THE RESPIRATION OF LEUCOCYTES.

by

SHEENAH K. BISSET, B.Sc. (St. And.)

from

The Department of Pathological Biochemistry
(Western Infirmary)
University of Glasgow.

Thesis submitted for the degree of Ph.D.

of

The University of Glasgow.

April, 1959.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS.

ACKNOWLEDGMENTS.

I should like to thank Dr. E. B. Hendry for his help and constant encouragement during the course of this research problem. I wish also to thank Professor J. N. Davidson for the interest which he has maintained in this work.

My thanks I extend to Dr. Malcolm Dixon, F.R.S., for his advice at the beginning of this research.

To my colleague, Dr. W. D. Alexander, I give my thanks for without his clinical knowledge and help, this work could not have been achieved.

Also, I would like to thank Miss J. Graham for preparing the figures for this thesis, and Miss C. Burness for typing it.

This work was started in an attempt to correlate total body metabolism with the metabolism at cell level. The work was carried out in the Biochemistry Department of the Western Infirmary, Glasgow, and the subjects were human patients. Because of this, the choice of cell was severely limited. Leucocytes were the most suitable since they have a high respiration rate and are nucleated. They can be obtained from the patient by simple venepuncture, and, with care, can be isolated as intact cells almost uncontaminated with erythrocytes. There is very little published work on the correlation between total body metabolism and cell respiration in the human subject. If a relationship could be shown to exist between the basal metabolic rate and the respiration of the leucocyte, it was intended to proceed with a study of the action of metabolic stimulants on the respiration rate of the leucocytes and correlate the results with the response of the body as a whole. Fortunately, it was found that a direct relationship did indeed exist between these two quantities and this thesis is a record of the experiments and thoughts which resulted.

INTRODUCTION.

Respiration of Leucocytes.

Circulating leucocytes are well suited for metabolic studies being the only easily accessible, free-floating, nucleated mammalian cells. They are readily obtainable in quantity, and can be counted with accuracy. Since they can be studied in the human patient they provide a tool for investigation of interest both to the clinician and to the biochemist. The only other cells that have been extensively used for research are spermatazoa and the cells of certain tumours such as the Ehrlich ascites tumour.

This work records an examination of the respiration of human leucocytes, under various conditions, including the effects of certain metabolic stimulants. In the examination of the data available in the literature, there are three main difficulties in attempting to compare the results with those obtained by other workers, and these difficulties are:-

(1) Different workers use leucocyte preparations which differ widely in origin. The literature records studies of both normal and leukaemic human leucocytes as well as leucocytes from other animal species,

(2) Although the analytical methods employed are reasonably/

/reasonably standardised, the media in which the leucocytes are suspended during respiration studies vary from one laboratory to another and experience has shown that the results obtained depend to some considerable extent on the composition of the medium employed, and,

(3) The protocols have been expressed in a variety of different ways, e.g., in terms of dry weight, cell numbers, and cell nitrogen content. Respiration in terms of cell numbers is thought to be the most suitable method of expressing the results and is the method employed here. Where the "Q" notation is used (indicating activity per mg. dry weight of cells per hour) Beck and Valentine (1953) and Bird, Clements and Becker (1951) have calculated that -

Q_{O_2} x 1,960 = microlitres O_2 per 10^{10} cells per hour, and this equation is useful in comparing results expressed by two common methods.

The earliest study of the respiratory activity of leucocytes was carried out by Grafe (1911) who tried to explain the marked increase in the total oxygen consumption of the body in leukaemia by studying the gas exchange of the leucocytes of normal and leukaemic patients. He isolated the leucocytes by a rather crude method involving/

/involving centrifugation of whole blood, removing and discarding the plasma, and re-suspending the red and white cells in a salt solution containing saponin. Using a Haldane-Barcroft apparatus, he obtained results equivalent to an oxygen uptake of 0.017 - 0.037 m-mole per 10^{10} leucocytes per hour in a mixed group of bloods from myeloid and lymphatic leukaemia patients. Apart from these determinations, Grafe made no comment on the usefulness of the method for research purposes.

In 1930, Warburg published his classical studies on cell metabolism, concluding that neoplastic transformation involved fundamental changes in the energy-supplying metabolic reactions. A large number of confirmatory papers strongly implied that here was one criterion for malignancy - high aerobic and anaerobic glycolysis and a moderately high respiratory rate. The later discovery of normal tissues possessing these characteristics (e.g., renal medulla and jejunal mucosa) resulted in an attempt to attribute the "cancer pattern" in normal tissue to injury to the cells. Thus began a large series of investigations to determine the fundamental difference between normal, injured and malignant tissue.

The field was reviewed by Kempner (1939) whose own studies on patients with myeloid and lymphatic leukaemia led/

/lead him to the conclusion that the occurrence of high aerobic glycolysis in mature leucocytes was a sign of ageing and dying. Victor and Potter (1938,a) found consistent increases in both aerobic and anaerobic glycolysis in the lymph nodes of mice with spontaneous and transmitted lymphocytic leukaemia , when compared with the corresponding values obtained using normal mouse lymph nodes. These findings were confirmed by Hall and Furth (1942), but at the same time, they showed that there was little difference between the oxygen consumption of the lymph nodes of leukaemia mice and that of normal mouse lymph nodes.

Daland and Isaacs (1927) and Glover, Daland and Schmitz (1930) showed that the respiratory and glycolytic rates of leukaemic cells were slightly higher than thoseof normal human cells, but the difference was not very great. Soffer and Wintrobe (1932) failed to confirm these findings, nor could they correlate the respiratory and glycolytic rates with the maturity of the cell population.

Ponder and Macleod (1938) evaluated the effects on metabolism of cell maturity by producing rabbit peritoneal exudates in rapid succession so that the cells became younger and younger due to bone marrow stimulation. The oxygen consumption of the first exudate was 0.18 m-mole oxygen per 10^{10} cells per hour, and/

/and that of the fourth exudate, containing many immature forms, was higher by 50%. An increase of 400% over the control value could be obtained by creating repeated exudates in rapid succession.

Keible and Spitzy (1951) reported studies on a group of normal and a group of leukaemic leucocytes. They removed the bulk of the erythrocytes by centrifugation and then isolated the leucocytes from the plasma, at the same time, showing that centrifugation was not harmful to the leucocytes. They then compared respiration, aerobic, and anaerobic glycolysis rates in groups of selected cases of varying differential white cell count. They claimed to be able to distinguish a difference in the aerobic glycolytic rates between a preparation containing 90% of lymphocytes and one containing 80% of lymphocytes, but since they presented only the mean results of their different groups and made no calculation of the standard deviation, their claim to such a degree of accuracy must be considered doubtful. In this paper, they also produced evidence showing that myeloid respiration rate, both normal and leukaemic, increased with increasing maturity of the cell population.

Perhaps the most important single factor in the study/

/study of leucocyte respiration rates is the composition of the medium in which the cells are finally suspended. Some workers employ serum; others use simple crystalloid solutions of varying composition. In many cases where serum is used, it is not clear in the text whether normal or leukaemic serum has been employed. And in most cases, no reason is given for any particular choice of medium.

Victor and Potter (1938,b) showed that mouse lymphoid and leukaemic cells have higher respiratory rates in normal serum than in Ringer's solution while the rates of anaerobic glycolysis are the same in both media. Typical results showed Q_{O_2} values for leukaemic cells of 6.1 (in serum) and 4.9 (in Ringer's solution). This has been confirmed by others including Macleod and Rhoads (1939) who studied suspensions of rabbit peritoneal exudate leucocytes. They found Q_{O_2} averaging 4.6 in Ringer's solution buffered to pH 7.0, and Q_{O_2} values averaging 7.0 in serum neutralised with hydrochloric acid to pH 7.0.

It has been reported by certain authors that leucocyte respiration is particularly susceptible to the effects of "crowding", i.e., the respiration rate of leucocytes is not proportional to the cell count beyond/

/beyond a certain limiting number of cells per unit volume, (Soffer and Wintrobe (1932), Barron and Harrop (1929)). This was not confirmed by Ponder and Macleod (1936) who were quite emphatic that centrifugation, cooling, and "crowding" did not interfere with leucocyte respiration.

More recently, Hartman (1952), using guinea pig exudate leucocytes in varying concentrations, found that a disproportion between oxygen uptake and cell numbers per unit volume occurred only when the cells were suspended in serum, but that these two quantities were proportional to each other over a wide range when the leucocytes were washed and suspended in a physiological salt solution. Beck and Valentine (1952, a, b) examined homogenates of human leucocytes in isotonic potassium chloride and did not observe any "crowding" effects.

Newer techniques of leucocyte isolation were brought out by Bird, Clements and Becker (1951). They examined the oxygen consumption, glucose utilisation, and lactic acid production in normal human leucocytes and in leukaemic leucocytes. The leucocyte suspension was prepared by means of the "bovine fibrinogen method". This consists of adding a 5% (w/v) solution of bovine fibrinogen in saline to the blood specimen; this brings about increased rouleaux formation in the erythrocytes leading/

/leading to a rapid sedimentation. Sedimentation enables a large volume of plasma to be obtained, and this plasma contains the bulk of the leucocytes in the original blood. The fibrinogen does not appear to interfere in any way with the respiration or metabolism of the leucocytes. This "bovine fibrinogen method" was first introduced by Buckley, Powell and Gibson (1950). The leucocytes so obtained were suspended in a medium containing Ringer's solution, plasma, and added glucose. Under these conditions, Bird, Clements and Becker found a mean oxygen consumption of 0.23 m-mole oxygen per 10^{10} cells per hour with normal leucocytes, and a mean oxygen consumption of 0.10 m-mole oxygen per 10^{10} cells per hour for the leucocytes obtained from cases of chronic myeloid and lymphocytic leukaemias. However, the standard deviation of each group was so large that the authors were not prepared to regard the difference as significant. They also observed a low glucose utilisation and lactic acid formation in the leucocytes from cases of lymphatic leukaemia and these they regarded as significantly different from the normal controls.

A later modification replaced the bovine fibrinogen by dextran which also increases the erythrocyte sedimentation rate. McKinney, Rundles and Martin (1952) introduced this method, and using it, they found a leucocyte/

/leucocyte mean oxygen uptake of 0.28 m-mole oxygen per 10^{10} cells per hour when the leucocytes were suspended in a medium containing plasma and Hank's solution.

Beck and Valentine (1952, a) studied the respiration and aerobic glycolysis of leucocytes isolated by the fibrinogen method (see above) from normal and leukaemic bloods. Three groups of patients were employed, (1) normals, (2) cases of chronic myeloid leukaemia (average leucocyte count 100,000 cells per cu.mm. with 96% myeloid cells) and (3) cases of chronic lymphatic leukaemia (average leucocyte count 124,000 cells per cu.mm. with 93% lymphocytes). The homogenised leucocytes were finally suspended in a medium of isotonic potassium chloride to which had been added a buffered (pH = 7.4) fortified system containing adenosine triphosphate, diphosphopyridine nucleotide, cytochrome C, magnesium and phosphate ions, with glucose as the main substrate. With normal leucocytes, they found an oxygen consumption of 0.40 $\pm$ 0.17 m-mole per 10^{10} cells per hour, which is rather higher than the values found by most other workers. The figures for cases of chronic myeloid and chronic lymphatic leukaemia cases were 0.12 $\pm$ 0.03 and 0.11 $\pm$ 0.10 respectively. With both types of leukaemia they also found a markedly reduced glucose utilisation rate and a markedly reduced lactic acid production rate.

The many reported biochemical differences (especially differences in oxygen consumption rates) between normal and leukaemic leucocytes suggest the possibility of different metabolic pathways in the two types of cell. The intermediate carbohydrate metabolism has been studied by a number of workers, especially by Wagner and Reinstein (1950). They investigated the breakdown of glycogen in leucocytes and found that the reaction was catalysed by a phosphorylase, identical as far as they could discover, with the phosphorylase occurring in other tissues. In leucocytes isolated by the fibrinogen method from citrated horse blood they demonstrated the formation of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate, and phosphoglyceric acid, and inferred that the catabolism of glycogen followed the same pathways as in other tissues.

McKinney, Martin,Rundles and Green (1953) studied the respiratory and glycolytic activities of human leucocytes and concluded that the carbohydrate breakdown involved the usual Krebs' cycle. They also gave evidence that intracellular oxidation may pass through the cytochrome system.

All the available evidence favours the conclusion that leucocytes have a pathway for oxidation similar to that found in other tissues, and that these cells have/

/have a high rate of aerobic glycolysis. McKinney et al. (1953) go further and state that the difference between intact and homogenised leucocytes to the effects of intermediate products of metabolism and to various inhibitors, may be correlated with cell permeability, and that the response of leucocytes to certain chemical agents parallels the pharmacological effects of these agents in the intact animal.

After 1953, there are very few reports of work on the respiration of leucocytes. Cooper and Fitzgerald (1958) examined rat lymphocytes suspended in a Krebs-Ringer-phosphate medium and found an oxygen consumption of 0.24 ± 0.03 μ mole oxygen per 10^7 cells per hour. Addition of glucose to a final concentration of 100 mg. per 100 ml. did not alter the respiration rate in the first two hours. The oxygen consumption was proportional to the cell concentration over the range 16-130 x 10^6 lymphocytes per ml. They also stated that 5 hours after incubation in Warburg flasks at 37°C., the lymphocytes still showed their characteristic pseudopodial movements.

The latest review of the subject was by Valentine (1956) who covered the work on leucocytes in the period 1953-56 and concluded by saying that studies on the biochemical activity of leucocytes are in an early formative period, and represent attempts to get inside and/

/and explore its metabolic machinery. It is apparent, he states, that morphologically identical cell populations such as those of chronic myeloid leukaemia and polycythaemia rubra vera in the leukaemoid phase, may continue to show widely different metabolic capacities. It is known that biochemical changes precede clinically detectable leukaemia as proved by studies on the atom bomb survivors of Hiroshima and Nagasaki (Moloney and Large, 1954). This work is a further exploration of the metabolism of leucocytes under various selected conditions.

Metabolic Action of Salicylate.

