ENZYMES OF CARBOHYDRATE METABOLISM IN NORMAL AND PATHOLOGICAL HUMAN TISSUES.

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by

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LIST OF ABBREVIATIONS.

ATP : adenosine triphosphate

E : extinction

LDH : lactate dehydrogenase (E.C.1.1.1.27.)

IDH : isocitrate dehydrogenase (E.C.1.1.1.42.)

PGDH : phosphogluconate dehydrogenase (E.C.1.1.1.44.)

SDH : succinate dehydrogenase (E.C.1.3.99.1.)

RNA : ribonucleic acid

DNA : deoxyribonucleic acid

tris : 2-amino-2(hydroxymethyl)-1:3-propanediol

NAD, NAD^+, NADH. : nicotinamide adenine dinucleotide, oxidised, reduced.

NADP, $NADP^+$, NADPH. : nicotinamide adenine dinucleotide phosphate, oxidised, reduced.

CONTENTS.

INTRODUCTION

In animal tissues the principal raw material for energy production is glucose, and it can be catabolised by three main pathways, the Embden Meyerhof Pathway of anaerobic glycolysis, the Citric Acid Cycle and the Hexose Monophosphate Pathway. It has been established that, in many tumours of human and animal origin, these pathways are frequently deranged, and, in particular, anaerobic glycolysis is increased. This thesis represents an exploration of this phenomenen in a spectrum of human tumours, and also comparisons with normal human tissues and non-malignant lesions of these tissues.

There is scanty information in the literature concerning the relative importance of the three pathways in human tissues, although there is rather more about animal tissues. Most of the studies which have been made compare the metabolism of normal tissue with that of tumour tissue, without making any attempt to study other pathological conditions, which may in many cases precede the appearance of a tumour.

This study has estimated one enzyme from each of the three pathways mentioned above. Each of the enzymes is linked in its function to a pyridine nucleotide coenzyme and can be conveniently measured under similar conditions. The effect of radiation on human cancer has also been studied by measurement of the three enzymes. It was hoped that this might yield results leading to knowledge of the alteration of cell metabolism induced by radiation.

From the measurements of the three enzymes in various pathological conditions of three types of human tissue, it was hoped that information concerning the relative operation of each pathway in the different tissues could be gained.

SURVEY OF THE LITERATURE ON CARBOHYDRATE METABOLISM IN TUMOUR TISSUE.

Since Warburg (1930) found that anaerobic glycolysis was raised in tumour cells, attention has been focussed on the carbohydrate metabolism and energy production of tumour cells compared with normal cells.

These two processes have been studied in various ways, by experiments on intact animals with induced neoplasms using radioactive tracer techniques, by similar experiments on tissue homogenates, tissue slices, or on tissue culture preparations; by histochemical, spectrophotometrical and manometrical measurement of key enzymes in the metabolic pathways.

Each method has its advantages and disadvantages. Results obtained using radioactive metabolites are rendered less conclusive by the variety of conversions to which the atoms of the compound are subjected. Conclusions drawn from experiments on animals with induced neoplasms cannot be applied to neoplasms of human tissues without some reservation. Tissue culture preparations would seem to give conditions approximating most closely to those in the intact animal, but it is not possible to say that the cells being grown in vitro behave as did the original living cells from which the preparation was grown. Enzyme preparations obtained from human tissues are also not ideal, as the various treatments applied to the tissue, in order to extract the enzyme, alter drastically the conditions under which the metabolic pathways function.

Histochemistry on frozen tissue sections probably provides the nearest approach to in vivo conditions, but as yet the techniques available do not give the precision in enzyme estimations that is possible with

estimations in tissue extracts.

The three enzymes studied in the present work, Lactate dehydrogenase (E.C.1.1.1.27.), Isocitrate dehydrogenase(E.C.1.1.1.42.), and Phosphogluconate dehydrogenase(E.C.1.1.1.44.) occur in three different pathways of carbohydrate metabolism and energy production.

The final enzyme of anaerobic glycolysis is lactate dehydrogenase, which catalyses the oxidation of pyruvic acid to lactic acid in the presence of NADH. This conversion from 1 mole of glucose to 2 moles of lactic acid supplies energy in the form of 2 moles of ATP. This represents a small energy yield, but has the advantage that the raw material for the reaction, glucose, is supplied to the tissues by the bloodstream, and the waste product, lactic acid, is removed by the bloodstream for further metabolism by the liver. Therefore, this reaction supplies a ready source of energy in the tissues. From these facts it would seem likely that a tissue with a high energy requirement would have increased lactate dehydrogenase activity. There is ample evidence in the literature for lactic acid accumulation in tumours. (de Roetth, 1957, and McBeth and Bekesi, 1962)

Isocitrate dehydrogenase is one of the enzymes of aerobic glycolysis via the citric acid cycle which can be measured spectrophotometrically. It catalyses the oxidation of isocitric acid to oxalosuccinic acid in the presence of $NADP^{+}$. The catabolism of 1 mole of pyruvic acid to carbon dioxide and water supplies 15 moles of ATP.

One of the enzymes of the Hexose Monophosphate Pathway is phosphogluconate dehydrogenase, which catalyses the oxidative

decarboxylation of 6-phosphogluconic acid to ribulose-5-phosphate in the presence of $NADP^+$. 1 mole of glucose can be converted stoichiometrically to 6 moles of carbon dioxide by this pathway, or it can be converted into triose phosphate and metabolised via the citric acid cycle under aerobic conditions. The Hexose Monophosphate Pathway does not operate to any extent in the liver but accounts for 10% of the glycolytic activity of muscle.

Manso, Sugiura and Wroblewski (1958) reported that increased serum lactate dehydrogenase activity followed the introduction of transplantable neoplasms in mice and rats, and postulated that it came from the tumours. Burgess and Sylveen (1962,1963) found increased lactate dehydrogenase activity in central and peripheral fluid obtained from solid mammary tumours and Ehrlich-Landschutz ascites tumours in mice, compared with normal peritoneal fluid, accompanied with increased serum lactate dehydrogenase activity. They concluded that a large part of the elevation arose from leakage of lactate dehydrogenase from the tumours. di Simone, Lorenzutti and Sapio (1962) reported increased lactate dehydrogenase activity in the cytoplasmic fraction of rat Shay hepatoma. Decreased lactate dehydrogenase activities in the supernatant fractions of rat Novikoff hepatoma were found by Boxer and Shonk (1960), which finding was confirmed by Weber and Morris (1961, 1963) who also stated that lactate dehydrogenase activity showed no correlation with the growth rate of experimentally induced tumours in rat liver.

Rees and Huggins(1960) found greatly increased lactate dehydrogenase activity in three types of experimental mammary cancers in mice. Weber, Banerjee, Levine and Ashmore (1961) found a significant increase in lactate dehydrogenase activity in chicken chorioallantoic membrane after the appearance of tumours caused by Rous Sarcoma virus.

Histochemical results have confirmed the general picture of increased lactate dehydrogenase content. Mori, Miyaji, Murata and Nagasuna (1962) found increased lactate dehydrogenase content in experimental carcinomas of hamster check pouch and Thiery and Willighagen (1964) showed an increase in lactate dehydrogenase content in experimental cervical cancers in the mouse.

In contrast with the large amount of information on animal tissues there is very little information on lactate dehydrogenase activity in human tissues. McBeth and Bekesi (1962) using respiratory techniques found increased anaerobic glycolysis in various human tumours when compared with normal tissue from the same patient.

Ames, Albaum, and Antopol (1964) found a significant increase in lactate dehydrogenase activity in adenocarcinoma of the colon, compared with uninvolved tissue of origin from the same patient. Shonk and Boxer (1964) and Shonk, Koven, Majima and Boxer(1964) have established patterns of glycolytic enzymes in the supernatant fractions of various normal human tissues and aim to establish patterns in pathological conditions. Goldman, Kaplan and Hall (1964) have reported increased lactate dehydrogenase activity

in the supernatant fractions of human neoplasms compared with uninvolved tissue of origin.

Isocitrate dehydrogenase has seldom been studied in the supernatant fractions of cells although some work has been done on mitochondrial preparations. Shepherd (1956, 1961) reported that isocitrate dehydrogenase occurs in the mitochondrial fraction of rat, mouse, rabbit and human liver.

More recently Tremblay and Pearse (1960), working on sections of human thyroid glands, reported increased isocitrate dehydrogenase activity, especially in the mitochondria. This occurred in thyrotoxic, adenomatous and Hashimoto glands where cells rich in mitochondria were found. Thiery and Willigtagen (1964) found increased isocitrate dehydrogenase activity in murine experimental cervical cancers.

Phosphogluconate dehydrogenase activity has also been studied to some extent in experimental animals with induced neoplasms. Abraham, Hirsch and Chaikoff (1951) found that the Hexose Monophosphate Pathway was increased in lactating mammary gland of the rat, which result was confirmed by Glock and McLean (1954) who found increased phosphogluconate dehydrogenase activity in lactating rat mammary gland and in lymphatic tissue in general. Weber, Banerjee, Levine and Ashmore (1961) showed that there was a non-significant increase in phosphogluconate dehydrogenase activity in the supernatant fraction of tumours of chicken chorioallantoic membrane infected with Rous Sarcoma virus. Weber and Morris (1963) found that phosphogluconate dehydrogenase activity showed no correlation with growth rate of experimentally induced hepatomas in the rat.

Chayen et al. (1962) found histochemically that phosphogluconate dehydrogenase was raised in proliferating cells of any type; one example being liver cells regenerating after starvation, confirming the findings of Fitch and Chaikoff (1960), other examples being malignancy induced by dimethyl-aminoazo-benzene, and regeneration in the livers where malignancy did not occur. Rees and Huggins (1960) found that in normal and abnormal mammary tissue in mice, lactate dehydrogenase activity per g. wet weight was greater than isocitrate dehydrogenase which was in turn greater than phosphogluconate dehydrogenase activity, except for lactating tissue in which the activity of phosphogluconate dehydrogenase activity exceeded that of isocitrate dehydrogenase. Scott, Morris, Reiskin and Pakoskoy (1962) reported that phosphogluconate dehydrogenase seemed to be linked more closely to hyperplasia than neoplasia in the artificial induction of tumours in hamster cheek pouch. All these findings have stimulated interest in the overall glucose metabolism of the living organism. Evidence has been collected by Boyd, Clapp and Finnegan (1962) to show that in human cancer patients, the handling of glucose is abnormal; this impairment may be linked to the ability of tumour tissue to take up essential metabolites from surrounding normal tissue, which theory has been put forward by Shrivastava and Quastel (1962).

The tissues studied were human thyroid gland, mammary gland and cervix uteri. The enzymes were estimated with respect to wet weight of the tissue studied and protein content of the fraction in which

they were estimated. The enzymes were measured in the particulate fractions of the tissues from which sufficient amounts were obtained.

A smaller series of estimations was also carried out on thyroid tissue in which DNA content was also measured, to give some idea of the relative cellularity of the types of thyroid tissue studied.

Thyroid tissue and breast tissue gave a variety of lesions capable of investigation and offered an opportunity to compare benign and malignant lesions of different types. Cervical samples were easily obtainable and offered a chance to study the effects of radiation on the protein and enzyme content of a human tumour.

It was hoped that this approach might lead to information regarding the metabolic behaviour of human neoplasms, and other pathological conditions, and might reveal abnormalities of enzyme content which could be exploited clinically.

MATERIAL AND METHODS

COLLECTION OF SPECIMENS.

Tissues were collected from the operating theatre directly into ice-cold distilled water. They were washed free of blood in distilled water and thoroughly dried on absorbent tissues. Mammary and cervical samples were also weighed. If the material had to be stored before use, it was frozen and stored at -20°.

NUMBER AND TYPE OF SPECIMENS.

Samples of thyroid tissue were obtained from patients undergoing operations for removal of various lesions. These were divided into two series; a Main series in which the enzymes were estimated with reference to protein content and wet weight of tissue used, and a Nucleic Acid series in which the enzymes were estimated with reference to DNA Phosphorus as well as protein content and wet weight of tissue. There were eleven samples of normal tissue in the Main series, of which seven were removed along with an adenoma, three along with a carcinoma, and one along with a Hurthle cell adenoma. The seven samples of normal tissue used in the Nucleic Acid series were removed post-mortem from patients with no thyroid abnormality. There were ten samples of adenoma in the Main series and five in the Nucleic Acid series, all removed surgically. Since patients suffering from thyrotoxicosis underwent varying treatments prior to surgery, a larger series of twenty samples was investigated. There were five thyrotoxic samples in the Nucleic Acid series.

The fifteen samples of normal cervix were obtained during

operative procedures for the repair of a prolapsed uterus. A portion of the amputated cervix was cut parallel to the inferior surface in such a way as to include as much of the epithelial layer and as little of the underlying fibrous tissue as possible. Although on naked eye examination the tissue appeared normal in all cases, in several instances microscopic examination revealed varying degrees of epithelialisation and inflammatory change. Twenty-three carcinoma biopsy specimens were obtained during the first insertion of a radium implant. In thirteen of these cases a second biopsy specimen was obtained during the second insertion of radium. A second biopsy was not available either where the tumour was extensively invasive, for in these cases the patients received only one insertion, or where the tumour was too small to permit a second biopsy. Five samples of normal breast were obtained from patients being operated on for the removal of a cyst or a fibroadenoma. In the five cases of chronic mastitis studied a representative portion of the excised breast was processed. Five samples of fibroadenoma were also studied, and five specimens of breast carcinoma of various types.

In all thyroid tissue series other than normal and carcinoma no sample weighing less than 3g. was used. The smallest samples in the normal and carcinoma series weighed 0.39g. and 0.55g. respectively.

In the normal cervix group the smallest sample weighed 0.35g., in the pre-radiation carcinoma series 0.045g., and in the post-radiation carcinoma series 0.015g.

Only in the normal breast series was a tissue sample weighing less than 1.8 g. used. The smallest sample of the normal series weighed 0.58 g.

PREPARATION OF TISSUES.

The tissues were prepared in such a way as to yield three fractions:-

1) Fraction M1 - sedimenting between 500g and 5,000g, for 20 min.
2) Fraction M2 - sedimenting between 5,000g and 35,000g, for 1 hr.
3) Supernatant - 35,000g, for 1 hr.

Thyroid tissue was minced and homogenised in five volumes ice-cold 0.25M sucrose solution in an M.S.E. Blendor for 3 min. at the maximum speed possible.

Cervical and mammary tissues were sliced to a thickness of 10 - 40 microns on a freezing microtome with carbon dioxide as freezing agent, and homogenised in a suitable volume of ice-cold 0.25M sucrose.

To remove the cell debris and the nuclei, the homogenate was spun at 500g for 10 min. in an M.S.E. Minor centrifuge at 4^{o}. In the case of breast tissue, this step also separated out the fat from the tissue, since it floated on the top and could be lifted off in the form of an intact pellet. The unsedimented layer was transferred as quantitatively as possible, avoiding nuclear contamination, and then spun at 5,000g for 20 min. in an M.S.E. Superspeed "17" centrifuge at 4^{o}, to bring down fraction M1 which is predominantly mitochondria. The unsedimented layer was then spun at 35,000g for 60 min. in an M.S.E. Superspeed "17" centrifuge at 4^{o}, to bring down fraction M2 which is predominantly microsomes. The sediments of M1 and M2 were washed by resuspending

in a large volume of 0.25M sucrose, by sucking up and down in a Pasteur pipette. They were then spun again to recover the particle fractions. These were suspended in a known volume of ice-cold distilled water (about 3 ml.)calculated according to yield, and ruptured in an M.S.E. Ultrasonic Disintegrator, for 90 sec. at 4°. In the case of Ml a coagulum of ruptured mitochondrial membranes which formed during the process was discarded and the two preparations were reddy for assay.

For the purposes of calculation, the yield of particles is taken as 100%, and the volume of the supernatant fraction is taken to be the volume of diluent sucrose. The three fractions were stored in ice overnight and the enzymes assayed the following day and protein assayed the day after.

TISSUE NUCLEIC ACID ESTIMATION.

Reagents.

0.15M Potassium Chloride

0.6N Perchloric Acid

0.2N Perchloric Acid

1% (v/v) Potassium Acetate in Ethanol(absolute)

Ethanol(absolute)

Chloroform A.R.

Diethyl Ether (Anaesthetic grade)

0.6N Potassium Hydroxide

The tissues were collected, chilled washed and weighed, as before. They were minced and homogenised in 10 volumes potassium chloride for

five minutes at the maximum speed possible. A sample of the homogenate was spun at 35,000g as before, to provide a supernatant fraction for enzyme assay.

The remainder of the homogenate was treated according to the methods of Fleck and Munro (1962), Hallinan, Fleck and Munro (1963), and Hutchison and Munro (1961). All procedures were carried out at 4^{o} unless otherwise stated.

1) Removal of Acid Soluble Substances.

4 ml. of the homogenate were pipetted into each of six centrifuge tubes containing 2 ml. 0.6N perchloric acid. The six tubes were stoppered and mixed and allowed to stand in ice for 10 min. after which time they were centrifuged for 10 min. and the supernatant layers were discarded. The precipitates were washed twice with 0.2N perchloric acid and drained by inverting over absorbent tissues.

1a) Lipid Extraction.

The precipitates of tubes 3 and 4 were extracted with,

1) 5 ml. 1% potassium acetate in ethanol
2) 5 ml. 3:1 ethanol-chloroform mixture
3) 5 ml. 3:1 ethanol-ether mixture
4) 5 ml. ether

} extracted at room temperature.

and the extracts were made up to 20 ml. with ether, for lipid phosphorus estimation.

2) Partial Alkaline Hydrolysis: to render RNA acid soluble while DNA remains acid precipitable.

To the six precipitates, 2 ml. distilled water and 2 ml. 0.6N

potassium hydroxide were added. After solubilisation the tubes were placed in a water bath at 37°. At the end of one hour, tubes 1 and 2 were removed for the estimation of RNA and Protein, and tubes 3 and 4 were removed for the estimation of DNA Phosphorus. At the end of eighteen hours, tubes 5 and 6 were removed for the estimation of DNA and Protein.

3) Separation of RNA.

a) To tubes 1 and 2 which were cooled in ice, 5 ml. 0.6N perchloric acid were added. 10 min. later they were centrifuged for 10 min. The precipitate was washed twice with 5 ml. 0.2N perchloric acid. The supernatant and washings were transferred to a 50 ml. measuring cylinder, and made up to the mark with 2 ml. 0.6N perchloric acid and distilled water. These solutions contained the RNA fraction in 0.1N perchloric acid.

b) Tubes 5 and 6 were treated in the same way as tubes 1 and 2, but the supernatants were discarded.

4) Solubilisation of DNA.

To the precipitates in tubes 3, 4, 5 and 6, 2.5 ml. distilled water and 2.5 ml. 0.6N potassium hydroxide were added, and the tubes were placed in a water bath at 37° for five hours. The solutions were transferred to 50 ml. measuring cylinders.

a) The lipid extracted samples, tubes 3 and 4, were made up to 20 ml. with distilled water prior to phosphorus estimation. These cylinders contained the lipid free DNA fraction.

b) The solutions 5 and 6, were made up to 50 ml. with 6 ml. 0.6N potassium hydroxide and distilled water. These solutions

F L O W D I A G R A M.

