct bilirubin in the mixture did not coincide with the theoretical. With decreased bilirubinemia in such mixtures, the actual rose progressively above the theoretical in the case of haemolytic jaundice serum, and fell progressively below the latter in the case of obstructive jaundice serum. Cantarow suggests that the capacity of serum bilirubin for giving the direct reaction is dependent in part at least, on factors in serum other than the bilirubin molecule or the nature of its combination with albumin.

Boyce (1) states of the van den Bergh reaction that it furnishes much the same information as does the simpler icteric index test, and that it is open to such possibilities of misinterpretation that many investigators are unwilling to use it at all as a test of liver function.

According to Mann and Bollman (22) true haemolytic jaundice, similar to that in humans, has not been produced in animals by injections of haemolysed blood or by injections of various haemolytic agents, for the bilirubin which accumulates in the blood after these procedures gives a direct reaction with Ehrlich's reagent: however, hepatectomy causes bilirubinemia similar to that of haemolytic jaundice, giving the indirect reaction.

Heilbrun and Hubbard (340) suggest that the measurement of the chloroform-soluble fraction of serum bilirubin in cases of severe jaundice (of non-haemolytic origin) should serve as an estimation of liver function. In their studies, the values for the chloroform-soluble fraction were in general higher in hepatogenous than in obstructive jaundice, presumably because of the more diffuse liver damage present.

e. Bilirubin clearance test

A mild liver injury may not cause hyperbilirubinemia because the liver cells may still be able to excrete the bilirubin, or because there may be a concomitant decrease in the production of bile pigments (331). Reasoning that if the functional load is increased, impaired ability of the liver to excrete a physiological substance should be more easily demonstrated, Eilbott (345) has devised a bilirubin clearance test in which the disappearance of injected bilirubin from the blood is measured.

In his original paper Eilbott gives a full description of the behaviour of colloidal dye stuffs injected into the blood stream, and states the two assumptions on which his test is based, viz. that only the liver eliminates bilirubin and that the length of time bilirubin remains in the blood depends on the condition of the liver. He found that in normal animals, bilirubin injected intravenously never gave a clinically detectable bilirubinemia. He found no correlation between the height of the blood bilirubin before

the test, and the time required for the clearance of the injected pigment, and felt that the blood bilirubin level in itself was not a good indicator of liver function.

The bilirubin test has been used clinically by many investigators and has been found to be a very sensitive test of impaired hepatic function (1). Many workers (331) (346) (347) have detected with this test slight liver insufficiency generally unrevealed by other commonly used methods. Liver function was also shown to be depressed during the last half of pregnancy (348), and in many cases after arsphenamine intoxication (84) (85).

Soffer and Paulson (349) and Kornberg (350) found that many persons long recovered from catarrhal jaundice show residual hepatic damage and an abnormal retention of bilirubin. Similarly, many otherwise healthy young men were discovered by the same test to have chronic non-haemolytic jaundice; Comfort and Hayre (351) confirmed this fact and found the bilirubin excretion test to be the only one showing abnormality in a group of persons with an elevated serum bilirubin. He has suggested that in these individuals there might be an inborn deficiency of the hepatic cells.

Weech and colleagues (352) have given the test added sensitivity by evaluating the rate of removal of injected bilirubin from the circulation as a "velocity constant function" which they consider to be a measure of the excretory function of the liver.

Investigators differ widely concerning the effect of hyperbilirubinemia upon the usefulness of the test. Eilbott (345) has claimed that the presence of endogenous bilirubin, direct or indirect, is of no importance, since the error introduced would be constant. Soffer (346) and Boyce (1), on the other hand, feel that the test is useful only if the bilirubin concentration is less than 1 mg. per 100 ml. of blood, since the presence of hyperbilirubinemia indicates that the liver cannot handle adequately the amount of bile pigment in the blood prior to the performance of the test. Weech et al (352) suggest that the test could be used if the van den Bergh reaction were indirect, since in such a case there would be no evidence of regurgitation of bile into the blood.

Elton (4) is the only investigator who has criticised unfavourably the sensitivity and specificity of the bilirubin clearance test. He claims that his data (unpublished) indicate that, in man, bilirubin injected intravenously in alkaline solvents is not only not excreted by the liver, but also fails to raise the serum bilirubin level proportionately to the amount injected.

Perhaps the greatest drawbacks in the performance of this test are the high cost of bilirubin, and during the last years of the war, its unavailability. Furthermore, it is difficult to obtain the degree of analytical precision needed to bring out small differences in blood bilirubin concentration.

In preference to the van den Bergh reaction, Eilbott (345) originally used the Ernst and Förste method (353) which involved comparison of the yellow colour of the pigment with a potassium dichromate solution. Weech et al (352) also prefer such a method, and have increased its sensitivity and accuracy by the use of a photoelectric colorimeter and a selective colour filter.

Modifications of the diazo method of van den Bergh have been proposed by Malloy and Evelyn (354), Scott (355), Hunter (356), Sepulveda and Osterberg (357) (361), and Ducci and Watson (358).

f. Biliverdin

Larson and Evans (359) have recently made a study of variations in the biliverdin concentration in human serum. In normal individuals, the level was less than 0.05 mg. per 100 ml. of blood; in a series of patients with jaundice the amount varied from 0 to 2.2 mg. per 100 ml. of blood. Although the highest values of biliverdin were usually associated with the highest bilirubin concentrations, it was found that the biliverdin content could fluctuate markedly in a manner quite unrelated to the course of icterus. Biliverdin was present in the blood in every one of twenty-six cases of carcinomatous obstruction of the common bile duct, and was never found in haemolytic jaundice.

g. Urobilinogen

When the bile reaches the intestine, bilirubin undergoes a reduction by bacteria to urobilinogen, a colourless compound which in turn is oxidized to urobilin, a brownish-yellow pigment closely related to, if not identical with, stercobilin, the colour imparting a brownish colour to the faeces. Both urobilinogen and urobilin normally are present in the faeces. In bile fistula animals urobilin gradually disappears from the excreted bile, reappearing when whole bile is fed to the animals.

The amount of urobilinogen formed reflects, in part, the rate of bile pigment metabolism. Miller and co-workers (360) have introduced a "haemolytic index" which they define as:

$$\frac{\text{Average daily output of fecal urobilinogen (mg.)} \times 100}{\text{Total haemoglobin}}$$

By this method they found that from 11.1 to 20.8 mg. of faecal urobilinogen is normally derived from 100 grams of circulating haemoglobin in twenty-four hours. Administration of sulfanilamide in customary therapeutic doses was found by Watson and Spink (195) usually to cause acceleration of haemoglobin metabolism, characterised by an increase of faecal urobilinogen. Wassermann and his associates (362), measuring urobilinogen in faeces and urine after blood transfusions in patients with aplastic anaemia, determined these levels to be directly proportional to the length of time of blood storage.

A considerable part of the urobilinogen is absorbed through the portal circulation, and returned to the liver. Some investigators think that it may be used in haemoglobin production, or reconverted into bilirubin. A part, however, is re-excreted in the bile. Normally only traces of urobilinogen reach the kidney. Such is not the case in jaundice, however. Excessive haemolysis of red cells leads to increased bilirubin production, and as a result, an increase in urobilinogen beyond the capacity of the liver to excrete it, so that it is found in the urine in increased amounts.

In complete biliary obstruction, bile pigment does not reach the intestine: therefore no urobilinogen is formed and none is found in either stool or urine. The latter, however, contains bilirubin.

Where the excretory function of the liver is seriously impaired as a result of hepatic disease involving liver cell damage, a portion of the urobilinogen which has been reabsorbed from the intestine fails to be re-excreted in the bile. It is carried by the blood stream to the kidney, which apparently possesses no threshold for urobilinogen, and appears in the urine in amounts roughly proportional to the extent of the liver cell damage. Mann and Bollman (22) suggest that the capacity of the liver to excrete urobilinogen is very easily disturbed since the compound appears in the urine in fevers and in many non-specific infections which do not appear to involve the liver.

The distribution of pigments in the various types of jaundice may be represented schematically:

	Urinary Bilirubin	Urinary Urobilinogen	Faecal Urobilinogen
Haemolytic jaundice	none	+	+++
Hepatitis	+	+++	+
Obstruction	+++	0 or +	0 or +

Steigman and Dyniewicz (363), in a study of urobilinogen, point out that there are exceptions to the above scheme.

Complete absence of urobilinogen from stool and urine is found only in complete obstruction on a malignant basis. In intrahepatic obstruction small quantities may be excreted intermittently. In some cases of severe toxic hepatitis, particularly in those due to arsenical intoxication, and in cases of obstructive cirrhosis, the urobilinogen in both urine and faeces may temporarily drop to very low levels, simulating a complete obstruction of neoplastic origin.

The relatively high urobilinuria in some cases of obstructive jaundice may reflect, on the one hand, incompleteness of the obstruction and on the other, impaired hepatic excretion of urobilinogen returned to the liver.

Watson (364) (365) has established the twenty-four hour excretion of urobilinogen in the normal adult to be 0.4 mg. in the urine and 40 to 280 mg. in the stool. Faecal urobilinogen is lowered by inanition, inactivity, or low grade infection, and increased by considerable fever, dependent on the type and severity of infection, pernicious

anemia, and in instances of leukemia and Hodgkin's disease.

Bodansky and Bodansky (366) draw attention to the fact that, in exceptional cases, urobilinogen is formed in sites other than the intestine. Bacteria present in infected biliary passages can transfer bilirubin into urobilinogen. Other extra-hepatic causes of increasedurobilinogen production and excretion are infarction (e.g. splenic, pulmonary), haemorrhagic ovarian cyst, haematoma due to ectopic pregnancy, and congestive heart failure with jaundice and ascites.

Jankelson (367) in one of the most recent studies of this subject, found that in the bile of normal humans urobilin, but not urobilinogen, was present. However, urobilinogen was detected in the bile in about half of the cases of organic liver or biliary tract diseases studied and the quantity of urobilinogen present seemed to be related in a general way to the amount of liver damage. Watson, in the discussion following (367), has proposed using the rate of removal of injected stercobilin from the blood as a liver function test. Soon after such an injection the bile was found to contain urobilinogen in relatively large amounts, and the stercobilin disappeared rapidly from the blood. In cirrhosis very large fractions of the injected material appeared in the urine because of the inability of the liver to excrete it.

There are three methods commonly used for the determination of urobilinogen in the urine and faeces. Of these,

both the Wallace and Diamond method and the Sparkman method must be performed on fresh specimens, since the urobilinogen is converted to urobilin on standing. As a result, the collection of twenty-four hour specimens is obviated. Watson, the author of the third method, considers a four-day collection to be the minimum in which to study the average daily excretion of urobilinogen (368), and other investigators (369) have found determinations on random samples to be inaccurate because of the variations in urobilinogen excretion through the day. The Sparkman, and Wallace and Diamond methods are apparently not sensitive enough to detect small but significant amounts of urobilinogen in either urine or faeces (368) (369). In the Watson procedure (370) (371), urobilin is converted back to urobilinogen which is concentrated by extraction with petroleum ether. In this way the sensitivity and specificity of the method is greatly increased (198).

Bang (381) has made a thorough study of several of the older methods for the determination of urobilinogen, and has investigated many of the factors which cause fluctuations in its excretion.

h. Measurement of bile in urine

The presence of bile in the urine may be detected by the qualitative methods of Maher (372) and Mueller (373) or the quantitative method of Singer (374). The "methylene

blue test" has been employed presumably to measure bilirubin in urine; the test consists of adding methylene blue to urine dropwise, a positive result being one which requires more than five drops of dye to convert the green colour formed originally to blue. Gellis and Stokes (375) found that the test became positive in patients with preicteric hepatitis from one to six days before scleral icterus appeared, but became negative again before the disappearance of the jaundice. The specificity of the test has been challenged, however; apparently the green colour produced when methylene blue is added to urine results from the subtraction of colour from the visible spectrum by the substances involved in the test. A positive result may be obtained with any distinctly yellow liquid or urine, by adding a blue pigment, or by holding a blue glass filter in front of the test-tube containing the sample (376) (377).

i. Porphyrin metabolism

Porphyrins form a group of pigments which are widely distributed throughout the plant and animal kingdoms. Only porphyrins of Types I and III are known to occur naturally. The liver is involved in both the metabolism and the excretion of these compounds, and in cases of hepatic insufficiency the excretion of porphyrins through the bile is reduced.

In a recent symposium, Watson (378) has discussed porphyrin metabolism in a number of pathological conditions,

including cirrhosis of the liver, chronic alcoholism, and haemolytic jaundice. Dobriner, also, has studied urinary porphyrins in disease (379).

Rosenblum (380) suggests that porphyrinuria, when present in pellagra, is probably a manifestation of the coincident existence of hepatic dysfunction. Palmer (275) has found a high incidence of repeated porphyrinuria in workers who are in close contact with TNT, and in whom jaundice and toxic necrosis of the liver occasionally develop.

6. Excretion of Dyes by the Liver a. Phenoltetrachlorphthalein

The parenchymal cells of the liver possess the ability to excrete into the bile many dyes which are injected into the blood stream. One of the early liver function tests (382) consisted of determining the amount of dye in the faeces after the intravenous injection of phenoltetrachlorphthalein. Because of the inaccuracy and tediousness of this procedure, Rosenthal investigated, instead, the problem of measuring the rate of disappearance of the dye from the blood-stream, and found evidence that the curves obtained paralleled the functional impairment of the liver (383) (384).

The sensitivity of the modified test was studied in animals by several investigators. Rosenthal (383) found that after chloroform poisoning in dogs, the initial

concentration of dye in the blood was higher, and the duration of the retention was longer, than in normal animals. When 12 per cent of the liver tissue was removed from rabbits, dye excretion became abnormal; if more tissue was excised, there was a close correlation between the quantity of tissue removed and the degree of functional impairment produced (385).

Lamson et al (163) employed the test in a study of the toxicity of carbontetrachloride in dogs. Two kinds of abnormal results were found in the poisoned animals: in some, there was a high initial concentration of dye, disappearing from the blood rapidly; in others, a normal initial concentration of dye remaining in the blood for a long period of time, was noted. The second type of result was more frequently associated with severe damage, as shown by clinical and pathological studies. However, Lamson et al state that before any of the functional tests employed indicated injury to the liver, necrosis could be detected.

MacNider (117) (386) applied the phenoltetrachlorphthalein test to dogs during acute alcoholic intoxication and found that the increase in the initial concentration of the dye, and the delay in removal of the dye from the blood, ran parallel to the course of the hepatic damage.

Rosenthal and White (387) studied the rate of removal of several phthalein dyes from the blood of rabbits in which about 80 per cent of the liver was ligated. It was found

that phenoltetrachlorphthalein diffused into the tissues, while sulfonated phthaleins did not. The studies of these investigators indicated that the best dye for testing the excretory capacity of the liver was bromsulfalein (phenol-tetrabromophthalein sodium sulfonate). As a result, this compound has been widely used for the past twenty years, and has entirely superseded the older dye for this purpose.

b. Bromsulfalein

Many workers have found the bromsulfalein test to be one of the most accurate, sensitive, and practical tests available for the study of the liver function (388) (389) (390). Within recent years there has been a critical study of the normal range of the test, and several modifications have been proposed. MacDonald (391) (392) found, by following the concentration of bromsulfalein in the blood at two minute intervals after the injection of 2 mg. of dye per kilogram of body weight, that all curves which fell to zero concentration within thirty minutes (this period being considered the normal value) did not do so by exhibiting a decreased retention at each time interval. Similarly, it was noted that the large majority of normal individuals were able to remove all the dye from the blood within eighteen rather than thirty minutes. MacDonald advocated determining the concentration of dye in the blood at frequent intervals in order to detect slight abnormalities of liver function.

Deutsch (393) made a similar suggestion, and found that a convex curve of dye concentration within the interval from five to fifteen minutes indicated liver damage, even when there was no dye present in the fifteen-minute specimen.

Mateer et al (394) regard twenty minutes as the normal time for complete disappearance of the dye from the blood when a dose of 2 mg. of the dye per kilogram body weight is given. The test is much more sensitive, however, if a large dose (5 mg. per kilogram of body weight) is used (394) (395). Although it was formerly believed that the normal liver required sixty minutes to remove the larger dose of dye from the blood, more recent studies have indicated that only thirty (395) to forty-five minutes (394) are required.

The bromsulfalein test was a sensitive indicator of liver injury in animals poisoned with chloroform (396), phosphorus (397), and atabrine (182), and in dogs with experimental hyperthyroidism (256). The test was of less value in detecting the damage caused by selenium ingestion (218) or by cholecystectomy (398).

c. Rose bengal and other dyes

Rose bengal (disodiumtetraiodotetrachlorofluorescein) is another dye which is specifically and rapidly removed from the blood by the liver; its retention in the blood stream has been found to correlate closely with the extent of liver cell damage (399) (400) (401) (402) (403). However,

it is removed so rapidly from the blood that the test must be carefully timed; another great drawback to its use is the fact that it renders the patient photosensitive for a time after its injection. Fuchsin-S (404) and azorubin-S (405) have also been employed in measuring the excretory capacity of the liver, but none of these dyes has met with such widespread popularity as has bromsulfalein.

d. The role of the R.E. system in dye excretion

Within recent years there has been much investigation into the mechanism of the removal of bromsulfalein from the blood. Schellong and Eisler (406) had found that removing the spleen with its large accumulation of R.E. cells, or blocking the R.E. cells of the liver and other organs by injections of India ink, caused an abnormal retention of phenoltetrachlorphthalein and azorubin-S in the blood-stream. A similar observation was made by Rosenthal and Lillie (407), who reported, however, that such procedures did not affect the removal of bromsulfalein from the blood. Nevertheless, subsequent workers (332) (408) (409) have shown that the excretion of this dye, too, is delayed after splenectomy or India ink injections. Mills and Dragstedt (410) found that obstruction of the biliary outflow had little effect on the removal of the dye from the blood; even after ligation of both the common bile duct and the cystic duct, there was no appreciable reduction in the rate of disappearance of the dye. Excretion of bilirubin however,

was immediately prevented by such a procedure. These observations all pointed to the R.E. cells as the agents directly responsible for the removal of bromsulfalein from the blood. A similar conclusion was reached by Cantarow and Wirts (411) (412) (413) who found that although the dye disappeared rapidly from the blood stream, it was excreted in the bile only slowly; these observers suggested that the first step, the rapid removal of the dye from the blood, was a function of the R.E. cells, while the second step was performed by the parenchymal cells of the liver.

Dragstedt and Mills produced hyperbilirubinemia in dogs by injection of bilirubin (333). Thirty to 180 minutes later, the excretion of bromsulfalein was delayed, proportional to the amount of bilirubin given, although presumably the bile pigment had not been taken up by the R.E. cells. A somewhat similar phenomenon was observed by Rosenthal and Lillie (407) who found that lipemia, spontaneous or experimentally produced, resulted in a slightly prolonged retention of bromsulfalein within the blood-stream, although there was no indication that the R.E. cells had ingested appreciable amounts of fat.

It is interesting to note that several years before American investigators had begun to study the problem of the relationship between the R.E. system and the removal of bromsulfalein from the blood, Herlitz (334) (414), and Schellong and Eisler (406) in Europe had suggested that dye

excretion tests were not tests of general liver function but tests of the activity of the R.E. system, and because the R.E. cells are injured in infections, the abnormal dye retention in such conditions (440) would reflect damage to the R.E. system, rather than to the liver.

7. Detoxication and Conjugation a. Metabolism and excretion of bile salts

The role of the liver in bile salt metabolism is a very complex one. A wealth of experimental evidence has accumulated from the many efforts to elucidate the different steps in the metabolism of the bile salts, and to determine the various causes for their appearance in blood and urine.

That the liver is the site of bile salt synthesis was shown conclusively by Bollman and Mann (415) who demonstrated that after hepatectomy, no bile salts were found in blood or urine, although bilirubinemia developed and bile pigment was found in the urine. It had previously been demonstrated by many workers that the extent of bile salt formation was influenced greatly by the condition of the liver cells. In animals with complete biliary fistula, the daily excretion of bile salts was found to be constant under constant dietary and physical conditions (416) but the output of bile acids in these animals could be influenced at will by regulation of the diet. A meat diet caused the highest output of bile acids (417) while on a carbohydrate diet the output was

much lower. Foster and colleagues (416) found that urinary nitrogen and taurocholic acid excretion showed a similarity in these animals, and felt that this indicated a relationship between protein metabolism and taurocholic acid production. It is now believed that the component taurine is derived in the body from the amino-acid cysteine; studies with carbon isotopes have shown that acetic acid may be utilized in the biosynthesis of steroids (418).

Slight hepatic injury may greatly reduce the amount of bile salts formed and excreted. Infection of the intrahepatic ducts, or damage by toxins such as chloroform or carbon tetrachloride, may reduce the bile salt excretion in fistula bile to less than one-tenth of its former value (21) (22). Greatest reduction occurs when hepatic injury is most severe, but marked reduction in the bile salt excretion may be obtained with doses of chloroform that do not cause histologically recognizable liver injury or clinical reaction (419). It would appear that the ability of the liver to form bile salts is easily impaired. Fistula bile from dogs with an Eck fistula contains only about half the normal amount of bile acid (19).

In humans undergoing drainage of the common bile duct, Gray (420) attempted to correlate the concentration of bile acids in the bile with the liver condition as indicated by liver function tests and by the appearance of the liver at operation. The bile acid concentration in the bile was

low in every case of hepatic damage.

In complete obstruction of the biliary outflow, the amount of bile salts excreted in the urine is only about half of that found in normal animals with biliary fistulae, presumably because of the resultant hepatic damage. Similarly, Greene et al (421) found in humans that biliary obstruction caused reduction or cessation in the bile acid production. The terminal decrease in urinary bile salts in continued obstructive jaundice is not reached until appreciable secondary hepatic injury is demonstrable; the decrease in urinary bile salts is obviously due to their decreased formation.

Mann (21) found that the bile salt concentration in the urine of dogs with complete biliary obstruction was a fairly accurate index of the condition of the liver. The greater the excretion of bile salts under these conditions, the better the state of the liver. Additional injury to the livers of these dogs diminished the bile salt excretion. Morrison and Swalm (422) (423) (424) advocate a study of the bile salt concentration in bile or urine as a "sensitive physiologic index to the function of the liver in health and disease".

The bile salts, after being synthesized, are excreted by the liver parenchyma into the bile, and in the normal animal only traces of bile salts are found in the urine and none is detectable in the normal blood. Biliary obstruction

or injury by hepatotoxins, sufficient to give rise to urinary excretion of bile pigment, is usually accompanied by the excretion of increasing amounts of bile salts in the blood and urine. Usually the concentration of bile acids in the blood is higher in obstructive jaundice than in hepatitis, partly owing to the maintained ability of the liver cells in obstruction to produce bile salts, and partly because of the hepatobiliolymphatic circulation appearing only in obstruction which carries the bile salts from the liver to the circulating blood (vide infra). As these animals recover, bile pigment may still be present in large amounts, but the bile salts are excreted in the bile and disappear from the blood and urine. Severe hepatic injury reduces the amount of bile salts found in the blood and urine, apparently by reducing the formation of these substances. Toluylenediamine (151) causes primarily a disturbance of the excretory function of the liver, with diminished excretion of bilirubin and bile acids in the bile. But in contrast to the action of other toxins, there is continued formation of bile acids and they appear both in the blood and urine in increased concentration.

The bile salts which reach the intestine in the bile are not excreted from the body, but are almost entirely resorbed from the gut, carried to the liver by the portal blood, and again excreted into the bile. Only a small fraction of the bile acids is lost in each of these turns,

and a correspondingly small fraction is synthesized to replace the lost part. The fate of the lost bile acids is obscure; some may be destroyed by the liver and some may be decomposed by the intestinal flora. Bollman and Mann (415) found that if bile salts were administered to a hepatectomised animal, they appeared in the urine within a few hours. If,however, the animal was instead suffering from obstruction of the biliary outflow, not all the administered bile salts were found in the urine. These workers felt that the liver cells destroyed some of the bile salts. Josephson (425), in his review of the circulation of the bile acids, criticises this theory and points out that the method used by Bollman and Mann for measuring urinary bile salts is extremely unspecific.