In 1916, Denis and Means first observed that administration of salicylate increased the oxygen consumption and the carbon dioxide production in man. This finding has been repeatedly confirmed in man, e.g. by Cochrane (1952, 1954), and in experimental animals, e.g. by Meade (1954), Tenney and Miller (1955) and Reid (1957). Although salicylate increases the minute pulmonary ventilation, it has been demonstrated that the increase in basal metabolic rate following salicylate administration is not dependent on the increase in respiratory work./

/work. Decapitated or curarized dogs, maintained in a total body respirator designed to keep constant ventilation, showed the same oxygen consumption in response to salicylate administration as the intact animal (Tenney and Miller, 1955). It has also been shown by Brody (1956), that the oxygen consumption of tissues removed from salicylate-treated animals is greater than the oxygen consumption of the corresponding tissues of control animals. Sproull (1954) and Smith and Jeffrey (1956) have shown that salicylate, added in vitro, increases the oxygen consumption of isolated tissue and of mitochondrial preparations. These increases in oxygen consumption are accompanied by reduction in the esterification of organic phosphate, i.e., salicylate uncouples oxidative phosphorylation (Brody, 1956). This is now believed to be one of the main pharmacological actions of salicylate.

The effect of salicylate is strikingly similar to that of 2:4-dinitrophenol which increases the basal metabolic rate in the intact animal (Cutting, Rytand and Tainter, 1934) (Simkins, 1937) and also increases the oxygen consumption in tissue slices (Goldberg, Wolff and Greep, 1955). Lardy and Wellman (1952) and Packer (1957) have shown that 2:4-dinitrophenol uncouples oxidative phosphorylation in/

/in isolated mitochondrial preparations.

Sproull (1954) has shown that sodium salicylate has two separate effects on the respiratory rate of mouse liver slices, (1) an initial stimulation followed by (2) a depression of respiration at higher salicylate concentrations. It cannot be assumed that these effects are universal, occurring in all tissues, but it is reasonable to suppose that they are not confined to the liver. In the past, such effects have been particularly associated with the action of 2:4-dinitrophenol. But stimulation of respiration is not the only common factor in the effects of salicylate and 2:4-dinitrophenol on tissue metabolism. Both substances are glycogenolytic (Manne, Mayer and Plantefol, 1933, Lutwak-Mann, 1942, Sproull, 1954); both are diaphoretic and increase nitrogen excretion in the urine. Body weight falls after repeated administration, and polyuria follows withdrawal (Manne et al., 1933, a, b); Reid, Watson and Sproull, 1950).

The action of salicylate in reducing hyperglycaemia, glycosuria and ketonaemia in some diabetic patients has long been known. In the rat, both sodium salicylate and acetyl-salicylic acid are capable of reducing the hyperglycaemia and the glycosuria of diabetes/

/diabetes induced by partial pancreatectomy (Ingle, 1950), or induced by alloxan (Smith, Meade and Bronstein, 1952), or induced by cortisone (Smith, 1952). The mechanism of this action of salicylate is not known. Salicylate is unlikely to act in diabetes by reducing gluconeogenesis, for in normal rats, salicylate administration produces a negative nitrogen balance (Winters and Morrill, 1955; Penmall, 1956).

Manchester, Randle and Howard-Smith (1958) re-investigated this effect by studying the action of salicylate on the uptake of glucose by the rat diaphragm in a medium buffered by bicarbonate. Their observations showed that sodium salicylate increased glucose uptake and potassium loss from the tissue, and it also decreased the incorporation of ^{14}C-labelled glycine, lysine and glutamic acid into the protein of the tissue. This finding supports the suggestion of Reid, Macdougall and Andrews (1957) that salicylate stimulates metabolism in peripheral tissues. These authors also observed that salicylate increases the basal metabolic rate of both diabetic and non-diabetic subjects.

In 1955, Brody carried out extensive investigations on the <u>in vitro</u> action of salicylate on mitochondrial preparations, and compared the results obtained with the effects of 2:4-dinitrophenol on similar preparations.

He/

/He found that a concentration of 2 x 10^{-3}M salicylate depressed the oxygen consumption by about 20% and completely inhibited the uptake of inorganic phosphate (P:O = 0). Qualitatively similar results were obtained with 2:4-dinitrophenol. Several nitrated and halogenated phenols, when added to mitochondrial preparations, accelerate the liberation of inorganic phosphate from adenosine-triphosphate (Hunter, 1951; Potter and Recknagel, 1951). It has been suggested that the effect of 2:4-dinitrophenol in uncoupling oxidative phosphorylation, and its effect in catalysing the liberation of inorganic phosphate from adenosine-triphosphate are related in some way, although the nature of the relationship is still obscure. Sodium salicylate, like 2:4-dinitrophenol, catalyses the hydrolysis of adenosine-triphosphate, but a higher concentration of salicylate is required than that needed to uncouple oxidative phosphorylation.

Thyroid Hormone.

The fundamental effect of thyroid hormone is to increase the speed of normal metabolic processes in all cells of the body except the thyroid, spleen, lymph nodes, thymus, gonads and brain (Barker, 1955). This effect can be demonstrated by the increase in oxygen consumption both in the intact animal and in surviving isolated tissue. Whether the increased oxygen consumption is itself the fundamental reaction, or whether it is increased speed of some specific enzyme process, has still to be decided. In the complete absence of thyroid hormone, cellular activity still proceeds, but the rate of oxygen uptake is about 40-45% lower than normal.

The principal secretion of the thyroid gland is thyroxine, but small and variable amounts of triiodothyronine are also excreted. These two amino acids are removed from the circulation mainly by the liver, but also to a small extent by the kidneys. There are reasons for believing that neither of these two compounds is the true thyroid hormone, and the question of the moment in thyroid biochemistry is the precise nature of the chemical compound which stimulates peripheral tissue and whose precursor had its origin in the thyroid gland.

It/

/It has long been known that there is a latent period after the administration of various thyroid preparations and substances isolated from the thyroid gland, before any metabolic stimulation can be observed. With dessicated thyroid gland, this latent period is of the order 10-14 days; with thyroxine, the latent period lasts for several hours. In the case of thyroxine, it was originally assumed that this latent period corresponded to the time required for the thyroxine to react with protein to give thyroglobulin, but in his general review of the subject, Harington (1945) pointed out that such a theory would only introduce an entirely unnecessary complication.

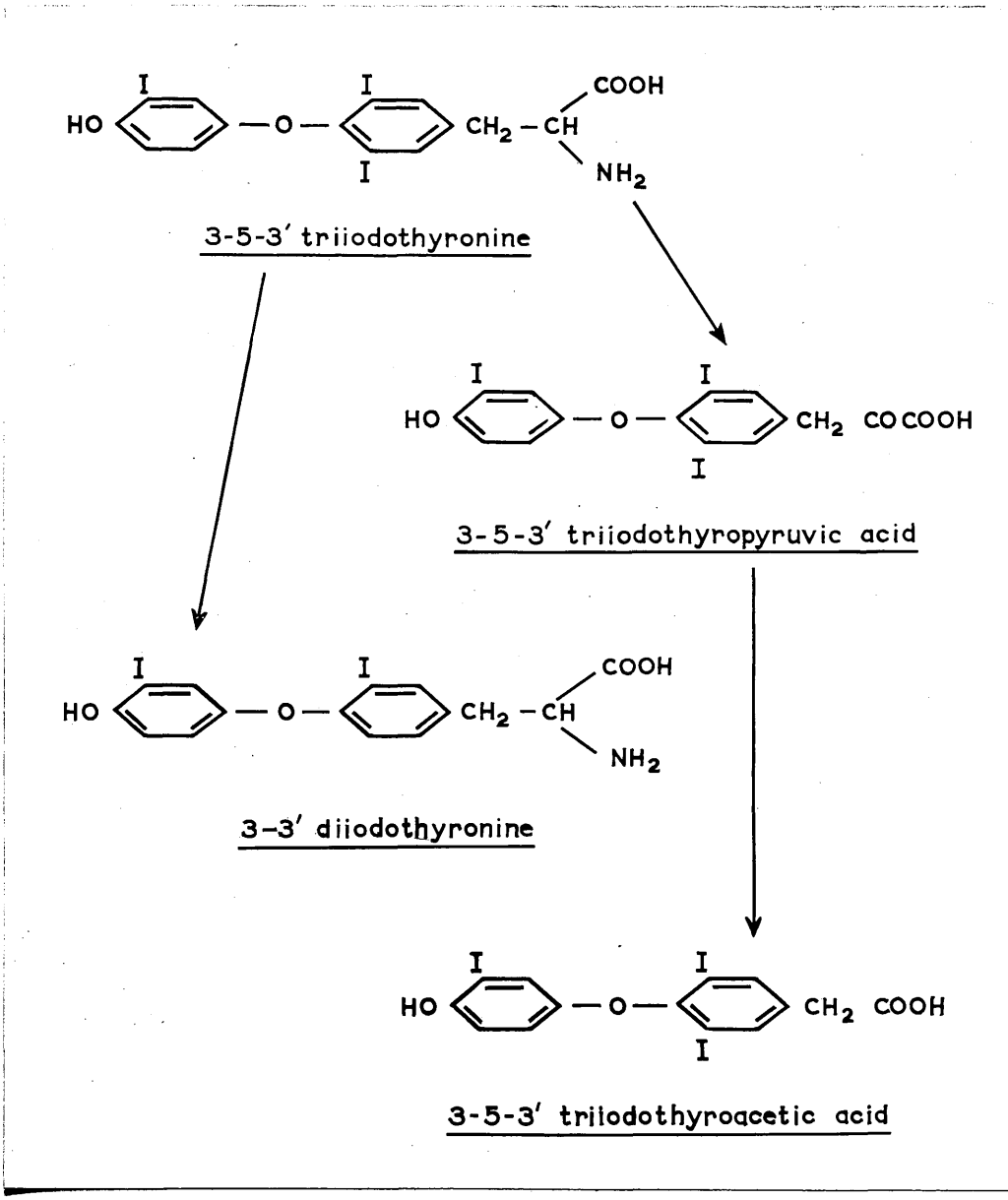
The discovery of triiodothyronine was one of the most important discoveries in the field of thyroid biochemistry during the last few years, and brought the hope of finding a solution to the problem of the latent period. After its identification in plasma and a study of its physiological properties by Gross and Pitt-Rivers (1952), it was thought that this compound might be the active form of the thyroid hormone which investigators had been seeking. By the same standards as are applied to thyroxine, triiodothyronine is/

/is a thyroid hormone. Both substances are formed in the thyroid gland and both are secreted into the blood stream; they have the same physiological actions but quantitatively, triiodothyronine is the more active. Gross, Pitt-Rivers and Thibault (1953) showed that triiodothyronine has exactly the same latent period as thyroxine. The problem of the latent period remained unsolved and Klemperer (1955) concluded that "the latent period might correspond to the formation of some active form of hormone, different from both thyroxine and triiodothyronine".

Harington and Pitt-Rivers (1952) and Pitt-Rivers (1953) succeeded in synthesising several acetic acid derivatives of the thyroid hormones. Pitt-Rivers (1953) showed that triiodothyroacetic acid (commonly contracted to TRIAC) has the same physiological actions as triiodothyronine. Thibault and Pitt-Rivers (1955, a, b) tested the effects of these acetic derivatives of thyroxine on the respiration of tissue slices and found that, in contrast to thyroxine and triiodothyronine, they produce an immediate effect on the respiration rate. They therefore concluded that the thyroid hormones (thyroxine and triiodothyronine) became metabolically active only after their conversion to/

/to the corresponding acetic derivatives.

It was later discovered that the muscle of thyroidectomised rats, after injection of physiological doses of 3:5:3'-triiodothyronine contained 3:5:3'-triiodothyroacetic acid and l-diiodothyronine.



The above formulae and interactions show the probable pathways for the metabolism of triiodothyronine, (1) partial deiodination without change in the alanine residue, and (2) oxidative deamination and decarboxylation of the amino acid side chain. Presumably the same interactions may take place with thyroxine giving rise to the corresponding acetic derivative 3:5:3':5'-tetraiodothyroacetic acid which has also been isolated in small quantity from plasma.

Triiodothyroacetic acid has been detected in the receptor cells of muscle and kidney. Either or both of these iodothyroacetic acids may be the active form of the thyroid hormone. The point has not yet been decided.

The actions of these acetic derivatives have been extended in other directions. Pitt-Rivers (1953) showed that they could reverse the thiouracil-induced goitre of rats; Bruce, Pitt-Rivers and Sloviter (1956) found that they restored the altered plumage of thyroidectomised birds to normal; Lerman and Pitt-Rivers (1955) administered these acids to human cases of myxoedema and observed a subsequent loss of weight and fall in the blood cholesterol concentration; Heimberg, Park, Isaacs and Pitt-Rivers (1955) showed that they produced an immediate stimulation of oxygen consumption and glycolysis in ascites-tumour cells.

The available evidence strongly supports the view that the latent period represents the time necessary for the conversion of the thyroid hormones to the metabolically active acetic derivatives. The enzymes necessary for this interconversion are normally present in all tissues. La Chaze and Thibault (1952) first identified the amino acid oxidase which can catalyse the conversion of all aromatic amino acids to the corresponding acetic acid derivatives. Certain tissues, e.g., thyroid, spleen, lymph nodes, etc., which are very rich a decarboxylase are probably poor in the amino acid oxidase necessary for this reaction, and these tissues are not sensitive to thyroid hormone (Barker, 1955).

EXPERIMENTAL METHODS.

The oxygen consumption of leucocytes was determined by the direct method using air as the gas phase at 37°C. in a Rotary Warburg apparatus made by Braun of Melsungen, Western Germany. The flasks and manometers were calibrated by the method described by Dixon (1943). The flasks were shaken at 80 oscillations per minute.

Isolation of Leucocytes:

Blood specimens were collected during the early forenoon from fasting and resting (basal) subjects. 40-50 ml. blood was withdrawn from the median basilic vein using a wide-bore needle and was transferred directly to a large siliconed tube containing heparin (100 units). Sedimentation of the erythrocytes was accelerated by the addition of 1 volume of Dextraven (Benger's dextran solution, 6% (w/v) solution of dextran in 0.9% sodium chloride solution) to 5 volumes of blood. The two were gently mixed and allowed to stand for 30 minutes at 4°C. The supernatant plasma (containing the leucocytes) was then transferred by a siliconed pipette into siliconed centrifuge tubes. The plasma was centrifuged at 50 g. for 10 minutes and the

supernatant/

/supernatant decanted and discarded. The sediment was re-suspended in a modified Krebs' buffer (see below) and so adjusted that the final suspension contained 50-70 x 10^6 leucocytes per 3.0 ml.

