PROCEEDINGS OF THE EUROPEAN SOCIETY FOR THE STUDY OF DRUG TOXICITY

VOLUME V, 1965

# ADVANCES IN TOXICOLOGICAL METHODOLOGY


INTERNATIONAL CONGRESS SERIES No. 90

EXCERPTA MEDICA FOUNDATION

AMSTERDAM / NEW YORK / LONDON

MILAN / TOKYO / BUENOS AIRES

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# FOREWORD

The assessment of the toxic effects of a substance following experiments in animals still depends to a very large extent on the results of a detailed histological examination conducted in a conventional way. It is a truism, of course, that a functional disturbance should precede observable morphological damage, but unfortunately it is equally true that, at the present time, functional tests are not as sensitive and as specific as we would like them to be. The purpose of the meeting of the European Society for the Study of Drug Toxicity held at Bad Homburg, Germany, in January 1965, the Proceedings of which are recounted in this volume, was to review knowledge in certain major areas of the field of functional tests. Additionally, some attention was given to electron microscopy and histochemistry to determine what help they might give in interpreting the results of histological examination.

Certain conclusions seem permissible:

(1) Present functional tests, when used in experimental toxicological investigations, still reveal little that is not revealed better at the detailed histological examination of the tissues of the experimental animals.

(2) Their conduct and their interpretation, whether in man or in experimental animals, require great care (one would wish that this lesson could be learned by the too many people who write letters to the medical press stating that this and that enzyme change was produced by this and that drug).

(3) Because of (a) the extremely high magnification given by the electron microscope, (b) the correspondingly extremely small volume of tissue which can be examined, and (c) the sensitivity of tissues to small nutritional changes, much care is required in the interpretation of electron microscopic appearances relative to *toxicological* changes.

Our Society will perform a valuable service if it encourages and stimulates much more work in the fields of functional tests, histochemistry and electron microscopy as applied to toxicological studies. This it intends to do and it can be taken for granted that more meetings will be given to these subjects.

D. G. DAVEY

# CONCERNING LIVER FUNCTION TESTS

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## INTRODUCTION

The biochemical assessment of alterations in liver function is difficult. Firstly, the reserve function of the liver is considerable so that the detection of moderate deficiencies may not be easy. Secondly, there is difficulty in finding tests which are solely dependent on changes within the liver. Thirdly, in the past there has been poor correlation between the clinical, histological and biochemical findings in relation to liver disease.

Because of these difficulties it has become fashionable, over the last two decades, to describe liver function tests as being of very limited use in assessing liver damage. Despite these limitations there have been, within the last few years, important developments which indicate that the search for more specific, sensitive and less empirical tests is becoming fruitful and there is less pessimism in this field of clinical biochemistry.

In general, investigation of liver function is confined to the detection of a blood or urine constituent whose excretion, catabolism or metabolism involves the liver, or to tests involving a stress of the organ by over-loading the liver with an agent administered in excess. More recently there have been added a number of tests where the levels of blood enzymes are determined because the source of these enzymes may be increased cellular breakdown or increased cell permeability within the liver.

This discussion will be concerned with those tests which are of particular use in the assessment of liver function following therapy with drugs with suspected hepato-toxic properties but will, of necessity, range into the clinical field of infective hepato-toxic agents and chronic liver disease. Much of the experience gained in the clinical field may be applicable to animals but species difference is a difficulty in this, as in many other fields.

The listing of all the tests of liver function which have been suggested would be a considerable task and, therefore, my comments will be limited to a dis-

cussion of those which have proved clinical value. Too often the technical difficulties of the suggested tests are not emphasised and for this reason under the heading of each test such difficulties are discussed. In addition, the expected values in normal subjects are stated and comments on the interpretation of abnormal results are given.

*Total serum bilirubin*

This is an important basic test of the liver's ability to excrete bilirubin into the intestine. Jaundice cannot be detected clinically until the serum bilirubin has reached a value above 2 mg./100 ml. and therefore the method is of particular importance in detecting abnormal values below 2 mg./100 ml. A method for the determination of serum bilirubin which is becoming widely used is that of Powell (1944). This method is of particular use at low levels of bilirubin because it is free of turbidities, only dilutes the serum 1 in 10, and gives an immediate colour with all forms of bilirubin. The method is dependent on the reaction of bilirubin with diazotised sulphanilic acid in the presence of benzoate and urea. Three possible sources of error with the technique are firstly, the use of nitrite reagent which has deteriorated, leading to low values of bilirubin; secondly, a failure to realise how sensitive bilirubin is to light, and this is particularly true at low levels; and thirdly, haemolysed serum which must not be used because haemolysis produces falsely low results by the Powell technique.