Josephson and his co-workers have made an intensive study of the metabolism of bile salts by studying their concentration in the blood and urine after oral or intravenous administration.

Normally most of the bile acids are conjugated with glycine or with taurine. If unconjugated acids are supplied, they will be conjugated to a certain degree. In patients with liver diseases, the bile may contain larger amounts of unconjugated bile acids (426). Whether these free bile acids can occur normally is still an open question.

Foster and colleagues (427) gave large amounts of free cholic acid to bile fistula animals, and found that it was

conjugated according to the supply of glycine or taurine available. When these supplies were exhausted, then free cholate appeared in the bile. Josephson et al (425) obtained different results after injecting comparatively larger amounts of cholic acid into cats and rabbits with the gall bladder ligated and with a cannula in the common bile duct. During the first half-hour after the injection, the bile salt excretion was large and nearly all the excreted cholate was unconjugated. Later on, as the bile salt excretion became less, more of the cholate appeared in the conjugated form. Similar results were found in humans, and two plausible explanations have been advanced. First, the enzymatic nature of the process of bile salt conjugation would prevent this conjugation from being instantaneous. Second, the time that elapses between an injection of free cholate and the appearance of conjugated acids may be the time required for mobilising or producing glycine or taurine for the conjugation.

Josephson (425) draws attention to the fact that the coupling of free cholic acid is very similar to the formation of hippuric acid from glycine and benzoic acid, and that it is quite conceivable that both these syntheses might be performed by the same enzymatic apparatus, at least in man, where the formation of hippuric acid takes place in the liver (see p. 98).

When bile salts are injected into a normal individual, they disappear rapidly from the blood (428). Most of the

bile salts have left the blood within four minutes after the injection and in thirty minutes the blood concentration is normal again. After an hour, most of the injected cholate can be recovered in the bile, partly in the unconjugated form. Even in patients or animals with hepatic cell damage, a large proportion of the injected bile salts disappears quickly from the blood (429) (430) and this first rapid disappearance cannot be referred solely to the liver. Josephson (425) excluded from the circulation the liver of an animal by ligation of the hepatic artery and portal vein, but found that the concentration of bile salts four minutes after injection was still only two-thirds of what might have been expected if the bile salts had been merely diluted by the blood. He suggests that the bile salts are absorbed on the walls of the blood vessels and gradually incorporated into normal circulation. This rapid disappearance of injected bile salts from the blood is normally followed by an almost quantitative excretion by the liver. In patients and animals with bile fistulas, ninety to one hundred per cent of a moderate amount of injected bile salts is recovered in the bile in a few hours after the injection. Similar results have been obtained with cholecystectomised patients.

Josephson et al (425) then found that if an injection of bile salts was undertaken on an animal with the liver excluded and having considerable icterus from a previous

ligation of the bile duct, the blood concentration of the bile salts rose enormously, and corresponded roughly to the simple dilution of the injected solution by the blood of the animal. These investigators suggested that in icterus, an adsorption of the injected salts on the walls of the blood vessels does not take place to the same degree as normally, because the walls of the blood vessels are already loaded with bile salts. Josephson believes that in obstructive jaundice, the injected bile salts are absorbed by the liver, and that very gradually they enter the hepatobiliolymphatic circulation, resulting in a comparatively rapid disappearance of the injected bile salts from the blood. In hepatogenous jaundice, on the other hand, where there is no bile circulation, the salts remain in the blood for a long time.

In jaundice, whether caused by obstruction or by parenchymatous damage of the liver cells, the normal enterohepatic circulation of bile acids is more or less completely broken. In obstructive jaundice, provided there is little or no secondary liver damage, the cells can still produce bile acids, and contact between the blood and the liver cells and between the liver cells and the bile capillaries, is still unbroken. However, the slight secretion pressure of the bile is enough to open the intercellular spaces between the parenchymal cells, and a new outflow is opened by which bile can escape from the bile capillaries, and reach the thoracic duct and the circulating blood in the lymph spaces and

vessels of the liver. Thus in obstructive jaundice the normal enterohepatic circulation of bile salts is replaced by the "hepatobiliolymphatic" circulation.

If the icterus is caused by hepatic cell damage, there is very little or no passage of bile through the parenchymal cells to the bile capillaries. The contact between the cells and the blood passing the liver is usually better, however. The inflamed liver has a great difficulty in absorbing bile salts from the circulation, and these salts remain for a long time in the circulating blood if injected intravenously into patients or animals with hepatitis.

When bile salts are injected intravenously into patients or animals with obstructive jaundice, their immediate disappearance is not as rapid as under normal circumstances, but the subsequent decrease in concentration is rapid as compared with that in hepatitis, because of the continued capacity of the liver cells to take up acids and keep them circulating. Thus the behaviour of intravenously injected cholates illustrates the difference in the circulation of the bile salts in obstructive jaundice and hepatogenous jaundice (425).

On the basis of these observations, Josephson (425) (428) has introduced a cholic acid test, in which sodium cholate is given and the blood cholates determined before, and five, thirty, and sixty minutes after the injection, and has applied the test to a large number of cases of liver injury.

In liver disease without jaundice, the cholates disappeared just as rapidly as in normal cases. In cases of biliary obstruction, the increase five minutes after the injection was usually noticeable, but after thirty minutes the concentration had already fallen to near the original level. When there was parenchymatous damage, the first increase was usually of the same order of magnitude as in obstruction, but the subsequent decrease of cholates was very slow. In phosphorus and carbon tetrachloride poisoning in cats and rabbits, there was usually good agreement between the results of the microscopic examination of the livers, and the delay in the decrease of the blood cholates after intravenous injection. Ottenberg (431) has suggested that the cholic acid test of Josephson may become a valuable diagnostic method.

In general, there is very little parallelism between the level of bile acids in the blood or bile, and the production and concentration of other substances such as cholesterol and plasma protein, which are manufactured in the liver (425) (432). Although the blood cholates are seldom increased and the results of the cholate elimination test are always normal when the blood bilirubin level is normal, yet in cases of liver disease the formation and circulation of the bile acids seem to be rather independent of the other functions of the liver. The complexity of the composition of the bile salts has led to the development of numerous methods for their determination, both qualitatively and

quantitative by. In 1919 Foster and Hooper (433) reviewed the old methods, and introduced a new one which measured the amino nitrogen in taurine after the hydrolysis of taurocholic acid, which is thepredominant bile acid in dog's bile.

Gregory and Pascoe (434) in 1929, introduced a colour reaction, which subsequent workers have found to be specific for cholic acid. Since human bile contains a relatively large proportion of desoxycholic acid, the Gregory-Pascoe reaction is of little value in this analysis when used by itself. The phosphovanillin reaction of Chabrol et al (435) (436) is also a test for cholic acid. Josephson and Junger (437), and Larrson (438) have greatly increased the specificity of the Pettenkofer reaction, but this reaction, too, is useful only for cholic acid and its derivatives, and is not practical for clinical use.

Doubilet (439) has studied the most reliable of the methods, and has conbined them so that they might be used in the differential quantitative analysis of bile acids in bile and duodenal drainage material. His method permits the analysis of bile for taurocholic, glycocholic, and total conjugated acids, cholic, desoxycholic, total bile acids, and free bile acids. Duodenal drainage material may be analysed for cholic, desoxycholic, and total bile acids.

Josephson (425) suggests that the surface activity of bile salts may be used as a means of following changes in their concentration, since the surface tension of the blood

or serum of an individual is very stable if no surface active substance is added. A bile salt injection causes an obvious change in the surface tension, which returns to the original level when the salts are eliminated from the blood. Josephson points out that although the method gives no idea of the real bile salt concentration in the blood, this absolute concentration is of little clinical interest. The method proposed by Morrison (423) for studying the concentration of the bile salts in bile and urine depends similarly upon measurement of surface tension.

b. Hippuric acid

That the body detoxifies benzoic acid by conjugating it with glycine to form hippuric acid, has been known for many decades. A great deal of confusion existed in the early literature, however, concerning the site of the formation of hippuric acid (3) (271) (441) (442) (443). The kidney appeared to play an important part in this conjugation but the evidence implicating the liver as a site was conflicting. As a result, the synthesis of hippuric acid after ingestion of benzoic acid was originally used as a kidney function test. Bryan (443) was probably the first to suggest that since the amino-acid glycine is synthesized in the liver, the detoxification of benzoic acid might be reduced in the presence of a damaged liver. Quick (444) made a study of the conjugation, and found that much of the early confusion

arose from the fact that in dogs, benzoic acid is largely conjugated with glucuronic acid, rather than with glycine. In man, however, only a small amount of the ingested benzoic acid is excreted as the glucuronic acid monobenzoate (444) (445), and within the last few years the "hippuric acid test" of liver function (446) has been widely used.

Originally, the test was considered merely as a measure of the capacity of the liver to synthesize glycine. Irrespective of the dose of benzoic acid given, the maximum amount of hippuric acid formed per hour was practically constant (447); when extra glycine was supplied, however, the synthesis of hippuric acid was usually greatly increased (448). Probstein and Londe (449), on the other hand, have reported that occasionally a subnormal synthesis of hippuric acid may be attributed to a deficiency in the conjugation mechanism.

Boyce and McFetridge (1) have pointed out that because the syntheses of glycocholic acid and hippuric acid appear to be closely related, and because the synthesis of an amino-acid such as glycine involves a precursor presumably derived from carbohydrate metabolism, the hippuric acid test measures a physiologic process and imposes no strain upon the liver.

In general, the hippuric acid test has proved to be a valuable and sensitive aid in estimating the condition of the liver, (1) (450) (451) (452) (453) (454), and has been useful in patients receiving arsenicals (76), and in those about to undergo surgical treatments (389) (455). Although

some workers have not found the test to be of value in the differential diagnosis of jaundice (389), others claim that if icterus of obstructive origin is of short duration, the results of the test will be normal (456) (457). Heilig and Kantiengar (458) found that the hippuric acid test indicated impairment of liver function during the menstrual period as compared with that recorded in the interval between periods.

Although originally sodium benzoate was administered orally, both Quick (447) (448) and Lipschutz (459) proposed a modification in which a smaller dose of the substance was injected intravenously. The intravenous test has been found to be more sensitive than the oral one (455). The establishment of normal values for the test is complicated by the fact that healthy persons excrete more hippuric acid following ingestion of a dose of sodium benzoate than do bed-ridden subjects with no evidence of liver disease (449) (460), and that there is a correlation between the excretion of hippuric acid and the size of the subject (461) (462).

Because the hippuric acid formed in the liver must be excreted by the kidneys, it has been suggested that before the hippuric acid test is performed, it should be ascertained that no kidney damage is present (451) (463). However, Probstein and Londe (449) consider the renal factor important only in the presence of marked diminution of function associated with increased amounts of urea in the blood.

Hippuric acid may be determined in the urine either by titration, or by weighing the acid which is precipitated upon acidification of the urine (446). Saturation of the urine with sodium chloride (464) or with ammonium sulfate (461) renders the precipitation more complete. Krause and Dulkin (465) have emphasized the necessity of adding an excess of mineral acid to the urine to reduce the solubility of hippuric acid. Several workers (451) (463) have recommended extraction of the compound from the urine with ether, followed by a formol titration as a more accurate method than the precipitation procedure of Quick (446), but Aldersberg and Minibeck (260) found that the results of the Quick method agreed within 5 per cent with the results of more complicated and presumably more accurate procedures.

c. Glucuronic acid

Many compounds which are metabolised with difficulty by the body can be detoxified by conjugation with glucuronic acid. Although the site of this process was assumed to be the liver, this was not proven until 1934 when Hemingway and colleagues (466) perfused various organs (liver, spleen and tissues of the hind limb) in combination with the kidney, and measured the excretion of glucuronic acid in the urine. These investigators concluded that the liver is the main site, and probably the only site, of the formation and conjugation of glucuronic acid in the body.

Lipschitz and Bueding (261) (467), a few years later, studied the conjugation of glucuronic acid with menthol, borneol, avertin, and phenol, by tissue slices from several animals. It was found that liver slices produced the acid in large quantities, that kidney slices formed only a small amount, and that other tissues produced none. The synthesis of glucuronic acid by normal liver slices could be significantly increased by the addition of sodium lactate or pyruvate to the medium. Liver slices from guinea pigs poisoned with phosphorus showed fatty infiltration and degeneration of tissue, with no signs of regeneration in the terminal stages. Addition of lactate to the medium did not cause an increase in glucuronic acid production, indicating that the mechanism of its synthesis had been disturbed. On the other hand, in the terminal stages of chloroform poisoning, although the liver slices showed fatty infiltration, there were signs of marked regeneration, and sodium lactate caused increased production of glucuronic acid equal to that observed with normal liver slices.

Studies of the enzymatic process of glucuronic acid production have shown that an oxidation is involved; the synthesis is catalysed by heavy metals, and depressed by cyanide, iodoacetate, or fluoride ion. Apparently one step is an esterification of phosphoric acid with organic material. Lipschitz and Bueding (467) believe that the compound is synthesized from 3- carbon compounds.

Long before these experiments were performed, workers had studied the metabolism of glucuronic acid in conditions of liver disease. In 1916 Roger (468) gave camphor to patients and determined the glucuronic acid level in the urine. The fact that no glucuronic acid could be detected in the urine after a forty-eight hour fast indicated the importance of the liver glycogen in its synthesis. In cases of cirrhosis, there was little or no glucuronic acid excretion, even after camphor ingestion. Obstructive jaundice was characterised by a temporary increase in glucuronuria, apparently caused by the presence of large amounts of products of intestinal putrefaction in the portal blood; as the liver gradually became damaged, the glucuronic acid excretion decreased.

In spite of the fact that glucuronic acid metabolism is primarily a function of the liver, there is today no satisfactory test based on this physiological fact, nor is there agreement among investigators as to the value of studies of glucuronic acid excretion in liver disease.

That such studies should be useful was indicated not only by Roger's work but by the results of Boku and Kin (469), who found that camphor ingestion raised the urinary level of glucuronic acid in normal humans but not in cases of catarrhal jaundice. It was also observed that in rabbits poisoned with phosphorus or carbon tetrachloride, there was a marked decrease in the production of camphor glucuronide.

Nasarijanz (470) used the conjugation of menthol as a liver function test, and found that the synthesis of menthol glucuronide was diminished by various diseases of the liver parenchyma. The greater the amount of hepatic injury, the less was the amount of menthol glucuronide synthesized.

Acetylsalicylic acid was employed by Salt (471) as a glucuronogenic drug, but although he suggested its use as a test of liver function, he did not submit any results of actual tests performed on patients suffering from liver disease. In normal humans, ingestion of the drug was followed within twelve hours by a sharp increase in the urinary glucuronide. Other workers have not found studies of urinary glucuronic acid to yield much information in hepatic damage. Mukerji and Ghose (146) (472) observed that during the early stages of liver injury in rabbits and dogs, there is usually a tendency towards an increased excretion of glucuronide, but later, when the pathologic changes in the cells are well advanced, there is a definite decrease in urinary conjugated glucuronic acid. These workers, believing that glucuronic acid excretion in itself held little promise as a test of liver function, turned their attention to a study of the metabolism of chloral. This compound was found to break down in the body into trichlorethyl alcohol which, in turn, is conjugated with glucuronic acid and excreted as urochloralic acid. In dogs with either acute or chronic liver damage from carbon tetrachloride, chloral

hydrate administration produced a well-marked increase in the level of free chloral in the urine, while in normal dogs, chloral ingestion resulted in only very slight excretion of free chloral.

The many methods used by different workers for the determination of glucuronic acid (471) (473) (474) are all modifications of Tollen's naphthoresorcinol reaction.

d. Other processes of detoxication

From time to time other tests of the detoxifying function of the liver have been proposed. Pelkan and Whipple (20) have studied phenol conjugation in dogs by determining the time required for the liver to conjugate a standard dose of p-cresol. The test was found to be inadequate for measuring the high limits of liver reserve, and in chloroform poisoning the output of conjugated cresol did not fall below normal until more than one-third of the liver parenchyma had been damaged. However, more severe liver damage was paralleled by increasing impairment of the liver's ability to conjugate phenols, and conjugation was prevented in animals by the elimination of the liver. Although the test is quite specific, p-cresol is too toxic for clinical use. Pelkan and Whipple suggest that it might be possible to find a similar compound more suitable for use in humans. Presumably, in their experiments, p-cresol was conjugated with glucuronic acid, but the authors determined analytically

only the phenol component of the conjugate, and did not suggest what the other component might be.

Foster and Kahn (475) have suggested that a study of the excretion of ethereal sulfates after the ingestion of thymol might be of value, because of the important role of the liver in sulfur metabolism.

Studying the conjugation of sulfanilamide by tissue slices, Harris and Klein (476) found that the reaction was effected by liver slices, but not by muscle, spleen, kidney, or blood. The conjugate was shown to be acetyl sulfanilamide. The acetylation of sulfonamide in the liver was further studied by Carryer and Swanson (477), who reasoned that since in humansthe drug is excreted in the urine in a state of partial acetylation, the functional state of the liver might be reflected by variations in the percentage of acetylated drug. Normal individuals were found to conjugate 40 to 60 per cent, and patients with hepatic disease 20 to 60 per cent of the dose of sulfanilamide given. The data did not correlate with the severity of liver disease as indicated by several other liver function tests (bromsulfalein excretion, prothrombin time, and the hippuric acid test) and the authors concluded that the extent of acetylation of sulanilamide was of no value as an indication of liver function.

Cholesterol is continually synthesized and broken down in the body; recently Bloch et al (418) demonstrated its formation in surviving rat liver slices by adding either

heavy water or acetic acid containing isotopic carbon to the buffer fluid. The esterification of cholesterol also takes place in the liver; normally, about 70 per cent of the cholesterol in the blood is esterified. In obstructive jaundice the total cholesterol (free and esterified) is usually elevated, because it cannot be excreted in the bile. A normal ester ratio, however, is maintained in the absence of severe hepatic damage. A fall in the percentage of esters to less than 50 per cent of the total cholesterol is strongly indicative of damage to the liver (532). The ester ratio is seldom depressed until the results of other function tests are abnormal however (119) (347) (388). Rimmerman et al (317) found an increase in the ester percentage in cirrhotics receiving a diet high in carbohydrate, protein, choline, and the vitamin B complex. Although Upham and Chaikin (713) found the total blood cholesterol and the ester ratio of value in the differential diagnosis of jaundice, White et al (389) report that the cholesterol ester ratio is of value only in the prognosis of acute liver disease.

That the liver plays an important part in the inactivation of steroid hormones has been shown by several workers. Liver damage in rats from carbon tetrachloride (478) (479) or from cirrhosis-producing diets (480) increased the activity of endogenous or exogenous oestrogens. Even a diet deficient in the vitamin B complex (481) (482) (483) caused sufficient liver injury to reduce markedly the inactivation

of oestrogens; androgens were little affected by such hepatic damage, however (484). Selye (485) utilized the anaesthetic action of certain steroids to show that these compounds had a much more pronounced activity in partially hepatectomised animals. The mechanism of the detoxification remains to be elucidated.

8. Nitrogen Metabolism a. Amino-acids, uric acid, and urea

The liver plays an important part in the metabolism of nitrogen-containing compounds. The deamination of amino-acids, the formation of urea from ammonia, and the conversion of uric acid to allantoin which takes place in all mammals except man and the higher apes, are known to be performed by the liver. Severe chloroform poisoning in the dog (21) (490) always causes an increase in the total nitrogen excreted; the blood and urinary amino-acid-nitrogen rises, and the blood and urinary nitrogen falls. There is a conspicuous increase in the excretion of uric acid, which may reach nearly five times the normal level. Apparently the mechanism of uric acid destruction is disturbed rather easily (486); the urea-forming capacity is lost only in extremely severe liver injury (487).

Most workers have found that liver injury caused little impairment in the ability of the liver to deaminate amino-acids. In humans with liver disease and in dogs poisoned with chloroform and phosphorus, the urinary and plasma

amino-nitrogen values remained within the normal range (488) (489). Witts (487) states that there is no evidence that the amino-acid content of the blood or urine is increased in liver diseases other than acute yellow atrophy. Polson (286) has used a glycine tolerance test in a study of experimental shale oil necrosis of the liver in rabbits. Progressive liver-cell damage was paralleled by progressive impairment of glycine tolerance in the consecutive daily tests. Such favourable results were not obtained in humans, however. Although there was a marked increase in amino-acid excretion following glycine ingestion, this increase was no greater in patients with hepatic disease than in normal persons, nor were there any significant differences in the plasma amino-nitrogen or urea concentration which might be of diagnostic value (487) (488). Lyttle et al (491) administered casein hydrolysate intravenously in ten per cent solution, and found that seldom was there a delayed removal of the amino-acids from the blood in liver diseases other than cirrhosis.

Recent workers, however, have drawn attention specificaly to the amino-acid tyrosine. A clue to its metabolism was provided by the clinical observation that tyrosine crystals are found in the urine in only two types of disease. In cases of rapidly degenerating tumors and extensive skin lesions, the amino-acid apparently comes from the tissues in amounts too large to be completely utilized or catabolised in the body. The fact that the crystals were found in the urine

in cases of liver disease indicated the importance of the liver in the metabolism of tyrosine (492).

Attention was then turned to the occurrence of free tyrosine in the blood (493) (494). In normal fasting individuals the amino-acid was found only in traces or not at all, depending largely on the sensitivity of the analytical method used. Tyrosinemia was demonstrated in 80 per cent of cases of liver disease; it was found more frequently in cases of diffuse liver damage than in focal injury to the liver.

The development of a tyrosine tolerance test followed quickly upon these observations. Jankelson and colleagues (492) had found that in normal dogs, large amounts of tyrosine injected intravenously disappeared from the blood filtrate within half an hour. The test was applied to humans by Bernhart and Schneider (495). Blood tyrosine was determined by a modified Millon reaction; the reaction is not specific for tyrosine, but for the characteristic phenolic hydroxyl group of tyrosine. The term "tyrosyl" was introduced to refer to tyrosine and closely related metabolic products which give the test. The normal fasting level was found to be from 1.0 to 1.8 mg. "tyrosyl" per 100 ml. of blood. In some instances of severe hepatic injury this level was significantly elevated beyond the upper normal limit. The new test appeared to be more sensitive than the bromsulfalein and other common tests of liver function, but no further work on the test has been reported.

Loeper et al (496) suggest that in cirrhosis of the liver there is a greater prognostic value in tyraminemia than in tyrosinemia; tyramine, produced in the intestine, is normally deaminated, oxidised, or conjugated in the liver. Felix and Teske (497) have proposed a liver function test based on the following reactions:

$$\text{d-tyrosine} \xrightarrow[\text{kidneys}]{\text{liver}} \text{p-hydroxyphenylpyruvic acid} \xrightarrow{\text{liver}} \text{acetoacetic acid} + \text{carbon dioxide.}$$

The test necessitates maintaining the patient on a standard diet for six or seven days; on the third day two grams of p-hydroxyphenylpyruvic acid is given and its concentration in the urine measured by the Millon reaction.

b. Plasma proteins

The wealth of conflicting evidence concerning the source of the plasma proteins has been reviewed recently by Madden and Whipple (498) (499), who have concluded that the liver plays an important part in their formation. Although there is little agreement at present as to whether the plasma contains more than one completely independent protein fraction, yet for clinical purposes, the plasma proteins are conveniently represented as a mixture of albumin, globulin, and fibrinogen. The three plasma proteins have been characterized through differences in their solubilities. Albumin is soluble in water but is precipitated by saturation of the solution with ammonium sulfate. Globulin may be salted

out from solution by half-saturation with ammonium sulfate; fibrinogen although resembling globulin, differs in being insoluble in a 0.75 molar solution of sodium sulfate, and in a half-saturated solution of sodium chloride. The globulin fraction may, in turn, be divided into two fractions, euglobulin and pseudoglobulin, the former being insoluble in 1.0 molar sodium sulfate solution. Prothrombin, a globulin, has been the subject of much study since the discovery of vitamin K, and a separate section will be devoted to a discussion of prothrombin and the other factors concerned in the coagulation of the blood (see p.124).