The technique does not effect complete separation of leucocytes and erythrocytes. The ratio of leucocytes to erythrocytes in the final suspension was of the order 1 : 2 but since the oxygen consumption of leucocytes is at least 100 times greater than the oxygen consumption of erythrocytes, the presence of these contaminating erythrocytes may be ignored. Other types of cell present in the suspension were reticulocytes and platelets. Reticulocytes are juvenile red cells; they contain remnants of the basophilic ribonucleoprotein which was present in larger amounts in the cytoplasm of the nucleated precursors from which they were derived. The reticulocyte count is 0.2 - 2.00% of the total red cell count. Since in the final suspension of leucocytes, the red cell:white cell ratio is 2:1, the reticulocyte:white cell ratio will be of the order of 0.001 - 0.010:1 and is so small that it may be disregarded.

The platelets in the blood are active with respect to repiration and carbohydrate metabolism (Campbell, Small, LoPilato and Dameshek (1957)). Their/

/Their oxygen uptake is of the order of 0.003 µm. per hour per 10^7 platelets (Campbell, Small and Dameshek (1956)). Since the platelet count is 150,000 - 400,000 as against 5,000 - 10,000 per cu.mm. for leucocytes, the question arises whether contamination with leucocytes will introduce a large error in determination of respiration of leucocytes. However, during the separation of the leucocytes, after the sedimentation of the erythrocytes, the supernatant plasma containing leucocytes plus platelets is centrifuged at 50 g. At this speed, the leucocytes are precipitated while the platelets are almost totally still suspended in the plasma. Thus in the suspension of leucocytes used in these studies, the few reticulocytes present do not significantly contribute to the total respiration of the cells in the Warburg flasks.

Each Warburg flask contained approximately 50×10^6 leucocytes (i.e., 3.0 ml. of the suspension) and all estimations of oxygen consumption were carried out in duplicate.

Buffer Medium:

The buffer solution used was a modification of the Krebs Medium II Buffer (Krebs, 1950). This is a calcium-free medium containing sodium, potassium, magnesium/

/magnesium, chloride and sulphate in approximately the same concentrations as occur in plasma and is recommended as the best medium to use for human cells or tissue. Phosphate is about 20 times higher and bicarbonate about 10 times lower than the corresponding concentrations in plasma. Krebs Medium II Buffer also contains pyruvate, fumarate and glutamate which were omitted from the modified buffer used in this work. In addition, the glucose concentration was increased to a final value of 100 mg. per 100 ml.

The modified buffer solution used had the following composition:-

83 volumes 0.90% (w/v) sodium chloride,

4 volumes 1.15% (w/v) potassium chloride,

1 volume 2.11% (w/v) anhydrous potassium dihydrogen phosphate,

1 volume 3.82% (w/v) magnesium sulphate heptahydrate,

3 volumes 1.30% (w/v) sodium bicarbonate,

18 volumes sodium phosphate buffer mixture.

The sodium phosphate buffer solution consisted of:-

100 volumes 0.1 M disodium hydrogen phosphate,

25 volumes 0.1 M sodium dihydrogen phosphate.

In order to minimise bacterial growth in the medium the glucose was not added to the medium until/

/until the day of use. Normally about 20 ml. of medium were required; to this was added 20 mg. of glucose thus bringing the concentration of glucose to 100 mg. per 100 ml. Compared with plasma, the greater part of the bicarbonate buffer is replaced by a phosphate buffer of the same pH and of approximately equivalent concentration. The solution has a relatively high concentration of phosphate - hence the omission of calcium.

The pH of the medium was finally adjusted to 7.40 $\pm$ 0.05 by the addition of iso-osmotic disodium hydrogen phosphate solution containing 1.725 g. of the anhydrous salt per 100 ml.

It is essential that the pH be maintained within the limits 7.35 to 7.45, otherwise the respiration of the leucocytes is grossly disturbed. The most critical factor is pH control. The buffer system suggested by McKinney, Rundles and Martin (1952) was not found to be satisfactory and the difficulty was traced to the pH which was always less than 7.30. In any experiment where the cells failed to respire properly, it was found that the pH was outwith the required limits of 7.35 to 7.45. This occasionally happened early in the work and was found to be due to/

/to inadequate removal of acid after the flasks, etc., had been siliconed.

Oxygen consumption of the subject was measured by the Benedict-Roth apparatus. White cell counts were carried out in a haemocytometer using the technique described by Whitby and Britton (1953), and taking the mean of two independent counts. Blood films for differential cell counts were stained by Leishmann's method and examined in the usual way. Plasma salicylate was determined by the method of Trinder (1954).

It was important to ensure that the relationship between oxygen consumption and cell numbers per flask was linear over the range likely to be encountered with the large numbers of cells obtainable from leukaemic patients. The primary object of such an experiment was to prove that the capacity of the buffer solution used, was sufficient to cope with the metabolic products of large numbers of cells, and at the same time, to show that with normal blood, there was a considerable reserve of buffering power. Leucocytes were isolated from three different patients suffering from leukaemia and it was shown that the oxygen consumption per hour per 10^7 cells was constant in each case when the number of leucocytes per/

/per flask was varied from 3.0×10^7 cells to 20.0×10^7 cells per flask. The results are given in Table I.

TABLE I. Effect of increasing the Number of Cells per Flask on the Oxygen Uptake per Hour per 10^7 Cells.

No. of cells per flask.	Oxygen uptake in μ moles per hour per 10^7 cells		
	Patient A.	Patient B.	Patient C.
3.0×10^7	0.125	0.103	0.152
6.0×10^7	0.129	0.101	0.157
9.0×10^7	0.125	0.108	0.150
12.0×10^7	0.127	0.105	0.157
20.0×10^7	0.123	0.107	0.152

The reproducibility of the method of determining oxygen consumption is shown by a comparison between duplicate analyses of the same cell preparation. Results are given in Table II.

TABLE II. Differences between Duplicate Estimation of the Rate of Oxygen Consumption.

All results are given in μ moles O_2 per hour per 10^7 leucocytes.

Duplicates.			Duplicates.		
Flask A.	Flask B.	Diff.	Flask A.	Flask B.	Diff.
0.126	0.120	0.006	0.073	0.080	0.007
0.050	0.049	0.001	0.095	0.093	0.002
0.122	0.114	0.008	0.130	0.116	0.014
0.116	0.110	0.006	0.124	0.113	0.021
0.178	0.188	0.010	0.094	0.099	0.005
0.220	0.200	0.020	0.120	0.110	0.010
0.134	0.116	0.018	0.063	0.062	0.001
0.205	0.160	0.045	0.098	0.099	0.001
0.103	0.101	0.002	0.144	0.146	0.002
0.210	0.186	0.024	0.136	0.132	0.004
0.078	0.070	0.008	0.128	0.135	0.007
0.095	0.094	0.001	0.173	0.181	0.008
0.077	0.076	0.001	0.086	0.090	0.004
0.084	0.083	0.001	0.116	0.103	0.013
0.086	0.086	0.000	0.071	0.072	0.001
0.166	0.162	0.004	0.090	0.091	0.001
0.130	0.116	0.014	0.121	0.120	0.001
0.122	0.118	0.004	0.065	0.068	0.003
0.111	0.116	0.005	0.179	0.183	0.004
			0.148	0.152	0.004

TABLE II. Mean of 39 differences..... 0.007

S.D. 0.011

The experiments tabulated in Table II cover the range of the great majority of estimations carried out in this work. Reproducibility over this range is satisfactory, and the accuracy of the method appears sufficiently good for the purposes for which it has been used.

Studies of the oxygen consumption of leucocytes were carried out on:-

(1) Normal healthy subjects (medical students),

(2) Patients with rheumatic fever who were being treated with sodium salicylate, and whose differential leucocyte count was within normal limits,

(3) Cases of hypothyroidism and of hyperthyroidism.

In vitro experiments were carried out with the metabolic stimulants, sodium salicylate at concentrations of 10 to 160 mg. per 100 ml. medium, and triiodothyroacetic acid (TRIAC) at concentration of 10 to 50 µg. per 100 ml. medium. These in vitro studies were carried out on:-

(1) Normal healthy subjects (medical students),

(2) Cases of chronic myeloid leukaemia and of chronic lymphatic leukaemia.

THE OXYGEN CONSUMPTION OF NORMAL LEUCOCYTES.

The rate of oxygen consumption of normal leucocytes from normal individuals was determined in a series of 16 cases. These controls were healthy young adults of both sexes. They came to hospital in a fasting state and were made to rest for at least 30 minutes. The basal metabolic rate was first determined and blood was taken by venepuncture immediately afterwards. In certain cases, the procedure was repeated on the same individual a few days later in order to assess the day-to-day variation. Results are shown in Table III.

TABLE III. The Oxygen Consumption of Normal Leucocytes.

All results are given in μ moles O_2 per hour per 10^7 cells.

Each figure quoted is the mean of two duplicate analyses. In 6 cases, the oxygen consumption was measured on two separate occasions at intervals of a few days.

TABLE III. The Oxygen Consumption of Normal Leucocytes.

Subject.	Cell oxygen consumption. First estimation.	Second estimation.
1.	0.079	0.081
2.	0.086	0.090
3.	0.086	0.103
4.	0.088	0.099
5.	0.091	0.088
6.	0.096	-
7.	0.097	0.093
8.	0.112	-
9.	0.113	-
10.	0.118	-
11.	0.123	-
12.	0.123	-
13.	0.125	-
14.	0.138	-
15.	0.145	-
16.	0.155	-
Mean of 16 values	0.111	
S.D.	0.023	

Results are expressed as μm. per hour per 10^7 cells.

THE EFFECT OF EXERCISE ON THE OXYGEN CONSUMPTION OF LEUCOCYTES.

During investigation of the respiration of human leucocytes it was observed that moderate exercise by the donor, prior to venepuncture, was associated with an increase in the rate of oxygen consumption. A short series of experiments was then carried out to determine the effect of such exercise on both the oxygen consumption of leucocytes and the metabolic rate of normal subjects. The subjects used were normal healthy young adults aged 18 to 29 years. They were in the fasting state, and rested for at least 30 minutes before the experiments began. The basal metabolic rate was then determined over a period of 5 minutes and blood was then collected by the method described above. The basal metabolic rate was determined a second time. The subjects then walked up and down two flights of stairs four times at the normal rate taking approximately 3-4 minutes. A third recording of the metabolic rate was made, and a second specimen of blood was taken. Finally, the metabolic rate was determined for a fourth time. The average of the first and second recordings was taken/

/taken as the basal metabolic rate, and the average of the third and fourth recordings was taken as the metabolic rate after exercise.

It is known that exercise may alter the number of circulating leucocytes; but the fact that results are expressed in μ moles oxygen per hour per 10^7 cells, eliminated any variation in the total white cell count. The data given in Table IV shows that the differential leucocyte count is not significantly altered.

TABLE IV. The Total Leucocyte Count and the Differential Leucocyte Count before and after moderate Exercise.

Total Leucocyte Count is expressed as cells per cu. mm. blood.

B = Before exercise.
A = After exercise.

Subject.		W.B.C.	Poly. %	Lymph. %	Mono. %	Eosin. %	Basoph. %
1.	B.	5,250	63	31	4	2	0
	A.	6,900	60	35½	3½	1	0
2.	No total or differential leucocyte counts were made.						
3.	B.	6,600	58	33	6	3	0
	A.	6,900	59	33	5	3	0
4.	B.	5,160	72	22	5	1	0
	A.	4,900	69	24	5	1	1
5.	B.	6,700	72	22	4	1½	½
	A.	10,600	70	24	5	1	0
6.	B.	5,480	66	28	5	1	0
	A.	5,700	68	27	4	1	0

The effect of this standard exercise test on the oxygen consumption of leucocytes is shown in Table V (a and b) where the effect is compared with the corresponding alteration in the metabolic rate.

TABLE V (a). Effect of Exercise on the Respiration of Leucocytes.

Leucocyte oxygen consumption μ moles O_2 per hour per 10^7 cells.

Subject.	Before exercise.	After exercise.	Percentage Increase.
1.	0.086	0.148	62%
2.	0.138	0.202	46%
3.	0.112	0.190	57%
4.	0.155	0.265	71%
5.	0.091	0.136	49%
6.	0.096	0.130	36%
		Average increase.....54%	

TABLE V (b). The corresponding Effect of Exercise on the Subject's Oxygen Consumption.

Subject's Oxygen Consumption ml. oxygen per minute.

Subject.	Surface Area. m^2.	Before exercise.	After exercise.	Percentage Increase.
1.	1.82	226	246	9%
2.	2.01	359	437	22%
3.	1.86	199	298	49%
4.	1.88	203	279	37%
5.	1.92	272	390	43%
6.	1.78	218	235	8%
			Average increase.....	28%

The effect of exercise on the respiration of leucocytes is twice as great as its effect on total body oxygen consumption. Further, more consistent results are obtained by measuring the oxygen consumption of the isolated cells.

The possibility that the change in leucocyte oxygen consumption was due in some way to venepuncture was considered. Blood samples were taken from three normal subjects, exactly under the conditions described above, except that the exercise was omitted. The change in/

/in oxygen consumption between the two samples was -11%, +5%, and -3%. Such figures cannot account for the changes observed in Table V (a).

Two conclusions were drawn from these results:-

(1) that a "substance" may appear in the blood as a result of moderate exercise, which has the property of stimulating the metabolic activity of the leucocytes <u>in vitro</u>, and,

(2) that in previous work on the respiration of leucocytes, the discordant results reported (e.g., see Beck and Valentine, 1953) may have been due to the fact that preceding muscular activity was not taken into account.

These two conclusions will be dealt with in the discussion (see p. 87).

METABOLIC STIMULATION BY SALICYLATE.

It is known that salicylate stimulates body metabolism. The plasma salicylate concentration has been directly correlated with the metabolic rate by Reid (1957) in the rabbit, and by Alexander and Johnston (1958) in man. With this in mind, experiments were designed to discover if there were any correlation between the plasma salicylate concentration and the oxygen consumption of leucocytes *in vitro* in man.

Two patients who were still on salicylate therapy, were selected as suitable subjects. Their total and differential leucocyte counts were within normal limits. In both cases, 10 different specimens of blood were collected at intervals as the dosage of acetyl-salicylic acid was increased, and the plasma salicylate concentration and the rate of oxygen consumption of the leucocytes were determined on each specimen.

The leucocytes were suspended in the usual buffer solution (p.27) which contained no salicylate, and it was observed repeatedly that the oxygen uptake at all plasma salicylate concentrations was linear over a period of 2-3 hours, indicating that therapeutic salicylate/

/salicylate had reached its site of action inside the cells.