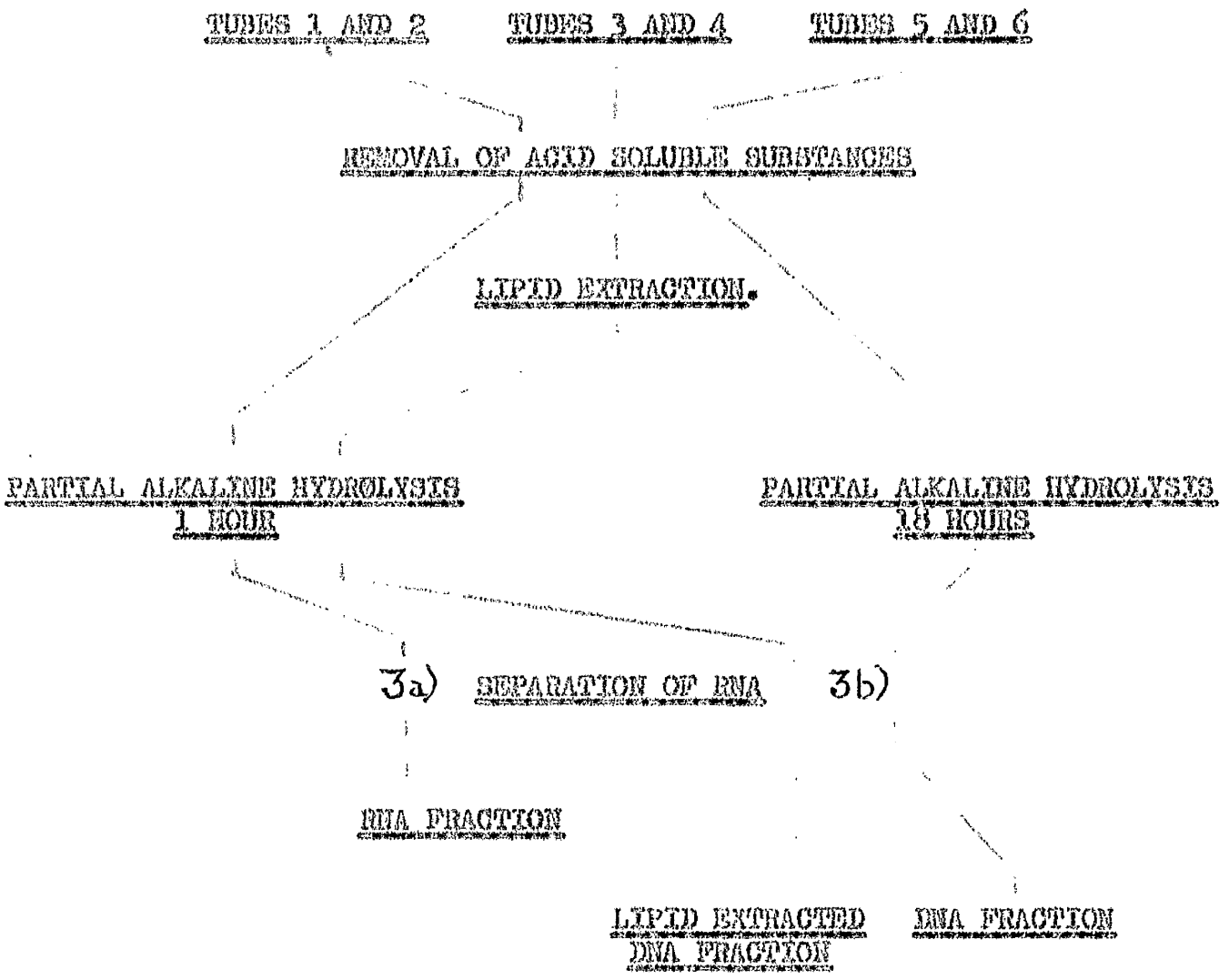

Fig. (1)

contained the DNA fraction in 0.1N potassium hydroxide.

These processes are explained more clearly in a flow diagram (Fig. 1).

PHOSPHORUS ESTIMATION.

Principle:-

The method used is a modification of that published by Allen (1940). The phosphorus containing substances were digested with acid to liberate the phosphorus in inorganic form. This was converted to a phosphomolybdic complex which was reduced to a blue pigment, the reducing agent used being different from the original. The blue colour was then estimated spectrophotometrically.

Reagents:-

10N Sulphuric Acid

8.3% (w/v) Ammonium Molybdate, $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$.

1% Amidol i.e. 1% (w/v) 2:4-Diamino-phenol Hydrochloride dissolved in 20% (w/v) Sodium Metabisulphite in distilled water; the mixture being filtered before use. This solution was stable in the dark at 4° for about 10 days.

Hydrogen Peroxide, 100 volumes.

Standard:-

The standard used was anhydrous Potassium dihydrogen phosphate.

Method:-

5 ml. of the two fractions, Lipid Phosphorus and DNA Phosphorus were pipetted into microkjeldahl flasks, each estimation being carried out in duplicate. The fifth flask contained distilled water for a reagent blank. To each flask were added one glass bead and a few

carborundum stones (nitric acid washed). 0.6 ml. of 10N sulphuric acid were added to each flask and the mixtures were digested over a low flame until thick white fumes appeared. The flasks were cooled and to each was added enough hydrogen peroxide to bleach the residues from digestion. The mixtures were then digested until the second cloud of white fumes appeared. The flasks were cooled and to each were added 10.83 ml. distilled water, 0.5 ml. ammonium molybdate, and 1 ml. amidol solution. The solutions were read at 640 mμ, after standing 10 min. at room temperature.

RNA ESTIMATION.

The extinction of the filtered RNA solution was read at 260 mμ, where an extinction of 1.00 is equivalent to 35.18 μg. whole rat liver RNA per ml.

LACTATE DEHYDROGENASE ESTIMATION.

Principle:-

Lactate dehydrogenase was estimated according to the method of Neilands (1957), whereby the change in extinction at 340 mμ, due to the conversion of NAD^+ to NADH is measured.

Reagents:-

0.02M NAD^+ (freshly prepared) C.F.Boehringer & Soehne, GMBH, Mannheim, Germany.

0.48M Sodium Lactate

0.1M Glycine Buffer, pH 10.0

The original method was formulated for use with purified enzyme solutions in a volume of 2 ml. As the enzyme solution used in the present study was a crude mixture, and the volume of the assay mixture

was 3 ml., the extra volume was made up with buffer solution.

The sodium lactate solution in the original method was made up to 96 ml. which gave a final molarity of 0.5M. In the present study the volume used was 100 ml. Therefore the solution was 0.48M.

Method:-

Measurements were carried out in a Hilger Watts spectrophotometer with a thermostatically controlled carriage ($25^{o}_{\pm}1$). The reaction took place in 3 ml. quartz cuvettes with a 1 cm. light path. The control cell contained 0.1 ml. NAD^{+} solution and 2.8 ml. buffer solution. The test cell contained 0.1 ml. NAD^{+} solution, 0.1 ml. sodium lactate and 2.7 ml. buffer solution. The enzyme solution was blown into the control and washed into the test cell, each cuvette being stirred after the addition of the enzyme solution. The extinction at 340 mμ was read each minute for 10 min. The enzyme solution was diluted to give a reading of not greater than 0.20 per min. 1 unit of enzyme activity is equivalent to a change of 0.01 in extinction per min. per ml. of enzyme solution in 3 ml. of reaction mixture.

Units:-

The Molar Extinction Coefficient of NADH is 6.22×10^{3} (Horecker and

$\therefore$ 1 mμmole in 1 ml. has an E. of 6.22×10^{-3} Kornberg, 1948)

$\therefore$ 1 mμmole in 3 ml. has an E of $\frac{6.22 \times 10^{-3}}{3}$

$$= 2.07 \times 10^{-3}.$$

$\therefore$ 1 E. unit = a change of 0.01 per min per ml. enzyme solution at 25^{o}.

$$= \frac{0.01}{2.07 \times 10^{-3}}$$

= 4.83 mμmoles NADH formed /min. /ml. of enzyme solution at 25^{o}.

ISOCITRATE DEHYDROGENASE ESTIMATION.

Principle:-

Isocitrate dehydrogenase was estimated according to the method of Plaut and Sung (1954) in which the change in extinction at 340 mμ, due to the conversion of $NADP^+$ to NADPH is measured.

Reagents:-

0.0015M $NADP^+$ sodium salt, (freshly prepared) C.F.Boehringer & Soehne.

0.08M dl-Isocitric Acid, trisodium salt.

0.02M Manganese Sulphate(A.R.)

0.1M Tris-HCl Buffer, pH 7.4 (2-amino-2(hydroxymethyl)-1:3-propanediol).

The original method was established for the assay of purified enzyme solutions. In the present study the volume of buffer solution in the reaction mixture was increased at the expense of distilled water.

Method:-

As for lactate dehydrogenase. The control cell contained 0.2 ml. $NADP^+$ solution, 0.1 ml. manganese sulphate solution, and 2.60 ml. buffer solution. The test cell contained 0.2 ml. $NADP^+$ solution, 0.1 ml. manganese sulphate solution, 0.05 ml. sodium isocitrate solution and 2.55 ml. buffer solution.

Units:-

As for lactate dehydrogenase, the Molar Extinction Coefficient of NADPH being 6.22×10^3. (Horecker and Kornberg, 1948)

∴ 1 E. unit = 4.83 mμmoles NADPH formed per min. per ml. of enzyme solution at 25^0.

PHOSPHOGLUCONATE DEHYDROGENASE ESTIMATION.

Principle:-

Phosphogluconate dehydrogenase was estimated according to the method of Horecker and Smyrniotis (1948), in which the change in extinction at 340 mμ, due to the conversion of $NADP^+$ to NADPH is measured.

As in the isocitrate dehydrogenase method, the reaction mixture contained more buffer solution at the expense of distilled water.

Reagents:-

0.0015M $NADP^+$, as before.

0.25M 6-Phosphogluconic acid, trisodium salt.

0.02M Magnesium Chloride.

0.025M Glycylglycine-NaCl Buffer, pH 7.4.

Method:-

As for lactate dehydrogenase. The control cell contained 0.2 ml. magnesium chloride solution, 0.2 ml. $NADP^+$ solution and 2.4 ml. buffer solution. The test cell contained 0.2 ml. $NADP^+$ solution, 0.2 ml. magnesium chloride solution, 0.2 ml. sodium 6-phosphogluconate solution and 2.2 ml. buffer solution.

During the estimation of the enzyme activity of the particulate cell fractions, certain of the reaction mixtures, control and test, became turbid and therefore unsuitable for use. In these cases the activity was determined using the Tris buffer prepared for isocitrate dehydrogenase estimation. A comparison of results obtained with the two buffer solutions will be made in the Results section.

Units:-

As for Lactate Dehydrogenase. The final reaction mixture had a volume of 2.9 ml. .'. 1 E. unit = 4.67 mμmoles NADPH formed per min. per ml. of enzyme solution at 25°.

PROTEIN ESTIMATION.

Principle:-

The method used was that of Lowry, Rosebrough, Farr and Randall, (1951) in which the Folin method for the estimation of tyrosine is made more sensitive by the addition of an alkaline copper reagent.

Reagents:-

2% (w/v) Sodium Carbonate (anhydrous) in 0.1N Sodium Hydroxide.

1% Copper Sulphate (w/v) ($CuSO_4 \cdot 5H_2O$)

2% (w/v) Sodium Tartrate $(CH(OH)COONa)_2 \cdot 2H_2O$.

Folin-Ciocalteau phenol reagent, diluted to give a 1N solution.

The working copper solution was made up just before use, by the addition of 1 ml. of a mixture of equal parts of copper sulphate solution and sodium tartrate solution to 49 ml. sodium carbonate solution. In the original method, the copper sulphate and the sodium tartrate were made up in the one solution, but this was found to be unstable.

Standard:-

The protein used was crystalline bovine serum albumin (Armour Pharmaceutical Co. Eastbourne)

Method:-

The solution to be estimated was diluted to give a protein concentration of 25 - 300 μg.per ml. To 1 ml. of the diluted solution

was added 5 ml. of the working copper solution and this mixture was left for 10 min. at room temperature. 0.5 ml. of the diluted phenol reagent for 10 min. at room temperature. 0.5 ml. of the diluted phenol reagent were added and the mixture was shaken immediately, within 1-2 sec. After 30 min. standing at room temperature, the extinction of the solution was read at 625 mμ.

SUCCINATE DEHYDROGENASE ESTIMATION (E.C.1.3.99.1.)

Principle:-

The method used was a modification of that of Jardetzky and Glick, (1956) in which the succinate dehydrogenase reduces neo-tetrazolium chloride to a pink pigment which can be measured by its extinction.

Reagents:-

0.5% (w/v) neo-tetrazolium chloride (L. Light and Co. Ltd.)

0.67M Sörenson's Phosphate Buffer, pH 7.7, containing 5.34×10^{-4}M Disodium Ethylenediaminetetraacetate

0.2M Sodium Succinate in 0.67M phosphate buffer

0.2M Sodium Malonate in 0.67M phosphate buffer

3:1 Carbon Tetrachloride (A.R.) : Acetone (Lab. grade)

Methods:-

The reaction mixture contained, 2 ml. buffer solution, 1 ml. neo-tetrazolium chloride, 1 ml. sodium succinate and 1 ml. enzyme solution. The control tube contained sodium malonate in the place of sodium succinate. The reaction was started by the addition of the enzyme solution and the tubes were incubated in the dark at 37° for 60 min. The solutions were then extracted with the carbon tetrachloride and acetone mixture. The organic layer was separated by centrifugation and its extinction was read in an S.P. 600 spectrophotometer at 520 mμ.

against a blank of the organic mixture.

Units:-

The unit of activity is 0.001 E/hr./mg. protein.

ESTIMATION OF DNA.

Principle:-

The method used was a modification of that of Ceriotti(1952) in which the DNA was hydrolysed by acid and the fragments produced were estimated by the extinction of the coloured product formed by their reaction with indole.

Reagents:-

0.04% (w/v) Indole (Lab. grade)

Hydrochloric Acid, specific gravity 1.18, (A.R.)

Chloroform (A.R.)

Method:-

The reaction mixture contained 2 ml. of test solution, 1 ml. hydrochloric acid, and 1 ml. indole solution. The control tube contained distilled water in place of the test solution. The tubes were stoppered and placed in a boiling water bath for 10 min. The solutions were then extracted with 40 ml. chloroform, and the aqueous layer was separated by centrifugation. The extinction of the yellow pigment in the aqueous layer was read at 490 mμ.

Standard:-

The standard used was a solution of the sodium salt of Deoxyribonucleic Acid, highly polymerised.(Sigma Chemical Co.)

CALCULATIONS.

The enzyme activities were expressed in International Units per g. wet weight, per mg. protein, and in the case of the Thyroid gland Nucleic Acid series, per mg. DNA Phosphorus.

The means of the activities of each fraction of each series were calculated, also the standard deviation.

$$\text{Mean } \bar{x} = \frac{\Sigma x}{n} \qquad \text{Standard Deviation } \sigma = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x = each value in the series and n = the number of values.

Student's t-test was applied to the results obtained from normal and abnormal tissues. The formulae used were given in, "Facts from Figures", M.J.Moroney, Penguin Books Ltd.,(1962), Harmondsworth, Middlesex. When both sets of results were obtained from the one set of patients, the following formulae were used;

$$s^2 = \frac{\Sigma x^2}{n} - \bar{x}^2 \qquad t = \frac{\bar{x}\sqrt{n - 1}}{s}$$

where x = the differences between the two results for each patient, and $\bar{x}$ = the mean of the "x" values.

When the normal and abnormal results were obtained from two sets of patients, the following formulae were used;

$$s^2 = \frac{\Sigma(x - \bar{x})^2 + \Sigma(y - \bar{y})^2}{n + n' - 2} \qquad t = \frac{\bar{x} - \bar{y}\sqrt{n \times n'}}{s\sqrt{n + n'}}$$

where the "y" values belong to the second set of results, and n' is the number of results in the second set.

The "P" values corresponding to the "t" values found were obtained

from the "Cambridge Elementary Statistical Tables", D.V.Lindley and J.C.P.Miller, Cambridge University Press,(1961). Results were considered to be significant when the "P" values were less than 0.05.

The tissue preparation , the measurement of the protein concentrations, and the estimation of the nucleic acids were carried out in conjunction with Dr. D. M. Goldberg.

RESULTS

In the presentation of the results, the first section deals with the validity of the cell fractionation procedures used. It will be shown that the methods used produces a supernatant fraction from which particles containing appreciable enzyme activity have been eliminated, an M1 fraction which is predominantly mitochondrial in nature and an M2 fraction, which is predominantly microsomal.

Technical considerations are described in the second section and deal with the effects of dilution on the enzyme activities, difficulties associated with storage of material, measurement of particulate PGDH and measurement of protein concentration.

The studies on thyroid tissue are then described. Here, there are two sets of results; the majority of the results were obtained without nucleic acid analysis of the tissue and are described as the Main series. In a smaller number of tests, the amounts of the nucleic acids in the tissues were measured and the enzyme activities were related to DNA content as a measure of cell number.

Results for the Main series of thyroid tissue experiments are presented in sections describing the supernatant fraction, the mitochondrial (M1) fraction and the microsomal (M2) fraction, obtained from normal, hyperplastic, and malignant thyroid tissue. Following these results are those obtained from samples of cervix uteri. These are divided into two parts, namely (a) a comparison between the supernatant fractions of the normal and carcinoma series, and (b) a comparison between each of the three subcellular fractions of tumour cells from the cervix uteri, before and after radiation. The results

obtained from the supernatant fraction of human mammary gland are then described, from series of normal mammary tissue, hyperplastic tissue and tumour of the gland.

The results obtained from the Nucleic Acid series of thyroid tissue studies are then presented in the following sections (a) a report of the enzyme activities of the tissues, which is divided into two parts, 1) a comparison of the enzyme activities of the thyrotoxic and adenomatous tissue with those of normal thyroid tissue, 2) a comparison of the Main and Nucleic Acid series of normal, adenoma, and thyrotoxic tissue, (b) the RNA, (c) the DNA, (d) the phospholipid and (e) the protein content of the three types of tissue and (f) the RNA to DNA phosphorus ratio of these tissues.

INVESTIGATION OF CENTRIFUGATION TECHNIQUES.

In order to demonstrate the adequacy of the cell fractionation techniques used, the following studies were made; (a) electron microscopy of the particles sedimented, (b) an examination of the Supernatant fraction obtained, in order to see whether any particles bound enzymes were still present, (c) a test to determine the amount of supernatant activity lost in the preparation of the particulate fractions, and (d) a study of the distribution of succinate dehydrogenase activity in the particulate fractions of the tissues, as a measure of the contamination of the microsomal fraction.

(a) Electron Microscopy of the Particulate Fractions.

Samples of the nuclear pellet, the M1 fraction and the M2 fraction

of a thyrotoxic thyroid gland were examined under the electron microscope. The nuclear fraction was found to be free of mitochondria, but contained some fragments of the endoplasmic reticulum. The M1 fraction was found to be predominantly mitochondria and the M2 fraction predominantly microsomes. The lysosomes were spread between the two particulate fractions. (Figures 2,3.)

(b) Enzyme Activity of those Particles that remain Unsedimented after Centrifugation at 35,000g for 1 hr.

Owing to limitations of apparatus, the maximum centrifugal force used was 35,000g for 1 hr. In order to determine whether any particulate enzyme activity remained in the supernatant fraction so obtained, samples of the supernatant fraction from two thyrotoxic glands were spun at 105,000g for 1 hr. in another department. With one of these glands, a small portion only of the supernatant fraction was spun to obtain a comparison with the 35,000g supernatant fraction. In the other gland, all except a small portion of the supernatant fraction was spun in order to obtain the sediment as quantitatively as possible. The precipitate obtained was gelatinous in consistency and red in colour.

The results are shown in Table 1. In the first gland there was no appreciable difference in enzyme activity of the supernatant fraction after spinning, nor was there any difference in protein concentration. In the second gland, about 1% of the total supernatant activity and about 2.5% of the supernatant fraction protein appeared in the pellet.