The liver appears to be the source of the plasma albumin, and much of the globulin (498). Under certain circumstances, however, almost any tissue may contribute to the globulin fraction. Partial hepatectomy in rats caused a depression of the plasma proteins within twenty-four hours; the albumin fraction remained low for a period of four weeks. Total hepatectomy in dogs (500) resulted in a small loss or dilution of the total protein, with a similar change of albumin content, a marked loss of fibrinogen and euglobulin, and an increase in the plasma pseudoglobulin content. For periods up to thirty hours after removal of the liver, there was little evidence of loss or addition of protein to the plasma. There was apparently no regeneration of plasma proteins after plasmapheresis in hepatectomised animals.

In the immature rat, carbon tetrachloride hepatitis

caused a lowering of the plasma proteins, almost entirely at the expense of albumin (501). Whereas pregnancy in rats resulted in lowered globulin, hepatitis induced during pregnancy was the cause of a low level of albumin and a high globulin level post-partum (502).

The experimental evidence indicates, therefore, that the albumin level is much more closely related to the condition of the liver than is the globulin concentration. In humans, the average normal value for the plasma albumin is 4.3 per cent, and for globulin 2.8 per cent; the normal ratio of these two proteins in the plasma is 1.5 to 1. When the albumin concentration is lowered in liver disease, the ratio is frequently inverted, and much emphasis has been placed upon the "A-G ratio" as a diagnostic aid.

The plasma protein level is closely dependent upon the nutritional status of the body (498), however, as well as on the condition of the liver. Food protein cures the hypoproteinemia and oedema of malnutrition, provided neither infection nor liver disease is present. Beef serum and liver proteins are particularly effective in the regeneration of plasma proteins.

On the other hand, a fasting dog may be kept in nitrogen equilibrium and even in weight equilibrium for many days, if plasma protein is given intravenously. There is no change in the "A-G ratio"; apparently the body uses both albumin and globulin at about the same rate to carry on its

normal internal protein metabolism. Therefore changes in the A:G ratio should be due more frequently to variations in production rather than to lack of use of the normal globulins.

A reversal of the A:G ratio has been observed on a basal diet containing only a small amount of vegetable protein (503). A liver diet raised the production and output of albumin, and brought the ratio back to normal.

The evidence for the presence of a reserve store of protein in the liver is overwhelming. In the dog, the quantity of materials available is at least sufficient to replace 40 to 60 per cent of the circulating mass of plasma protein originally present. It appears that the plasma proteins are part of a balanced system of body proteins, and that there is a steady state or "ebb and flow" between them and a portion of the cell and tissue body proteins (498).

There has been much clinical evidence pointing to the liver as a source of the plasma proteins. Hypoalbuminemia resulting in a diminution or inversion of the A:G ratio has been found in almost every case of chronic advanced liver disease (388) (504) (505) and occasionally in cases of acute liver disease (506). Neither the level of the serum proteins nor the A:G ratio, however, has met with general favour as an indicator of liver function because of the severe pathological changes required to alter the normal levels as measured by chemical fractionation.

Stacey (505) in a study of cirrhosis of the liver in the people of Iraq, found that the serum euglobulin was well above normal in all the cirrhotics studied. This investigator has suggested that the serum euglobulin and the euglobulin-albumin ratio may be a more sensitive index of abnormal protein formation in cirrhosis than the total globulin or the A:G ratio.

Within recent years several workers (507) (508) have undertaken studies of the serum proteins by means of electrophoretic analysis, in the hope of achieving a less artificial separation of the proteins than that resulting from the usual methods of fractional precipitation. It was found that the electrophoretic process separated from albumin certain globulin components which are present in the chemically-precipitated albumin fraction, with the result that lowered albumin and higher globulin fractions, and a lower A:G ratio, were obtained.

The globulin component is subdivided by electrophoresis into several components, designated as alpha, beta, gamma, and phi globulins. In many cases of liver disease, electrophoresis has shown both quantitative and qualitative changes in the serum proteins, when the results of chemical fractionation have been normal. The quantitative differences were reflected in a decreased albumin and a decreased A:G ratio, very frequently found in acute parenchymatous damage in which the proteins, as determined by chemical methods,

were usually normal. The qualitative changes in the globulin fraction are of special interest. Gray and Barron (508) found the most characteristic change in liver disease in humans to be a large increase in the alpha globulin; significant alterations in the beta-globulin fraction were also noted. The changes were greater and more frequent in cirrhosis than in acute parenchymatous disease. Zeldis and Alling (507) report that in dogs with acute and chronic cholangitis, the serum albumin was low, and the beta-globulin level was abnormally high, when determined by electrophorectic analysis.

The electrophoretic studies are of interest not only because of the greater sensitivity of the electrophoretic method in detecting changes in the protein components, but also because of the contributions which have been made in determining the mechanism of the flocculation of colloidal gold and cephalin-cholesterol emulsions (vide infra).

c. Flocculation tests i. Takata-Ara test

The condition of the liver has been studied by the use of several flocculation tests, the results of which appear to be related to qualitative or quantitative differences in the serum proteins. One of the oldest of these is the Takata-Ara test, in which varying dilutions of serum are treated with measured volumes of sodium carbonate and mercuric chloride solution, and the degree of precipitation

observed. Although it was hoped at first that the test would serve to differentiate between cirrhosis of the liver and other liver diseases (509), many workers have found the results of the test to be positive in almost any chronic severe liver disease, in some cases of acute hepatocellular damage, and in malignancy involving the liver (388) (390) (494) (504) (510) (511) (512) (513).

Various attempts have been made to elucidate the mechanism of the flocculation reaction. Magath (390) found some degree of correlation between the results of the test and changes in the A:G ratio, but Horejsi (514) did not find any such definite relationship. Bowman and Bray (512) report that the test gave positive results when the total protein or the A:G ratio was low, while Kirk (513) states that flocculation may be obtained in any disease in which the globulin level is elevated.

Horejsi (514) found that the reaction could be changed by the addition of albumin, and by small quantities of cysteine. This amino-acid not only prevented flocculation, but the floccules already formed were easily dissolved when cysteine was added to the solution. On the basis of these results, Horejsi has proposed a theory to explain the mechanism of the reaction.

ii. Cephalin-cholesterol flocculation test

In 1938 Hanger (515) reported that sera from normal and from diseased persons differed in their power to

flocculate a cephalin-cholesterol emulsion. The emulsion was seldom flocculated by normal sera or by sera from patients without hepatic disease, but was usually flocculated by sera from patients with liver damage. In catarrhal jaundice, the degree of flocculation(graded from one - to four - plus) was found to parallel the severity of active liver disease. These observations were soon confirmed by a number of workers (516) (517) (518) (519) (520) (521) (522). The evidence concerning flocculation in cases of obstructive jaundice or focal lesions of the liver is conflicting, but, as Rosenberg (516) has pointed out, flocculation in these cases will depend largely on the amount of parenchymatous damage present.

The test appears to be an index of the degree of disturbance of the liver parenchyma, rather than a test of hepatic function (523). Many investigators (524) have found it to be a more sensitive index of hepatic disease than many of the functional studies. While Hanger (515) reported that the results of the test did not run parallel to changes in the blood bilirubin, cholesterol, phosphatase, or Takata-Ara test, Lawson and Engelhart (518) found that in most of their cases, the results of the cephalin-cholesterol and the Takata-Ara tests agreed. Furthermore, Pohle and Stewart (517) claim that flocculation is usually accompanied by a lowered prothrombin level and decreased formation and excretion of hippuric acid.

The problem of the "false positives" in normal controls has caused much confusion. Whereas Pohle and Stewart (517) encountered none in nearly three hundred healthy individuals, Lawson and Engelhart (518) found one-plus reactions in fourteen per cent of their normal controls, and Lippman and Bakst (519) consider all one- and two-plus reactions doubtful. The occurrence of false positive reactions has been frequently attributed to the use of "unripened" or "unoxidized" cephalin (518) (522) (524) (525).

The mechanism of the flocculation reaction is not entirely clear. Hanger (523) originally suggested that the presence of liver damage might alter the globulin components of the plasma in such a way that they attach themselves to the colloidal elements of the emulsion, with the result that there is a change in the surface potential of the colloidal particles, leading to flocculation. Recently Kabat, Hanger, and colleagues (526) have separated gamma-globulins from normal and pathological human sera by electrophoresis; these proteins showed marked activity in flocculating colloidal gold (see p. 120) and cephalin-cholesterol emulsions. Addition of electrophoretically separated albumin to the gamma-globulin inhibited the colloidal gold reaction, but did not significantly inhibit cephalin-cholesterol flocculation.

Mirski and von Brecht (527) have drawn attention to an anomaly discovered in their studies of Brüger's "fractional" cephalin-cholesterol flocculation test (521). Whereas with

0.2 ml. of undiluted normal blood serum a negative reaction occurred in nearly every instance, a positive reaction was noted when 0.1 ml. or less of saline-diluted serum was employed. Flocculation was almost invariably noted with greater dilutions of serum. It is clear from all these results that further work is needed to explain the mechanism of the flocculation of cephalin-cholesterol emulsions by normal and pathological sera.

iii. Colloidal gold test

That the colloidal gold test frequently yields positive results in liver damage was shown by Gray (528) (532) in 1939, when he obtained positive results in 89 out of 96 cases of hepatic disease, and negative results in 73 out of 75 patients with various extrahepatic diseases.

Noth and Loew (529) found the colloidal-gold test in liver disease to be less specific and less sensitive than did Gray, but MacLagan (530) (531), modifying the technique by the introduction of a buffer solution, reported that the test was a valuable indicator of liver damage, and of use in the differential diagnosis of jaundice.

No relationship has been found between the results of the test and quantitative changes in the serum globulin level or albumin-globulin ratio. Gray (528) believed the euglobulin content of the blood serum to be the determining factor in the precipitation of colloidal gold, and the serum

albumin a protective agent against precipitation. The work of Kabat et al (see p. 119) lends weight to these assumptions.

d. Prothrombin and other factors involved in blood coagulation

i. Nature of the coagulation process

The mechanism of blood coagulation has not yet been entirely elucidated, even after half a century of research. It is clear, however, that at least four constituents of normal blood are required for clotting. For the purpose of our discussion we may represent their relationship by the following classical scheme:

$$\text{Prothrombin} \xrightarrow{\text{thromboplastin} + Ca^{++}} \text{thrombin}$$

$$\text{Fibrinogen (sol)} \xrightarrow{\text{thrombin}} \text{fibrin (gel)}$$

It is now suspected by certain authorities that a thromboplastin activator is also necessary in the first stage of this mechanism.

Normally, thromboplastin is confined to the blood platelets and tissue fluids, and is liberated when the platelets and tissue cells disintegrate. Macfarlane (533) and others claim that the platelets are a relatively unimportant source of thromboplastin, but rather act as nuclei for the formation of fibrin. Studies on thromboplastin (534) (535) (536) have led to the isolation of a water-soluble lipoprotein which shows thromboplastic activity in quantities as small as 0.008 gamma of protein. The phospholipids of the prosthetic group are "an exdeedingly complex mixture".

Both protein and phosphatide components are necessary for maximal thromboplastic activity. Heparin displaces the lipid constituent of the protein to form a heparin-protein complex with marked anticoagulant properties. Alcohol or ether extracts of tissues contain thromboplastic lipids which are associated with the crude cephalin fraction; the most active preparations are soluble in alcohol and are not identifiable with any of the known phosphatides (537) (538).

Prothrombin and fibrinogen are proteins which have been prepared in an almost pure state (539) (540), and have been made available commercially in practically pure form by Cohn's laboratory at Harvard. Quick (541) has recently published evidence which indicates that prothrombin is composed of calcium ions, and two separable components designated as A and B, differing in their lability towards oxidation, and in their ease of adsorption on aluminium hydroxide. Apparently dicumarol poisoning (see p. 144) results in the disappearance of the B component from the plasma.

Whereas Quick considers calcium ion to be an essential part of the thromboplastin molecule, Loomis and Seegers (542) believe it to be merely a catalyst in Reaction I above. These workers, and Richards and Johnson (543), have substituted strontium chloride for calcium chloride, and have found the reaction rate to be much slower, and the optimum molar concentration much higher, when strontium is used.

Prothrombin and thrombin are non-dialysable, carbohydrate-containing proteins. Solubility curves of purified thrombin indicate the presence of two active components with different solubilities. Chargaff (544) says that the best evidence supports the assumption of an enzymatic effect for thrombin. Chargaff (544) and Brinkhous (545) have reviewed the present knowledge concerning the nature of the coagulation factors and their roles in the clotting of blood.

Several other substances have activating or coagulating properties. Trypsin clots blood in the presence of oxalates which remove the calcium ions. Tagnon et al (546) (547) have prepared from chloroform-treated human plasma a proteolytic enzyme which, like trypsin, can change prothrombin into thrombin in the absence of calcium or thromboplastin, and have expressed the belief that this enzyme plays a primary role in normal blood coagulation.

Venoms have a thromboplastin-like action. Papain and some venoms are able to clot fibrinogen. Certain organic compounds such as chloramine-T, alloxan, and ninhydrin, can coagulate blood in the absence of calcium or thromboplastin.

Ferguson (548) has recently proposed a new theory of blood clotting, in which he postulates that a colloidal disturbance causes activation of blood tryptase. This enzyme, optimally in conjunction with ionized calcium and free phospholipid, converts prothrombin into thrombin. Macfarlane (549) has suggested that the tissues contain a venom-like

enzyme and a lipoid factor, which together constitute "thromboplastin". However, since these theories were put forth, Chargaff has prepared from lung tissue the thromboplastic protein previously mentioned. Highly purified, extremely potent preparations of this protein showed no proteolytic activity.

Because of the advances which have been made in purifying the various components of the clotting process, great strides should be made within the next few years in solving the problem of blood coagulation.

ii. Prothrombin, the liver and vitamin K

The bleeding tendency associated with many cases of jaundice and other liver diseases puzzled investigators for many years. The question was of great practical importance, and most workers concentrated their efforts on studying variations in the calcium (550) and fibrinogen levels of the blood, without much success. Judd et al (551) reviewed in 1935 the experimental data concerning the part played by these substances in the coagulation defect. As the authors state, the tendency to bleed varies in general with the duration and the degree of jaundice, and can occur in hepatic disease in the absence of clinical jaundice, being associated most frequently with severe damage to the liver parenchyma.

About this time several workers turned their attention to prothrombin which, unlike the other coagulation factors,

lacked an accurate method for its quantitative estimation. Procedures were worked out which were of necessity presumptive, because of the difficulty in separating prothrombin from the plasma; they are based on the assumption that the coagulation time in the presence of excess thromboplastin and an adequate amount of calcium is proportional to the prothrombin concentration. The inadequacies of this assumption have been discussed by Macfarlane (549).

Studies of the prothrombin concentration in the blood led to the important discovery that the bleeding tendency found in liver disease is caused by a low level of prothrombin. Warner et al (552) produced liver damage in dogs by administering chloroform, and reported that in twenty to thirty hours after chloroform poisoning the plasma prothrombin had fallen to less than five per cent of normal; recovery was complete within one week. The fall in fibrinogen was less extreme. Phosphorus poisoning, resulting in more severe liver damage, caused an equally great fall in prothrombin before death (553). The same workers (554) subjected rats to partial hepatectomy; the plasma prothrombin decreased markedly, gradually returning to normal during the period required for restoration of the liver to its normal weight. Postoperative fibrinogen values were found to be normal or high. Total hepatectomy resulted in a rapid fall in the plasma prothrombin concentration, accompanied by a decline in fibrinogen (555). These workers

failed to produce a prothrombin deficiency in dogs through haemorrhage, plasmapheresis, laparotomy, or three hours of ether anaesthesia.

Lord, however, showed that massage of the liver for 25 minutes resulted in a profound decrease in plasma prothrombin, with a slow recovery lasting for six days (556).

About 1937 it was shown by many workers that vitamin K is necessary for the formation of prothrombin in the liver, and conversely, that the liver is necessary for the action of vitamin K in promoting prothrombin formation (557). The liver plays a further role in forming bile salts, which are necessary for the absorption of fat and fat-soluble vitamins, including vitamin K (679).

Flynn and Warner (558) produced a bleeding tendency in rats by a combination of dietary regulation and bile duct ligation, and cured the condition by the administration of vitamin K. On a diet containing 20% mineral oil, which is non-assimilable and carries fat-soluble vitamins with it into the faeces, rats developed a prothrombin deficiency within five weeks, corrected either by administration of large amounts of the vitamin, or by a return of the animals to the control diet (559).

Lockhart et al (560) apparently caused an unexpected avitaminosis K by feeding rats a synthetic diet containing a large amount of dihydroxystearic acid; within a month the animals developed haemorrhages which could not be cured with,

or prevented by, administration of the vitamin. The subject of experimental hypoprothrombinemia has been reviewed by Rhoads (561) and by Andrus (562).

Clinicians, too, reported lowering of plasma prothrombin levels in various pathological conditions, and administered vitamin K in the hope of raising these levels. It rapidly became obvious that while such treatment was of great benefit in cases such as obstructive jaundice or biliary fistula, where absence of bile from the intestine prevented the absorption of the fat-soluble vitamins, yet in many conditions such as cirrhosis, the low prothrombin concentration was a consequence primarily of defective hepatic function, and not of vitamin K deficiency. By studying the prothrombin levels before and after the administration of vitamin K and bile salts or, later, one of the water-soluble compounds having vitamin K activity, valuable information has been obtained concerning the condition of the liver (187) (563) (564) (565((566) (567) (568) (569) (570) (571) (572) (641) (642) (680) (681). Pohle and Stewart (567) were among the first to suggest that in the absence of obstruction, external biliary fistula, vomiting, diarrhea, starvation, or abnormal intestinal absorptive surface, the plasma prothrombin concentration should serve as a measure of liver function. Numerous papers appeared in the literature, some favouring the use of the prothrombin level as a test of liver function (647) (680), others condemning it

(566) (575) (576) (577). Wilson (573) (574) found a correlation between the plasma prothrombin concentration as measured by the two-stage method, and the hippuric acid excretion, but no such correlation was found between these tests and the other liver function tests performed on the same patients. Other workers (575) (576) (577), using one-stage methods, found no correlation between prothrombin levels and hippuric acid excretion, or the pathological condition of the liver. Grueber and Dyckerhoff (572) report that they obtained no relation between the Takata-Ara reaction and the prothrombin level.

Sweet and colleagues (578) report a better correlation between the prothrombin concentration and the histological appearance of the liver, than between the prothrombin concentration and the results of the hippuric acid test. Delor and Reinhart (579) have found that as liver function is diminished, the prothrombin, bilirubin, and total cholesterol levels of the blood, and the results of the hippuric acid and bromsulfalein tests, roughly paralleled each other. Andrus and Lord (565) and Brinkhous (545) have reviewed the physiology of the plasma prothrombin and its relation to liver function.

Morlock and Hall (580) have suggested that factors other than the concentration of prothrombin may be of significance in explaining the abnormalities in blood coagulation in severe liver damage; they found a definite decrease in

platelets in one-sixth of eighty cases of cirrhosis.

Honorato and Acosta (588) prepared thrombin from the plasma of cirrhotics and found it to be of maximum potency, so that the actual prothrombin concentration appeared to be normal; these workers suggest that the apparent decrease in prothrombin was actually due to an excess of anticoagulants.

Apparently a nutritional deficiency of vitamin K is a rather rare occurrence, for very few cases have been reported in the literature (581) (582) (583). Javert and Macri (584) observed, however, a lowering of the prothrombin concentration in two-thirds of their patients taking mineral oil daily, and in one-third of those taking it intermittently. Murphy and Clark (585) have reported a case of apparent idiopathic hypoprothrombinemia. Although the liver was apparently normal, administration of K was without effect in raising the prothrombin.

In haemophilia, aplastic anaemia, and thrombocytopoenic purpura, the prothrombin concentration is not reduced (570) (586) but leukaemia and burns are associated with low prothrombin and a poor response to vitamin K, indicating concomitant liver damage (597). Reduced plasma prothrombin levels are found in many other diseases, such as pneumonia, active tuberculosis, relapses in pernicious anaemia, chronic debility, chronic ulcerative colitis, and many other infections (589) (688) (689). A reduction of plasma prothrombin has been reported following administration of sulfonamides

(187) (188), and arsenicals (590), Barnes (591) found a temporary decrease in plasma prothrombin in rabbits whose bodies had been subjected to large doses of X-rays.

Anaesthesia in animals has not been found to cause a lowered prothrombin concentration, even when chloroform was used, provided no surgical procedures were undertaken (592) (593). In humans, neither ether nor cyclopropane anaesthesia affected the prothrombin level, although chloroform caused a fall in prothrombin (594). On the whole, it seems therefore that the lowered level of prothrombin following operation on the liver or biliary tract is a result of other factors, such as trauma to the liver, and blood loss at operation; that the normal liver function is depressed may be inferred from Quick's observation that the response to vitamin K is markedly reduced after operation (595). Reid (568) points out that the restricted post-operative diet may be deficient in vitamin K.

It is interesting to note that Shapiro and colleagues (596) found the level of prothrombin to increase, about six to ten days after operation, concomitant with the occurrence of thrombocytosis and thrombo-embolisation in these patients. Stewart (187) has reported thrombosis in two patients with a prothrombin concentration of more than 110 per cent of the normal level.

Only recently have workers succeeded in producing a state of hyperprothrombinemia by the administration of vitamin

K and bile salts. Neither Rhoads et al (561) nor Stewart (187) were able to cause such a condition in humans. Field and colleagues (597), however, have found increased prothrombin levels, above the pre-test normal values, in dogs, rabbits, and rats, after single oral doses of vitamin K.

Field et al (598) have induced hyperprothrombinemia in animals by giving the methylxanthines caffein, theobromine, or theophylline. In the dog the effect lasted from four to five days and counteracted the action of dicumarol. The drugs reduced the hepatotoxic activity of chloroform as reflected by changes in prothrombin, and the investigators have suggested that there is produced a functional stimulation of the hepatic tissue. Choline and methionine were inactive, indicating that the methyl groups were not responsible for this effect; other purines, pyrimidines, and related compounds were also without activity.

Numerous studies of the prothrombin level in infants have led to the conclusion that the concentration of prothrombin in the blood falls to its lowest level on the second to the fifth days of life (599) (600) (601) (602) (603) (604) (605), gradually returning to normal at the end of a week or ten days. Administration of vitamin K to the babies (601) (602) (603) will cause an increase in prothrombin to the normal level. If given early enough, even if administered to the mother prior to or during delivery (602) (606) (607), the vitamin will prevent the customary fall in prothrombin.

Increases in prothrombin level in humans during pregnancy have been reported by Adams (608), Brambel and Loker (609), and by Javert and Macri (610). The problem has been studied in rats by Field et al (611), who found that the prothrombin was high during the last week of pregnancy, and that in both pregnant and lactating rats there was an abnormal resistance to the hypoprothrombinemia induced by dicumarol. These workers suggest that this phenomenon may be related to the increase in liver weight occurring during pregnancy and lactation.

iii. Methods for the determination of prothrombin

The many methods for the determination of prothrombin concentration are in general modifications of the two original procedures; the "one-stage method" of Quick (612) (613), and the "two-stage method" of Warner et al (552) (553) (614). The controversy over the relative merits of each procedure still rages, and the issue is obscured by the present day presumptive and empirical nature of the whole problem. The two-stage method consists of dividing the coagulation process into, first, the conversion of prothrombin into thrombin, and second, the measurement of the thrombin concentration. In the first step, a globulin fraction of plasma, presumably containing almost all of the prothrombin, is precipitated from plasma, redissolved in oxalated saline, and dialysed. Addition of an optimal concentration of calcium and an excess

of thromboplastin causes the prothrombin to be converted completely into thrombin. The thrombin concentration is determined by titration with fibrinogen solutions. Quick's one-stage method, which has been modified by many workers, consists merely in adding calcium and thromboplastin to plasma or blood, and measuring the time required for coagulation. From the standpoint of practicability alone, workers are unanimous in choosing the one-stage method, but many feel that actually the two-stage procedure is more reliable and sensitive (187) (570) (615) (616).

