THE APPLICATION OF BIOCHEMICAL METHODS INVOLVING ENZYME ASSAYS IN THE STUDY OF CERTAIN PATHOLOGICAL CONDITIONS. STUDIES WITH ISOCITRATE DEHYDROGENASE AND β-GLUCURONIDASE.

by

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Summary of

Thesis submitted for the degree of Ph.D

of

The University of Glasgow.

August, 1965

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The thesis consists of two separate studies on two enzymes which have potentially useful applications in the biochemical study of various human diseases.

In Part 1, the activity of the enzyme, isocitrate dehydrogenase, in serum of patients with diseases involving the liver or biliary tract, was studied and compared with established biochemical tests of liver function, in order to assess its application to the differential diagnosis of jaundice. Particular attention was paid to results on patients with obstructive jaundice as previous authors have reached differing conclusions of the ultimate usefulness of this enzyme, due to the equivocal results shown in these case

Very high values were found in severe, acute breakdown of liver cells. These coincided with large increases in activity of two other enzymes used in the diagnosis of acute hepatic damage - serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase. The serum isocitrate dehydrogenase was extremely sensitive in detecting liver cell damage, even at a subclinical level, and was comparable and perhaps slightly superior to the serum transaminases in this respect, while all three enzymes were very much more sensitive than other conventional tests of liver function.

In chronic liver damage, variable values for serum isocitrate dehydrogenase were found, with only small to moderate increases which could not be related to the clinical state of the patient. Higher values were found in biliary

cirrhosis than in portal cirrhosis. The enzyme results were significantly correlated with the transaminase results, but not with the other liver function tests.

In obstructive jaundice, the enzyme was elevated in the serum of over 50% of the cases, and particularly high values were associated with secondary involvement of the liver in malignant cases, and acute inflammation of the biliary tract in benign obstructive jaundice. The enzyme could not be used to differentiate malignant from non-malignant cases, and was not related to the severity or duration of the biliary retention. In these cases, the enzyme was not closely related to serum transaminase values.

It was concluded that the enzyme did not offer great advantages in the differential diagnosis of jaundice due to the abnormal values which may be found in obstructive jaundice. However, it was an easily estimated enzyme, very sensitive for detecting and assessing acute damage to liver cells, and could be a useful adjunct or alternative to the serum transaminases.

Part 2 involved the study of the enzyme, β-glucuronidase in various biological material taken from patients with carcinoma of the cervix uteri.

Biopsy specimens of cervical carcinoma were obtained and fractionated by homogenisation and differential centrifugatio into three cytoplasmic fractions - mitochondria, microsomes and soluble supernatant. β-Glucuronidase activity was

measured in each fraction and the results compared with specimens of non-malignant cervix treated in the same way. The activity of the carcinoma specimens was found to be considerably higher than non-malignant specimens in the majority of cases. Increases in β-glucuronidase activity were mainly in the soluble supernatant fraction and not in particle enzyme, even though the enzyme had previously been described as located mainly within cytoplasmic particles. There was no correlation with the enzyme activity and degree of malignancy of the cervical lesions.

Samples of cervical carcinoma were taken before and after treatment of the lesion by irradiation from radium implants, and their β-glucuronidase activity compared. There was not a uniform change in enzyme activity following radiation due to large unexplained increases and decreases being observed.

The excretion of β-glucuronidase in urine of patients with cervical carcinoma receiving radiotherapy was followed over the full course of treatment. Pronounced increases in excretion of the enzyme following radium treatment were observed in the majority of patients studied, and the pattern of β-glucuronidase excretion could be related to the enzyme activity of the tissue being irradiated. There was also increases in β-glucuronidase activity of the serum in these patients, and the results indicated that radiation of the lesion caused mobilisation of the enzyme into the patient's circulation, which was then cleared by the kidneys into the ur

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Thesis submitted for the degree of Ph.D.

in the

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August, 1965

ACKNOWLEDGEMENTS.

The author would like to express his sincere gratitude to Dr. E.B. Hendry for his interest and advice during the preparation of this thesis, and for the excellent facilities provided for the practical investigations. He expresses his thanks to Professor J.N. Davidson and Professor H.N. Munro for their advice at various stages of the work. He is indebted to Dr. D.M. Goldberg for arranging the supply of tissue specimens, his advice on various medical aspects of the work, and his interest in the preparation of the manuscript. Many of the tissue fractionation procedures were carried out conjointly with Dr. Goldberg and Miss H.A. Ayre. His thanks are due to the technical staff of this department for their assistance in performing some of the routine tests of liver function; Mr. G. Donald for his advice and facilities in the preparation and photography of the Figures; to the nursing staff and patients in various wards of the Western Infirmary for their co-operation in the collection of specimens, and finally, his appreciation to Mrs. C. Watts for her help in the typing of the manuscript.

TABLE OF CONTENTS.

ABBREVIATIONS.

DNP-	: 2:4 dinitrophenyl-
EDTA	: ethylenediaminetetra-acetic acid
g.w.w.	: g. wet weight of tissue
I.U.	: International Units
K-A	: King-Armstrong
NADP, $NADPH_2$	: nicotinamide adenine dinucleotide, oxidised and reduced forms
S-GOT	: serum glutamic-oxalacetic transaminase (L-aspartate:2-oxoglutarate aminotransferase)
S-GPT	: serum glutamic-pyruvic transaminase (L-alanine:2-oxoglutarate aminotransferase)
S-ICD	: serum isocitrate dehydrogenase (L-isocitrate:NADP oxidoreductase - decarboxylating)
Tris	: 2-amino-2(hydroxymethyl)-propane-1,3 diol
n.s.	: not significant

STATISTICAL CALCULATIONS.

In both Parts 1 and 2, the Mean and Standard Deviation (S.D.) on a group of results were calculated by the formulae:-

$$\text{Mean } \bar{x} = \frac{Sx}{n} \qquad \text{S.D.} = \sqrt{\frac{Sx^2 - \frac{(Sx)^2}{n}}{n-1}}$$

where x = individual values, and n = number of observations in the group.

Student's t Test of Significance between groups of unpaired samples was applied by the formula:-

$$t = \frac{\bar{x} - \bar{y}\sqrt{n_x \times n_y}}{S\sqrt{n_x + n_y}} \qquad \text{where } S^2 = \frac{Sx^2 - \frac{(Sx)^2}{n_x} + Sy^2 - \frac{(Sy)^2}{n_y}}{n_x + n_y - 2}$$

$$S\sqrt{n_x + n_y}$$

$$n_x + n_y - 2$$

re $\bar{x}$ and $\bar{y}$ are the means of the two groups, and n_x and n_y are the number of samples in each group.

Degrees of Freedom = $n_x + n_y - 2$

In groups with paired samples, a t value was obtained from the differences of the paired samples by the formula:-

$$t = \frac{(\bar{x} - 0)\sqrt{n}}{S} \qquad \text{where } S^2 = \frac{Sx^2 - \frac{(Sx)^2}{n}}{n - 1}$$

x = the difference between paired samples, $\bar{x}$ the mean difference, and n the number of paired samples.

Degrees of Freedom = n - 1

P values corresponding to the t values were obtained by reference to Documenta Geigy Scientific Tables (1962). Results were considered significantly different when P was

less than 0.05.

Correlation Coefficients (r) between two groups of results were obtained from the formula:-

$$r^2 = \frac{S(x'y')}{Sx^2 - \frac{(Sx)^2}{n}} \times \frac{S(x'y')}{Sy^2 - \frac{(Sy)^2}{n}}$$

where $S(x'y') = S(x \times y) - \frac{Sx \times Sy}{n}$

The significance of a correlation coefficient was given by the formula:-

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

where n = number of paired results, and n - 2 = Degrees of Freedom (Fisher, 1954)

P values corresponding to t were obtained from the Student t tables.

GENERAL INTRODUCTION.

"Enzymes are organic catalysts produced in, or through, the agency of living cells of both animals and plants." (Chambers Encyclopaedia, 1955). Investigations which eventually led to this definition were initiated in the 19th Century by Thénard, Schwann, Kützing, Pasteur and others. Their studies on the fermentation of sugars by yeasts led to the concept of biological catalysts, present in living cells, which could promote a wide range of naturally occurring chemical reactions. Further work showed that these substances could be extracted in an active form from cells (Buchner, 1897). Parallel studies on the digestion of food in the stomach led to the conclusion that this was also a process governed by enzymes (Schwann, 1836; Bernard, 1856).

From these early beginnings, the study of enzyme activities in biological materials progressed rapidly, and has culminated in the last three decades in the recognition and description of individual enzymes which mediate in the various steps of intermediary metabolism in living tissues.

It was inevitable that these enzyme studies should be extended to pathological processes as well as normal physiological ones. Early work in this direction was concerned mainly with digestive enzymes, and the first important diagnostic enzyme assay to emerge was the estimation of amylase in the urine for the diagnosis of acute pancreatitis (Wohlgemuth, 1910). Studies on fermentation indicated that enzymes were involved in the formation and

hydrolysis of sugar phosphates, and this led to Robison (1923) finding high concentrations of an enzyme in growing bone which hydrolysed glucose-phosphate; he called this enzyme alkaline phosphatase. Subsequent investigations were then directed to studying the activity of this enzyme in the serum of patients with various bone diseases (Kay, 1929).

Since the birth of clinical enzymology, the number and complexity of enzymes being used as routine clinical diagnostic tests, or as research tools, in the study of pathological conditions has increased enormously. The subject has grown to such an extent that textbooks have been written entirely devoted to this one aspect of clinical biochemistry (Abderhalden and Oesper, 1961; King, 1965). In a recent attempt to assess the extent to which enzymes were being used by clinical biochemists, mainly in the British Isles, no less than 46 enzymes were being utilised for routine or research purposes (Gowenlock, 1965).

The activity of enzymes may be measured for clinical purposes in the patient's serum, urine, cerebrospinal fluid, digestive juices, abnormal body fluids or tissue biopsy specimens. By far the majority of diagnostic enzymes are determined in blood serum. Bodansky (1962) has listed 33 serum enzymes which have been reported to have diagnostic potentialities; the most widely used and well-established of these are acid and alkaline phosphatase, amylase,

cholinesterase, glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase.

Enzymes are infrequently measured in urine, the only established ones being amylase and, possibly, uropepsin. Various enzyme activities have been measured in cerebrospinal fluid (Delank, 1963; Mellisk and Bassett, 1964), and in abnormal body fluids, e.g. malignant and benign effusions (Brauer, West and Zimmerman, 1963).

The investigation into enzyme activities in specimens of tissue from normal and pathological material is still in its infancy. Histochemistry, which involves the localisation of enzymes within tissues by staining of histological preparations, has progressed forward to include many enzymes. Results from these techniques, however, must be viewed with caution as many factors, e.g. permeability of the tissue to the enzyme substrate, tissue autolysis, or naturally occurring activators or inhibitors, may influence results in an unpredictable way. It is only recently that results of detailed investigations into the quantitative measurement of enzymes in various human tissues have appeared, e.g. Shonk, Koven, Majima and Boxer, 1964. Little work has been carried out on the distribution of enzymes among intracellular components - nucleus, mitochondria, microsomes and cell sap - in normal and diseased human tissues.

The clinical use of enzymes covers a broad spectrum of disease states, but there are two fields of human disease

which have attracted a vast amount of investigations on many different enzymes. Firstly, there are diseases involving the liver. Hepatic cells are a rich source of many enzymes due to their diverse metabolic capabilities, and, consequently, damage to these cells in liver disease has led to studies on the appearence of abnormal amounts of these enzymes in the blood stream of jaundiced patients (Baron, 1963). Secondly, enzymology has emerged as one of the foremost instruments in the search for the cause, diagnosis, prognosis and cure of the numerous forms of cancer. The concept that malignant cells show changes in their metabolism compared with normal cells has been proposed by Warburg (1956), and many investigations since then have indicated that neoplastic cells can have pronounced differences from normal cells in various enzyme activities. This subject has been extensively reviewed by Douglas (1963). Besides studies on enzymes in malignant tissues, intensive investigations have been, and are being, undertaken into their activities in other biological material from cancer patients, e.g. serum, blood cells, urine, cerebrospinal fluid and other body fluids. These studies are directed mainly towards assessing the enzyme as a diagnostic and prognostic aid, and its use as an index of the progression of the disease during treatment.

The present investigation consists of two separate studies, each one being an example of the clinical use of

an enzyme in the two fields of human disease mentioned above.

In Part 1, the activity of the enzyme, isocitrate dehydrogenase, has been studied in the serum of jaundiced patients, in order to assess its potential as a diagnostic aid in the differential diagnosis of jaundice.

Part 2 consists of a study into the β-glucuronidase activity of tissue specimens, urine and serum of patients with carcinoma of the cervix uteri, some of whom received radiotherapy, again with a view to assessing its diagnostic or prognostic applications.

PART 1.

STUDIES ON ISOCITRATE DEHYDROGENASE IN HEPATIC DISEASE.

INTRODUCTION.

Many biochemical tests have been devised for assessing the functioning and integrity of the liver in patients with suspected hepatic disease. Some of these tests have involved the determination of the activity of enzymes present in venous blood. Three of these enzymes have become established as useful tools for the differential diagnosis of jaundice. Firstly, serum alkaline phosphatase (E.C. 3.1.3.1.) has become one of the standard tests for diagnosing jaundice due to obstruction of the biliary tract. Gutman, Olson, Gutman and Flood (1940) found raised values for this enzyme in 98% of patients with obstructive jaundice. A value of over 30 King-Armstrong (K-A) units/100 ml. for the alkaline phosphatase in a jaundiced patient is one of the surest guides of an obstruction to the flow of bile.

Two other enzymes, serum glutamic-oxalacetic transaminase (S-GOT, E.C. 2.6.1.1.) and serum glutamic-pyruvic transaminase (S-GPT, E.C. 2.6.1.2.), have now become widely used as sensitive indices of acute hepatocellular necrosis (Wróblewski, 1958; Agress, 1959). Very high values are found in conditions causing acute breakdown of liver cells, e.g. infectious hepatitis, homologous serum jaundice, and toxic liver damage caused by chemicals (Molander, Sheppard and Payne, 1957; Wróblewski and LaDue, 1956a,b). The determination of these two enzymes also has a prognostic and epidemiological value in acute hepatitis (Wróblewski, Jervis and LaDue, 1956; Goldberg and Riddell Campbell, 1962).

Both the S-GOT and S-GPT may be raised to varying degrees in chronic liver damage and obstructive jaundice (Molander et al., 1957; Chinsky, Wolff and Sherry, 1957), but not to high values found with acute liver cell damage.

Isocitrate Dehydrogenase.

The activities of other enzymes in the serum of jaundiced patients have been studied by many investigators. One of these is isocitrate dehydrogenase (E.C. 1.1.1.42.). This is one of the enzymes concerned in the aerobic breakdown of carbohydrate in the citric acid cycle, and catalyses the reaction:-

$$\text{1-isocitrate} + \text{NADP} \underset{Mn^{2+}}{\rightleftharpoons} \text{2-oxoglutarate} + \text{NADPH}_2 + CO_2$$

(Colowick and Kaplan, 1955). It is widely distributed in animal tissues and found in high concentrations in heart, liver, kidney and adrenal glands, moderate amounts in ovary and intestine, and smaller amounts in muscle, brain, lung, testis and spleen (Adler, Euler, Günther and Plass, 1939). It also has measurable activity in sera taken from apparently healthy people (Wolfson and Williams-Ashman, 1957a).

Serum Isocitrate Dehydrogenase (S-ICD) in Liver Disease.

Values of S-ICD to be found with most types of hepatic disease have been described.

a) Acute Damage to Liver Cells.

White (1958) stated that the highest concentrations of the enzyme in mouse tissues were to be found in liver cells. Liver homogenates from rats have also been found to have the highest isocitrate dehydrogenase activity with 30% more activity than the next highest tissue- heart - which, in turn, was very much more active than other rat tissues (Nachlas, Davidson, Goldberg and Seligman, 1963). These facts are reflected in human disease where greatly abnormal amounts of isocitrate dehydrogenase in serum are found in conditions causing acute damage and breakdown of liver cells. Wolfson and Williams-Ashman (1957b) showed that very high values for S-ICD were obtained in acute hepatitis, e.g. infectious hepatitis and homologous serum jaundice. Since then, all other authors reporting on S-ICD have stated that the highest levels of activity were encountered in these conditions. Sterkel, Spencer, Wolfson and Williams-Ashman (1958) stated that in cases of infectious hepatitis, high S-ICD values of approximately 15 times the normal mean were found within 10 days following the onset of jaundice; these returned to normal values approximately 10 days later. Okumera and Spellberg (1960) investigated 20 patients with acute hepatitis, 12 due to infectious hepatitis, 6 with homologous serum jaundice and two with toxic hepatitis due to the drug, iproniazid. They stated that the highest S-ICD values were to be seen early

in the disease, but that the S-ICD had no direct relationship to the patient's clinical condition or to the duration of the illness. There was no correlation with the S-ICD and other tests indicating hepatocellular damage. Bell, Shaldon and Baron (1962) showed that in 48 cases of infectious hepatitis all had raised S-ICD values at some stage of the disease. In general, peak values occurred 5-10 days after onset of symptoms and usually returned to normal by the third to fourth week of illness.

In some instances the S-ICD has been compared with one or both of the serum transaminases in acute hepatitis. Sterkel et al. (1958) indicated that S-GPT showed similar changes to S-ICD, the rises in S-GPT activity often being of greater magnitude than those of S-ICD. Kerppola, Nikkilä and Pitkänen (1959) showed that S-ICD mirrored the S-GOT in acute liver cell damage. Hargreaves, Janota and Smith (1961) showed an incidence of abnormal values of 85% for S-ICD and both transaminases in acute hepatitis. They demonstrated that the S-ICD tended to return to normal rather more quickly than the transaminases. Bodansky, Schwartz, Krugman, Giles and Jacobs (1960) undertook a sequential study of S-ICD activity in children submitted to inoculation with viral hepatitis and compared the results with S-GOT. They found that in 9 cases which developed clinical evidence of hepatitis, 8 showed elevations in S-ICD and S-GOT on the same day. They expressed the rise

in enzyme activity as $\frac{\text{Maximal activity recorded}}{\text{Upper limit of normal}}$ and showed that the S-GOT rose correspondingly higher than the S-ICD. The above ratio for S-ICD varied from 11-38, while for S-GOT the corresponding values were 16-50. The authors concluded that the S-GOT was to be preferred to the S-ICD. Franken, Brauns, Storck and Kazmier (1960) claimed that the S-ICD was not so significantly raised as the transaminases in acute hepatitis. Cohen, Potters and Bowers (1961) stated that S-ICD was raised on average to 16 times the upper limit of normal and that the S-GPT was a slightly more sensitive indicator of acute hepatic damage than the S-ICD and S-GOT, these last two being comparable in sensitivity.

Bell et al. (1962) showed that in acute liver damage caused by hepatotoxic drugs, the S-ICD was elevated in many cases before the S-GOT and both enzymes showed abnormal results before the other standard tests of liver function. They concluded that the S-ICD was a very sensitive test for detecting liver damage caused by drugs.

b) Chronic Liver Damage.

Variable S-ICD values are found in chronic diseases of the liver. Wolfson, Spencer, Sterkel and Williams-Ashman (1958) stated that normal S-ICD activities were usually found in portal cirrhosis. Okumera and Spellberg (1960) found normal or slightly elevated values (up to twice the

upper limit of normal) in a group of 18 patients with portal cirrhosis. Two cases of hepatic coma had normal values.

In 10 cases in the series of Kerppola et al. (1959), 4 showed abnormal S-ICD values and all of these were stated to be in the 'active' phase of the disease, characterised by considerable jaundice and inflammatory changes in the liver. Cohen et al. (1961) supported this by stating that the majority of 19 'active' cases had raised S-ICD values which agreed with the clinical impression of the activity of the condition, whilst 5 'inactive' cases showed normal S-ICD values. Opposed to this, Sterkel et al. (1958) found 5 raised S-ICD values in 27 results on 19 patients with portal cirrhosis and stated that there was no relationship between the S-ICD, S-GPT and the degree of hyperbilirubinaemia or clinical evidence of activity of the cirrhotic process. Franken et al. (1960) stated that the S-ICD was variable in cirrhosis and not related to the clinical state of the patient. Bell et al. (1962) found the S-ICD elevated to varying degrees in 28 of 39 cases and divided these into compensated and decompensated patients, defining a 'decompensated' case as one with a serum bilirubin above 2.0 mg./100 ml. and a serum albumin of less than 3.0 g./100 ml. They found that no correlation existed between the S-ICD and degree of compensation of the patient, nor with the severity of the cirrhosis as judged by the presence of ascites or hepatic coma.

There are few ~~incidences~~ instances of the S-ICD being compared with transaminases in chronic liver damage. Hargreaves et al. (1961) found abnormal values for S-ICD and S-GPT in 50% of cases compared with 65% for S-GOT. Cohen et al. (1961) claimed that the S-GOT and S-GPT gave essentially the same results as S-ICD in their 'active' and 'inactive' cases.

c) Malignant Disease in the Liver.

In patients with neoplastic diseases, increases in S-ICD activity have been associated with secondary involvement of the liver (Wolfson and Williams-Ashman, 1957b). White (1958) showed that 13 cases of metastatic cancer with no hepatic secondaries had normal S-ICD values, while 4 patients with metastatic invasion of the liver all had high S-ICD values - up to three times normal. Sterkel et al. (1958) stated that elevations of S-ICD in an individual with malignancy appeared to indicate liver metastases. Cohen et al. (1961) found raised S-ICD values in 70% of cases with carcinoma of the liver, compared with abnormal transaminase values in 64% of the cases. They stated that the S-ICD approached the alkaline phosphatase in sensitivity as an index of liver metastases. Tan, Cohen, West and Zimmerman (1963) in a very large series of metastatic liver cases (284 patients), found that the S-ICD was raised in the majority of cases up to twice the upper limit of normal, and above this in 20% of the cases. The S-ICD was elevated in

a higher proportion of cases than were the transaminases.

d) Obstructive Jaundice.

Conflicting reports have been published concerning the significance of S-ICD in obstructive jaundice. Wolfson et al. (1958) claimed that the S-ICD was seldom raised in extra-hepatic obstruction of the biliary tract. Sterkel et al. (1958) claimed that the enzyme could be used to differentiate between intra-hepatic jaundice and extra-hepatic obstructive jaundice, basing this on 12 S-ICD determinations on 6 cases of extra-hepatic obstruction, three benign and three malignant, which showed normal S-ICD values. The S-GPT showed 5 abnormal results in 12 determinations and they claimed that the S-ICD would yield more specific information than the S-GPT in the differential diagnosis of intra- and extra-hepatic jaundice. Bell et al. (1962) supported this statement, saying that S-ICD was normal in 19 of 20 cases of uncomplicated obstructive jaundice of short duration, whereas the S-GOT was raised in 20% of these cases. They stated, however, that long-standing obstruction leading to liver cell damage resulted in raised S-ICD values up to three times the upper limit of normal.

In opposition to this, Cohen et al. (1961) found raised values in 4 of 6 cases of extra-hepatic obstruction, the average of the 6 cases giving a rise in S-ICD of 1.6 times

the upper limit of normal. Kerppola et al. (1959) found normal S-ICD in 7 cases of cholelithiasis, but stated that increased S-ICD in obstructive jaundice pointed to malignant disease. Okumera and Spellberg (1960) found S-ICD values ranging from 2-4.5 times normal in 4 of 5 cases of malignant obstructive jaundice, these cases being classed separately from those with liver metastases. In data presented by Saad, Steigman and Dubin (1963), only one case out of 12 with benign obstructive jaundice, and only 4 of 19 malignant cases had normal S-ICD values. Norberg (1961) noted a 36% incidence of increased S-ICD values in patients with symptoms of acute cholecystitis, with or without accompanying jaundice. Okumera and Spellberg (1960) showed high S-ICD in two out of 17 cases of acute and chronic cholecystitis. Hargreaves et al. (1961) claimed that the S-ICD was raised in 30% of 25 cases of obstructive jaundice, while the S-GPT was raised in 60% and the S-GOT in 80%, but their series included 6 cases with biliary cirrhosis. Bell et al. (1962) found raised S-ICD values in 21 of 27 cases of biliary cirrhosis.

It is of interest to note that Okumera and Spellberg (1960) reported that three cases of extra-hepatic neonatal jaundice (two with atresia of the common bile duct, and one caused by a choledochal cyst) had S-ICD values 1.5-5 times normal. The S-ICD fell to normal 5 days after surgical removal of the cyst and relief of the obstruction. Komiya

(1963), studying S-ICD in infancy and childhood, found that 10 of 13 cases of common bile duct atresia and one case of choledochal cyst had moderate elevations of S-ICD. Wolfson and Williams-Ashman (1957b) stated that an increase in S-ICD activity followed bile duct ligation in rats, and Okumera and Spellberg (1960) found moderate elevations after ligation of one hepatic duct in dogs and also following an experimental biliary fistula.

Outline of the Present Investigation.

This present work was undertaken to assess the rôle that S-ICD can play in the diagnosis of hepatic disease, and compare the results, wherever possible, with the two serum transaminases, alkaline phosphatase and other biochemical tests of liver function. S-ICD was determined in a large group of patients with various diseases affecting the hepatic and biliary system. Particular attention was paid to cases of extra-hepatic biliary obstruction in the hope of clarifying the S-ICD results which are found in this condition, since the overall status of the enzyme as a test in the differential diagnosis of jaundice depends to a large extent upon its response to biliary retention as opposed to liver cell damage.

PART 1.

MATERIAL AND METHODS.

All serum S-ICD and other enzyme analyses were estimated on unhaemolysed serum specimens. The analyses were carried out with minimal delay after withdrawal of the specimen, but material that could not be handled immediately was stored at -20° and the analysis completed in less than two weeks. The serum could be kept at -20° for this period with minimal loss of S-ICD, transaminase and alkaline phosphatase activity.

Estimation of Isocitrate Dehydrogenase in Serum.

The enzyme was estimated in serum by the colorimetric method described by Bell and Baron (1960), which depends upon measurement of the coloured DNP-hydrazone of α-oxo-glutaric acid in alkaline solution.

Reagents:-

1. 0.1M-Tris buffer, pH 7.5

2. Buffered substrate. 1.845g. of dl-trisodium isocitric acid were dissolved in 70ml. tris buffer, 15ml. N-NaOH added and the solution stood overnight. The pH was adjusted to 7.5 with N-HCl and the volume made to 100ml. with tris buffer; the solution was stored at -20°.

3. 0.013M-NADP. 10mg. of NADP (Boehringer) dissolved in 1ml. of 0.15M-NaCl. This was made up fresh for each batch of analyses.

4. 0.03M-Manganese chloride. 0.597g. of $MnCl_2.4H_2O$ dissolved in 100ml. of 0.15M-NaCl.

5. 0.19M-EDTA. 7.1g. of the disodium salt of EDTA dissolved in 100ml. water.

6. 0.001M-DNP-hydrazine. 19.8mg. dissolved in 100ml. N-HCl

7. 0.4N-NaOH

8. α-oxoglutaric acid standard. A stock solution was made by dissolving 70mg. α-oxoglutaric acid (B.D.H.) in 100ml. water. This was made up fresh every two weeks.

Procedure:-

The following reagents were pipetted into test tubes placed in a 37° water bath:

Serum test - 0.3ml. of 0.15M-NaCl, 0.1ml. of 0.03M-$MnCl_2$, 0.5ml. of buffered substrate, 0.1ml. of 0.013M-NADP.

Serum control - 0.4ml. of 0.15M-NaCl, then as the serum test but minus the NADP.

NADP blank - 1.0ml. of 0.15M-NaCl, 0.1ml. of $MnCl_2$, 0.1ml. of 0.013M-NADP.

The reagents were allowed to equilibrate to 37°, then 0.2ml. serum was added to serum test and control, and incubated for exactly 60 minutes. The enzyme reaction was stopped by adding 1 ml. of 0.19M-EDTA to complex the Mn^{2+}, followed immediately by 1 ml. of 0.001M-DNP-hydrazine. The tubes were shaken and left at room temp. for 20 minutes. Ten ml. of 0.4N-NaOH were then added to each tube, left for 15 minutes, and serum test read against control at 390 mμ on a Unicam SP600 spectrophotometer. The NADP blank was

read against a reagent blank and the extinction subtracted from each test reading.

A calibration curve was made from different dilutions of the stock α-oxoglutarate solution. 0.1ml. of 0.03M-$MnCl_2$, 1 ml. of 0.19M-EDTA and 1 ml. of 0.001M-DNP-hydrazine were added to 1 ml. volumes of the diluted standards, the tubes left for 20 minutes, 10ml. of 0.4N-NaOH added and the extinction read against a reagent blank at 390 mμ. A new calibration curve was made for each new batch of DNP-hydrazine and NaOH solutions.

A unit of S-ICD activity was expressed as the formation of 1 mμmole of α-oxoglutarate/min./ml. at 37°. Enzyme units/ml. serum were obtained from the calibration curve by the equation $\frac{\mu g.\ \alpha\text{-oxoglutarate} \times 5 \times 1000}{146 \times 60}$ = S-ICD units/ml. These units may be converted to I.U./litre by dividing by a factor of 3 to allow for the change in reaction rate at 25° instead of 37°.

Estimation of Transaminases in Serum.

S-GOT was measured by the method of Cabaud, Leeper and Wróblewski (1956), and S-GPT by that of Wróblewski and Cabaud (1957). These standard methods involve measuring the coloured DNP-hydrazone of pyruvate, which is formed directly by the enzyme S-GPT from the reaction:-

l-alanine + α-oxoglutarate $\rightleftharpoons$ pyruvate + glutamate

and indirectly in the estimation of S-GOT from the reaction:-

aspartate + α-oxoglutarate $\rightleftharpoons$ oxalacetate + glutamate.

The oxalacetate is converted to pyruvate by aniline citrate. These methods involve the precipitation of proteins to stop the enzyme reaction and the pyruvate DNP-hydrazone being extracted into toluene before colorimetric assay.

A unit of transaminase activity is defined as that amount of enzyme in 1 ml. of serum which forms 1 µg. of pyruvic acid in 20 minutes at 25° under the test conditions. To convert these units to I.U./litre, they should be multiplied by 0.57.

Estimation of Alkaline Phosphatase in Serum.

This was estimated using the standard method first described by King, Haslewood, Delory and Beall (1942). It involves the colorimetric determination of phenol liberated by the enzyme from sodium phenyl phosphate.

A unit of enzyme activity is defined as that amount of enzyme contained in 100ml. of serum which will liberate 1 mg. of phenol in 15 minutes at 37° under the test conditions. These units must be multiplied by 7.1 to convert them to I.U./litre (King and Campbell, 1961).

Other biochemical tests of liver function were estimated by standard routine methods. Serum bilirubin was measured by the method of Malloy and Evelyn (1937), thymol turbidity

and flocculation as described by Maclagan (1944) and zinc sulphate turbidity by the method of Kunkel (1947).

PART 1.

RESULTS.

Methodology of Isocitrate Dehydrogenase Estimation.

Bell and Baron (1960), in their original publication on the colorimetric measurement of S-ICD, undertook a detailed investigation into the effects of various reaction conditions, e.g. kinetics of the reaction with time and temperature, substrate and coenzyme concentrations, metal ion cofactor requirements, and quantities of test material (serum) used.

In the present investigation, it was felt necessary to check on the reproducibility of the method by performing duplicate analyses and also varying the amount of serum and incubation time, in order to cope with specimens with high S-ICD activity.

A number of specimens were analysed in duplicate at the commencement of the investigation and results on 10 sera covering a wide range of enzyme activity are shown in Table 1. Very good reproducibility occurred over the whole range of activity studied.

Specimens with moderate to high activities were also measured using reduced amounts of serum; results are shown in Table 1 where it can be seen that reproducible results were again obtained.

High activity specimens were also analysed by varying the incubation time and it is shown by the data in Table 1 that the reaction is linear with time over the 60 minute incubation period.

TABLE 1.

DATA ON THE ESTIMATION OF S-ICD TO SHOW THE REPRODUCIBILITY OF THE METHOD, AND VARIATION IN REACTION CONDITIONS.

Part A:- S-ICD results of duplicate analyses.

Specimen	Serum ml.	Incubation time min.	S-ICD units/ml. Analysis no.1	Analysis no.2
1.	0.2	60	4	5
2.	"	"	8	8
3.	"	"	12	13
4.	"	"	13	14
5.	"	"	15	15
6.	"	"	25	26
7.	"	"	30	30
8.	"	"	33	34
9.	0.1	"	55	53
10.	"	30	100	102

Part B:- S-ICD results using different aliquots of serum.

			ml. of serum 0.2	0.1	0.05
11.	-	60	21	22	20
7.	-	"	30	32	32
12.	-	30	46	48	48

Part C:- S-ICD results using different incubation times.

			Incubation time(min.) 60	30	15
8.	0.2	-	33	34	35
12.	"	-	47*	46	48
13.	"	-	52*	54	54

*0.1ml. serum used.

Determination of a Normal Range for Serum Isocitrate Dehydrogenase.

Blood samples taken from 40 healthy people, aged from 20-80 years, were used to determine a normal range for S-ICD activity. They included laboratory workers, medical students and pre-operative specimens taken from apparently healthy people admitted for minor surgery, e.g. repair of hernia. These gave a normal range of approximately 3-10 units/ml. (mean 6.0, S.D. 1.7), which agrees almost exactly with the range quoted by Bell et al. (1962).