These findings confirmed that centrifugation at 35,000g for 1 hr. is a satisfactory way of preparing a supernatant fraction from human

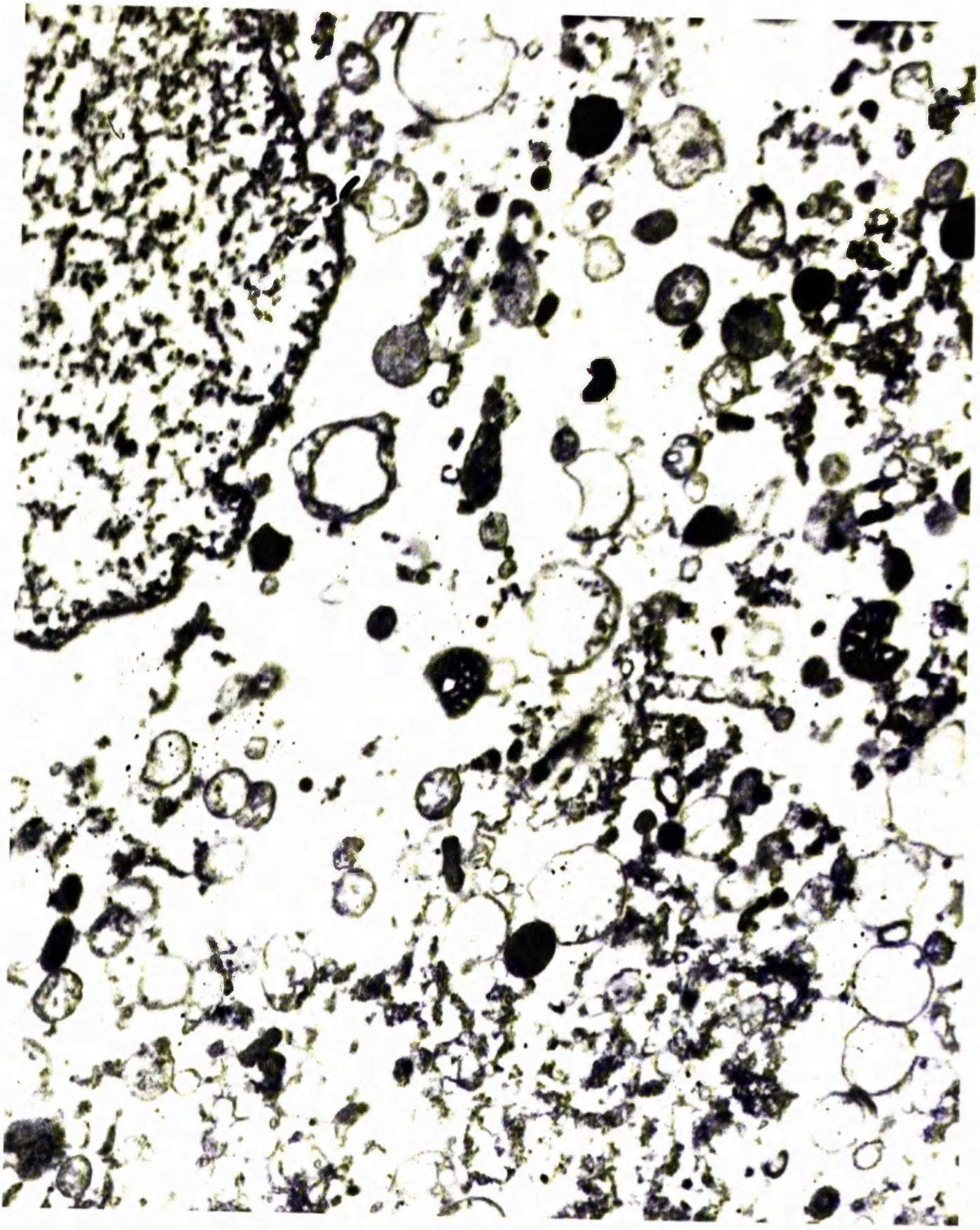

Figure 2. A sample of the M1 fraction of a human thyrotoxic thyroid gland, x 22,000. The fraction consists predominantly of mitochondria. Very few microsomes are present, but an occasional lysosome may be identified. This field shows a nucleus at the upper left corner, but it was the only one of several examined to show nuclear contamination.

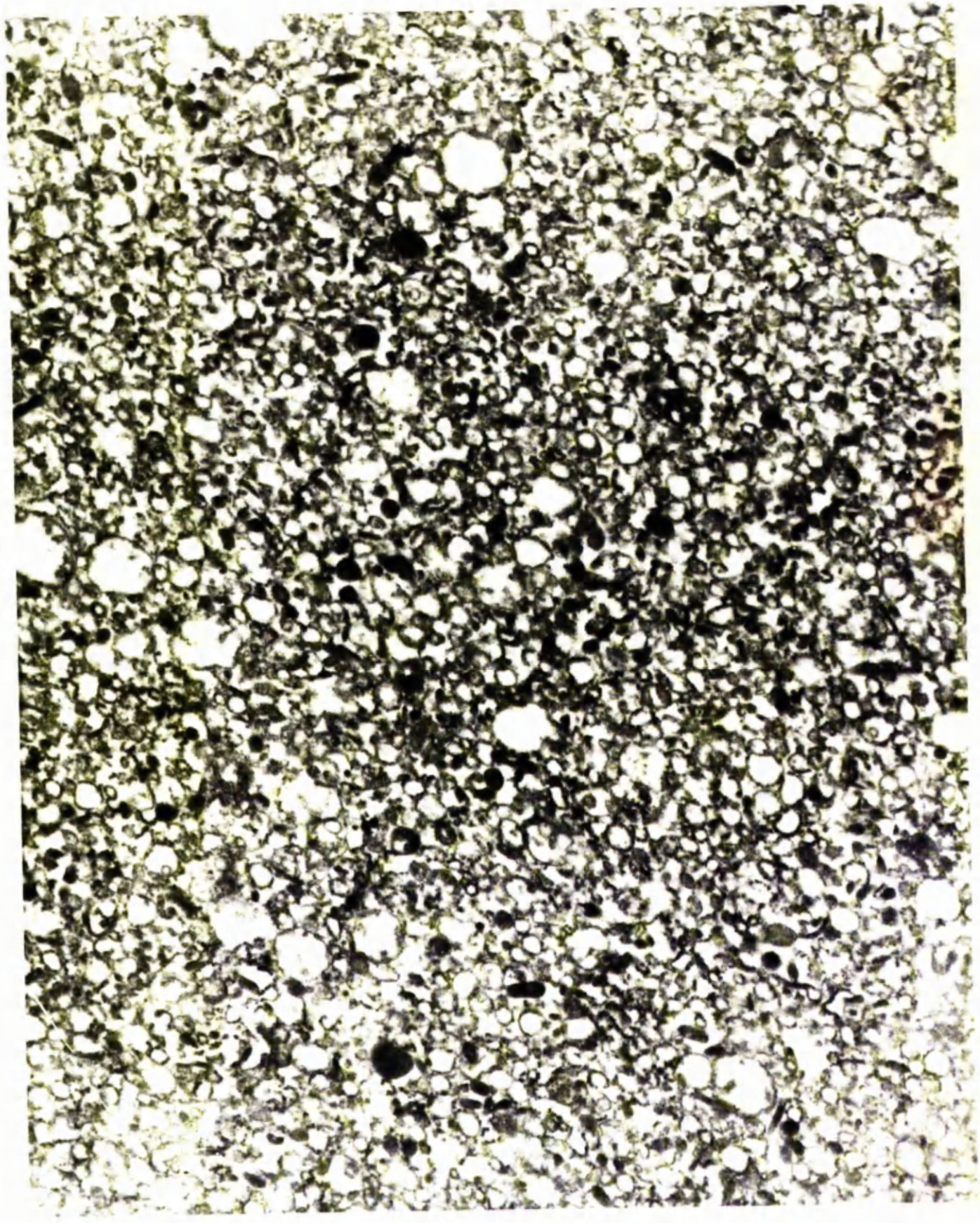

Figure 3. A sample of the M2 fraction of a human thyrotoxic thyroid gland, x 22,000. The fraction consists predominantly of microsomes. Occasional mitochondria may be seen, as may a very few dense bodies, which could be small lysosomes.

TABLE 1. EFFECT OF CENTRIFUGATION AT 105,000g ON THE SUPERNATANT FRACTIONS OF THYROTOXIC THYROID GLANDS.

	Original Supernatant Fraction	105,000g Supernatant Fraction	105,000g Pellet
GLAND I			
LDH	8210	8210	
IDH	1360	1360	
PGDH	1340	1270	
Protein	99	99	
GLAND II			
LDH	7970		30
IDH	1200		12
PGDH	1260		14
Protein	102		2.5

Enzyme activities expressed in mumoles substrate transformed/min./g. wet weight of tissue.

Protein concentrations expressed in mg./g. wet weight of tissue.

tissues, whose enzyme and protein content is essentially the same as that of more rigorously prepared cell sap.

(c) Quantitative Removal of Unsedimented Layers.

In order to separate the supernatant fraction cleanly, some fluid was left above the particle layer after centrifuging. The particles were then washed with sucrose. In one specimen of carcinoma of cervix, the enzyme activities of the sucrose used for washing the two particulate fractions were estimated. Each volume of rinsing sucrose contained 3% of the total supernatant fraction activity. Within the limits of accuracy of the method, this activity was negligible.

(d) Succinate Dehydrogenase Estimation.

(d) Succinate Dehydrogenase Estimation. Since the microsome fraction contained appreciable numbers of mitochondria, SDH was estimated in the M1 and M2 fractions of the thyrotoxic thyroid gland used for electron microscopy. The particulate material had been deep frozen immediately after removal of samples for electron microscopy, which caused some rupture of the particles. The estimation was not wholly successful in the M1 fraction, as the reaction appeared to take place on fragments of the mitochondrial membranes and the pink colour proved difficult to extract. However, duplication of results gave good agreement, notwithstanding the amount of dye that could not be extracted, i.e., 41 and 39 E units per mg. protein. In the M2 fraction the fragments of membrane again appeared to be the locus of the reaction, but these were finer fragments,

than those of the M1 fraction, and the pink colour proved more easily extractable. Duplication of results was not so good with this fraction, the two results being, 149 and 98 E units per mg. protein. However, the activity of the M2 fraction was much greater than that of the M1 fraction. This finding suggests either that the M2 fraction contained mitochondria, which conflicts with the results of electron microscopy, or that SDH occurs in the M2 fraction of human thyrotoxic thyroid glands.

SDH was also estimated on samples of particulate fractions which had been disrupted ultrasonically in the normal way for enzyme assay. These preparations were, therefore, free of fragmented membranes, through coagulation(see Material and Methods section). The activity found was too small to measure quantitatively, but the M1 fraction showed greater activity than the M2 fraction.

INVESTIGATION OF ENZYME MEASUREMENTS.

In order to evaluate the accuracy of the methods used for the estimation of enzyme activity in the tissue fractions, various tests were carried out. These showed, (a) that the methods used gave reproducible results, (b) that alteration of the dilution medium made no difference to the activities recorded, (c) that slight inaccuracies were introduced by the different dilution factors required by the different tissue samples, (d) that the alteration of the buffer solution used in the measurement of the particulate PGDH did not affect the enzyme activities, and (e) that the alteration in the activities of the enzymes caused

by storage of the supernatant fraction at -20° were small enough to make the storage for one week, necessary in the Nucleic Acid series of thyroid tissue, permissible.

(a) Duplication of Results.

At the beginning of this study, certain enzyme assays were carried out in duplicate, where material permitted. The results (see Table 3.) obtained gave good agreement, in many cases within 2.5%. The greatest divergence found was 10%, and thereafter, single estimations only were carried out.

(b) Effect of Differing Dilution Media on Enzyme Results.

Enzyme activities were estimated on a sample of thyrotoxic tissue and a sample of breast cancer(Table 2.). The supernatant fractions were diluted prior to estimation in a variety of media, namely, the appropriate buffer solution, sucrose, distilled water, and 0.15M potassium chloride. The enzyme activities obtained were essentially the same in all the media, and sucrose was chosen.

(c) Effect of Varying Dilution Factors on Enzyme Results.

Enzyme activities of various types of tissue were estimated on differing dilutions of the supernatant fraction, where material and time permitted. Where possible the assays were carried out in duplicate (Table 3.). In most of the samples studied, the activity corrected for dilution, was greater in the more dilute solutions, but in two samples the opposite effect was recorded. However, the values obtained from the most dilute solution varied from 90% to 125% of the estimate obtained from the most concentrated solution. This variability was considered to lie

TABLE 2. EFFECT OF DIFFERING DILUTION MEDIA ON ENZYME RESULTS.

		Buffer Solution	Sucrose	Distilled water	Potassium chloride
THYROTOXIC	LDH	240	246	252	258
GLAND	LDH	40	42	44	40
	PGDH	28	34	32	30
BREAST	LDH	44	52	54	48
CANCER	LDH	46	53	47	56
SAMPLE	PGDH	11	15	10	10

Enzyme activities expressed as extinction units per ml. of supernatant fraction.

TABLE 3. EFFECT OF VARYING DILUTION FACTORS ON ENZYME RESULTS.

ENZYME	TISSUE	DILUTION FACTORS 1/5	1/7	1/10	1/15	1/20	1/30	1/40	1/50
LDH	Thyrotoxic I						180) 180)	152) 140)	
	Thyrotoxic II			77) 88)		82) 84)		92) 92)	90) 96)
	Hashimoto					242	279	292	280
	Breast cancer			59) 60)		66) 70)			
	Cervical cancer				124	134	138		155
IDH	Thyrotoxic I	37) 36)		39) 40)		42) 42)	42		
	Hashimoto	76		71		68			
	Breast cancer			47) 50)		50) 54)			
	Cervical cancer	40		40	45	42			
PGDH	Thyrotoxic I	27) 27)		27) 29)		32) 35)	33		
	Normal thyroid		78) 73)		83	92			
	Cervical cancer	35		37	44	34			

Enzyme activities are expressed as extinction units per ml. of supernatant fraction at 25°. Duplicate results are indicated by brackets.

within the limits of accuracy of the experimental procedure.

(d) Estimation of PGDH in the Particulate Fractions.

As was noted in the Material and Methods section, the addition of the particulate fractions to the reaction mixture used for the estimation of PGDH, caused turbidity, making the measurement of the reaction rate impossible. This turbidity was eliminated by using tris buffer in the place of glycylglycine buffer. The tris buffer used in the IDH method proved suitable for this purpose. In order to confirm that this substitution was valid, ten samples of the supernatant fraction of various tissues were taken from storage at -20^{0} and estimated for PGDH activity using both buffer solutions. The "t" value found when the two sets of results were compared was 1.13 (P less than 25%). This experiment was repeated using the tris buffer diluted 1/4 so that the molarity would correspond to that of the glycylglycine buffer. The "t" value found in this case was 0.006 (P less than 25%). The diluted tris buffer was, therefore, substituted for the glycylglycine buffer in the estimation of particulate PGDH.

(e) Effect of Storage at -20^{0} on the Enzyme Activities of the Supernatant Fraction of Human Thyroid Gland.

The enzyme activities of the supernatant fraction of the tissue samples in the Nucleic Acid series were measured when the other estimations were complete. The supernatant fractions were, therefore, stored at -20^{0} for one week. Insufficient material was available for studying the effects of storage on the enzyme activities in these specimens. However, enzyme activities were determined on

other samples of supernatant fraction of thyroid gland, which had been prepared in the Main series, and stored at -20°, where excess material was available, for periods of time ranging from two months to a year. The enzyme activities after storage were expressed as a percentage of the original activity. The mean percentage LDH activity after storage was 92% of the initial value. The mean percentage IDH activity after storage was 84% and the mean percentage PGDH activity was 66%. It is unlikely that storage for one week would have a greater effect on the enzyme activities than the changes observed over the 2 - 12 month periods examined.

INVESTIGATION OF THE LOWRY PROTEIN METHOD.

Various aspects of the Lowry method of protein estimation were considered, these being 1) at which wavelength the colour should be read to give the best range of protein concentrations, 2) the effect of perchloric acid, used in the preparation of the RNA fraction in the Nucleic Acid series, on the measurement of protein and 3) the stability of the copper reagent.

1) Measurement of Colour.

The measurement of the blue complex was carried out at 625 mμ in order to extend the range of the estimation to 300ug. per ml.

2) Measurement of Protein in 0.1N Perchloric Acid Solution.

The protein concentrations found when the standard protein solutions were dissolved in 0.1N perchloric acid were about 6% lower than the true concentrations. This difference was considered to be negligible.

3) Stability of Copper Reagent.

It was found that, on standing, this reagent showed formation of blue crystals after one day, and one month later a red precipitate appeared. A standard protein solution was estimated at different times using the same copper reagent ($CuSO_4$ / Sodium Tartrate) diluted to give a working solution each time. The concentration of the protein appeared to decrease, reaching 87% of the initial value in 34 days. (Figure 4, Experiment 1.)

This experiment was modified to eliminate the possibility of deterioration of the protein solution as a cause of the above affect. The copper sulphate and the sodium tartrate solutions were made up separately. The combined reagent was made up periodically during one month, by mixing equal parts of the two solutions. At the end of this time, a protein solution was estimated using different working copper solutions obtained by dilution of the copper reagents of varying ages. The protein concentration given by the 31 day-old reagent was 85% of the concentration given by the freshly made copper reagent. (Figure 4, Experiment 2.)

A third experiment was carried out to verify that different copper reagents freshly prepared would give comparable results. This was found to be the case (Table 4.). Thereafter, the working copper solution used for the estimation of protein was obtained by dilution of a freshly made copper reagent.

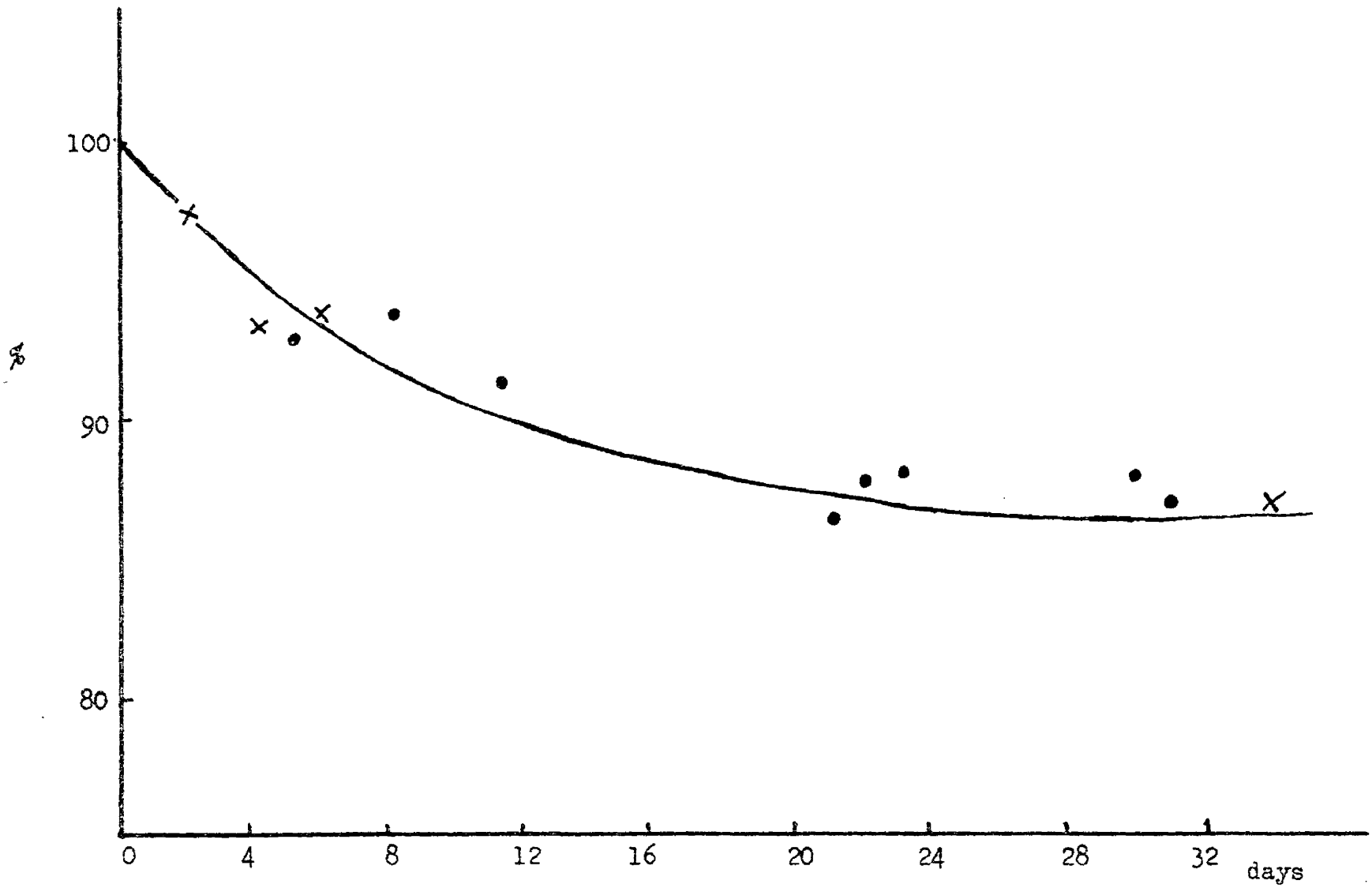

Figure 4. Effect of Age on Copper Reagent of Lowry Protein Estimation.

x Experiment I. Average of 9 estimations

• Experiment II. Average of 2 estimations

TABLE 4.