The results of the two methods agree fairly well (617), and the simpler one-stage procedure is considered adequate for the detection of the tendency to bleed which requires vitamin K therapy (574) and for the disclosure of severe liver damage. However, under certain circumstances there is a definite discrepancy between the results obtained by the two procedures (589). Quick (618) has presented evidence which indicates that the rabbit has a concentration of prothrombin in the blood five times as great as the concentration in human blood. By the one-stage method, rabbit plasma diluted 1:5 has the same "prothrombin time" as whole human plasma, and 10 per cent rabbit plasma has the same "prothrombin time" as 50 per cent human plasma. Warner et al (619) feel that there is not actually a quantitative difference in prothrombin between the two species, but that the conversion of prothrombin into thrombin is five times as

rapid in rabbit plasma as in human plasma. During the coagulation reaction, oxalate was added to remove the calcium ions, stopping the conversion of prothrombin to thrombin at varying stages. Under these conditions it was found that thrombin was formed much more rapidly in rabbit plasma than in human plasma. Even when the plasma in the various samples was diluted so that the concentration of prothrombin was uniform, the conversion was more rapid in the rabbit than in the human. The elimination of known inhibitors of clotting by ammonium sulfate fractionation of prothrombin did not affect the results (619).

The fact that the two-stage method indicates lowered prothrombin levels in many human subjects with moderate liver damage where the results of the one-stage test are normal (620), has led to the application of this concept of varying conversion rates to such cases; Owen (621) has postulated that this is a compensatory mechanism whereby clotting times are normal despite insufficient prothrombin. Similarly, the one-stage tests do not show a difference in the prothrombin concentration of the young and of the adult; the two-stage method indicates that the prothrombin level rises only slowly to the adult level, over a period of many months (622). Quick and Grossman (605) suggest that the plasma proteins of the newborn may differ from those of the adult, resulting in a difference in the antithrombin content. The concept of variations in the convertability of prothrombin has been

neither widely accepted, nor rejected, and further discoveries of the nature of the coagulation process are required before the validity of the concept can be established.

Both methods have been criticized from many angles. Warner et al (553) and Owen et al (600) point out that the total clotting time is made up of the prothrombin conversion time, which may be affected by many variables of unpredictable importance, and of the time required for the thrombin formed to react with the fibrinogen present. In the one-stage tests, the thrombin phase overlaps the conversion phase to a varying degree and "the uncontrolled summation of these two reactions gives a clotting time which is very difficult to interpret in terms of prothrombin concentration " (553). Macfarlane (549) sums up a long attack by branding the one-stage methods as being sensitive to slight changes in the reactivity of the prothrombin, but relatively insensitive to changes in the prothrombin concentration; he claims that while the tests as usually performed may measure prothrombin, they certainly measure many other variables as well. This investigator states that the two-stage methods avoid many of the fallacies of the single-stage methods, but are so elaborate and introduce so many pitfalls of their own, that they are not yet available for ordinary laboratory practice. A similar criticism is expressed by Campbell et al (623), who feel that during the purification of the various components involved in the coagulation mechanism, variables other

than prothrombin may possibly be admitted. Quick (618), a staunch supporter of the one-stage procedures, points out that it has not yet been demonstrated that the thrombin obtained in the final dilution of the two-stage method is equal to the total that the prothrombin in the original mixture can yield. He also queries whether a substance such as prothrombin can be diluted several hundred times without undergoing a certain amount of degeneration, especially because of the disturbance of the protective colloids.

Ferguson (624) however, has studied by the two-stage method the assay recovery of prothrombin added to plasma, and has obtained excellent recoveries. The problem is still a complex one, with many theoretical as well as practical difficulties to be solved.

The two-stage method of Warner et al (552) (553) (614) has been modified by Stewart (563) Herbert (625), and Jacques (626).

The original one-stage procedure for the determination of prothrombin in the blood was proposed by Quick in 1935. The "prothrombin time" was measured by adding to oxalated plasma equal amounts of calcium chloride solution and thromboplastin, and observing the time required for coagulation. The relationship between the clotting time and the concentration of the prothrombin was not linear, but could be expressed by the curve

$$t = a + \left(\frac{k}{c}\right)$$

where c = concentration of prothrombin

t = time

a and k are constants which vary with the species and with the thromboplastin preparation used (612) (613) (627). Numerous modifications have been made in the original procedure. Scherber (628) advocates the use of a special constant temperature bath. Hause and Tocantins (629) have added a table for the calculation of prothrombin concentrations higher than 100 per cent of normal values. Owen and Toohey (630) and Innes and Davidson (631) have reduced the procedure to a micro scale; the latter workers have used Russell viper venom (see p. 140) as a source of thromboplastin. Oxalated or citrated whole blood has been employed by many (603) (607) (632) (633) (634), with results essentially the same as those obtained with plasma. In Abramson's modification (635), however, unoxalated capillary blood is added directly to a mixture of calcium chloride and thromboplastin.

Ziffren et al (617) in 1939 introduced the "bedside test", which employs only whole blood and thromboplastin. The results of this method agreed within fifteen per cent with those obtained on the same patients by the two-stage procedure, and it was considered a satisfactory measure of the bleeding tendency caused by low prothrombin levels. The test has been modified to a micro scale by several workers (604) (634) (636) (637) (638) (639) whose methods vary only in the relative amounts of reagents used; by these procedures

prothrombin may be measured in capillary blood, rather than venous blood.

In Quick's method, the prothrombin time is decreased only two or three seconds by a fall in prothrombin concentration from 100 per cent to 40 per cent of normal (640). Since there may be individual variations of two or three seconds in the normal prothrombin time, and since successive determinations do not check within less than one or two seconds (690), it is impossible to determine accurately by this method variations in prothrombin concentration within the range of fifty to one hundred per cent of the normal level. While this is not important from the viewpoint of merely detecting a bleeding tendency, it severely limits the use of the determination as a measure of liver function. The curve of the prothrombin time flattens out, however, over the range of concentrations below 50 per cent, and many workers realized that more reliable results might be obtained by measuring the coagulation time of plasma diluted to 25 per cent or less. Thus developed the use of "serial dilutions" (581) (582) (593) (609) (631) (643) (644) (645) (646) (647) (648) (682). It was found that frequently the study of diluted plasma showed variations in prothrombin concentration which were undetected when whole plasma was used (609) (647).

Shapiro et al (649) found that the difference between the prothrombin time of whole and dilute plasma was a more reliable indication of prothrombin activity than was the

prothrombin time of either alone. This difference was fairly constant in normal individuals; when prolonged it indicated hypoprothrombinemia, and when reduced, it suggested the presence of abnormally high prothrombin levels, and was frequently followed by thrombo-embolisation. That hyperprothrombinemia may be the cause of such a condition has been suggested by several workers (609) (649) (see p.130).

Thromboplastin solutions have been prepared from ox and dog lungs, but brain tissue is most frequently used. Brains from the chicken, rat, rabbit, sheep, calf, horse, and stillborn foetus have been employed by various workers (559) (568) (628) (650) (651) (652) (653) (654) (655) (656). Although Kaump and Greenwood (653) found rabbit brain to be superior to calf brain and to rabbit or calf lung as a source of thromboplastin, horse brain is recommended by Kazal and Arnow (656) as a more economical source of the coagulation factor.

That there is a difference in the activity of thromboplastin from different tissues, and from different animals, and that there is a difference also in the response of the blood of these animals to thromboplastins, was shown by Quick (651) and by Copley (650). Apparently thromboplastins are most active upon their homologous plasma, and less active upon the plasma of other species. Even thromboplastins prepared by different methods from the same tissue may vary in activity. Quick's method (652) of making a saline extract of the

dried acetone-extracted rabbit brain results in a very active preparation which will coagulate rabbit plasma in 6 seconds and human plasma in 12 to 15 seconds. When a simple saline extract of rabbit brain is used, the prothrombin times are delayed to almost twice these values. Souter and Kark (654) have recommended using the less active preparation, because a difference of one or two seconds in the prothrombin time would represent a smaller change in prothrombin concentration than when the much more active extract was used.

According to Quick (652), thromboplastin is rather unstable under ordinary conditions. It may be stored for several months at 5° C., as a saline solution in stoppered vials or as the dried powder, without loss of potency (654) (657) (658).

Many workers have preferred to use commercial Russell viper venom as the thromboplastin reagent in the one-stage estimation of prothrombin, obviating the necessity of preparing tissue extracts and affording a reagent of standardized activity (631) (647) (659) (660) (661) (662), yet producing comparable results. Lecithin used in conjunction with venom accelerates clotting, and its use is advocated by some workers (646) (660), but Page et al (663) found that the reduction in prothrombin time was greater when coagulation was prolonged, than when normal, so that the use of lecithin reduced the diagnostic possibilities of the test.

Variations between different batches of venom have been shown to be insignificant (664). The dried powder, in nitrogen-filled ampoules, is stable for more than five years, and solutions of the venom retain their activity for four months at room temperature (665) (666).

The venom can be dissolved directly in the calcium chloride solution, making the coagulation end point sharper (667) (668) (669) (683).

Several workers have shown that excess calcium inhibits the coagulation mechanism (616) (660) (670). The optimum range apparently falls between M/30 and M/200. Although Pohle and Stewart (671) advocate determining the optimum calcium concentration for each analysis, Hobson and Witts (660) have found no evidence that the optimal calcium concentration varies appreciably with the sample of plasma, and Stein (616), in a study of the problem, reports that the usual concentration employed (M/40) falls sufficiently well within the optimal range to make this concentration adequate for routine use. The calcium concentration will, of course, be affected by variations in the amount of oxalate used as an anticoagulant.

Cheney (672) has proposed a test in which no thromboplastin is used, but varying amounts of calcium are added to oxalated plasma and the shortest clotting time noted. The test, he claims, is a "simple clinical test to determine the desirability of administering vitamin K therapeutically".

It is not, of course, an accurate measure of prothrombin concentration.

Although prothrombin in plasma or in solution is gradually destroyed on standing (540) (673) (674) (675) (676), even at low temperatures, this destruction is so slow that it is not important in the routine determination of prothrombin in blood or plasma when tests are carried out with reasonable promptness. Workers have found no decrease in prothrombin concentration in plasma allowed to stand at room temperature (678) or in an incubator (599) for an hour, or in a refrigerator for twenty-four hours (660) or for a week (623).

Page et al (662) report that the prothrombin time of oxalated, uncentrifuged blood usually increased after standing for two hours at room temperature, but was occasionally decreased, presumably as a result of the release of lecithin through haemolysis. Plasma prothrombin time increased slightly in plasma standing at room temperature for one and a half to five hours, but such increases were usually small.

Exposure of plasma to an air current at 38° C., was followed by a definite loss of activity (677) (678). Tocantins (677) reported that the addition of carbon dioxide corrected this loss, but Quick (678) found carbon dioxide to be of no benefit, and believed this to be a case of direct oxidation of the prothrombin to an inactive compound.

iv. Fibrinogen

The formation of fibrinogen in the liver is not as easily depressed as is prothrombin synthesis. Chronic chloroform intoxication (553) and partial hepatectomy in dogs (554), procedures which profoundly depress the prothrombin level, seldom cause a decrease in fibrinogen. In fact, small doses of hepatotoxins cause a rise in fibrinogen, just as tissue injury elsewhere in the body does. Foster and Whipple (684) believe this high level of fibrinogen to be a response to dead tissue and inflammation. Large doses of damaging agents cause a fall in fibrinogen, however, if sufficient injury to the liver parenchyma results. In clinical jaundice, the fibrinogen concentration is rarely diminished, and may even be elevated (640). In chronic ulcerative colitis, the prothrombin concentration is lowered, but fibrinogen is above the normal level (685). No consistent relationship has been found between the plasma fibrinogen and prothrombin concentrations in normal humans and in those with a variety of diseases (563) (573) (686).

The question has arisen whether a decreased fibrinogen concentration in the blood interferes with the accurate determination of prothrombin. Quick (612) found that the addition of purified fibrinogen to plasma did not alter the speed of clotting. Witts cites a case of congenital fibrinopoenia, in which the blood was found to contain no fibrinogen. When this blood was mixed with normal plasma, the prothrombin

time was not significantly affected until the fibrinogen concentration was less than 30 per cent of the normal level. It is generally believed that a lowered fibrinogen concentration is seldom a factor in prolonging the prothrombin time of blood (555) (644) (687).

v. Dicumarol

The prothrombin concentration of the blood is decreased to very low levels by dicumarol (3,3'-methylenebis-(4-hydroxycoumarin)), the compound causing haemorrhagic sweet clover disease in cattle. The "anticoagulant" is active in prolonging the coagulation of blood only in vivo, and the maximal effect is not reached until twelve to twenty-four hours after ingestion of the drug (691) (692). The hypoprothrombinemia is transitory (693), even after repeated doses. McGinty et al (694) report the occurrence of temporary hyperprothrombinemia in dogs during the recovery period.

Because dicumarol is less expensive than heparin, it has been employed in the prevention and treatment of thrombosis (693) (695) (696). Several workers (691) (693) (697) found that vitamin K was not effective in counteracting the hypoprothrombinemia produced by dicumarol, and it was believed that a blood transfusion was necessary to restore the prothrombin level quickly to normal. McGinty et al (694) reported that purified beef prothrombin given by vein to dogs served to counteract the effects of dicumarol. However, it was soon found that large doses of vitamin K given before,

with, or twenty-four hours after dicumarol, reduced the hypoprothrombinemic action of the drug (692) (693) (698) (699) (700) (701). Dicumarol is more effective in cases of hepatic or renal injury (695) (702), or vitamin K deficiency (692). Jaques and Dunlop (699) found that sodium phthalate in very large doses caused a transient lessening of the hypoprothrombinemia induced by dicumarol in dogs, but the effect was transient, and the compound differed from vitamin K in being active in the absence of the liver. The magnitude of the increased prothrombin time resulting from the administration of dicumarol was found to be very sensitive to changes in the calcium concentration used in the analysis (703).

Many studies of the toxicity of dicumarol have been made. Function tests have rarely demonstrated the presence of damage to the liver or kidney following repeated administration of the drug (693) (696) (697) (704) (705). Lehmann (693) gave moderate doses of the drug daily to rabbits for a month, and found no toxic effects on the circulation, respiration, composition of the blood, or on any of the viscera. Bollman and Preston (695) witnessed no harmful effects in dogs maintained for six weeks with very low prothrombin levels.

Pathological studies in animals, however, have shown that repeated large doses of dicumarol will cause death, with some necrosis of the liver (696) (706). Richards and

Cortell (706) point out, however, that their animals suffered from severe anaemia, which itself probably contributed to the liver lesions. Several investigators (691) (707) have noted congestion of the liver and generalised vasodilatation in dogs following the administration of a lethal dose of dicumarol.

The mechanism by which dicumarol acts in reducing the prothrombin concentration of the blood is not known. Link et al (708) have proposed several possible explanations to account for the latent period; one of these is that dicumarol must undergo a chemical change in the body before it is effective. A study of various analogues of dicumarol and structurally related compounds revealed that the only compounds showing anti-coagulant action were those which theoretically might yield salicylic acid or an o-hydroxybenzoic acid derivative, on breaking down. It was found that salicylic acid produced in animals a temporary hypoprothrombinemia, comparable in all respects to that caused by dicumarol, and preventable by large doses of vitamin K (708) (709) (710). Hypoprothrombinemia was also frequently discovered in humans receiving salicylate medication (709) (710) (711). Kabat et al (712) report that a number of indanedione derivatives have an effect similar to dicumarol in decreasing the prothrombin concentration of the blood.

e. Enzymes in the blood i. Alkaline phosphatase

The phosphatases hydrolyze the linkage between phosphoric acid and the hydroxyl group in a wide variety of compounds; of particular importance is the phosphorylation of glucose to glucose-1-phosphate and the reverse reaction, which take place in the liver. There are two phosphatases in blood; the one which has its optimum activity around pH 9 is commonly referred to as "alkaline" phosphatase, and that which is most active between pH 5 and 6 is known as "acid" phosphatase. It must be noted that the concentration of an enzyme is determined only by the amount of substrate broken down in a definite length of time; it is impossible to tell whether one is measuring an increased concentration or merely an increased activation of the enzyme.

Variations in the concentration or activity of "alkaline" phosphatase in the blood have been frequently studied in a variety of liver diseases. The enzyme is apparently excreted in the bile; it has been found in extremely high amounts in fistula and gall-bladder bile (714) and in the faeces (715).

An increase in the alkaline phosphatase activity of the blood of patients with jaundice was first reported by Roberts (716). Subsequent workers have confirmed this finding in cases of obstructive jaundice (714) (715) (717) (718), but in jaundice due to hepatitis, the results of determinations of serum phosphatase recorded by different observers vary

widely. Some claim that serum phosphatase is low in such cases, and that this affords a basis for the differential diagnosis of the two conditions (717) (718) (719) (720) (721) (722). However, many other workers have found elevated values of serum phosphatase in all types of jaundice except haemolytic jaundice (715) (715) (723) (724) (725).

Experiments with dogs have afforded similar data. After obstruction to the common bile duct, the serum phosphatase rose to very high levels (143) (715) (726), falling gradually to the initial value after relief of the obstruction. The production of toxic hepatitis with carbon tetrachloride, neoarsphenamine, and other drugs caused an elevation in the serum phosphatase (143) (726) (727) (728). Although the rise following obstruction of the biliary outflow was usually higher than that found in hepatitis, there was frequently some overlapping of values in the two conditions. An increase in the serum phosphatase was often one of the earliest signs of damage to the liver, and one of the last to disappear during the recovery period (727) (728). Cholecystectomy in dogs was followed by an increased retention of bromsulfalein and a rise in the serum phosphatase (398). Hyperthyroid dogs also had an abnormal retention of dye paralleled by an increased concentration of serum phosphatase (257) (258).

Hough et al (320) (321) found that the removal of protein from the diet of dogs was soon followed by an increase in serum phosphatase and retention of rose bengal. Addition

of protein to the diet reversed these findings.

Although the original hope that the determination of serum phosphatase activity might be a valuable aid in distinguishing between obstructive and non-obstructive jaundice has largely been abandoned, it is felt by many workers that the phosphatase level, when considered along with other tests, will provide valuable information about the condition of the liver.

The origin of the alkaline phosphatase in the serum has been the subject of much speculation. The bones and the liver have been suggested as sites of its formation (143). Maddock and colleagues (729) (735) found that hepatectomy in dogs resulted in a rise in serum phosphatase activity much more rapid than that found after obstruction of the biliary outflow. Evisceration caused only a slight terminal rise; removal of all the organs except the liver and kidneys was followed by no rise in serum phosphatase. These investigators concluded that the enzyme is made in the intestines (and perhaps in the kidneys and bones), and that it is utilized by the liver in the dephosphorylation of hexose-1-monophosphate. In the absence of the liver, the enzyme accumulates in the blood more rapidly than when only its excretion in the bile is prevented. In 1938 Freeman and Chen (730) made the curious observation that the serum phosphatase of a dog could be raised for several days by transfusing the animal with blood from a dog with obstructive jaundice.

Thannhauser et al (731) reported a similar phenomemon in vitro; sera with high phosphatase values were able to increase the hydrolysis of the substrate when mixed with normal serum in equal proportions. They suggested that some substance was present in these sera which caused an increased activation of the enzyme, and not an actual increase in concentration.

Cloetens (732) has isolated from liver and other tissues two alkaline phosphatases with somewhat different properties. Phosphatase I is inactive in the absence of magnesium ions; its activity is inhibited by fluoride, but not by cyanide. On the other hand, phosphatase II does not require magnesium, but is inactive in the presence of cyanide. Drill and his colleagues (733) (734) have investigated the effects of these ions on the alkaline phosphatase of the serum. It was found that the addition of sodium cyanide to the substrate reduced the increased phosphatase values in humans and dogs with hepatic damage to the normal level, but not below this level. Magnesium and fluoride had little effect on the activity of the enzyme. It has been suggested that the increase in phosphatase in liver disease is due to an increase in Cloetens' "alkaline phosphatase II". The authors failed to mention whether the study included any cases of obstructive jaundice; the results, however, promise to shed new light upon the origin and behaviour of serum phosphatase in disease.

11. Diastase and choline esterase

Diastase or amylase hydrolyzes starch to maltose. The concentration of the enzyme in the blood is largely independent of nutritional factors and is remarkable constant in any one individual. Liver damage is associated with a decrease in the diastatic activity of the blood (736) (737) (738); Gulzow (737) has suggested that this fall results from the impaired metabolism of carbohydrates in the damaged liver. Bartlett (739) believes the decrease in blood diastase in thyrotoxicosis to be evidence of impaired liver function.

McArdle (740) has found the determination of choline esterase in serum useful in the estimation of liver function and in the differential diagnosis of jaundice.

9. Carbohydrate Metabolism a. Galactose and fructose tolerance

The liver is the site of the conversion of many sugars into glycogen. It is reasonable to suppose that damage to the liver should interfere with this glycogenesis, and for many years the metabolism of fructose and galactose has been studied as a guide in the detection of pathological lesions of the liver. Galactose appears to be the sugar of choice for such a test. Fructose can be transformed into glucose by the stomach and the intestines (141) (741) as well as by the liver. There is a renal threshold for fructose, and very little is excreted in the urine, even after large

quantities have been ingested (742). On the other hand, galactose is only slowly utilized by the body, and in the absence of a renal threshold, is excreted to a large extent in the urine (743). In the hepatectomised animal, a much larger portion of the administered galactose is found in the urine (141). The utilization of galactose by tissues other than the liver is negligible (742).

Corley (272) found that the removal of fructose from the blood of rabbits was little affected by slight injury to the liver, but was prolonged after severe hepatic damage had been produced. Herbert and Davidson (741) have recommended a study of the changes in blood fructose following oral administration of the sugar, not as a test for the existence of hepatic disease, but rather as an indication of the extent of liver damage. Rennie (744) found that such a test frequently gave positive results in cirrhosis, but was, on the whole, less sensitive than the hippuric acid test.

The capacity of the liver to metabolize carbohydrates is apparently disturbed with difficulty. Bollman et al (141) found that animals with extensive cirrhosis, and animals from which 50 to 70 per cent of the liver had been removed, excreted essentially normal amounts of galactose following ingestion of the sugar. The utilization of galactose was definitely impaired, however, in the presence of acute hepatic damage produced by hepatotoxins such as phosphorus and carbon tetrachloride. Decreased galactose utilization or

"tolerance" was accompanied by hyperbilirubinemia and increased retention of bromsulfalein in the blood. Only when the animal was in the premortal stages from extensive hepatic destruction, however, was the amount of galactose excreted comparable to that excreted by a hepatectomised animal.

Clinically, the galactose tolerance test is usually performed by measuring the excretion of sugar in the urine after a 40-gram oral dose of galactose. In the presence of hepatic injury, incomplete conversion of this sugar into glycogen results in glycosuria above the normal level of 2.5 to 3 grams of sugar in five hours following galactose ingestion. After years of clinical use, the test has not found favour as a general test of liver function, although some workers have found it of value in distinguishing between hepatocellular and obstructive jaundice (389) (745) (746) (747). The results of the test, although usually strongly positive in severe and diffuse acute liver damage, were often negative in chronic liver disease (389). Because galactosuria is frequently accompanied by glucosuria, several investigators (745) (748) have advocated the differential fermentation of glucose in the urine with yeast before estimation of the sugar content.

Within the past few years various modifications of the galactose tolerance test have been proposed. Several workers (141) (749) (750) (751) found that the wide variations in

the rate of intestinal absorption greatly influenced the amount of galactose excreted. MacLagan (752) reported that the galactose excretion depended too much on the rate of urinary flow to be a reliable guide. As a result, many investigators have advocated following the disappearance of galactose from the blood after the intravenous injection of the sugar (749) (750) (751). The test has been of great value in the differential diagnosis of jaundice (751) (753), and has been of some use as a general test of liver function (754).

In thyreotoxicosis, the results of the galactose tolerance test following oral administration of the sugar are frequently abnormal. This abnormality has been attributed to both an increased intestinal absorption of the sugar, and impaired liver function (253) (753) (755).

b. Glucose tolerance

In the presence of liver damage, there is a disturbance in the normal rate of removal of glucose from the blood. Hall (228) found a diminished glucose tolerance in dogs after administration of acacia, and Soskin et al (756) believed the abnormal sugar tolerance curves found in toxemia to be evidence of concomitant liver damage. Pachman (757), however, in a study of acute hepatitis, found no correlation between the degree of icterus and the type of glucose tolerance curve; in many cases there was a decreased tolerance to

orally administered glucose when the response to intravenously administered glucose was normal. When the intravenous test showed a decreased tolerance, the results of the oral test were always abnormal. The glucose tolerance test has on the whole been little used as a general test of liver function, because of the fact that the level of the blood sugar is intimately regulated by an interplay of endocrine factors.