ISOCITRATE DEHYDROGENASE IN HEPATIC DISEASE.

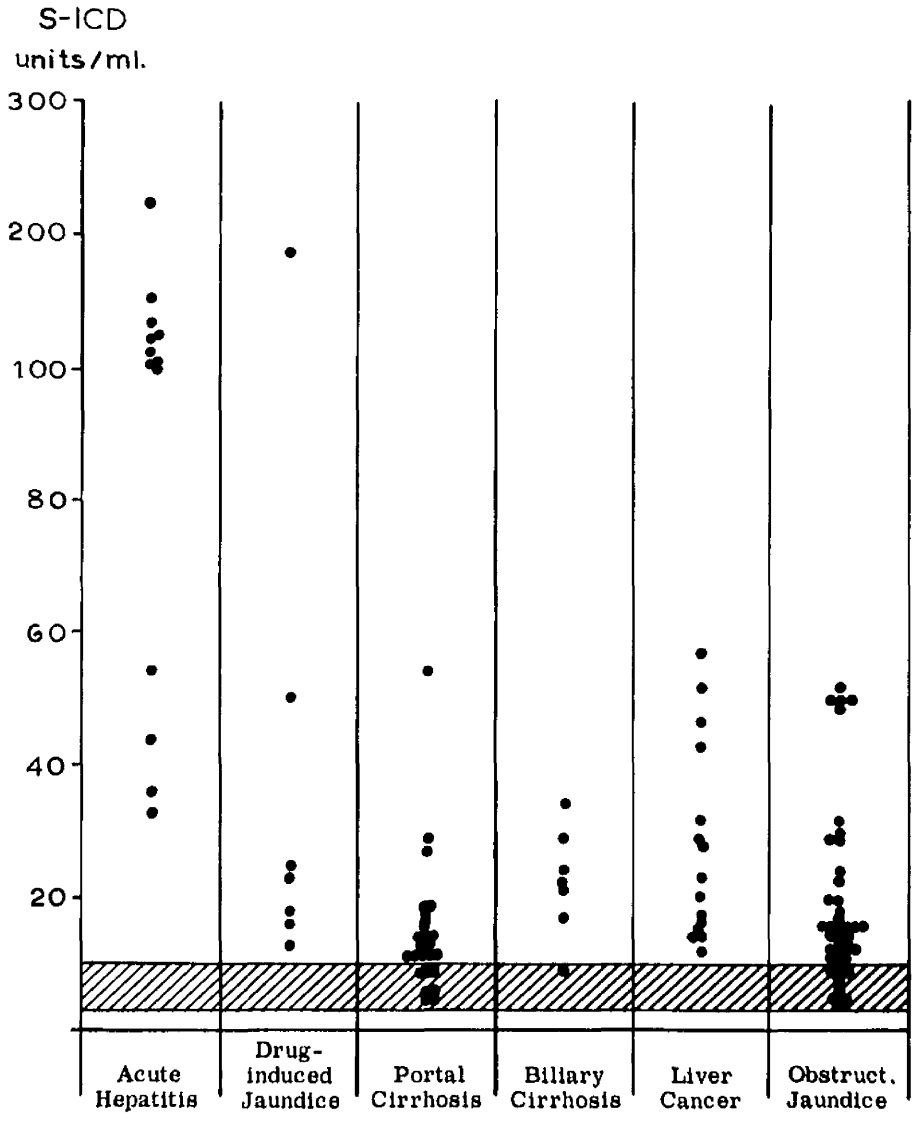
S-ICD activities in cases of various hepato-biliary diseases are classified in Fig. 1. Each point represents one S-ICD determination on a single case. The distribution of S-ICD values found in the various groups is shown in Table 2 where abnormal values are expressed as multiples of the upper limit of normal - 10 units/ml.

a) Acute Hepatitis.

The patients were all suffering from infectious hepatitis and all had high values ranging from 33-224 units/ml. Specimens were obtained within 14 days of the onset of symptoms except in two cases which gave S-ICD values of 104 units/ml. at 22 days in one case, and 54 units/ml. at 27 days in the other.

FIGURE 1.

S-ICD VALUES IN DIFFERENT TYPES OF HEPATO-BILIARY DISEASE.



The normal range for S-ICD of 3-10 units/ml. is shown by the shaded area.

TABLE 2.

DISTRIBUTION OF S-ICD RESULTS IN VARIOUS HEPATIC DISEASES.

		Cases with normal S-ICD	Cases with raised S-ICD				
			1-1.5x	1.5-2x	2-3x	3-4x	>4x
			upper limit of normal				
Units/ml.		3-10	11-15	16-20	21-30	31-40	>40
Acute hepatitis	(13)	0	0	0	0	2	11
Portal cirrhosis	(25)	7	11	4	2	0	1
Biliary cirrhosis	(7)	1	0	1	4	1	0
Obstructive jaundice	(53)	9	15	12	7	3	7
Subdivided into:-							
Malignant with liver secondaries	(11)	0	3	2	3	1	2
Malignant without liver secondaries	(14)	5	4	4	1	0	0
Non-malignant without acute inflammation	(20)	4	8	5	3	0	0
Non-malignant with acute inflammation	(8)	0	0	1	0	2	5

Figures in parenthesis denote total no. of cases in each group.

b) Drug-Induced Jaundice.

There were three cases of jaundice attributed to the drug chlorpromazine, one due to promazine, and one case of poisoning by a phenolic disinfectant ('Sanizal'). Biochemical data on these cases are shown in Table 3. The specimen on the first case (M.K.) was taken three weeks after commencing chlorpromazine therapy (50mg. t.i.d.); the second (M.M.) was obtained 7 days after a single injection of 50mg. chlorpromazine, and the third (H.G.) following a two week period of treatment with the drug.

The specimen from patient R.N. was taken when jaundice was noticed approximately 10 days following a dose of promazine. Subsequent laparotomy showed no mechanical obstruction in the biliary tract, but liver biopsy produced histology consistent with obstructive jaundice.

The jaundice caused by phenolic poisoning in patient J.W. was found by haematology and subsequent post mortem to be haemolytic. Liver morphology and histology was described as normal. The high alkaline phosphatase was probably due to the patient also having extensive bone fractures. The raised S-ICD in this case does not appear, therefore, to be associated with liver damage or biliary retention.

The two highest S-ICD values in this group (188 and 50 units/ml.) were found in two cases of liver necrosis caused by a hypersensitivity reaction to p-aminosalicylic

TABLE 3.

S-ICD VALUES AND OTHER BIOCHEMICAL DATA ON DRUG-INDUCED JAUNDICE CASES.

Case	Serum bil.	Alk. phos.	TT/TF	$ZnSO_4$ Turb.	S-GOT	S-GPT	S-ICD
	(0.2-1.7	4-12	0-2/0	3-12	5-50	0-50	3-10)
	mg.%	K-A units%	units	units		units/ml.	
M.K.*	6.8	24	0/0	5	-	204	25
M.M.*	6.3	64	1/0	10	-	64	18
H.G.*	2.7	15	0/0	3	76	165	16
R.N.†	6.8	44	1/0	2	-	-	13
J.W.¶	20.8	35	1/0	3	-	-	23

Normal range shown in parenthesis.

* Chlorpromazine jaundice

† Promazine jaundice

¶ Phenol-induced jaundice

acid. One of these cases was investigated in detail. This was a man, aged 20, who developed acute liver failure 28 days after starting on 12g. p-aminosalicylic acid daily. He was severely ill for two weeks, but eventually made a slow recovery. Biochemical tests showing the course of the acute hepatic upset are shown in Table 4. As can be seen, the patient became jaundiced and showed ample evidence of severe liver damage. The turbidity and flocculation tests were grossly abnormal and this was reflected by the changes in serum proteins where there was an increase in the globulins (mainly γ globulin) giving an inverse albumin/globulin ratio. The alkaline phosphatase showed a high value indicative of biliary obstruction. The transaminases, however, reached values found only with acute hepatocellular damage, indicating extensive liver cell damage. The S-ICD was also considerably raised, this fitting in very well with the other biochemical findings.

Once the patient had fully recovered, it was decided to confirm the diagnosis of a hypersensitivity reaction by giving a challenge dose of p-aminosalicylic acid. This offered the opportunity for investigating, biochemically, the occurence of possible hepatocellular damage.

The patient was given a 3g. dose of p-aminosalicylic acid and serial blood samples were withdrawn at time intervals shown in Table 5. There was an increase in the activities of S-ICD and S-GPT to abnormal values, and the

TABLE 4.

BIOCHEMICAL DATA ON A CASE OF LIVER DAMAGE CAUSED BY p-AMINOSALICYLIC ACID.

	Bili-rubin	TT/TF*	$ZnSO_4$ Turb.	Proteins Alb./Glob.	Alk. phos.	S-GOT	S-GPT	S-ICD
	mg.%	units	units	g.%	K-A units%		units/ml.	
Normal Range	0.2-1.7	0-2/0	3-12	3.9-/1.7 5.5 3.0	4-12	5-50	0-50	3-10
Days†								
28	2.5	2/0	3	3.4/2.0	34	-	-	-
33	8.3	4/+1	8	4.0/2.6	53	432	728	50
40	1.8	10/+4	24	3.5/4.2	23	214	500	18
46	0.5	5/+3	27	4.5/3.7	16	42	186	5
54	0.5	6/+3	15	5.0/2.7	11	20	44	-
76	0.4	4/0	14	4.7/3.7	9	21	25	-
158	0.5	2/0	9	4.8/2.3	7	13	16	5

* Thymol turbidity/thymol flocculation

† Days after commencing p-aminosalicylic acid therapy

TABLE 5.

BIOCHEMICAL DATA ON LIVER CELL DAMAGE FOLLOWING A TEST DOSE OF p-AMINOSALICYLIC ACID.

Time*	Serum bilirubin	Alkaline phosphatase	S-GOT	S-GPT	S-ICD
	(0.2-1.7 mg.%	4-12 K-A units%	5-50	0-50 units/ml.	3-10)
0 hours	0.5	7	13	16	5
2 "	-	7	21	18	5
6 "	-	5	25	16	6
12 "	-	6	6	10	5
24 "	0.4	5	11	14	7
2 days	0.7	6	42	43	14
3 "	-	6	38	58	13
4 "	0.6	6	25	59	11
7 "	-	6	32	54	11
9 "	0.6	6	22	31	7

Abnormal values are underlined.

*Time following the administration of a 3g. dose of p-aminosalicylic acid.

S-GOT also seemed to show a slight rise, although never to abnormal values. Two other enzymes not reported here - sorbitol dehydrogenase and adenosine deaminase - also produced abnormal values over this period (Watts and Griffiths, 1964). The serum bilirubin showed no evidence of subclinical jaundice and the alkaline phosphatase did not increase. But the response of the enzymes coincided with symptoms of general malaise and liver tenderness and were believed to be conclusive evidence of subclinical liver cell damage.

c) S-ICD in Cirrhosis.

Twenty-five cases of portal cirrhosis showed variable S-ICD results. Seven cases (28%) had a normal value, the majority of the abnormal results were only minimal rises (below 15 units/ml.), while only three cases gave values above 20 units/ml. Bell et al. (1962) defined a decompensated case of cirrhosis as one with a serum bilirubin greater than 2.0mg./100ml. and a serum albumin less than 3.0g./100ml., and attempted to correlate the S-ICD with the degree of compensation as assessed by these criteria. In the present series, the S-ICD values were not related to the compensation of the patient.

d) Biliary Cirrhosis.

All these cases were diagnosed with the aid of liver

biopsy. Only one of the 7 cases had a normal S-ICD, and subsequent specimens taken from that case produced S-ICD values of 15 and 12 units/ml.

e) Malignant Invasion of the Liver.

Fifteen cases of invasive cancer of the liver all gave raised S-ICD values ranging from 12-57 units/ml. These included 11 cases with liver metastases from different primaries, two reticulosarcomas and one lymphadenoma. One case of primary liver hepatoma had an S-ICD of 47 units/ml.

f) S-ICD in Obstructive Jaundice.

All analyses in this group were carried out on pre-operative blood specimens. In no case was there clinical, biochemical or operative evidence of concomitant liver cell injury, although in the majority of cases liver biopsy specimens were not taken to confirm this. In those cases where biopsy specimens were obtained, the histology was compatible with uncomplicated biliary obstruction. Fifty-three cases were investigated. Of these, only 9 (17%) had normal S-ICD activity. Fifteen cases (28%) had minimal increases (up to 1.5 times the upper limit of normal), 19 cases (36%) had moderate increases ranging from 16-30 units/ml., and the remainder (19%) had high values up to 52 units/ml.

Cases of obstructive jaundice were divided into 4 groups

as shown in Table 2 (p.29):-

1. Malignant obstructive jaundice with metastatic spread to the liver. These cases were classified with the liver cancer cases in Fig. 1. They were diagnosed at operation and/or post mortem.

2. Malignant obstruction with no evidence of liver metastases. Again the diagnosis was made at operation or autopsy.

3. A non-malignant, non-inflammatory group - comprising cholelithiasis and chronic cholecystitis with little or no evidence of an acute inflammatory process occurring at the time of blood withdrawal, when applying the criteria stated below.

4. Non-malignant obstruction accompanied by acute inflammation at or near the time of blood withdrawal. The criteria for inclusion in this group were, operative or pathological evidence of acute inflammation of the gall bladder or biliary tract, or pyrexia over 100°F or pronounced leucocytosis associated with jaundice when no other cause was apparent when the blood specimen was taken.

The distribution of S-ICD values in these groups is shown in Table 2, and statistical analysis of the data is presented in Table 6. It can be seen from the latter Table that secondary invasion of the liver in malignant obstructive jaundice gave significantly higher S-ICD values, but this was not invariably so as there was some overlap

TABLE 6.

SERUM ISOCITRATE DEHYDROGENASE IN OBSTRUCTIVE JAUNDICE.

Group	No. of cases	S-ICD units/ml. Mean ± S.D.	t	P
Malignant with liver secondaries	11	27 ± 14.7		
			3.244	<0.005
Malignant without liver secondaries	14	13 ± 6.8		
			0.928	n.s.
Non-malignant without acute inflammation	20	15 ± 5.8		
			7.938	<0.001
Non-malignant with acute inflammation	8	42 ± 12.3		

t values were obtained by applying the Student t test between the means of the various groups.

between groups 1 and 2 (Table 2). There was no significant difference between S-ICD values found in uncomplicated malignant and non-malignant cases (groups 2 and 3), and the spread of results was similar in the two groups. Although the group with acute inflammation was small compared with the non-inflammatory group, the S-ICD values in the former group showed a high degree of significance from those of the latter ($P < 0.001$). As can be seen from Table 2 (p.29) there was very little overlap between these groups, only one case from the inflammatory group having an S-ICD as low as 20 units/ml. and falling within the range of the non-inflammatory group. It should be mentioned that three cases were not included in either of these groups as their classification was equivocal. Two had very high S-ICD values (47 and 52 units/ml.) and also showed some evidence of acute inflammation, but not sufficient to satisfy the criteria for inclusion in the inflammatory group. When the blood specimens were taken, both had pyrexia just below $100^{o}F$ and one also had a slight neutrophilia. There was no evidence of an acute inflammatory process at operation 14 days later in one case and 5 days later in the other. The third case had an S-ICD of 8 units/ml., and although there was no clinical evidence of inflammation when the blood specimen was taken, pus was found in the biliary tract at operation 8 days later.

Fig. 2 shows serial analyses of S-ICD, transaminases and alkaline phosphatase on one of the cases in the non-inflammatory group. This man had an operation for mild obstructive jaundice which proved to be due to multiple adhesions surrounding the gall bladder. There was no evidence of acute inflammation. He developed, post-operatively, an infection associated with jaundice which persisted for 8 days. The S-ICD showed a very pronounced rise to 58 units/ml., whereas the transaminases, whilst giving a sharp increase, remained within the range generally seen in obstructive jaundice (both below 400 units/ml.). The alkaline phosphatase was also greatly elevated. The turbidity tests and serum proteins remained normal. It seems likely that this man developed a post-operative cholangitis.

Table 7 shows the S-ICD results on the non-inflammatory benign cases and malignant obstructive jaundice cases with no liver metastases divided on the basis of duration of symptoms. The onset of symptoms was taken in its widest sense, i.e. the first indication of abdominal or biliary upset, however vague or non-specific. It seems obvious that the S-ICD results were distributed irrespective of duration of symptoms.

In some of these cases, the S-ICD was determined post-operatively after successful surgical relief of the biliary obstruction. The S-ICD returned to normal fairly

FIGURE 2.

SERUM ENZYMES IN A CASE OF POST-OPERATIVE JAUNDICE ASSOCIATED WITH INFECTION.

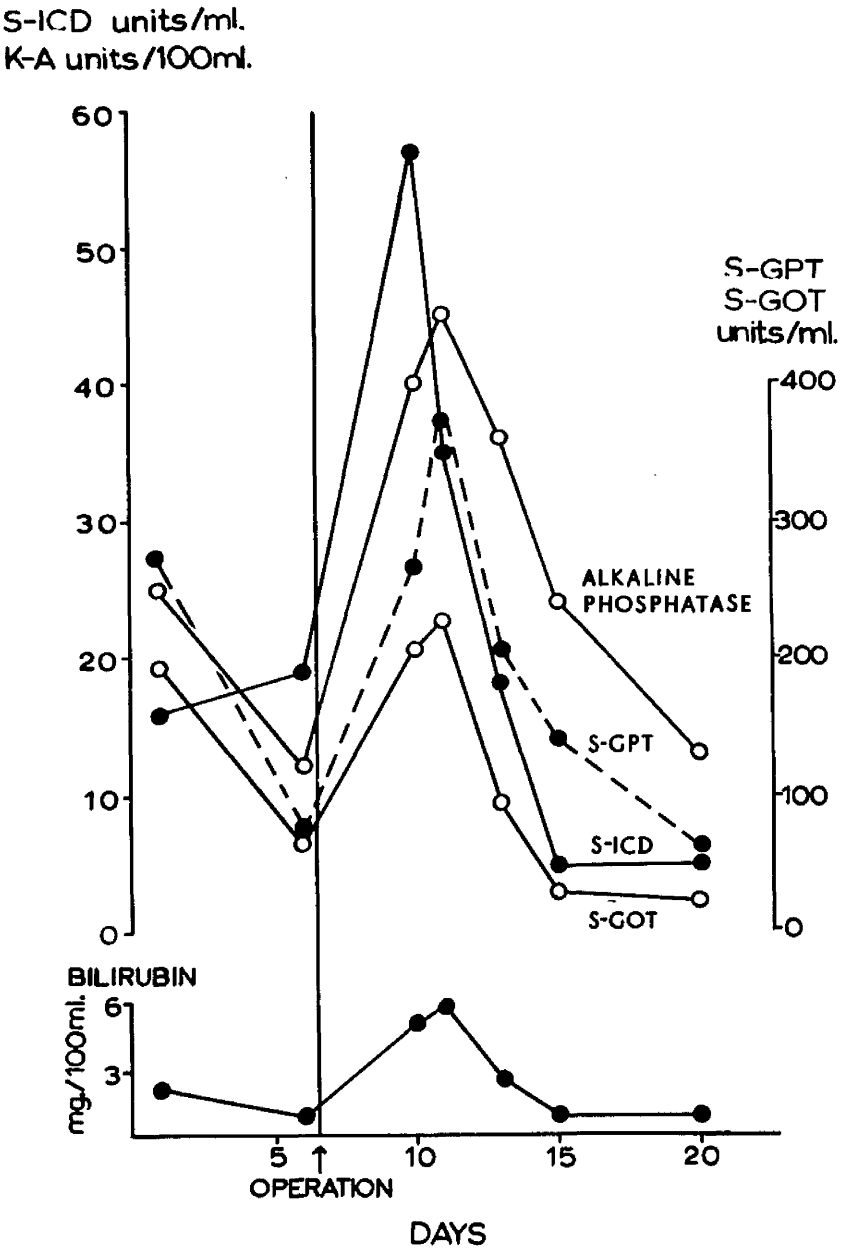


TABLE 7.

S-ICD IN OBSTRUCTIVE JAUNDICE CASES WITH VARYING DURATION OF SYMPTOMS.

Group	No. of cases	S-ICD range (units/ml.)	Mean
Patients with symptoms of 1 week or less duration	5	5-29	14
Patients with symptoms of 2-3 weeks duration	9	11-16	14
Patients with symptoms of 1-3 months duration	11	5-29	14
Patients with a long past history of biliary upset or abdominal symptoms	9	4-24	13

Cases included are those with malignant obstruction without liver metastases and non-malignant without acute inflammation.

quickly in those cases which showed a raised pre-operative S-ICD.

SERUM TRANSAMINASE RESULTS.

In many of the patients studied, either one or both of the transaminases were measured. The distribution of results for the S-GOT and S-GPT are shown in Tables 8 and 9. It can be seen that, as expected, both transaminases produced very high values in acute hepatitis with all values well above 400 units/ml. The S-GPT usually showed greater increases than the S-GOT (Fig. 3, p.49).

There was a wide scatter of results for both transaminases in portal cirrhosis. The S-GPT was normal in 60% of cases and the S-GOT in 40%. Elevations in activity were in general only moderate and values for both S-GOT and S-GPT were usually well below 200 units/ml., except in two cases which produced S-GOT results of 204 and 212 units/ml. These results in no way correlated with the clinical status of the patient.

In obstructive jaundice, the results were again very variable and were scattered fairly uniformly over the range 0-400 units/ml. A normal S-GPT was found in 13% of the cases and normal S-GOT in 21%. There was a random distribution of abnormal results in the 4 groups of extra-hepatic biliary obstruction. Statistical analysis of these results is shown in Table 10 for the S-GPT and Table 11 for

TABLE 8.

DISTRIBUTION OF S-GPT RESULTS IN VARIOUS HEPATIC DISEASES.

		Cases with normal S-GPT	Cases with raised S-GPT					
			1-1.5x upper limit of normal	1.5-2x upper limit of normal	2-3x upper limit of normal	3-4x upper limit of normal	4-8x upper limit of normal	>8x upper limit of normal
Units/ml.		0-50	51-75	76-100	101-150	151-200	201-400	>400
Acute hepatitis	(13)	0	0	0	0	0	0	13
Portal cirrhosis	(20)	12	2	2	3	1	0	0
Biliary cirrhosis	(7)	1	0	1	1	2	2	0
Obstructive jaundice	(39)	5	4	7	8	5	10	0
Subdivided into:-								
Malignant with liver secondaries	(9)	1	3	0	3	1	1	0
Malignant without liver secondaries	(10)	4	0	1	2	0	3	0
Non-malignant without acute inflammation	(13)	0	1	5	3	1	3	0
Non-malignant with acute inflammation	(7)	0	0	1	0	3	3	0

Figures in parenthesis denote total no. of cases in each group.

TABLE 9.

DISTRIBUTION OF S-GOT RESULTS IN VARIOUS HEPATIC DISEASES.

		Cases with normal S-GOT	Cases with raised S-GOT					
			1-1.5x upper limit of normal	1.5-2x upper limit of normal	2-3x upper limit of normal	3-4x upper limit of normal	4-8x upper limit of normal	>8x upper limit of normal
Units/ml.		5-50	51-75	76-100	101-150	151-200	201-400	>400
Acute hepatitis	(13)	0	0	0	0	0	0	13
Portal cirrhosis	(21)	8	4	2	4	1	2	0
Biliary cirrhosis	(7)	0	0	1	2	2	2	0
Obstructive jaundice	(28)	6	5	3	6	4	4	0
Subdivided into:-								
Malignant with liver secondaries	(6)	2	0	1	1	1	1	0
Malignant without liver secondaries	(8)	2	2	0	1	1	2	0
Non-malignant without acute inflammation	(9)	2	2	2	2	1	0	0
Non-malignant with acute inflammation	(5)	0	1	0	2	1	1	0

Figures in parenthesis denote total no. of cases in each group.

TABLE 10.

SERUM GLUTAMIC PYRUVIC TRANSAMINASE IN OBSTRUCTIVE JAUNDICE.

Group	No. of cases	S-GPT units/ml. Mean ± S.D.	t	P
Malignant with liver secondaries	9	112 ± 87		
			0.643	n.s.
Malignant without liver secondaries	10	143 ± 131		
			0.188	n.s.
Non-malignant without acute inflammation	13	135 ± 71		
			2.108	n.s.
Non-malignant with acute inflammation	7	231 ± 125		

t values were obtained by applying the Student t test between the means of the various groups.

TABLE 11.

SERUM GLUTAMIC OXALACETIC TRANSAMINASE IN OBSTRUCTIVE JAUNDICE.

Group	No. of cases	S-GOT units/ml. Mean ± S.D.	t	P
Malignant with liver secondaries	6	126 ± 104		
			0.377	n.s.
Malignant without liver secondaries	8	124 ± 99		
			0.957	n.s.
Non-malignant without acute inflammation	9	88 ± 49		
			1.700	n.s.
Non-malignant with acute inflammation	5	165 ± 124		

the S-GOT. No significant difference could be detected between the groups for either of the transaminases. The S-GPT, however, just missed significance at the 5% level between the non-inflammatory and inflammatory groups and a difference may well have shown up with a greater number of inflammatory cases.

Fig. 3 shows the ratio of S-GOT to S-GPT in patients who had either one or both of the transaminases raised above normal. It shows clearly the diagnostic advantage of this ratio over the absolute values for the transaminases in chronic liver damage where it is almost invariably greater than 1.0. Only one of 13 cases showed a ratio less than this (0.92). It can be seen that in the majority of cases of other types of hepatic disease, the S-GPT was usually higher than the S-GOT.

ALKALINE PHOSPHATASE RESULTS.

Serum alkaline phosphatase was determined in all the patients studied, and the results obtained are shown in Table 12. In all but two of the cases with acute and chronic liver cell damage, the alkaline phosphatase was below 30 K-A units/100ml. One case of viral hepatitis had an alkaline phosphatase of 42 K-A units/100ml., and one case of portal cirrhosis had a value of 54 K-A units/100ml. In biliary cirrhosis, the alkaline phosphatase tended to be higher, with 4 out of 6 cases having values

FIGURE 3.

RATIO OF S-GOT TO S-GPT IN VARIOUS HEPATO-BILIARY DISEASES.

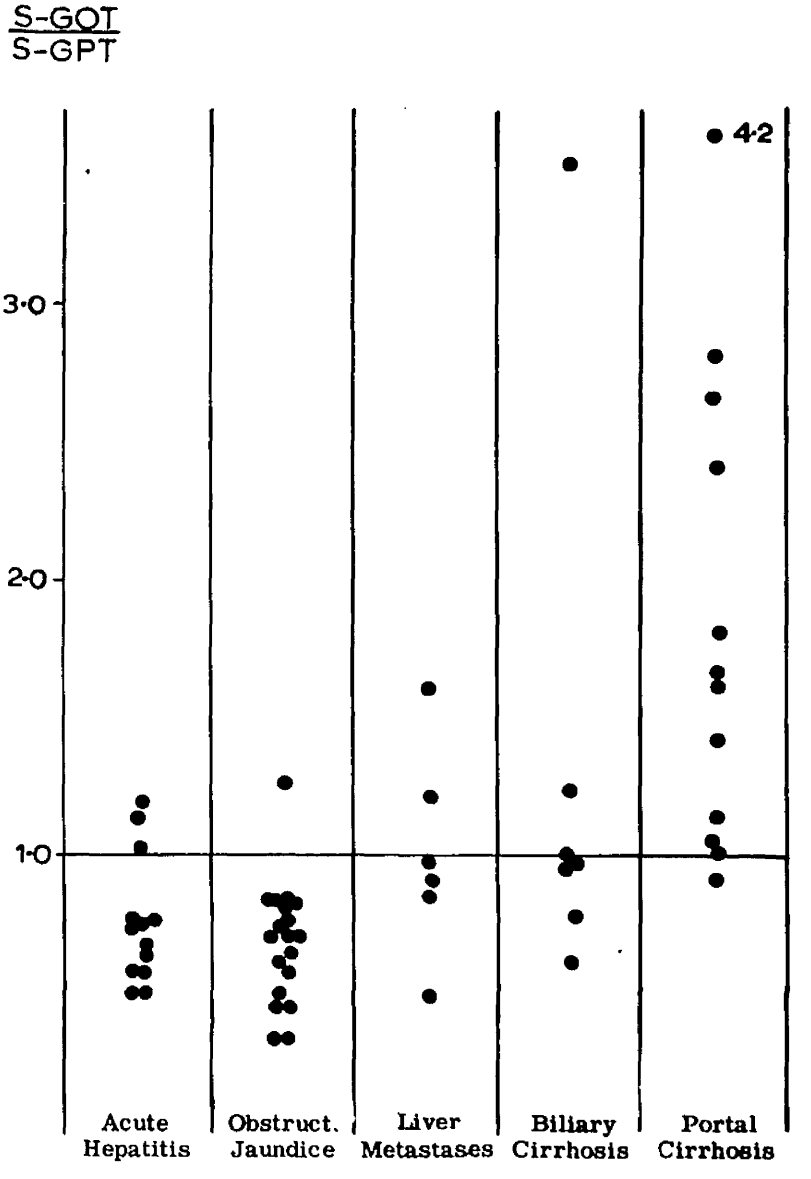


TABLE 12.

DISTRIBUTION OF SERUM ALKALINE PHOSPHATASE RESULTS IN VARIOUS HEPATIC DISEASES.

		Cases with normal alk. phosphatase	Cases with raised alk. phos.				
			1-1.5x	1.5-2x	2-3x	3-4x	>4x
			upper limit of normal				
K-A units/100ml.		4-12	13-18	19-24	25-36	37-48	>48
Acute hepatitis	(12)	1	6	4	0	1	0
Portal cirrhosis	(25)	10	9	2	3	0	1
Biliary cirrhosis	(6)	1	1	0	1	0	3
Obstructive jaundice	(53)	3	3	5	26	4	12
Subdivided into:-							
Malignant with liver secondaries	(11)	0	0	0	4	1	6
Malignant without liver secondaries	(14)	0	0	2	8	2	2
Non-malignant without acute inflammation	(20)	1	1	3	12	0	3
Non-malignant with acute inflammation	(8)	2	2	0	2	1	1

Figures in parenthesis denote total no. of cases in each group.

above 30 K-A units/100ml., and three of these 4 had very high values of 80, 90, and 104 K-A units/100ml.

The enzyme had normal activity in only 6% of obstructive jaundice cases. Twenty-two cases (42%) had values below 30 K-A units/100ml.; 11 of these, however, were between 25 and 30 K-A units/100ml. In considering the different types of obstructive jaundice, it is clear that the presence of liver secondaries gave, in general, higher alkaline phosphatase values than in the other groups. This is shown in Table 13 where statistical analysis between the groups gave a significant difference between malignant cases with secondary liver involvement and those without liver metastases ($P < 0.02$). Five of the cases with liver secondaries had extremely high values ranging from 63-152 K-A units/100ml.

COMPARISON OF S-ICD RESULTS WITH SERUM TRANSAMINASES.

S-ICD values could be compared with one or both of the transaminases when these enzymes were determined in the same blood specimen; this applied in the majority of cases.

Acute Hepatitis.

S-ICD was compared with both S-GOT and S-GPT in the 13 cases of viral hepatitis. In all of them, high S-ICD values were invariably accompanied by high transaminase activities. It can be seen from Fig. 4, however, that the correlation for S-ICD with respect to S-GPT - 0.49 ($P < 0.02$),

TABLE 13.