EXTINCTIONS OBTAINED USING FRESHLY MADE COPPER REAGENTS IN THE ESTIMATION OF TWO PROTEIN SOLUTIONS.

	Copper Reagent	Average Extinction	Extinction Readings
PROTEIN I	1	0.339	0.340, 0.335, 0.342
	2	0.356	0.356, 0.372, 0.342
	3	0.358	0.372, 0.346, 0.356
PROTEIN II	4	0.360	0.368, 0.353
	5	0.364	0.374, 0.356, 0.361

ENZYME ACTIVITIES IN THE HUMAN THYROID GLAND - MAIN SERIES.

The enzyme results of the human thyroid gland are divided into three sections dealing with, 1) the supernatant fraction, 2) the mitochondrial (M1)fraction and 3) the microsomal (M2) fraction. The classes of thyroid tissue studied were, normal thyroid, thyrotoxic thyroid, adenoma, carcinoma and Hashimoto's disease. In each section the results are expressed in tabular form, as the mean of each series ± the standard deviation. The results related to weight of tissue are tabulated on different pages from those related to protein content. The results for each of the abnormal groups are compared with the normal group using Student's t-test. The results of the t-test are reported both in the text and in tables. One sample of Hürthle cell adenoma was also studied, but the results recorded were so unlike those of the other samples of adenoma of thyroid, that they are reported separately.

The precentage activity of each enzyme in each fraction is also reported, the combined activities of all three subcellular fractions being taken as 100%.

The range of weights of tissues used in each series is also reported in table form, in order to give some indication of the representative nature of the specimens.

1) Supernatant Fraction. (Tables 5-9)

In all the tissues studied, LDH activity is greater than IDH activity which is greater in turn than PGDH activity. The activity of the Hürthle Cell adenoma is similar to the mean of the carcinoma series, except for higher IDH activity.(Tables 5,7.)

LDH activity is significantly higher than normal, at the 5% level in the thyrotoxic series, at the 0.1% level in the carcinoma and Hashimoto series, but is not significantly different in the adenoma series, when the results are expressed in units per g. wet weight. When the LDH activities are expressed in units per mg. protein, there is a significant increase above normal, at the 0.1% level in the thyrotoxic, carcinoma and Hashimoto series, while in the adenoma series there is no significant difference (Tables 6,8.). In the thyroid carcinoma series, there was one sample of spheroidal cell carcinoma, which had a very high LDH activity, 43370 mumoles substrate transformed per min. per g. wet weight of tissue. Histologically it was classed as very malignant.

IDH activity in units per g. wet weight, is significantly higher than normal, at the 0.1% level in the carcinoma series, at the 0.2% level in the Hashimoto series, and at the 5% level in the thyrotoxic and adenoma series. The Hürthle Cell adenoma result is about 5 times the normal mean. When the results are expressed in units per mg. protein, IDH activity is not significantly different from normal in the thyrotoxic and adenoma series, but is significantly higher in the carcinoma series and the Hashimoto series, at the 0.1% level. The Hürthle Cell adenoma result is about 12 times the normal mean.(Tables 5,6,7,8.)

PGDH activity expressed as units per g. wet weight is significantly higher than normal, at the 0.1% level in the thyrotoxic series, at the 5% level in the carcinoma series,,and at the 2% level in the Hashimoto series, but is not significantly different from

normal in the adenoma series. When the results are expressed as units per mg. protein, there is a significant increase above normal, at the 0.1% level in the thyrotoxic and carcinoma series, and at the 2% level in the Hashimoto series, but there is no significant difference in the adenoma series (Tables 6,8.).

The mean percentage of LDH appearing in the supernatant fraction of the normal, thyrotoxic and adenoma series is so close to 100%, that the difference could lie within the accuracy of the methods used (Table 9.). The mean percentage of IDH activity appearing in the supernatant fraction is lower than that of LDH in all the tissues studied (Table 9.), and this is due mainly to appreciable activity in the M1 fraction (see Tables 9,14,19.). The mean percentage supernatant fraction activity decreases in the tissue series in the following order; normal, thyrotoxic, adenoma, Hashimoto, carcinoma and Hürthle Cell adenoma. Thus, in the sample of Hürthle Cell adenoma the supernatant activity is only 53% of the total measured activity. The mean percentage of PGDH activity occurring in the supernatant fraction is again less than that of LDH (Table 9.). In decreasing order of supernatant activity, the tissues are as follows; Hashimoto, thyrotoxic, adenoma, Hürthle Cell adenoma and carcinoma.

TABLE 5. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./g. wet weight of tissue, at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	11	6130 ± 3050	880 ± 340	300 ± 130
TOXIC	20	9050 ± 3710	1500 ± 880	960 ± 790
ADENOMA	10	6900 ± 4590	1290 ± 900	580 ± 660
CARCINOMA	5	21600 ± 1340	2590 ± 480	920 ± 480
HASHIMOTO	4	9100 ± 2400	2260 ± 950	580 ± 230
" HURTHLE CELL ADENOMA	1	20600	4300	790

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	11 + 20	2.21	<0.05	147	2.44	<0.05	170	4.95	<0.001	320
NORMAL V ADENOMA	8 x 2	0.76	-	-	2.40	<0.05	147	1.22	<0.25	-
NORMAL V CARCINOMA	11 + 5	6.71	<0.001	351	8.4	<0.001	294	2.45	<0.05	367
NORMAL V HASHIMOTO	11 + 4	5.72	<0.001	148	4.16	<0.002	257	2.92	<0.02	193

$$* \% = \frac{\text{Mean of abnormal results x 100}}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per g. wet weight of tissue.

TABLE 7. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	11	66.3 ± 28.5	8.4 ± 3.6	3.2 ± 1.8
TOXIC	20	101 ± 59	16.7 ± 13.2	10.1 ± 6.0
ADENOMA	10	83.1 ± 37.2	15.5 ± 12.6	7.9 ± 11.9
CARCINOMA	5	326 ± 214	35.3 ± 13.5	11.1 ± 4.3
HASHIMOTO	4	226 ± 31.0	56.1 ± 18.4	14.0 ± 2.3
HÜRTHLE CELL ADENOMA	1	488	100	4.0

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	11 + 20	5.57	<0.001	152	2.00	<0.1	-	3.84	<0.001	152
NORMAL V ADENOMA	8 x 2	1.94	<0.1	-	1.68	<0.25	-	1.41	<0.25	-
NORMAL V CARCINOMA	11 + 5	4.11	<0.001	492	6.37	<0.001	421	5.12	<0.001	352
NORMAL V HASHIMOTO	11 + 4	9.92	<0.001	341	8.10	<0.001	667	2.98	<0.02	444

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per mg. protein.

TABLE 8.

TABLE 9. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

% of total measured activity in cytoplasm.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	99.0 ± 0.8	96.7 ± 2.9	90.7 ± 9.4
TOXIC	20	99.0 ± 0.6	93.6 ± 2.9	95.7 ± 4.1
ADENOMA	10	99.1 ± 1.1	93.0 ± 3.2	94.7 ± 3.0
CARCINOMA	4	98.8	87.7	83.6
HASHIMOTO	4	9815	92.5	96.3
HÜRTHLE CELL ADENOMA	1	98.7	53.0	87.6

Results expressed as Mean ± Standard Deviation.

2) M1 Fraction of Human Thyroid Gland. (Tables 10 - 14)

In this fraction the order of the enzyme activities is again varied, IDH activity becoming more important. In the normal series no consistent pattern emerges, but in the other series, the pattern IDH > LDH > PGDH is predominant.

LDH activity is not significantly different from normal in any of the tissues studied. No LDH activity was detected in the M1 fraction of the Hürthle Cell adenoma (Tables 11,13.).

IDH activity expressed as units per g. wet weight is significantly higher than normal, at the 0.1% level in the adenoma series and at the 5% level in the carcinoma series (the mean being about 10 times the normal mean) but is not significantly different in the thyrotoxic and Hashimoto series. The Hürthle Cell adenoma result is about 150 times the normal mean. IDH activity, expressed as units per mg. protein is significantly higher than normal, at the 0.1% level in the carcinoma series (the mean being about 8 times the normal mean),and at the 1% level in the thyrotoxic, adenoma and Hashimoto series. The Hürthle Cell adenoma result is about 40times the normal mean (Tables 10,11,12,13.).

PGDH activity expressed as units per g. wet weight, is significantly higher than normal, at the 0.1% level in the adenoma series, but is not significantly different from normal in the thyrotoxic, carcinoma and Hashimoto series. When the results are expressed as units per mg. protein, PGDH activity is significantly higher than normal, at the 0.1% level in the thyrotoxic series, but is not significantly different from normal in the adenoma, Hashimoto and carcinoma series (Tables 11,13.).

TABLE 10. ENZYME ACTIVITIES OF THE M1 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./g. wet weight of tissue, at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	18 ± 15	23 ± 25	8.9 ± 8.4
TOXIC	20	35 ± 31	58 ± 54	20 ± 23
ADENOMA	9	26 ± 17	82 ± 52	34 ± 31
CARCINOMA	4	217	222	51
HASHIMOTO	4	43.5	113	17
" HÜRTHLE CELL ADENOMA	1	0	3600	17

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE M1 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	5 + 20	1.21	< 0.25	-	1.43	<0.25	-	0.99	-	-
NORMAL V ADENOMA	5 + 9	1.46	< 0.25	-	7.41	<0.001	360	5.37	<0.001	383
NORMAL V CARCINOMA	5 + 4	1.56	<0.25	-	2.35	=0.05	959	1.39	<0.25	-
NORMAL V HASHIMOTO	5 + 4	1.12	-	-	1.77	<0.25	-	0.35	-	-

$$* \% = \frac{\text{Mean of abnormal results x 100}}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per g. wet weight of tissue.

TABLE 14.

TABLE 12. ENZYME ACTIVITIES OF THE M1 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	8	5.8 ± 3.4	4.8 ± 3.4	1.4 ± 1.9
TOXIC	20	8.7 ± 4.3	15 ± 9.7	5.6 ± 7.9
ADENOMA	9	6.3 ± 2.4	20 ± 13	2.6 ± 1.5
CARCINOMA	4	30	32	7.0
HASHIMOTO	4	7.7	18.9	2.3
HÜRTHLE CELL ADENOMA	1	0	200	6.3

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE MI FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	11 + 20	1.43	<0.25	-	3.09	<0.01	313	5.24	<0.001	400
NORMAL V ADENOMA	8 + 9	0.34	-	-	3.28	<0.01	417	1.59	<0.25	-
NORMAL V CARCINOMA	8 + 4	1.60	<0.25	-	5.58	<0.001	670	1.79	<0.25	-
NORMAL V HASHIMOTO	8 + 4	2.04	<0.1	-	3.00	<0.01	394	0.66	-	-

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where P} \quad 0.05$$

Calculations carried out on activities per mg. protein.

TABLE 13.

TABLE 14. ENZYME ACTIVITIES OF THE M1 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

% of total measured activity.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	0.3 ± 0.2	2.5 ± 2.3	6.3 ± 7.4
TOXIC	20	0.4 ± 0.2	3.0 ± 2.8	2.4 ± 2.9
ADENOMA	5	0.5 ± 0.5	4.8 ± 2.7	2.6 ± 2.7
CARCINOMA	4	0.7	8.0	6.0
HASHIMOTO	4	0.5	3.9	2.0
"HURTHLE CELL ADENOMA	1	0	45.4	12.4

Results expressed as Mean ± Standard Deviation.

3) M2 Fraction of Human Thyroid Glands.(Tables 15 - 19)

Like the M1 fraction, this fraction shows alteration in the pattern of enzyme activities, IDH activity becoming more important. In the normal series, no consistent pattern emerges, but in the other series the pattern IDH > LDH > PGDH is predominant.

LDH activity expressed as units per g. wet weight is not significantly different from normal in any of the tissues studied, but the result obtained from the sample of Hürthle Cell adenoma is about 7 times the normal mean. However, when the results are expressed in units per mg. protein, there is a significant increase, at the 5% level in the thyrotoxic series and the Hürthle Cell adenoma result is about 4 times the normal mean.(Tables 15,16,17,18.)

IDH activity expressed in units per g. wet weight is significantly higher than normal at the 5% level in the thyrotoxic, carcinoma and Hashimoto series, but is not significantly different from normal in the adenoma series. The result for the Hürthle Cell adenoma is about 13 times the normal mean. When the data are expressed per mg. protein, IDH activity is significantly higher than normal, at the 0.1% level in the thyrotoxic series, at the 2% level in the adenoma and carcinoma series, and at the 5% level in the Hashimoto series. The result obtained from the Hürthle Cell adenoma is about 6 times the normal mean (Tables 15,16,17,18.).

PGDH activity is not significantly different from normal in any of the tissues studied either in units per g. wet weight or in units per mg. protein. No PGDH activity was detected in the M2 fraction of the Hürthle Cell adenoma (Tables 15,16,17,18.).

TABLE 15. ENZYME ACTIVITIES OF THE M2 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./g. wet weight of tissue, at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	6	40 ± 43	10 ± 7.3	17 ± 23
TOXIC	20	60 ± 53	46 ± 37	15 ± 14
ADENOMA	10	27 ± 21	27 ± 21	8.9 ± 6.5
CARCINOMA	4	184	121	93
HASHIMOTO	4	73.5	103	12.6
HÜRTHLE CELL ADENOMA	1	280	129	0

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE M2 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	6 + 20	0.78	-	-	2.11	<0.05	460	0.21	-	-
NORMAL V ADENOMA	6 + 9	1.13	-	-	1.83	<0.1	-	0.98	-	-
NORMAL V CARCINOMA	6 + 4	1.37	<0.25	-	2.79	<0.05	1190	2.08	<0.10	-
NORMAL V HASHIMOTO	6 + 4	1.35	<0.25	-	2.91	<0.02	1030	1.01	-	-

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per g. wet weight of tissue.

TABLE 16.

TABLE 17. ENZYME ACTIVITIES OF THE M2 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

mμmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	6	8.7 ± 5.8	2.9 ± 1.9	3.7 ± 4.7
TOXIC	20	15 ± 8.2	13 ± 5.8	5.1 ± 5.1
ADENOMA	10	7.3 ± 2.4	6.8 ± 1.2	2.6 ± 1.3
CARCINOMA	5	20	12	8.9
HASHIMOTO	4	14.0	15.9	2.3
" HÜRTHLE CELL ADENOMA	1	37	17	0

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE M2 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	9 + 20	2.18	<0.05	172	4.70	<0.001	448	0.71	-	-
NORMAL V ADENOMA	9 + 9	0.69	-	-	2.83	<0.02	234	0.72	-	-
NORMAL V CARCINOMA	9 + 5	0.43	-	-	3.00	<0.02	400	0.44	-	-
NORMAL V HASHIMOTO	9 + 4	1.56	<0.25	-	2.40	<0.05	548	0.57	-	-

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per mg. protein.

TABLE 19. ENZYME ACTIVITIES OF THE M2 FRACTION OF HUMAN THYROID GLANDS - MAIN SERIES.

% of total measured activity.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	6	0.6 ± 0.5	0.8 ± 0.7	5.1 ± 6.4
TOXIC	20	0.6 ± 1.3	2.5 ± 1.5	1.8 ± 2.6
ADENOMA	10	0.6 ± 0.7	2.8 ± 1.8	1.6 ± 0.9
CARCINOMA	4	0.6	4.3	10.7
HASHIMOTO	4	0.9	3.7	1.7
" HURTHLE CELL ADENOMA	1	1.3	1.6	0

Results expressed as Mean ± Standard Deviation.

TABLE 20. RANGE OF WEIGHTS OF SAMPLES OF HUMAN THYROID GLANDS - MAIN SERIES.

	Number of samples	Weight in g.
NORMAL	11	0.39 - 5.15
TOXIC	20	4.70 - 22.4
ADENOMA	10	3.04 - 15.9
CARCINOMA	5	0.55 - 16.2
HASHIMOTO	4	3.95 - 12.3
" HÜRTHLE CELL ADENOMA	1	3.02

Protein Content of the Human Thyroid Gland.

The protein content of the supernatant fraction of normal thyroid tissue is 106 ± 53 mg. per g. wet weight of tissue, of thyrotoxic tissue is 104 ± 26 mg. per g. wet weight and of adenomatous tissue is 97 ± 41 mg. per g. wet weight. The protein contents of the supernatant fractions of the carcinoma and Hashimoto series are significantly different from normal at the 1% and 0.5% levels respectively, the values being 57.9 ± 14.9 and 40.6 ± 10.3 mg. per g. wet weight.

The concentrations of protein in the M1 fractions of human thyroid tissues are as follows; normal 4.4 ± 1.7, thyrotoxic 3.5 ± 1.7, adenomatous 3.8 ± 2.0, carcinomatous 6.5 ± 2.9 and Hashimoto 5.6 ± 2.9 mg. per g. wet weight of tissue, none of the pathological tissue series having values significantly different from normal.

In the M2 fraction of thyroid gland the protein content of normal tissue is 4.8 ± 3.5 mg. per g. wet weight and of thyrotoxic tissue is 3.4 ± 1.4 mg. per g. wet weight. This value is significantly lower than normal at the 5% level. None of the other series have values differing from normal, the results being, in the adenoma series 3.4 ± 1.9 mg. per g. wet weight of tissue, in the carcinoma series 8.0 ± 1.7 mg. per g. wet weight and in the Hashimoto series 5.6 ± 2.4 mg. per g. wet weight.

ENZYME ACTIVITIES IN THE HUMAN CERVIX UTERI.

The enzyme results of the human cervix uteri are divided into sections dealing with, 1) a comparison between the supernatant fractions of the normal and carcinomatous cervix uteri, there being inadequate particulate material for analysis in normal cervical tissue, and 2) a comparison of the samples of carcinoma of cervix taken from patients before and after radiation. This section is divided into three parts describing the effects of radiation on, 2a) the supernatant fraction, 2b) the M1 fraction and 2c) the M2 fraction. The series of carcinoma specimens with which the normal series is compared is larger than that with which the biopsies taken after radiation are compared. This is due to the fact that certain of the patients receiving radiation did not have a second biopsy taken.

The results are again expressed as the mean of each group ± the standard deviation, and the results related to the weight of the tissue sample are tabulated on different pages from those related to protein content. Since the amounts of this tissue available were very small a list of the weights of tissue samples used is again presented, in order to give some estimate of the size of samples on which the conclusions are based.