TABLE VI. Relation between Time and Oxygen Uptake of Leucocytes from Patients on Salicylate Therapy.

(Sodium salicylate concentration of plasma:- 40 mg. per 100 ml.)

Time in mins.	Δ t.	Flask 1.	Δ Reading.	Flask 2.	Δ Reading.
Zero	-	150.0	-	150	-
10	10	144.0	6.0	145.0	5.0
20	10	138.5	5.5	140.0	5.0
30	10	132.0	6.5	134.5	5.5
40	10	126.0	6.0	131.0	4.5
50	10	120.5	5.5	125.0	5.0
60	10	114.0	6.5	120.0	5.0
90	30	96.5	17.5	104.0	16.0
120	30	78.0	18.5	89.0	15.0
150	30	60.5	17.5	64.5	15.5
180	30	42.0	18.5	50.5	14.5

If the salicylate were exerting its effect at the surface of the cell, it would be expected that, as the cell was bathed in a salicylate-free buffer medium, any salicylate molecules would be washed off the membrane and the oxygen consumption of the system would steadily decrease.

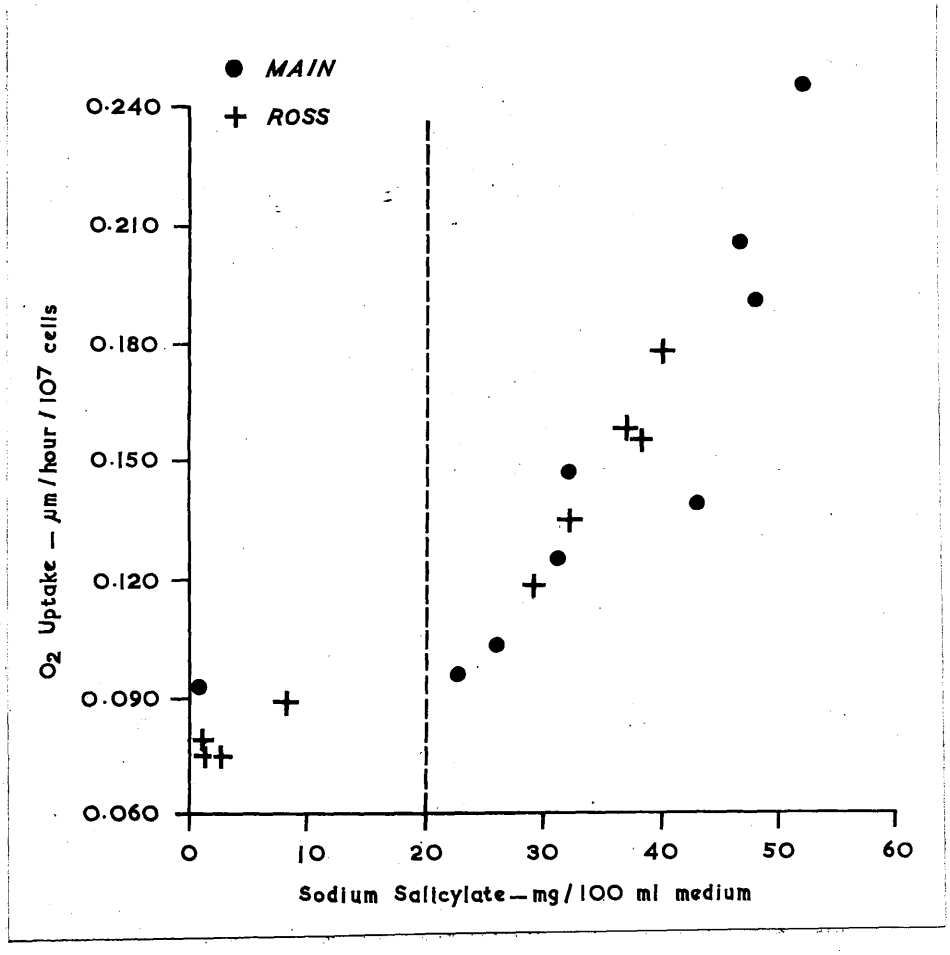
Results of these two experiments are shown in Figure 1, p. 44.

The oxygen consumption of the leucocytes does not begin to increase until the concentration of salicylate in the patient's plasma has exceeded 20 mg. per 100 ml. This agrees with the finding of Alexander and Johnston (1958) that the metabolic rate did not begin to increase significantly on salicylate administration until the plasma salicylate concentration reached 20 mg. per 100 ml. Lester, Lolli and Greenberg (1946) studied the ability of human plasma to bind salicylate in vivo and in vitro. They found that the proportion of bound salicylate in plasma decreases with increasing concentrations of total salicylate. At a level of 4 mg. per 100 ml., 90% was bound; at 16 mg. per 100 ml., 70% was bound; at 70 mg. per 100 ml., 54%.

When the plasma salicylate concentration lies in the range 20-60 mg. per 100 ml., the increase in oxygen consumption is linear. When a plasma concentration/

Figure 1.

In vivo effect of salicylate on the oxygen uptake of leucocytes.



/concentration of 60 mg. per 100 ml. is exceeded, symptoms of salicylism (dizziness, tinnitus, nausea, etc.) develop rapidly.

It is also interesting to note that the minimal therapeutic plasma concentration of salicylate is generally taken to be about 20 mg. per 100 ml., and relief of rheumatic symptoms does not occur, or is not maintained, unless the plasma concentration is above this level.

Effect of Salicylate in vitro.

The effect of salicylate on the respiration of leucocytes was further studied by determining the oxygen consumption of leucocytes from normal blood after they had been re-suspended in buffer containing various concentrations of sodium salicylate. Blood was obtained from eight healthy subjects (medical students) whose total and differential leucocyte count was within normal limits (see Table VII).

TABLE VII. Total and Differential Leucocyte Counts of the Normal Subjects whose Leucocytes were used for in vitro Studies with Salicylate.

(Cf. Table VIII and Figure 2).

Total leucocyte count in cells per cu. mm. blood.

Subject.	Total. W.B.C.	Poly. %	Lymph. %	Mono. %	Eosin. %	Basoph. %
1.	6,940	72	22	5	1	0
2.	7,200	71½	23½	3½	0	1½
3.	5,250	50	41	7	1½	½
4.	5,420	62½	33½	2½	1½	0
5.	5,020	48	41	6½	4½	0
6.	5,920	64	31½	3½	1	0
7.	6,260	66	28½	4	1½	0
8.	4,360	56	36	6	2	0
	Means	61	32	5	2	0

In each experiment, Warburg flasks were set up containing zero, 10, 20, 40, 60, 80, and 160mg. sodium salicylate per 100 ml. of buffer medium, and to each flask was added an equal number of leucocytes. The rate of oxygen consumption was then determined in the usual way. Results are shown in Table VIII, and the combined/

/combined result of all eight experiments is shown graphically in Figure 2.

TABLE VIII. The in vitro Effect of Sodium Salicylate on the Oxygen Consumption of Normal Human Leucocytes.

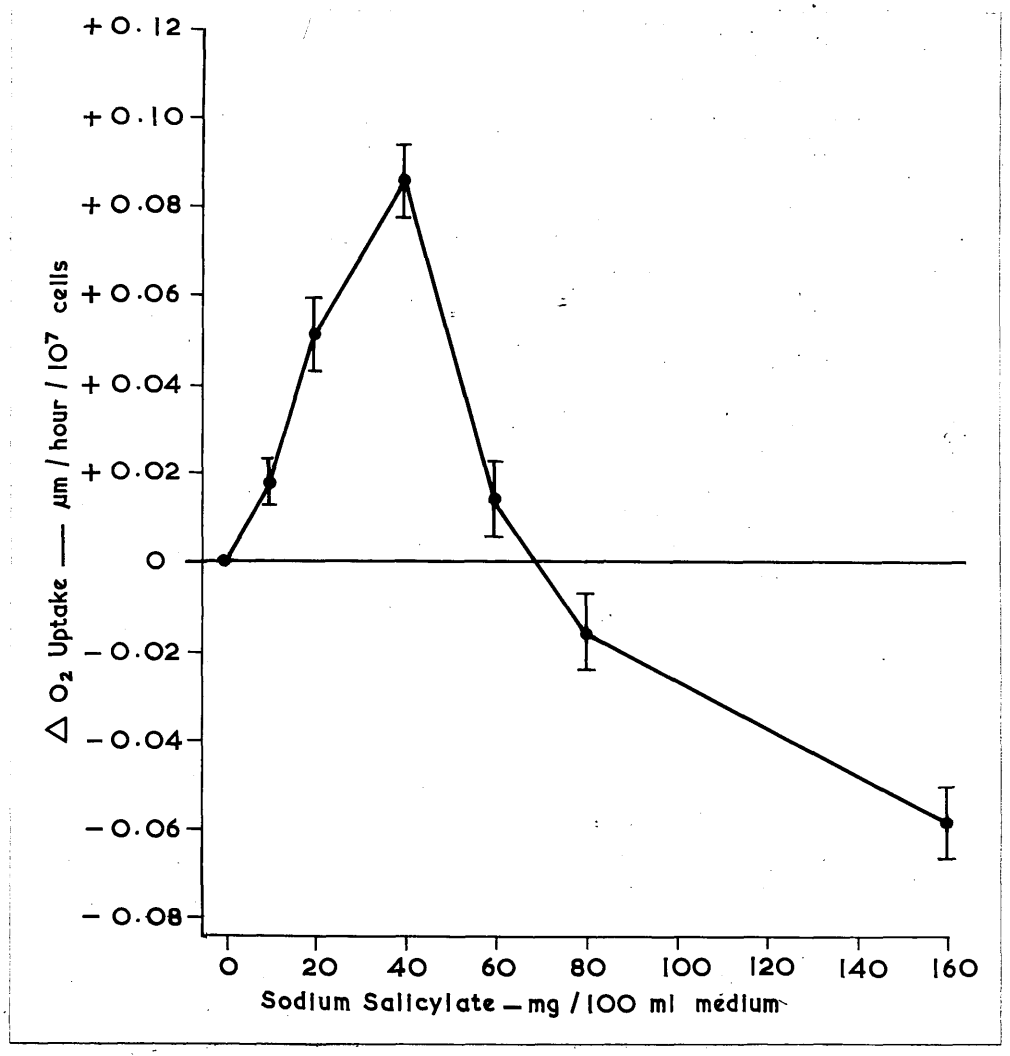
Results expressed in μ moles O_2 per hour per 10^7 leucocytes.

	Salicylate concentration in mg. per 100 ml.						
Subject.	Zero	10.	20.	40.	60.	80.	160.
1.	0.137	0.146	0.156	0.196	0.100	0.069	0.050
2.	0.097	0.104	0.149	0.168	0.115	-	0.044
3.	0.086	0.089	0.128	0.106	0.097	0.076	0.050
4.	0.125	0.148	-	0.240	0.144	0.113	0.085
5.	0.107	-	0.165	0.196	0.150	0.104	0.074
6.	0.110	0.140	0.158	0.197	-	0.113	0.085
7.	0.106	-	0.132	0.164	0.114	0.084	0.046
8.	0.109	0.150	0.199	0.230	0.152	0.098	-

Figure 2 shows the changes in the rate of oxygen consumption, compared with zero time, at the corresponding salicylate concentrations. Each point on the graph represents the mean change in oxygen consumption corresponding to the salicylate concentration; the feet represent the standard errors of each mean.

Figure 2.

Effect of sodium salicylate *in vitro* on oxygen uptake of normal human leucocytes.



The oxygen consumption increased up to a maximum corresponding to a salicylate concentration of 40 mg. per 100 ml. medium, and thereafter, progressively decreased. Concentrations of salicylate greater than 70 mg. per 100 ml. medium depressed the normal rate of oxygen consumption.

The importance of maintaining a constant pH in the Warburg flasks has been referred to above (p. 28). Sodium salicylate is slightly acid in aqueous solution (pH = 5-6). The buffer solution used is sufficiently powerful to maintain the pH constant to within $\pm$ 0.02 unit when the sodium salicylate concentration is increased up to the maximum value used - 160 mg. per 100 ml. The changes shown in Figure 2 are not, therefore, due to alteration of the pH.

The only previously recorded work on the in vitro effect of salicylate on O_2 uptake of cells, is the dose-response curve using mouse liver slices, reported by Sproull (1954). Sproull's curve is very similar to that shown in Figure 2. The oxygen consumption of the mouse liver slices increased with increasing salicylate concentration, reaching a maximum between 32-48 mg. salicylate per 100 ml. Thereafter, as the salicylate concentration was further increased, the oxygen consumption decreased, and/

/and at concentrations greater than 80 mg. salicylate per 100 ml., the rate of oxygen consumption was less than normal.

With mouse liver slices, Sproull found that the maximum stimulation by salicylate resulted in an increased rate of oxygen consumption of +22%. Using leucocytes, maximum stimulation at the salicylate concentration of 40 mg. per 100 ml., was +72% which is more in keeping with the increase in the total oxygen consumption of the subject during salicylate administration (Alexander and Johnston, 1958) which may increase the metabolic rate by as much as +45%. There are several possible reasons for this: (a) the method of preparation of the suspension of leucocytes results in little damage to the cells, (b) each leucocyte is independently bathed by the medium so that there is no danger of anoxia, and this is demonstrated by the fact that leucocytes respire at a constant rate for at least three hours under the conditions of these experiments.

When salicylate is administered to the subject, the rate of oxygen consumption of the leucocytes increases over the range 20-60 mg. salicylate per 100 ml. plasma (Figure 1, p. 44); when salicylate is added to the system <u>in vitro</u>, the rate of oxygen consumption of the leucocytes increases over the range

0/

/0-40 mg. salicylate per 100 ml. medium (Figure 2, p. 48). In the former case, there is evidence that the circulating salicylate is partly bound to the plasma protein, and therefore not available to the cells (Lester, Lolli and Greenberg, 1946) whereas in the latter case, the method of preparation of the leucocyte suspension involves removal of almost all the plasma protein (see p. 24) and the cells are then suspended in what is virtually a protein-free salt solution.

RELATIONSHIP BETWEEN THE OXYGEN UPTAKE OF LEUCOCYTES AND THE BASAL METABOLIC RATE.