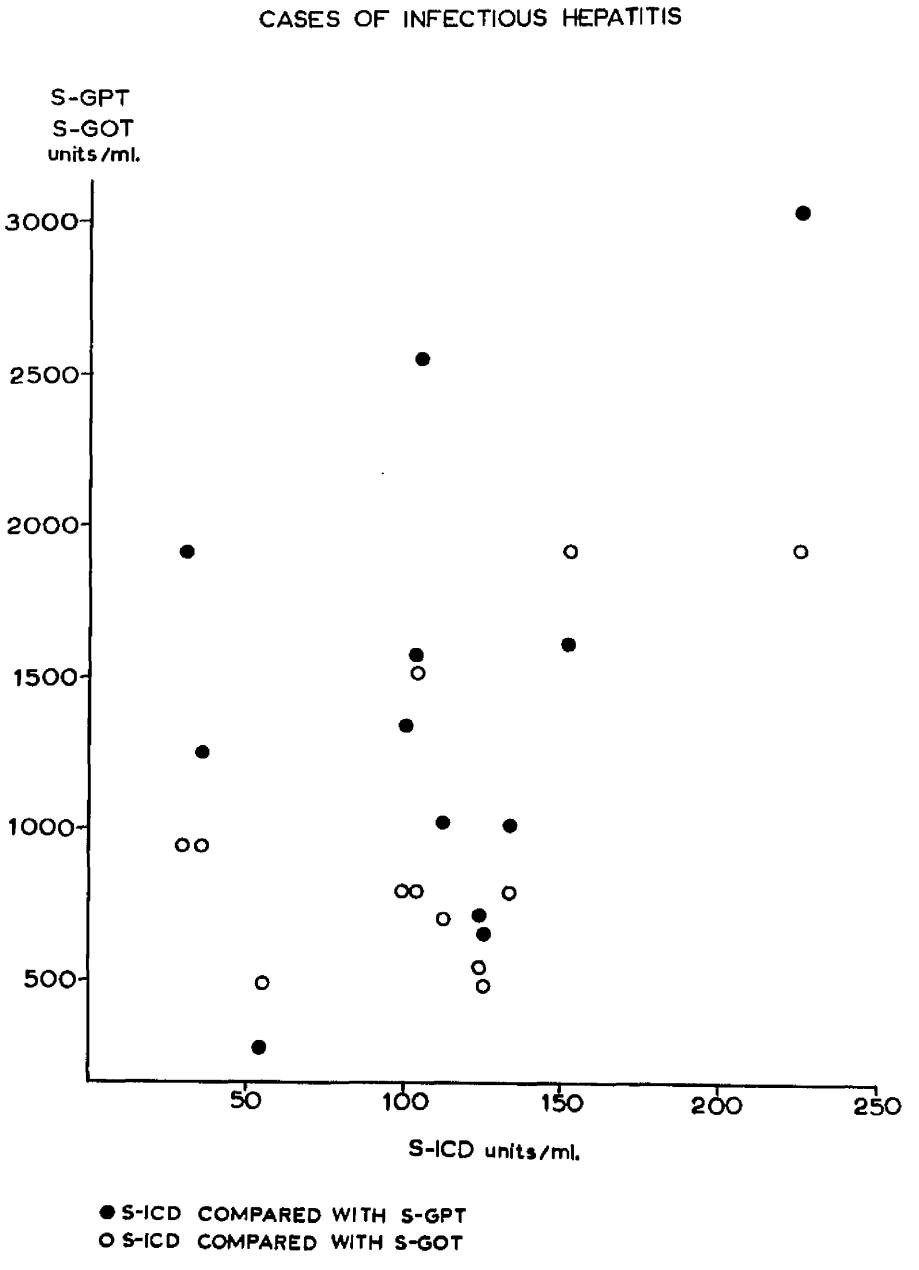
SERUM ALKALINE PHOSPHATASE IN OBSTRUCTIVE JAUNDICE.

Group	No. of cases	Mean ± S.D. (K-A units/100ml.)	t	P
Malignant with liver secondaries	11	67 ± 39		
			2.736	<0.02
Malignant without liver secondaries	14	35 ± 15		
			0.378	n.s.
Non-malignant without acute inflammation	20	33 ± 15		
			0.391	n.s.
Non-malignant with acute inflammation	8	30 ± 25		

t values were obtained by applying the Student t test between the means of the various groups.

FIGURE 4.

RELATIONSHIP OF S-ICD AND SERUM TRANSAMINASES IN ACUTE HEPATITIS.



and with respect to S-GOT, 0.55 ($P < 0.005$), was low.

On the other hand, Fig. 5 shows how the S-ICD can mirror the transaminase results. These sequential enzyme values were obtained from a young girl who had a severe attack of viral hepatitis with a very slow recovery period. The response of the S-ICD and transaminases was almost identical. The first specimen was obviously taken early in the developement of liver damage, as the peaks of enzyme activity had not been reached. The initial rise and fall of S-ICD was very similar to that of the transaminases, and this is also shown very clearly in the period 20-30 days, when there was a secondary rise in enzyme values due to the premature ambulation of the patient. All the enzymes were markedly elevated after 70 days and indicated the probable onset of chronic liver damage.

The extent to which each enzyme rises may be expressed by the ratio - $M = \frac{\text{maximum activity measured}}{\text{upper limit of normal}}$ after Bodansky et al. (1962). This was determined for S-ICD, S-GPT and S-GOT in all the infectious hepatitis cases. The comparative rise in S-ICD activity was then compared with that of S-GPT and S-GOT; this is shown in Fig. 6 and illustrates the fact that in all but two cases, both serum transaminases produced a higher response than the S-ICD.

In the other two cases of acute hepatocellular damage, caused by p-aminosalicylic acid, high S-ICD values again coincided with high transaminase results; this is shown for

FIGURE 5.

SERUM ENZYMES IN A CASE OF SEVERE INFECTIOUS HEPATITIS.

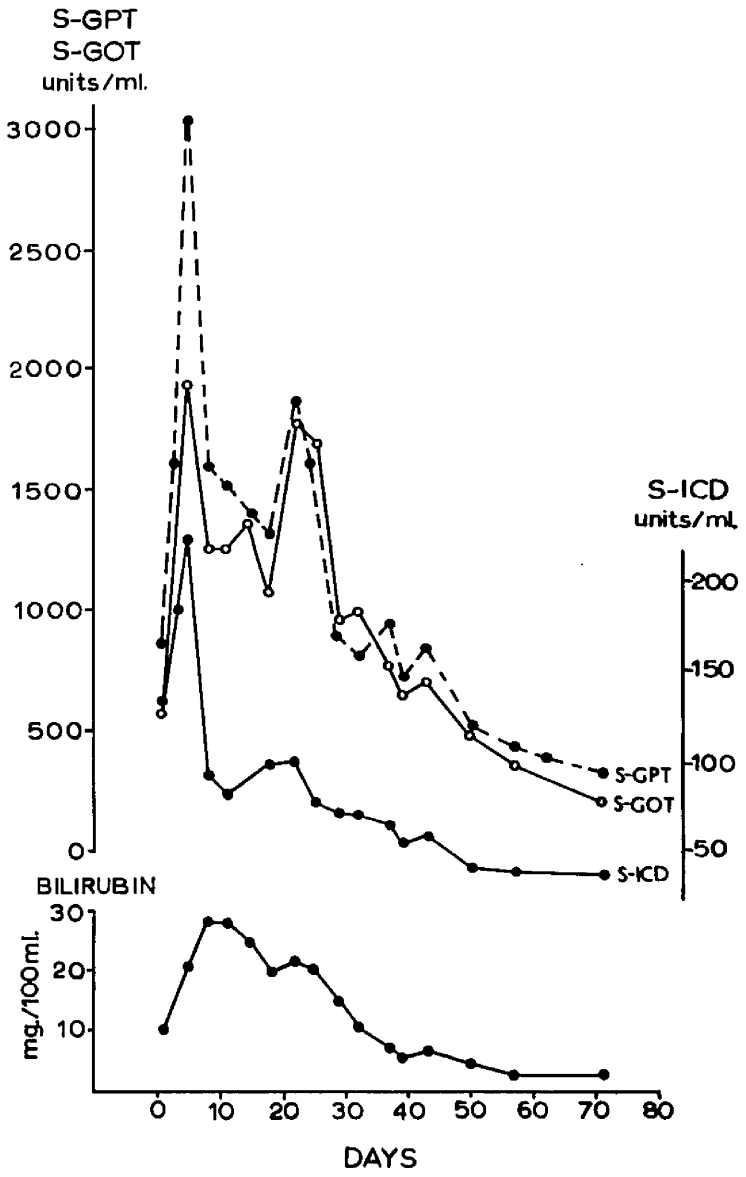
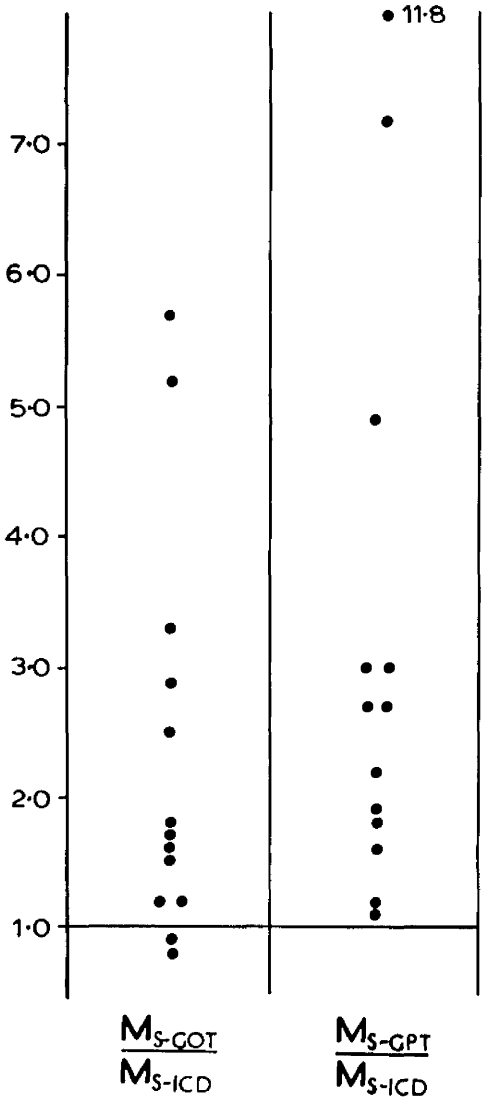


FIGURE 6.

COMPARISON BETWEEN THE RISE IN S-ICD AND TRANSAMINASE ACTIVITIES IN ACUTE HEPATITIS.

CASES OF INFECTIOUS HEPATITIS



M for each enzyme = $\frac{\text{maximum activity measured}}{\text{upper limit of normal}}$

one of the cases in Table 4 (p. 33). The S-ICD fell back to normal values much sooner than the S-GPT. In the subsequent experiment involving the test dose of p-amino-salicylic acid, the S-ICD was probably slightly more sensitive than the S-GPT, and definitely more so than the S-GOT, in reflecting the subclinical hepatic dysfunction (Table 5, p. 34). In the other case, the very high S-ICD value of 188 units/ml. corresponded to an S-GPT of only 1035 units/ml. and an S-GOT of 573 units/ml. Eight days later, the S-ICD had fallen to 32 units/ml., the S-GPT to 342 and the S-GOT to 140 units/ml.

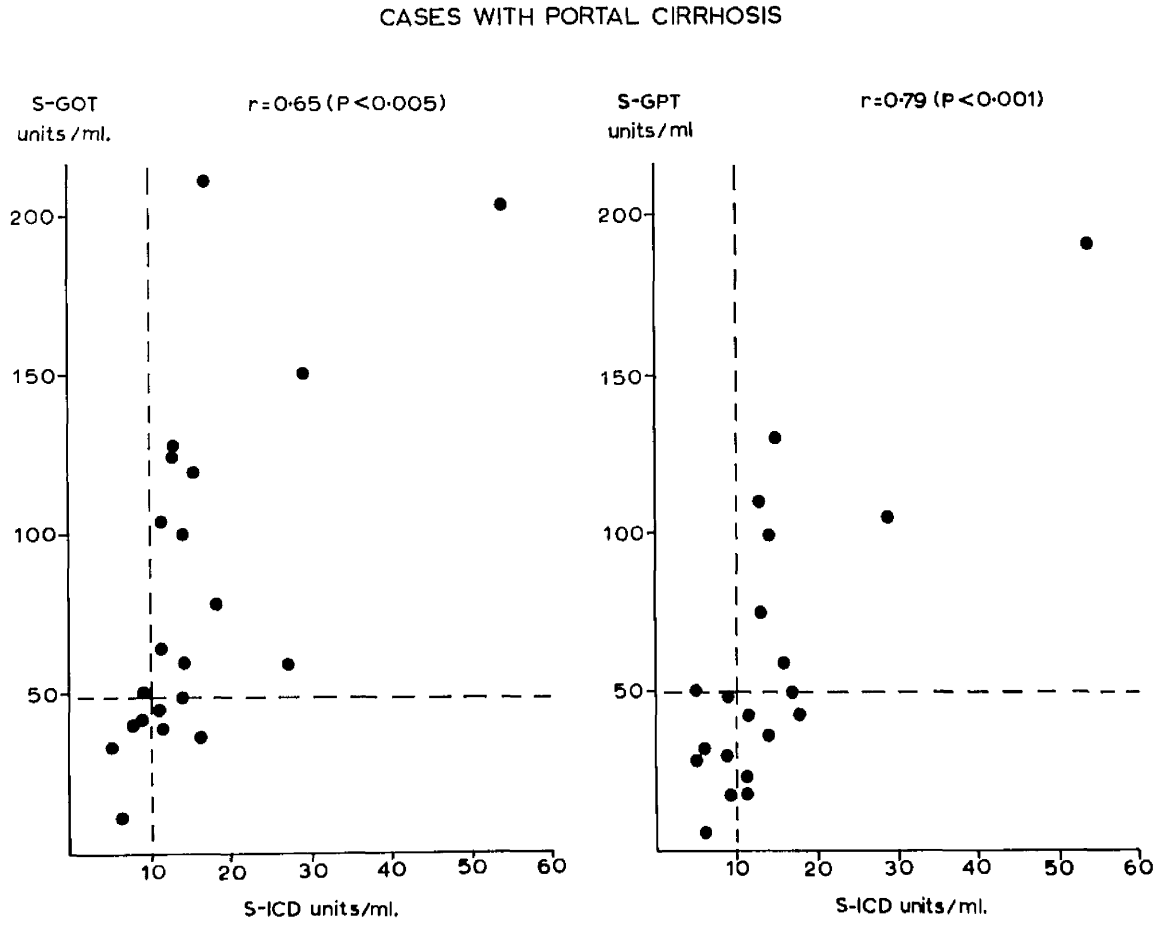
Portal Cirrhosis.

In chronic liver damage, cases showing raised S-GOT or S-GPT activities usually had an S-ICD above normal, the only exceptions being two cases with minimal rises in serum transaminases who had normal S-ICD values. The correlation between S-ICD and both transaminases is shown in Fig. 7. Statistical analysis of the data showed that there was a slightly closer relationship between the S-ICD and S-GPT, with a significant correlation coefficient of 0.79 ($P < 0.001$), than between the S-ICD and S-GOT with a significant coefficient of 0.65 ($P < 0.005$). None of these enzymes were related, however, to the clinical state of the patient.

It should be noted that the ratio of S-GOT to S-GPT was greater than 1.0 in all but one case, and gave a more

FIGURE 7.

RELATIONSHIP OF S-ICD AND SERUM TRANSAMINASES IN PORTAL CIRRHOSIS.



r = correlation coefficient

Broken lines indicate the upper limit of normal for each enzyme.

consistent pattern than the wide variability of the individual S-ICD and transaminase values.

Biliary Cirrhosis.

The S-ICD was closely related to both transaminases in all 7 cases of biliary cirrhosis. This is shown in Fig. 8, which includes two sets of results from one patient. Statistical analysis gave very good correlation coefficients of 0.93 ($P < 0.001$) for S-ICD with respect to S-GPT and 0.82 ($P < 0.02$) for S-ICD with respect to S-GOT.

Obstructive Jaundice.

The S-ICD could be correlated with the S-GPT in 20 cases of benign biliary obstruction; 7 of these were in the acute inflammatory group. In the malignant cases, the S-ICD and S-GPT were both estimated in 19 cases - 9 with liver metastases and 10 without. The correlation between the two enzymes is shown in Fig. 9, and, as can be seen, is not very pronounced. In the cases of uncomplicated obstructive jaundice (the non-inflammatory benign group and malignant obstruction without liver metastases), the correlation coefficient for S-ICD and S-GPT was 0.43 ($P < 0.05$).

In the non-malignant group, the higher S-ICD values associated with acute inflammation were reflected by the S-GPT in only two cases. In addition, no significant difference could be shown statistically between the S-GPT

FIGURE 8.

RELATIONSHIP OF S-ICD AND SERUM TRANSAMINASES IN BILIARY CIRRHOSIS.

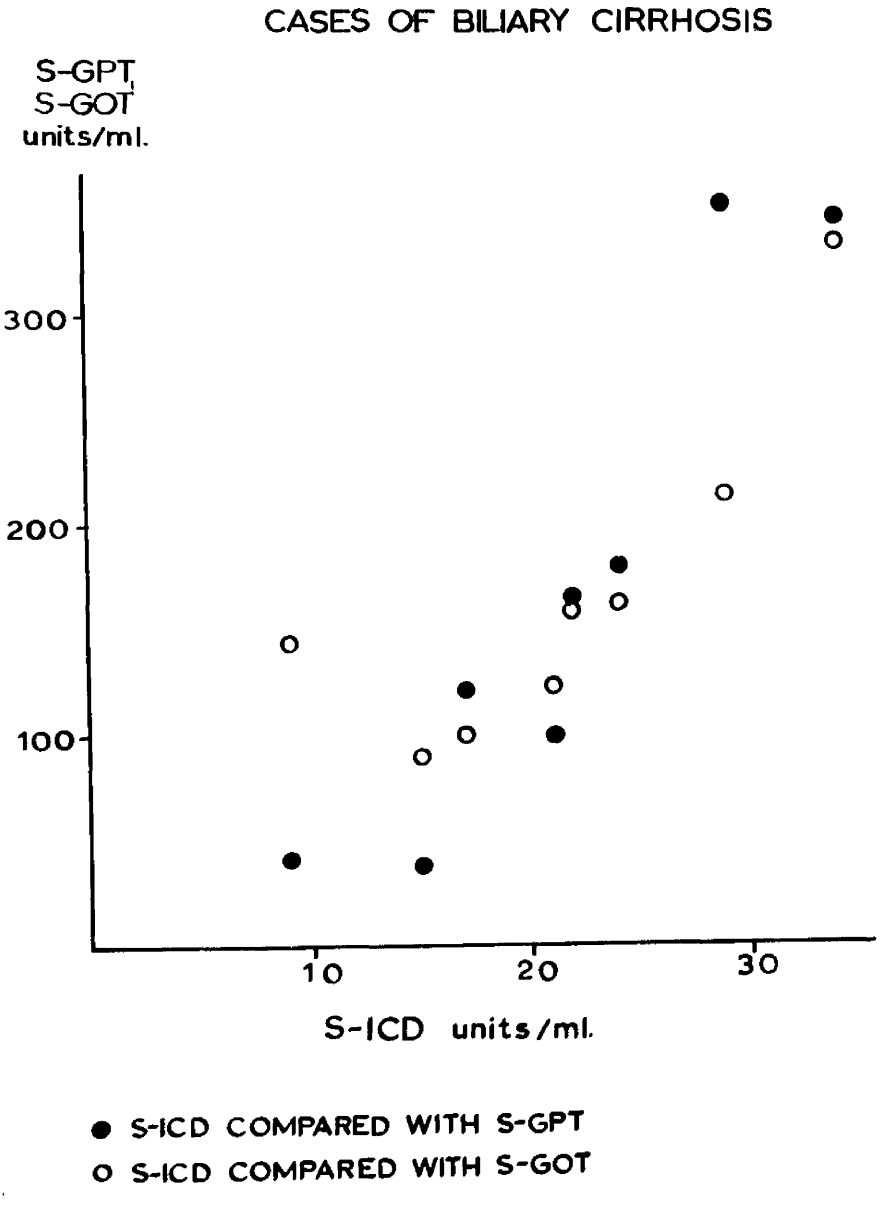
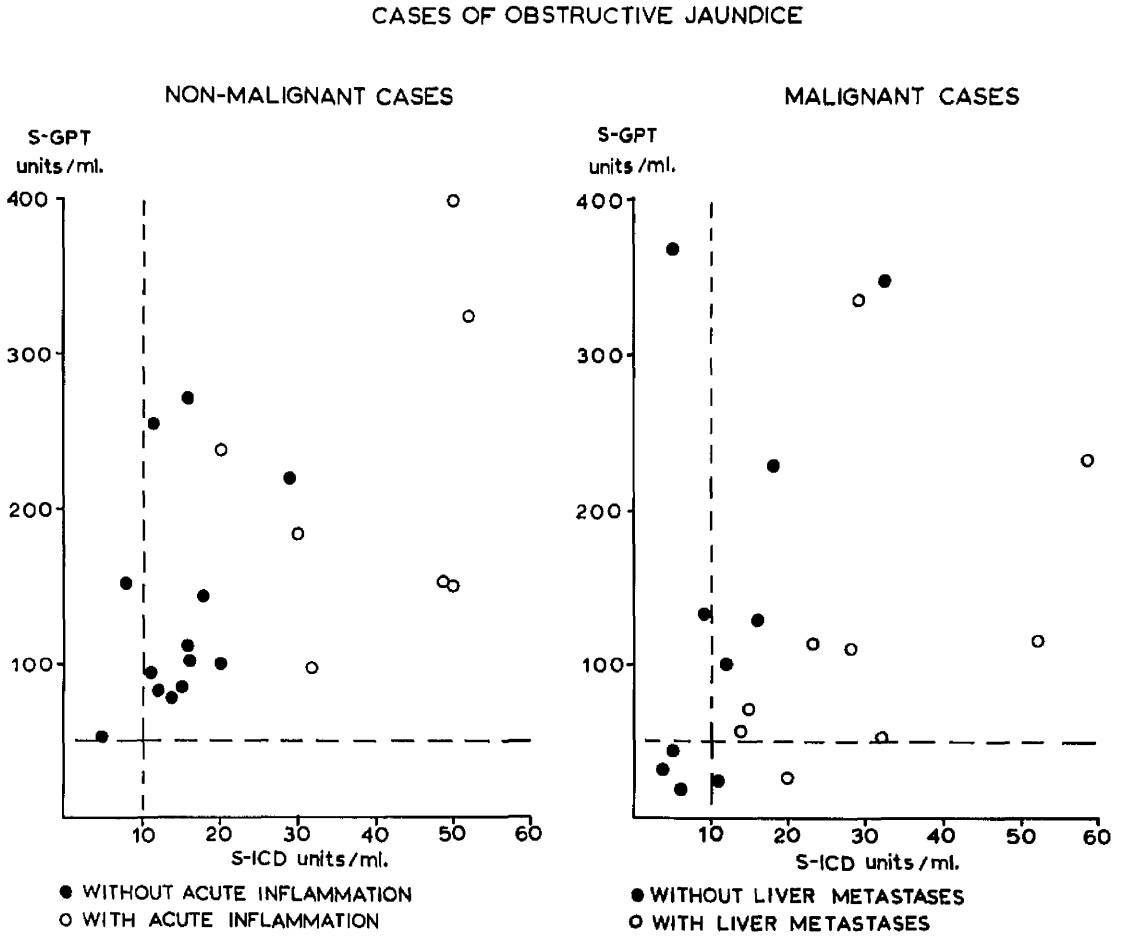


FIGURE 9.

RELATIONSHIP OF S-ICD AND S-GPT VALUES IN OBSTRUCTIVE JAUNDICE.



Broken lines indicate the upper limit of normal for each enzyme.

results obtained from the inflammatory and non-inflammatory groups (Table 10, p.46), whereas the S-ICD values in these two groups showed high significance (Table 6, p.38).

In the malignant cases, it can be seen from Fig. 9 that S-ICD tends to give comparatively higher results than the S-GPT when there is secondary involvement of the liver. There was not sufficient cases, however, to show a statistically significant difference between the regression coefficients for the two groups as shown under the malignant cases in Fig. 9.

There were fewer cases in which the S-ICD and S-GOT could be correlated; these were 14 non-malignant cases (5 with acute inflammation) and 14 malignant cases, 6 of which had liver metastases. The S-GOT and S-ICD results on these cases are shown in Fig. 10. There were insufficient results to show a significant correlation between the two enzymes, but it can be seen that these results mirror those found for the S-ICD and S-GPT shown in Fig. 9.

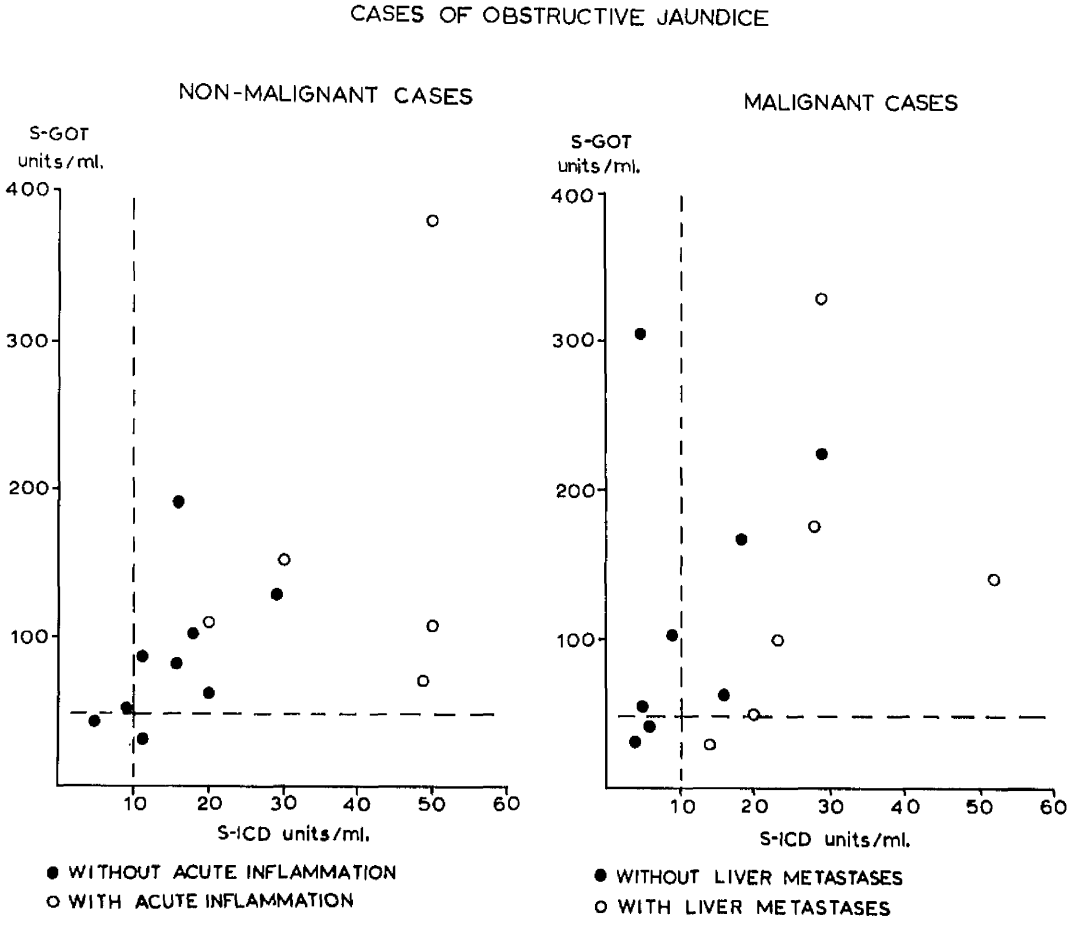
S-ICD RESULTS COMPARED WITH ALKALINE PHOSPHATASE AND OTHER TESTS OF LIVER FUNCTION.

a) Alkaline Phosphatase.

There was little point in considering these two enzymes in relationship to each other except in cases of obstructive jaundice, where the alkaline phosphatase might

FIGURE 10.

RELATIONSHIP OF S-ICD AND S-GOT VALUES IN OBSTRUCTIVE JAUNDICE.



Broken lines indicate the upper limit of normal for each enzyme.

be considered as a possible index of severity of biliary retention. This is illustrated in Fig. 11 and shows that there is no correlation between the two enzymes. Although both enzymes showed abnormal values in all cases with liver metastases, the alkaline phosphatase tended to give greater increases in activity than the S-ICD. Acute inflammation in the benign group did not affect alkaline phosphatase values in the same way as it did the activity of S-ICD (cf. Table 6, p.38and Table 13, p.52).

b) Serum Bilirubin.

In the cases of acute and chronic liver damage there was no correlation between the S-ICD values and degree of hyperbilirubinaemia. In obstructive jaundice, the concentration of serum bilirubin may also be considered as another index of degree of biliary retention. Fig. 12 shows that there is no correlation between S-ICD and bilirubin in the uncomplicated cases of extra-hepatic obstruction.

c) Thymol Turbidity and Flocculation Tests, and Zinc Sulphate Turbidity Test.

These tests of liver cell integrity depend upon quantity and quality of proteins produced by the hepatic cell and passed into the blood stream. They show up damage to liver cells when abnormal amounts of protein enter the circulation and cause turbidity when serum is added to certain buffered solutions. Consequently, these tests

FIGURE 11.

COMPARISON OF S-ICD AND ALKALINE PHOSPHATASE RESULTS IN CASES WITH UNCOMPLICATED OBSTRUCTIVE JAUNDICE.

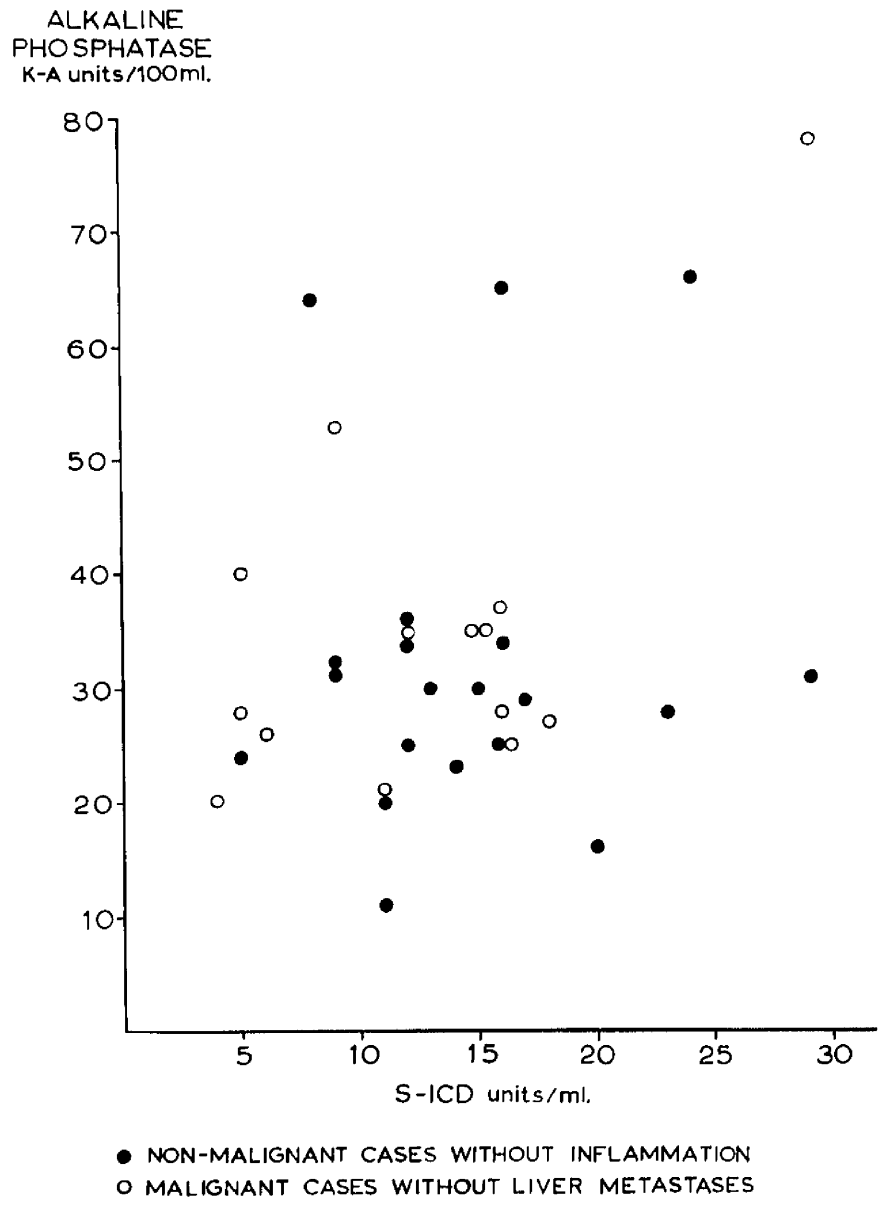
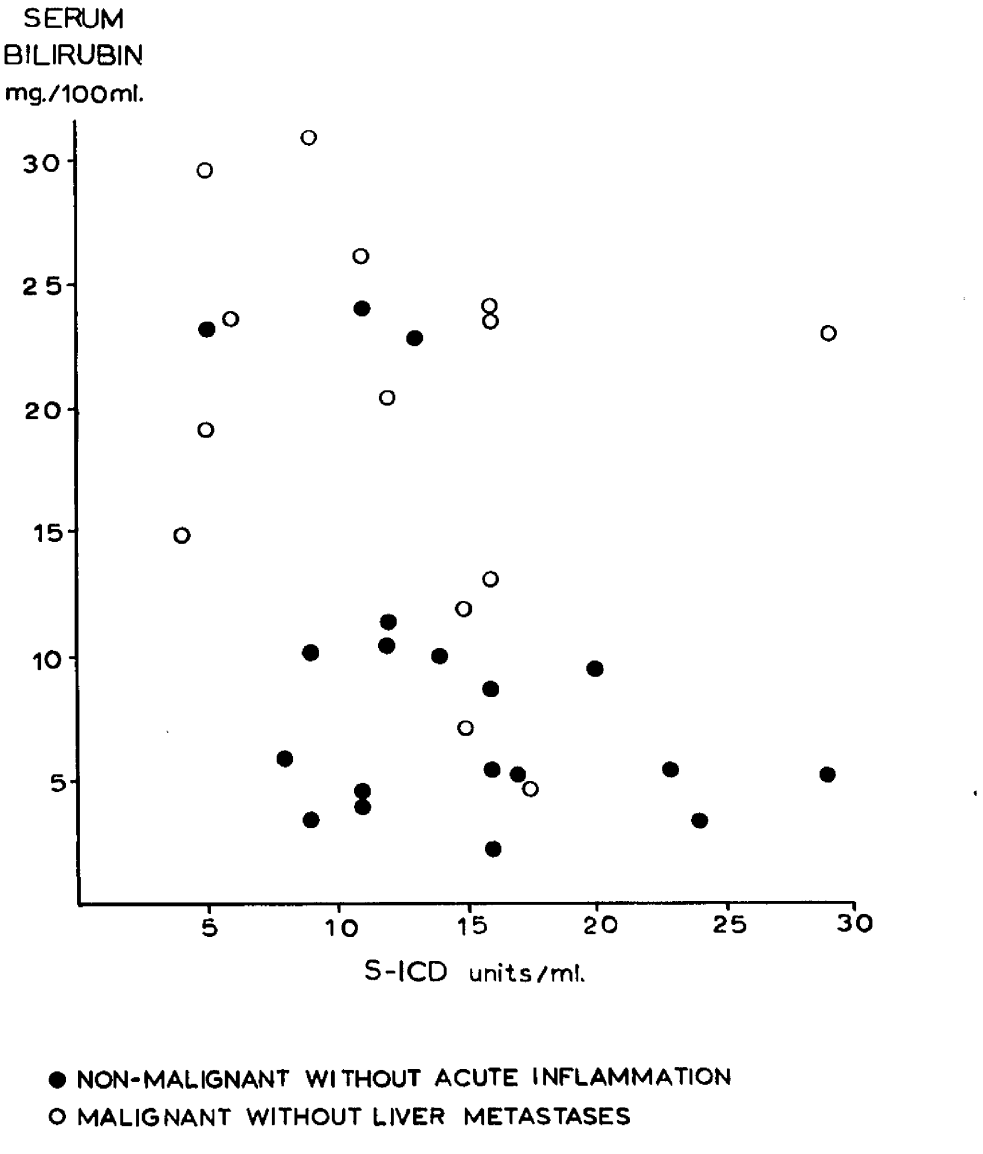


FIGURE 12.