1) Supernatant Fraction of the Normal and Carcinoma Series. (Tables 21 - 24)

The enzyme pattern of the supernatant fraction of these two series is the same as that of the thyroid gland, i.e. LDH > IDH > PGDH, excepting four samples of carcinoma which have the pattern LDH > PGDH > IDH. These patterns are the same when the results are expressed either in units per g.

wet weight or per mg. protein (Tables 21, 23).

Certain of the carcinoma specimens were classed histologically as being more malignant than others, but these differences were not linked with higher enzyme activities in all cases. One very malignant specimen had a very high LDH activity but another specimen with even higher LDH activity was classed as only moderately malignant.

When the carcinoma series was divided into two groups, those with a second specimen after irradiation and those without, the two series were found to differ. When the results were expressed as units per g. wet weight, the IDH activity and the PGDH activity were significantly higher at the 2% level in the group which had a second specimen taken, and were usually more advanced carcinomas, although some very malignant samples also did not have a second biopsy taken. The normal series was compared throughout with the combined group in order to obtain a comparison with the full range of carcinoma of cervix.

LDH activity in the carcinoma series is significantly higher than normal, at the 0.1% level when the results are expressed as units per g. wet weight and at the 0.2% level when the results are expressed as units per mg. protein. The results show a very high variance (Tables 22, 24).

IDH activity and PGDH activity in the carcinoma series are significantly higher than normal, at the 0.1% level when the results are expressed either in units per g. wet weight or in units per mg. protein (Tables 22, 24).

The protein content of the supernatant fraction of normal cervix

TABLE 21. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN CERVIX UTERI.

mμmoles substrate transformed/min./g. wet weight of tissue, at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	15	2900 ± 1690	690 ± 320	210 ± 140
CARCINOMA	23	6860 ± 7780	1820 ± 1050	1120 ± 560

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF NORMAL AND CARCINOMATOUS HUMAN CERVIX UTERI.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V CARCINOMA	15 + 23	6.07	<0.001	236	12.6	<0.001	264	18.5	<0.001	545

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per g. wet weight of tissue.

TABLE 22.

TABLE 23. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN CERVIX UTERI.

mμmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	15	102 ± 41	24.6 ± 9.7	11.2 ± 6.1
CARCINOMA	23	140 ± 125	36.7 ± 17.4	29.0 ± 22.9

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF NORMAL AND CARCINOMATOUS HUMAN CERVIX UTERI.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V CARCINOMA	15 + 23	3.49	< 0.002	139	7.63	<0.001	149	9.30	<0.001	259

$$* \% = \frac{\text{Mean of abnormal results x 100}}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per mg. protein.

TABLE 24.

uteri is 28.0 ± 7.5 mg. per g. wet weight of tissue and of carcinoma of cervix uteri is 46.3 ± 34.2 mg. per g. wet weight, which value is significantly higher than normal at the 5% level.

2a) Effects of Radiation on the Supernatant Fraction of Carcinoma of the Human Cervix Uteri (Tables 25, 26).

The supernatant fraction enzyme patterns of the post-radiation carcinoma series fall into two groups, 10 having LDH > IDH > PGDH and 4 having LDH > PGDH > IDH. Whichever pattern occurs, does so when the results are expressed either in units per g. wet weight or per mg. protein.

LDH activity is significantly lower after radiation, at the 5% level, when the results are expressed either in units per g. wet weight or per mg. protein (Tables 21, 22). IDH activity is significantly lower after radiation, at the 1% level, when the results are expressed in units per g. wet weight, but there is no significant difference when the results are expressed per mg. protein (Tables 21, 22). PGDH activity is significantly lower after radiation, at the 0.1% level when the results are expressed in units per g. wet weight, but there is no significant difference when the results are expressed in units per mg. protein (Tables 25, 26).

The protein content of the supernatant fraction of cervical tissue is lowered significantly at the 2% level after radiation from a mean value of 59.9 mg. per g. wet weight to a mean value of 32.9 mg. per g. wet weight. The percentage protein content of the supernatant fraction of the total protein of the three fractions is significantly lowered at the 5% level after radiation, from 77.2% to 67.1%.

TABLE 25. COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./g. wet weight of tissue at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	9180	1600-32100	2.5	<0.05
	After Radiation	3420	575-10400		
IDH	Before Radiation	2220	414-3930	3.13	<0.01
	After Radiation	1170	125-4670		
PGDH	Before Radiation	1330	336-2290	5.45	<0.001
	After Radiation	746	266-1730		

14 pairs of samples were processed.

TABLE 26. COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./mg. protein at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	166	46-531	2.26	<0.05
	After Radiation	99	40-203		
IDH	Before Radiation	40.6	13.5-94.7	1.47	<0.25
	After Radiation	34.3	7.7-97.6		
PGDH	Before Radiation	26.2	8.4-46.7	0.40	-
	After Radiation	43.4	8.9-55.5		

14 pairs of samples were processed.

2b) Effect of Radiation on the M1 Fraction of Human Cervix Uteri.(Tables 27,28.)

In the pre-radiation carcinoma series, 1 out of 7 samples has the enzyme pattern LDH > PGDH > IDH. In the other samples LDH is the most active enzyme , but in two specimens no IDH activity was detected, and in these and in one other specimen no PGDH activity was detected. These patterns occur when the results are expressed either in units per g. wet weight or per mg. protein.

In the post-radiation carcinoma series, 1 out of 7 samples has the pattern LDH > PGDH > IDH and this specimen before radiation had the more usual pattern of LDH > IDH > PGDH. 4 out of the 7 samples had no detectable IDH activity and 5 had no detectable PGDH activity.

LDH activity is significantly increased after radiation at the 5% level when the results are expressed in units per g. wet weight, but there is no significant difference between the two groups when the results are expressed in units per mg. protein (Tables 27,28.).

There is no significant difference between the two groups in IDH or PGDH activity when the results are expressed either in units per g. wet weight or per mg. protein (Tables 27, 28.).

There is no significant difference in the protein content of the M1 fraction after radiation, mean values 8.7, and 9.6 mg. per g. wet weight of tissue, but there is a significant increase at the 1% level in the percentage of the total cytoplasmic protein occurring in the M1 fraction, from 12.4% to 18.2%.

TABLE 27. COMPARISON OF ENZYME ACTIVITIES IN THE M1 FRACTION OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./g. wet weight of tissue at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	99	51-184	2.62	<0.05*
	After Radiation	109	52-215		
IDH	Before Radiation	18.6	0-68.6	1.84	<0.25
	After Radiation	6.3	0-22.2		
PGDH	Before Radiation	13.1	0-54.1	0.29	-
	After Radiation	10.7	0-46.7		

7 pairs of results were obtained.

* A significant INCREASE is indicated.

TABLE 28. COMPARISON OF ENZYME ACTIVITIES IN THE M1 FRACTION OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./mg. protein at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	12.1	3.9-17.4	1.02	-
	After Radiation	14.0	6.8-27.1		
IDH	Before Radiation	2.4	0-5.8	1.18	-
	After Radiation	0.5	0-1.9		
PGDH	Before Radiation	1.4	0-4.7	1.24	-
	After Radiation	1.4	0-5.6		

7 pairs of results were obtained.

3e) Effect of Radiation on the M2 Fraction of Human Cervix Uteri (Tables 29, 30).

In the pre-radiation carcinoma series, 4 out of 7 samples have the enzyme pattern LDH > PGDH > IDH, 1 has IDH > PGDH > LDH, and 2 have LDH > IDH > PGDH.

In the post-radiation carcinoma series, 4 out of 7 samples have the enzyme pattern LDH PGDH IDH and 3 of these had this pattern before radiation also. The other three samples had no detectable IDH or PGDH activity. These patterns occur both when the results are expressed in units per g. wet weight or per mg. protein.

There is no significant difference between the two series in any of the enzymes studied when the results are expressed either as units per g. wet weight or per mg. protein (Tables 29, 30).

Neither the protein content (mean values 8.9 and 8.3 mg. per g. wet weight of tissue) nor the percentage protein content (11.4% and 14.5%) of the M2 fraction is altered by radiation.

TABLE 29. COMPARISON OF ENZYME ACTIVITIES IN THE M2 FRACTION OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./g. wet weight of tissue at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	154	11-532	0.76	-
	After Radiation	106	29-286		
IDH	Before Radiation	29.0	0-151		
	After Radiation	10.6	0-56.0	1.28	<0.25
PGDH	Before Radiation	16.3	0-34.1	0.51	-
	After Radiation	24.3	0-114		

7 pairs of results were obtained.

TABLE 30. COMPARISON OF ENZYME ACTIVITIES IN THE M2 FRACTION OF CARCINOMA OF HUMAN CERVIX UTERI BEFORE AND AFTER TREATMENT BY RADIATION.

mμmoles substrate transformed/min./mg. protein at 25°.

		MEAN	RANGE	t	P
LDH	Before Radiation	31.9	4.5–155	0.95	-
	After Radiation	12.6	8.2–19.8		
IDH	Before Radiation	2.4	0–10.2	1.01	-
	After Radiation	1.0	0–3.9		
PGDH	Before Radiation	2.3	0–4.2	0.19	-
	After Radiation	2.3	0–5.6		

7 pairs of results were obtained.

TABLE 31. RANGE OF WEIGHTS OF SAMPLES OF HUMAN CERVIX UTERI.

	Number of samples	Weight in g.
NORMAL	15	0.35 - 7.61
CARCINOMA Before Radiation	23	0.045 - 2.58
CARCINOMA After Radiation	14	0.015 - 1.67

ENZYME ACTIVITIES OF THE SUPERNATANT FRACTION OF HUMAN MAMMARY GLANDS.

Adequate amounts of particulate material could not be obtained from the human mammary gland specimens, and therefore, results for the supernatant fraction only are presented. The types of tissue studied were normal breast, chronic mastitis (a hyperplastic condition), fibroadenoma and carcinoma. The results are reported in tabular form as before, being related to weight of tissue and protein content of the supernatant fraction and expressed as the mean of each group ± the standard deviation. The range of weights of the tissue samples is also listed, in order to give some index of the representative nature of the specimens.

The supernatant enzyme pattern is the same as that of thyroid gland (LDH > IDH > PGDH) in all the breast samples studied except for two samples of chronic mastitis, in which the pattern is LDH PGDH IDH.

LDH activity is significantly higher than normal in the carcinoma series at the 5% level when the results are expressed in units per g. wet weight, but is not significantly different when the results are expressed in units per mg. protein. There is no significant difference from normal in the LDH activity of either the fibroadenoma or the chronic mastitis series (Tables 33,35.).

IDH activity is significantly higher than normal at the 2% level in the chronic mastitis series, when the results are expressed in units per mg. protein, but there is no significant difference when the results are epxressed per g. wet weight. IDH activity is significantly higher than normal in the carcinoma series, at the 1% level both when the results are expressed in units per g. wet weight or per mg. protein. There is no significant difference from normal in the fibroadenoma

TABLE 32. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN MAMMARY GLANDS.

μmoles substrate transformed/min./g. wet weight of tissue at 25°.

	NUMBER OF SAMPLES	LDH	ICDH	PGDH
NORMAL	5	1250 ± 790	230 ± 140	91 ± 23
CHRONIC MASTITIS	5	920 ± 260	430 ± 230	48 ± 36
FIBROADENOMA	5	1890 ± 920	480 ± 340	51 ± 33
CARCINOMA	5	3610 ± 820	1110 ± 580	191 ± 117

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN MAMMARY GLANDS.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V CHRONIC MASTITIS	5 + 5	0.91	-	-	0.16	-	-	1.52	< 0.25	-
NORMAL V FIBROADENOMA	5 + 5	1.12	-	-	1.95	< 0.1	-	0	-	-
NORMAL V CARCINOMA	5 + 5	2.67	< 0.05	208	4.01	< 0.01	480	2.67	< 0.05	373

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}} \quad \text{where } P < 0.05$$

Calculations carried out on activities per g. wet weight of tissue.

TABLE 33.

TABLE 34. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN MAMMARY GLANDS.

μmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	46 ± 34	10 ± 6	2.3 ± 1.4
CHRONIC MASTITIS	5	60 ± 10	28 ± 12	28 ± 37
FIBROADENOMA	5	87 ± 48	21 ± 14	2.3 ± 1.9
CARCINOMA	5	115 ± 74	41 ± 16	7.0 ± 2.3

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN MAMMARY GLANDS.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V CHRONIC MASTITIS	5 + 5	0.83	-	-	2.92	<0.02	271	1.59	<0.25	-
NORMAL V FIBROADENOMA	5 + 5	1.51	<0.25	-	1.60	<0.25	-	0	-	-
NORMAL V CARCINOMA	5 + 5	1.85	<0.25	-	3.92	<0.01	400	3.77	<0.01	300

$$* \% = \frac{\text{Mean of abnormal results x 100}}{\text{Mean of normal results}}$$ where P 0.05

Calculations carried out on activities per mg. protein.

TABLE 35.

series (Tables 33,35.).

PGDH activity expressed in units per g. wet weight is not significantly different from normal in either the fibroadenoma or the chronic mastitis series, both when the results are expressed in units per g. wet weight and per mg. protein. PGDH activity is significantly higher than normal in the carcinoma series, at the 5% level when the results are expressed in units per g. wet weight and at the 1% level when the results are expressed in units per mg. protein (Tables 33,35.).

The protein contents per g. wet weight of the supernatant fractions of the fibroadenoma and carcinoma series are not significantly different from normal, but that of the chronic mastitis series is significantly lower at the 1% level, the values being, normal breast 21.9 $\pm$ 2.5 mg. per g. wet weight of tissue, fibroadenoma 22.1 $\pm$ 4.9, carcinoma 27.4 $\pm$ 14.9 and chronic mastitis 15.4 $\pm$ 3.2 mg. per g. wet weight.

TABLE 36. RANGE OF WEIGHTS OF SAMPLES OF HUMAN MAMMARY TISSUE.

	Number of samples	Weight in g.
NORMAL	5	0.58 - 5.03
CHRONIC MASTITIS	5	1.82 - 8.78
FIBROADENOMA	5	4.84 - 10.5
CARCINOMA	5	2.40 - 14.6

COMPARISON OF THE ENZYME ACTIVITIES OF THE SUPERNATANT FRACTIONS OF HUMAN THYROID, UTERINE CERVICAL AND MAMMARY TISSUE.

In this section the results reported in detail for the supernatant fractions of each tissue are summarised and compared in Tables 37, 38, and 39. The particulate fraction data are too incomplete, and have too slight an activity to merit this treatment.

The least active of the three normal tissues studied is breast tissue. Expressing the mean activities per g. wet weight of the other two normal tissues as a percentage of the mean activity of normal breast tissue for each enzyme, we find for normal cervix uteri and thyroid gland activities for LDH of 230% and 490%, for IDH of 300% and 380%. and for PGDH of 410% and 590% respectively.

An alternative method of comparison which includes the abnormal tissues as well as the normal tissues was formulated, the mean of each abnormal type being expressed as a percentage of the mean of the normal series of that tissue. LDH activity shows a clear increase in the carcinoma and Hashimoto series of thyroid tissue. In cervical tissue a clear increase is again obvious in the carcinoma series and a decrease after radiation is evident. In breast tissue the carcinoma series only has much higher activity than the normal series. IDH and PGDH activities show the same overall pattern as LDH activity.

THE MEAN SUPERNATANT LACTATE DEHYDROGENASE ACTIVITY OF ABNORMAL TISSUES

EXPRESSED AS A PERCENTAGE OF THE MEAN NORMAL VALUE FOR THAT TISSUE.

CLASSIFICATION	THYROID TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	CERVIX TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	BREAST TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*
HYPERPLASTIC	Toxic	147	152	-	-	-	Chronic mastitis	74	130
BENIGN NEOPLASM	Adenoma	113	125	-	-	-	Fibroadenoma	151	189
MALIGNANT NEOPLASM	Carcinoma	351	492	Carcinoma	236	139	Carcinoma	208	250
OTHER	Hashimoto	148	341	Post Radiation Carcinoma	118	97	-	-	-

* per g. wet weight and per mg. Protein.

TABLE 37.

THE MEAN SUPERNATANT ISOCITRATE DEHYDROGENASE ACTIVITY OF ABNORMAL TISSUES

EXPRESSED AS A PERCENTAGE OF THE MEAN NORMAL VALUE FOR THAT TISSUE.

CLASSIFICATION	THYROID TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	CERVIX TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	BREAST TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*
HYPERPLASTIC	Toxic	170	199	-	-	-	Chronic mastitis	187	280
BENIGN NEOPLASM	Adenoma	147	185	-	-	-	Fibroadenoma	208	210
MALIGNANT NEOPLASM	Carcinoma	294	421	Carcinoma	264	149	Carcinoma	480	400
OTHER	Hashimoto	257	667	Post Radiation Carcinoma	170	139	-	-	-

* per g. wet weight and per mg. Protein.

TABLE 38.

THE MEAN SUPERNATANT PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY OF ABNORMAL TISSUES EXPRESSED AS A PERCENTAGE OF THE MEAN NORMAL VALUE FOR THAT TISSUE.

CLASSIFICATION	THYROID TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	CERVIX TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*	BREAST TISSUE	ACTIVITY per g.WW	ACTIVITY per mg.P*
HYPERPLASTIC	Toxic	320	152	-	-	-	Chronic mastitis	94	1210
BENIGN NEOPLASM	Adenoma	193	247	-	-	-	Fibroadenoma	100	100
MALIGNANT NEOPLASM	Carcinoma	367	352	Carcinoma	545	259	Carcinoma	373	300
OTHER	Hashimoto	193	444	Post Radiation Carcinoma	355	386	-	-	-

* per g. wet weight and per mg. Protein.

TABLE 39.

THE NUCLEIC ACID SERIES OF HUMAN THYROID GLANDS.

The normal thyroid tissue controls for this series were obtained post-mortem, and the pathological samples at operation. The results are divided into various sections dealing with (a) enzyme activities, (b) RNA content, (c) DNA phosphorus content, (d) lipid phosphorus content, (e) protein content and (f) the RNA to DNA phosphorus ratio of the tissues studied.

In section (a), the results are further divided into two parts, namely, 1) the comparison between the supernatant fractions of the normal thyroid and the adenoma and thyrotoxic series, and 2) the comparison between the two series, i.e. the Nucleic Acid Series, and the Main Series of each type of tissue. The tables are drawn up according to the same format as that used in the preceding sections.

Section (b) describes the results obtained for the RNA content of the three types of tissue.

In section (c), the DNA phosphorus content of the thyroid tissues is reported and an evaluation of the Ceriotti method of DNA estimation in thyroid tissue is made.

Section (d) reports the results of the estimation of the lipid extracted phosphorus of the tissues which is a measure of their phospholipid content.

The protein contents of the tissues recorded in section (e) are presented in two forms, firstly the results are related to the weight of the tissue sample, and secondly to the DNA content of the tissue sample. This provides an interesting contrast between protein content

per unit weight of tissue and protein content per cell.

In section (f), the mean RNA to DNA phosphorus ratio for each tissue is reported, in order to determine whether the RNA content per cell of thyrotoxic tissue is higher than it is in normal tissue.

(a) Enzyme Results. 1) Supernatant Fraction of the Thyrotoxic and Adenoma Series compared with the Normal Series (Tables 40-45).

The enzyme pattern LDH > IDH > PGDH observed in the Main series occurs again in the Nucleic Acid series.

There is no significant difference between the normal and adenoma series in any of the enzymes measured, when the results are expressed in units per g. wet weight or per mg. protein or per mg. DNA phosphorus.

In thyrotoxic tissue LDH activity is significantly higher than normal, at the 2% level when the results are expressed as units per g. wet weight and at the 1% level with the results as units per mg. protein. IDH activity is significantly higher than normal at the 2% level when the results are expressed in units per g. wet weight, but there is no significant difference when the results are expressed as units per mg. protein. PGDH activity is significantly higher than normal at the 0.1% level when the results are expressed per g. wet weight and at the 1% level when they are expressed per mg. protein. There is no significant difference from normal in any of the enzyme activities in thyrotoxic tissue when the activities are expressed per mg. DNA phosphorus.