It has been shown (Figure 1, p. 44) that there is a direct relationship between the oxygen uptake of leucocytes and the concentration of salicylate in the plasma; it has also been shown by Alexander and Johnston (1958) that there is a direct relationship between the concentration of salicylate in the plasma and the metabolic rate of the subject. The next development is the investigation of the direct relationship between oxygen uptake of the leucocytes and the metabolic rate. For this purpose, a series of hyperthyroid and myxoedematous patients, and a series of euthyroid control subjects, have been investigated. In each case, the patients and control subjects were studied under basal conditions. The basal metabolic rate was first determined by the Benedict-Roth method. Immediately afterwards, blood was taken for determination of the oxygen consumption of the leucocytes.

The patients were classified by the usual clinical methods supported by laboratory examinations, including the estimation of the basal metabolic rate. Results are set out in the Tables immediately following.

TABLE IX. Rate of Oxygen Consumption of Leucocytes and Basal Metabolic Rate.

Hyperthyroid Cases.

Oxygen Consumption is expressed in μ moles O_2 per hour per 10^7 leucocytes.

Basal Metabolic Rate is expressed in Cals. per m^2 per hour.

Subject.	Sex.	Age.	Basal Metabolic Rate.	Oxygen uptake of Leucocytes.
A.	F.	41.	58	0.240
B-1.	F.	44.	49	0.181
B-2.	F.	49.	51	0.238
C.	F.	52.	48	0.192
F.	F.	23.	70	0.119
L.	F.	34.	53	0.150
M.	F.	46.	50	0.198
P.	F.	57.	45	0.144
Q.	F.	37.	51	0.182
S-1.	F.	55.	47	0.134
S-2.	F.	49.	56.	0.194
T.	F.	24.	47	0.183
W.	F.	71.	54	0.210
S.	F.	38.	57	0.230
Means of 14 cases			53	0.185
Standard deviations			6	0.037

TABLE X. Rate of Oxygen Consumption of Leucocytes and Basal Metabolic Rate.

Myxoedematous Cases.

Oxygen Consumption is expressed in μ moles O_2 per hour per 10^7 leucocytes.

Basal Metabolic Rate is expressed in Cals. per m^2 per hour.

Subject.	Sex.	Age.	Basal Metabolic Rate.	Oxygen Uptake of Leucocytes.
B.	F.	48.	27	0.063
F.	F.	64.	30	0.097
McC.	F.	59.	26	0.094
McF-1.	F.	38.	26	0.084
McF-2.	F.	38.	30	0.077
McI.	F.	59.	24	0.077
McK.	F.	64.	23	0.095
McL.	F.	54.	26	0.074
P.	F.	66.	24	0.118
R.	M.	35.	19	0.050
Means of 10 cases			26	0.083
Standard deviations			2	0.016

Total and differential white cell counts were carried out on eight cases of hyperthyroidism (Table IX) and on eight cases of myxoedema (Table X). All values were found to be within the normally accepted limits.

TABLE XI. Rate of Oxygen Consumption of Leucocytes and Basal Metabolic Rate.

Euthyroid (control) Subjects. (Cp. Table III, p. 34).

Oxygen Consumption is expressed in μ moles O_2 per hour per 10^7 leucocytes.

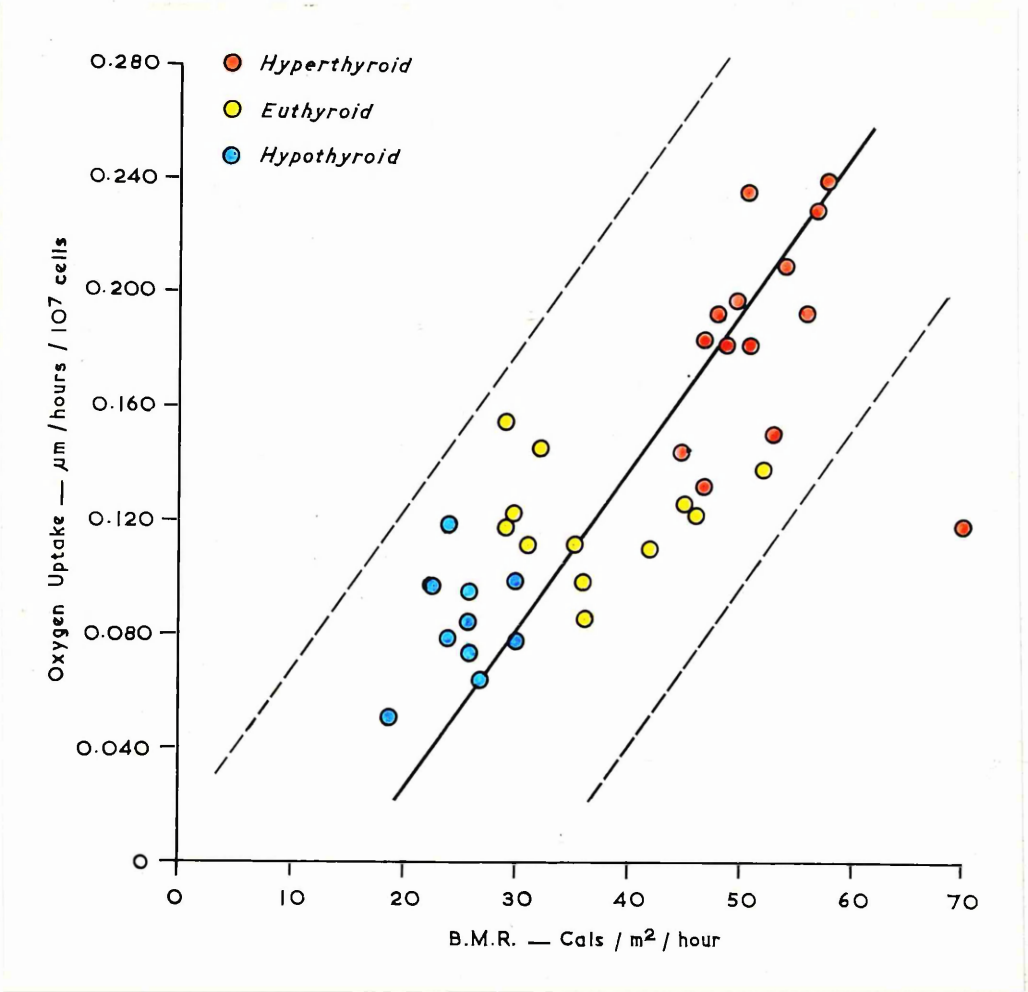
Basal Metabolic Rate is expressed in Cals. per m^2 per hour.

Subject.	Sex.	Age.	Basal Metabolic Rate.	Oxygen Uptake of Leucocytes.
A.	M.	29.	36	0.086
E.	M.	20.	52	0.138
F.	F.	17.	46	0.123
G.	M.	28.	31	0.112
McD.	M.	30.	29	0.155
M.	M.	25.	42	0.091
P-1.	F.		32	0.145
P-2.	F.	66.	30	0.123
R-1.	M.	26.	36	0.096
R-2.	M.	35.	29	0.118
S.	F.	60.	35	0.113
W.	F.	68.	45	0.125
Means of 12 subjects			37	0.119
Standard deviations			8	0.021

Note: Subject R-2 in the above table is the same as Case R in Table X. The values given above were those obtained after the patient had been successfully treated with thyroxine.

Figure 3.

Correlation of leucocyte oxygen uptake and B.M.R. in Hypothyroid, Euthyroid and Hyperthyroid cases.



$Y = 182x + 15.23$

$r = 8.77$ $\sigma r = 0.74$

95% confidence limits $\pm$ 17.8

The data in Tables IX, X, and XI, are shown graphically and compared in Figure 3. The correlation between basal metabolic rate and the oxygen consumption of the leucocytes is very satisfactory with one single exception.

Having determined this relationship, the point was further examined by following the changes in basal metabolic rate and in leucocyte oxygen consumption in myxoedematous and hyperthyroid patients before and after appropriate treatment. Three cases of myxoedema were selected at random and treated in the usual way with l-thyroxine until the basal metabolic rate of each had reached the normal range. Similarly, three cases of hyperthyroidism were treated with radioactive iodine. The changes in basal metabolic rate and in leucocyte oxygen consumption resulting from therapy are shown graphically in Figure 4. The actual protocols of Figure 4 are given in Table XII (p. 59).

In all six cases, the basal metabolic rate and the leucocyte oxygen consumption change in the same direction under treatment. The thyroxine is converted to "thyroid hormone" which increases both quantities; the radioactive iodine suppresses production of "thyroid hormone" in the thyroid gland and thereby leads to a decrease in both quantities. The matter will be more fully dealt with in the discussion (see p. 94).

Figure 4.

Effect of treatment on oxygen uptake of leucocytes from hyperthyroid and myxoedematous patients.

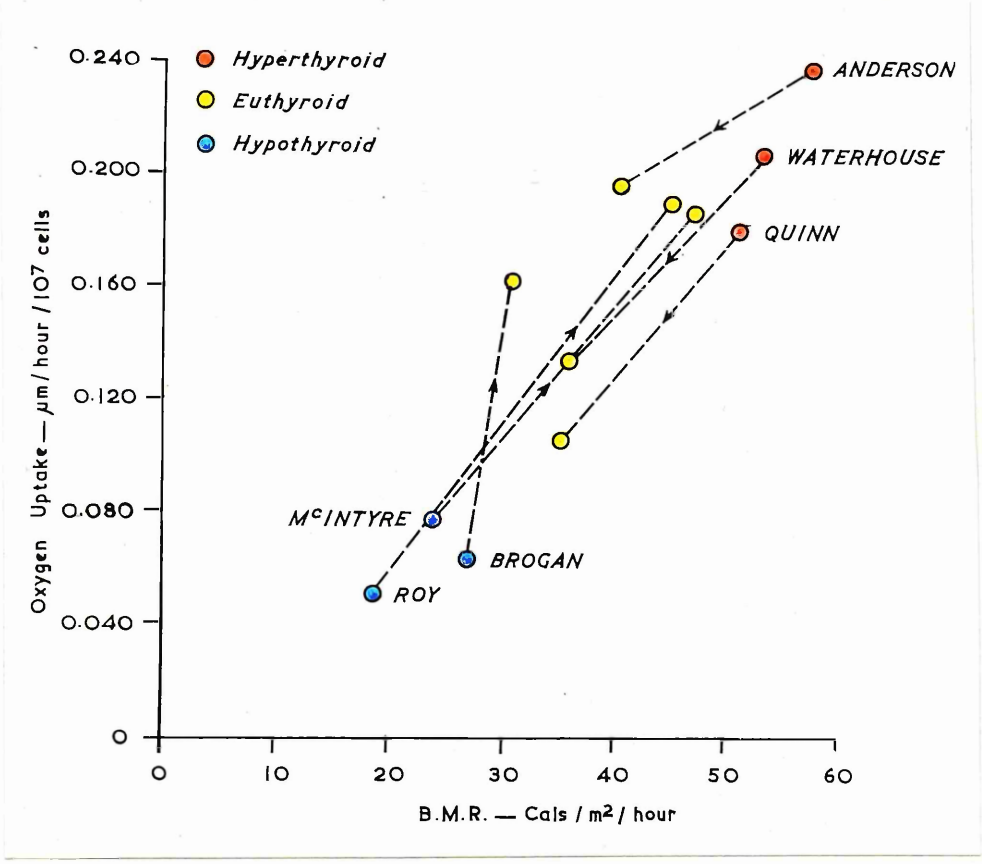


TABLE XII. The Effect of Treatment on the Basal Metabolic Rate and on Leucocyte Oxygen Consumption in Cases of Myxoedema and Hyperthyroidism.

(Cp. Figure 4, p. 58).

Oxygen Consumption is expressed in μ moles O_2 per hour per 10^7 leucocytes.

Basal Metabolic Rate is expressed in Cals. per m^2 per hour.

Hyperthyroid Cases: Treated with ^{131}I.

Patient:	Anderson.	Quinn.	Waterhouse.
B.M.R. before therapy.	57.9	51.4	54.0
Leucocyte oxygen uptake before therapy.	0.240	0.182	0.210
Dose of ^{131}I.	13 mc.	12 mc.	10 mc.
B.M.R. after therapy.	40.5	35.9	36.2
Leucocyte oxygen uptake after therapy.	0.196	0.106	0.135

Myxoedema Cases: Treated with l-Thyroxine.

Patient:	Brogan.	McIntyre.	Roy.
B.M.R. before therapy.	27.0	24.0	19.0
Leucocyte oxygen uptake before therapy.	0.063	0.077	0.050
Dose of l-Thyroxine.	0.2 mg. per day.	0.2 mg. per day.	0.3 mg. per day.
B.M.R. after therapy.	31.5	47.0	45.0
Leucocyte oxygen uptake after therapy.	0.163	0.187	0.190

The Effect of Triiodothyroacetic Acid on the Oxygen Uptake of the Leucocytes of Myxoedematous Patients.

It has been demonstrated (1) that the leucocyte oxygen uptake is directly proportional to the metabolic rate of the patient being high in hyperthyroidism and low in myxoedema (p. 56), and (2) that leucocyte oxygen consumption varies with the thyroid activity of the patient, being increased in cases of myxoedema treated with l-thyroxine, and decreased in cases of hyperthyroidism treated with ^{131}I (p. 58).

The acetic acid analogues of l-thyroxine and triiodothyronine (namely, tetraiodothyroacetic acid and triiodothyroacetic acid respectively) have been found to raise the metabolic rate of thyroidectomised and hypophysectomised rats without delay (Thibault and Pitt-Rivers (1955); Thibault (1955); and Donhoffer (1956)).

In hypophysectomised rats, intravenous injection of a few micrograms of TRIAC is invariably followed by a marked rise in total body oxygen consumption and in body temperature, while only about half of the thyroidectomised, and none of the intact rats showed a similar response (Várnai (1957)).

The/

/The next experiment in this series was designed to observe the effect of TRIAC on the oxygen consumption of the leucocytes in cases of myxoedema. It is not an easy matter to obtain suitable patients for such experiments. Cardiac lesions are not uncommon in myxoedema and an injection of TRIAC exerts a considerable strain on the patient's heart muscle and may produce a severe fibrillation. Consequently, such experiments are confined to relatively young subjects in whom no secondary cardiac lesion is demonstrable. Most hospital cases of myxoedema are elderly and not uncommonly have cardiac lesions demonstrable by electrocardiography, and in such cases, intravenous injection of TRIAC is not without danger.