COMPARISON OF S-ICD AND SERUM BILIRUBIN RESULTS IN CASES WITH UNCOMPLICATED OBSTRUCTIVE JAUNDICE.



usually show abnormal values in conditions causing dysfunction of hepatic cells.

Eleven of the 13 cases of infectious hepatitis showed abnormalities in one or both of the turbidity tests on the blood specimen taken for S-ICD estimation. However, the degree of upset of these tests was in no way related to the S-ICD value obtained. This also applied to the cases of chronic liver damage. Whereas the S-ICD showed variable results in portal cirrhosis and 28% of the cases had normal values, all but one of the 25 cases had upset in the protein turbidity tests. The S-ICD was not, therefore, related to these indices of liver cell damage. This is also illustrated in Table 4 (p.33) concerning the patient with hepatocellular damage from p-aminosalicylic acid, where it can be seen that the maximal response of the S-ICD, S-GPT and S-GOT preceded that of the turbidity tests and upset in serum proteins, which remained at abnormal concentrations for a long period after the enzymes had returned to normal.

By definition of obstructive jaundice without biochemical evidence of hepatocellular complications, all the cases in the obstructive jaundice group had normal turbidity tests, and they could not, therefore, be compared with S-ICD results.

PART 1.

DISCUSSION.

In putting forward any new test of liver function, enzymic or otherwise, two factors are of prime importance for the test to be of real use as a diagnostic tool, a) that it should have specificity for certain types of liver dysfunction, i.e. it can be used in the differential diagnosis of jaundice, and b) the test should preferably be sensitive so that early diagnosis of the liver upset can be obtained.

The set of liver function tests now normally employed in most clinical chemistry laboratories have been selected primarily for the above reasons. These tests consist of the determination of serum bilirubin for the diagnosis of jaundice, gross or subclinical, and for following the progression of the jaundice; protein precipitation tests, e.g. thymol turbidity and flocculation tests and zinc sulphate turbidity tests, which reflect changes in quantity and quality of plasma proteins which occur with chronic or acute liver cell damage; alkaline phosphatase because of its specificity in differentiating between intra- and extra-hepatic jaundice.

These tests are not 100% specific for a particular condition; this applies to the majority of biochemical tests in clinical use. Thus, increases in serum bilirubin can occur without damage to the liver or interference with biliary excretion, e.g. haemolytic jaundice due to excessive breakdown of red blood cells; the turbidity and flocculation

tests may be abnormal in other conditions causing changes in plasma proteins without damage to liver cells, e.g. Hashimoto's disease, long-standing infections; alkaline phosphatase, which may be raised in any type of liver condition, but with a value above 30 K-A units/100ml. in a jaundiced patient diagnostic of obstruction in the biliary tract, can show high values also in certain bone diseases, e.g. Paget's disease or bone metastases. However, in the jaundiced patient, the composite application of these tests can give a useful index of the probable type of liver pathology except in complicated or atypical cases.

Adjuncts to these basic tests are the estimation of serum transaminases, S-GOT and S-GPT, which have become widely used as tests of liver function. Their importance arises mainly from their great sensitivity in detecting acute necrosis of liver cells. This may be gross as in viral or drug-induced hepatitis where 100% diagnostic values for both transaminases will be found, or minimal where these enzymes can be used for detecting hepatic damage before the onset of jaundice, or abnormalities in turbidity tests, or clinical symptoms. The value of the transaminases in the differential diagnosis of jaundice is diminished somewhat because the enzymes are excreted into the bile and consequently raised values are seen in obstructive jaundice. In addition, variable values may be encountered in chronic liver damage.

In assessing the rôle of S-ICD in the diagnosis of different types of hepatic disease, the results to be found in the three main types of hepatic dysfunction, i.e. acute damage to liver parenchyma, chronic disease of the liver, and obstructive jaundice, must be studied in detail and the results compared with the information obtained from other liver function tests. It is obvious from previous publications that the enzyme should be compared closely with the transaminases, as their rôles as liver function tests will be very similar.

METHODOLOGY.

When considering a new analysis for introduction as a routine diagnostic test in clinical chemistry, it must be a method which will give reliable, reproducible results without presenting too many or severe practical problems. It is also desirable with enzymatic procedures that the conditions of analysis should be strictly adhered to and a check made against results obtained by other workers using the same procedure. It is quite possible for an enzyme method to give varying results from different establishments using the same procedure, due, perhaps, to differences in source or preparation of substrate or coenzymes, or apparatus used in the analysis.

The colorimetric determination of S-ICD, as described by Bell and Baron (1960), was undertaken with no difficulty.

Very reproducible results were obtained which were comparable with Bell and Baron's original data on duplicate analyses, variations in incubation time, and volume of serum used. This was also reflected in obtaining an almost identical normal range as that quoted by Bell et al. (1962) using the same method. Besides giving reliable results, the procedure presented no analytical problems. The reagents were very stable, including the enzyme substrate when stored at 4^{o}, except for the NADP solution which was made up fresh for each batch of analyses. A calibration curve using α-oxoglutaric acid was easily constructed and standards could be run along with tests as a check on reagents.

Serum enzymes could be stored at 4^{o} or -20^{o} for at least 14 days with negligible loss of S-ICD activity. Wolfson and Williams-Ashman (1957a) stated that sera could be stored safely for many days at 4^{o}, and Bodansky et al. (1960) stated that S-ICD was stable in a deep-freeze for 21 days.

From a practical point of view, the estimation of S-ICD by the colorimetric method is comparable, and in some ways, better than that for the transaminases. The method was definitely superior to the colorimetric method used in this investigation for both S-GOT and S-GPT, as the toluene extraction of the DNP-hydrazones was laborious and time-consuming. However, there are other improved methods for

both transaminases which do not involve protein precipitation and toluene extraction (Reitman and Frankel, 1957). The S-ICD determination has advantages over these methods as the calibration curve is more linear and easier to construct, and very high enzyme activities are more accurately obtained by the S-ICD method using shorter incubation times and reduced sample size, than by equivalent procedures with the transaminase methods. Bodansky et al. (1960) favoured the S-GOT in preference to the S-ICD, in part because the S-GOT was stable in the deep-freeze up to one year, whereas the S-ICD was stable for only 21 days. This, however, is not a valid practical consideration as it is unusual and unlikely that periods of three weeks should elapse between withdrawal and analysis of the specimen.

It can be stated from the outset, therefore, that when considering the S-ICD as a biochemical test of liver function, and particularly when comparing it with the serum transaminases, the practical estimation of the enzyme presents no problems, and, if anything, is somewhat superior to the transaminases in this respect.

ISOCITRATE DEHYDROGENASE IN ACUTE HEPATITIS.

The highest S-ICD values recorded in this investigation all fell within this group and this confirms the findings of previous authors, e.g. Merten and Solbach (1961) who found that acute hepatitis produced S-ICD values far in

excess of those found in other disease states. In the infectious hepatitis group studied here, 9 of the 13 results were above 100 units/ml. The 4 lower ones, under 60 units/ml. (Fig. 1, p.28) were probably due to the S-ICD having reached its peak prior to the blood specimen being taken, and the activity was probably falling back to normal. The initial rise of this enzyme takes place early in the disease, often before the onset of jaundice (Okumera and Spellberg, 1960), and tends to fall fairly rapidly back to normal values. Bell et al.(1962) stated that the S-ICD returned to normal usually 3-4 weeks after the onset of symptoms.

S-ICD in infectious hepatitis gives very much the same information as the S-GOT and S-GPT. High values for one enzyme always coincided with high values for the others. As can be seen from the case which showed fluctuations in enzyme activity over a long period (Fig. 5, p.55), all three enzymes produced very similar results. Sterkel et al. (1958) remarked on the similarity of the behaviour of S-ICD and S-GPT in acute hepatitis; Kerppola et al. (1959) commented on S-ICD and S-GOT, and Cohen et al. (1961) paired the S-ICD and S-GOT with having the same degree of sensitivity for reflecting acute hepatocellular damage, while the S-GPT was slightly superior to both of these.

From Fig. 4 (p.53), it is clear that there is not a good correlation between the S-ICD and both transaminases

on direct comparison of results. This can be explained by the fact that it has been reported that the fall from the initial peak of S-ICD activity tends to be somewhat sharper than for the transaminases (Hargreaves et al., 1961; Cohen et al., 1961). It is probable, therefore, that in the cases of infectious hepatitis, the S-ICD activity may be in decline while the transaminases may still be rising, or falling at comparatively slower rates. In these circumstances, a direct comparison between these enzymes on isolated specimens would be expected to give only a moderate or poor correlation.

Bodansky et al. (1960), in their study of S-ICD and S-GOT in experimentally induced viral hepatitis, commented on the superiority of S-GOT over S-ICD because it rose to higher values. This has been shown in the present work (Fig. 6, p.56), where in all but two cases, both transaminases showed higher increases in activity than the S-ICD. However, this is not a good way of expressing the sensitivity of an enzyme. The important factor in assessing the comparative sensitivity is to consider the time at which the enzyme starts to show detectable abnormal values in relation to symptoms and other tests of pathological upset, and not so much the absolute peak value eventually reached. Thus, Bodansky et al. (1960) found that in 8 of 9 cases, the S-ICD and S-GOT became elevated on the same day during the developement of hepatitis. Okumera and Spellberg (1960)

found in one of their cases that the S-ICD reached its peak activity before S-GOT.

Bell et al. (1962), commenting on the usefulness of S-ICD in detecting acute liver damage caused by hepatotoxic drugs, found that elevations in S-ICD activity occurred in many cases before elevations in S-GOT activity, and both enzymes showed superior sensitivity over the commonly used liver function tests. The results on the patient with liver damage caused by p-aminosalicylic acid supports these findings. The S-ICD and both transaminases showed very high values during the initial liver upset; the S-ICD fell to normal values more quickly than the S-GPT and possibly the S-GOT (Table 4, p.33). However, when using these enzymes to detect minimal liver cell damage following the test dose of p-aminosalicylic acid (Table 5, p.34), the S-ICD was the enzyme to show the first abnormal values and it was slightly more sensitive than the transaminases.

From these facts, it can be concluded that, while the transaminases produce, in most cases, values higher than the S-ICD in acute hepatitis, the sensitivity of these enzymes in detecting and following the course of acute hepatic damage is very similar, and although the results obtained from S-ICD estimations do not show any definite advantages, they are very comparable and in no way inferior to those of the transaminases.

SERUM ISOCITRATE DEHYDROGENASE IN DRUG-INDUCED JAUNDICE.

The widely-used drug, chlorpromazine, is known to produce hepatic dysfunction, with or without jaundice, in a small percentage of patients taking the drug (Sherlock, 1958). The jaundice has been classed as cholangiolitic due to intrahepatic cholestasis giving high alkaline phosphatase values. Promazine has been stated to produce a similar picture (Waitzkin, 1957). Some reports have indicated that there may be mild hepatocellular damage in this condition; Zelman (1959) found increased transaminase values with histological evidence of mild liver cell necrosis in all of 9 patients. The S-ICD results on the patients with chlorpromazine and promazine jaundice in this present study, are in agreement with what would be expected from the histological description of the liver pathology.

The S-ICD obviously does not have a diagnostic use in these cases, but its importance in other drug-induced liver conditions has been shown (p.32), and Bell et al. (1962).

In haemolytic jaundice, normal S-ICD values would be expected. Bell et al. (1962) found normal values in 10 cases of chronic haemolytic jaundice. They stated, however, that high values might be found in haemolytic crises and this would explain the value of 23 units/ml. found in the case of phenolic poisoning, where there was extensive breakdown of red blood cells.

SERUM ISOCITRATE DEHYDROGENASE IN CIRRHOSIS.

S-ICD values found in patients with portal cirrhosis were, in all but one case, very much lower than those found in acute hepatic damage. Twenty-eight percent of cases had normal values, 64% had minimal increases (below 20 units/ml.) and of the remaining three cases, only one had a high value of 54 units/ml. These results agree with previously reported data, of which Wolfson and Williams-Ashman (1957b), Wolfson et al. (1958), and Sterkel et al. (1958) all stated that the S-ICD tended to be normal in the majority of cases of cirrhosis, and increases when encountered, were only moderate. Okumera and Spellberg (1960) found increases of up to two times normal; results in this report are similar to their findings.

The variation in S-ICD values in the cirrhotic patients was not predictable. Kerppola et al. (1959) and Cohen et al. (1961) stated that raised S-ICD values were associated with 'active' phases of the disease, where there was active breakdown or regeneration of liver cells. The former authors used criteria of severity of jaundice and inflammatory changes in the liver seen in needle biopsy specimens to assess the degree of activity; the latter assessed their cases by the degree of jaundice, ascites and upset of liver function tests, and histological evidence of active regeneration, inflammation or necrosis of the liver tissue. In the cases investigated here, no liver biopsy specimens

were taken and, therefore, no histological indication of the activity of the cirrhotic process in the liver could be obtained. The case with the high S-ICD value of 54 units/ml. had ample evidence of active cirrhosis, with a bilirubin of 9.8mg./100ml., high transaminase activities and a very poor clinical condition. In the majority of other patients, however, S-ICD values could not be correlated with certain indices of active cirrhosis. There was no relationship between S-ICD values and degree of hyperbilirubinaemia or the clinical state of the patient. This agrees with Sterkel et al. (1958), who found no relationship with S-ICD and the amount of jaundice or clinical evidence of activity, and Bell et al. (1962) who found no correlation between S-ICD and the presence of jaundice and ascites in their patients. Franken et al. (1960) stated that there was no correlation between S-ICD values and compensation of the patient; Bell et al. (1962) agreed with this, defining a decompensated case as one with a bilirubin greater than 2.0mg./100ml. and a serum albumin less than 3.0g./100ml. On investigating the present series from this viewpoint, similar results were obtained.

On the other hand, if the serum transaminases are taken as indices of the activity of cirrhosis (Wróblewski and LaDue, 1956; Baron, 1964), then the S-ICD must also reflect activity, as results of these enzymes were significantly

correlated (Fig. 7, p.58), and, in the main, abnormal values for the transaminases coincided with raised S-ICD values. Cohen et al. (1961) stated that S-ICD, S-GOT and S-GPT were all elevated in their 'active' cases. Sterkel et al. (1958) found 5 abnormal S-ICD values in 27 estimations on 19 patients, compared with one slightly elevated S-GPT result in 20 estimations on 12 cases, and stated that S-GPT, along with S-ICD, was not related to biochemical and clinical evidence of active cirrhosis. Hargreaves et al. (1961) quoted an incidence of abnormal results similar for all three enzymes in cirrhosis, but did not split their cases into 'active' and 'inactive' types.

S-ICD determinations in portal cirrhosis appear, therefore, to give a picture similar to that produced by the transaminases, with variable increases which are in some cases unspecific in nature. However, the transaminases do have a diagnostic advantage over the S-ICD in chronic liver damage. Wróblewski and LaDue (1956) found that in cases of portal cirrhosis which showed abnormal transaminase values, the S-GOT was always higher than the S-GPT. Thus, the ratio S-GOT/S-GPT would always be greater than 1.0. They suggested that this fact might be of use in the diagnosis of chronic liver damage. Baron (1964) has refuted this by saying that the ratio has not been proved to have a diagnostic value. In the cases presented here, which showed abnormal values for the transaminases, the S-GOT

was higher than the S-GPT in 11 of 13 cases (Fig. 3, p.49), the other two cases producing values of 1.0 and 0.92 for the S-GOT/S-GPT ratio. Therefore, although the transaminases can produce variable results, this ratio would appear to be a more specific indicator of chronic liver damage than the absolute values of either S-GOT, S-GPT or S-ICD.

It is not unreasonable to expect the S-ICD to show variable and unpredictable results in a liver condition such as portal cirrhosis where damage to, and regeneration of liver cells, and the progress of the disease, may fluctuate and make a true assessment of the changes taking place inside the diseased organ at a particular time very difficult.

SERUM ISOCITRATE DEHYDROGENASE IN OBSTRUCTIVE JAUNDICE.

The activity of S-ICD in patients with extra-hepatic biliary obstruction, whatever the cause, is important, as the overall status of this enzyme as a useful factor in the differential diagnosis of jaundice stands or falls by its ability to distinguish jauudice due to extra-hepatic causes from intracellular liver damage. In 1958, Sterkel *et al.* suggested that the S-ICD could be used to differentiate between intra- and extra-hepatic jaundice, and Baron (1963) stated that S-ICD had advantages over the transaminases because of normal values seen in obstructive jaundice. This has been reiterated by the same author (1964) in a

textbook in which he states that S-ICD is generally normal in biliary obstruction. There are, however, authors who have found variably raised activities in obstructive jaundice (Hargreaves et al., 1961; Cohen et al., 1961; Saad et al., 1963).

In assessing S-ICD in acute and chronic liver cell damage, and comparing the results with serum transaminases, it has been found that the S-ICD is sensitive and comparable with the transaminases as an indicator of acute hepato-cellular damage. In chronic liver damage, the S-ICD has little or no diagnostic use, whereas the transaminases, with the S-GOT/S-GPT ratio, have. S-GOT and S-GPT also have the disadvantage of producing variable increases in obstructive jaundice and, therefore, the S-ICD would be useful as an adjunct to these enzymes if it produced more specific results in this pathology.

The S-ICD results found in this present series of obstructive jaundice cases, however, do not support Baron's contention (1964) that the S-ICD gives essentially normal values with obstruction. In considering all the cases which had proven extra-hepatic biliary retention, only 17% produced normal S-ICD values (Table 2, p.29). Admittedly, abnormal values in some of the cases could be explained by secondary complications, e.g. the presence of liver metastases in malignant obstruction and acute inflammation in benign cases, but there still remained a high proportion

of cases, which could be classified only as 'typical' obstructive jaundice, i.e. uncomplicated by involvement of liver cell damage, in which the S-ICD showed some increase. In many cases this was only minimal and values above 20 units/ml. were infrequently encountered.

Uncomplicated Obstructive Jaundice.

These cases consisted of 14 with malignant obstruction and 20 with benign biliary retention caused by stone in the bile duct, with or without chronic cholecystitis. The cases were all proven at operation and had no concomitant evidence of liver cell damage, e.g. clinically, there was no evidence of hepatomegaly or chronic liver damage, and biochemical tests of liver function gave a picture of obstructive jaundice with, usually, a raised alkaline phosphatase and, in every case, normal thymol turbidity and flocculation and zinc sulphate turbidity tests. Furthermore, the serum transaminases, where estimated, always fell within the accepted range for obstructive jaundice, no values for either S-GOT or S-GPT above 400 units/ml. being found. Cases which showed adverse liver morphology at operation were excluded from this group.

It can be seen from Table 6 (p.38) that there was not a statistical difference between the S-ICD results obtained from the malignant and non-malignant groups, and the distribution of S-ICD values in the two groups was very

similar (Table 2, p.29). Okumera and Spellberg (1960) found only two abnormal S-ICD values in 17 cases of acute and chronic cholecystitis, but 4 out of 5 cases with malignant obstruction had raised S-ICD values; Kerppola et al. (1959) claimed that the S-ICD could be used to differentiate malignant from non-malignant obstructive jaundice, but they based this on only a small number of cases. It is obvious from the present results that no such distinction can be made.

Considering the malignant and non-malignant cases together, there were only 9 of the 34 cases (26%) which had normal S-ICD values. Twelve cases (35%) had minimal rises (below 15 units/ml.), a further 26% had moderate elevations (15-20 units/ml.), and 4 cases (13%) had higher values, but all were below 30 units/ml. These results support data put foward by Cohen et al. (1961) who found 4 of 6 cases of obstructive jaundice with raised S-ICD values and of Saad et al. (1963) who found only 6 normal results in 21 S-ICD estimations on uncomplicated malignant and non-malignant obstructive cases. Hargreaves et al. (1964) quoted an incidence of 30% for abnormal S-ICD values in their obstructive jaundice group, but Bell et al. (1962) pointed out that these cases included biliary cirrhosis which would produce raised values. However, in this present work, cases of biliary cirrhosis have been treated separately. Bell

et al. (1962) claimed that the S-ICD would be normal in cases of obstructive jaundice uncomplicated by liver cell damage. It must be stated that they classified their 19 cases in this way with the aid of liver biopsy. This was not possible in the present study except in a few isolated cases where liver material was taken at operation and histology was compatible with biliary retention. However, Bell et al. (1962) also stated that they found normal S-ICD values in obstructive jaundice cases only when they were of short duration, and that long-term obstruction leading to liver cell damage produced raised S-ICD values up to 30 units/ml. They did not define, however, what constituted short and long-term duration of biliary retention. It can be seen in this study (Table 7, p.42) that the duration of symptoms did not appear to influence the distribution of S-ICD values. The time of onset of symptoms was taken with a liberal view, i.e. in each case, the duration was timed from the first indication of jaundice, where stated, or abdominal symptoms, however vague or non-specific. Whilst it must be accepted that liver cell damage can, and probably does, occur with prolonged biliary retention, this cannot be put forward as the reason for increases in S-ICD activity in these cases, particularly as there was no other evidence, clinical or biochemical, for the existence of concomitant liver cell injury. It should also be noted here that the S-ICD could not be related to the severity

of the obstruction when using the serum bilirubin and alkaline phosphatase as independent indices of this (Fig. 11 and 12, p.65,66).

If what Bell et al. (1962) say is true, that the S-ICD will be normal in uncomplicated short-term obstructive jaundice, then these present results can be explained only by the fact that liver cell damage occurs to some extent in the majority of cases of obstructive jaundice and is not related to the duration or severity of the obstruction. This may be so, but can be ascertained only to any extent by knowing the state of the liver parenchyma in each case, and this would involve obtaining liver biopsy material. Since the S-ICD is being considered as a potential test for the differential diagnosis of jaundice, the results must be able to be interpreted without having to resort to this technique. It would appear from these results that abnormal S-ICD values (below 30 units/ml.) may be expected in uncomplicated obstructive jaundice, and the enzyme will not necessarily yield specific information as to severity, cause or liver cell complications of the obstructive lesion.

The increases in S-ICD in these cases were poorly correlated with the transaminases. There was a significant correlation between the S-ICD and S-GPT ($r = 0.43$, $P < 0.05$), but this was much lower than that found in acute and chronic hepatitis cases. This could be explained by the fact that in acute hepatitis, both enzymes are reflecting active liver

cell damage; in chronic hepatic upset they are probably responding to changes in the liver at the cellular level, whereas in obstructive jaundice, it is likely that the enzyme increases could be due, in part, to interference with their biliary excretion. There is evidence that the transaminases are excreted into the bile (Reichard, 1959), and Wolfson and Williams-Ashman (1957b) stated that S-ICD was increased after bile duct ligation in the rat, and Okumera and Spellberg (1960) observed moderate elevations following ligation of one of the hepatic ducts in the dog. It could well be that removal of S-ICD via the biliary tract is independent of the processes affecting transaminase removal, and, therefore, a good correlation between these enzymes in biliary retention would not be expected. However, this hypothesis is not supported by the results obtained in biliary cirrhosis, where the S-ICD showed the highest correlation with both transaminases (Fig. 8, p. 60). The values for S-GOT and S-GPT are usually higher in biliary than in portal cirrhosis (Molander et al., 1957). This could be due to the composite effect of liver cell damage plus biliary retention giving high transaminase values, but it is difficult to explain why the S-ICD should follow the transaminases so closely when there is such a poor correlation in extra-hepatic obstruction.

Obstructive Jaundice Associated with Liver Cancer.

The presence of metastatic liver involvement in cases of obstructive jaundice due to malignant lesions, tended to give higher S-ICD values (Table 2, p.29) which were significantly different from the non-metastatic malignant cases ($P < 0.005$, Table 6, p.38). There was, however, some overlap between the two groups, and the S-ICD was not definitive in separating them. Previous authors have commented on secondary involvement of the liver in neoplastic disease causing raised S-ICD values (White, 1958; Kerppola et al., 1959). Sterkel et al. (1958) concluded that S-ICD could be useful for detecting liver metastases in malignancy. Others have found S-ICD raised in a high proportion of cases of liver cancer; Cohen et al. (1961) reported an incidence of 70%; Bell et al. (1962) found raised values in 19 of 23 cases, and Tan et al. (1963) found increases in S-ICD in the majority of 289 patients with liver metastases, although only 20% had values above 20 units/ml. All the cases reported here had raised values with over 50% above 20 units/ml. However, Okumera and Spellberg (1960) reported that the rise in S-ICD was governed by the extent of liver involvement, as normal S-ICD values were found in cases where there were minimal small metastatic lesions in the liver. Although it was not possible to grade the present cases according to the extent of metastatic deposits in the liver, none of them could be said to have minimal lesions, and three of the cases,

which were reported as having huge liver secondaries, had high S-ICD values (28, 32 and 57 units/ml.).

The S-ICD seems to respond in a more specific way to metastatic liver involvement in obstructive jaundice than does the S-GOT and S-GPT. No significant differences could be seen between the transaminase results in the metastatic and non-metastatic groups (Tables 10 and 11, p.46,47), and Fig. 9 shows that S-ICD gives comparatively higher values in the metastatic group, whereas the S-GPT does not. These facts agree with those of Tan et al. (1963) who stated that S-ICD was raised in a greater proportion of cases with liver metastases than were the transaminases, and Cohen et al. (1961) found an incidence of 64% for raised transaminases compared to 70% for S-ICD in cases of liver cancer.

These same authors also stated that the S-ICD approached the alkaline phosphatase in sensitivity as an index of hepatic involvement in malignancy. This present study also shows that the alkaline phosphatase gives significantly higher results in the presence of liver metastases (Table 13, p.52), which agrees with previous findings for this enzyme (Shay and Siplet, 1954). Mendelsohn and Bodansky (1952) stated that there appeared to be a relationship between the degree of hepatic involvement and rise in alkaline phosphatase. Okumera and Spellberg (1960) suggested this applied also to S-ICD, but when comparing the two enzymes in this study, no correlation was found between them, (Fig.

11, p.65). Therefore, although both enzymes produced raised values in every case of liver metastases, which were significantly different from the results on cases of obstructive jaundice without liver metastases, it cannot be said that the two enzymes were comparable indices of liver involvement. The alkaline phosphatase tended to give higher rises in activity than the S-ICD in the majority of these cases, but for both enzymes there was an overlap in results obtained from the non-metastatic and metastatic groups, which decreased the specificity and, therefore, the usefulness of these enzymes in diagnosing liver metastases in the jaundiced patient.

Obstructive Jaundice Complicated by Acute Inflammation.

On reviewing the cases of non-malignant obstructive jaundice, it was found in three of them with high S-ICD values, that they showed signs of having had acute inflammation near to the time at which the blood specimen was taken. Consequently, all the cases were classified as inflammatory or non-inflammatory using the criteria for acute inflammation mentioned previously (p.37), except for three cases whose classification was equivocal. It was found after separating the cases on the above basis, that S-ICD values were much higher in 7 of the 8 inflammatory cases. There was a high degree of significance between the two groups ($P < 0.001$, Table 6, p.38).

Except for one case discussed below, the inflammatory cases presented with a history suggestive of obstructive jaundice with acute cholecystitis. They had no clinical or biochemical evidence of active liver cell damage occuring along with the biliary retention. Norberg (1961) reported essentially normal S-ICD values in patients with symptomless gall bladder dysfunction, but raised values occurred in some patients with symptoms of acute cholecystitis. Only 7 of 30 cases had very high values in the latter group. The author did not, however, give any criteria for classification into this group. At least 8 out of 34 patients did not have clinical jaundice and 17 of 31 cases had only normal or moderate rises in alkaline phosphatase. It is, therefore, likely that some of the cases in that series would not have been classed in the inflammatory group in the present study. Okumera and Spellberg (1960) found two abnormal values in 17 cases of benign obstructive jaundice. One of those, with an S-ICD value 4 times the upper limit of normal, had acute cholecystitis, diabetes mellitus and acute pancreatitis. Saad et al. (1963) also found a very high S-ICD (approximately 10 times normal) in a case shown to have a stone in the common bile duct and acute cholecystitis, but liver biopsy showed a high degree of focal necrosis which may have been responsible, in part, for the very high S-ICD activity.

The other case in the present study, was a women diagnosed as having cholangitis associated with stones in the common

bile duct of approximately two weeks duration. She had very high S-ICD values, up to 6 times the upper limit of normal, on frequent occasions, pre- and post-operatively. In addition to this case, was the patient who developed a post-operative infection with jaundice, and whose S-ICD activity rose to a high value. This man was thought to have a post-operative cholangitis. Wolfson and Williams-Ashman (1957b) reported that cholangitis gave raised S-ICD values, although they gave no details. The second case in the benign obstructive jaundice series of Okumera and Spellberg (1960) which showed a high S-ICD value, had chronic cholecystitis, pericholecystitis and intra-hepatic cholangitis, and Cohen et al. (1961) stated that the patient with the highest S-ICD value in their obstructive jaundice group (4 times the upper limit of normal) had ascending cholangitis.

There is a distinct possibility in cases with ascending infection that large numbers of hepatic cells may become damaged, and might produce high S-ICD values. This would be expected to give high transaminase values too. This was shown in the man with post-operative infection, where the sharp increase in S-ICD activity coincided with peaks of transaminase activity. However, there was also an equally sharp and impressive rise in alkaline phosphatase which would indicate an acute exaberation of biliary retention, and it is, therefore, impossible to say if the rise in S-ICD

and transaminases was due to liver cell damage. The woman with cholangitis who produced very high S-ICD values, did not show such a pronounced increase in transaminase values; the S-GPT never went above 200 units/ml., and the highest recorded value for S-GOT was only 110 units/ml. The thymol turbidity and flocculation and zinc sulphate turbidity tests did not show any abnormal results, even after a prolonged period of infection, and liver biopsy showed only small foci of acute liver cell degeneration. From this, an increased S-ICD value would be expected, but perhaps not to such a degree as was found.

There was no reason to suspect hepatic cell complications in the other cases with acute inflammation of the biliary tract. If the high S-ICD values are not explained by liver cell damage, there is the possibility that the enzyme is released in large amounts at areas of inflammation where there will be a large influx of mononuclear, polymorph and lymphocytic cells. High S-ICD values have been reported in many cases of infectious mononucleosis. In this condition liver histology shows only a small degree of focal parenchymal damage, but with intensive invasion of the liver by mononuclear and polymorph cells (Sherlock, 1963). Bell et al. (1962) reported that S-ICD values in infectious mononucleosis covered the same range as those found in infectious hepatitis. All their cases showed raised S-ICD values at some stage of the disease, the highest values being seen in cases with

biochemical or other overt clinical evidence of liver involvement, but some cases showed raised S-ICD values with no other evidence of liver damage. It may well be that raised S-ICD values are a reflection not only of hepatocellular dysfunction but are associated with the presence in the liver of large populations of inflammatory cells.

S-ICD values in the inflammatory group were not reflected to the same extent by the transaminases, where no significant differences could be shown between the inflammatory and non-inflammatory groups for both S-GPT and S-GOT (Tables 10 and 11, p.46, 47). Two of the cases did have high S-GPT values (300-400 units/ml.), but the other cases showed only moderate increases as might be expected in simple cases of obstructive jaundice. This would lend weight, therefore, to the hypothesis that S-ICD activity in these inflammatory cases may have arisen, in part, from extra-hepatocellular sources.

ISOCITRATE DEHYDROGENASE COMPARED WITH SERUM TRANSAMINASES.