TABLE 40. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

mμmoles substrate transformed/min./g. wet weight of tissue, at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	5990 ± 680	1170 ± 430	470 ± 110
TOXIC	5	11600 ± 1400	1840 ± 330	1170 ± 370
ADENOMA	5	4410 ± 1120	770 ± 280	340 ± 90

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	5 + 5	4.50	0.02	194	3.14	0.02	157	4.75	0.001	249
NORMAL V ADENOMA	5 + 5	0.94	-	-	0.99	-	-	1.55	0.25	-

$$* \% = \frac{\text{Mean of Abnormal results x 100}}{\text{Mean of normal results}}$$ where P 0.05

Calculations carried out on activities per g. wet weight of tissue.

TABLE 41.

TABLE 42. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

mμmoles substrate transformed/min./mg. protein at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	67.2 ± 25.6	13.1 ± 8.2	5.1 ± 0.7
TOXIC	5	108 ± 21.3	17.4 ± 12.6	11.2 ± 4.2
ADENOMA	5	43.0 ± 14.5	7.7 ± 5.3	3.3 ± 1.4

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	5 + 5	3.61	0.01	161	1.41	0.25	-	3.83	0.01	220
NORMAL V ADENOMA	5 + 5	2.08	0.1	-	2.05	0.1	-	0.79	-	-

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}}$$ where P 0.05

Calculations carried out on activities per mg. protein.

TABLE 43.

TABLE 44. ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

mμmoles substrate transformed/min./mg. DNA Phosphorus at 25°.

	NUMBER OF SAMPLES	LDH	IDH	PGDH
NORMAL	5	32200 ± 22600	5750 ± 4250	2240 ± 1240
TOXIC	5	38200 ± 4550	6030 ± 2810	3880 ± 1120
ADENOMA	5	24500 ± 2800	4250 ± 1150	1870 ± 330

Results expressed as Mean ± Standard Deviation.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTION OF HUMAN THYROID GLANDS - NUCLEIC ACID SERIES.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL V TOXIC	5 + 5	0.59	-	-	0.15	-	-	2.18	0.1	-
NORMAL V ADENOMA	5 + 5	2.28	0.1	-	1.77	0.25	-	1.17	-	-

$$* \% = \frac{\text{Mean of abnormal results} \times 100}{\text{Mean of normal results}}$$ where P 0.05

Calculations carried out on activities per mg. DNA Phosphorus.

TABLE 45.

(a) Enzyme Results. 2) Comparison between the Main and Nucleic Acid series of normal, adenomatous and thyrotoxic thyroid tissue (Tables 46, 47).

In the normal thyroid gland there is no significant difference in LDH or IDH activity between the two series. However, when the PGDH activity is expressed in units per g. wet weight, there is a significant difference at the 1% level between the two series, the Nucleic Acid series having the higher values. There is no significant difference when the results are expressed as units per mg. protein.

There is no significant difference between the two thyrotoxic groups in any of the enzymes studied.

There is a significant difference at the 5% level between the two adenoma series in LDH activity, the Nucleic Acid series having the lower values, when the results are expressed in units per mg. protein, but there is no significant difference when the results are expressed in units per g. wet weight. There is also a significant difference in IDH activity at the 1% level, both when the results are expressed per g. wet weight and per mg. protein, the Nucleic Acid series again having the lower values. There is no significant difference in PGDH activity between the two adenoma groups.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF THE MAIN AND NUCLEIC ACID SERIES OF HUMAN THYROID GLAND.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL	11 + 5	0.11	-	-	1.33	0.25	-	3.12	0.01	62
TOXIC	20 + 5	1.46	-	-	0.73	-	-	1.00	-	-
ADENOMA	10 + 5	1.14	-	-	3.08	0.01	167	0.81	-	-

$$* \% = \frac{\text{Mean of nucleic acid series result} \times 100}{\text{Mean of main series result}}$$ where P 0.05

Calculations carried out on activities per g. wet weight of tissue.

TABLE 46.

COMPARISON OF ENZYME ACTIVITIES IN THE SUPERNATANT FRACTIONS OF THE MAIN AND NUCLEIC ACID SERIES OF HUMAN THYROID GLAND.

	NUMBER OF SAMPLES	LDH			IDH			PGDH		
		t	P	%*	t	P	%*	t	P	%*
NORMAL	11 + 5	0.65	-	-	0.86	-	-	1.70	0.25	-
TOXIC	20 + 5	0.83	-	-	0.03	-	-	0.49	-	-
ADENOMA	10 + 5	2.26	0.05	193	3.74	0.01	200	0.87	-	-

$$* \% = \frac{\text{Mean of nucleic acid series result} \times 100}{\text{Mean of main series result}}$$ where P 0.05

Calculations carried out on activities per mg. protein.

(b) Estimation of RNA in the Human Thyroid Gland.

The RNA content of the tissue fractions was estimated as described in the Material and Methods section, and a spectrum was plotted for each fraction obtained. The minimum absorption was found to lie between 235 mμ and 242 mμ, which indicated (Fleck and Begg (1965)) that there was a certain amount of protein contamination (Figure 5). The protein content of the tissue fractions was determined by the Lowry method, and the tissue RNA extracts gave values ranging from 16 - 40 μg. per ml. A correction was applied to the extinction at 260 mμ in order to compensate for that due to the protein. The correction factor applied to each solution was given by Fleck and Begg (1965) and is equivalent to an extinction of 0.0017 per μg. protein per ml. of solution.

The RNA content of normal thyroid tissue is 0.92 ± 0.22 mg. per g. wet weight of tissue. The RNA content of adenoma tissue is 0.68 ± 0.15 mg. per g. wet weight of tissue, which value is not significantly different from that of the normal series. The RNA content of thyrotoxic tissue is 1.68 ± 0.23 mg. per g. wet weight of tissue, which value is significantly higher than normal at the 0.1% level.

(c) Estimation of DNA in the Human Thyroid Gland.

In preliminary experiments, DNA estimations were carried out using the colorimetric method of Ceriotti (1952). Together with each batch of test solutions standard solutions and a reagent blank were run, and the amount of DNA in the test solutions was calculated by reference to the extinction of the standard, the phosphorus content

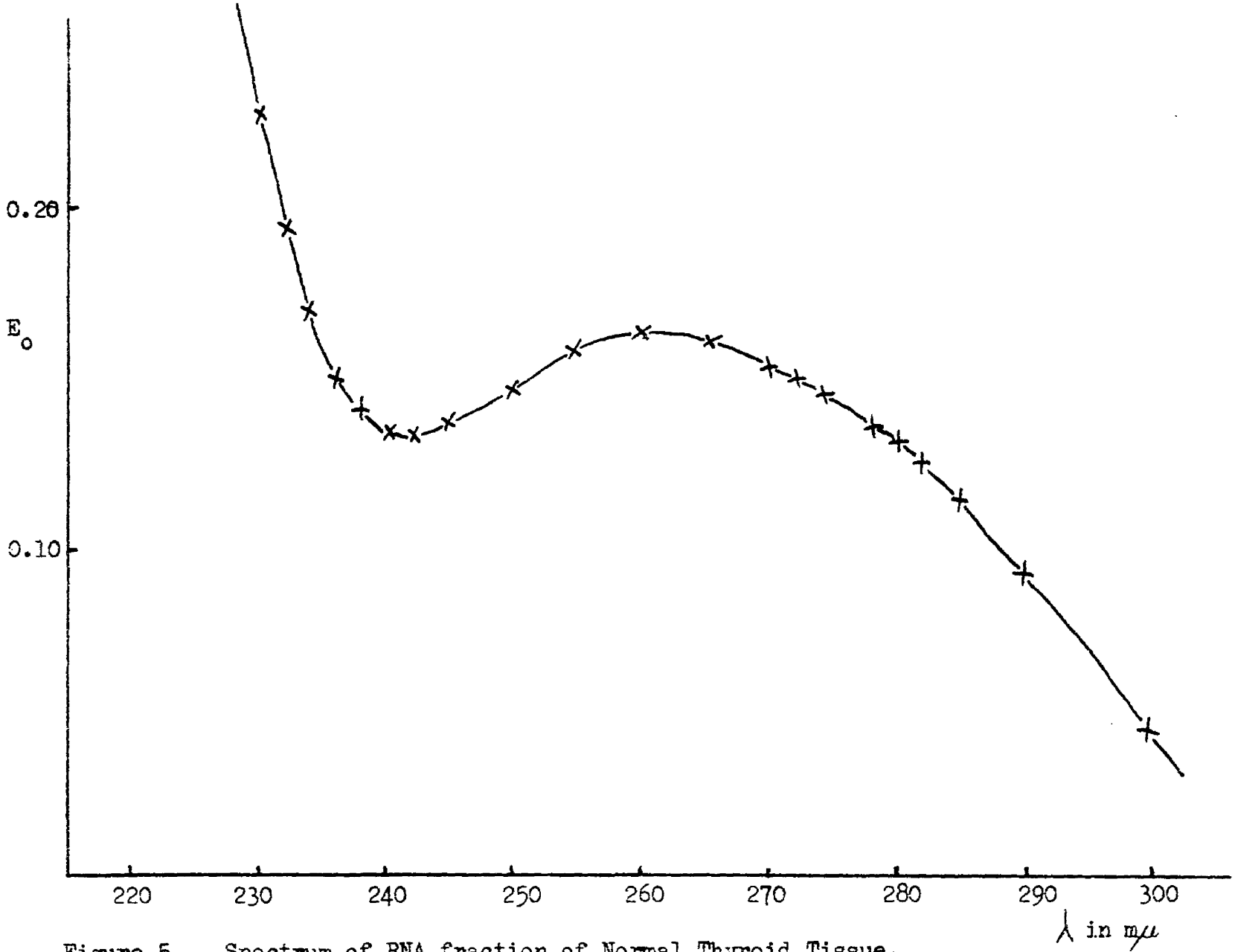

Figure 5. Spectrum of RNA fraction of Normal Thyroid Tissue.

of which had been previously determined.

Several features of the estimation were considered to be unsatisfactory. These were, firstly the poor reproducibility shown by the duplicate analyses of the test material and the standard (Table 48) and secondly the difference between the spectrum of the test material and the standard (Figure 6). This difference was best expressed by considering the ratio E_{490}/E_{460}. In solutions obtained from the standard DNA the ratio gave values between 2.13 and 2.47, whereas in the solutions obtained from the normal tissue fractions the ratio gave values between 1.40 and 1.63.

It was thought that there might be a certain amount of colour produced during the Ceriotti reaction by the interaction of the tissue constituents with the hydrochloric acid. An acid blank was therefore carried out for each test solution to be estimated, and consisted of the test solution, distilled water in the place of the indole solution and hydrochloric acid. When the results obtained for the acid blank were subtracted from those of the Ceriotti reaction, it was found that although the actual values were lower the ratio E_{490}/E_{460} was higher, giving values for normal tissue of 1.70 - 1.77, for adenoma tissue of 1.51 - 1.90 and for thyrotoxic tissue of 2.02 - 2.12.

Another modification was introduced in which the stage of partial alkaline hydrolysis was extended to 18 hours so that as much as possible of the tissue protein would be eliminated from the DNA fraction. This brought about a sharpening of the peak due to DNA and a lessening of the subsidiary peak, thus improving

TABLE 48. EXTINCTIONS OBTAINED FROM DUPLICATE ANALYSES OF THE CERIOTTI REACTION ON VARIOUS MATERIALS.

Tissue Fraction 1	0.135		0.160	
Tissue Fraction 2	0.126		0.144	
Tissue Fraction 3	0.141		0.219	
Tissue Fraction 4	0.137		0.221	
Standard Calf	0.209	0.212	0.113	0.148
Thymus DNA	0.155	0.147	0.151	0.116
	0.170	0.224		
Thyroid Tissue	0.198	0.216	0.227	0.217
DNA	0.180	0.202	0.220	0.231
	0.221	0.184		

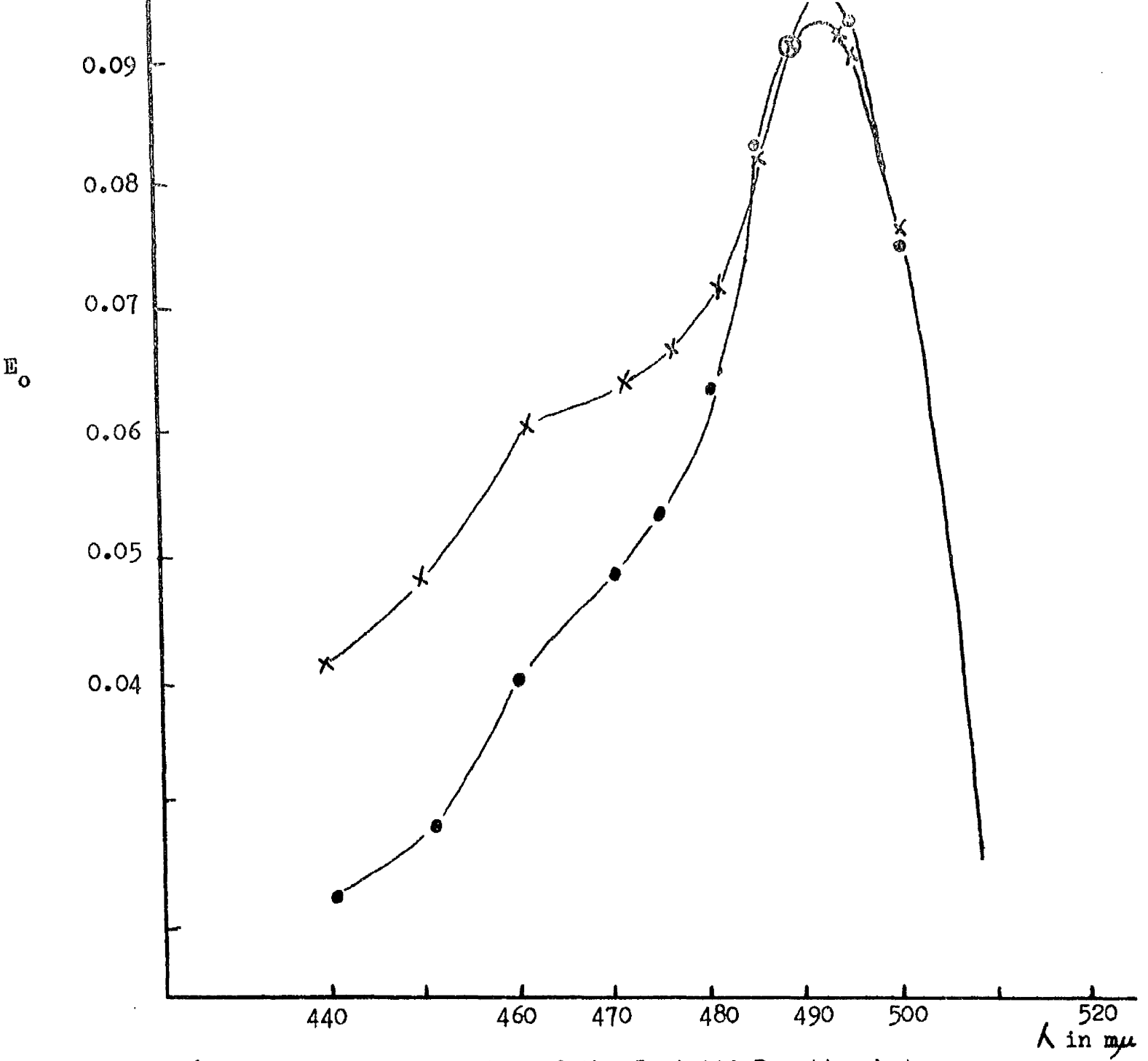

Figure 6. Comparison of Spectra of the Ceriotti Reaction between a Standard Solution and Normal Thyroid DNA Fraction.

x Normal tissue sample $\frac{E_{490}}{E_{460}} = 1.57$

o Standard calf thymus DNA $\frac{E_{490}}{E_{460}} = 2.29$

the actual value obtained, and the ratio E_{490}/E_{460} (Figure 7). For normal tissue the ratio gave values between 1.75 and 1.85. When both modifications were applied to normal tissue the ratio gave values between 1.83 and 1.97 and in adenoma tissue 1.89 - 2.16.

These two modifications were applied to all subsequent analyses on the preliminary series and the results obtained in the tissue fractions were acceptable, although they did not show the high degree of reproducibility which was possible with the phosphorus estimations.

The possibility that the poor duplication of estimations carried out on the standard solution might be due to incomplete hydrolysis was investigated by treating the standard solution in various ways, these beings the complete procedure for tissue preparation, the tissue preparation after the stage of RNA separation, and hydrolysis by hot perchloric acid. The extinction at 490 mμ in the Ceriotti reaction was measured and the results gave an interesting picture. The standard solution which had been hydrolysed by the perchloric acid gave much higher values than the others, but when the reactions were repeated on the following day on the same solutions, the values obtained were quite different (Table 49). In order to verify one or other of the two sets of results, the complete experiment was repeated, but the results obtained were scattered over a very wide range, and the duplicate estimates on the same solutions were not acceptable (Table 49).

It was then thought that the highly polymerised DNA standard

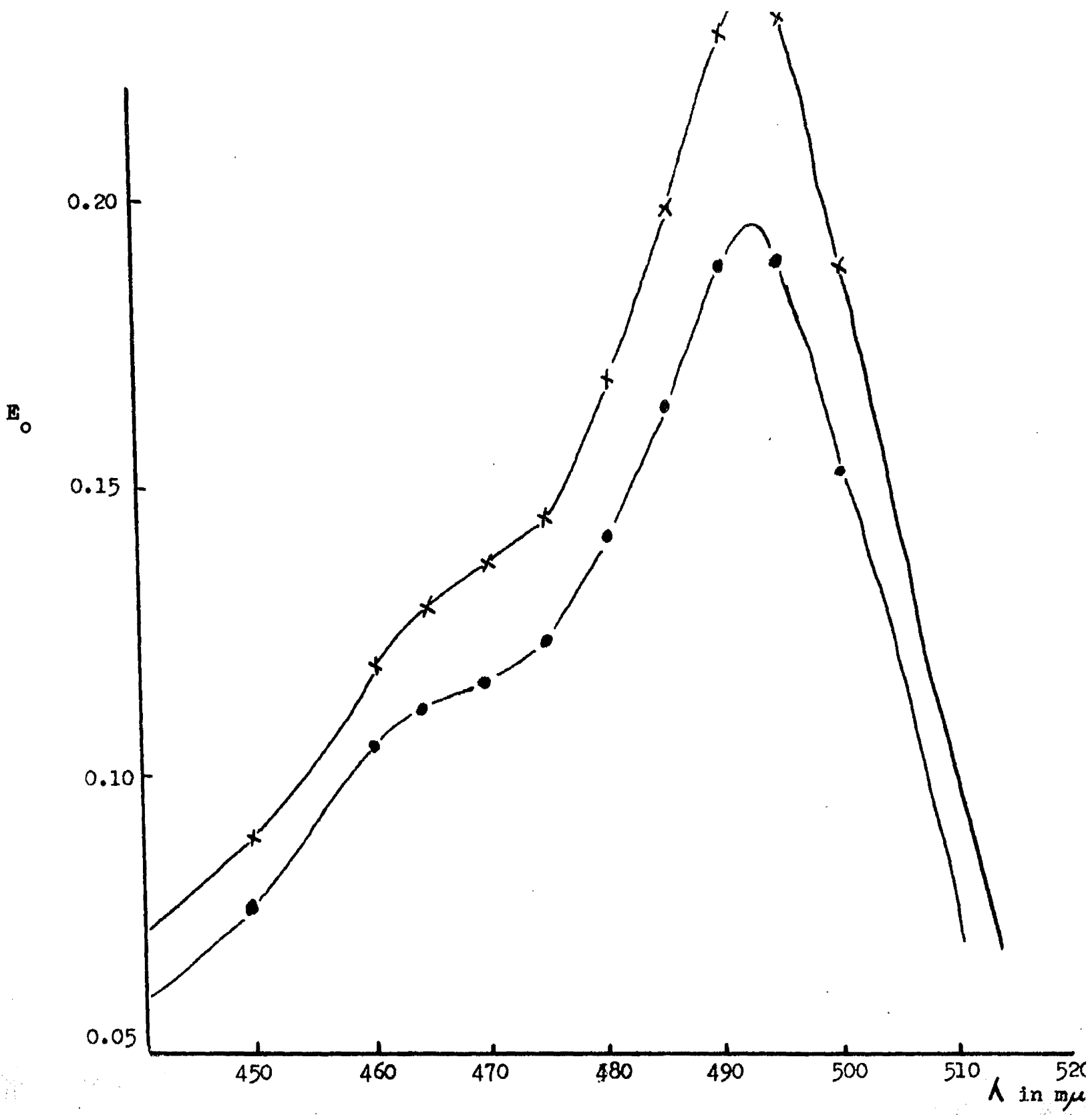

Figure 7. Comparison of Spectra of Ceriotti Reaction of Normal Tissue DNA Fraction after 1 hr. and 18 hr. Partial Alkaline Hydrolysis. (Acid Blanks subtracted)

		Acid Blank	No Acid Blank
x 18 hrs.	$\frac{E_{490}}{E_{460}}$	1.97	1.85
o 1 hr.	$\frac{E_{490}}{E_{460}}$	1.75	1.63

TABLE 49. EFFECT OF VARYING TREATMENTS ON THE EXTINCTION PRODUCED BY THE CERIOTTI REACTION ON CALF THYMUS DNA.