The first case was a young woman aged 30. The patient had fasted and rested for 12 hours, then the basal metabolic rate was determined, and immediately afterwards, blood was withdrawn to determine the basal rate of oxygen consumption of the leucocytes. 5.0 mg. of triiodothyroacetic acid (in the form of the ethanolamine salt) in saline solution was injected intravenously. The basal metabolic rate was determined 60, 120 and 390 minutes after injection, and blood was taken for determination of the leucocyte oxygen uptake 30, 60, 120 and 180 minutes after injection. Results are shown in Table XIII.

TABLE XIII. Effect of Injection of TRIAC in a Case of Myxoedema.

Case 1. (see also Figure 5, p. 63).

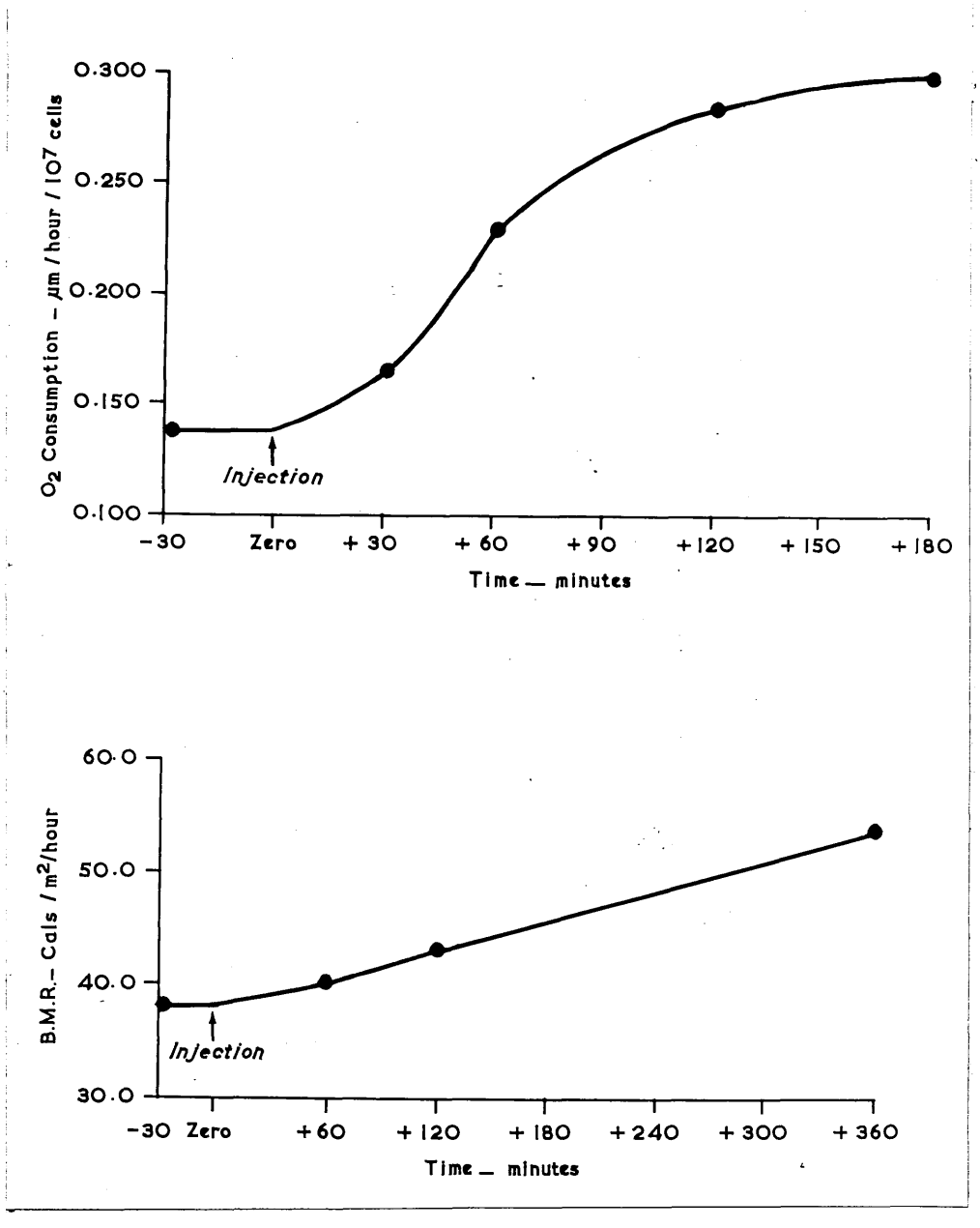
Oxygen uptake is expressed in μ moles O_2 per hour per 10^7 leucocytes.

Metabolic Rate is expressed in Cals. per m^2 per hour.

Time relative to the injection.	Oxygen uptake of leucocytes.	Metabolic Rate.
- 30 minutes.	0.138	37.9
+ 30 minutes.	0.165	-
+ 60 minutes.	0.230	40.1
+ 120 minutes.	0.285	43.0
+ 180 minutes.	0.300	-
+ 390 minutes.	-	54.0

Figure 5.

Effect of injection of TRIAC on the oxygen uptake of leucocytes and B.M.R. of a myxoedematous patient.



The second case was a young woman aged 20. The experiment was repeated as above, the only difference being the timing of the observations. The basal metabolic rate was determined 60, 120, 180 and 240 minutes after injection of TRIAC, and blood was taken for leucocyte oxygen uptake 60, 120 and 180 minutes after injection. Results are shown in Table XIV.

TABLE XIV. Effect of Injection of TRIAC in a Case of Myxoedema.

Case 2. (see also Figure 6, p. 65).

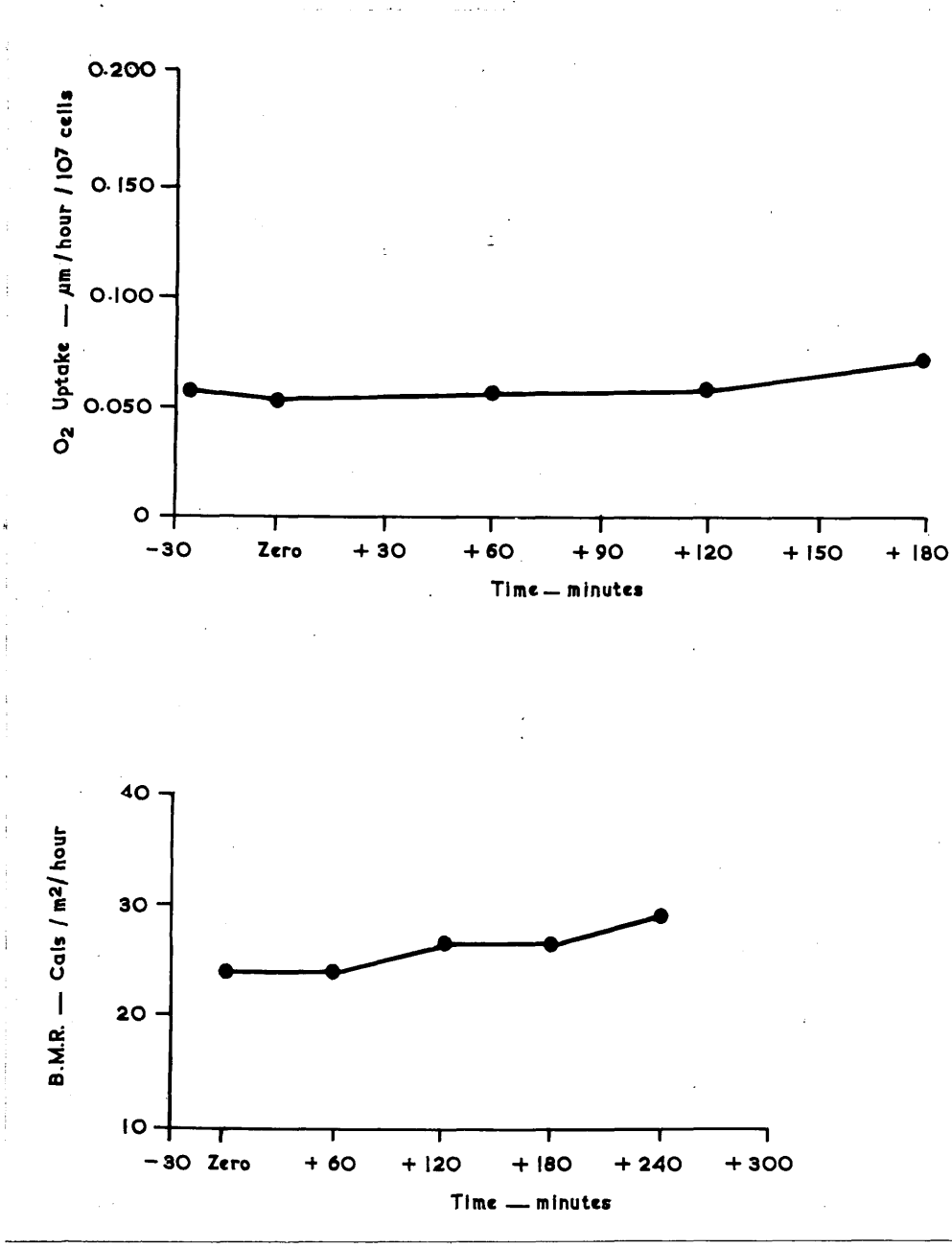
Oxygen uptake is expressed in μ moles O_2 per hour per 10^7 leucocytes.

Metabolic Rate is expressed in Cals. per m^2 per hour.

Time relative to the injection.	Oxygen uptake of leucocytes.	Metabolic Rate.
- 30 minutes.	0.059	-
Zero.	0.054	24.0
+ 60 minutes.	0.056	24.0
+ 120 minutes.	0.059	26.7
+ 180 minutes.	0.081	26.7
+ 240 minutes.	-	29.1

Figure 6.

Effect of an injection of TRIAC on the oxygen uptake of leucocytes and B.M.R. of a myxoedematous patient.



The limiting factor in this section of the work was the availability of suitable patients, and few conclusions can be drawn from only two experiments. The oxygen uptake of the leucocytes corresponds with the basal metabolic rate in each patient, and the different response of the two patients is far beyond the limits of experimental error of both determinations. Consequently, one is forced to conclude that the two patients have responded quite differently to the injection of TRIAC. This may possibly correspond with the variations in response found by Várnai (1957), described above.

In the second patient, the response to TRIAC is only small (but significant at 180 minutes) and is considerably delayed. This may be associated with the fact that the second patient was much more hypothyroid than the first, and might have required a larger dose of TRIAC to produce the increases obtained with the first patient. But at the time of injection, it was felt that 5.0 mg. TRIAC was the maximal dose that could be given with safety.

These matters require further experimentation, and this in turn, depends on the available number of suitable patients.

THE EFFECT OF TRIIODOTHYROACETIC ACID ON THE OXYGEN UPTAKE OF NORMAL HUMAN LEUCOCYTES.

Thibault and Pitt-Rivers (1955) obtained an immediate stimulation of oxygen consumption of kidney slices from thyroidectomised rats by addition to the system of the compound 3:5:3'-triiodothyroacetic acid (TRIAC). Donhoffer, Várnai and Szeibert-Horvath (1958) recorded an immediate increase in metabolic rate and in body temperature by injecting TRIAC into hypophysectomised rats. Having already studied the metabolic stimulation of the oxygen consumption of normal leucocytes *in vitro* with sodium salicylate (above, p. 48), it was of interest to determine whether TRIAC would also act as a metabolic stimulant of the oxygen consumption of normal leucocytes *in vitro*.

The procedure was similar to that carried out using sodium salicylate as stimulant. Normal leucocytes were isolated by the method previously described from the blood of 8 normal healthy subjects. These leucocytes were then suspended in the modified Krebs' No. 2 buffer. Warburg flasks were set up containing zero, 10, 15, 20, 25, 35 and 50 micrograms TRIAC per 100 ml. medium. The cell suspensions were added to these flasks and the rate of oxygen consumption was determined. The results obtained are shown in Table XV, and Figure 7, p. 67a.

Figure 7.

The effect of TRIAC on the oxygen uptake of normal human leucocytes in vitro.

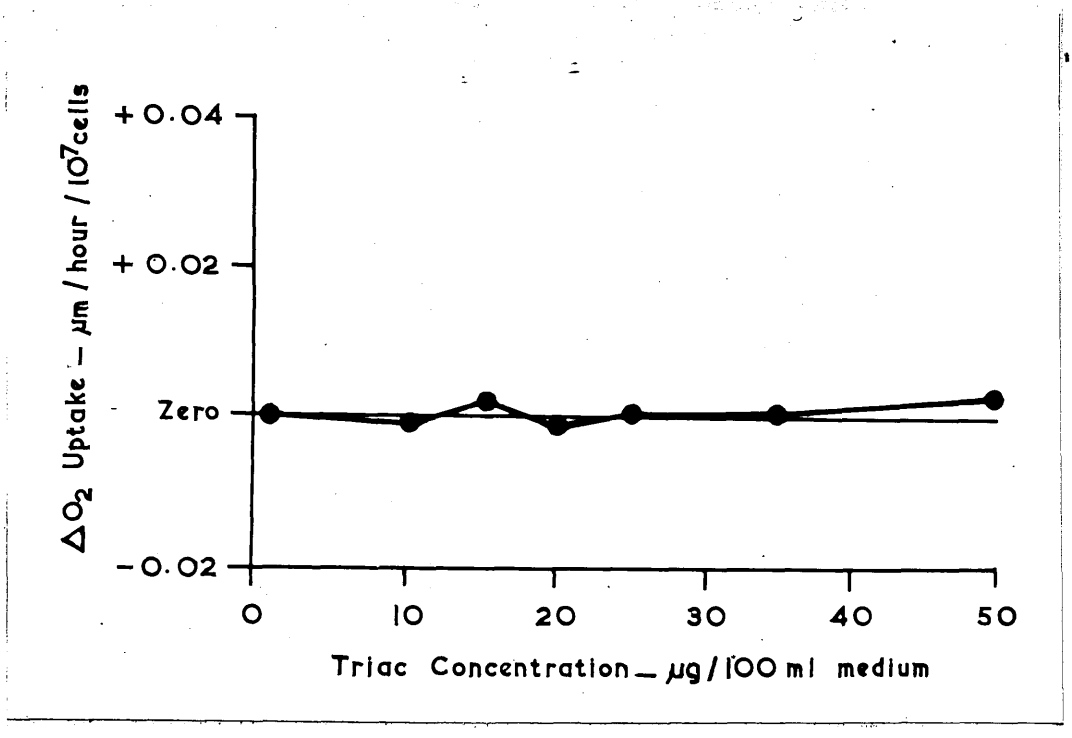


TABLE XV. The in vitro Effect of Triiodothyroacetic Acid on the Oxygen Consumption of Normal Human Leucocytes.