In previous studies where the S-ICD has been compared with one or both of the serum transaminases, the opinions of various authors have differed in the final assessment of their relative value of their estimation in the jaundiced patient. Sterkel *et al.* (1958) indicated that the S-ICD yields more specific information than S-GPT because of normal values seen in obstructive jaundice. Bell *et al.* (1962)

stated that S-ICD was a more specific test of liver cell necrosis than S-GOT for the same reason, and Baron (1963, 1964) has also suggested S-ICD has advantages over the transaminases because of normal values found in obstructive jaundice. Opposed to this are the views of Kerppola et al. (1959) who, on considering the clinical significance of S-ICD, did not think that it provided any more useful information than the combined use of S-GOT and alkaline phosphatase: Cohen et al. (1961) said that, although S-ICD was a sensitive and rather specific indicator of hepatocellular damage, but slightly less so than S-GPT, it had no significant diagnostic advantages compared with the serum transaminases.

Sterkel et al. (1958) proposed that S-ICD had some advantages over the transaminases in acute hepatitis because of its ease of measurement, whereas Bodansky et al. (1960) preferred S-GOT to S-ICD because of the former's higher increases in acute hepatitis, and the latter's relative instability on storage of serum specimens.

The results of this present investigation support the view of Cohen et al. (1961) that S-ICD does not have any significant diagnostic advantages over the serum transaminases in the differential diagnosis of jaundice. Raised S-ICD values are to be expected in obstructive jaundice and, therefore, the premise of Bell et al. (1962) and Baron (1963, 1964) cannot be upheld by this work.

There is no doubt, however, that S-ICD is a sensitive indicator of acute liver cell damage, is comparable with both transaminases in this respect, and is, perhaps, somewhat superior to the S-GOT as indicated by Bell et al. (1962). The conclusions of Bodansky et al. (1960) are not supported as, firstly, the sensitivity of S-ICD in detecting acute hepatocellular damage was assessed here by its time of response and not the overall magnitude of the increase in activity, and secondly, the stability of S-ICD on storage was not considered to be a practical problem or make S-ICD inferior to S-GOT or S-GPT. The practical estimation of the enzyme was in some ways better than that for transaminases which agrees with Sterkel et al. (1958).

In cirrhosis, the transaminases, expressed as the S-GOT/S-GPT ratio, had distinct diagnostic advantages over the S-ICD, the latter having no real value in this condition as it could not be related to the clinical state of the patient. Rises in S-ICD activity with liver metastases were more pronounced than with either transaminase, and supported Tan et al. (1963) who found raised S-ICD values in a greater proportion of cases than elevations of S-GOT and S-GPT.

In making an overall assessment of S-ICD compared with the serum transaminases, it can be stated that S-ICD can be readily used as a useful adjunct to the transaminases, but it is not likely that the enzyme would ever supersede them.

ISOCITRATE DEHYDROGENASE COMPARED WITH OTHER TESTS OF LIVER FUNCTION.

S-ICD was compared with alkaline phosphatase only in cases of obstructive jaundice; there was no correlation between the two enzymes. Acute inflammation affected only S-ICD results and did not produce higher alkaline phosphatase values. The presence of liver metastases tended to give higher values for both enzymes. Cohen et al. (1961) stated that S-ICD was comparable with alkaline phosphatase as a sensitive index of secondary liver involvement, but it is not possible to reach the same conclusion from the present work, as increases in S-ICD in malignant obstructive jaundice were not specific for secondary liver involvement, although S-ICD values obtained in the metastatic group were significantly higher than the non-metastatic group. The same can be said of the alkaline phosphatase results, but increases in this enzyme activity with liver metastases were comparatively higher than those of S-ICD.

S-ICD could not be correlated with serum bilirubin in any of the groups of patients studied. In acute hepatic damage, peaks in enzyme activity usually precede that for bilirubin, and it has previously been observed that S-ICD and transaminases are more sensitive than standard tests of liver function in detecting acute breakdown of liver cells (Bell et al., 1962). Results obtained in the case showing liver damage caused by p-aminosalicylic acid indicated the

superior sensitivity of S-ICD and the transaminases over the bilirubin in detecting minimal damage to hepatic tissue. In chronic liver damage, there was no correlation between S-ICD and degree of hyperbilirubinaemia, which agrees with Sterkel et al. (1958). This also applied to cases of obstructive jaundice.

The protein turbidity and flocculation tests used in the diagnosis of acute or chronic damage to the liver cell were not directly comparable with S-ICD. These tests usually show abnormal values at a later stage, and remain abnormal for a longer period, than serum enzymes in acute hepatocellular upset. This is illustrated by the data in Table 4 (p.33). In chronic liver damage, the turbidity tests were a more reliable index of cell damage than the variable results encountered with S-ICD and serum transaminases.

CONCLUSIONS.

S-ICD is an enzyme which can be easily measured in serum, and may be of some use in the diagnosis of hepatic disease. It is a very sensitive indicator of liver cell damage and shows very high results in conditions involving acute breakdown of liver cells. Because of its sensitivity, it will be useful in detecting subclinical hepatic damage caused, for example, by hepatotoxic drugs. In these respects it compares favourably with the serum transaminases.

It is of little or no diagnostic value in chronic liver

conditions, where extremely variable and non-specific values are found. S-ICD results are again related to transaminase values, but the S-GOT/S-GPT ratio coupled with other tests of chronic cell damage, e,g. turbidity and flocculation tests, are to be preferred.

Raised values are sometimes seen in obstructive jaundice which cannot be related to severity or duration of symptoms, and the enzyme cannot be used to differentiate malignant from non-malignant cases. High S-ICD values are to be expected when secondary liver invasion occurs in malignant obstructive jaundice. High values occurring in benign obstructive jaundice are associated with an acute inflammatory condition of the biliary tract.

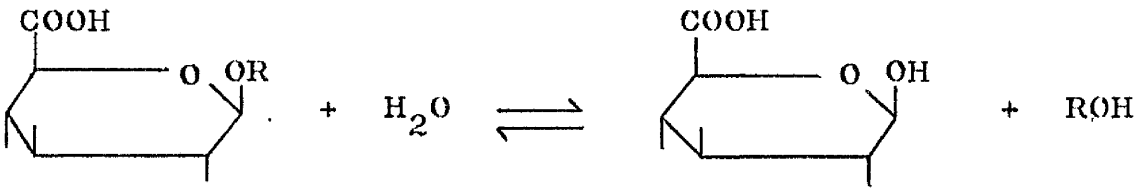
The enzyme will give essentially similar information as the serum transaminases for most important diagnostic uses, differing only in some details which are probably not of great practical consequence. S-ICD may be used as an adjunct to the transaminases without difficulty and may yield more specific information in those conditions mentioned above which produce high S-ICD values. Its sensitivity as an index of active liver cell damage will be an asset in detecting subclinical hepatic dysfunction.

PART 2.

STUDIES ON β-GLUCURONIDASE IN CERVICAL CANCER.

INTRODUCTION.

The enzyme, β-glucuronidase (E.C. 3.2.1.31) hydrolyses aliphatic, alicyclic, phenolic and ester β-D-glucosiduronic acids (Marsh and Levvy, 1958). It has a wide range of specificity and will split a large number of natural and synthetic substrates. The basic reaction may be summarised as:-



β-D-glucosiduronic acid

β-D-glucuronic acid

It seems likely that more than one protein complex may exist with β-glucuronidase activity in a given tissue (Mills, Paul and Smith, 1953), but most of the mammalian β-glucuronidases from different species have a pH optimum between 4.5-5.3. There are no known coenzymes or cofactors, but the purified enzyme can be activated by a number of substances including protamine, bovine serum albumin, DNA from various sources, gelatin, lysine and ornithine; it is inhibited by other substances, e.g. hydroxycarboxylic acids, ascorbic acid and heparin.

The enzyme can readily be assayed by using a β-D-glucosiduronic derivative of a substance which can be measured colorimetrically after being liberated by enzymic hydrolysis; the one most commonly used is phenolphthalein-mono-β-glucuronic acid and estimation of released phenolphthalein.

PHYSIOLOGICAL FUNCTION OF β-GLUCURONIDASE.

Although this enzyme has such a widespread distribution in animal tissues, and a great deal of investigation has been done on the *in vivo* action of the enzyme, its exact rôle in living tissue is still not fully understood.

Fishman (1940) observed an increase in β-glucuronidase activity of liver, kidney and spleen in dogs fed a terpene alcohol (borneol) and in mice fed menthol, both compounds being excreted in the urine as glucuronides. It was thought, therefore, that the enzyme might in some way be related, in these tissues, with detoxication processes involving the formation of glucuronide excretion products. However, the equilibrium of the enzyme reaction lies far in favour of hydrolysis (Levvy and Marsh, 1963), and it is not conceivable that the enzyme could play a major rôle in the synthesis of glucuronides. The enzyme has, however, been shown to possess a transferase activity of glucuronyl radicals *in vitro* (Fishman and Green, 1957), but as a route of synthesis of glucuronides *in vivo* this cannot compare in efficiency with the established pathway employing UDP-glucosyluronic acid and the very different enzyme, glucuronyl transferase (Dutton and Storey, 1954).

In most mammalian tissues, the enzyme activity appears to be related to the presence or function of various steroid hormones. This is particularly evident in tissues of the female reproductive tract which come under the influence of

sex hormones, mainly oestrogens, and which have, in general, a high β-glucuronidase content which is sensitive to hormone stimulation.

Harris and Cohen (1951) showed that the uterine β-glucuronidase of ovariectomised mice was significantly less than that of normal mice, and a return to normal levels could be brought about by oestrogen administration. This effect of oestrogen upon the tissue β-glucuronidase was stated to be a specific one and not to have resulted from oestrogen-induced tissue proliferation, as progesterone inhibited the oestrogen stimulation of β-glucuronidase but not the growth of the tissue.

Beyler and Szego (1954) discovered that the preputial (clitoral) gland of the rat was rich in β-glucuronidase and this fluctuated during pregnancy, apparently in response to circulating hormones. Androgens have been found to increase the activity of β-glucuronidase in renal tubule cells and its excretion in urine (Riotten and Fishman, 1953).

In human studies, the β-glucuronidase activity of tissues from the female reproductive tract have shown variation associated with changes in oestrogen production. Alterations in tissue β-glucuronidase show a cyclic variation relative to events of the menstrual cycle (Fishman and Mitchell,1959); higher β-glucuronidase activities are found in pregnant cervix taken at term than in normal cervix (Odell, Burt and Bethea, 1949). These tissue findings are reflected in elevated

serum β-glucuronidase values found in pregnancy (McDonald and Odell, 1947), and were interpreted as showing enhanced conjugation of oestrogens and other hormones. Fishman, Kasdon, Donner, Fishman and Homburger (1951) showed that serum β-glucuronidase was sensitive to oestrogens, e.g. the serum values doubled in post-menopausal women treated with as little as 5 mg. stilboestrol per day.

It appears, therefore, that the enzyme is closely connected with the presence or functioning of steroids in tissues and must be concerned with either the synthesis or hydrolysis of steroid glucuronide compounds, but its ultimate rôle is not as yet known.

DISTRIBUTION OF β-GLUCURONIDASE.

The enzyme is widely distributed in living organisms, mainly in the animal kingdom, but it has been reported to exist in certain plants (Levvy, 1954), and is fairly widely distributed in bacteria (Beuhler, Katzman, Doisy and Doisy, 1949; Jacox, 1953). In the animal world, the enzyme is widespread and is found in birds, fish, amphibia, insects and molluscs. It is found in the majority of mammalian tissues and has been extensively studied in man, rat, mouse, rabbit, dog and cat tissues.

It occurs in most species in largest amounts in liver, kidney and spleen, and is also found extensively in tissues of the reproductive and endocrine systems; the richest known

source is from the female rat clitoral gland (Boyler and Szego, 1954).

Seligman, Tsou, Rutenburg and Cohen (1954) undertook a comprehensive investigation into the distribution of the enzyme in rat tissue, using histochemical techniques. They concluded that β-glucuronidase was confined, in general, to cells of epithelial origin; mesodermal cells showed a poor staining reaction for the enzyme. The greatest concentrations were seen in liver, spleen, epithelia of the gastro-intestinal tract and uterus, thyroid and white matter of nervous tissue. The distribution of the enzyme did not vary from one rat to another, but the intensity of the staining reaction did, and this was not related to sex, age or size of the animal. Fishman and Baker (1956) also described a histochemical study of β-glucuronidase in 30 tissues of the adult rat, and showed that epithelial tissue was the most active. Kawakatsu and Mori (1963), again using histochemical techniques, showed that β-glucuronidase was distributed throughout the cells of normal mucosae and in squamous epithelium of normal uterine cervix.

Besides these histochemical studies, there have been a few quantitative assessments of various normal tissues by determination of β-glucuronidase activities of whole tissue homogenates. Talalay, Fishman and Huggins (1946) gave comparative figures for 12 rat tissues and found β-glucuronidase in all the homogenates, with the highest activity/mg. wet

weight of tissue in spleen, liver, ovary and uterus. Fishman and Anlyan (1947) assayed homogenised human tissues and found the highest β-glucuronidase activity in liver, colon, rectum and endometrium.

The enzyme can, therefore, be detected in most tissues from mammalian sources and is found with highest activity mainly in epithelial cells.

INTRACELLULAR LOCALISATION OF β-GLUCURONIDASE.

β-Glucuronidase in the Nucleus.

The histochemical studies of Seligman et al. (1954) and Fishman and Baker (1956) showed that only the cytoplasm of normal epithelial cells stained for β-glucuronidase, with no reaction in cell nuclei. Fishman, Baker and Borges (1959) found that cell nuclei of neoplastic cells from human tumours were negative or only weakly positive, and Oka, Okamoto, Omachi and Mori (1964) found the enzyme confined to the cytoplasm of cells from oral neoplasms. Fishman, Mitchell, Dimitrakis and Hayashi (1963), however, found intensely staining nuclei in some histological sections taken from one case of adenocarcinoma of the human endometrium. Siebert (1963) stated that isolated nuclei from rat cancer cells showed considerable β-glucuronidase activity which was not due to cytoplasmic contamination of the preparations. The author did not state, however, what proportion of the tissue enzyme was found in the nuclear fraction.

In most tissue fractionation procedures, the nuclei are centrifuged down from homogenates along with cell debris and unruptured cells, and in studies on the β-glucuronidase distribution in subcellular fractions, this is usually referred to as the 'nuclear' fraction. Various figures have been quoted for the percentage of the total β-glucuronidase activity of a tissue homogenate which resides in the 'nuclear' fraction, and depends, naturally, on the homogenisation conditions which will influence the number of unruptured cells and other tissue debris (Levvy and Conchie, 1964). When using recognised fractionation techniques, the β-glucuronidase activity in the 'nuclear' fraction rarely exceeds 20% of the total tissue activity (Plaut and Fishman, 1963).

Cytoplasmic β-Glucuronidase.

The greater part of β-glucuronidase of most cells, normal and neoplastic, would appear, therefore, to lie in the cytoplasm of the cells. The distribution of the enzyme in the cytoplasmic fractions of the cell - mitochondria, microsomes, lysosomes and free soluble enzyme - can be variable from one tissue to another. Nearly all the work on the intracellular localisation has been done on rat and mouse tissues, mainly liver and kidney cells (deDuve, Wattiaux and Baudhuin, 1962). Fishman (1960) indicated that the enzyme was found in cellular fractions as follows:-

Nucleus	Mitochondria	Lysosomes	Microsomes	Supernatant
-ve	not stated	++	++	±

Walker (1952) stated that in mouse liver cells, the greater part of β-glucuronidase was located in the cytoplasmic granules (mitochondria and microsomes) and assumed there was little in nuclei and the soluble fraction of cytoplasm. deDuve, Pressman, Gianetto, Wattiaux and Appelmans (1955) put forward the hypothesis that β-glucuronidase was bound, along with various other hydrolytic enzymes, in separate discreet cytoplasmic organelles which they termed lysosomes. The particles would separate out along with mitochondria and microsomes in most tissue fractionation procedures. Microsomes were also found to be high in β-glucuronidase activity.

β-Glucuronidase has, therefore, been regarded as primarily a cytoplasmic enzyme, bound mainly within particles. The distribution between the various particulate fractions and supernatant can, however, vary with the tissue (Levvy and Conchie, 1964). In mouse kidney cells, Conchie and Levvy (1959) found approximately 21% of the total activity in the non-particulate cytoplasmic fraction (supernatant), with 22% in the mitochondrial and 21% in the microsomal fractions. Roth, Bukovsky and Eichel (1962) found a vastly different distribution in rat spleen with approximately 70% supernatant, 11% mitochondrial, 5% microsomal and 9% in the nuclei. The lysosomal β-glucuronidase would be included with the particulate fractions, probably mainly with the mitochondria. There appears to be no data on cells from human tissues.

There is a suggestion that cancer cells may have a different distribution of β-glucuronidase, although there is slight evidence of this - Conchie and Levvy (1959) found that in mouse S37 tumour, over half of the enzyme activity of the cytoplasm was free and not confined to particles. Levvy and Conchie (1964) reported similar findings in Ehrlich and T2146 tumours.

β-GLUCURONIDASE IN CANCER TISSUES.

Fishman, Anlyan and Gordon (1947) compared β-glucuronidase activity of homogenates of various human carcinomas with corresponding uninvolved tissue and found higher activities of the enzyme in nearly every malignant tissue. Striking elevations were found in malignant neoplasms of breast, stomach, colon, uterus and lung. Fishman and Anlyan (1947) compared β-glucuronidase activity of normal, tumour and lymph node tissue of surgical patients, and found higher β-glucuronidase in cancer tissues than in non-involved tissues. Fishman and Bigelow (1950) compared the histology and β-glucuronidase of 44 gastrointestinal neoplasms, and although they found consistently increased β-glucuronidase in these neoplasms, they could not correlate the activity with a single morphological characteristic. Their impression was that increased β-glucuronidase might be associated with greater cellularity of the lesion, and to a slight extent with mitotic activity, but this was not definitely

established. They stated that tissue changes, only secondary to malignancy, e.g. inflammation, necrosis, and formation of fibrous stroma, could be excluded as causes of increased β-glucuronidase activity.

This work on tissue homogenates has been supported by various histochemical studies. Fishman et al. (1959) found consistently that neoplastic cells of human tumours stained for β-glucuronidase in the cytoplasm with little or no reaction in the nuclei. They stated that neoplastic cells were good sources of β-glucuronidase, that these cells were the major contributors to the β-glucuronidase activity of the whole tumour tissue and not non-malignant elements such as leucocytes, fibroblasts and fibrocytes. Monis, Banks and Rutenburg (1960) investigated β-glucuronidase activity in 121 human tumours, 100 of which were epithelial in origin. They found activities varying from low to very high in the epithelial tumours, while very low or negative reactions were found in tumours of mesodermal origin. The β-glucuronidase activity could not be correlated with histological grades of differentiation. Oka et al. (1964), in an investigation into oral neoplasms of epithelial origin, found that the tumour parenchyma reacted more intensely for β-glucuronidase than the surrounding healthy tissue; the activity in the tumour cells was confined to the cytoplasm. Lehrer (1962) investigated a number of enzymes in glial tumours and found that β-glucuronidase was the only one which showed a consistent

and marked elevation in all tumours.

β-Glucuronidase is found, therefore, in a wide variety of tumour tissues and usually with increased activities over corresponding normal tissues. It is found mainly in tumours of epithelial origin and by histochemical techniques is found only in the cytoplasm.

β-GLUCURONIDASE IN CERVICAL CARCINOMA.

Great interest has centred upon β-glucuronidase in malignant and benign lesions of the female genital tract since Odell and Burt (1950) and Kasdon, Fishman and Homburger (1950) indicated that the determination of β-glucuronidase in vaginal fluid was a useful diagnostic aid for uterine malignant growths, especially of the cervix uteri, where elevated values were found in over 80% of cases with cancer.

Odell *et al.* (1949) undertook β-glucuronidase assays on whole tissue homogenates in water of various female genital tumours. They compared 13 non-malignant cervices with 6 squamous cell cervical carcinomas, and found that the range of β-glucuronidase activity as units/g. wet weight of tissue of the latter group was very much higher than the non-malignant group. Fishman, Kasdon and Homburger (1950) compared 22 specimens taken from untreated cervical cancer and found the mean activity for the group was over 10 times the mean obtained from 46 specimens of non-malignant cervix.

Fishman and Mitchell (1959) studied the distribution of

β-glucuronidase in normal specimens of vagina, cervix and uterus by histochemical techniques, and described β-glucuronidase as showing strong activity always in the basal layer of epithelium and often in several layers of cells above, whilst there was variable activity in the superficial layers. There was little or no reaction in the stroma of the tissues. The same group (Fishman, Mitchell, Borges, LaDue and Hayashi, 1963) then studied the enzymorphology of cervical cancer and described results on three cases - one carcinoma in situ, one early invasive, and one advanced epidermoid carcinoma. In the carcinoma in situ, β-glucuronidase was found in healthy zones confined to basal and superficial layers of the epithelium, while in the carcinomatous areas, the basal epithelium reacted strongly, and so did the stroma. Similar findings were described for the early invasive carcinoma. In the epidermoid tumour, the distribution of the enzyme was more widespread and generalised, with a highly active stroma. They concluded that in non-invasive epithelium, the β-glucuronidase remains localised more or less in the basal layer, whereas in invasive neoplasm many more of the cancer cells are positive. Starzewski, Waronski and Steplewski (1963), in a study on 10 cervical cancer specimens, stated that β-glucuronidase and acid phosphatase are high in the border areas of the cancer and low or absent in the centre of the tumour. Kawakatsu and Mori (1963) found in squamous cell carcinoma, that

β-glucuronidase activity was more marked in the invasive and peripheral areas of epidermoid cancer than in necrotic or keratinised portions.

The intensity of β-glucuronidase staining in histochemistry of cervical neoplasms may vary greatly, and attempts to correlate enzyme activity with the degree of malignancy have not been very successful. Hatzimichael (1962) compared vaginal fluid and cervical mucus in relation to cancer of the cervix and uterus and found high β-glucuronidase activity in the mucus of 20 out of 21 proven cancer patients. Although it could not be established, the author's general impression was that β-glucuronidase activity of the cervical mucus paralleled the degree of malignancy. On the other hand, Rauramo (1959), although finding high β-glucuronidase in most cases of cervical carcinoma, could not correlate the activity with the spread of cancer (cf. Fishman and Bigelow, 1950, p. 109).

β-Glucuronidase is, therefore, found with increased activity in cervical carcinoma. This is associated with increased enzyme activity in vaginal fluid and cervical mucus of tumour patients. Increases appear to occur mainly in the squamous cells of the carcinoma, although stromal enzyme has been described. There does not seem to be a definite relationship between enzyme activity and severity of the malignant lesion.

There have been no reports on the intracellular

distribution of the enzyme in cervical carcinoma, other than histochemical studies stating that the enzyme is confined to the cytoplasm of malignant cells (Hopman, 1961; Kawakatsu and Mori, 1963). It is not known if the large increases in enzyme activity detected in homogenates of cervical cancers are due to increases in soluble cytoplasmic enzyme or in the particulate enzyme within the cell.

TISSUE β-GLUCURONIDASE AND RADIATION.

As β-glucuronidase shows a high activity in malignant cervical tissues and the treatment consists of irradiating the lesion, it is of interest to know how the enzyme in the tissue responds to this procedure. Little data are available on this subject.

Odell and Burt (1950) stated that after irradiation of cervical tumour tissue with radium and roentgen rays, the tissue β-glucuronidase tended to decrease in the absence of a recurrence of malignant growth. Odell et al. (1949) and Odell, Priddle and Burt (1950) stated that radiation treatment also caused a reduction in β-glucuronidase activity of vaginal fluid as well as tissue removed from the tumour site. These observations, however, were made on specimens removed approximately two months after therapy, and it is probable that the reduction in activity was due to malignant tissue being replaced by fibrous tissue comparatively weak in β-glucuronidase activity. Kasdon et al. (1950) made

observations on tissue specimens following irradiation of two months and longer, and could produce no evidence that radiation caused a decrease in β-glucuronidase. Rauramo (1959) studied 10 patients and observed changes in β-glucuronidase of whole tissue homogenates after treatment of the cervical lesion with a single radium implant. This author found that in two of the specimens there was increased β-glucuronidase activity and in eight a decrease occurred. Hatzimichael (1962) made observations on cervical mucus from eight patients receiving radium treatment. In four of these, the mucus became negative for β-glucuronidase activity, and the other four remained positive.

It is difficult to ascertain from these facts if the β-glucuronidase in cervical tissue is affected in a uniform way by radiation treatment, especially on a short-term basis. Reduction in β-glucuronidase over a long period probably reflects the reduction in malignant cells in the tissue specimen.

Studies on radiation of other tissues, mainly from animal experiments, have been reported, though their relevance to the conditions pertaining during therapeutic radiation of the human subject is hard to assign. Pellegrino and Villani (1957) studied the effect of whole-body x-ray irradiation on β-glucuronidase activity in lymphatic tissues in rats. They found that the activity/g. wet weight of tissue increased following radiation, but

this was associated with decrease in weight of the tissue due to loss of protein, and the effect was probably secondary to radiation, as the same result could be produced by other experimental conditions, e.g. fasting. Roth et al. (1962) carried out similar experiments on rat spleen, but determined β-glucuronidase activity of various tissue fractions - nuclei, mitochondria, microsomes and supernatant. They found an increase in the percentage of the total activity in the supernatant fraction 64 hours after giving a 700 rad whole-body dose. This was accompanied by an increase in specific activity of β-glucuronidase which was attributed to loss of non-enzyme protein nitrogen which occurs in the spleen after irradiation. Rahman (1962) obtained similar results which were interpreted on the basis of selective nitrogen loss. The author also indicated that the enzyme was not released from lysosomes by the radiation dose. Bacq and Alexander (1960) stated that a dose of 10,000 rads was required before leakage of hydrolytic enzymes from isolated lysosomes could be detected. Sottocasa, Glass and de Bernard (1965) showed that when lysosomal preparations (contaminated with mitochondria) from beef heart were irradiated in vitro with massive radiation doses up to 440,000 rads, there was no release of enzymes from the lysosomes. However, when heart tissue slices, with lysosomes intact in the cell, were irradiated with doses from 10,000-440,000 rads, there was an increase in homogenate specific activity which was shown to

be a true increase in β-glucuronidase and not due to loss of nitrogen; this was caused by release of enzyme from lysosomes. There was not, however, an increase in homogenate activity with radiation doses below 10,000 rads.

URINARY EXCRETION OF β-GLUCURONIDASE BY CANCER PATIENTS.

The excretion of β-glucuronidase in the urine has been shown to be increased in some patients with various malignant lesions (Boyland, Gasson and Williams, 1957). Cases of cancer of the larynx, bronchus, oesophagus, prostate and testis tended to show the most significant increases. The excretion of this enzyme has been investigated most fully, however, in patients with bladder carcinoma, where increased values have been found in the majority of these patients (Boyland, Wallace and Williams, 1955; Lewis and Plaice, 1960; Melicow, Uson and Lipton, 1961; Haije and van der Werf-Messing, 1962; Kerr, Barkin, D'Aloisio and Menczyk, 1963). Interest has arisen in these patients as it was thought that β-glucuronidase might play a vital rôle in the genesis of bladder tumours by its hydrolytic action upon glucuronide conjugates of carcinogenic substances in urine, e.g. ortho-amines (Boyland, Wallace and Williams, 1957).

Although the urine enzyme has been found to be increased in a wide variety of malignant lesions, there has been little evidence put forward that the malignant lesion itself is the primary source of this increase. Kerr *et al.* (1963),

investigating bladder tumour, showed that surgical removal of the tumour resulted in a fall in urine enzyme from previously high values to normal values. Haije and van der Werf-Messing (1962) did not find elevated values in a group of bladder carcinoma patients who, after radiotherapy, showed no evidence of tumour at follow up, whereas patients with active bladder tumour showed increased β-glucuronidase excretion. There have been no reports on the effects of treatment of other malignant lesions upon urinary β-glucuronidase excretion. A study by Takiguchi (1963) on the activity of β-glucuronidase in serum of patients with cervical cancer who underwent radiotherapy is the only report pertinent to this question. The author stated that serial determinations of serum β-glucuronidase and lactic dehydrogenase might permit an estimation of the radiosensitivity of cervical cancer.

OUTLINE OF THE PRESENT INVESTIGATION.

An opportunity existed for obtaining tissue specimens of cervical carcinoma. It was decided to investigate their β-glucuronidase activity and study the distribution of the enzyme in the cytoplasmic subcellular fractions of tissue homogenates. The results were compared with a series of non-malignant cervical specimens. In some cases, changes in the tissue β-glucuronidase of the tumours following irradiation of the lesion by radium implantation were

investigated.

The excretion of β-glucuronidase in the urine of patients with cervical cancer, undergoing radiation treatment, was studied to see if the urine enzyme responded to destruction of the lesion. It was expected that this would give an indication if the enzyme in the urine was produced by the cervical tumour or was a secondary systemic effect. Concomitant studies on serum β-glucuronidase activities were also carried out on these patients. The urinary excretion of β-glucuronidase was studied, for comparison, in two groups of patients receiving radiotherapy on other malignant lesions - patients with bladder carcinoma, and a group of patients with lesions in the thoracic region.

PART 2.

MATERIAL AND METHODS.

For this investigation, biopsy material was obtained from patients with carcinoma of the cervix uteri who underwent a course of radiotherapy involving radium implants and/or a period of deep x-ray irradiation. For comparison, normal cervical tissue was obtained from patients undergoing surgical repair for prolapsed uterus.

Investigations into the excretion of β-glucuronidase in urine during a course of radiotherapy were undertaken in selected patients with cervical, bladder and thoracic tumours.

TISSUE SPECIMENS.

Carcinoma of the Cervix Uteri.

Nineteen specimens were obtained from patients with proven cervical carcinoma, taken under anaesthesia just prior to insertion of the first radium implant. After removal, the specimens were placed immediately into iced water, blood clots and inflammatory exudate carefully dissected away, and the specimen washed, dried on adsorbant paper, weighed and stored at -20^{o} until further processing.

It was possible to classify each tumour from histology obtained from biopsy material taken prior to radium treatment. There were two adenocarcinomas and the others were squamous carcinomas of the cervix.

'Normal' Cervical Tissue.

Twelve samples of cervical tissue were obtained at operation on patients undergoing repair of uterine prolapse.

The specimens were taken in such a way as to include as much of the epithelial layer as possible with minimal amounts of underlying fibrous tissue. Although these specimens were classed as normal, microscopic evidence of varying degrees of chronic cervicitis were seen in some of the specimens, but this was unavoidable.

Pre- and Post-Radiation Specimens.

In 12 of the patients in the Carcinoma Series, further biopsy specimens were taken after radium had been implanted for about two days. They were obtained at the time of insertion of the second radium implant. These 12 specimens constituted the Post-Radiation Series, and the specimens taken before radium treatment on these 12 patients made up the Pre-Radiation Series.

URINE STUDIES.

Cervical Carcinoma Group.

Twenty-four hour collections of urine were taken periodically throughout a full course of radiotherapy on selected cases with carcinoma of the cervix uteri. These were patients who could, and would, readily co-operate with collection of specimens, were not incontinent of urine, showed no signs of urinary infection, and had good renal function at the time of presentation.

Two 24-hour collections were made before commencement of treatment, followed by collections while radium implants

were in situ, collections taken 2-3 days following radium withdrawal, and further collections taken 2-3 times per week over a course of supervoltage therapy which lasted usually 3-4 weeks.

Blood samples were also taken during therapy on days corresponding to urine collections. They were withdrawn early in the morning with the patient in a fasting state.

Two further groups of patients receiving radiotherapy were also studied by taking 24-hour urine collections before and during therapy. These were:-

Bladder Carcinoma Group - patients receiving radiation of the renal tract, comprising 8 cases (3 males, 5 females) who had not received previous radium therapy.

Thoracic Carcinoma Group - these were patients receiving radiation away from the urogenital tract. They consisted of 5 patients with carcinoma of the bronchus (3 male, 2 female), one female with carcinoma of the oesophagus, one with epiglottal carcinoma and one with carcinoma of the breast.

RADIATION DOSAGE.

Patients with carcinoma of the cervix uteri received total radiation doses ranging from 5000-8500 rads. These were given, in part, as radium implants inserted for periods of 48 to 100 hours as one or two insertions, followed by a course of supervoltage therapy from the A.E.I. Linear

Accelerator or Orbitron Co^{60} Unit administered over a 3-4 week period.

Patients in the Bladder Carcinoma Group received doses ranging from 3500-7000 rads. Radium implants were not used in this group, and all doses were given by supervoltage therapy. This also applied to the Thoracic Carcinoma Group where the tumour radiation dose ranged from 2500-7000 rads.

TISSUE FRACTIONATION.

All tissue specimens, normal and cancerous, which had previously been washed and weighed and stored at -20^{o}, were fractionated by the following method.