	Day 1	Day 2	Day 3
Complete Tissue Preparation	0.197	0.165	0.264
Procedure	0.187	0.220	0.226
1 Hour Alkaline Hydrolysis	0.168	0.188	0.246
	0.202	0.260	0.255
18 Hours Alkaline Hydrolysis	0.199	0.182	0.247
	0.205	0.245	0.273
Digestion by hot Perchloric Acid	0.336	0.199	0.247
	0.344	0.213	0.238

might not be suitable for estimation by this micromethod, and that a standard solution derived from the tissue itself might be more suitable. A large portion of thyroid gland was processed in the normal way to give a concentrated DNA fraction. This solution was standardised against phosphorus with perfectly satisfactory results, but again poor duplication was obtained using the Ceriotti method (Table 48).

Various other experiments were also carried out to try to improve the duplication of the method. These proved, firstly that the poor results were not caused by deterioration of the reagents, secondly that varying amounts of light did not affect the results, and thirdly that different methods of washing the reaction tubes did not produce any improvement in the duplication of results.

The DNA content of the tissues in the Nucleic Acid series was therefore estimated by the phosphorus content of the tissues after lipid extraction.

The DNA Phosphorus content of normal thyroid tissue is 0.230 ± 0.063 mg. per g. wet weight of tissue. The DNA Phosphorus content of adenoma tissue is 0.179 ± 0.040 mg. per g. wet weight of tissue, which value is not significantly different from that of the normal series. The DNA Phosphorus content of thyrotoxic tissue is 0.385 ± 0.064 mg. per g. wet weight of tissue, which result is significantly higher than normal at the 1% level.

(d) Estimation of Lipid Phosphorus in Human Thyroid Glands.

The lipid phosphorus content of normal thyroid tissue is 0.225 ± 0.055 mg. per g. wet weight of tissue. The lipid phosphorus content of adenoma tissue is 1.71 ± 0.039 mg. per g. wet weight of tissue, which is not significantly different from normal. The lipid phosphorus content of thyrotoxic tissue is 0.385 ± 0.064 mg. per g. wet weight of tissue, which is significantly higher than normal at the 0.2% level.

(e) Estimation of Total Protein in Human Thyroid Glands.

The total protein content of each sample was obtained by the addition of the protein content of the RNA fraction of tubes 1 and 2 to the protein content of the DNA fraction of tubes 1 and 2. The total protein content of normal thyroid tissue is 140 ± 16 mg. per g. wet weight of tissue. The total protein content of adenoma tissue is 115 ± 28 mg. per g. wet weight of tissue, and of thyrotoxic tissue is 114 ± 22 mg. per g. wet weight of tissue, neither of which results is significantly different from normal.

The total protein content of each tissue sample was expressed per mg. DNA phosphorus. The mean protein content of the normal tissue is 647 mg. per mg. DNA phosphorus, of adenoma tissue is 640 mg. per mg. DNA phosphorus and of thyrotoxic tissue is 377 mg. per mg. DNA phosphorus.

(f) RNA/DNA Ratio.

Assuming that the phosphorus content of thyroid tissue DNA is 10%, the RNA/DNA ratios of the tissues were calculated and the mean for each series obtained; these are, for normal tissue 0.46, for adenoma tissue 0.42 and for thyrotoxic tissue 0.59.

D I S C U S S I O N

The main findings of this survey can be very briefly stated. The enzymes studied, lactate dehydrogenase, isocitrate dehydrogenase and phosphogluconate dehydrogenase were found to be located predominantly in the supernatant fraction of both thyroid and cervical cancer tissue. The three enzymes were elevated in the supernatant fractions of all the tissues studied in the case of malignant, but not in the case of benign neoplasia, by reference to control tissue samples. The enzyme activities were also increased in hyperplasia but not to such a degree as in the carcinoma specimens.

The discussion of these results will be presented in a series of sections, firstly, the validity of comparing pathological and normal tissues, secondly, the location of the enzyme activities in the subcellular fractions and thirdly the significance of changes in enzyme activity under pathological conditions.

VALIDITY OF COMPARISONS BETWEEN TISSUE SAMPLES, COMPARING NORMAL WITH PATHOLOGICAL CONDITIONS.

In this investigation, we were faced with a comparison between normal andpathological tissues. This could either be done by comparing normal and abnormal tissues taken from a series of patients, or by comparing normal tissue from one set of patients with abnormal tissue taken from a different set of patients. A discussion of the relative merits of each approach follows.

1) Samples of Normal and Abnormal Tissue from the Same Patient.

During surgical removal of thyroid carcinoma and adenoma and breast fibroadenoma, portions of normal tissue from the same patient were taken, which were large enough for analysis. It was not possible, however, to

obtain normal tissue from patients with conditions involving the whole gland, such as thyrotoxicosis and chronic mastitis.

If such paired specimens from one patient are obtained biological variation between patients is eliminated, but two unknown factors are introduced. Firstly, it is possible that the reduction of functional tissue brought about by the growth of a neoplasm will cause compensatory hypertrophy of the unaffected tissue in order to meet the needs of the body, e.g. in the case of thyroid gland, by increased hormone production. This problem does not arise with breast tissue as none of the samples were removed from actively secreting tissue. Secondly, it is possible that the metabolism of the tumour tissue causes some alteration in that of the host tissue (Boyd et al., 1962).

The theory of Shrivastava and Quastel (1962) concerning the glucose metabolism of normal and cancer tissue is interesting in this context. They suggested that the high rate of glycolysis in tumour tissues causes a slight depletion of glucose in the surrounding tissues and since it is known that glucose is required by normal tissue in the uptake of certain nutrients, e.g. amino acids, this would cause a flow of nutrient substance from the normal tissue to the tumour.

The findings of White (1958a) suggest that tumour tissues can have effects on far distant parts of the body, e.g. muscle wasting in order to provide protein for the growth of the tumour. Increases in dietary protein in patients with cancer who had high serum lactate dehydrogenase activities were followed by a decrease in the activities of the enzyme (White 1958b). Kabakow et al. (1962), correlating these and other facts (Wu, 1959) suggested that the lowered nutritional state caused by the metabolism of the tumour might cause release of enzymes from normal cells

by altering their permeability. All the effects mentioned above might well occur in the portion of remaining functional tissue, causing it to differ from normal tissue.

2) Samples of Normal and Abnormal Tissue from Different Patients.

In view of the factors mentioned above true "normal" tissue can only be obtained from patients with no pathological condition whatever. Since this is rarely possible in clinical practice, material unaffected by the changes occurring in the lesions being studied, was used. Samples of "normal" cervical tissue obtained from patients undergoing operative procedures for the repair of a prolapsed uterus, while not strictly normal, fall into this category.

It was not possible to obtain adequate amounts of the particulate fractions of normal cervix uteri, whereas high yields were obtained from specimens of carcinoma. This could have been predicted from the electron microscope findings of Luibel, Sanders and Ashworth (1960) that the mitochondria of carcinoma cells of the cervix uteri are more numerous than those of normal cells and larger in size.

A comparison of the enzyme activities of the normal thyroid tissue obtained surgically and post-mortem showed that samples of the latter type i.e. those in the Nucleic Acid series, had significantly higher phosphogluconate dehydrogenase activity. It was thought originally that this arose from difference in preparation of the tissue in both series. The possibility that the presence of chloride ions in the homogenisation medium might cause increased activity of the enzyme was considered. However, in the Results section it was shown that dilution of concentrated supernatant fractions in potassium chloride or sucrose

made no difference to the final activity. Another possibility was that the increased homogenisation time might cause some rupture of particles and increase the enzyme content of the supernatant fraction. However, since the activity of the particulate fractions of normal thyroid was very small, this idea was discarded. The difference in phosphogluconate dehydrogenase activity did not occur when comparisons were made between the two thyrotoxic and adenoma series and is therefore more likely to be linked with the difference in source of the normal tissue. It is not possible, however, to decide whether the lower activity of the surgically removed specimens was due to the effects of the neoplasms on the "normal" tissue or whether the higher activity of the post-mortem specimens was due to alterations in the tissue after the death of the patient.

Some additional problems arise in tissue sampling. Certain of the tissue samples were obtained at times unsuitable for immediate preparation and assay. These were stored therefore at -20° for periods up to one month. Shonk et al. (1964) reported that such storage of intact tissue samples weighing over 100 mg. does not have any effect on the activity of glycolytic enzymes.

Again, the samples of carcinoma of cervix were contaminated in varying degrees by blood clots which proved difficult to remove completely. It was noted that the vascularity of the lesion, as gauged by the colour of the supernatant fraction, varied considerably and there was a tendency for high protein concentration to be associated with depth of colour of the supernatant fraction.

LOCATION OF ENZYMES IN THE SUBCELLULAR FRACTIONS OF NORMAL TISSUES.

1) Lactate Dehydrogenase.

De Duve, Wattiaux and Baudhiun (1962) have reviewed the intracellular location of enzymes in animal tissues. In certain tissues, such as rat liver, lactate dehydrogenase appears to be a totally cytoplasmic enzyme, whereas in others, such as the ascites form of Novikoff hepatoma, its location is primarily microsomal.

The finding of lactate dehydrogenase in particles is considered by some workers to be an artefact (Paigen and Wenner, 1962, Keck and Choules, 1962). They found particulate activity when the tissue homogenates were prepared in a medium of low ionic strength, but this activity did not occur when a solution of electrolyte was added to the medium. Bonting, Pollack, Muehrcke and Kark (1960) found 93.3% of the lactate dehydrogenase of human kidney in the supernatant fraction. The values found in this study were not expressed as a percentage of the total homogenate activity, since the activity of the nuclear fraction was not determined. This was found by Bonting et al.(1960) to be only 1% of the total activity of the human kidney homogenate.

The work of this study shows that in normal thyroid tissue, 99% of the cytoplasmic lactate dehydrogenase activity is to be found in the supernatant fraction. The presence of true particulate activity is, therefore, doubted.

2) Isocitrate Dehydrogenase.

De Duve et al.(1962) described the intracellular location of

isocitrate dehydrogenase as being primarily cytoplasmic, but probably also partly mitochondrial. Shepherd (1961) reported that isocitrate dehydrogenase was present in the mitochondrial fraction of rat, mouse, rabbit and human liver. Hogeboom and Schneider (1950) reported on the intracellular location of isocitrate dehydrogenase in mouse liver. They found 82% of the activity in the supernatant fraction, 3% in the nuclear fraction, 12% in the mitochondrial fraction and 1% in the microsomal fraction. The work of this study shows the presence of isocitrate dehydrogenase in the mitochondria of cervical carcinomas and in all types of thyroid tissue. In normal thyroid tissue, 97% of the cytoplasmic enzyme activity occurred in the supernatant fraction, the remainder of the activity being associated mainly with the M1 fraction.

3) Phosphogluconate Dehydrogenase.

De Duve et al.(1962) described the location of phosphogluconate dehydrogenase as being purely in the supernatant fraction of animal tissues. In opposition to this view, Yamada and Shimazono (1961) reported the presence of phosphogluconate dehydrogenase in the particulate fractions of guinea pig brain, but this enzyme had to be separated from the particle membranes before the activity could be measured, since the membranes appeared to reoxidise the NADPH formed, thus making the measurement of the reaction impossible. They concluded that this would explain the lack of activity found in particulate fractions by other workers. They did not draw any conclusions, however, about the site of the enzyme, i.e. whether it was present within the particles or adsorbed on to the particle

surface. In this study, definite activity has been found in the particulate fractions of the tissues studied, using methods similar to those of Yamada and Shimazono (1961), but with ultrasonic disruption of the particles replacing solubilisation of the enzyme by Triton, the M1 and M2 fractions of normal thyroid tissue containing respectively, 6% and 5,6 of the cytoplasmic activity. Consequently this is more than mere contamination.

SIGNIFICANCE OF CHANGES IN LACTATE DEHYDROGENASE ACTIVITY.

This section will be divided into parts related to the changes in the enzyme activities of pathological thyroid, mammary and uterine cervical tissues compared with normal tissue controls. Following this there will be a discussion of similar and contradictory findings reported by other workers.

1) Thyroid Gland.

In the supernatant fraction there is an increase in lactate dehydrogenase activity in the thyrotoxic, Hashimoto and carcinoma series, the last being the most active, but the adenoma series does not differ significantly from normal. The sample of Hürthle Cell adenoma also shows increased activity. The comparison of normal thyroid with adenoma and carcinoma samples has also been made by Goldman et al.(1964), on a smaller series of samples, and their findings are in agreement with those stated above.

In the M1 fraction there is no significant difference from normal in any of the tissues studied.

In the M2 fraction of the thyrotoxic series there is an increase in enzyme activity compared with normal, but the other series show

a high variance and thus the finding is likely to be of no value.

2) Cervix Uteri.

In the supernatant fraction, there is a significant increase in lactate dehydrogenase activity in malignant tissue, which shows a significant decrease following radiation to values more similar to those of normal tissue. Thiery and Willighagen (1964) found a similar increase in activity in experimental cervical cancers in the mouse, using histochemical techniques.

In the M1 fraction, there is a significant increase in lactate dehydrogenase activity per g. wet weight of tissue, but not per mg. protein following radiation. This would indicate either an increase in lactate dehydrogenase and a parallel increase in protein in the M1 fraction, or a decrease in the other tissue constituents following radiation.

An increase in the mitochondrial lactate dehydrogenase and protein content could be explained by increased absorption of protein on to the mitochondrial membrane after radiation, However, in opposition to this Thomson and Rahman (1962) found that radiation seemed to have little effect on the mitochondria of rat liver. Also from the findings of Paigen and Wenner (1962) and Keck and Choules (1962) previously reported in this section (Page 65), it would seem likely that if absorption of lactate dehydrogenase were to take place it would also occur in the M2 fraction. However, no increase in microsomal lactate dehydrogenase was found in this work.

Depletion was found in the protein content of the supernatant and M2 fractions following radiation, so that it would seem likely that the apparent rise in the lactate dehydrogenase of the M1 fraction

is due to loss of other tissue constituents.

3) Breast.

There is no significant difference from normal in the supernatant fraction of the fibroadenoma or chronic mastitis series, but there is a significant increase in the carcinoma series. Similar findings were reported by Goldman et al.(1964).

4) Comparison of the Results of this Study with those of other Workers.

Rees and Huggins (1960) found lactate dehydrogenase increased in experimental mammary cancers in the rat, the cancer series being compared with a control series of pregnant or lactating rats, depending on the condition of the rats in the cancer series. In preliminary studies comparing normal mammary material from pregnant and lactating animals, lactate dehydrogenase was found to be increased in lactation. Lactation can be classed as hyperplasia of the acinar cells, and the state of the tissue during lactation is more similar to thyrotoxicosis than it it to chronic mastitis, since in that condition, the fibrous tissue of the breast is hyperplastic, there being only slight and irregular hyperplasia of the acinar tissue. From this it follows that there should be an increase in lactate dehydrogenase activity in thyrotoxicosis, but that this increase need not necessarily occur in chronic mastitis. In the present study, increased activity was found in thyrotoxicosis, but no increase was found in chronic mastitis.

Dow and Allen (1961) found that the overall operation of the Embden Meyerhof Pathway was increased in the hyperthyroid rat. Although their results cannot be directly compared with those of the present work, it is interesting that increased lactate dehydrogenase

activity was found in the series of thyrotoxic samples studied.

One sample of spheroidal cell carcinoma of thyroid was studied by the present investigator, and had a much higher lactate dehydrogenase activity than the other samples in the series. Histologically it was classed as being the most malignant sample studied. This bears out reports mentioned by Shrivastava and Quastel (1962) that high malignancy is associated with high lactate dehydrogenase activity. However, no such agreement between lactate dehydrogenase activity and malignancy could be found in carcinoma of the cervix uteri. This coincides with the findings of de Roetth (1957).

It is interesting that the Hashimoto series of the present investigations has lactate dehydrogenase activity similar to that of thyrotoxic series, when the results are expressed per g. wet weight, but is more like the carcinoma series both in protein content and in activity per mg. protein. The low protein content of Hashimoto and carcinoma tissue may best be explained by the difference in the cell type predominating in these two lesions.

SIGNIFICANCE OF CHANGES IN ISOCITRATE DEHYDROGENASE ACTIVITY.

The discussion of the changes of isocitrate dehydrogenase activity in abnormal human tissues is organised along lines similar to those of the preceding section.

1) Thyroid Gland.

There is an increase in isocitrate dehydrogenase activity in the supernatant fraction of all the tissue types studied, including the sample of Hürthle Cell adenoma. Schussler and Ingbar (1961) found that addition of NADPH or isocitrate dehydrogenase to homogenates of sheep thyroid gland produced an increase in organic

iodination. This is of interest, since in the tissue with increased organic iodination, i.e., thyrotoxic tissue, increased isocitrate dehydrogenase activity has been found in this study. Tremblay and Pearse (1960), using histochemical techniques, found increased isocitrate dehydrogenase activity in adenoma, thyrotoxic and Hashimoto thyroid glands, but did not state clearly whether this activity belonged to the supernatant fraction or was limited to the mitochondrial.

In the M1 fraction, large differences in isocitrate dehydrogenase activity were recorded. The tissue types were, in increasing order of activity, normal, adenoma, thyrotoxic, Hashimoto, carcinoma, and Hürthle Cell adenoma. Similar results were also found by Tremblay and Pearse (1960) in adenoma, thyrotoxic and Hashimoto samples. They located the high activity in Askanazy cells or in a similar type of cell which they named a mitochondrion-rich cell. It is interesting that by far the highest activity recorded in the present study occurred in the sample of Hürthle Cell adenoma, which is a tissue type composed of Askanazy cells.

In the M2 fraction, findings similar to those of the M1 fraction were recorded. Dumont (1960) reported that NADPH is necessary for the deiodination of iodotyrosine by an unspecified microsomal enzyme, so that increased activity of isocitrate dehydrogenase might be essential in tissue with increased thyroxine production.

2) Cervix Uteri.

In the supernatant fraction, there is a significant increase in isocitrate dehydrogenase activity in malignancy and a significant decrease following radiation.