(Cp. Table VIII, p. 47).

Results expressed in μ moles O_2 per hour per 10^7 leucocytes.

	TRIAC conc. in micrograms per 100 ml. medium.						
Subject.	Zero.	10.	15.	20.	25.	35.	50.
1.	0.073	0.077	0.072	0.071	0.076	-	0.072
2.	0.122	0.102	0.106	0.116	0.112	0.104	0.116
3.	0.190	0.174	0.200	0.170	0.180	0.184	0.186
4.	0.115	0.116	0.111	-	0.104	0.109	0.106
5.	0.110	0.114	0.112	0.116	0.130	0.114	0.126
6.	0.095	0.099	0.108	-	0.107	0.105	0.108
7.	0.072	0.076	0.069	0.081	0.078	0.076	0.078
8.	0.123	0.134	0.140	0.126	0.120	0.134	0.134

It is clear from these results that TRIAC does not cause any stimulation of normal leucocyte respiration in vitro, thereby differing completely from sodium salicylate.

THE OXYGEN CONSUMPTION OF LEUCOCYTES IN CASES OF LEUKAEMIA.

Leukaemia is a morbid condition of unknown aetiology and fatal termination which is characterised by widespread proliferation of leucocytes and their precursors in the tissues of the body. In particular, it is associated with both qualitative and quantitative changes in the circulating white cells of the blood. It is probable that the disorder is fundamentally one of growth control, but it is also likely that abnormalities occur in the metabolism of the leukaemic cell itself and a study of these metabolic abnormalities may yield further knowledge of the elementary difference between normal and leukaemic leucocytes.

Since the effects of two metabolic stimulants, sodium salicylate and triiodothyroacetic acid, on the oxygen uptake of normal human leucocytes had been studied, the effects of these two stimulants on leukaemic leucocytes were then investigated.

Two groups were studied - chronic myeloid leukaemia and chronic lymphatic leukaemia. Eight patients from each group were available. Because of the high white cell counts, only 20-30 ml. of blood were required from each case, a considerable technical advantage. Since two experiments were carried out on each patient, two

control/

/control values for the oxygen uptake of the leucocytes were obtained on each subject. The patients came up to hospital as out-patients and were not always in a completely basal state before blood was withdrawn. All the control values have been collected together and are shown in Table XVI.

TABLE XVI. The Oxygen Consumption of Leukaemic Leucocytes.

All results are given in μ moles O_2 per hour per 10^7 cells.

Chronic myeloid leukaemia.		Chronic lymphatic leukaemia.	
Case.	Oxygen uptake.	Case.	Oxygen uptake.
1 a.	0.182	1 a.	0.176
b.	0.145	b.	0.169
2 a.	0.097	2 a.	0.182
b.	0.221	b.	0.125
3 a.	0.102	3 a.	0.164
b.	0.112	b.	0.100
4 a.	0.104	4 a.	0.194
b.	0.096	b.	0.147
5 a.	0.130	5 a.	0.145
b.	0.116	b.	0.248
6 a.	0.101	6 a.	0.148
b.	0.127	b.	0.149
7 a.	0.152	7 a.	0.126
b.	0.116	b.	0.195
8 a.	0.136	8 a.	0.142
b.	0.126	b.	0.143
Means.	0.126		0.160
S.D.	0.039		0.032

It is not considered that these values are significantly different either from each other or from the values obtained for normal human leucocytes (Table III, p. 34). This is in accordance with the findings of Beck and Valentine (1953) and Bird, Clements and Becker (1951).

The leucocyte counts were as follows:-

Chronic myeloid leukaemia:

W.B.C. 80,000 to 720,000 per mm^3. (average = 210,000)

Myeloid cells. 96% of the total.

Chronic lymphatic leukaemia:

W.B.C. 79,000 to 282,000 per mm^3. (average = 160,000)

Lymphocytes. 92 - 97% of the total. (average = 94%)
Lymphoblasts. 1 - 4% of the total. (average = $2\frac{1}{2}$%).

METABOLIC STIMULATION BY SALICYLATE. LEUKAEMIC CELLS.

It will be recalled that with normal human leucocytes, addition of salicylate *in vitro* first stimulates and then depresses the oxygen consumption (Table VIII, p. 47, and Figure 2, p. 48). The technique was exactly the same as that used in the study of normal leucocytes (see p. 46). The only minor difference was that these leukaemic patients were not strictly basal when the blood was withdrawn, but since the values obtained were referred to the control for each subject, it is not considered that this difference had any significant bearing on the conclusions reached. In this connection, it has long been known that the basal metabolic rate is increased in both chronic myeloid and in chronic lymphatic leukaemias (Riddle and Sturgis, 1927, Krantz and Riddle, 1928, and many others).

Results with cases of chronic myeloid leukaemia are shown in Table XVII and in Figure 8; with cases of chronic lymphatic leukaemia in Table XVIII and in Figure 9.

TABLE XVII. The in vitro Effect of Sodium Salicylate on the Oxygen Consumption of Human Leucocytes from cases of Chronic Myeloid Leukaemia.

Results are expressed in μ moles O_2 per hour per 10^7 cells.

	Salicylate concentration in mg. per 100 ml.						
Subject.	Zero.	10.	20.	40.	60.	80.	160.
Gilmour.	0.176	0.210	0.263	0.245	0.230	0.224	-
Murray.	0.182	0.172	0.182	0.213	0.222	0.215	0.200
Gibb.	0.164	0.176	0.190	-	0.200	0.235	-
Rafferty.	0.194	0.200	0.215	0.225	0.247	0.268	0.230
Cattanach.	0.145	0.161	0.170	0.175	0.190	-	0.177
Wood.	0.149	0.162	0.168	0.178	0.192	0.175	0.160
Lamberton.	0.195	0.205	0.220	0.240	0.188	0.152	-
Smith.	0.142	0.205	0.188	0.156	0.149	-	0.140

Figure 8 shows the changes in the rate of oxygen consumption, compared with zero time, at the corresponding salicylate concentrations. Each point on the graph represents the mean change in oxygen consumption corresponding to the salicylate concentration; the feet represent the standard errors of each mean.

Figure 8.

The effect in vitro of sodium salicylate on the oxygen consumption of human leucocytes from cases of Chronic Myeloid Leukaemia.

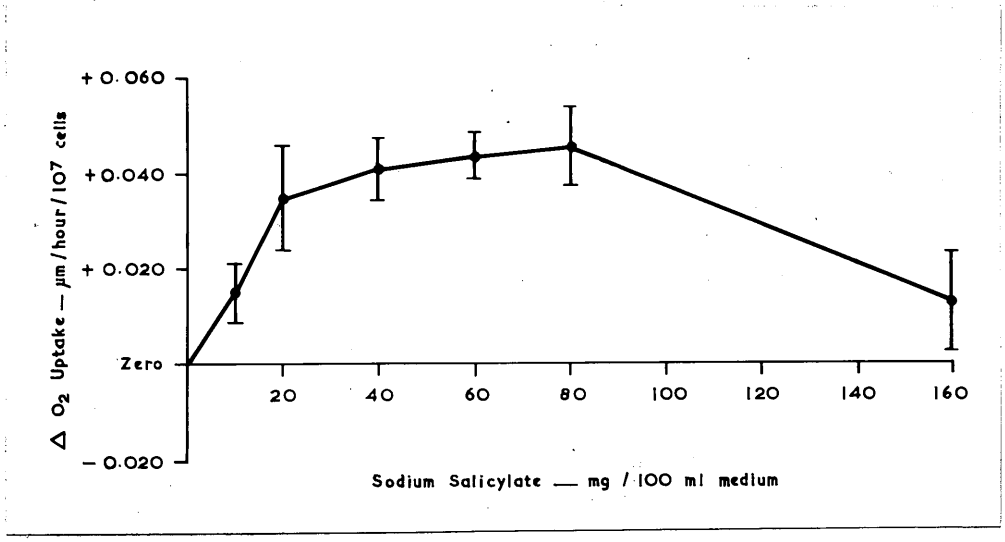


TABLE XVIII. The in vitro Effect of Sodium Salicylate on the Oxygen Consumption of Human Leucocytes from cases of Chronic Lymphatic Leukaemia.

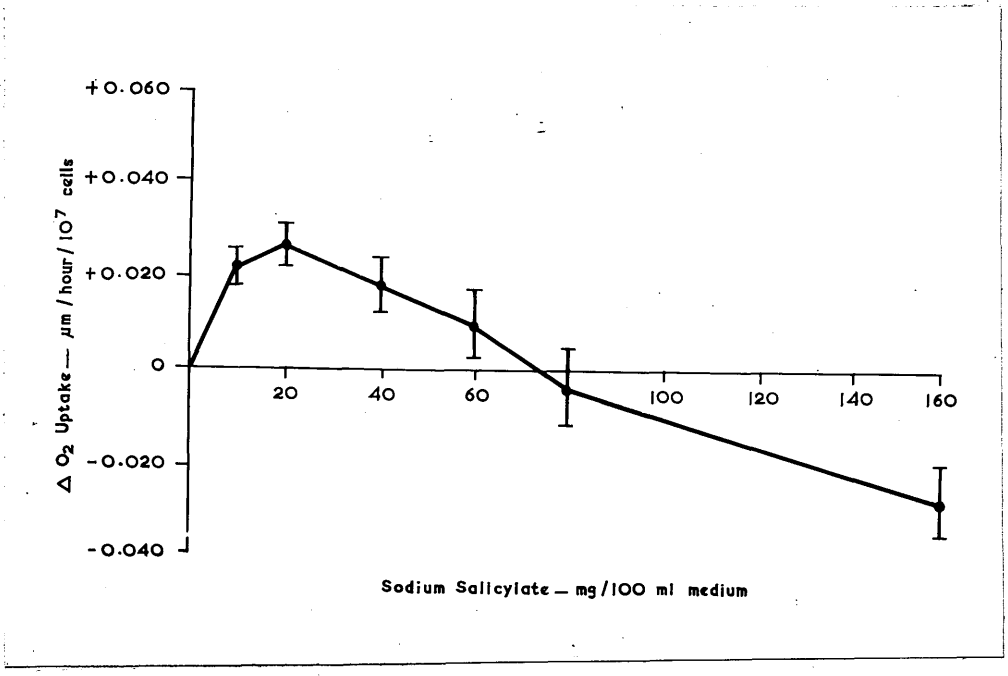
Results are expressed in μ moles O_2 per hour per 10^7 cells.

	Salicylate concentration in mg. per 100 ml.						
Subject.	Zero.	10.	20.	40.	60.	80.	160.
McLaughlin.	0.182	0.192	0.204	0.220	0.215	0.192	0.145
McMillan.	0.102	0.112	0.120	0.120	0.102	0.093	0.069
Walker.	0.104	0.130	0.136	0.123	0.111	0.086	0.081
McCluskey.	0.094	0.116	0.120	0.124	0.131	0.134	0.143
Smith.	0.130	0.147	0.161	0.117	0.102	0.102	0.071
Darner.	0.101	0.136	0.112	0.108	-	0.099	0.076
McCutcheon.	0.152	0.186	0.203	0.175	0.145	0.117	0.104
Stewart.	0.136	0.155	0.157	0.168	0.156	-	0.144

Figure 9 shows the changes in the rate of oxygen consumption, compared with zero time, at the corresponding salicylate concentrations. Each point on the graph represents the mean change in oxygen consumption corresponding to the salicylate concentration; the feet represent the standard error of each mean.

Figure 9.

The effect *in vitro* of sodium salicylate on the oxygen uptake of human leucocytes from cases of Chronic Lymphatic Leukaemia.



The behaviour, under these conditions, of the leucocytes from myeloid leukaemic blood is quite different from normal human blood (Cp. Figures 8 and 2, p. 74 and p. 48). Salicylate stimulates myeloid cells to a much smaller extent than normal cells, but whereas the maximal stimulation with normal cells is reached at a concentration of 40 mg. salicylate per 100 ml., it is not reached with myeloid cells until a concentration of 80 mg. per 100 ml. by which level of concentration respiration is depressed when normal cells are used. Furthermore, even at a concentration of 160 mg. salicylate per 100 ml., the myeloid cells are still respiring at a rate which is greater than normal - a concentration which severely inhibits the oxygen consumption of normal leucocytes.

Lymphatic leukaemic leucocytes under these conditions, are also different from normal leucocytes (Cp. Figures 9 and 2, p. 76 and p. 48) but resemble their behaviour more closely than do myeloid leucocytes. Comments on these differences will be found in the Discussion.

METABOLIC STIMULATION BY TRIIODOTHYROACETIC ACID. LEUKAEMIC CELLS.

It has already been shown that triiodothyroacetic acid (TRIAC) *in vitro* does not stimulate the oxygen consumption of normal human leucocytes (p.67a) and Table XV, p. 68). The experiments with normal cells leading to that conclusion have been repeated using leucocytes from cases of chronic myeloid and chronic lymphatic leukaemia.

Results with cases of chronic myeloid leukaemia are shown in Table XIX and in Figure 10; with cases of chronic lymphatic leukaemia in Table XX and in Figure 11.

TABLE XIX. The in vitro Effect of Triiodothyroacetic Acid on the Oxygen Consumption of Leucocytes from Cases of Chronic Myeloid Leukaemia.

Results are expressed in μ moles O_2 per hour per 10^7 cells.

	TRIAC concentration in micrograms per 100 ml.						
Subject.	Zero.	10.	15.	20.	25.	35.	50.
Gilmour.	0.169	0.183	0.188	0.195	0.207	0.205	0.205
Murray.	0.125	0.131	0.149	0.139	0.132	0.128	0.117
Gibb.	0.100	0.100	0.105	0.108	-	0.114	0.126
Rafferty.	0.149	0.155	-	0.170	-	0.168	0.160
Cattenach.	0.248	0.264	0.278	0.288	0.275	-	0.240
Wood.	0.148	0.151	0.160	0.159	0.168	0.150	0.150
Lamberton.	0.126	0.130	0.134	0.144	0.156	-	0.146
Smith.	0.143	0.152	0.156	0.160	0.162	0.167	0.143

Figure 10 shows the changes in the rate of oxygen consumption, compared with zero time, at the corresponding TRIAC concentration. Each point on the graph represents the mean change in oxygen consumption at that concentration; the feet represent the standard error of each mean.

Figure 10.

The effect in vitro of TRIAC on the oxygen uptake of leucocytes from cases of Chronic Myeloid Leukaemia.