Homogenates of 1 in 10 or 1 in 20 (w/v) in isotonic sucrose were made depending on the type and weight of the tissue specimen. The frozen tissue was sliced into sections, 10-40 microns thick, on a freezing microtome; these were then placed in an appropriate volume of ice-cold 0.25M-sucrose and homogenised in an M.S.E. Blendor for 3 minutes, using crushed ice as the refrigerant. The homogenate was then spun at 500 g for 10 minutes in an M.S.E. 'Minor' centrifuge at 4^{o} to remove cell debris and nuclei. The non-sedimented fraction was then spun at 5000 g for 20 minutes in an M.S.E. Superspeed 17 Refrigerated Centrifuge at 4^{o}. The remaining fluid fraction was pipetted off from the sedimented particulate fraction and centrifuged at 35,000 g at 4^{o} for 60 minutes. The supernatant layer was removed from the

second particulate fraction and both particulate fractions were washed with approximately 10 ml. of ice-cold 0.25M-sucrose and centrifuged down again.

The three cytoplasmic fractions obtained by this procedure were designated - Supernatant, and M1 and M2 particulate fractions. It was not possible to ascertain the precise composition of each fraction, but relevant information was obtained from electron micrographs of similar fractions obtained from thyroid tissue fractionated under identical conditions (Goldberg, 1965). It has been assumed that fractions of cervical tissue would produce a similar picture. The three fractions were described as:-

Supernatant - an optically clear solution containing predominately cytoplasmic or soluble cell material with minimal contamination by intact particulate material.

M1 Fraction - particles centrifuged down between 500-5000 g. These were mainly well-preserved mitochondria. Some microsomes were seen, but there were few lysosomes.

M2 Fraction - particles centrifuged down between 5000-35,000 g. These were predominately microsomes but occasional mitochondria and mitochondrial-like fragments were seen. There appeared to be about twice the lysosomal contamination seen in the M1 Fraction.

It was estimated by visual study of the electron micrographs, that the mitochondria were distributed approximately 75% in the M1 Fraction and 25% in the M2, with the reverse

for the microsomes.

The two particulate fractions were suspended in ice-cold water of known volume (usually about 3 ml.) and the particles disrupted by sonication at 20Kc/sec. under refrigeration in an M.S.E. Ultrasonic Disintegrator Model 60W. A coagulum of mitochondrial membranes was formed in the M1 Fraction which was removed to leave an opalescent solution. The M2 Fraction formed a clear solution without formation of a coagulum.

It was not possible to obtain particulate fractions on some of the specimens due to the small amount of material available. This applied especially to normal cervical tissue where the particle yield was very small. In some of these cases, the particles centrifuged down at 5000 g were combined with the fraction sedimented at 35,000 g; the particulate material was then termed '<u>Combined Particles</u>'.

There were certain technical complications regarding the separation of particles from normal cervix which were unavoidable. These arose from the large amount of collagen fibres in the normal tissue which were spun down with cell debris and nuclei at 500 g. This matrix of fibres would trap some particles and prevent their recovery, and might account for the small yield of particles obtained from normal cervical tissue. There was comparatively little collagen material in the carcinoma specimens and particle yields were, on the whole, satisfactory.

ESTIMATION OF β-GLUCURONIDASE.

In Tissue Fractions and Serum.

The method used for the estimation of the enzyme in test material other than urine, was essentially that described by Talalay *et al.* (1946), but using a different preparation of enzyme substrate, and an 80% reduction in volumes of substrate and test solution to allow an economical assay of the enzyme.

This method involves the photocolorimetric measurement of phenolphthalein in alkaline solution, liberated by the enzyme from phenolphthalein mono-β-glucuronic acid.

Reagents

0.1M-acetate buffer, pH 4.5 - as described by Talalay *et al.*

0.4M-glycine buffer, pH 10.4 - " " " " "

0.01M-phenolphthalein-glucuronic acid substrate. 50 mg. of phenolphthalein mono-β-glucuronic acid* were dissolved in 5 ml. water, neutralised with 0.1N-NaOH, and diluted to 10 ml. The solution was stored in a dark bottle at 4° with one drop of toluene as preservative.

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* Obtained from the Sigma Chemical Company Ltd. Although the suppliers stated that variation in hydrolysis rates by β-glucuronidase might occur with different batches of this substrate, this was never found to be a complication when changing from one lot no. to another.

Phenolphthalein Standard. 25 mg. 'Analar' phenolphthalein were dissolved in 100 ml. of 80% ethanol. 1 ml. contained 250 µg. phenolphthalein.

Procedure.

0.8 ml. acetate buffer and 0.1 ml. of test solution were pipetted into two conical centrifuge tubes - test and control - and placed in a water bath at 37°. 0.1 ml. of substrate was added to the test and the mixture incubated for a known time. Four ml. of glycine were then added to each tube followed by 0.1 ml. of substrate to the control. The tubes were centrifuged to remove small amounts of precipitated protein and the supernatant decanted into 1 cm. cuvettes. The test was read against the control on a Unicam S.P.600 spectrophotometer at 550 mµ.

A phenolphthalein calibration curve was made using 0.1 ml. of various dilutions of the phenolphthalein standard with 0.8 ml. acetate buffer, 0.1 ml. water and 4 ml. glycine buffer. These were read against a reagent blank. The calibration curve was linear over a wide range of extinction.

Precise reaction conditions for different test material were:-

Serum - 0.1 ml. serum with an incubation of 24 hours.

Tissue fractions - 0.1 ml. of suitable dilutions of the various fractions with incubation times varying between 1-6 hours.

A unit of β-glucuronidase activity is expressed as the

formation of 1 µg. phenolphthalein in 1 hour at 37° under the test conditions. Results are expressed as units/ml. for tissue fractions, and units/100 ml. for serum. To convert to I.U./litre at 37°, the tissue results must be multiplied by 0.05, and the serum results by 0.0005.

Urine β-Glucuronidase.

The excretion of β-glucuronidase in urine was estimated using the method described by Melicow et al. (1961), but with slight modifications. The method also involves the measurement of liberated phenolphthalein from phenolphthalein mono-β-glucuronic acid substrate.

Urine collections.

Twenty-four hour urine specimens were collected by the patient voiding the bladder at a known time, usually early morning, and collecting every specimen from then up to and including a specimen passed at the same time 24 hours later. All specimens passed in the 24 hours were combined in a Winchester bottle containing 10 ml. chloroform as a preservative. The collections were kept at room temperature.

The 24-hour collection was thoroughly mixed and the volume measured. The pH was recorded and an aliquot of the collection taken and spun vigorously for 10 minutes. The clear supernatant urine was pipetted off and stored in a refrigerator at 4° until analysed.

Reagents.

1. 0.2M-Acetate buffer, pH 4.5. 5.785 g. sodium acetate ($CH_3COONa.3H_2O$) and 3.25 ml. glacial acetic acid were made up in 450 ml. water, the pH adjusted to 4.5 and the final volume made to 500 ml.

2. 0.01M-phenolphthalein-glucuronic acid substrate. This was the same as that used in the tissue and serum method.

3. 10% (w/v) sodium carbonate. 10 g. anhydrous sodium carbonate were dissolved in 100 ml. water.

4. Phenolphthalein Standard - as in the tissue and serum method.

Procedure.

Two 10 ml. graduated tubes were set up - test and control. To 1 ml. of urine in each tube were added 1.1 ml. acetate buffer. The tubes were placed in a water bath at 37° and 0.1 ml. substrate added to the test. The tubes were then incubated for exactly 18 hours, when 1 ml. 10% sodium carbonate was added to both test and control, followed by 0.1 ml. substrate to the control. The volume was then made to 10 ml. with water and the extinction of the test read against the control on the Unicam S.P.600 spectrophotometer at 550 mμ with a 1 cm. light path.

A calibration curve was made by using 0.05, 0.1 and 0.2 ml. volumes of the phenolphthalein standard and adding 1 ml. buffer, 1 ml. urine, 1 ml. sodium carbonate and making to 10 ml. with water. The extinction was read against a reagent plus urine blank. Melicow et al. (1961) stated that a

calibration curve should be made for each urine specimen, but in practice this was not found necessary as the calibration curve did not alter appreciably with different urines of normal pH. Only when urines with a high pH above 8 were encountered, was a special calibration curve made using that particular urine.

When urines of high β-glucuronidase were suspected or encountered, 0.5 ml. of urine was used in the estimation. However, 0.5 ml. of boiled urine was also added to the incubation mixture so as to counteract effects due to dilution of heat-stable inhibitors or activators caused by using a reduced volume of urine.

One unit of enzyme activity was defined as that amount of enzyme which liberates 1 µg. phenolphthalein at 37° under the test conditions and is essentially the same as that used in tissue and serum studies. The results are expressed as units/ml. urine or units excreted/24 hours. These results are to be multiplied by 0.05 to convert them to I.U./litre at 37°.

TISSUE PROTEIN.

Protein in tissue fractions was measured by the method of Lowry, Rosebrough, Farr and Randell (1951), with a slight modification by Ayre (1965) to increase the sensitivity of the reaction.

PART 2.

RESULTS.

METHODOLOGY OF THE β-GLUCURONIDASE ESTIMATION.

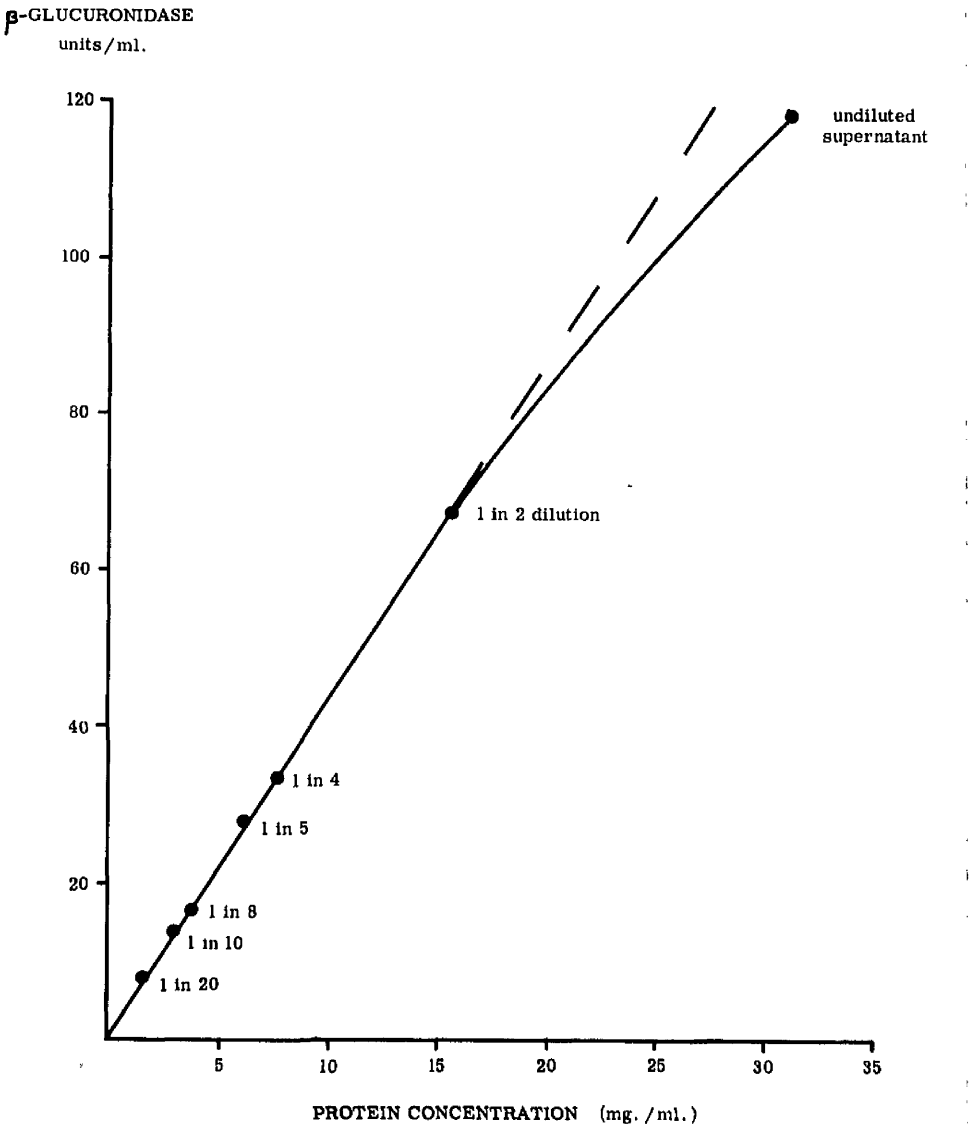
The estimation of serum and urine β-glucuronidase presented no problems, and reproducible results were readily obtained.

In the tissue studies, however, it was expected that wide ranges of β-glucuronidase activity would be encountered, and it was felt necessary to undertake preliminary experiments to ensure that variation in reaction conditions, to cope with high enzyme activities, would still produce reliable results.

First, the effect of dilution of tissue fractions was investigated to observe possible effects due to dilution of activators or inhibitors of β-glucuronidase. Fig. 13 shows results obtained from various dilutions of an active preparation of a supernatant fraction from cervical carcinoma. The supernatant, made up in 0.25M-sucrose, was diluted with water. It can be seen that dilution of the fraction had an effect upon the observed β-glucuronidase activity only at the 1 in 2 dilution, and further dilution produced linear reduction in β-glucuronidase activity. This effect would be due, presumably, to dilution of inhibitors present in the undiluted sample but which would not be effective on increased dilution. It might also be caused by inadequate substrate concentration at the high enzyme activity of the undiluted sample, but it was found that increasing the concentration of substrate did not effect the result obtained on the undiluted sample. The effect of dilution is again shown in

FIGURE 13.

EFFECT OF DILUTION OF A CERVICAL SUPERNATANT FRACTION ON ITS β-GLUCURONIDASE ACTIVITY.



The protein concentration of each dilution was calculated from the protein concentration estimated in the undiluted sample.

Table 14, where it can be seen that dilution of supernatant from another cervical carcinoma produced slightly higher β-glucuronidase activity. This effect was not seen in the particulate fractions made up in water and, if anything, dilution caused slight inhibition of the enzyme. It is possible that the sucrose in the supernatant causes this slight inhibition, but the effect is not as pronounced as that stated by deDuve (1963), who claimed that 0.25M-sucrose caused a 30% inhibition of β-glucuronidase.

The effects produced by dilution of supernatant and particulate fractions are only minimal and would not appreciably affect measured β-glucuronidase activities. To minimise any of the above effects, fractions obtained from different specimens were diluted wherever possible at least 1 in 2, and the variation in reaction conditions achieved mainly by changes in incubation time (see below). Some specimens of normal cervix, however, could not be diluted due to low β-glucuronidase activity.

The second factor considered in the estimation of tissue β-glucuronidase was linearity of the enzyme reaction with time. Talalay *et al.* (1946) showed that the reaction was linear with time using different tissue homogenates, but it was felt that a check should be made using the tissue fractions being studied in the present work.

The supernatant and particulate fractions of a cervical carcinoma, at 1 in 2 dilutions, were used to follow the course

TABLE 14.

EFFECT OF DILUTION OF TISSUE FRACTIONS UPON β-GLUCURONIDASE ACTIVITY.

Tissue:- Cervical Carcinoma

Tissue Fraction	Dilution	Observed β-gluc. units/ml.	β-Gluc.corrected for dilution units/ml.
SUPERNATANT (in 0.25M-sucrose)	0	150	150
	1 in 2	77	154
	1 in 4	39	156
M1 FRACTION (in water)	0	32	32
	1 in 2	15	30
	1 in 4	7	28
M2 FRACTION (in water)	0	36	36
	1 in 2	16	32
	1 in 4	8	32

All dilutions were made in water.

of the enzyme reaction with time. Fig. 14 illustrates that the enzyme reaction was linear over at least a 6 hour period for the supernatant and M1 fractions, and for the M2 fraction, the reaction was linear at least up to 5 hours; it was not tested beyond this because of the high β-glucuronidase activity of the M2 specimen.

It was possible, therefore, to vary incubation times quite safely from 1 to 6 hours, but in practice it was found that incubation times in excess of 4 hours were not required.

By varying the dilution of the tissue fractions and incubation times, it was possible to estimate specimens of low and very high β-glucuronidase activity and produce reliable results.

β-GLUCURONIDASE ACTIVITY OF NORMAL AND CANCEROUS CERVICAL TISSUE.

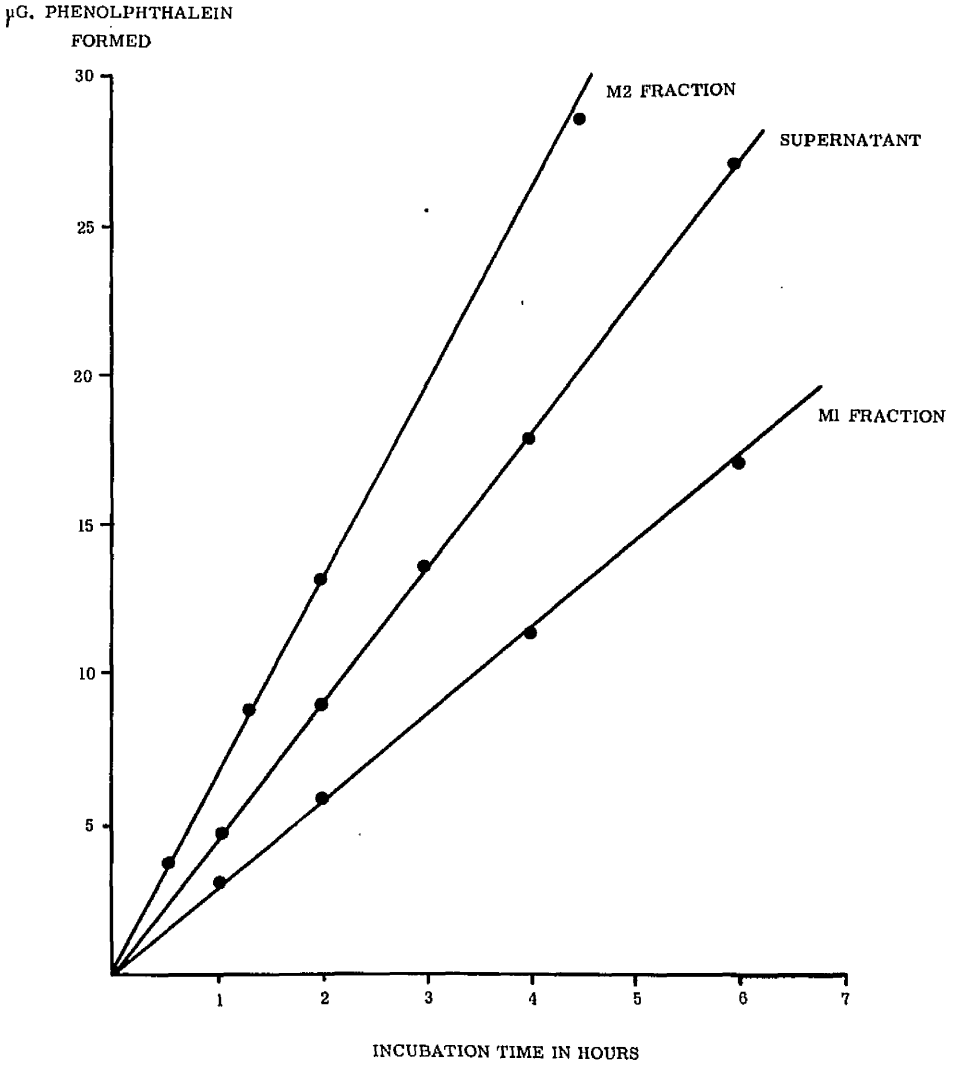
The enzyme activities of the tissue fractions were expressed in two ways, (a) as β-glucuronidase units/g. wet weight of tissue - units/g.w.w. - and (b) as specific activities, i.e. units/mg. tissue protein as measured by the method of Lowry *et al.* (1951).

Normal Cervical Tissue.

The β-glucuronidase activities recorded on 12 specimens of cervical tissue classed as normal but including, in some cases, non-malignant changes consistent with chronic cervicitis,

FIGURE 14.

LINEARITY OF THE RATE OF HYDROLYSIS OF SUBSTRATE BY β-GLUCURONIDASE IN THREE CYTOPLASMIC FRACTIONS OF CERVICAL CARCINOMA.



The substrate was phenolphthalein mono-β-glucuronic acid; the amount of phenolphthalein liberated was measured and plotted against time of incubation at 37°.

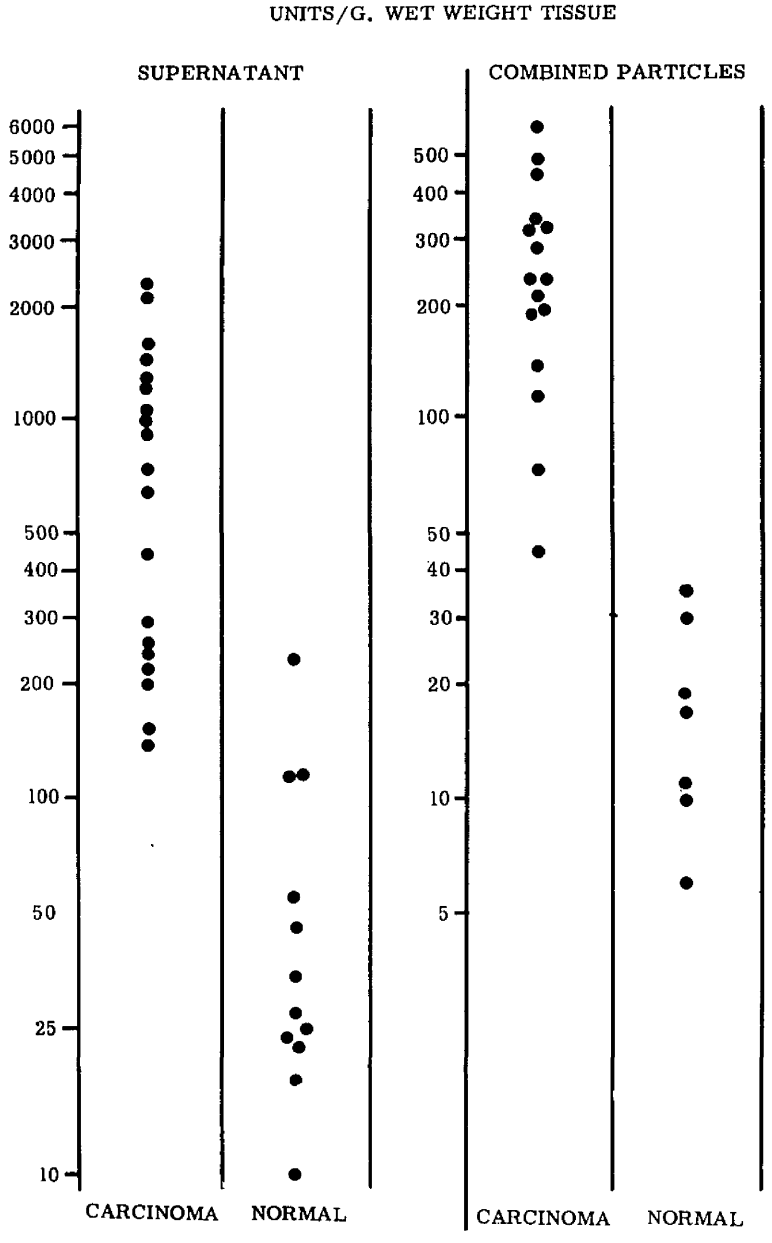
are shown in Fig. 15 as units/g.w.w. and Fig. 16 as specific activities, and the data pertaining to normal cervix from these Figures is summarised in Table 15. Particulate material was obtained in only 7 of the 12 specimens, and this could be divided into M1 and M2 fractions in only 4 of these. Consequently, the 'Combined Particle' fraction in Fig. 15 and 16 and Table 15 includes the results obtained on three specimens with M1 and M2 fractions combined before enzyme analysis, together with summated M1 and M2 results on the other 4 specimens.

The β-glucuronidase activity was low in the majority of these tissues. On a unit/g.w.w. basis, the measured activity appeared to be mainly in the supernatant fraction in these tissues in which particles were obtained (Table 15). However when the results were expressed as specific activities, the particles appeared to be more active than the supernatant.

Three of the specimens with supernatant β-glucuronidase activities of 235, 115 and 114 units/g.w.w. were much higher than any of the others (range 10-55 units/g.w.w.). There was no histological feature in these three specimens to account for their comparatively high enzyme values. Comparable findings were obtained when the activity was expressed as specific activity, indicating that this was a real increase in tissue enzyme in these specimens and not due to increased soluble tissue protein. Combined particles were obtained on two of the specimens with a higher β-glucuronidase activity,

FIGURE 15.

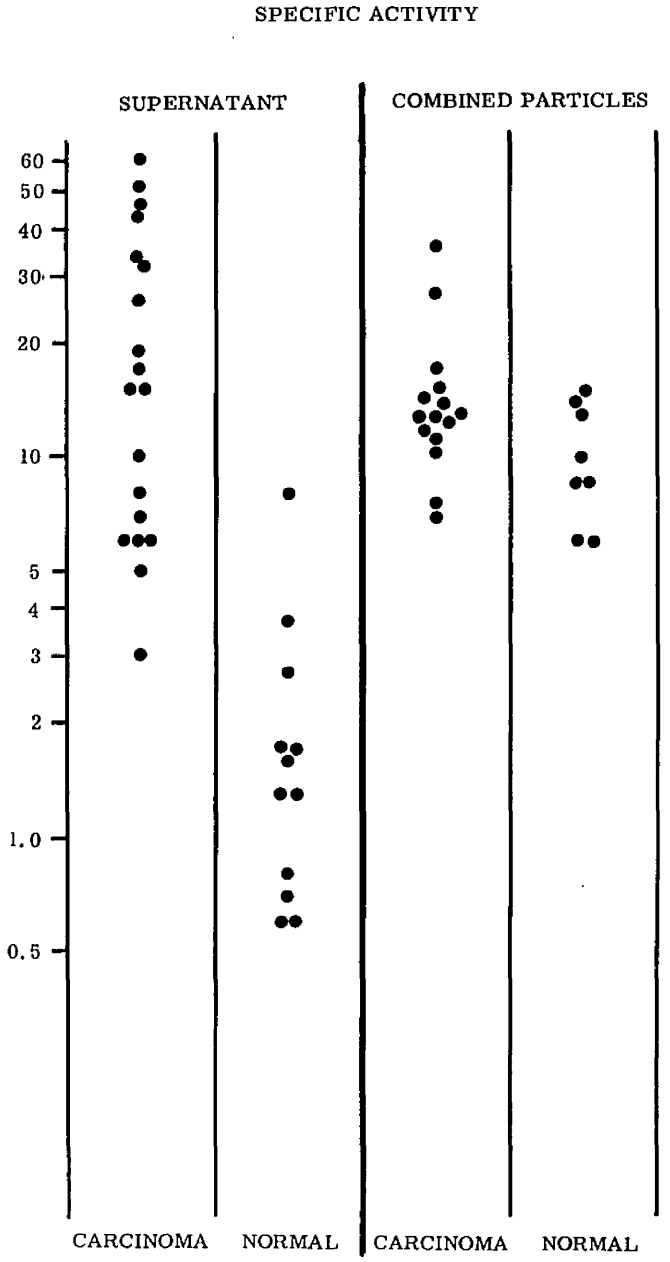
β-GLUCURONIDASE CONTENT OF SUPERNATANT AND PARTICULATE FRACTIONS OF MALIGNANT AND NON-MALIGNANT CERVICAL TISSUES.



Results are plotted on a semi-log scale.

FIGURE 16.

β-GLUCURONIDASE SPECIFIC ACTIVITIES IN SUPERNATANT AND PARTICULATE FRACTIONS OF MALIGNANT AND NON-MALIGNANT CERVICAL TISSUES.



Results are plotted on a semi-log scale.

TABLE 15.

β-GLUCURONIDASE ACTIVITY OF CYTOPLASMIC FRACTIONS OF NORMAL CERVICAL TISSUE.

Tissue:- Normal Cervix

	Tissue β-glucuronidase	
	units/g. wet weight tissue	specific activity
	Mean ± S.D.	Mean ± S.D.
SUPERNATANT (12)	60 ± 65	2.1 ± 2.0
M1 FRACTION (4)	6 ± 1.7	9 ± 4.1
M2 FRACTION (4)	8 ± 3.5	10 ± 4.2
COMBINED PARTICLES (7)	18 ± 11	10 ± 3.5

	% of total activity
	Mean ± S.D.
SUPERNATANT (7)	71 ± 10.3
M1 FRACTION (4)	13 ± 2.0
M2 FRACTION (4)	19 ± 9.5
COMBINED PARTICLES (7)	28 ± 7.7

Figures in parenthesis denote number of samples.

and their activity/g.w.w. was higher (35 and 30 units/g.w.w.) than that recorded on the other particulate specimens (6-18 units/g.w.w.). However this was not mirrored by the specific activities and would indicate a higher particle yield in these two specimens than in the others.

Cervical Carcinoma.

The β-glucuronidase activities obtained in the 19 carcinoma specimens are shown also in Fig. 15 (p.140) and Fig. 16 (p.141), and the data on these specimens are summarised in Table 16. M1 and M2 particulate fractions were obtained in 14 specimens; in one, the particle fractions were combined. The 'total activity' presented in Table 16 was calculated by combining the supernatant and particulate results. It is, therefore, an assessment of the total cytoplasmic activity obtained from the tissue, and not a representation of the total β-glucuronidase activity of the tissue, as no account has been taken of nuclear or residual β-glucuronidase left in the cell debris after homogenisation.

As can be seen, a wide range of β-glucuronidase activities was obtained. Values for the total activity ranged from 211-2590 units/g.w.w. and 5-44 units/mg. protein. The activity was mainly in the supernatant fraction (74%± 13.1); the distribution of activity was very similar to that found in normal cervical specimens. The particulate activity was fairly evenly divided between the M1 and M2 fractions, the M2

TABLE 16.

β-GLUCURONIDASE ACTIVITY OF CYTOPLASMIC FRACTIONS OF CERVICAL CARCINOMA TISSUE.

Tissue:- Malignant Cervix

	Tissue β-glucuronidase	
	units/g. wet weight tissue	specific activity
	Mean ± S.D.	Mean ± S.D.
SUPERNATANT (19)	855 ± 669	22 ± 18
M1 FRACTION (14)	114 ± 56	14 ± 7.0
M2 FRACTION (14)	142 ± 69	16 ± 8.8
TOTAL ACTIVITY* (15)	1190 ± 809	18 ± 11.8

	% of total activity
	Mean ± S.D.
SUPERNATANT (15)	74 ± 13.1
M1 FRACTION (14)	12 ± 6.7
M2 FRACTION (14)	14 ± 8.2

* Obtained by combining the Supernatant and Particulate results, including one additional case which only yielded Combined Particles on fractionation.

fraction showing a slightly higher activity in all but 4 of the specimens when the results were expressed as units/g.w.w. or as specific activities.

There was not such a striking difference between the supernatant and particle fractions when the β-glucuronidase of each fraction was expressed as specific activity instead of units/g.w.w. This is because there is approximately three times the amount of soluble protein in the supernatant as in the particulate fractions.

Comparison of Normal Cervix with Cervical Carcinoma.

It is clear that tissue from carcinoma of the cervix uteri, whilst having great variability in β-glucuronidase, usually has higher enzyme activity than normal cervical tissue. (Fig. 15 and 16, p.140, 141). The results on M1 and M2 fractions from the carcinoma specimens have been combined for purposes of comparison with the normal specimens. There was a large difference between the normal and carcinoma series with only one normal case lying in the carcinoma range of supernatant β-glucuronidase activity, and there was no overlap in the particulate enzyme results. Statistical analysis of these data is shown in Table 17 with the results as units/g.w.w. and Table 18 as specific activities. The differences for both supernatant and particle fractions on a unit/g.w.w. basis are highly significant (Table 17), but the specific activities of the particle fractions of the two series are very similar

TABLE 17.

COMPARISON OF β-GLUCURONIDASE ACTIVITY OF NORMAL AND MALIGNANT CERVICAL TISSUE.

SUPERNATANT

Tissue	No. of samples	β-glucuronidase (units/g.w.w.) Mean ± S.D.	t	P
NORMAL	12	60 ± 65	4.052	<0.001
CARCINOMA	19	855 ± 669		

COMBINED PARTICLES

NORMAL	7	18 ± 10.8	4.671	<0.001
CARCINOMA	15	242 ± 126		

TABLE 18.

COMPARISON OF β-GLUCURONIDASE SPECIFIC ACTIVITIES OF NORMAL AND MALIGNANT CERVICAL TISSUE.

SUPERNATANT

Cervical tissue	No. of samples	β-glucuronidase (units/mg. protein) Mean ± S.D.	t	P
NORMAL	12	2.1 ± 2.0	3.805	<0.001
CARCINOMA	19	22 ± 18		

COMBINED PARTICLES

NORMAL	7	10 ± 3.5	1.802	n.s.
CARCINOMA	15	15 ± 7.3		

and there was no demonstrable difference between them (Table 18). This indicates that the differences in particulate β-glucuronidase/g.w.w. between the normal and cancer specimens are due to differences in particle yield, and are caused, in part, by the poor yield of particles from normal tissue for the technical reasons previously mentioned (p.126).

DISTRIBUTION OF CYTOPLASMIC β-GLUCURONIDASE IN CERVICAL CARCINOMA.

On comparing the results on the tissues with high β-glucuronidase and those with low, there was a difference in the distribution of the enzyme between the supernatant and particulate fractions. Consequently, the specimens were split into two well separated groups: those with a total cytoplasmic β-glucuronidase of over 1000 units/g.w.w. - the High-Content Group, and those with a total activity of under 500 units/g.w.w. - the Low-Content Group. On this basis, only one case with a total activity of 749 units/g.w.w. was not placed. The distribution of β-glucuronidase in this case was 71% in the supernatant, 13% in the M1 and 16% in the M2 fraction.