Althausen and co-workers (758) have made a study of a modified glucose tolerance test in which the blood sugar level was followed for three hours after the administration of insulin, glucose, and water. In humans, the presence of liver damage caused a terminal hypoglycemia, and these investigators reported that the test was able to detect latent functional impairment of the liver; the results of the test paralleled those of the Rose Bengal test.

The modified test was further studied in rabbits, and epinephrine, as well as insulin, was administered (759) (760). Fasting rabbits showed a reduced tolerance and a diminished response to epinephrine, both apparently due to the low liver glycogen concentration. This decreased liver glycogen level, however, did not produce the typical hypoglycemia found at the end of the test in humans with liver damage. In both phosphorus and chloroform poisoning, the first functional deficiency to appear was a failure to maintain the normal blood sugar level. Later, there was a progressive decrease in sugar tolerance, caused, at least in part, by the

deposition of only small amounts of liver glycogen. Epinephrine hyperglycemia was finally abolished entirely. During recovery, the blood sugar level, sugar tolerance, and response to epinephrine returned to normal.

There is a complex relationship between the liver glycogen and liver damage; low levels of glycogen increase the susceptibility of the liver to damaging agents, and these agents in turn decrease the liver glycogen (see p. 20). Althausen (761) showed that insulin administered to rabbits increased the glycogen content in a normal liver when it was low, but failed to do so when the liver was damaged.

Staub (762) reported in 1922 that fasting resulted in a decreased glucose tolerance, both in the intact animal, and in the perfused liver. He suggested that the low level of glycogen in the liver might be the cause of the decreased tolerance. Ikushima, too, found a decreased tolerance to glucose and fructose in rabbits in which the amount of glycogen in the liver was diminished, and an enhanced tolerance when the liver glycogen concentration was high (763) (764).

Meyer (41) claims, however, that the power of the liver to form glycogen is not impaired when its glycogen concentration is low. The rise in blood sugar following the administration of fructose to the fasted animals was the result of little or no formation of glycogen in the muscles; the fasted animals stored more glycogen in the liver in the four-hour period after fructose ingestion, than did the non-fasted

animals. Meyer suggests, however, that metabolic changes in the liver may influence the anabolism of glycogen in the muscles, and thus be the cause of the decreased fructose tolerance.

Butsch (765) showed that the continued intravenous administration of glucose to a fasted dog resulted finally in a definite sharp break in the glucose tolerance, at a point which corresponded to the maximum capacity of the animal for the storage of glycogen. After the test, the liver cells were distended with glycogen. The maximum amount of glycogen found in the liver was 20 per cent; the muscles contained from 3 to 4 per cent of glycogen.

Treadwell et al (766) (767) found decreased glucose tolerance in rats in which fatty livers had been produced by maintenance of the animals on a high fat, low protein diet. However, there was no direct relationship between glucose tolerance and either the fat or glycogen content of the liver.

10. Miscellaneous Tests a. Blood iodine content

De Courcy (771) (772) has drawn attention to the fact that iodine ingested in the food is excreted by the liver. In chronic cholecystitis the blood iodine value is increased, reaching extremely high levels if the common bile duct is obstructed. Moderately high values are found in cancer of the liver, but for some unexplained reason the blood iodine concentration is normal in cases of advanced cirrhosis of the liver.

function tests may be adapted to such a procedure. The use of a "composite test" appears to be a great step forward in the search for a simple procedure which will evaluate the condition of the liver.

EXPERIMENTAL PROCEDURES

The description of the experimental work falls naturally into two sections: (1) modification of procedures and analytical methods, and (2) production of liver damage in animals and the evaluation of the tests.

1. Procedures, analytical methods, and normal values for the tests. a. Galactose tolerance test.

The determination of the concentration of galactose in the blood after its intravenous administration has been found by many to increase the sensitivity and value of the galactose tolerance test. King, Harrison and Delory (144) used such a test in rabbits following carbon tetrachloride poisoning, and because the test in this form is easily performed on animals, it seemed worthy of further investigation.

A solution of galactose (50 per cent, weight/volume) was prepared by placing the sugar in a volumetric flask, dissolving it by heating the flask on a water bath, and, when cool, adding enough water to make the desired volume. The solution was filtered, sterilized, and dispensed aseptically into test tubes (30 ml. capacity) provided with plastic screw tops. The tubes were protected from the dust by covering with brown paper. To prevent the precipitation of galactose, the solution was kept at room temperature rather

than in the refrigerator. In all the tests performed, 2 ml. of this galactose solution (equal to one gram of galactose) per kilogram of body weight was injected into the marginal vein of the rabbit's ear.

The concentration of galactose in the blood was determined by the method of MacLagan (752) which required only 0.2 ml. of blood. The glucose in the blood was first destroyed by fermentation with yeast.

A suspension of one part of Fleischmann's yeast to two parts of water was made, and the yeast cells were washed several times by allowing them to settle out and resuspending them in fresh portions of distilled water. Such a yeast preparation was found to retain its activity after four months in the refrigerator. It is necessary to test each new lot of yeast to make certain that it is unable to ferment galactose.

Estimation of galactose

Reagents

Alkaline Copper Reagent: Dissolve 25 g. of anhydrous sodium carbonate, 20 g. of sodium bicarbonate, and 25 g. of Rochelle salt (sodium potassium tartrate) in 600 cc. of water. Dissolve 7.5 g. of copper sulphate ($CuSO_4.5H_2O$) separately in about 100 cc. of water. Introduce the copper solution into the carbonate-tartrate solution through the funnel, the tip of which rests on the bottom of the beaker, stirring the solution well during the addition to prevent loss of carbon dioxide. Add to the mixture 5 g. of potassium iodide and 0.175 g. of potassium iodate. Dilute to 1 litre.

Isotonic sodium sulphate (3%)

10% sodium tungstate

7% copper sulphate

1 N sulphuric acid (30 ml. pure concentrated sulphuric acid, d. 1.84. Pour cautiously and slowly into about 3 to 4 volumes water; cool, mix thoroughly, and dilute to one litre. Standardize against sodium hydroxide with phenolphthalein)

0.002 N sodium thiosulphate. This must be freshly prepared each day by accurately diluting N/10 sodium thiosulphate 50 times.

1% starch solution in saturated sodium chloride

Yeast suspension (see p. 161).

Procedure

One centrifuge tube of 15 ml. capacity is needed for each determination and one or two for the controls. Pipette 1 ml. of yeast suspension into each tube. Centrifuge the tubes and decant the supernatant liquid. Dry the tubes by draining and remove any remaining liquid by wiping the inside with pieces of filter paper. Add 2.2 ml. of isotonic sodium sulfate.

Pipette 0.2 ml. of blood into each tube, and mix the contents thoroughly with a fine glass rod. Let the tubes stand at room temperature for fifteen minutes. At the same time prepare a blank (preferably in duplicate) with normal blood, to check the completeness of the removal of glucose.

After fifteen minutes precipitate the proteins by adding 0.3 ml. of 7 per cent copper sulphate and 0.3 ml. of sodium tungstate. Stopper the tubes and shake after each addition. Centrifuge the tubes for five minutes.

Pipette 2.0 ml. of the centrifugate into a test-tube (125 mm x 20 mm) and add 2 ml. of alkaline copper reagent. Mix the contents of the tube by shaking and stopper the tube with cotton wool. Place the tube in a boiling water bath for exactly ten minutes, cool under running water for two minutes, and add 2 ml. of normal sulphuric acid. Titrate the contents of the tube immediately with 0.002 N. sodium thiosulphate, using a 5 ml. microburette. One drop of starch solution is added as an indicator.

The blank requires 4.8 to 4.9 ml. of thiosulphate. The difference between the blank and the unknown titre is

used in calculating the galactose.

A standard curve is prepared by setting up a series of yeast tubes containing 2.0 ml. of isotonic sodium sulphate and 0.2 ml. of galactose-free blood. Samples of 0.2 ml. of galactose solutions of known concentration are added to the tubes and the analysis is completed as described above. In this laboratory a straight line relationship was found between the galactose concentration and the difference between the blank and the thiosulphate titre, from 0 to 220 mg. of galactose per 100 ml. of blood.

Since the first blood sample taken after the injection of galactose usually contained more than 200 mg. of galactose per 100 ml. of blood, it was necessary to use 0.1 ml. of this blood and 0.1 ml. of galactose-free blood for the determination. The blood samples (about 0.5 ml. of blood to allow for duplicate determinations) were collected in tiny test-tubes coated on the inside with oxalate.* Each tube was stoppered, mixed well with the oxalate to prevent clotting, and kept in the refrigerator until all the samples had been collected.

The concentration of galactose in the blood of a rabbit was determined at approximately fifteen minute intervals following the injection. The disappearance of the galactose from the bloodstream is illustrated in Figure 1. The results of two tests on the same rabbit are shown.

* The anticoagulant used throughout this investigation was made up of 2 parts of potassium oxalate and 3 parts of ammonium oxalate in 2 per cent solution. For each 2 ml. of blood to be collected, 0.1 ml. of the solution was used. The use of this oxalate solution is recommended by Heller and Paul (775) to prevent alteration in the red blood cells.

The galactose disappeared gradually from the blood over a period of three hours.

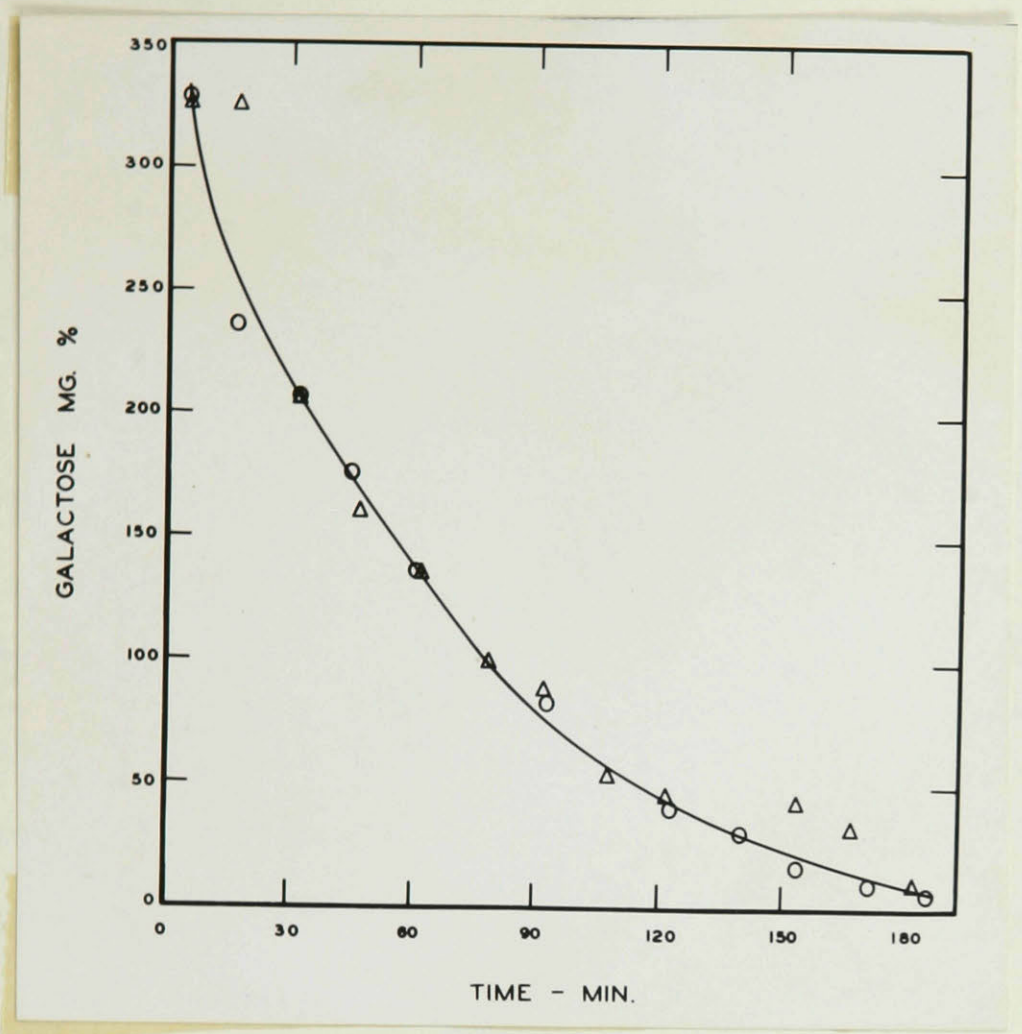

Figure 1.
Disappearance of galactose from the blood

Seventeen galactose tolerance tests were performed on normal rabbits by collecting blood samples at five minutes, one, two, and three hours respectively after the injection of galactose, according to the procedure of King et al. On the whole, higher values for the blood galactose concentration were found in these experiments than in King's. However, when those investigators simplified the test by replacing the one- and two-hour samples by one taken ninety minutes after the injection, the values found in the ninety-minute samples were higher (rather than lower) than the values found

at sixty minutes in the longer test. These ninety-minute values corresponded very closely with those found in the three such tests which were performed in this laboratory. It is possible that the inconsistency in King's results may be due to the use of a different strain of animals or to the performance of some of the tests during a different season of the year. Our experiments, which gave consistent results, were carried out during the colder part of the year (between November and March).

In Table I will be found a summary of the results of the tests performed on normal rabbits, and a comparison of the average, the highest, and the lowest of these values with similar values from the work of King et al.

TABLE I

Concentration of galactose in the blood after injection

	5 min.	1 hr.	1½ hr.	2 hr.	3 hr.
This laboratory					
Average value	304	122	72	51	10
Highest value	412	148	104	94	22
Lowest value	200	82	54	18	0
King et al					
Average value	208	57	73	24	4
Highest value	270	85	106	50	21
Lowest value	154	35	51	5	0

When the results of all the test performed were plotted in graphs, it became apparent that whereas the blood galactose values fell sharply during the first hour after the injection, the galactose concentration thereafter fell at a constant rate. It was obvious that the test could be simplified without sacrificing accuracy by determining the blood galactose concentration at five minutes, one, and three hours, rather than at five minutes, one and a half, and three hours as suggested by King et al.

MacLagan has introduced the term "galactose index" to represent the sum of the four blood galactose values found at thirty, sixty, ninety, and one hundred twenty minutes respectively after an oral dose of 40 g. of galactose in humans. The "galactose index", which affords an excellent method of expressing the results without requiring a curve, has been applied in this investigation to the sum of the blood galactose values, in milligrams per 100 ml. of blood, found at five minutes, one and three hours respectively after the intravenous administration of one gram of galactose (in 50 per cent solution) per kilogram of body weight. The normal values for the galactose index, calculated from the normal galactose tolerance curves determined in this laboratory, ranged from 362 to 566.

b. Bromsulfalein excretion test

The bromsulfalein test as usually performed presents

certain analytical difficulties. The usual procedure is to add a drop of sodium hydroxide solution to about 5 ml. of serum and compare the purple colour with a set of standards. Not only does this method require a large sample of blood but it cannot be used in the presence of haemolysis.

Several workers have used acetone or alcohol to precipitate the serum proteins in determining the concentration of rose bengal (320) and azorubin S (406) but Rosenthal and Lillie (407) reported in 1931 that acetone is not satisfactory for the extraction of bromsulfalein from serum. Robinson, on the other hand, used acetone precipitation with satisfactory results (776).

Several protein precipitants were tested in this investigation. Both trichloroacetic acid and zinc hydroxide were found to precipitate the dye along with the proteins. A simplification of Robinson's method was worked out and was found satisfactory.

Estimation of bromsulfalein

Reagents

20 per cent sodium hydroxide
Acetone

Procedure

Add 1 ml. of plasma or serum to a mixture of 3 ml. of distilled water and 8 ml. of acetone in a 15 ml. centrifuge tube. Stopper the tube and mix the contents well. Let the tubes stand for at least one hour, and then centrifuge for three minutes. Pipette 5 ml. of the supernatant liquid into a colorimeter tube, and make an initial "blank" reading for each tube in the photoelectric colorimeter. Add one drop of 20 per cent sodium hydroxide

> solution, mix the contents of the tube by shaking, and make another reading. The purple colour develops rapidly upon addition of the alkali, and is stable for several hours. Filter #565 (or #580) and the 6 ml. aperture are used.
>
> Bromsulfalein is available in 5 per cent solution in water. In the determination of the standard curve, a series of solutions of bromsulfalein in serum ranging in concentration from 0 to 10 mg. per 100 ml. of serum are used. The latter concentration represents complete retention when the customary dose of 5 mg. of dye per kilogram of body weight is given. The standard curve is a straight line from 0 to 10 mg. of dye per 100 ml. of serum; this range extends over the whole accurate range of the colorimeter.

Since acetone is rather volatile, it was necessary to keep the tubes stoppered. When this precaution was observed, reproducible results were obtained. In later work, it was found quite feasible to perform the determination with half the quantities originally employed, that is, with only 0.5 ml. of serum, 1.5 ml. water, and 4 ml. acetone. A 5 ml. aliquot was still taken for the colorimeter reading. The smaller blood sample also permitted more accurate timing of the blood samples.

Originally, the tubes were centrifuged almost immediately after the precipitation of the plasma proteins. Occasionally, however, the supernatant was slightly turbid, which resulted in a high centre setting in the colorimeter. It was noted that if the centrifuge tubes were allowed to stand for an hour or two before or after centrifugation, the turbidity was not found. When plasma was used, a precipitate, interfering with the colorimeter reading, occasionally formed upon the addition of the alkali to the acetone solution.

This precipitate was formed when a smaller than the desired amount of blood was obtained from the rabbit, and was traced to the excess of oxalate present in the sample. With serum or with plasma collected with a minimum of oxalate, this difficulty was never encountered.

In the performance of the bromsulfalein excretion test on rabbits, it was necessary to dilute the commercial bromsulfalein solution 1 to 10, to give a solution with a concentration of 5 mg. of dye per ml. A dose of 5 mg. of dye (or 1 ml. of solution) per kilogram of body weight was injected. Samples were usually taken five and twelve minutes following the injection; it was not always possible to obtain samples at exactly these times, but the time of the sample was noted, and from a curve of the normal results, the correct values could be calculated. The blood samples were collected from the ear vein into small beakers containing dried oxalate, or into graduated centrifuge tubes in which the blood was allowed to clot and the serum obtained. The latter procedure is preferable because of the ease with which the correct amount of blood is obtained.

The first dye excretion tests were performed on fed animals, and the normal values established. The normal retention of dye in the blood of the rabbit was found to vary between 25 and 35 per cent at five minutes and was usually below 10 per cent twelve minutes after the injection of 5 mg. of dye per kilogram of body weight. These results agree

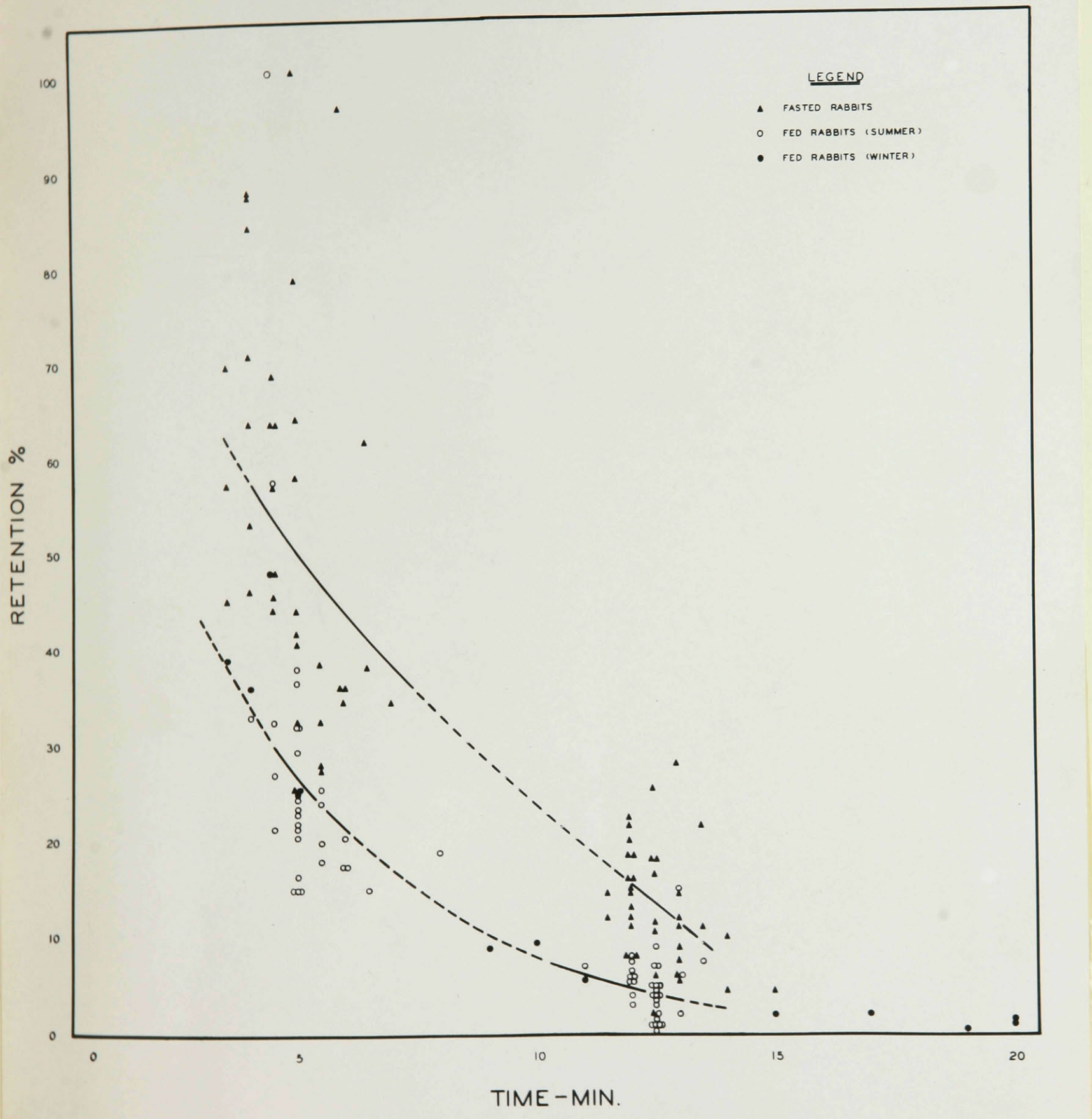

Figure 2
Retention of Bromsulfalein in the blood

well with those of Rosenthal and White (384). The degree of retention is calculated in the following manner: the plasma volume of the rabbit is assumed to be 50 ml. per kilogram of body weight; then if the amount of dye injected is "x" mg. per kilogram, 100 per cent retention would cause a concentration of 2x mg. per 100 ml. of plasma.

During the investigation, a series of tests was performed on fasted animals, and different degrees of abnormal retention were found. The same animals, when fed, were able to excrete the dye with ease. When fasted again, abnormal results were again obtained. The results of all these tests on fasted and fed normal rabbits are shown in Figure 2. The rate of dye excretion in fed animals was fairly constant; the values fell within a small range. The fasted animals showed great individual variations, however.

In Figure 3 are shown the results of the dye excretion test in one animal during periods of fasting, compared with the normal results obtained when the animal was fed. These results are typical of those found in ten of a series of twelve rabbits. Each rabbit showed a rather characteristic degree of dye retention when fasted. The degree of abnormality of dye retention upon fasting varied, however, from only a slight rise in some animals, to a very pronounced increase in others.