A variety of normal values is to be found in the literature. In the author's laboratory, two surveys have shown that the normal value is 0.8 mg./100 ml. or less. A value above 0.8 mg./100 ml. is worthy of further study, as outlined below.

Abnormal values may indicate (a) an extra-hepatic obstruction of bile secretion; (b) a cellular failure within the liver preventing normal bilirubin handling; (c) an over-production of bilirubin as a result of increased haemolysis of red cells. Further tests will help in detecting the source of the bilirubin.

*Conjugated and non-conjugated bilirubin*

It has been known for fifty years that bilirubin can exist in serum in two forms. One form reacts directly with diazotised sulphanilic acid, the so-called "direct" reacting bilirubin of Van den Bergh and Muller (1916) and characteristically present when bilirubin is failing to be excreted because of intra- or extra-hepatic obstruction. The second form only reacts with diazotised sulphanilic acid in the presence of such chemical reagents as alcohol and benzoate and urea. This is the "indirect" reacting bilirubin of Van den Bergh and Muller (1916) and is found in patients who are jaundiced due to overproduction of bilirubin from haemolytic processes. Work by Lathe and his co-workers (Billing, Cole and Lathe, 1957) has shown that this difference in

bilirubin is not due to protein binding of the bilirubin, as first thought, but that the bilirubin is present in the serum either in a conjugated form (the "direct" reacting pigment) or in a free form (the "indirect" reacting pigment of haemolytic jaundice).

The Van den Bergh reaction performed qualitatively is of little use and a quantitative determination is essential for correct interpretation. The method of Powell (1944) can be used to determine quantitatively the conjugated and un-conjugated forms. The presence of a predominantly conjugated bilirubin (greater than 50% of the total) is indicative of obstructive or hepatogenous jaundice whilst the presence of more than half the total bilirubin in the free form is indicative of haemolytic jaundice. The bilirubin normally present in serum is in the free form. Differentiation of the serum bilirubin at low levels is difficult because the colour produced in the reaction is not strong and colorimeter readings are therefore small. Some patients with chronic liver disease have increased red cell breakdown and patients with haemolytic disease may, by forming pigment gall stones, have biliary obstruction; but interpretation of results is possible in the vast majority of jaundiced patients.

There are numerous techniques available for the further investigation of patients with suspected haemolytic jaundice and it is rare that the aetiology of the jaundice cannot be ascertained if it is present due to the increased haemolysis of red cells.

*Tests for bile pigments*

Too often the very useful qualitative or semi-quantitative tests for bile pigment in urine are either not done or their simplicity is technically abused. Bilirubin can only be excreted by the kidneys in the conjugated form and is, therefore, not present in the urine in haemolytic jaundice. In liver disease or extra-hepatic obstruction of the bile bilirubin in the urine may be one of the earliest signs, occurring before jaundice is detected and before the excretion of the pigment urobilinogen. Normally, bilirubin is not present in the urine. The most satisfactory method of detection is the tablet test suggested by Tatlack and Sherlock (1954); this can detect bilirubin of the order of 0.1 mg. per 100 ml. of urine.

Urobilinogen can only be produced by bacterial reduction of bilirubin in the intestine. It is re-absorbed and is a normal constituent of urine but in very low concentration. If, due to extra- or intra-hepatic obstruction of bile flow, there is no bile reaching the intestine then no urobilinogen will be produced, none re-absorbed and none excreted in the urine. In liver disease the urobilinogen re-absorbed into the entero-hepatic circulation may not be adequately dealt with by the liver and will be excreted in the urine. The method of Watson, Schwarz, Sborov and Bertie (1944) as modified by Varley (1962)

gives a quantitative result calibrated in arbitrary units, but it is of special use to those who are not experienced in making semi-quantitative measurements on urines for urobilinogen content. There are three important points: (a) the excretion of urobilinogen is highest in the afternoon and a 14.00—16.00 hours urine specimen should be used; (b) urobilinogen is light sensitive and therefore a fresh specimen is essential; (c) porphobilinogen and certain food dyestuffs give colours with this reagent. Values above 2 units are abnormal, 90% of normal subjects having values below 1 unit. Urinary urobilinogen tests are extremely useful in showing the pre-obstructive, post-obstructive, and recovery phases of infective hepatitis.