The percentage of enzyme activity occurring in each of the three fractions in both groups is shown in Table 19, and it can be seen that there is a highly significant difference in the distribution of the enzyme in the two groups.

TABLE 19.

DISTRIBUTION OF β-GLUCURONIDASE ACTIVITY IN CERVICAL CARCINOMA OF HIGH AND LOW ENZYME CONTENT.

Percentage of Total Activity in each Fraction

	High-Content Group (8)	Low-Content Group (5)		
	Mean ± S.D.	Mean ± S.D.	t	P
SUPERNATANT	82 ± 5.3	61 ± 13.4	4.038	<0.005
M1 FRACTION	8 ± 2.3	18 ± 7.4	3.645	<0.005
M2 FRACTION	10 ± 3.3	21 ± 10.5	2.810	<0.025

Figures in parenthesis denote number of cases in each group.

t and P values were obtained by comparing the equivalent fractions of the two groups.

In the High-Content Group, a greater proportion of the enzyme activity is found in the supernatant fraction than in the Low-Content Group. This indicates that most of the increase of β-glucuronidase in these tissues was in soluble cytoplasmic enzyme rather than in particulate β-glucuronidase. However there could be an alternative explanation of this finding in terms of variation in particle yield in the two groups. This could be due to (a) tissue specimens in the Low-Content Group giving a higher particle yield than the High-Content Group, (b) the particles in the Low-Content Group having more β-glucuronidase activity than the High-Content Group or (c) differences in the fragility of the particles in the two groups resulting in particulate contamination of the supernatant in the High-Content Group.

However, the most likely explanation can be arrived at when the specific activities and the protein content of the specimens in the two groups are compared. Table 20 shows the protein content as mg./g.w.w. of the two groups. There is not a great difference in particulate protein between the High-Content and Low-Content groups. The particulate protein/g.w.w. can be taken as an index of the yield of particles from the tissue, and it is clear, therefore, that there is not a great difference in this respect between the groups. There is in fact, an indication of a slightly smaller particle yield from the Low-Content Group which is the reverse of what would be needed to explain the difference

TABLE 20.

PROTEIN CONTENT OF CERVICAL CARCINOMA SPECIMENS OF HIGH AND LOW β-GLUCURONIDASE CONTENT.

Protein Content of each Fraction
(mg./g.w.w.)

	High-Content Group	Low-Content Group		
	Mean ± S.D.	Mean ± S.D.	t	P
SUPERNATANT	56 ± 13.0	36 ± 8.1	4.146	<0.001
M1 FRACTION	9 ± 3.2	8 ± 5.4	0.663	n.s.
M2 FRACTION	11 ± 3.8	7 ± 2.0	2.143	n.s.

in β-glucuronidase distribution on this basis.

Table 21 shows the specific activities found in the two groups. From this, it can be seen that there are no pronounced differences in β-glucuronidase activities of the particulate material in both groups and, if anything, the High-Content Group was more active in this respect than the Low-Content Group; this finding is again the opposite of that required to account for the different distribution of the enzyme in the two groups by differences in particle activities.

It is also shown in Table 21 that the supernatant specific activity in the High-Content Group is considerably higher than the particulate specific activities. It is not possible, therefore, to explain the increase in supernatant β-glucuronidase in this group in terms of breakdown of particles during fractionation, to release the enzyme into the supernatant. There would have to be a selective release of β-glucuronidase-rich protein from the particles to give such large increases in supernatant specific activity in view of the fact that protein released from particles would be greatly diluted due to the very large protein content of supernatant relative to particles.

From these considerations, it can be concluded that increases in the majority of specimens of cervical cancer are due mainly to increases in soluble cytoplasmic enzyme with only slight increases in particulate β-glucuronidase.

Another fact to be observed from Table 20 (p.151) is that

TABLE 21.

SPECIFIC ACTIVITIES OF FRACTIONS OF CERVICAL CARCINOMA SPECIMENS OF HIGH AND LOW β-GLUCURONIDASE CONTENT.

Specific Activity of Each Fraction

	High-Content Group	Low-Content Group		
	Mean ± S.D.	Mean ± S.D.	t	P
SUPERNATANT	30 ± 13.0	7 ± 4.6	3.761	<0.005
M1 FRACTION	17 ± 8.2	10 ± 2.0	1.860	n.s.
M2 FRACTION	19 ± 10.1	11 ± 4.1	1.647	n.s.

there was significantly more supernatant protein/g.w.w. in the High-Content Group. This could be a reflection of more epithelial tissue being obtained in these specimens than in the Low-Content Group, where it is likely that there was more stromal material which would not yield cytoplasmic protein. This would also explain the higher β-glucuronidase content of the tissues in this group, as the enzyme is usually confined mainly to epithelial cells with little or no activity in the stroma.

β-GLUCURONIDASE AND THE DEGREE OF MALIGNANCY OF CERVICAL CARCINOMA.

Because there was such a wide range of β-glucuronidase activities in the carcinoma series, it was felt necessary to see if this was associated with the clinical and histological assessment of the severity of the disease. The cases were graded into the following three groups using the clinical staging of the disease together with histology reports: Severe cases, with a widespread, highly malignant lesion; Mild cases, with an early lesion; Intermediate group comprising those cases which could not be placed with certainty in the other two groups. This classification must needs be arbitrary, but the main purpose was to separate the distinctly advanced cases from those with early lesions. The clinical and histological classification was made independently by a colleague with no knowledge of the enzyme

results. On this basis, 9 of the 19 cases were classed as Severe, with 5 Mild and 5 in the Intermediate group. The supernatant β-glucuronidase activity of the specimens obtained from these patients in each of the groups is shown in Table 22. There was a significant difference between the Mild and Severe cases with the results expressed as units/g.w.w. and as specific activities. This difference was due to all 5 cases in the Mild group having very low β-glucuronidase activities with none higher than 250 units/g.w.w., whilst cases with higher activities were spread over the Severe and Intermediate groups. There was no difference between the Severe and Intermediate cases. Particulate material was obtained on only three of the cases in the Mild group. The combined particle activity for these three cases - 138, 73 and 48 units/g.w.w. - were very much lower than for the Severe group - 198-487 units/g.w.w. This difference was not apparent, however, with the results expressed as specific activities and was, therefore, probably due to a small particle yield in the Mild cases.

Table 23 shows the protein content of normal and malignant cervical specimens. Comparing these values with those in Table 20 (p.151), it is seen that the carcinomas with low β-glucuronidase activity approach the normal tissues in their supernatant protein content. Also it is shown that those specimens classed as Mild in Table 22 lie perhaps even nearer to the normal group. This does not apply to the

TABLE 22.

SUPERNATANT β-GLUCURONIDASE RESULTS ON CASES OF CERVICAL CARCINOMA CLASSIFIED BY SEVERITY OF THE MALIGNANT LESION.

		β-Glucuronidase (units/g.w.w.) Mean ± S.D.	t †	P
Severe *	(9)	1044 ± 675	2.739	<0.02
Intermediate *	(5)	1159 ± 625	3.378	<0.01
Mild	(5)	212 ± 46		
		Specific Activity		
Severe *		24 ± 16.1	2.469	<0.05
Intermediate *		33 ± 21.7	2.510	<0.05
Mild		6 ± 1.1		

† t values obtained by reference to the Mild Group.

* The Severe and Intermediate Groups were not statistically different.

TABLE 23.

PROTEIN CONTENT OF NORMAL CERVIX AND CERVICAL CARCINOMA.

Protein (mg./g.w.w.)

	Samples	Mean ± S.D.
NORMAL CERVIX		
SUPERNATANT	12	27 ± 7.9
COMBINED PARTICLES	7	1.8 ± 0.9
CERVICAL CARCINOMA		
SUPERNATANT	19	43 ± 17.6
COMBINED PARTICLES	15	17 ± 6.9
MILD CASES*		
SUPERNATANT	5	33 ± 6.5
COMBINED PARTICLES	3	11.0, 4.0 and 10.0
SEVERE AND INTERMEDIATE* CASES		
SUPERNATANT	14	47 ± 18.9
COMBINED PARTICLES	12	19 ± 6.8

*Cervical carcinoma cases divided as in Table 22 (p.156)

particle fractions where the protein in the normal specimens is very much lower than that of the cancer specimens; this reflects the much lower yield of particles from normal cervical specimens.

EFFECT OF RADIATION ON TISSUE β-GLUCURONIDASE.

Specimens of cervical cancer tissue were obtained before and after radiation in 12 patients; particulate material was compared in 10 of these.

There was not a uniform change in β-glucuronidase in these tissues following treatment by radium, with the results expressed as units/g.w.w. or as specific activities. Three cases showed large increases in supernatant β-glucuronidase, while four showed large decreases. The remaining five cases showed small variations which probably arose from tissue sampling techniques.

Changes in particulate enzyme were also very variable, and they did not correlate with changes in supernatant enzyme.

The increases and decreases seen following radiation could not be correlated with the classification of the cases into Severe, Mild or Intermediate with respect to the degree of malignancy, or with the response of the patient to the radiation treatment.

BLOOD AND URINE β-GLUCURONIDASE FOLLOWING RADIOTHERAPY.

URINE STUDIES.

Results on the urinary β-glucuronidase excretion in the three groups of patients studied - those with cervical, bladder or thoracic carcinoma - were expressed as 24 hour excretion and as urine enzyme activity in units/ml.

A normal range for urine β-glucuronidase was obtained by taking 24-hour urine collections from 20 healthy subjects, aged 19-67 years. These gave a mean excretion of 1520 units/24 hours, S.D. 410, with an observed range of 950-2450 units/24 hours, and mean urine enzyme activity of 1.29 units/ml., S.D. 0.36, with an observed range of 0.74-1.93 units/ml.

Patients with Carcinoma of Cervix.

In order to group the results on all the patients together for statistical analysis, the scheme of radiotherapy treatment for each patient was divided into phases, and the mean β-glucuronidase excretion and activity was taken from the results on all 24-hour urine specimens collected in each phase. These phases were as follows:-

1. Pre-treatment - comprising two consecutive 24-hour collections taken before treatment commenced; the average of the results was taken as a baseline for β-glucuronidase excretion for each patient.
2. Radium treatment - in five of the patients, these comprised four collections, two following insertion of the first and

second radium implants, and two following withdrawal of these; in the remaining four patients, there was only one insertion of radium and, therefore, only two collections were made following radium insertion and withdrawal. The mean was taken of the results on all specimens from each patient over this period.

3. Supervoltage therapy - this was divided into three separate weekly periods; the mean of the β-glucuronidase results on the 2-3 collections taken in each week on each patient were used.

The collective data on the 9 patients in this group are shown as 24 hour excretion of β-glucuronidase in Table 24, and as units/ml. in Table 25. As can be seen from the standard deviations on the results during each treatment, there was a large patient variation in β-glucuronidase output before and after radiotherapy. The difference between base-line values and those obtained during different treatments were analysed statistically, and it is shown in Table 24 that there was a significant increase in 24 hour excretion of enzyme which occurred during and following radium treatment. The same was found with urine β-glucuronidase expressed as units/ml., indicating a true increase in enzyme output and not simply increases in urine volume.

There was, however, variation between the patients in the group. The larger increases in β-glucuronidase output occurred in patients who had an abnormally high 24 hour excretion of enzyme before radiotherapy. This is shown in

TABLE 24.

24-HOUR URINE β-GLUCURONIDASE DURING RADIOTHERAPY OF PATIENTS WITH CARCINOMA OF CERVIX.

	Daily β-glucuronidase excretion (units/24 hours) Mean ± S.D.	t	P
Before treatment	2270 ± 1216		
Radium treatment	3577 ± 1975	3.644	<0.01
Supervoltage Week 1	4310 ± 3070	3.107	<0.02
Week 2	3360 ± 3400	1.286	n.s.
Week 3	3380 ± 4310	0.948	n.s.

Number of patients = 9

t values were obtained by comparing the means for each period of treatment with the before-treatment value.

TABLE 25.

URINE β-GLUCURONIDASE ACTIVITY DURING RADIOTHERAPY OF PATIENTS WITH CARCINOMA OF CERVIX.

	β-Glucuronidase activity (units/ml. urine) Mean ± S.D.	t	P
Before treatment	1.66 ± 0.76		
Radium treatment	2.71 ± 1.34	3.841	<0.01
Supervoltage Week 1	2.84 ± 1.52	2.725	<0.05
Week 2	2.33 ± 1.44	1.478	n.s.
Week 3	1.86 ± 1.14	0.645	n.s.

Number of patients = 9

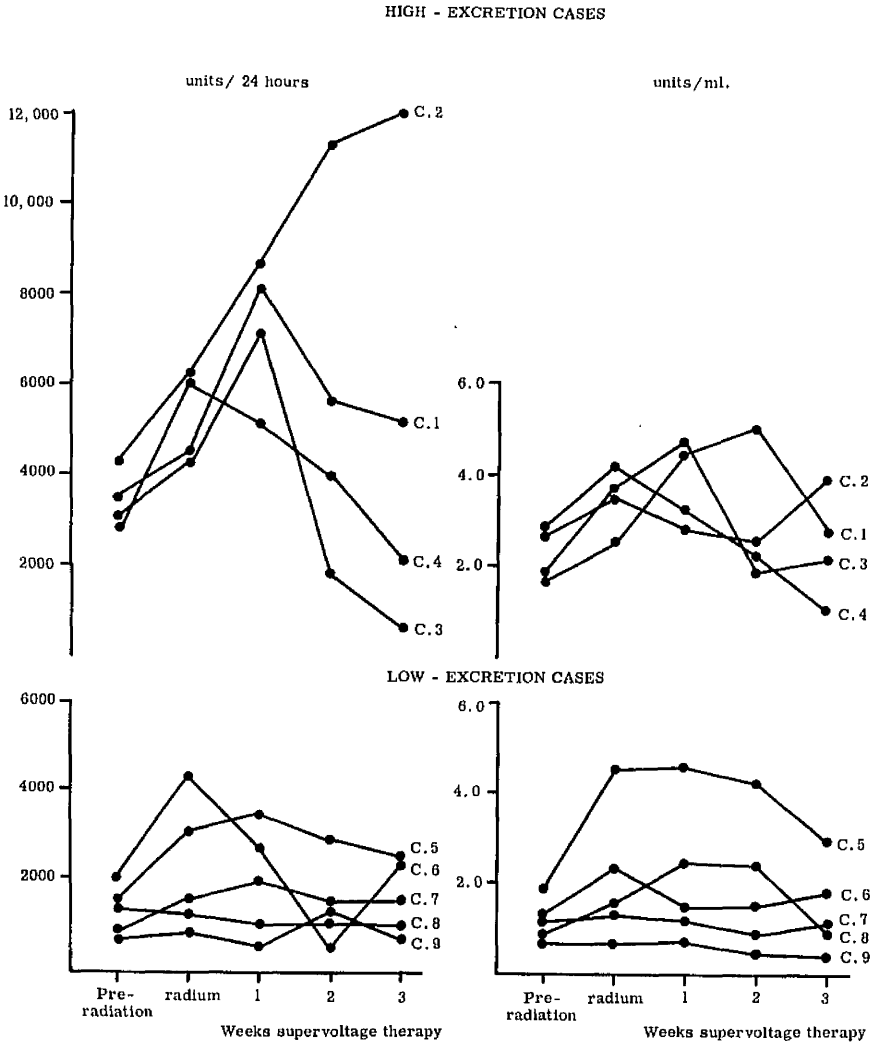
Figure 17. The cases were divided into those with a high pre-treatment β-glucuronidase excretion and those with normal β-glucuronidase values. All 4 cases in the high-excretion group showed pronounced increases in 24 hour enzyme output occurring during radium treatment and over the period directly following. This was reflected in the urine activity as units/ml. The large increases in 24 hour β-glucuronidase excretion in Case C.2 were accompanied by a diuresis occurring in the second and third weeks of supervoltage therapy. There was, however, an increase in enzyme activity in units/ml. in the third week. Collections were taken on this patient for a further two weeks of radiotherapy, and it was found that the enzyme excretion fell over this period to approximately 7000 units/24 hours.

Of the 5 patients with normal pre-treatment β-glucuronidase excretion, two (Cases C.5 and C.6) showed a definite increase in enzyme output occurring over the same period as the 4 high-excretion cases. Case C.7 appeared also to show a slight increase in enzyme excretion, although not to values far outside the normal range. The other cases - C.8 and C.9 - showed no response to radiation and produced remarkably constant urine values over the whole course of therapy.

It is clear that 7 of the 9 cases showed increases in β-glucuronidase excretion which in 6 were very pronounced. The increases occurred during implantation of radium into the cervical carcinoma and extended for some time afterwards

FIGURE 17.

EXCRETION OF β-GLUCURONIDASE IN URINE OF PATIENTS WITH CERVICAL CARCINOMA RECEIVING RADIOTHERAPY.



during supervoltage therapy. The enzyme output usually fell thereafter by a varying extent. The largest increases in enzyme excretion were associated with those cases which had an abnormally high β-glucuronidase excretion prior to radiotherapy.

There was not a direct correlation between the severity of the disease and β-glucuronidase response. The 4 patients in the high-excretion group were all classed as Severe by previous criteria (p.154). However, in the low-excretion group, Cases C.5 and C.6 which showed the largest response were Mild cases, and Case C.8 which produced no response was Severe.

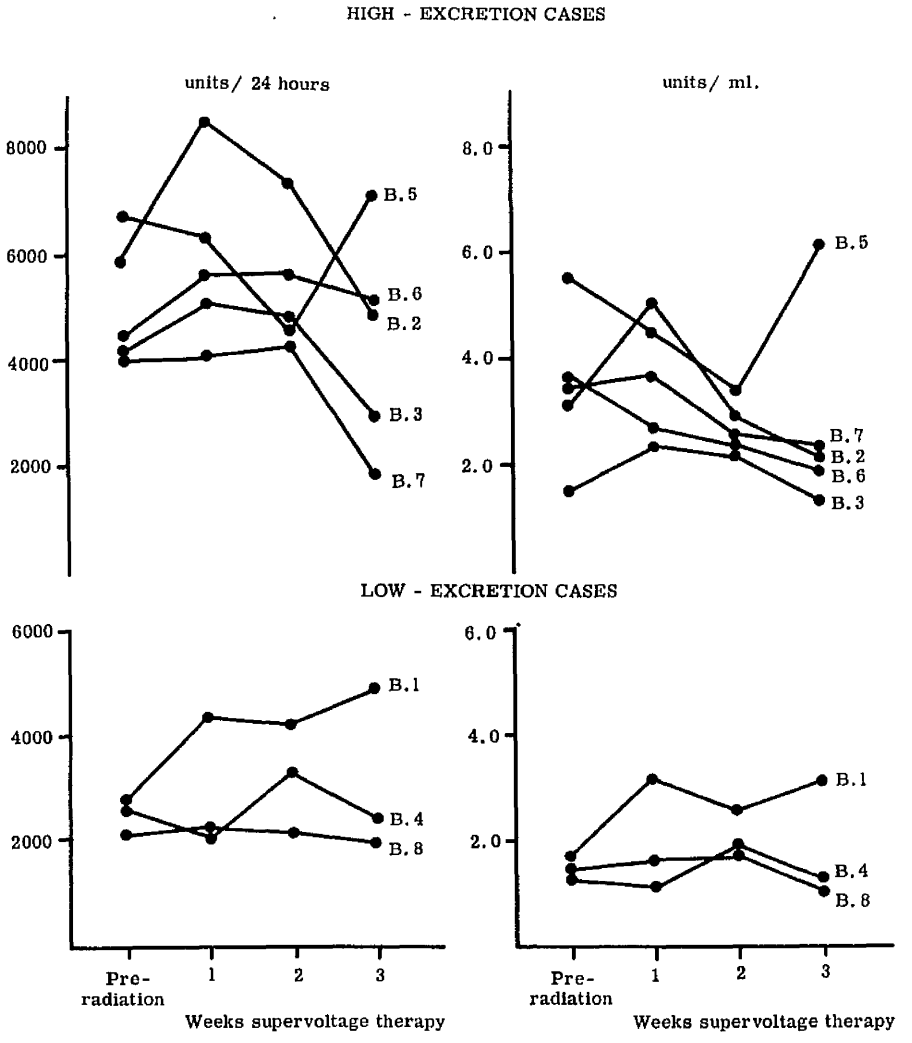
Patients with Bladder Carcinoma.

Urine collections were taken at intervals over the whole period of supervoltage therapy, and the results on the collections grouped into three separate weekly periods as previously described with the cervical carcinoma patients.

A statistical difference between the baseline values and those for each period of treatment could not be found. Results on individual cases are shown in Fig.18. It can be seen that there was not a consistent response of the patients to supervoltage therapy, even though 5 of the 8 patients had very high β-glucuronidase excretion before radiotherapy. Only two cases - B.1 and B.2 - showed pronounced increases during radiation, while the others showed only slight or

FIGURE 18.

EXCRETION OF β-GLUCURONIDASE IN URINE OF PATIENTS WITH BLADDER CARCINOMA RECEIVING RADIOTHERAPY.



non-specific changes.

Patients with Thoracic Carcinoma.

Urinary β-glucuronidase results on this group were obtained in a similar way to those in the bladder carcinoma group. A statistical difference between the results on different stages of the treatment could not be shown.

Fig. 19 shows the individual cases. Only two, both with carcinoma of the bronchus, had high pre-treatment β-glucuronidase values; both showed increased enzyme output over the first two weeks of treatment which fell to initial values during the third week. Of the other 6 cases, only Case T.7 (carcinoma of oesophagus) showed increased excretion, this in spite of the patient developing oliguria due to poor fluid intake during treatment.

Urine β-Glucuronidase and Radiation Dosage.

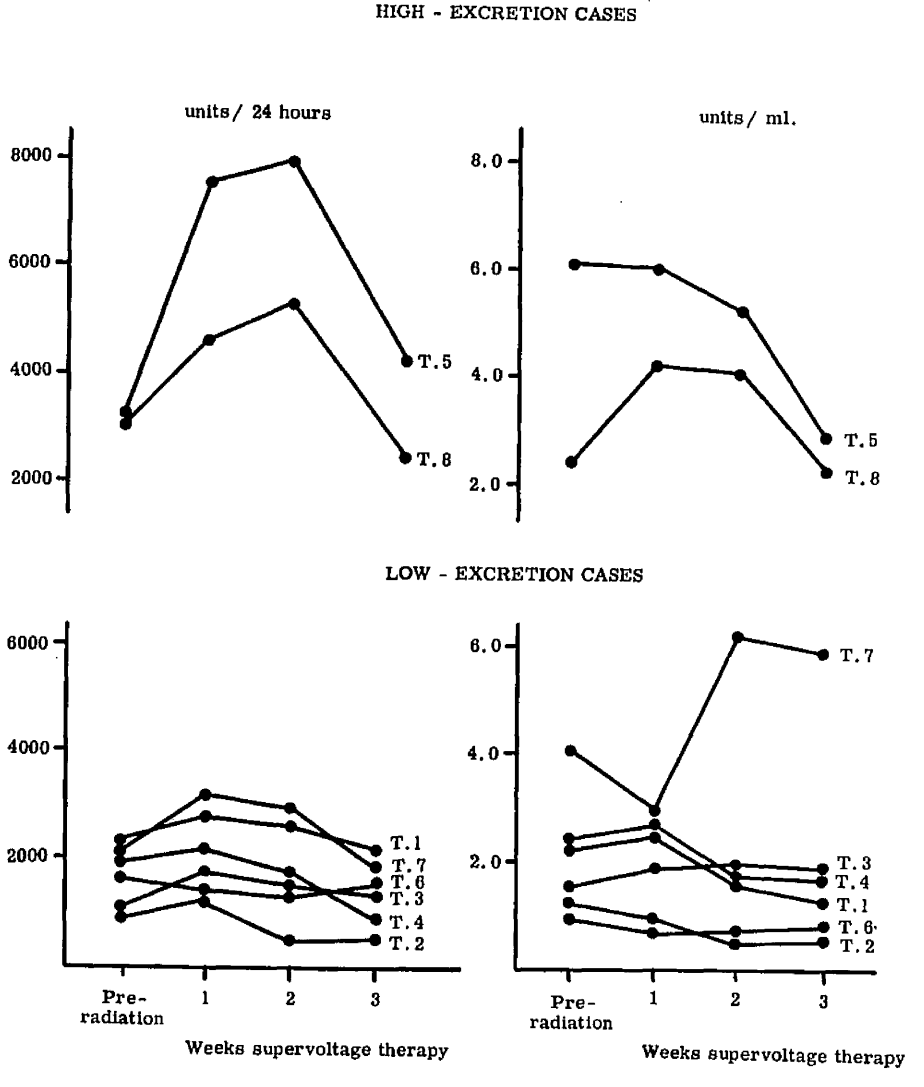
The pattern of β-glucuronidase excretion was not related to the total radiation dose given to the tumour tissues in any of the three groups. In the cervical carcinoma group, however, the radium implants appeared to be the major cause of the increases in enzyme excretion.

SERUM β-GLUCURONIDASE IN CERVICAL CANCER PATIENTS.

Blood specimens were taken along with urine collections in the cervical carcinoma group, and serum β-glucuronidase

FIGURE 19.

EXCRETION OF β-GLUCURONIDASE IN URINE OF PATIENTS WITH THORACIC CARCINOMA RECEIVING RADIOTHERAPY.



analyses carried out. The results on these patients were grouped in the same way as the urine results and, therefore, the values for serum β-glucuronidase in each patient during the different periods of treatment are the mean of 2-3 or sometimes 4 analyses of β-glucuronidase in different serum samples. All except one case showed an increase in serum β-glucuronidase during radium treatment; the results are shown statistically in Table 26. Thereafter, results were unpredictable, but in three cases there were further increases to high values over the course of supervoltage therapy. Four of the other cases showed subsequent decreases in enzyme activity. These results are shown in Fig.20.

No connection could be found between the serum and urine β-glucuronidase over the whole course of therapy in the majority of these patients. One fact that emerges, however, is that during radium treatment, increases in urine enzyme output were accompanied by increases in serum β-glucuronidase in 7 of the cases. Case C.8 which did not produce an increase in urine activity, also failed to produce a rise in serum β-glucuronidase during radium implantation.

Two cases, shown in Fig. 21, showed a reciprocal relationship between serum and urine β-glucuronidase. Both cases showed a coincident increase in urine and serum enzyme during radium therapy. Case C.5 then produced a dramatic fall in urine enzyme output to low values of 200-400 units/

TABLE 26.

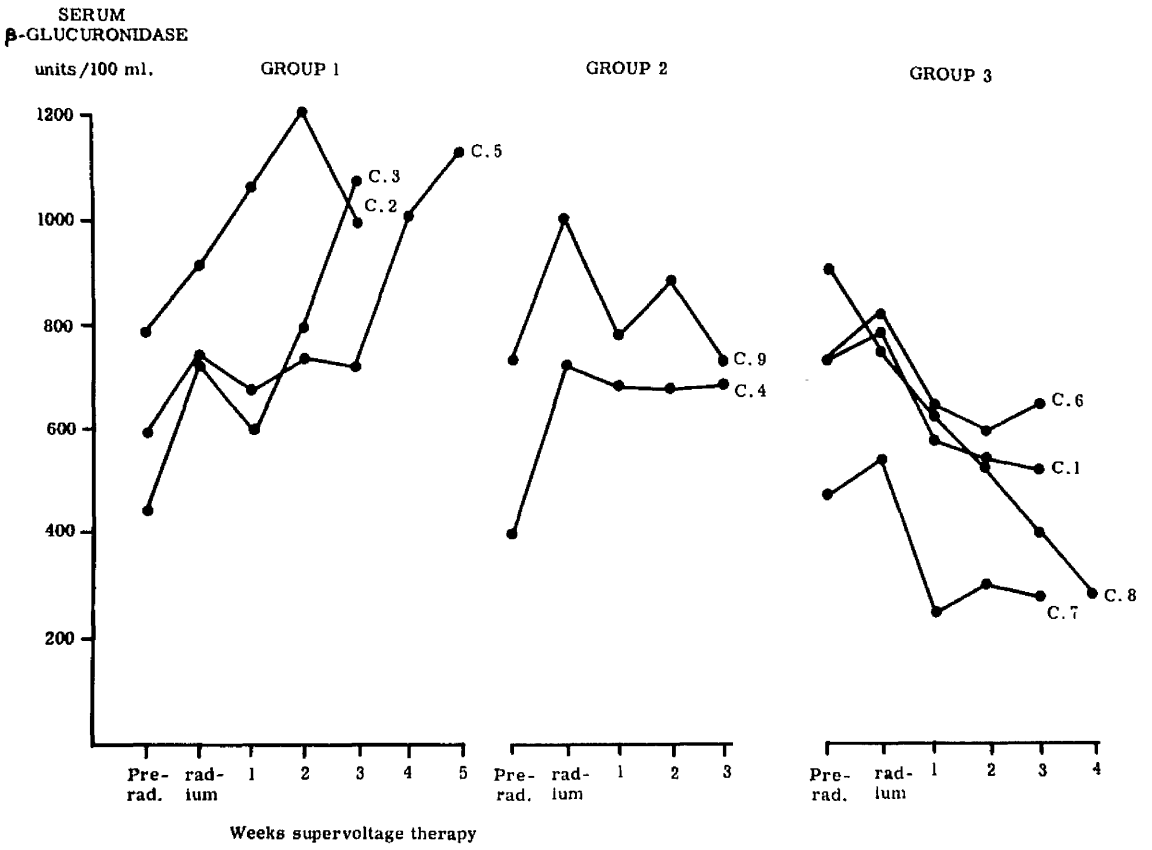
SERUM β-GLUCURONIDASE ACTIVITY DURING RADIOTHERAPY OF PATIENTS WITH CARCINOMA OF CERVIX.

	Serum β-glucuronidase (units/100 ml.) Mean ± S.D.	t	P
Before treatment	646 ± 174		
Radium treatment	794 ± 128	3.552	<0.01
Supervoltage Week 1	662 ± 220	-	n.s.
Week 2	701 ± 252	-	n.s.
Week 3	676 ± 254	-	n.s.

No. of patients = 9

FIGURE 20.

SERUM β-GLUCURONIDASE IN PATIENTS WITH CERVICAL CARCINOMA RECEIVING RADIOTHERAPY.



Group 1 - cases showing increased enzyme activity over supervoltage

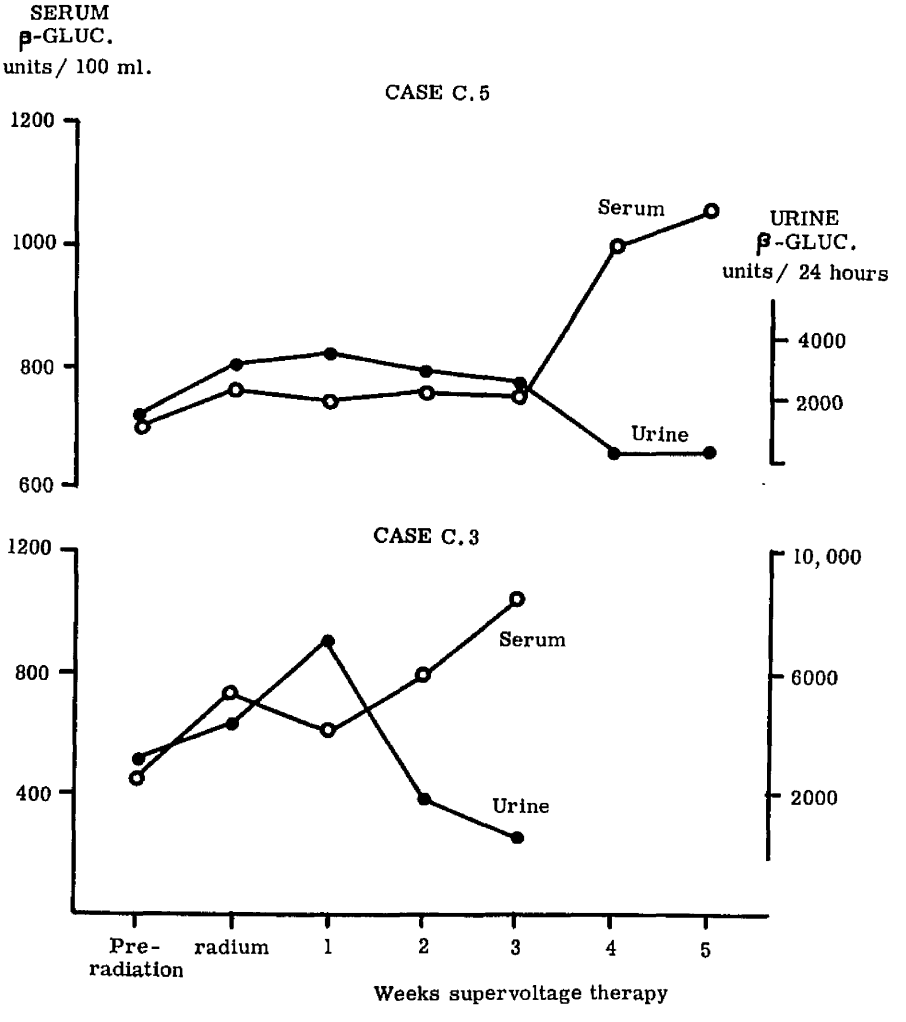
Group 2 - cases showing no specific change

Group 3 - cases showing decreases over supervoltage therapy

Case numbers refer to those in Fig. 17 (p.164).