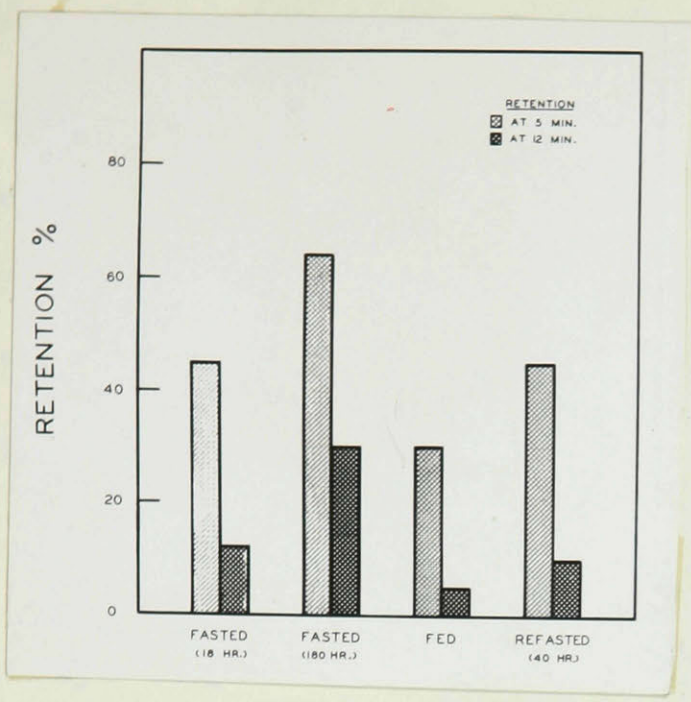

Figure 3

Dye retention in fasted and fed animals

An analysis of variance (Table 3), using the values in Figure 2 for retention of dye in the time intervals of $3\frac{1}{2}$ to 5 minutes, and $11\frac{1}{2}$ to 13 minutes after the injection, showed that the increased retention upon fasting was highly significant at the 0.1 per cent level.

TABLE II

Analysis of variance

Source of variance	Degrees of freedom	Mean square
$3\frac{1}{2}$ to 5 minute samples		
Between treatments (fasted and fed)	1	14102.29***
Within treatments	40	129.18
$11\frac{1}{2}$ to 13 minute samples		
Between treatments	1	1353.01***
Within treatments	46	17.02

*** Significant at 0.1 per cent level

The degree of retention after a fast of 7 to 8 days (180 to 204 hours) was almost invariably higher than that following a fast of 18 to 42 hours. Analysis of variance, Table III, shows that the increase is highly significant at the 5 per cent level in the case of samples taken at $11\frac{1}{2}$ to 13 minutes after injection of the dye.

TABLE III

Retention of dye $11\frac{1}{2}$ to 13 minutes after injection

	Fasted 18 to 24 hours	Fasted 180 to 204 hours
Rabbit #1	14.5 per cent	20.0 per cent
2	11.5	28.0
3	18.5	18.0
4	13.0	14.5
5	5.5	22.5
6	12.0	21.5
7	8.0	12.0
8	11.0	18.0
9	18.0	18.5

Analysis of variance

Source of variance	Degrees of freedom	Mean square
Length of fast	1	206.72*
Replicates (rabbits)	8	18.344
Length of fast x Replicates	8	21.004

* Significant at 5 per cent level

The slower removal of dye from the blood of fasted animals has been related to the low concentration of liver glycogen in these animals (45). The increased retention of dye in the second test would indicate that an eighteen- to

twenty-four-hour fast did not suffice to deplete the liver glycogen in these animals. It is obvious that in normal animals, consistent results in dye excretion tests are obtainable only when the animals are well-fed. Even an overnight fast is sufficient to cause a significant degree of abnormal dye retention in many animals.

c. Serum alkaline phosphatase

There are two standard methods for determining serum alkaline phosphatase activity. Bodansky's method (777) (778) measures the phosphate liberated from a sodium glycerophosphate substrate buffered with veronal. King and Armstrong's method (779) determines the liberated phenol from a buffered sodiumphenylphosphate substrate. The King-Armstrong method, as modified by Greenberg and colleagues (780), was tried in this laboratory, and proved to be very satisfactory. The Greenberg modification introduces the use of ammonium chloride-ammonium hydroxide buffer which was found to keep well for at least a month at room temperature. Another innovation is the use of a standard solution of tyrosine, instead of phenol, in the estimation of the phenol liberated. The method was adapted for use with the Evelyn photoelectric colorimeter by employing part of the method worked out for the determination of tyrosine in the blood. The quantities of reagents were reduced, and other modifications were introduced which simplified the procedure without reducing its accuracy.

Determination of serum alkaline phosphatase

Reagents

Buffer solutions: 0.5 M. ammonium chloride: Dissolve 2.675 g. of pure ammonium chloride in distilled water and make up to 100 ml. (or 26.75 g. to 1.0 litre).

0.5 M. ammonium hydroxide: Dilute 28 ml. of C.P. concentrated ammonium hydroxide to 1.0 litre and determine the exact strength by titration with standard acid solution using methyl red or methyl orange as an indicator. This reagent should be kept in a stoppered bottle to prevent loss of ammonia.

Buffer substrate mixture: Introduce 1.0 g. of disodium phosphate, 20 ml. of 0.5 M. ammonium chloride, and 20 ml. of 0.5 M. ammonium hydroxide into a 100 ml. volumetric flask. Dilute to volume with distilled water and mix.

Trichloroacetic acid, approximately 1.5 M. (24.5 per cent)

Sodium hydroxide, 2.3 per cent: This solution is used to neutralize the trichloroacetic acid introduced into the sample in order to precipitate the serum proteins. Titrate 3 ml. of the trichloroacetic acid with the sodium hydroxide and adjust their strengths so that 1.0 ml. of trichloroacetic acid is equivalent to 2.60 ml. of sodium hydroxide. It is necessary to do this titration only once for each set of solutions.

Sodium hydroxide, 20 per cent

Folin-Ciocalteu reagent: Transfer 100 g. of sodium tungstate ($Na_2WO_4.2H_2O$) and 25 g. of sodium molybdate ($Na_2MoO_4.2H_2O$) together with 700 ml. of distilled water to a 1500 ml. Florence flask. Add 50 ml. of 85 per cent phosphoric acid and 100 ml. of concentrated hydrochloric acid. Connect to a reflux condenser, protecting the stopper with tin foil, and boil gently for 10 hours. At the end of this time, add 150 g. of lithium sulphate, 50 ml. of water, and a few drops of liquid bromine. Boil without the condenser for 15 minutes to remove the excess bromine. Cool, dilute to 1.0 litre, and filter. There should be no greenish tint. Keep well-protected from dust.

Standard tyrosine solution: Weigh accurately 100.0 mg. of pure dry tyrosine and dissolve in approximately 0.1 M. hydrochloric acid solution to a final volume of 500 ml. In this solution, 5 ml. will equal 1.0 mg. of tyrosine.

A drop of merthiolate may be added to inhibit the growth of mold.

Procedure

Cell-free serum is used for the determination. The serum may be kept over-night in the refrigerator if necessary.

Into 10 ml. test-tubes pipette 4 ml. of substrate. Stopper the tubes and warm them in a water bath at 37° C. When the tubes have reached the temperature of the bath, pipette into each tube 0.2 ml. of serum and mix the contents well. At the same time, a series of control tubes containing only substrate are prepared. Incubate the tubes at 37° C. for one hour. At the end of this period, remove the tubes from the bath and cool them in ice water for five minutes. To each control tube add 0.2 ml. of serum. Precipitate the proteins by the addition of 1.0 ml. of trichloroacetic acid and centrifuge them after five minutes.

To measure the tyrosine equivalent of the phenol liberated, pipette into a test-tube marked at the 25 ml. level 2 ml. of the supernatant from the precipitation of the proteins. Neutralize the acid by the addition of 1.0 ml. of sodium hydroxide (2.3 per cent). Add about 15 ml. of water, 1.0 ml. of 20 per cent sodium hydroxide, and 1.0 ml. of Folin-Ciocalteu reagent. Add enough water to make the volume up to 25 ml. and mix the contents of the tube by pouring them back and forth into a colorimeter tube. Read the tubes in the colorimeter in two minutes, using Filter #660. The color fades rather quickly.

In the determination of the standard curve the standard tyrosine solution is diluted 1 to 10 with distilled water, and aliquots up to 10 ml. (equal to 0.10 mg. of tyrosine) are used. The standard curve is a straight line.

The phosphatase unit is defined as the amount of enzyme in 100 ml. of serum which will liberate in one hour under the prescribed conditions a quantity of phenol giving a color equivalent to that produced by 1 mg. of tyrosine. The amount of tyrosine found in the colorimetric determination is multiplied by a factor of 1300, and the difference between the test sample and the control sample is the phosphatase activity in units.

The original directions for the phenol reagent called for lithium sulphate. During the preparation of the second lot of this reagent, lithium sulphate was not obtainable.

However, it was called to our attention that Professor Folin had later omitted the lithium sulphate, and had prevented cloudiness in the final blue solution read in the colorimeter by adding urea to the alkaline solution used in the development of the colour. The reagent was prepared in the usual way, with the omission of lithium sulphate. It was found that turbidity prevented reading of the solutions in the colorimeter, and that the addition of 30 g. of urea to each 100 ml. of 20 per cent sodium hydroxide prevented the turbidity. Certain advantages were immediately noticed. The colour which developed was deeper, and did not fade as quickly as did the colour originally obtained. It was found that the maximal colour development was reached not in two minutes as before, but in five minutes, persisting for fifteen minutes without any appreciable degree of fading, and after that, fading very slowly. The increased stability of the colour greatly facilitated the routine determination of the phosphatase activity. However, on standing, the strong alkali decomposed the urea solution, with the liberation of ammonia. It was necessary to prepare two solutions; one of sodium hydroxide (20 per cent) and one of urea (30 per cent) and to add 1 ml. of each of these to the tubes in which the tyrosine was being determined.

The attempt to establish normal values for serum phosphatase was attended by many difficulties (Table IV). The values found covered a wide range (from 3 to 38 units) and

occasionally no phosphatase activity at all was demonstrable. Since these negative results were always associated with high values in the other determinations performed simultaneously, and since the determinations were always done in duplicate, it seems that any inactivating substance present must have been in the serum, rather than in the substrate or on the glassware.

TABLE IV

Serum phosphatase values in normal rabbits

Rabbit	1	2	3	4	5	6	7	8	9	10
June 11	None	17.6		19.0						
" 12							21.1	20.9	14.4	9.8
" 14	7.2	29.3	None	8.1						
" 21	None	20.1					4.3	5.2		
July 4							11.7		11.4	10.4
5	7.8	38.4	None	22.1						
9					7.1	6.5			6.5	
10	None		None					13.0		
12					4.6	None	None	4.0		

It has been shown by several workers that the serum phosphatase level is influenced by the diet. Bodansky and Jaffe (781) found that the phosphatase activity was higher in young rats maintained on a meat diet, than on the Sherman diet. Freeman and Farmer (782) (783) reported, however, that in adult rats these findings were reversed. In dogs, too, a meat diet favoured a lower serum phosphatase activity

than did a carbohydrate diet, presumably because of the importance of the enzyme in the metabolism of carbohydrate (783) (784). Fasting lowered the serum phosphatase in rats, guinea pigs, and dogs, and bleeding caused a decrease in the phosphatase level in rabbits.

Because of the wide variations among the phosphatase values in a series of rabbits on any one day, and in any one rabbit over a period of weeks, in spite of the fact that all the rabbits were subjected to the same treatment, it was impossible to establish any correlation between the serum phosphatase values and the food given, the amount of bleeding, or any environmental changes. The extreme heat and the obvious discomfort of the rabbits during the summer would affect the food intake and the water balance of the animals. The variations were probably the result of a summation of one or more of these factors in any one rabbit. In the course of the damaging experiments, the same variations were noted. Only in the presence of severe damage to the liver could any significance be noted in the fluctuations of the serum phosphatase values.

d. Plasma prothrombin

The methods used for the determination of plasma prothrombin, and their presumptive nature, have been described previously (see p. 132). Variations in prothrombin time between different individuals and daily variations in the same

person, have been observed by many workers (635) (649) (665). Hause and Tocantins (629) report that some normal men may have consistently high plasma prothrombin concentrations. Animals, also, show appreciable variations in prothrombin as is readily seen by an examination of some of the figures given in the literature. Campbell et al (623) report that although the prothrombin time of plasma diluted with seven parts of saline is relatively constant for any one rabbit, the normal range for a large number of animals extended from 20 to 40 seconds. Elliot et al (559) working with rats found the average prothrombin time for 50 per cent rat plasma to be 69.8 seconds, with a standard deviation of 9.8 seconds. Holmboe's values (655) range from 55 to 85 seconds, with an average of 70 seconds.

Quick (612) originally published a curve in which prothrombin concentration was plotted against time for coagulation by his method. The curve was obtained by combining the results obtained by measuring the prothrombin time of plasma diluted to various concentrations with saline or prothrombin-free plasma. Other workers have been unable to duplicate this curve, and since such a curve would vary according to the thromboplastin preparation used, and, in fact, would differ from one animal to another, values for prothrombin time taken from such a graph cannot be considered accurate, especially in the range from 50 to 100 per cent of normal. To report the prothrombin time only in seconds is

almost meaningless to other workers, and it is impossible to compare results from different laboratories where techniques and reagents may not be the same. The problem of a satisfactory method of expressing results has been frequently discussed (615) (616) (627) and many have advocated the use of one or more normal controls to check on the technique and the strength of the thromboplastin (567) (629) (635) (691) (653) (705). In view of the difficulty of accurately setting forth values in terms of a "normal", because of individual variations and variations from day to day in the same individual, and the factors of the thromboplastin preparation and the technique employed, the problem becomes exceedingly complex and indeed virtually impossible to solve with the present one-stage methods. In our investigation, enough thromboplastin was prepared to last throughout our experiments, and the potency of this preparation was checked at regular intervals. Normal values were established for each rabbit before liver damage was imposed. The values are expressed merely in seconds and while such results cannot be compared quantitatively with those obtained in other laboratories, they provide a better basis for determining variations from the normal in each animal.

The method used for the determination of plasma prothrombin concentration was essentially Campbell's modification (623) of Quick's procedure. About 1.5 ml. of blood from the rabbit's ear vein was collected in a small beaker

containing the dried oxalate mixture. The blood was centrifuged and the plasma removed. If more than a trace of haemolysis was present, the sample was discarded.

Determination of prothrombin time

Reagents

0.9 per cent sodium chloride ("saline")

M/40 calcium chloride solution

Thromboplastin suspension. Quickly remove the brain tissue from a freshly killed rabbit and free it from blood vessels by removing the meninges with a forceps. Grind the tissue to a paste in a mortar and extract three times with acetone. Dry the remaining tissue over-night and grind it up to form a powder which should be stored in the refrigerator.

To prepare enough thromboplastin for three determinations, suspend 0.1 g. of dried thromboplastin in 2 ml. of saline. The solution is stirred until a uniform suspension is obtained. Heat the suspension with stirring at 55° C. in a water bath for ten minutes to destroy prothrombin activity. Cool the suspension and add 2 ml. of calcium chloride solution. After stirring the suspension for 4 minutes, centrifuge the mixture for 4 minutes at 1700 r.p.m. to remove the large particles. Pipette the clear or slightly turbid supernatant solution into another test tube. Warm the reagent in the water bath to 37° C. before using it in the prothrombin determination.

Procedure

Dilute an aliquot of 0.1 ml. of plasma to 0.8 ml. with saline. Into each of three test tubes (10 ml. capacity) pipette 0.1 ml. of whole plasma. Into three more test tubes pipette 0.1 ml. of diluted plasma. The tubes are warmed in a water bath at 37° C. The prothrombin time is taken as the interval between the time when 0.2 ml. of thromboplastin-calcium chloride reagent is blown into the plasma, and the time when a clot is formed which can be caught on the end of the nichrome wire with which the plasma-thromboplastin mixture is continuously stirred.

The triplicate determinations almost invariably checked within 0.5 seconds for whole plasma, and 1.0 seconds for

diluted normal plasma. When the prothrombin time was prolonged beyond 25 seconds, the end point became less sharp and poorer checks were obtained.

A wide range of values was found for the prothrombin time of diluted and whole plasma. The former varied from 10.0 to 23.5 seconds, the latter from 8.0 to 14.5 seconds. However, inspection of Table V shows that for any one rabbit, the difference between the prothrombin time of whole and diluted plasma was relatively constant in spite of these variations, although occasionally the agreement was not as close as in the following examples.

TABLE V

Prothrombin times in normal rabbits

	Whole plasma	Diluted plasma	Difference
Rabbit #13	10.0	16.5	6.5
	11.5	18.5	7.0
	10.0	17.0	7.0
	11.5	17.0	5.5
Rabbit #16	11.5	18.0	6.5
	11.5	18.0	6.5
	9.0	15.5	6.5
	14.5	21.0	6.5
	11.0	18.0	7.0
	10.5	19.5	4.0
	13.0	19.5	6.5
	10.0	17.0	7.0
Rabbit #18	10.0	13.0	2.5
	11.0	14.5	3.5
	11.5	15.0	3.5
	13.5	16.0	2.5
	12.0	15.0	3.0
	13.0	16.0	3.0

Several times it was found that there was almost no difference (0 to 1.5 seconds) between the prothrombin time of diluted and whole plasma. This anomaly was noted three times in one rabbit, and once in two others. Somewhat similar aberrations have occasionally been reported by other workers.

Allen et al (644) found several patients whose prothrombin time was prolonged when whole plasma was used, but when the plasma was diluted, the prothrombin time approximated that of the diluted plasma of the normal control. Shapiro et al (649) encountered a rather similar condition, in which the prothrombin time of whole plasma was prolonged to fifty-one seconds, while that of diluted plasma was only forty seconds, a value within the normal range. The two-stage method indicated that the prothrombin concentration was indeed normal, and the phenomenon was attributable to the liberation in the blood of an excess of anticoagulant substances, which are rendered ineffective by dilution. Ferguson (624) has postulated that clotting inhibitors may cause clinical anomalies in prothrombin time determinations. Macfarlane (549) draws attention to the fact that the addition of thromboplastin to plasma from animals receiving dicumarol may actually inhibit coagulation. With the present methods of prothrombin determination, anomalous results are unavoidable.

A possible source of inaccuracy in our method lies in

the collection of the blood in a beaker to which is added first the amount of oxalate required by one and one half ml. of blood. Because of the difficulty in estimating the exact amount of blood which is collected, there is a certain variation in the proportion of oxalate present. However, Stein (616) has found that the calcium concentration may vary within rather wide limits without influencing the prothrombin time. Probably the variations resulting in this manner are much smaller than the normal daily variations.

e. Tyrosine tolerance test

In the application of the tyrosine test to rabbits, the chief modification necessary was in the analytical method. Jankelson (493) originally used the Millon reaction, and obtained a pink colour which was graded from one-plus to three-plus. Such a method would be of little value in a tolerance test. Bernhart and Schneider (493) developed a more accurate method using the Millon reaction and a photoelectric colorimeter. However, their method requires at least 5 ml. of blood for each determination, and is therefore unsuitable for repeated use in a tolerance test in small animals.

The possibility of using Folin's phenol reagent was studied, and a suitable method was worked out in which the quantity of blood needed is small, the procedure simple, and the degree of accuracy sufficient.

Determination of tyrosine in blood

Reagents

N/12 sulphuric acid

10 per cent sodium tungstate

5 M sodium hydroxide

Folin-Ciocalteu reagent (see p.175)

Standard tyrosine solution

Procedure

To 1.6 ml. of N/12 sulphuric acid in a centrifuge tube, add 0.2 ml. of blood from a blood pipette, and rinse the pipette. Add 0.2 ml. of 10 per cent sodium tungstate and mix thoroughly. Centrifuge the tubes and decant the supernatant liquid. To a test tube graduated at 25 ml., add 1 ml. of supernatant, about 15 ml. of water, 1 ml. of 5 M. sodium hydroxide, and 1 ml. of phenol reagent. Make the volume up to 25 ml. with distilled water and mix by pouring back and forth from the test tube into the colorimeter tube. The tubes are read after 2 minutes in an Evelyn colorimeter, using Filter #660. The discussion concerning the stabilization of the colour which follows the alkaline phosphatase method is equally applicable here (see p.177). The standard curve is prepared as described on page 176.

Up to 100 ml. of tyrosine per 100 ml. of blood may be determined by this method, with an error of no more than 5 per cent. Tyrosine added to blood was quantitatively recovered. In studying the recovery of tyrosine, however, it was necessary to use a solution of tyrosine in water, rather than in hydrochloric acid, as the small amount of acid added in this manner was sufficient to cause incomplete precipitation of the serum protein.

In humans, only traces of tyrosine are normally found in the blood. In rabbits, this is not the case. Values

between 18 and 35 mg. of tyrosine per 100 ml. of blood were consistently found, and there was no significant difference in the blood tyrosine in rabbits fasted, on a normal diet or on an oatmeal diet.

Tyrosine is very insoluble in pure water (solubility 0.04 g. per 100 ml.). It is therefore impractical to inject tyrosine intravenously in solution; it must be given orally. It was found that a suspension of tyrosine in water was absorbed as effectively as a solution of the amino acid in alkali or acid judging by the changes in blood tyrosine during the period of absorption.

There was no significant change in the blood tyrosine level during the three hour period following the ingestion of 0.1 g. of tyrosine per kg. of body weight when samples were taken at thirty minute intervals. A dose of 0.3 g. per kg. of body weight was usually followed by a temporary rise in the tyrosine concentration in the blood.

It was difficult to establish normal values for the concentration of tyrosine in the blood following its ingestion. The shape of the curves of tyrosine utilization varied considerably (see Figure 4). One animal persistently showed a decreased concentration of blood tyrosine thirty minutes following the ingestion of the amino acid, but showed a return to the normal value, or even an increase beyond it within an hour. The concentration of blood tyrosine three hours following its ingestion was sometimes higher, sometimes

lower, than the initial value.

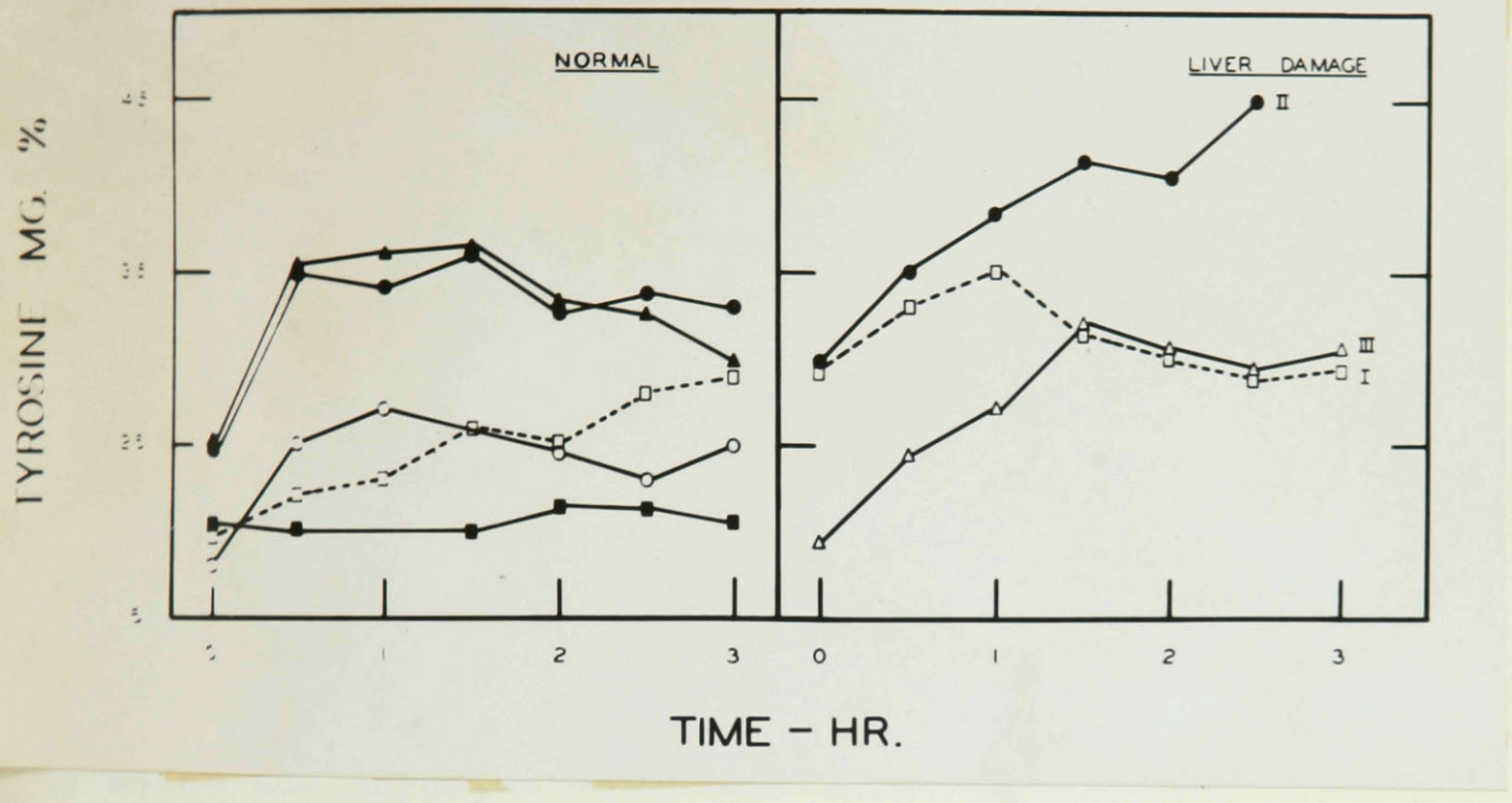

Figure 4
Tyrosine tolerance curves

The results obtained in animals subjected to damaging were also variable. In Figure 4 are shown several tyrosine tolerance curves determined in an animal which was given an oatmeal diet and a daily dose of 20 ml. of 24 per cent ethanol. Bromsulfalein excretion tests were performed for the purpose of comparison. After seven doses of ethanol, there was an increase in the dye retention, but the tyrosine tolerance curve was similar to that found in the animal before poisoning. After six more doses of ethanol, the bromsulfalein retention, still abnormal, had decreased slightly, while the blood tyrosine level three hours after the ingestion

of the amino acid had increased 30 mg. per 100 ml. above the pre-test value. The third bromsulfalein test, after another seven doses of alcohol, showed the same degree of dye retention, but the shape of the tyrosine tolerance curve was completely different, and the rise was much less. When the animal was killed one week later, pathological studies of the liver showed marked vacuolation and cloudy swelling throughout the lobules. There was no correlation between the degree of dye retention and the shape or height of the tyrosine tolerance curves, nor was any regular pattern evident in these curves. In the limited number of experiments in which the tyrosine tolerance test was used, the results were not promising.