Tests of faecal urobilinogen have not been useful in the early detection of liver disease.

*Bromsulphthalein (B.S.P.) test*

This test has grown in popularity and established itself as the most sensitive available today for the testing of one function of the liver.

Hepatic vein catheterisation has shown that injected B.S.P. is rapidly removed by the liver and excreted in the bile. In the dog and rat the B.S.P. is conjugated in the liver with glutathione and then excreted in the bile. Thus, the test is useful in assessing the ability of the liver to conjugate a 'foreign substance' and excrete it in the bile as the conjugate. The enzyme responsible for conjugation has been isolated from the liver by Combes and Stakelum (1961). The test involves injecting 0.1 ml. of a 5 g. per 100 ml. B.S.P. solution per kg. of body weight. Thirty minutes later a specimen of blood is taken from the other arm and the serum separated. A measured portion of the serum is made alkaline and compared with a 10 mg. per 100 ml. B.S.P. solution which is regarded as the 100% value. Normal subjects should retain only 10% of the standard 30 minutes after injection (Sherlock, 1963). There are many normal values quoted in the literature and confusion has resulted from a failure to state the timing of the blood specimen. When results are given the time interval used must be stated. The 30 minute value is the most useful. The patient should be fasting. Haemolysis in the serum, even in small amounts, gives falsely high results as pointed out by Shoemaker (1961). When haemolysis is present, and ideally in all cases, Shoemaker's technique, which is not affected by haemolysis or slight turbidities, should be used.

If jaundice is present and the bilirubin is conjugated, then a B.S.P. test is not necessary because there is already evidence of a failure to excrete bile. The B.S.P. test is most useful when jaundice cannot be demonstrated.

*Serum protein and turbidity tests*

The alteration in serum proteins resulting from liver disease has been the subject of many studies. The introduction of sophisticated methods of protein

separation such as electrophoresis and 'Sephadex' chromatography have yielded little information beyond that which can be obtained by a crude separation of albumin and globulin by salt precipitation, and gamma globulin determination by zinc sulphate turbidity (Kunkel, 1947). There is no doubt that the serum albumin is produced by the liver and in chronic liver disease there is a decrease in serum albumin. There is also an increase in the globulin fraction in chronic liver disease, particularly the gamma fraction, but the reason for this is unknown. These changes only occur slowly after the liver is damaged and it must also be remembered that such changes may occur as the result of dysfunction in other systems. Protein studies have little place in the detection of acute changes in liver function following liver damage.

The turbidity tests of liver function are predominately based on protein changes in the serum although other substances may be involved (e.g. lipids). The tests are unspecific and of little use in acute studies. Probably the most commonly used and most useful turbidity test is the thymol turbidity test of Maclagan (1944). Normal subjects give values of 3 units or less.

*Plasma ammonia*

There are very small amounts of ammonia present in plasma and its determination requires considerable analytical skill, but in our experience it is worth acquiring this skill and using plasma ammonia determination in cases of suspected liver dysfunction. The method of Fenton (1962) works well in our hands. The results have been disappointing in studies of acute toxic hepatitis, whether drug induced or of infective origin, but a rise in blood ammonia may occur in chronic liver disease and is almost certainly due to 'shunting' of blood away from the liver.

Although protein studies and blood ammonia determination have been criticised as tests for acute liver damage, we would use them as tests of function prior to treatment of patients with drugs possessing suspected hepatotoxic properties, in order to be certain that there is no hepatic disease present prior to treatment.

*Serum enzyme changes in liver disease*

*Alkaline phosphatase*

Since Roberts (1933) first reported the changes in serum alkaline phosphatase in liver disease, there has been considerable use of the test in the detection of liver dysfunction. It is still uncertain whether the rise in serum alkaline phosphatase is due to failure of excretion or to increased production by the liver. The enzyme is not, of course, confined to the liver, bone disease being characterised by an increase in serum activity. The King-Armstrong (1934) method of determination in serum is satisfactory and the normal concentration