FIGURE 21.

RECIPROCAL RELATIONSHIP OF BLOOD AND URINE β-GLUCURONIDASE IN TWO PATIENTS WITH CERVICAL CARCINOMA.



24 hours in the fourth and fifth weeks of deep x-ray therapy which was accompanied by a rise in serum activity to abnormal values. Case C.3 also showed a sharp fall in urine excretion of β-glucuronidase, with the serum values rising in a correspondingly sharp manner.

COMPARISON OF TISSUE AND URINE β-GLUCURONIDASE STUDIES IN PATIENTS WITH CERVICAL CARCINOMA.

There were only 5 patients with cervical carcinoma on whom biopsy specimens were obtained, who subsequently had urine studies performed. This was due to difficulty in selecting patients suitable for the urine studies. A post-radiation tissue specimen was obtained in only one of the 5 cases.

Biopsy material was taken on Cases C.1,2,3,5, and 8 shown in Fig. 17 (p. 164). Of the cases with a high initial excretion of β-glucuronidase, Case C.2 had a very high tissue supernatant β-glucuronidase - 2300 units/g.w.w. and specific activity of 44 - and produced a good increase in urine enzyme excretion following radiation; Case C.3 had a moderate tissue supernatant enzyme activity of 650 units/g.w.w., but a high specific activity of 46, and also produced a good response in urinary β-glucuronidase following irradiation of the tissue.

In the cases with normal β-glucuronidase excretion, Case C.5 had a moderate to high supernatant β-glucuronidase -

1000 units/g.w.w. with a specific activity of 17, and showed an increase in urine β-glucuronidase excretion following radiation, although not so marked as Cases C.2 and C.3. Case C.8, which showed no change in urine enzyme output following irradiation, had a very low tissue supernatant enzyme activity - 220 units/g.w.w. and a specific activity of 7. A post-radiation specimen was obtained in this case, and this showed no significant change from the pre-radiation specimen.

From a consideration of the results on these 4 cases, there appears to be a definite relationship between the output of enzyme in the urine following radiation of the tissue by radium implants, and the β-glucuronidase activity of the carcinoma tissue being irradiated. However, Case C.1 does not appear to uphold this hypothesis. This case had a high initial excretion of β-glucuronidase in urine which increased after radium therapy. The corresponding tissue specimen, however, showed only a low supernatant β-glucuronidase - 290 units/g.w.w. with a specific activity of 6. The total cytoplasmic activity was much higher - 490 units/g.w.w. - due to a high particulate enzyme content. This anomality might be explained by the tissue specimen obtained at biopsy having a high proportion of fibrous material and not being truly representative of the carcinoma. This view is supported by the fact that the case was classed from clinical and histological considerations as a Severe one, but the tissue

enzyme results were more consistent with those obtained from the Mild cases where tissue specimens were likely to contain a higher proportion of stromal material than the Severe cases.

PART 2.

DISCUSSION.

β-GLUCURONIDASE IN CERVICAL TISSUE.

Normal Cervical Tissue.

The 12 specimens of non-malignant cervix showed low β-glucuronidase activity. The observed range for the supernatant β-glucuronidase - 10-235 (mean 60) units/g.w.w. - compares with values quoted by Odell et al. (1949) of 23-330 (mean 142) units/g.w.w. for aqueous preparations of cytoplasmic contents of non-malignant cervical specimens. Fishman et al. (1950) used tissue homogenates in 0.1M-acetate buffer and quoted a range of 0-533 (mean 153) units/g.w.w. for non-malignant cervix uteri. Rauramo (1959) found a range of 20-270 units/g.w.w. using tissue homogenates in water.

The homogenates used by all these authors are likely to give a higher range than that quoted for supernatant β-glucuronidase in the present work. Their figures will include particulate enzyme, and from this study, it can be estimated that this would give at least a further 30% increase over the supernatant figures (Table 15, p. 142). Furthermore, homogenisation in water or 0.1M-acetate buffer is likely to cause disruption of particulate material and perhaps nuclei by osmotic effects; the homogenates in this study were prepared in isotonic sucrose solution to maintain the integrity of particulate material and, therefore, this will lead to lower values for particle-free supernatant fractions than crude tissue homogenates.

Although these cervical specimens were classed as normal

there were in some cases, benign changes characteristic of chronic or acute cervicitis. However, the severity of these changes in no way influenced the β-glucuronidase activity. This was borne out by Odell and Burt (1950) who found low β-glucuronidase activity in specimens with cervicitis, nabothian cysts and cervical erosion, which did not differ from normal.

Cervical Cancer Tissue.

The range of β-glucuronidase activity of the cervical cancer specimens was very much higher than that of non-malignant specimens. Supernatant β-glucuronidase values ranged from 138-2300 (mean 855) units/g.w.w., which compare with results on whole tissue homogenates of 543-2790 (mean 1274) units/g.w.w. obtained by Odell and Burt (1949) and 555-5950 (mean 1647) units/g.w.w. by Fishman et al. (1950). Rauramo (1959) described a range of 140-2820 units/g.w.w. with 50% of the cases having values above 600 units/g.w.w. If the particulate results are included with the supernatant results in this work, the range becomes higher - 211-2600 units/g.w.w. (mean 1190).

THE NATURE OF INCREASED β-GLUCURONIDASE IN CANCER SPECIMENS.

The cervical carcinoma group as a whole shows a much higher β-glucuronidase/g.w.w. than the normal cervical specimens. The precise nature of the increased activity

has not, as yet, been explained. Two possibilities present themselves:- 1. an increased activity of β-glucuronidase in the malignant cells, or 2. as cervical carcinoma is mainly due to a proliferation of the epithelium of the cervix, the increase in β-glucuronidase in the tissue may be due to more cells, with approximately the same β-glucuronidase activity as normal cells, being found in the biopsy specimen of tumour, than in normal samples.

Fishman et al. (1959) reported that cancer cells contain β-glucuronidase in an amount that exceeds the activity seen in non-neoplastic cells. On the other hand, Hopman (1961) upholds suggestion 2., by stating that the marked quantitative increase in β-glucuronidase in cervical carcinoma in situ was due to a greater amount of epithelium in the malignant tissue, and that the cancer cells did not stain any more strongly than basal cells of normal epithelium.

The results from this present study indicate that both possibilities contribute to the increased β-glucuronidase activity of cervical carcinoma. The amount of soluble protein obtained in the supernatant fraction of the tissue homogenates can be taken as an indication of the cellularity of the specimen, as this protein will be derived mainly from soluble intracellular protein and not from structural proteins of fibrous connective tissue. It can be seen from Table 23 (p. 157), that the carcinoma group had a higher supernatant protein content/g.w.w. than the normal group,

indicating that the malignant specimens had more epithelial tissue in the biopsy specimens than normal and, therefore, upholding the views of Hopman (1961).

However, when the supernatant enzyme activity is expressed as specific activity, i.e. with reference to the supernatant protein content, the carcinoma group is again very much more active than the normal group (Table 18, p.147). This substantiates the view of Fishman et al. (1959) that cancer cells have a higher β-glucuronidase activity than normal epithelial cells.

THE INTRACELLULAR DISTRIBUTION OF β-GLUCURONIDASE IN CERVICAL TISSUE.

The total activity of β-glucuronidase obtained from the tissue homogenates in this work is, in reality, the cytoplasmic activity, as no account has been taken of any enzyme that may be present in nuclei. Levvy and Conchie (1964) have shown that the time of homogenisation and method employed will influence the distribution of the enzyme in the subcellular fractions. They demonstrated that a blendor device, as used here, tends to disintegrate granules, and a better means of homogenisation was the Potter-Elvehjem homogenisor. However, this is suitable only for soft tissues and could not be used with cervical specimens. Levvy and Conchie (1964) also showed that in some tissues studied, if the homogenisation time was increased from one to three minutes, there was a

reduction in the percentage activity in the nuclear fraction and, in some cases, small reductions in the mitochondrial and microsomal fractions, with corresponding increases in the supernatant β-glucuronidase activity. They suggested that the prolonged homogenisation disrupted nuclei and other cytoplasmic particles. However, the reduction in β-glucuronidase of the sedimented nuclear fraction can equally be explained by a decrease in the number of unbroken cells found in this fraction on further homogenisation.

When fractionating tissue specimens, conditions must be selected which will give adequate breakdown of cellular material, and must be a compromise between this and an attempt to keep disruption of particles to a minimum. The conditions selected for the cervical tissue were based on these considerations.

In interpreting the distribution of β-glucuronidase between the supernatant and particulate fractions of the normal and malignant tissues, two factors must be considered. First, particles can be trapped within the fibrous network formed from disrupted connective tissue of the specimen. These will be centrifuged down with the nuclei and cell debris and rendered unobtainable. It is probable that this occurred extensively in the samples of normal cervix where fibrous material formed a high proportion of the total specimen, and accounted for the poor yield of particles. In the malignant tissue, however, this factor was very much less, due to the

high cellularity of the cancer specimens. Secondly, disruption of particles, with release of their contents into the supernatant fraction, can occur during tissue fractionation due to (a) mechanical disruption of particles in the blendor, and (b) disintegration of particles as a consequence of the freezing and thawing of the specimen during storage and subsequent slicing of the tissue on the freezing microtome. This factor will be common to both normal and malignant tissue preparations.

It is difficult to assess the extent to which the disruption of particles interferes with the distribution of β-glucuronidase in the cytoplasmic fractions. It is unlikely that this would invalidate the observed distribution to a notable degree for the following reasons. In the first place, the specific activity of the supernatant in the carcinoma group is considerably higher than that of the particles (Table 16, p.144), whereas the specific activity of the normal supernatant is only one-fifth that of the corresponding particles (Table 15, p. 142). If the distribution were affected by factors common to both groups of tissues, one would not expect such a remarkable difference between the supernatant/particle specific activity ratios. In the second place, the protein content of the cancer supernatant was approximately 50% higher than that of the normals (Table 23, p. 157); the specific activities of the particulate enzyme in the two groups was not widely different. If it was assumed

that all the supernatant enzyme was derived from the particles in both tissues, it can be calculated from the mean values of specific activity and protein content of each fraction, that 3/4 of the normal particles, and 4/5 of the cancer particles would need to be ruptured to give the observed results.

While the former possibility cannot be excluded on theoretical grounds, in view of doubts about the recovery of the particles, the latter is quite unacceptable, as this would require that approximately 85 mg. of particulate protein/g. of tissue had escaped into the supernatant, the entire protein content of which is considerably lower than this (mean 43 mg./g.w.w.). From these considerations, it seems likely that the supernatant enzyme is a separate entity from the particulate enzyme, and that only small modifications of this distribution occurred as a consequence of the techniques employed.

It can, therefore, be stated that, while the distribution between supernatant and particles for the normal specimens will be far from the true intracellular distribution *in vivo* due to loss of particles in the stroma, the distribution in the malignant group will be more reliable. It is obvious from the literature, that the distribution of β-glucuronidase in the cytoplasm can vary from tissue to tissue (Conchie and Levvy, 1959; deDuve *et al.*, 1962). Values as low as 14% for the percentage of total enzyme activity to be found in the supernatant from mouse liver (Levvy and Conchie, 1964) to

70-90% in rat spleen (Roth et al., 1962), have been reported.

When the specimens of cervical carcinoma were divided into those with high β-glucuronidase (above 1000 units/g.w.w.) and those with low β-glucuronidase (below 500 units/g.w.w.), it was shown that the increase in β-glucuronidase was mainly in the soluble supernatant fraction (Table 19, p.149); the supernatant specific activities were also greatly increased (Table 21, p.153). The particles from the two groups did not show any differences in enzyme activity, and also the yield of particles in the High-Content and Low-Content Groups were not significantly different when the particulate protein was used as an index of this (Table 20, p. 151). From this, it appears that the raised β-glucuronidase in malignant cervical tissue is primarily due to soluble enzyme free in the cytoplasm and not bound to particles. The particles in the malignant specimens with different total cytoplasmic β-glucuronidase activity do not show pronounced differences in enzyme activity, and indeed, the particles from malignant cervical cells do not vary greatly from normal cervical cells in their specific activities.

These findings would seem to support those of Conchie and Levvy (1959) who stated that in S37 mouse tumour, a large percentage of β-glucuronidase was free in the cytoplasm of the tumour cells, and Levvy and Conchie (1964) reported similar results from Ehrlich and T2146 tumours.

The estimation of β-glucuronidase activity in vaginal

secretions has proved to be the most profitable clinical application of the enzyme in the diagnosis of cervical cancer. Fishman and Mitchell (1959) stated that there were a number of possible sources of β-glucuronidase in vaginal fluid, including vaginal mucosa, uterine cervix and endometrium, bacterial flora, protozoa, blood, lymph or tissue fluids, and cancer cells. Watkins and Lawson (1963) stated that the source of the enzyme in vaginal fluid was unknown, and they were doubtful if cervical carcinoma contributes directly to the enhanced activity observed in the majority of these patients. From their work, it appeared that the enzyme was partly free in solution and partly bound in an insoluble form in the vaginal fluid, although whether this is due to being confined within cells or bound to mucopolysaccharides, insoluble under the conditions used, is not known. It is uncertain whether the increases in cervical carcinoma patients are in the free or bound forms.

It is, therefore, of interest to note in the present investigation, that the increased β-glucuronidase activity of malignant cervical cells occurred in the cytoplasmic supernatant and not bound within particles. It is feasible that, with this distribution, the enzyme could more readily escape from the malignant cells and enter vaginal secretions, than if the β-glucuronidase had been enclosed within cytoplasmic particles.

When comparison was made between the particulate

β-glucuronidase of normal and carcinomatous cervical specimens, there was a far higher β-glucuronidase content in the particles from the carcinoma cells. This has already been explained partly in terms of poor particle yield from the normal specimens due to technical difficulties. However, one fact should be borne in mind when considering these results; Leubel, Saunders and Ashworth (1960), in a study of carcinoma in situ and invasive carcinoma of the cervix uteri, with the electron microscope, described the cancer cells as being richer in mitochondria than the normal epithelial cell. The differences between the normal and cancer cells seen in this present work, may, therefore, in part be due to a higher content of mitochondria from the tumour specimens.

In the particle fractions of the tumour cells, the β-glucuronidase appears to be evenly divided between the M1 and M2 fractions (Table 16, p. 144). The enzyme has been referred to as a 'lysosomal' enzyme by deDuve et al. (1955), although they found comparable activity in microsomes. Levvy and Conchie (1964) have shown that it is present in mitochondrial and microsomal fractions. During any tissue fractionation procedure, cross-contamination of the particle fractions is difficult to avoid, and lysosomes will contaminate both M1 and M2 fractions. Levvy and Conchie (1964) showed that mitochondrial and microsomal fractions from various tissues prepared by a method similar to the present one, had roughly equal β-glucuronidase activity.

It is likely that cross-contamination of the particle fractions occurred and, therefore, as the β-glucuronidase activity was found to be very similar in the M1 and M2 fractions, nothing could be deduced about the particulate distribution of the enzyme in these tissues. The fact that the M2 fraction had slightly higher activities than the M1 fraction in most specimens, can be explained by the likelihood that the former would contain more lysosomes than the latter under the conditions of centrifugation employed.

TISSUE β-GLUCURONIDASE AND SEVERITY OF THE MALIGNANT LESION.

It was hoped that the wide range of β-glucuronidase activities of the cervical carcinoma specimens might be explained in terms of degree of malignancy of the lesion or clinical assessment of the disease. Previous authors have not been able to relate β-glucuronidase activity of tumour tissue to the histological appearance (Monis et al., 1960). Rauramo (1959) stated that the spread of a malignant cervical lesion did not seem to affect the β-glucuronidase content of the tissue to any notable degree. He did, however, find a higher incidence of normal values (below 300 units/g.w.w.) in Stage 0-1 incipient cases (38%) than in Stage 2 (13%) and Stages 3 and 4 (7%). In this present work, the Mild cases had significantly lower β-glucuronidase than the more advanced Intermediate and Severe cases (Table 22, p.156). Rauramo (1959) explained the low values seen in early cases by the

fact that normal cervical tissue would be removed readily along with tumour tissue in the biopsy specimen. The results found here confirm that this is the most likely explanation. The results on the Mild cases do, in fact, fall between those of the normal cervical specimens and the more advanced malignant specimens. For example, although they have a low supernatant β-glucuronidase, the range is still somewhat higher than that for the normal group (cf. Table 15, p.142 and Table 22, p.156). The supernatant protein of the Mild cases approaches, but is still a little higher than, the supernatant protein of the normal group. The protein content of the Intermediate and Severe groups was much higher (Table 23, p.157). This would indicate the higher cellularity of the specimens from the more advanced cases, and would fit in very well with the concept of normal tissue, containing less epithelial cells, being taken along with tumour tissue in the specimens from early lesions. There is no evidence from the present data to suggest that carcinoma cells from an early lesion would have less β-glucuronidase activity than cells from a more advanced case.

EFFECT OF RADIATION UPON TISSUE β-GLUCURONIDASE.

Results obtained from the Pre- and Post-Radiation Series did not show a specific pattern of response by tissue β-glucuronidase to radium treatment.

One of the major difficulties in comparing results on

two samples taken from the same tissue such as malignant cervix after a specific treatment, is the heterogeneous nature of the lesion. Because of this, a wide variation in results on the two specimens may arise from tissue sampling. It must be accepted that in a tissue of multiple histological elements where β-glucuronidase activity is confined mainly to one cell type - epithelium - there is likely to be an appreciable difference in β-glucuronidase/g.w.w. from one sample to another depending on the amount of epithelial tissue obtained in the biopsy. It is impossible to assess to what extent this will occur without examining a carefully controlled group of samples taken from patients receiving no treatment. This was not possible in the present work and, therefore, no means was available for determining to what extent tissue sampling had caused the very variable alterations in β-glucuronidase activity following radiation.

These variable results agree with those reported by Rauramo (1959) on 10 pre- and post-radiation specimens taken from patients receiving a single radium implant. The author found that β-glucuronidase/g.w.w. from whole tissue homogenates fell in eight cases and increased in two. Hartiala, Näntö, Rinne and Savola (1960) found that the β-glucuronidase activity of rat gastric mucosa and liver increased up to 50% three days after a 400 rad radiation dose and then returned to normal values. Sottocasa et al. (1965) also produced increases in β-glucuronidase specific activities of heart tissue slice

homogenates after large doses of irradiation; this was shown to be a true increase in enzyme and not a consequence of loss of protein. It could well be, therefore, that in cervical tissue two simultaneous processes occur - stimulation of β-glucuronidase within the cell to give increased activity in the tissue, and death of neoplastic cells tending to reduce the β-glucuronidase activity. Variable results would then arise depending on the relative extent of the two processes. From cytological studies, it is known that there is a variation in response of different lesions to radiation (Graham and Graham, 1955).

It has been suggested that radiation of tissues results in damage to lysosomes with concomitant release of their enzymes (Errera, 1959). However, it has since been demonstrated that very large radiation doses are required to cause leakage of hydrolytic enzymes from lysosomal preparations; Bacq and Alexander (1960) stated that a dose of 10,000 rads was required on *in vitro* preparations, and Sottocasa *et al.* (1965) found doses of over 10,000 rads were needed to release enzymes from lysosomes *in vivo*. There was no evidence from the particulate results on the Pre- and Post-Radiation specimens in this work to suggest that there had been any damage to lysosomes or other particles.

EXCRETION OF β-GLUCURONIDASE IN URINE OF PATIENTS RECEIVING RADIOTHERAPY.

There are no similar studies on the urinary excretion of β-glucuronidase following radiation with which to compare the present work. Although β-glucuronidase has been found to be elevated in the urine of patients with various malignant lesions (Boyland et al., 1957), there is little evidence that the malignant lesion is the major contributor to the increased β-glucuronidase in the urine. Kerr et al. (1963) showed that bladder carcinoma was a direct source of β-glucuronidase in the urine, as surgical removal of the tumour resulted in fall of β-glucuronidase excretion to normal values. This is not unexpected due to the location of the malignant lesion. It is of interest to know if other lesions outwith the urinary tract are the primary source of increased β-glucuronidase found in the urine of some of these patients. An alternative possibility is that these increases arise from a systemic reaction of the patient to the carcinoma, as the enzyme can show increased activity in the urine in some systemic body reactions such as pyrexia (Boyland and Williams, 1956).

The results obtained in the present study on patients with cervical cancer receiving radiation indicate that the lesion in the cervix is the source of increased β-glucuronidase in the urine of some of the patients. Insertion of radium into the lesion was followed by increases in the excretion of β-glucuronidase in 7 of the 9 patients (Fig.17, p. 164), which lasted for approximately 2-3 weeks and fell back to, and often below, the pre-radiation values. Furthermore, the

patients with an abnormal excretion of β-glucuronidase before radiotherapy produced the most pronounced increases.

The rises in β-glucuronidase excretion in the urine were accompanied by increases in serum β-glucuronidase activity. This suggests that the enzyme is liberated from the cancer tissue, enters the circulation, and is then cleared by the kidneys. It has been suggested that a proportion of the β-glucuronidase found in normal urine is derived from the urethra and bladder mucosa (Kerr et al.,1963), but the results found in the cervical cases in this work cannot be explained other than as a renal clearance of the enzyme. In two cases, the reciprocal relationship between serum β-glucuronidase and excretion of the enzyme in the urine (Fig.21, p. 172) can also be explained as an inadequate renal clearance of the enzyme causing an increase in circulating enzyme in the blood. In this respect, it is of interest to note that in some independent studies on one of the cases, the patient's renal function,as assessed by the Van Slyke urea clearance, fell from a pre-radiation value of 59% of normal to 40% after the full course of radiotherapy.

The group of patients with bladder carcinoma were studied to investigate the effect of radiation of a tumour known to be rich in β-glucuronidase and having direct access to the urinary tract. The patients were not, however, receiving radium implants. Suprisingly, the results within the group were not consistent and a regular pattern of β-glucuronidase

excretion, as seen in the cervical carcinoma group, was not shown. Five of the 8 patients had very high β-glucuronidase excretion before radiotherapy, but this was not increased to any noticeable degree except in one case. The other anticipated effect may have been a decrease of enzyme excretion towards the end of the therapy due to death of the malignant cells by radiation, but a decrease in enzyme output was not detected. However, decreases may have occurred at a much later date, as the effects of radiation upon tissues are not always immediate (Errera, 1959).

The third group of patients were studied with a view to observing any changes which might occur in urinary β-glucuronidase excretion in patients with malignant lesions receiving radiation distant from the urinary tract. Two of the patients, both with carcinoma of the bronchus, had abnormally high β-glucuronidase excretion before therapy, and both produced a pronounced increase in urine activity following radiation by supervoltage therapy (Fig.19, p. 168). Seligman et al.(1954) have described bronchial epithelia as having a high β-glucuronidase activity and Fishman et al. (1947) have indicated that lung carcinoma has high β-glucuronidase activity. The other patient who showed increases in β-glucuronidase excretion had an oesophagal carcinoma. Monis et al. (1960) showed that epidermoid carcinomas of the oesophagus were very rich in β-glucuronidase activity.

These results on this group, therefore, support the idea

that radiation of carcinomas rich in β-glucuronidase activity can cause an increased excretion of the enzyme in the urine of these patients.

It is unfortunate that tissue specimens could not be obtained from the thoracic and bladder carcinomas, particularly as the results from the cervical group indicate a connection between the β-glucuronidase activity of the malignant lesion and the excretion of urinary β-glucuronidase following irradiation of the tumour. Two patients, with a high pre-radiation β-glucuronidase excretion and showing a large increase in enzyme excretion following radium therapy, had high β-glucuronidase activity in their tissue biopsy specimens, one case with a moderate tissue activity showed urine increases slightly less pronounced, while one of the cases which produced a normal pre-radiation excretion and no radiation response, had low tissue β-glucuronidase activity. Thus, 4 of the 5 patients in which such an assessment was possible support the hypothesis of the cancer tissue being the source of the abnormal amounts of β-glucuronidase in the urine.

Serum β-Glucuronidase.

Takiguchi (1963) studied β-glucuronidase in serial blood specimens taken from patients with carcinoma of the cervix receiving radiotherapy. The author found variable patterns of response during radiation treatment, similar to those shown in Fig.20 (p.171), which he related to the clinical and

radiation response of the patient. It was not possible, however, in this work to correlate the serum enzyme pattern with the patient's progress.

CONCLUSIONS.

The results of this study have established that the malignant cells of cervical cancer tissue have higher β-glucuronidase activity than normal cervical epithelial cells, and that this increase occurs mainly in the free soluble fraction of the cytoplasm and not in the particles. The nature of this increase could well account for increased amounts of this enzyme which are found in vaginal secretions in the majority of these patients.

The estimation of β-glucuronidase activity of cervical biopsy specimens would be of no diagnostic advantage over histology, however, as there was such a wide scatter in results in the carcinoma series which could not be related, except in early lesions, with the severity of the disease.

The effect of radiation upon the malignant tissues of the cervix clearly seems to mobilise β-glucuronidase from the cells into the patient's circulation from whence it is eventually excreted in the urine. This seems to be caused mainly by implantation of radium into the tissue. It is possible that this also occurred in two patients with cancer of the bronchus and one patient with carcinoma of the oesophagus. However, radiation of bladder carcinoma, a

lesion usually associated with high β-glucuronidase activity and in close contact with urine, did not result in pronounced increases in enzyme content of the urine, even though there was a high pre-radiation excretion of the enzyme. This anomalous finding cannot be readily explained, but specific changes occurring in the bladder could not be assessed. It has been reported that inhibitors of β-glucuronidase are excreted in the urine of most subjects, including patients with bladder carcinoma (Lewis and Plaice, 1960). The source and nature of these inhibitors is not known, but it is within the bounds of possibility that radiation of the bladder mucosa could cause release of inhibitors of β-glucuronidase along with the enzyme. Future work could be readily undertaken to test this.

Changes in tissue β-glucuronidase in malignant cervical specimens following radium treatment did not produce any fruitful information. Results were very variable, and it may well be that this arose from taking the post-radiation specimen too soon, before specific changes in enzyme activity could be detected.

Odell et al. (1950) stated that β-glucuronidase activity of vaginal fluid tended to decrease following radiation of the cervical lesion. Hatzimichael (1962) claimed that malignant tumours of the cervix, which responded to radiation, were associated with decreased activity of the enzyme in cervical mucus. Unfortunately, in this present work, there were no facilities for obtaining vaginal secretions to study

along with tissue biopsy material. Similarly, there was no means of assessing the radiation response of the lesions and, therefore, the tissue, urine and serum β-glucuronidase results could not be considered from a prognostic viewpoint. Takiguchi (1963) claimed that serial serum β-glucuronidase and lactic dehydrogenase determinations might yield an index of radiation response in patients with cervical cancer. It is hoped that, in the future, a controlled study of comparative β-glucuronidase activities of vaginal secretions, tissue biopsies and excretion of the enzyme in the urine of patients with cervical cancer, before and after radiation, may be undertaken, with a view to assessing the response of the patient to treatment and ultimately arriving at a prognosis. There is every reason to believe that useful data might be obtained from further studies in this direction.

SUMMARY.

Separate studies on two enzymes have been included in this thesis.

In Part 1, the activity of isocitrate dehydrogenase was measured in the serum of patients with various diseases of the liver or biliary tract. The usefulness of this enzyme as a diagnostic aid was assessed by comparing the results with established biochemical tests of liver function, namely, serum bilirubin, protein turbidity and flocculation tests, serum alkaline phosphatase, and especially serum glutamic-oxalacetic transaminase (S-GOT) and serum glutamic-pyruvic transaminase (S-GPT).

Cases of hepato-biliary disease were divided into five groups:- (a) acute liver damage due to infectious hepatitis, (b) drug-induced jaundice, (c) chronic liver disease - portal and biliary cirrhosis, (d) cancer invasion of the liver, and (e) obstructive jaundice due to malignant and benign lesions.

(a) With acute damage of liver cells, very high values for serum isocitrate dehydrogenase (S-ICD) were found in every case. These high values coincided with very high transaminase values, and the three enzymes responded in a similar way. They were very sensitive in showing the onset and progression of infectious hepatitis.

(b) Two cases of acute liver cell damage caused by the drug, p-aminosalicylic acid, gave very high S-ICD values. Further investigations on one of these cases showed that S-ICD was extremely sensitive in detecting subclinical liver upset, and was probably slightly superior to the transaminases in this respect. The enzyme could be usefully employed as a screening test in patients receiving drugs which may cause toxic damage to liver cells. Chlorpromazine and promazine jaundice produced only small variable increases in S-ICD.

(c) In portal cirrhosis, 28% of the patients had normal S-ICD values. Increases in activity, where seen, were only moderate and could not be related to the clinical state of the patient. The S-ICD results were significantly correlated with both serum transaminases.

Patients with biliary cirrhosis produced generally higher S-ICD values than those from patients with portal cirrhosis; these values were closely correlated to S-GOT and S-GPT values.

(d) Fifteen cases of invasive cancer of the liver all gave raised S-ICD values; large increases were associated with widespread secondary deposits in the liver.

(e) In obstructive jaundice of different types, variable

S-ICD values were found; increased values were found in 83% of cases and were not related to the severity or duration of the obstruction.

In cases with malignant obstructive jaundice, the presence of secondary liver involvement caused significantly higher S-ICD values than those found with non-metastatic patients.

In cases with benign obstructive jaundice, the presence of an acute inflammatory process in the biliary tract produced high S-ICD values which were not necessarily associated with damage to liver cells.

There was no significant difference in S-ICD results from non-metastatic malignant cases and benign obstructive jaundice cases uncomplicated by acute inflammation.

S-ICD was not closely correlated with the transaminases in cases of obstructive jaundice, and neither the S-GOT nor S-GPT reflected the high S-ICD values seen with liver metastases in malignant cases and inflammation of the biliary tract in benign cases.

It was concluded that, while the S-ICD did not offer great diagnostic advantages over the serum transaminases, it could be a useful complementary or alternative test to them. It was an enzyme, easily estimated, very sensitive for detecting acute damage to liver cells, and could provide useful information in some cases of biliary obstruction.

Part 2 consisted of investigations on β-glucuronidase activity of specimens of cervical tissue, urine and serum taken from patients with cervical carcinoma.

Tissue specimens of carcinoma of the cervix uteri were fractionated by homogenisation and separation of cytoplasmic contents into cell sap (supernatant) and two particulate fractions designated M1 - consisting mainly of mitochondria, and M2 - consisting mainly of microsomes. β-Glucuronidase was measured in each fraction, and the results compared with those from similar fractions prepared from non-malignant cervical specimens.

β-Glucuronidase activity of the malignant cervical specimens was, in general, much higher than that of normal or non-malignant cervix. There was a wide range of activities in the malignant series, but values could not be related to the degree of malignancy, except in specimens from an early lesion which always gave low β-glucuronidase activities approaching those found in non-malignant specimens.

From the intracellular distribution of β-glucuronidase in the malignant specimens, it was found that increases of β-glucuronidase were mainly in free supernatant enzyme and not in particulate enzyme, although the enzyme has previously been described as located mainly within cytoplasmic particles.

This work appears to be the first study on the intracellular distribution of β-glucuronidase in human tumour tissue; the results are supported by similar findings in certain mouse tumours.

In twelve of the patients with cervical carcinoma, further tissue specimens were obtained after treatment of the lesion by an implant of radium for approximately two days. β-Glucuronidase activity of these specimens was compared with the pre-radiation value. No specific change in β-glucuronidase activity could be detected following radium treatment. In three cases there were large increases, and in four there were large decreases in β-glucuronidase activity. In the other cases, smaller variable changes occurred which may have arisen from tissue sampling.

In nine patients, the urinary excretion of β-glucuronidase was studied throughout a full course of radiotherapy. Results showed that there was a significant increase in the 24-hour excretion of the enzyme in seven patients following insertion of radium. Patients with abnormally high excretion of the enzyme before radiotherapy produced the most pronounced increases.

Rises in urinary enzyme output following radiation were accompanied by increases in serum β-glucuronidase. Results indicated that there was probably a renal clearance

of the enzyme.

It was seen on comparing the urinary excretion of β-glucuronidase and the activity of the enzyme in the tissue specimen from the same patient, that radiation of lesions with high β-glucuronidase activity produced large responses in urine output of the enzyme. Results suggested that increased β-glucuronidase in the urine of these patients was derived directly from the malignant lesion, and did not result from a systemic reaction of the patient.

β-Glucuronidase excretion was also studied in patients with bladder carcinoma receiving radiotherapy. These were studied as a group with a lesion usually described as having high β-glucuronidase content and closely associated with the urinary tract. Suprisingly, there was not a uniform response in urine β-glucuronidase following radiation of the lesion.

Another group of patients receiving radiation of malignant lesions well away from the urogenital tract were also studied. Some patients showed increased β-glucuronidase excretion; the most pronounced responses were found in two patients who had high pre-radiation enzyme excretion. This group offered further evidence that radiation of lesions high in β-glucuronidase could lead to increased excretion of the enzyme in the urine.

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