2. Production of liver damage in animals. a. General considerations.

In our attempts to produce a slowly progressing type of damage, the powerful hepatotoxins carbon tetrachloride, chloroform, and phosphorus, were purposely avoided. A number of less drastic damaging agents was tried. Gum acacia was found impractical for prolonged experiments; large quantities had to be injected daily, causing such injury to the ear veins that it was impossible to perform more than a few liver function tests on the animals. Gum acacia, therefore, was abandoned. Neoarsphenamine was found to cause necrosis, followed by blocking of the vein, if the drug found its way into the tissue surrounding the vein. However,

neoarsphenamine proved to be a good damaging agent, if injected carefully into the vein, for it was necessary to administer the drug only once or twice weekly.

In general, a poison that can be given by stomach tube is preferable to one which must be injected, in long term experiments such as were undertaken in this work, where injections had to be made frequently in the performance of the tests, and blood samples taken repeatedly. On the whole, the administration of drugs by stomach tube has proved very satisfactory. With a little practice it is possible for one person to administer a liquid such as alcohol to a rabbit quickly and easily.

In this investigation, the susceptibility of the liver to damage was increased by fasting the animals for varying periods prior to the period of hepatotoxin administration, and by maintaining the animals on a deficient diet during this period. Because previous experiments in this laboratory had shown that an oatmeal diet increased the toxicity of certain compounds in the rabbit, the animals used in this investigation were fed oatmeal* during the damaging period and a well-balanced diet (hay, carrots, and Purina Chow) during the period of recovery. A supply of drinking water was always available.

* "Quaker Oats"

b. Galactose tolerance and bromsulfalein tests

In the first group of experiments several hepatotoxins were given to rabbits, and bromsulfalein excretion and galactose tolerance tests were compared.

i. Sulfanilamide

One gram of sulfanilamide* per kilogram of body weight, administered daily by stomach tube to two rabbits as a suspension in water, proved very toxic.

Rabbit #8 After a fast of five days, the animal was given a diet of oatmeal. On the sixth to ninth days, 3.5 g. of sulfanilamide was administered daily. The rabbit became very sick on the ninth day and on the morning of the eleventh day it died. An autopsy showed that the liver was pale orange in colour, the depot fat under the skin was practically depleted, the stomach was full of hard undigested material, and in the wall of the stomach were many haemorrhages. In a pathological study, the liver showed marked fatty changes throughout, especially in the central areas. There were definite degenerative changes in the tubular epithelium of the kidney, but no necrosis. The changes were not typical of the massive necrosis found in true sulfanilamide damage in humans.

Rabbit #7 The animal was placed on an oatmeal diet after a four-day fast. From the fifth to the eighth day, 3.7 g. of sulfanilamide was given daily. On the eight day the animal was very lethargic. The performance of a bromsulfalein test

* "Streptocide" C.E. Frosst & Sons

was attempted, but it was impossible to get more than a few drops of blood to flow from the ear vein because of circulatory impairment. Instead, a test was done using blood obtained by heart puncture. The results were only slightly abnormal (21 per cent retention at 6 minutes and 6½ per cent at 13 minutes; normal values - 20 per cent at 6 minutes, 5 per cent at 13 minutes). The animal died on the tenth day, having lost much weight during the period of administration of the drug. Its normal weight was 3.99 kg.; after a fast of 4 days it weighed 3.74 kg., and at death its weight had fallen to 3.15 kg. At post mortem examination the liver appeared dark red in colour. The stomach was full, and the intestines almost empty. Pathological studies showed a suggestion of early degenerative changes in the central liver cells, but this was indefinite and was confined to the periphery. There was slight degeneration of the tubular epithelium of the kidney.

ii. Alcohol

Among the most profitable experiments in this investigation were those in which alcohol was used as a hepatotoxin. Ninety-five per cent alcohol, diluted approximately 1 to 4 with water, was administered daily by stomach tube to two rabbits and the results of the bromsulfalein excretion and galactose tolerance tests were studied.

Rabbit #3 Before any alcohol was given to this rabbit, the results of both tests were found to be normal. The animal

was fasted for ten days, then given the customary diet of Purina Chow, hay, and carrots. Alcohol was given daily in increasing amounts, and on the eleventh day the diet was changed to oatmeal. The animal ate very little food and lost weight rapidly.

Galactose tolerance tests were performed weekly, starting on the nineteenth day, and on the day following each galactose test, a bromsulfalein excretion test was performed. Figure 5 shows the amount of alcohol given daily, the percentage change in weight, and the results of the tests. In the upper graph the galactose index is plotted, and the bromsulfalein test is represented by two columns, the first being the retention of the dye at five minutes, the second at twelve minutes after injection. In the lower graphs, the concentration of galactose in the blood and the retention of dye are plotted against the time after the injection. The figures above each curve show the day on which that test was performed. In the lefthand graph, the solid black line represents the normal galactose tolerance curve found in this animal before damaging was begun. The curve in the righthand graph represents the normal excretion of bromsulfalein from the blood. Because only two samples were taken in the routine performance of the dye excretion test, the results are represented by straight lines.

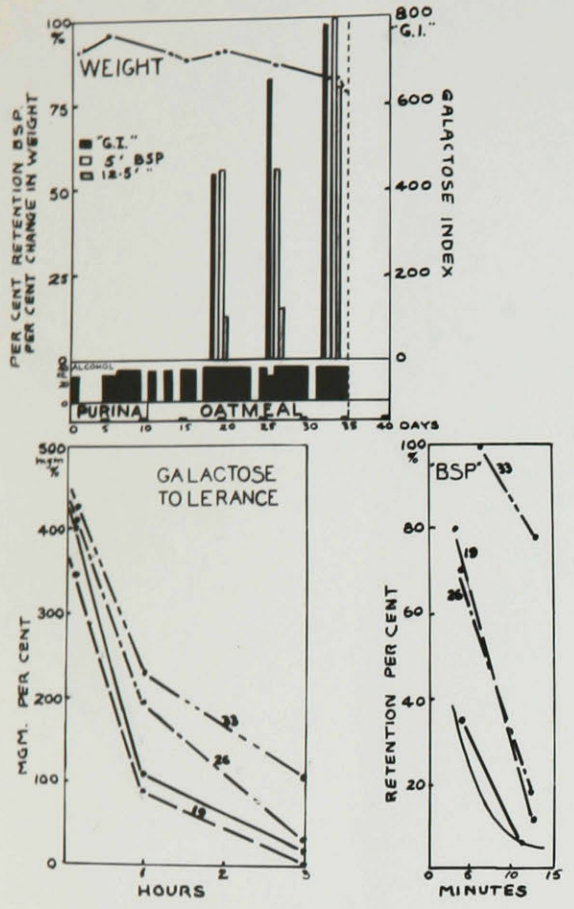

Figure 5 Rabbit #3

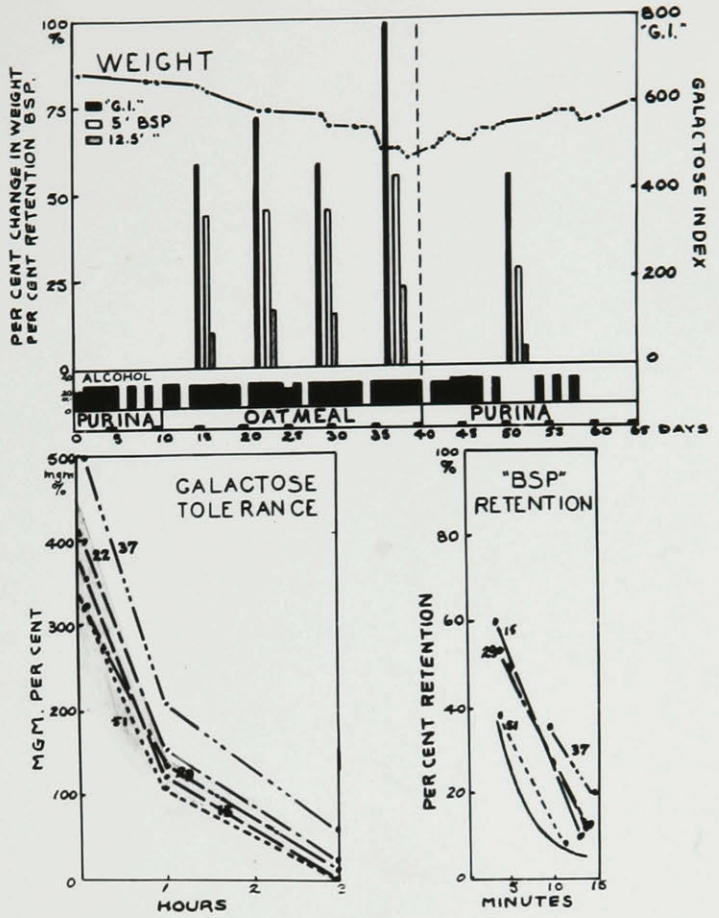

Figure 6 Rabbit #4

Rabbit #3 died on the thirty-sixth day, having received a total of 986 ml. of 24 per cent alcohol, an average dose of 38 ml. per day for 26 out of 36 days. At post mortem examination the liver was observed to be pale, small, and very friable, and the kidneys appeared slightly enlarged. Pathological studies showed extensive damage throughout the whole liver and kidneys. The liver was full of fat, and in addition showed necrotic changes. The convoluted tubules of the kidney also showed a much increased fat content.

TABLE VI

Rabbit #3

Day	Total Amount of Alcohol given	Bromsulfalein Test	Galactose Test	Galactose Index
Before	-	Normal	Normal	542
19	436 ml.	Slightly abnormal	Normal	440
26	666	Slightly abnormal	Slightly abnormal	656
33	906	Very high retention	Very high retention	780
36	986	Rabbit died		

Rabbit #4

The animal was fasted for fourteen days, then placed on a diet of Purina Chow, hay, and carrots, which was changed a week later to oatmeal. Alcohol was given daily, and starting on the fifteenth day, galactose and bromsulfalein tests were performed weekly. The results may be seen in Figure 6 and Table VII. The animal ate very little food and lost weight continually. On the thirty-seventh day the results of both tests were abnormal, and as it was feared the animal might die, the original adequate diet was again given. The animal gained weight once more, although alcohol was still being given, and after twelve days on this diet, showed normal galactose and bromsulfalein clearances. The weight levelled off somewhat and did not reach the normal level. The animal was not studied further after the tests returned to normal. The animal eventually died (136 days from the beginning of the experiment) when the investigator was absent, and no post mortem examination was performed.

TABLE VII

Rabbit #4

Day	Total amount of Alcohol given	Bromsulfalein Excretion	Galactose Tolerance	Galactose Index
15	317	Abnormal	Normal	474
22	497	No change	Normal	575
29	672	No change	Normal	468
37	852	No change	Abnormal	844
51	1207	Normal	Normal	437

iii. Neoarsphenamine

Neoarsphenamine was first administered in a dosage of 0.05 g. per kg. of body weight. The dose was increased when it was found to be fairly well tolerated. The drug was given intravenously every third or fourth day to two animals, and the results of the galactose and bromsulfalein tests followed.

Rabbit #5 The animal was fasted for fourteen days, then given Purina Chow, hay, and carrots for seven days; the diet was then changed to oatmeal. After a slight preliminary rise in weight, the animal lost weight continually. On the twenty-ninth and thirty-sixth days tests were done (see Table VIII). The rabbit died on the thirty-eighth day, and at post mortem examination the liver was small, yellowish, and very friable. A pathological study showed very diffuse fatty changes throughout the lobules of the liver, with patches of necrosis. The epithelium of the convoluted

tubules of the kidney was very much swollen and vacuolated, but there was no necrosis.

TABLE VIII

Rabbit #5

Day	Weight	Dose of Neo-arsphenamine	Bromsul-falein Excretion	Galactose Tolerance	Galactose Index
Before fast	3.49				
1	2.94	0.15 mg.			
8	3.09	0.15			
19	3.09	0.30			
23		0.30			
26	2.93	0.30			
29	2.88		Abnormal	Slightly abnormal	618
30	2.94	0.30			
34	2.71	0.30			
36	2.65		Very high	Very high	694
38	2.54	Animal died			

Rabbit #6 The animal was fasted for 14 days, then given Purina chow, hay, and carrots for seven days; on the eighth day the diet was changed to oatmeal. This rabbit was unusual among those being poisoned, in that its appetite was tremendous and it gained weight until it reached its pre-fasting level, and did not lose weight again until the dose of neoarsphenamine was raised to three times the original level (see Table IX).

TABLE IX

Rabbit #6

Day	Weight	Dose of Neo-arsphenamine	Bromsulfalein Excretion	Galactose Tolerance	Galactose Index
Before fast	2.82				
1	2.07	0.15 g.			
8	2.52	0.15			
19	2.72	0.27			
23		0.27			
26	2.85	0.30			
29	2.81		Slightly abnormal	Normal	402
30	2.83	0.30			
34	2.76	0.30			
36	2.81		No change	Normal	424
37	2.82				
39	2.82	0.45			
43	2.77	0.45			
46	2.78	0.45	No change	Normal	524
52	2.71		High retention	Normal	536

At this point unfortunately the rabbit's ears were so badly damaged that it proved impossible to carry out any further tests; however, the animal had begun to lose weight, and the change in bromsulfalein retention indicated that liver damage had occurred.

c. Prothrombin time, serum phosphatase and bromsulfalein test

The second group of experiments was performed during the summer months. Efforts to control the conditions adequately failed because of a number of deaths among the animals.

Several rabbits died in the early evening or during a week-end, and because considerable decomposition had occured when the deaths were discovered, it was useless to perform pathological studies. However, in a few rabbits excellent results were obtained. Variations in prothrombin time, serum alkaline phosphatase activity, and bromsulfalein excretion were studied in these animals.

i. Ethyl alcohol and oatmeal

Control tests were performed on two animals, and after an eight-day fast, oatmeal was given and the daily administration of 20 to 30 ml. of 24 per cent alcohol was begun. Rabbit #11 went into a coma and died after the second dose of alcohol was given. No post mortem examination was made. Rabbit #12 died immediately after the ninth dose of alcohol. No liver function tests had been performed. The animal's normal weight was 4.5 kg. After the fast it weighed 4.0 kg., and at death its weight had fallen to 3.6 kg. At post-mortem examination, the lungs were found to be somewhat haemorrhagic. The liver showed marked vacuolation of the hepatic cells, chiefly in the midzonal region, but extending throughout the lobule. Some nuclei were pyknotic, others swollen. The cytoplasm was granular.

ii. Methyl alcohol and oatmeal

Control tests were made on two animals. After an eight-day fast, the rabbits were given an oatmeal diet and daily administrations of approximately 25 ml. of 1 per cent methanol.

Rabbit #13 The animal died after 21 doses of methanol, on the twenty-eighth day after the first administration. The animal's weight had fallen from 2.8 kg. (before fasting) to 1.7 kg. at the time of death. The haemoglobin concentration just before death was only 8.3 g./100 ml. of blood.

Some liver function tests had been performed on this animal. On the thirteenth day the dye excretion showed the same degree of abnormality as in the fasting animal. Retention was increased on the nineteenth day, and on the twenty-seventh day, two days before death, the retention was 75 per cent at five minutes, and 40 per cent at twelve minutes. Prothrombin times were never prolonged; the prothrombin time of whole plasma varied from 10.0 to 11.5 seconds, of dilute plasma from 16.5 to 18.5 seconds and the difference from 5.5 to 7.0 seconds. The animal's serum showed no phosphatase activity both during the control period and on the thirteenth day of methanol administration. On the nineteenth day the phosphatase value was fifteen units, and two days before death it had fallen to five units.

An autopsy immediately after the animal's death indicated that the rabbit had died as a result of intestinal obstruction. The large intestine was distended with gas. The small intestine contained frothy yellow bile. The liver was small but not friable. Pathological studies showed only very mild degenerative changes in the central vein area; the swollen parenchymal cells had granular cytoplasm and swollen, pale nuclei.

Rabbit #14 The animal received 22 doses of methanol in 29 days, and tests were performed on the thirteenth, nineteenth, twenty-seventh, and thirty-fifth days. The weight fell from 4.0 kg. to 2.5 kg. during the experiment. The haemoglobin, however, normally between 10 and 12 g. per 100 ml. of blood, did not go below 9 g. Serum phosphatase activity in this animal before methanol was given was 19.8 and 22 units (see Table IV). During the administration of the poison, the serum phosphatase level varied from 5 to 7 units, and finally no activity was demonstrable. The difference between the prothrombin time of dilute and whole plasma fell, too, from 5.5 seconds to 3.5, and finally to 1.0 second. The first and second dye excretion tests showed a retention similar to that found in this animal when fasted; in the third and fourth tests the retention had decreased to the normal level.

The animal died on the thirty-ninth or fortieth day of the experiment (during a week-end) and because of the extent of decomposition, no pathological studies were made.

iii Alcohol and Purina Chow

Rabbit #17 After normal values had been established for this animal and it had undergone an eight day fast, a well-balanced diet of Purina Chow, hay, and carrots was given, and the daily administration of ethyl alcohol (20 ml. of 24 per cent ethanol daily) was commenced. The animal died during the middle of the night following the fourth dose of alcohol. The cause of death was not apparent.

Rabbit #18 This animal, undergoing the same treatment as #17, was fasted for eight days and then given a diet of Purina Chow, hay, and carrots. Starting on the ninth day, administration of alcohol was begun. On the thirty-eighth day alcohol administration was stopped, and on the forty-eighth day a diet of oatmeal was given. Seven days later alcohol was once more administered, but the animal died on the sixty-first or sixty-second day (during a weekend) and no autopsy was performed.

The results of this experiment are shown in Figure 7. The prothrombin times varied only slightly and never became abnormal. Serum phosphatase activity was rather constant, and was never appreciably higher than the value of 13 units found in the fasted animal. Bromsulfalein retention was never high, but there was possibly less retention after alcohol administration was stopped. This animal showed only a slight increase in dye retention when fasted, and the degree of retention in the fasted animal resembled strongly the retention found in the animal after alcohol ingestion, and at five minutes in the test performed after oatmeal was given instead of Purina. (Unfortunately the tube containing the twelve-minute sample was broken in the centrifuge).

The animal regained much of its original weight during the period of alcohol and oatmeal ingestion, but the weight levelled off and was never higher than ninety-three per cent of the pre-fasting weight. On the oatmeal diet, the animal rapidly lost weight.

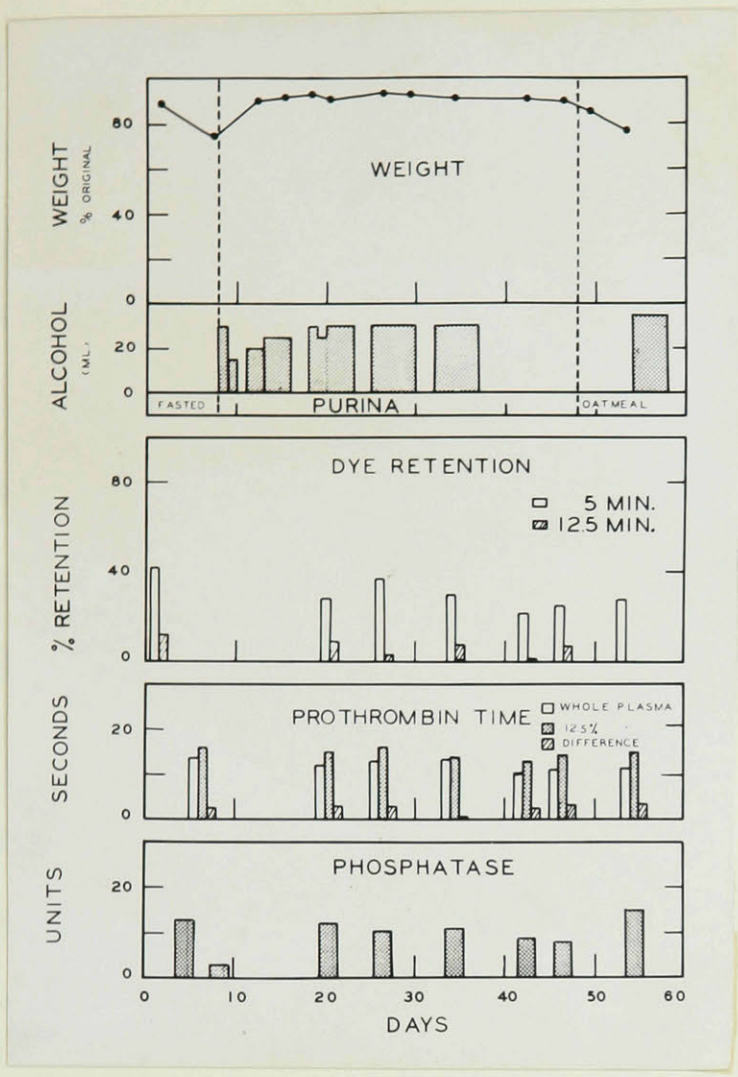

Figure 7 Rabbit #18

iv. DDT

Because of the widespread use of DDT as an insecticide, it was felt that a study of its effects on the liver should prove of interest.

Three grams of DDT was dissolved in 100 ml. of hot ethanol. The compound was precipitated in finely divided form by adding 0.15 ml. of the solution to about 20 ml. of water, in which the compound is insoluble. The water suspension was administered by stomach tube daily to two rabbits which were on an oatmeal diet following an eight-day fast.

Rabbit #19 died after only three doses of DDT. At this point a bromsulfalein test was performed on Rabbit #20. The animal retained 40 per cent of the dye fourteen minutes after the injection, when normally only traces of the dye should be present. After three more doses of DDT, the animal's serum was greenish-yellow in colour, and the haemoglobin concentration had fallen to 6.6 g. per 100 ml. of blood. The phosphatase activity was normal (7 units) and the difference between the prothrombin time of dilute and whole plasma, always low in this animal, fell almost to zero. The dye retention, still abnormal, was only 27 per cent at thirteen minutes, a value less than that found in the previous test. The animal was very weak on the day following these tests; its weight had decreased from 4 kg. to 2.8 kg. It died that afternoon, and a series of unavoidable incidents prevented an autopsy.