In both particulate fractions there is no significant difference

in isocitrate dehydrogenase activity before and after radiation.

3) Breast.

In the supernatant fraction no significant difference was found between normal and fibroadenoma tissue, but there was a significant increase in the carcinoma series. In chronic mastitis there is a significant increase in the enzyme activity when related to protein content, but this is due to the decreased protein content of the supernatant fraction of this tissue compared with normal breast tissue.

4) Comparison of the Results of this Study with those of Other Workers.

Rees and Huggins (1960) working on experimental mammary cancers in the rat, found that between the states of pregnancy and lactation there was no significant difference in isocitrate dehydrogenase per g. wet weight of tissue. If the analogy between lactation and thyrotoxicosis can be pursued, there should be no increase in isocitrate dehydrogenase activity in thyrotoxicosis. In the present study, however, an increase has been found, but this could well be explained by the presence of Askanazy cells which are rich in isocitrate dehydrogenase (Tremblay and Pearse, 1960) and, therefore, the increased isocitrate dehydrogenase activity may not arise from the acinar cells of the thyroid gland.

The work of Schussler and Ingbar (1961) in sheep thyroid glands did not prove which fractions of the cell contributed to the increased organic iodination. Dumont (1960) found that an enzyme concerned in the deiodination of iodotyrosine was active in the microsomal fraction of thyroid tissue and that this enzyme required the presence of NADPH ^{9}

which he considered to be the most efficient coenzyme for the binding of iodine to protein in the thyroid homogenate. It is possible that increased activitiy of the supernatant fraction enzymes producing this coenzyme might be required for increased iodination. This could be another explanation for the fact that isocitrate dehydrogenase is increased in thyrotoxic tissue.

The posibility of linking these two reports is provided by the work of Baker and Newburgh (1963) who suggested that supernatant fraction isocitrate dehydrogenase activity is linked with reductive synthesis, whereas the mitochondrial isocitrate dehydrogenase activity is more likely to be necessary for oxidative metabolism. Both these processes could well be increased in thyroid gland in the pathological states, either in the functional tissue or in the Askanazy and mitochondrion-rich cells.

As with lactate dehydrogenase, isocitrate dehydrogenase activity per mg. DNA phosphorus does not differ from that of normal tissue in the adenoma or thyrotoxic thyroid gland. It appears that the amount of enzyme present in each cell of these tissue types is constant.

SIGNIFICANCES OF CHANGES IN PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY.

The discussion of the activity of phosphogluconate dehydrogenase follows the same format as that of the other two enzymes.

1) Thyroid Gland.

In the supernatant fraction, phosphogluconate dehydrogenase activity is not significantly different from normal in the adenoma series, but is increased in all other lesions.

In the M1 fraction there is an apparent increase in activity

per g. wet weight in the adenoma series, and increased activity per mg. protein in the thyrotoxic series. These findings are considered to arise from the low activity and high variance of the tissue groups studied.

In the M2 fraction there is no significant difference from normal in any of the groups studied.

Dow and Allen (1961) found, by measurements of isotopically labelled expired carbon dioxide derived from labelled glucose metabolised by the hyperthyroid rat, that the Hexose Monophosphate Pathway was suppressed. This effect was thought to be due to lack of available NAD^+. In the thyrotoxic tissue studied in the present work no such suppression was found. This can be explained by the excess of oxidised coenzyme added to the reaction mixture. A direct comparison cannot be made between the results of Dow and Allen (1961) and those of the present study, since prior to removal of the tissue, various clinical procedures had been employed to counteract the metabolic effects of excess thyroxine.

2) Cervix Uteri.

In the supernatant fraction there is a significant increase in malignancy and a significant decrease after radiation. Bonham and Gibbs (1962) reported that increased activity of phosphogluconate dehydrogenase in human vaginal fluid gave a good correlation with cytological examination of gynaecological cancer, mainly cancer of the cervix uteri. They also carried out measurements on supernatant fractions of biopsy material which also showed high activity. However, Muir, Canti and Williams (1964) repeated this work on a larger series ^{o}

of patients and found a large proportion of false positives which they thought might be due to hyperplasia of the cervical tissue.

In the particulate fractions there is no difference in activity following radiation.

3) Breast.

In the supernatant fraction there is a significant increase only in the carcinoma series.

Rees and Huggins (1960) found increased phosphogluconate dehydrogenase activity between normal mammary and carcinoma tissue of pregnant rats, although the increase found was not so great as that in lactate dehydrogenase activity. In the present study the increase was found to be greater than that of lactate dehydrogenase.

4) Comparison of the Results of this Study with those of Other Workers.

Rees and Huggins (1960) also found a greater increase in phosphogluconate dehydrogenase activity during lactation. This finding of increased activity during lactation has also been reported by Abraham, Hirsch and Chaikoff (1957) and Glock and McLean (1954). Chayen et al.(1962) suggested that phosphogluconate dehydrogenase activity is increased in cell proliferation of any type, including regeneration of liver cells after starvation, this finding having been previously reported by Fitch and Chaikoff (1960). Scott et al. (1962) working on experimental tumours in hamster cheek pouch, suggested that increased phosphogluconate dehydrogenase activity was linked more with hyperplasia than with neoplasia.

The increase found in the thyrotoxic series agrees well with the hypothesis that increased phosphogluconate dehydrogenase activity is

associated with hyperplasia rather than with neoplasia. In fact the activity found in the hyperplastic thyroid tissue is very similar to, and perhaps slightly greater than, that of the thyroid carcinoma series. However, no such increase was found in the chronic mastitis series. All the hyperplastic tissues studied by the workers mentioned above have been epithelial, and it is, therefore, possible that in chronic mastitis, where the fibrous tissue of the breast has become hyperplastic, that the same considerations do not apply and that the same results need not necessarily be found.

Glock and McLean (1954) found that phosphogluconate dehydrogenase activity was increased in lymphatic tissue in general. It is possible that this might explain the higher activity of the Hashimoto series, since this tissue is invaded by lymphocytes.

As with the two other enzymes studied, there is no significant difference in phosphogluconate dehydrogenase activity per mg. DNA phosphorus in the three tissue types studied. Therefore, the concentration of the enzyme in thyroid tissue is related to the number of cells per unit weight of tissue.

EFFECTS OF RADIATION ON CANCER OF THE CERVIX UTERI.

In her review of the biochemical effects of ionising radiations, Holmes (1957) reported that depletion of tissue protein and of certain enzymes occurred following radiation of normal tissues. The glycolytic enzymes did not show this depletion as a whole. DuBois, Raymond and Hietbrink (1962) reported that phosphogluconate dehydrogenase activity is lowered by 25% in the livers of normal rats following radiation. Neither of these reports included information on the effects of

radiation on neoplastic tissues.

The activity of lactate dehydrogenase in the supernatant fraction of carcinoma of the cervix uteri is reduced following radiation to values similar to those of normal cervical tissue, both when the results are expressed per g. wet weight of tissue and per mg. protein.

Isocitrate dehydrogenase and phosphogluconate dehydrogenase activities are reduced subsequent to radiation when the results are expressed per g. wet weight, but not per mg. protein. This indicates that these two enzymes are depleted in the same proportion as the other tissue proteins, whereas the lactate dehydrogenase is depleted more than the tissue proteins. This agrees with the finding that the activities of isocitrate dehydrogenase and phosphogluconate dehydrogenase following radiation, though reduced, are still much higher than those of normal tissues.

The activities of the enzymes in the particulate fractions were not reduced following radiation; indeed, the lactate dehydrogenase activity was increased. The significance of this finding has been previously discussed in this section.(see Page 68)

COMPARISON OF THE THREE ENZYME ACTIVITIES IN THE THREE TYPES OF NORMAL TISSUE.

The practice of measuring different enzymes under a standard set of conditions so that their relative activities can be compared has been advocated by Shonk and Boxer (1964). In this study, isocitrate dehydrogenase and phosphogluconate dehydrogenase have been measured under the same conditions of pH and temperature in cationic buffer solutions. Such similarity of conditions has been recognised by Shonk

and Boxer (1964) as giving results from which the amounts of enzymes present can be compared. Lactate dehydrogenase has also been measured under the same conditions of temperature as the other two enzymes, but the pH was different. However, the reaction measured at pH 10 is the reverse of the reaction that takes place at pH 7 (Neilands, 1955), and the results for lactate dehydrogenase can, therefore, be compared with those of the other two enzymes.

In all the normal tissues the activity of lactate dehydrogenase is much greater than that of isocitrate dehydrogenase, which is in turn about three times as great as that of phosphogluconate dehydrogenase. The relative activities of the three enzymes in normal tissues are much less variable than in pathological tissues.

Of the three normal tissues studied, thyroid tissue was the most active. This could well reflect the fact that this tissue is metabolically very active and under constant pituitary stimulation. Cervical tissue is the next most active, being mainly epithelial and, therefore, in a state of constant renewal. Of the three breast tissue is the least active, being in a non-functional state in all the patients examined.

THE NUCLEIC ACID SERIES OF THYROID GLAND.

1) Enzyme Activities.

The enzyme activities per g. wet weight and per mg. protein followed exactly the pattern found in the Main series. However, the results per mg. DNA phosphorus showed no difference from normal in the adenoma or thyrotoxic series. This indicates that the amounts of the

enzymes in thyroid tissue are related to the cellularity of the tissue rather than to its functional state.

The comparison between the normal tissues in the Main series and in the Nucleic Acid series has already been described in this section. (see Page 63) When a comparison was made between the two thyrotoxic series, no significant difference was found in the activities of the enzymes, whatever way they were expressed. When a comparison was made of the two groups of adenoma tissue, significant differences in lactate dehydrogenase and isocitrate dehydrogenase activities were found, but not in phosphogluconate dehydrogenase activity. These differences can be explained by the high variance of the Main series and the low variance of the Nucleic Acid series, and merely reflect the greater variability of adenoma tissue compared with the other two types of tissue studied in the Nucleic Acid series.

2) RNA Content.

The RNA content of adenoma tissue is not significantly different from that of normal tissue, but the RNA content of thyrotoxic tissue is much higher.

3) DNA Content.

Again no significant difference exists between normal and adenoma tissue, but there is in the thyrotoxic series. The thyrotoxic tissue has less storage of colloid and the cell size is smaller than normal, both factors accounting for the increased DNA content (as measured by the DNA phosphorus).

4) RNA / DNA Ratio.

RNA in living cells is closely connected with protein synthesis,

increase in RNA reflecting an increase in protein synthesis (Brachet,1955). The increase in RNA found in the thyrotoxic tissue could be due to the increased number of cells per unit weight. In order to test this hypothesis, the ratio of RNA/DNA was determined for each tissue sample, and the mean value of the ratio was calculated for each tissue series. Thyrotoxic tissue showed a much higher ratio than normal or adenoma tissue. This indicates a true increase in RNA per cell and can be related to the increased thyroglobulin production in thyrotoxic tissue.

5) Lipid Phosphorus Content.

The lipid phosphorus content of normal and adenoma tissue is very similar to the DNA phosphorus content, but in thyrotoxic tissue it is somewhat higher, i.e. there is more phospholipid in the thyrotoxic cell than there is in the normal cell.

Since phospholipid occurs in various cell structures, e.g. the cell membrane, the mitochondria, and the endoplasmic reticulum, it is not possible to decide where the increase found in thyrotoxic tissue originates.

6) Protein Content.

The protein concentrations of the three tissue types were found to be very similar, therefore, the storage of colloid by the normal thyroid tissue does not appear to affect the protein concentration of the tissue when it is expressed per unit weight of tissue. If, however, the protein content of each tissue sample is expressed per mg. DNA phosphorus, in order to give a measure of the protein content per cell, the protein concentration of thyrotoxic tissue was found to be about two thirds of that of normal and adenomatous tissue, again reflecting the lack of

stored colloid in thyrotoxic tissue.

GENERAL CONCLUSIONS.

The object of this work was to determine the activities of three enzymes in the particle-free supernatant fraction of various normal and pathological human tissues, and in the particulate fractions where such were available in adequate amounts, in order to see whether there were any striking differences in enzyme content associated with any one lesion of a particular tissue, or with any condition of the types of tissue studied.

The activities of all three enzymes were found to be increased in all the pathological states studied, excepting certain benign lesions, and this increase in enzyme activity is considered to reflect abnormal metabolic activity in the tissue in question.

In mammary tissue there is increased activity of all three enzymes in the cancer series, but there is no significant difference from normal in the fibroadenoma or the chronic mastitis series. An increase in activity in the chronic mastitis series would have been consistent with the fact that the tissue is hyperplastic, but possibly hyperplasia of fibrous tissue does not present the same enzymic picture as hyperplasia of epithelial tissue.

In the thyroid tissue, the activities of lactate dehydrogenase and phosphogluconate dehydrogenase are increased in the supernatant fraction of all the lesions studied except adenoma of thyroid, but the increases are not specific to any one condition. The activity of isocitrate dehydrogenase is increased in all the pathological types of thyroid

tissue, possibly due to the presence of Askanazy cells, which are rich in this enzyme, especially in the mitochondrial fraction. In the thyroid gland, the measurement of the nucleic acid content of normal, adenomatous and thyrotoxic tissue makes the interpretation of the results more clear. Thyrotoxic tissue has increased protein production and less protein storage compared with normal tissue. It also has more cells per unit weight of tissue, and these cells are of a smaller size. The adenomatous tissue does not differ from normal tissue in any of these values. The amount of the respiratory enzymes, related to DNA content, is not significantly different from normal in the adenomatous and thyrotoxic tissue, indicating that the amounts of enzyme present in these tissues are related to the cellularity of the tissues. The work of Rees and Huggins (1960) has presented an enzymic picture of lactation in rats which shows that the activities of lactate dehydrogenase and phosphogluconate dehydrogenase are raised, whereas the activity of isocitrate dehydrogenase is not. From the point of view of this study, thyrotoxicosis can be likened to lactation, since both are brought about by hyperplasia of secretory cells. In thyrotoxicosis the activities of lactate dehydrogenase and phosphogluconate dehydrogenase are increased; so also is the activity of isocitrate dehydrogenase, but this is possibly due to the presence of Askanazy cells, and may not arise from the acinar cells.

The clarification of the results obtained from thyroid tissue brought about by the measurement of DNA content shows that it would be of value to measure the nucleic acid content of all tissues being studied enzymically. However, DNA content by itself would not provide a good basis of comparison of normal with tumour tissue, since the

cancer cells, which are more rapidly dividing, do not attain the full cytoplasmic volume of normal cells, also the DNA content of tumours does not give a measure of their cellularity, due to the presence of cells with several nuclei. Therefore, comparisons based on enzyme activities related to weight and protein content are also helpful.

In uterine cervical tissue, the increase in activity of all three enzymes recorded in the supernatant fraction of the cancer series is effectively reversed by radiation.

In reviewing the whole body of evidence from this study and from the literature it seems that measurement of the activity of a single enzyme does not give an index of the true activity of a pathway *in vivo* unless the enzyme is rate-limiting with respect to that pathway, but gives some idea of the potential activity of the reaction measured. In this study, the three enzymes were measured under similar conditions, which makes a comparison of their relative activities possible, certainly with respect to isocitrate dehydrogenase and phosphogluconate dehydrogenase. However, the activities of these two enzymes were increased similarly in the supernatant fraction of all the lesions studied, and no information concerning the relative operation of the Citric Acid Cycle and the Hexose Monophosphate Pathway could be gleaned from their activities. Indeed, none of the enzymes studied shows increased activity specific to either neoplasia or hyperplasia. To obtain a truer comparison of the metabolic activities of pathological and normal cells, the activities, related to unit weight, protein content and DNA content, of rate-limiting enzymes measured under similar conditions without the addition of cofactors would be necessary.

This study on human malignant and hyperplastic tissues thus supplements the picture that emerges from the published work on experimental animals. The increase in enzyme activity in the malignant cell is also present in the hyperplastic cell, as shown particularly in the series of results on thyroid tissue reported in this work. Consequently, the thesis of a specific increase in respiratory enzymes in malignant tissue is not supported by this work.

S U M M A R Y

SUMMARY.

Three enzymes of carbohydrate metabolism, lactate dehydrogenase, (NAD-dependent), isocitrate dehydrogenase (NADP-dependent), and phosphogluconate dehydrogenase (NADP-dependent) have been estimated in normal and pathological samples of three types of human tissue, namely thyroid gland, mammary gland and cervix uteri. The enzymes have been measured in the particle-free supernatant fractions of these tissues and in the particulate fractions where these were available in adequate amounts. In thyroid tissue of all types and in carcinoma of the cervix uteri, the location of the enzymes was mainly in the supernatant fraction, lactate dehydrogenase being found exclusively in the supernatant fraction in the case of normal thyroid tissue. In the supernatant fractions of most of the tissue samples lactate dehydrogenase activity was greater than isocitrate dehydrogenase activity which was greater in turn than phosphogluconate dehydrogenase activity.

A comparison of the three normal tissues studied, with respect to the activities of all the enzymes studied, indicated that thyroid tissue was the most active, followed by uterine cervical tissue, and mammary tissue was the least active of the three tissues studied.

With regard to changes in enzyme activity in the thyroid gland as a result of the onset of pathological lesions, two series of studies were made. First, there was the Main series, in which the enzyme activities were related to the weight of the tissue sample analysed and to the protein content of the subcellular fractions. Fifty-one samples of surgically removed thyroid tissue were studied,

comprising 11 samples of normal tissue, 10 samples of adenomatous tissue, 20 samples of thyrotoxic tissue, 5 samples of carcinoma of thyroid, 4 samples of Hashimoto's disease and 1 sample of Hürthle Cell adenoma. Secondly, there was the Nucleic Acid series, in which the enzyme activities were related to the DNA content of the tissue samples as well as to weight and protein content. Fifteen samples of thyroid tissue were studied, 5 normal tissue samples, 5 samples of adenoma of thyroid and 5 samples from cases of thyrotoxicosis. In the supernatant fraction of the various pathological types of thyroid tissue, isocitrate dehydrogenase activity was increased, and lactate dehydrogenase and phosphogluconate dehydrogenase activities were increased in all lesions other than adenoma of thyroid. In the particulate fractions, lactate dehydrogenase and phosphogluconate dehydrogenase activities were not significantly different from normal, but isocitrate dehydrogenase activity was strikingly increased in all the pathological conditions studied, especially in the sample of Hürthle Cell adenoma. When the enzyme results of the Nucleic Acid series were related to DNA content, no significant difference from normal tissue was found in the thyrotoxic and adenoma series with respect to any of the enzymes studied. In thyrotoxicosis the RNA content of the tissue was increased, also the DNA content and the RNA/DNA ratio. The phospholipid content was also increased, but the protein content per cell was decreased.

Fifteen samples of normal cervical tissue were studied and compared with 23 samples of carcinoma of cervix uteri, of which

14 samples were compared with biopsies removed following irradiation of the tumour. The enzyme activities of the supernatant fraction were found to be increased in the cancer series and to be decreased following radiation to values similar to those of normal tissue. There was no significant decrease in the enzyme activities of the particulate fractions following radiation.

Twenty samples of mammary material were analysed, comprising 5 samples of normal tissue, 5 samples of fibroadenoma, 5 samples of tissue from patients with chronic mastitis and 5 samples of carcinoma. In the supernatant fraction the activities of all three enzymes were increased only in the cancer series, there being no increase in any of the enzyme activities in the two benign lesions studied.

This investigation thus indicates that increased activity of respiratory enzymes occurs in a variety of tissues with the onset of malignancy, but that this also takes place in some non-malignant lesions such as hyperplasia, and is thus non-specific. On the other hand benign neoplasia fails to raise activity, as in the cases of adenoma of thyroid and fibroadenoma of breast. Thus there is evidence from the present investigations that changes in enzyme activity caused by tumour transformation of cells are not peculiar to the neoplastic process.

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