Because of the extremely toxic effects manifested by DDT, an attempt was made to repeat these experiments. Two more animals were fasted for five days, thenplaced on a diet of oatmeal. Over a period of one month DDT was given daily and the dose was gradually increased to 4.0 mg. per day. Rabbit #24 lost very little weight and its haemoglobin remained within the normal limits. Towards the end of the period of DDT administration, the dye retention was very slightly increased and the prothrombin time slightly prolonged. When DDT administration was stopped and the Purina diet

given again, the animal quickly regained the weight it had lost and its dye retention returned to normal, but there was no change in the prothrombin time.

Rabbit #25 showed essentially the same changes as #24, although the decrease in weight and haemoglobin was somewhat greater. Dye retention after a month of daily DDT administration was 12 per cent at twelve minutes, a value only slightly above normal. When the administration of DDT was stopped and a well balanced diet was given, dye excretion, weight, and haemoglobin returned to normal, but the prothrombin time remained prolonged.

v. Oatmeal alone

Because of the possibility that an oatmeal diet in itself might cause damage to the liver, two rabbits were fed oatmeal for several weeks, and prothrombin time, serum phosphatase, and excretion of bromsulfalein were studied.

Rabbit #15 After an eight-day fast, the animal was given an oatmeal diet, and tests were performed on the eleventh, eighteenth, twenty-fifth, thirty-second, thirty-ninth, forty-sixth, fifty-sixth, and sixtieth days. On the twenty-fifth day the greenish-yellow colour of the serum showed the presence of jaundice, which was found to be more severe the following week.

In Figure 8 will be seen the results of the tests in Rabbit #15. Each five-day period is marked off by a small black rectangle. The top graph shows the changes in weight,

calculated as per cent of the weight of the animal before fasting, and the degree of jaundice. The second graph shows the bromsulfalein retention at five and 12 minutes following injection of the dye. In the third graph, the white column represents the prothrombin time of whole plasma, the black column that of dilute plasma, and the grey column the difference between the two. The fourth graph shows the serum phosphatase activity expressed in units.

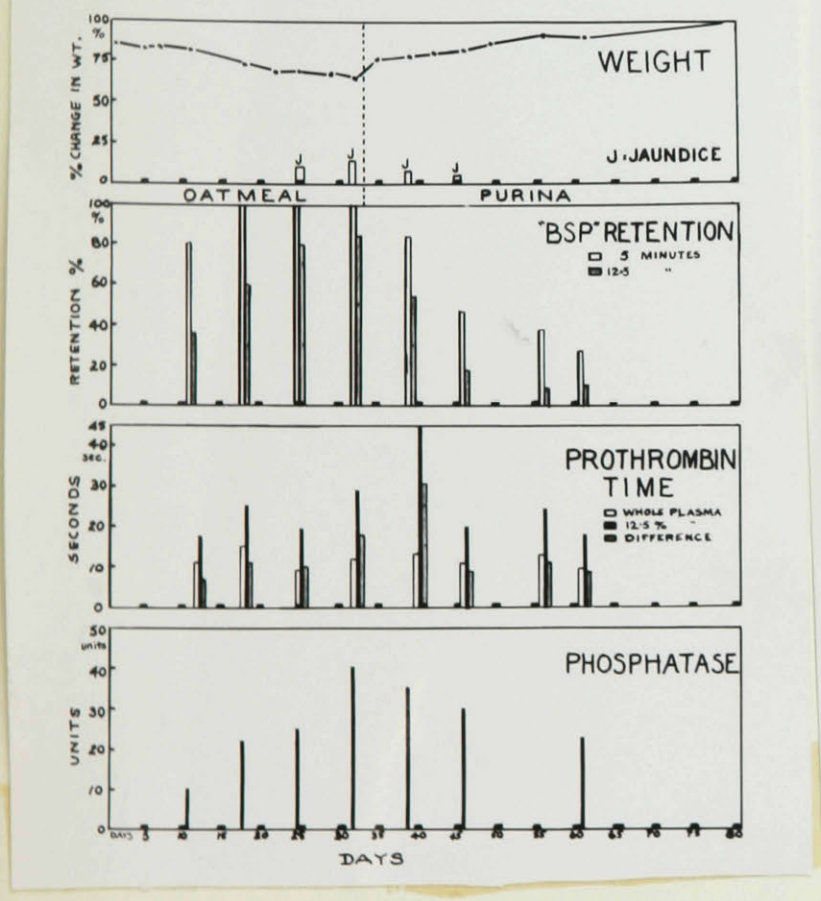

Figure 8 Rabbit #15

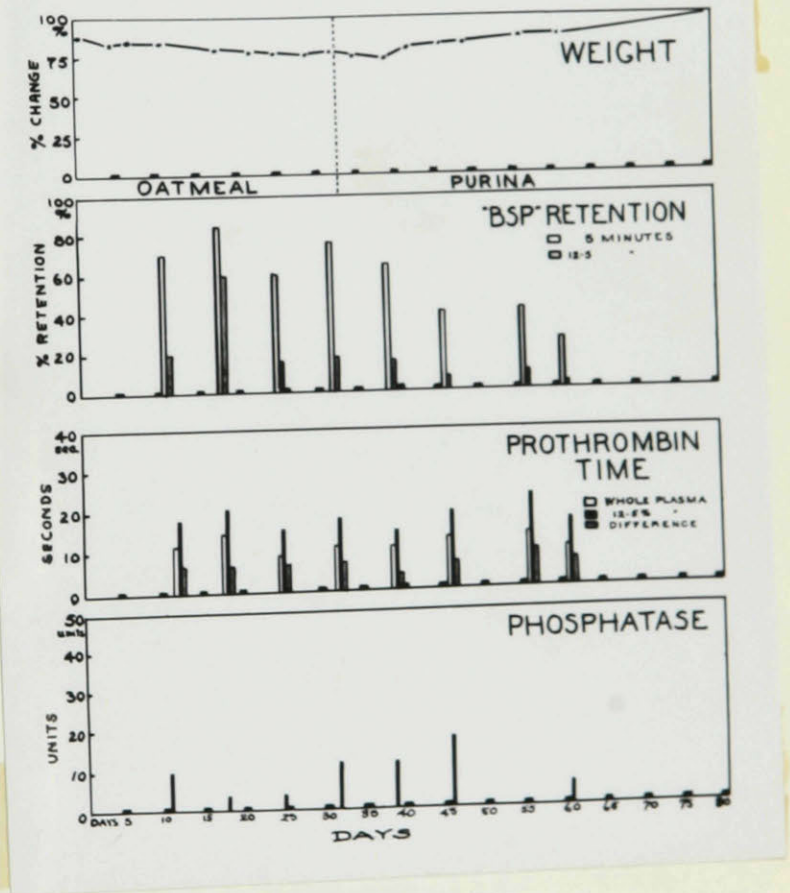

Figure 9 Rabbit #16

Rabbit #15 lost weight rapidly when fed only oatmeal. The retention of bromsulfalein became very high, and the serum phosphatase activity gradually increased. On the

eighteenth day the prothrombin times were prolonged, and although they were lower the following week, the difference between the prothrombin time of the dilute and whole plasma was prolonged to almost the same degree (ten seconds). On the thirty-second day the difference was seventeen seconds, and a week later it had risen to thirty-one seconds.

On the thirty-second day the well-balanced diet was given again. The rabbit's weight gradually returned to normal, jaundice disappeared, dye retention diminished, and the prothrombin time was shortened. Although the difference between the prothrombin times of dilute and whole plasma was eleven seconds on the fifty-sixth day, control tests on normal animals showed that the activity of the thromboplastin preparation had diminished. A new batch of thromboplastin was prepared, and the prothrombin times, although longer than those found before oatmeal was given, were found to be of the same magnitude as that of a normal animal. The serum phosphatase level fell only slowly, and on the eighty-fourth day was 16 units, a value well above the normal level of 5 to 7 units for this animal.

Rabbit #16 Although this rabbit was maintained on exactly the same regime as Rabbit #15, it was much more capable of withstanding the adverse effects of a deficient diet. Less weight was lost, and jaundice was never noted (Figure 9). The prothrombin times never exceeded the normal limits of variation, the prolongation observed in the last two tests

being due to changes in the thromboplastin preparation. Serum phosphatase activity decreased during the period of damage, and increased during the recovery period. The dye retention, however, became abnormal when the oatmeal diet was given. Although the amount of retention did not parallel the loss in weight during the period of damage, it did decrease in a regular fashion during the recovery period.

DISCUSSION OF RESULTS

The difficulties inherent in the problem of studying the functions of the liver have been summarized by Mann (21)

> "The capacity of the liver to carry on its many functions is greatly in excess of the normal needs of the organism. The capacity of the liver to perform its functions is dependent on many factors, and may change with extreme rapidity. A decrease in one of the functions of the liver does not necessarily mean that all functions, or any other one of its many functions, are equally impaired or even at all injured. There seems to be a dissociation of functions in the liver, so that it is possible for one to be defective in an otherwise normally functioning organ ... The liver maintains all its known and therefore all its possible measurable functions with extreme stubbornness".

The tests studied in this investigation measure several different functions of the liver, namely, the ability of the liver to excrete substances into the bile, and to catabolize and anabolize carbohydrates and proteins. Because these functions are presumably performed by different enzyme systems, it should be possible partially to inhibit one of these systems without affecting the others.

In our investigation, there was rather good agreement between the results of the galactose tolerance and the bromsulfalein excretion tests. There was, however, no absolute correlation between the degree of dye retention and the height of the galactose index. In Rabbit #3 (see p. 194) a galactose index of 780 was found when the retention of

dye twelve minutes after the injection was 78 per cent. Rabbit #4, on the other hand, had a galactose index of 844, when the retention of dye, although abnormal, was only 25 per cent at twelve minutes.

The bromsulfalein test was always abnormal before any change was found in the clearance of galactose. At times, there would be little change from week to week, in the rate of removal of bromsulfalein from the blood once this function was shown to be abnormal, while the galactose test would show a distinct change in the carbohydrate metabolic capacity of the liver. However, never was the ability to remove bromsulfalein from the blood found to be improved while the galactose test indicated an increase in liver damage. The bromsulfalein test detected liver damage before the galactose test showed any abnormality, but in cases of severe liver damage the galactose test was always abnormal. In the two rabbits (#3 and #5) which died during the experiment, the detention of dye and the galactose index immediately before death were very high, and pathological examination of sections of the liver confirmed that the organ had been severely damaged.

In the second group of experiments the sensitivity of the bromsulfalein excretion test was again demonstrated. The prothrombin time, on the other hand, was significantly prolonged only in Rabbit #15, when the animal suffered from jaundice (Figure 8). The greatest prolongation of prothrombin

time in this rabbit was found during the period of recovery, after the oatmeal diet had been discontinued. However, in this case the prothrombin time was determined on the day following the performance of the other tests, and the animal's haemoglobin concentration had fallen to 7.0 g. per 100 ml. of blood, presumably because of haemodilution. In the normal animal, prothrombin lost by haemorrhage or by plasmaphoresis is rapidly regenerated (555) (614). Warner et al (553) bled a dog so that the haematocrit was kept at or below the level of 27 per cent for over a month; the prothrombin concentration as measured by the two-stage method varied between 93 and 108 per cent of the normal value. Elliott (559) found that weekly bleeding did not significantly affect the prothrombin time in rats. It is reasonable to assume, however, that in an animal with a severely damaged liver (indicated by the presence of jaundice, a high degree of dye retention, and increased serum phosphatase activity), the prothrombin lost through haemorrhage would not be replaced as rapidly as in the normal animal.

The variations in serum phosphatase found in the normal rabbits (see p. 178) were also evident in the animals during the period of poisoning. Only in Rabbit #15 was there a significant increase in the phosphatase activity during the period of damaging, and a decrease in the activity during the recovery period. In other rabbits a decreased phosphatase activity was noted during the damaging period, increasing to

normal values after the cessation of the treatment. On the whole, little significance could be attached to the variations in the serum phosphatase level. The normal variations in serum phosphatase activity in our animals were so great that apparently the presence of extensive damage was necessary before the phosphatase level rose significantly.

In the calculation of the retention of bromsulfalein in the blood, the assumption is made that the blood volume is 10 per cent, and the plasma volume 5 per cent of the body weight. A decrease in the haematocrit tends to decrease the apparent degree of retention, while a decrease in the total blood volume has the opposite effect. The importance of these variations in plasma and blood volume cannot be ignored, especially in severe liver damage. Because of its role in the synthesis of the plasma proteins, the liver plays an important part in the regulation of the blood volume. Soffer (87) observed a marked haemoconcentration in dogs in which severe liver necrosis had been produced by administration of arsphenamine. This haemoconcentration is probably related to the acidosis which is found in many cases of liver damage. Daft et al (175) report that severe liver damage is accompanied by a conspicuous shrinkage in the blood volume. Adler et al (785) found that animals and humans with severe liver damage suffered more severe haemodilution after a standard dose of fluid than did normals. That the R.E. cells, too, are concerned in water metabolism is indicated by the singular

observation (786) that intravenous injections of colloidal silver result temporarily in an alteration in the water balance of the body. Because of the importance of water shifts in the regulation of body temperature, variations in the plasma volume will be found especially during the summer months. Water shifts have also been reported in animals in response to changes in the barometric pressure (791).

In humans, liver disease of long standing is commonly associated with anaemia. It is generally assumed that the presence of liver damage interferes with the storage or metabolism of the haematopoietic principle. In our experiments, the rabbits whose livers were damaged showed evidence of blood destruction, imparied regeneration of red cells, or a combination of these factors. In many cases, after a long period of poisoning, the haematocrit decreased to about one-half of its normal value. Presumably there was also a shrinkage of blood volume, for in several of these animals a concentration of dye was found in the blood which would correspond to a retention of more than 100 per cent of the dye injected. In such cases, the abnormally high retention was considered as 100 per cent, and the concentration of bromsulfalein in the second sample was recalculated accordingly. While this procedure is not entirely accurate, in the cases where it was used the condition of the animal was apparently so poor that a slight degree of inaccuracy was not misleading.

The possibility of changes in the haematocrit and the plasma and blood volumes is very real in both normal and damaged animals. The suggestion of Robinson (776), that in the clinical use of the bromsulfalein test the blood volume of the patient should be determined, is an excellent one.

There is a difference of opinion concerning the value of dye excretion tests in jaundice. Interference with the excretion of bilirubin in the bile would presumably hinder the excretion of bromsulfalein. In catarrhal jaundice, however, complete retention of dye in the bloodstream is not invariably found (789) (384). Greene (787) reported that in jaundice, the retention of phenoltetrachlorphthalein ran closely parallel to the increase in serum bilirubin concentration. Piersol and Bockus (788), however, did not find such a correlation. While the results of the bromsulfalein test in jaundice are difficult to interpret, it is of interest to note that in Rabbit #15, the degree of dye retention paralleled the severity of the jaundice.

The recent studies which have demonstrated the importance of the R.E. cells in the removal of bromsulfalein from the bloodstream (see p. 85) raise the question as to whether the bromsulfalein test should be considered a test of liver function. Little work has been done to study the effects of hepatotoxins on the cells of the R.E. system. A close relationship between the R.E. cells and the parenchymal cells of the liver is indicated, however, by the observation that

a depletion of liver glycogen results in an increased retention of dye in the bloodstream.

Here the important question arises as to whether a glycogen-depleted liver is a damaged liver. Certainly such a liver functions less efficiently than when the liver glycogen concentration is high, and it is more susceptible to the effects of toxic agents. The degree of dye retention in the first bromsulfalein test performed after administration of the hepatotoxins was begun was similar in several animals to that found in the same animal during a fast. Further damage resulted in an increase in dye retention above this level. These findings suggest that the first effect of a hepatotoxin is the depletion of the liver glycogen.

A diet of oatmeal alone was sufficient to cause jaundice in Rabbit #15. The cause of the toxicity of oatmeal was not determined. There are, however, several possible reasons for its damaging effect. Although this foodstuff contains a high proportion of carbohydrate and is rich in the vitamin B complex, the fact that its protein is of plant origin suggests that it may have been deficient in some of the necessary amino acids. No data concerning the content of sulphur-containing amino-acids were available. Because oatmeal contains a large amount of phytic acid and a high ratio of phosphorus to calcium, not only is its calcium of little value to the body, but it tends to deplete the calcium stores of the body. The relation between calcium and liver damage

is not entirely clear, but the recent work of Cantarow et al (168) indicates that liver damage is less severe when the body contains an adequate supply of calcium.

Oatmeal is an acid-forming food in the body and as a result causes a depletion of the liver glycogen (790). The relationship between liver damage and acidosis (see p. 42) is another problem worthy of investigation.

The high toxicity of DDT in the first experiments performed during the summer was unexpected. The possibility that the jaundice shown by Rabbit #10 was of haemolytic origin is suggested by the observation of a concomitant low haemoglobin concentration of 6.6 g. per 100 ml. of blood, and a dye retention of only 27 per cent thirteen minutes after injection.

In the autumn, a second pair of rabbits was subjected to much more severe treatment than that which caused the death of the first pair, yet these rabbits showed few signs of damage to the liver. Although the haemoglobin fell to about 7 g. per 100 ml. of blood in both rabbits, jaundice was never noted. The same solution of DDT was used in both series of experiments.

Two possible explanations may be offered for the great differences in susceptibility noted above. Not only is the mortality rate of untreated animals higher in summer than in winter, but the toxicity of many chemical substances varies widely from season to season (792). Second, whereas the

first two animals were "Flemish Giants" (large white rabbits weighing about 5 kg.), it was impossible to obtain more of these animals for the second experiment, and the more common, small, stocky type of rabbit (weighing about 2 kg.) was used. All of the animals which died for no apparent reason during the series of experiments in which prothrombin time and serum phosphatase were studied, were "Flemish Giants". It is possible that this variety of rabbit is less rugged than the animals usually used, especially during the summer months.

Sulfanilamide in the amounts used, was too toxic to be of value as a hepatotoxin. The drug is a strongly alkaline compound; one of the rabbits (#8) showed symptoms of tetany shortly after receiving the fourth dose of the drug. Methanol in the dose used in this investigation showed little toxicity for the liver. Both neoarsphenamine and ethanol, when administered to rabbits on a defficient diet, were capable of producing severe liver damage.

Throughout this investigation, a close relationship was evident between the loss of appetite (and consequently weight) and the presence of liver damage as judged by the results of the tests. Rabbit #6, which ate heartily and gained weight while on an oatmeal diet, showed no abnormality of dye retention after administration of neoarsphenamine until its weight began to decrease. Rabbit #5, receiving the same treatment, lost weight continually and suffered severe liver damage. Prior to the experiments described on

page 205 rabbits #5 and #16 were subjected to a two-week fast, followed by a two-week period on a diet of oatmeal and a similar period on the ordinary well-balanced laboratory diet of Purina Chow, hay and carrots. It was noted that whereas Rabbit #16 was able to maintain its weight while being fed oatmeal for two weeks, its partner lost weight almost as rapidly as during the fasting period. The degree of damage and the loss of weight later induced in each rabbit by prolonged periods on an oatmeal diet bore an interesting relationship to the preliminary experiments in that Rabbit #15 withstood much more poorly than #16, the deleterious effects of an oatmeal diet. Another example of the relationship between loss of weight and susceptibility to liver damage is provided by Rabbit #18, which suffered little injury to the liver from the daily administration of ethanol when fed a well-balanced diet; during this period there was little change in the animal's weight.

In the experiments in which liver damage and loss of weight are associated, it is difficult to say which is the cause and which the effect. Quite possibly there is a reciprocal action, in that the combination of a poor diet and a decreased food intake is incapable of replacing adequately the stores depleted by fasting, and the liver damage produced is not conducive to hearty appetite.

Throughout this investigation great variation was noted in the resistance of the animals to the effects of hepatotoxins. Whereas an oatmeal diet alone was sufficient to produce

severe liver damage in one animal, others were resistant to the damaging effects of such a diet even when accompanied by the administration of a damaging agent. Because of the time-consuming nature of the experiments, it was not possible to collect sufficient data to permit an accurate analysis of the parts played by the diet and the toxic substances administered. That the diet was important in the production of liver damage cannot be denied. However, since neither Rabbit #4 nor #18, on a well-balanced diet, was able to regain entirely its pre-fasting weight when ethanol was administered repeatedly, it is probable that alcohol itself exerted a depressing effect upon the liver.

There are two important factors to be considered in the evaluation of a liver function test. The first is the specificity and sensitivity of the test, and the second, the ease with which it may be performed. This latter includes both the period of time during which the samples must be taken, and the simplicity of the analytical procedure.

In small animals, factors enter which are of little importance clinically. It is useless to perform the cephalin-cholesterol test, for the serum of almost every animal yields a strongly positive result. In the performance of a test such as the hippuric acid test, it is the amount of benzoic acid detoxified within the first hour or two after its administration that indicates the condition of the liver; in small animals it is difficult to secure complete urine

samples at definite time intervals. The collection of twenty-four hour urine specimens (e.g. for the determination of urobilinogen) necessitates the use of a metabolism cage. The most satisfactory type of test for use on an animal is one in which a substance is estimated in the blood, rather than the urine. In small animals, the size of the blood sample required is of great importance.

The bromsulfalein test is easily and quickly performed and the analysis is simple, requiring only 0.5 ml. of serum. Seeler and Juna (263) have devised a micro method for the determination of bromsulfalein serum but the method described on p. 167 is more useful in that it may be used in the presence of haemolysis.

Although the galactose tolerance test requires very small blood samples, it is time-consuming and cumbersome.

For routine work, both the galactose and the bromsulfalein tests may be simplified. In the latter test, the retention of dye in the twelve-minute sample is more significant than that in the sample taken four to five minutes after the injection, when high values, presumably the result of incomplete mixing of the dye with the blood, are occasionally found. In the galactose tolerance test, the five-minute sample of blood yields little information the other two samples do not give. In fact, the concentration of galactose in the blood three hours after the injection is frequently a good indicator of the animal's tolerance to galactose, although in borderline cases the one-hour sample

may be of value.

From the viewpoint of simplicity, the prothrombin time and serum phosphatase determinations are well suited for use with animals. No injections are required, a single sample may be used, and the analytical procedures are relatively simple.

In this investigation, a combination of tests such as was employed in the second set of experiments proved quite practical. After sufficient blood had been obtained from the rabbit's ear for the estimation of haemoglobin, serum phosphatase, and prothrombin time, bromsulfalein was injected and samples collected. With care, it was possible to obtain all the samples for one group of tests from one cut in the marginal vein of the ear, with the result that excess scarring was avoided. In one animal, the group of tests was repeated weekly twelve times with no difficulty. Although the serum phosphatase activity prothrombin time were little influenced by mild liver damage, in animals receiving severe treatment such a combination of tests should prove of value. The addition of a galactose tolerance test (using only one blood sample) would yield more information with little extra effort.

In the repeated performance of a number of liver function tests in a small animal such as the rat, it is essential to use a group of determinations which can be performed on a single sample of blood. The difficulties attendant upon

collecting blood from a rat's tail necessitate the use of heart punctures, with the result that tolerance tests become impractical, unless it is possible to perform other analyses upon the sample of blood containing the injected material. The work done in this investigation shows that the quantities of blood required for most determinations may be greatly reduced.

CONCLUSIONS

The galactose tolerance and bromsulfalein excretion tests, and the determination of prothrombin time and serum phosphatase activity can be performed easily on rabbits and require small quantities of blood. Such a group of tests may possibly be carried out on a smaller animal such as the rat.

Slowly progressing liver damage (detected by liver function tests and by pathological studies) may be produced in rabbits after periods of fasting by a deficient diet alone, or in combination with alcohol or neoarsphenamine. Loss of body weight is closely associated with the progress of the liver lesions. The toxicity of a diet of oatmeal may lie in its capacity to produce acidosis in the body.

Of all the tests studied, the bromsulfalein test is the most sensitive in the detection of liver damage. However, when interpreting dye retention data, the influence of changes in blood and plasma volume cannot be ignored. Retention of dye is increased during fasting; although the degree of dye retention is rather constant in fed animals, fasted animals show a wide variation in the removal of dye from the blood stream. However, the retention is rather constant for any one animal.

Galactose tolerance and prothrombin time are markedly abnormal when the liver is severely damaged. Fluctuations in serum phosphatase activity are of significance only in the presence of severe hepatic damage.

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