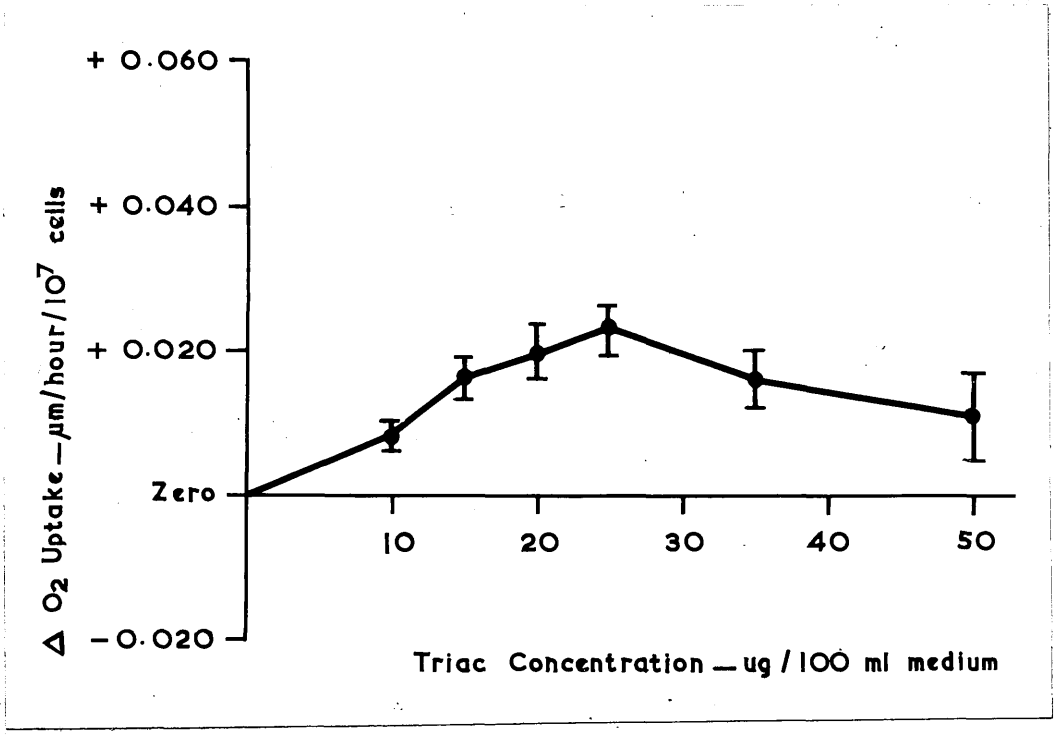


TABLE XX. The in vitro Effect of Triiodothyroacetic Acid on the Oxygen Consumption of Leucocytes from Cases of Chronic Lymphatic Leukaemia.

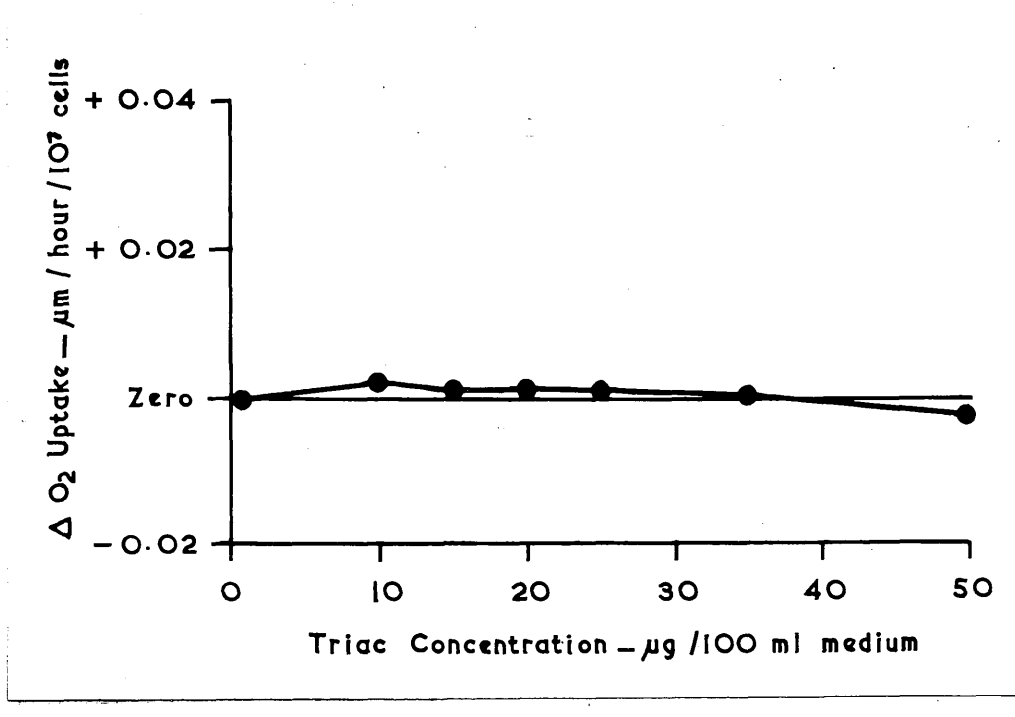
Results are expressed in μ moles O_2 per hour per 10^7 cells.

	TRIAC concentration in micrograms per 100 ml.						
Subject.	Zero.	10.	15.	20.	25.	35.	50.
McLaughlin.	0.145	-	0.149	0.153	0.156	0.153	0.142
McMillan.	0.112	0.115	0.110	0.114	0.116	0.105	-
Walker.	0.096	0.102	0.104	0.102	0.099	0.102	0.102
McCluskey.	0.221	0.225	0.213	0.216	0.204	0.201	0.190
Smith.	0.116	0.118	0.119	0.115	0.115	-	0.118
Darner.	0.127	0.123	0.136	0.126	0.131	0.127	0.128
McCutcheon.	0.116	0.122	0.123	0.128	0.128	0.127	0.124
Stewart.	0.126	0.128	0.123	0.124	0.126	0.132	0.110

Figure 11 shows the changes in the rate of oxygen consumption, compared with zero time, at each TRIAC concentration. Each point on the graph represents the mean change in oxygen uptake at that concentration; the feet represent the standard error of each mean.

Figure 11.

The effect in vitro of TRIAC on the oxygen uptake of leucocytes from cases of Chronic Lymphatic Leukaemia.



When TRIAC was added to chronic myeloid leukaemic leucocytes, a positive response was obtained. The myeloid cells showed a significant increase in oxygen consumption at all concentrations from 10 - 35 micrograms TRIAC per 100 ml. of medium, maximum stimulation occurring at a concentration of 25 micrograms per 100 ml. At this point, the oxygen consumption was 15% above the control value. Furthermore, with myeloid cells, stimulation of oxygen consumption occurred at all concentrations of TRIAC which were studied.

Over the same range of TRIAC concentrations, chronic lymphatic leukaemic leucocytes showed no significant response. At 4 of the 6 concentrations of TRIAC, oxygen uptake was slightly, but not significantly, above the control value; in the other 2 concentrations, it was slightly, but again not significantly, below the control value. It is most likely that these results are simply variations about a mean.

Thibault and Pitt-Rivers (1955) obtained an immediate effect with TRIAC in vitro using kidney slices taken from thyroidectomised rats. Donhoffer, Várnai and Szeibert-Horvath (1958) also obtained an immediate effect with TRIAC in vivo using thyroidectomised and adrenalectomised rats. Heimberg, Park, Isaacs and Pitt-Rivers (1955) also obtained an immediate response to TRIAC in vitro using mouse ascites tumour cells.

DISCUSSION.

This work was approached with two main objects in view. One was an attempt to evolve some method of estimating total body metabolism, and the other, to examine respiration at cell level and to observe the effects of metabolic stimulants on this respiration.

Total body metabolism is measured by determining the basal metabolic rate, or the metabolic rate, and this determination is notoriously liable to be affected by numerous intrinsic and extrinsic factors, of which we need only mention age, sex, diet, excitability (in the normal individual), metabolic disease, respiratory difficulties in the common Benedict-Roth method of estimating the basal metabolic rate, and so on. It is generally recognised that the first two or three estimations of a patient's basal metabolic rate are worthless, and that it is not until the fourth or fifth attempt, that reliable results can be obtained. All clinical biochemists are aware of the impossibility of attempting a differential diagnosis between hysteria and a mild or moderate hyperthyroidism in a young female, by means of the Benedict-Roth apparatus. The problem then arose, whether or not one could obtain living cells from the human body, whose respiration

was/

/was representative of the respiration of the body as a whole.

The only cells which are readily available for this purpose are those circulating in the blood. The erythrocytes are of no use since, apart from the question of whether they are "living" or not, their respiration rate is so small that there is some doubt if they respire at all. Furthermore, they are not to be regarded as typical body cells since the mature erythrocyte is the only cell in the body which is non-nucleated. One must therefore fall back on the leucocytes. These cells are free-floating, can be easily counted in any system, have a high rate of respiration, and a priori, are in some form of chemical equilibrium with all the tissues of the body. On the other hand, leucocytes are easily damaged by laboratory manipulation and are difficult to separate undamaged from erythrocytes. There are also several different cell types which, although relatively constant in health and in many diseases, may be drastically altered in other diseases, e.g. the leukaemias.

It is not the object of this work to provide an alternative to routine hospital determinations of the basal metabolic rate. In spite of the disadvantages of the basal metabolic rate as a method of investigation, it/

/it is unlikely that the Benedict-Roth apparatus will ever be replaced routinely by the Warburg apparatus, whatever the advantages of the latter. In all probability the Warburg apparatus will always remain a research tool, and it was with this in mind that the work presented here was attempted. Its outstanding advantage as a method of investigations is that its use eliminates subjective variations in the determination of the rate of metabolism.

Respiration at cell level can be followed only in an apparatus such as the Warburg apparatus which is eminently suitable for the present purposes - namely the effect of added metabolic stimulants, whether added in vivo or in vitro. This apparatus also makes easy the study of day-to-day variations, especially of patients undergoing treatment.

Little need be said here about the actual technique of using the Warburg apparatus, or its standardisation. The most important practical point with the system employed was the adequate control of the pH. The only tehcnical difficulty encountered was traced to the use of a basic medium, the pH of which was too low, and this was counteracted by the addition of disodium hydrogen phosphate to a final pH of 7.40 $\pm$ 0.05. Preliminary experiments showed that the oxygen consumption per hour per 10^7 leucocytes did not depend

on/

/on the total number of cells present in the flask (Table I, p. 30); replicate estimations gave very good agreement in the great majority of cases (Table II, p. 31); and in a series of 16 normal subjects, the average oxygen consumption of the leucocytes was found to be 0.111 μ moles of oxygen per hour per 10^7 cells, with a standard deviation of 0.023.

Perhaps the greatest single difficulty in determining the basal metabolic rate is the problem of getting the patient in a truly "basal" condition. The use of an isolated cell system will certainly eliminate many variables, but such a system may still be affected by hormonal stimulants poured out into the blood in response to environmental changes. The total white cell count, but not the differential white cell count, varies with exercise. Typical changes are shown in Table IV (p. 37). The changes are irregular, and are largely eliminated by expressing results in values per 10^7 cells, since changes in the differential white cell count are insignificant.

The effect of exercise on the oxygen uptake of leucocytes is shown in Table V (a) (p. 38) and is compared with the corresponding effect of exercise on the subject's metabolic rate (Table V (b), p. 39). Both are increased - the former much more than the latter. This is to be expected since the leucocytes are one of the first types of cell to be exposed to

any/

/any changes in the body chemistry. Since exercise increases the oxygen consumption of isolated leucocytes, it is still necessary to obtain standards for reference while the subject is in the basal state, but this is comparatively easy when nothing more than venepuncture is the only manipulation involved. There is no evidence that venepuncture causes any significant change in the leucocyte oxygen uptake (see p. 40).

The data in Table V (a) and (b) show that the increase in leucocyte oxygen consumption is almost twice the increase in body oxygen consumption after exercise. Presumably this leucocyte stimulation is due to the secretion of adrenaline or some compound of similar biological activity. In a paper on the metabolism of leucocytes by Beck and Valentine (1953) the rate of oxygen consumption by leucocytes has been reviewed in detail, but no mention is made of blood specimens having been collected from subjects while they were in the "basal" state, and the wide divergence of results recorded by various authors clearly points to this omission as one of the main causes of the variation of the reported normal values.

Experiments with Salicylate.

In order to be useful to the body, and not be directly lost as heat, oxidative energy is employed to esterify inorganic phosphate into high-energy phosphate bonds during the process of electron transfer to oxygen. The efficiency of oxidation is often expressed as the "P:O ratio", i.e., the number of moles of phosphorus esterified divided by the number of moles of oxygen utilised. The level of high-energy phosphate present, the amount being utilised and hence the availability of some acceptor for such energy (e.g. glucose-hexokinase) is believed to control the rate of tissue oxidation. In other words, the rate of most tissue oxidations is normally dependent upon, or coupled to, the formation of energy-rich phosphate bonds.

The in vitro action of salicylate is qualitatively similar to that of 2:4-dinitrophenol. Both are believed to uncouple oxidative phosphorylation, to stimulate the respiration of an acceptor-deficient system, to inhibit fatty acid oxidation, and to stimulate the hydrolysis of adenosine triphosphate. (Meade, 1954; Smith and Jeffrey, 1956; Brody, 1956; Pennial, 1956). In particular, Brody (1956) showed that at high concentration of sodium salicylate, the P:O ratio was reduced almost to zero, i.e., at high salicylate/

/salicylate concentrations, the production of energy-rich phosphate bonds was greatly reduced and the cell (in this case, kidney mitochondria) no longer produced the energy necessary for survival.

The drug-response curve obtained with human leucocytes over the range 0 - 160 mg. sodium salicylate per 100 ml. medium is similar in shape to that obtained with mouse liver slices by Sproull (1954). Figure 2 (p. 48) shows that the oxygen uptake of leucocytes increases in direct proportion to the salicylate concentration up to 40 mg. per 100 ml. medium, and thereafter, the respiration rate decreases. Sproull found maximal stimulation at a concentration of 32-48 mg. salicylate per 100 ml., and further increase in salicylate concentration resulted in a decrease of oxygen uptake.

The considerable similarity between the responses of two entirely different cell systems is sufficiently encouraging to suggest that the action of sodium salicylate on different cells has a common basis.

Since salicylate stimulates leucocyte respiration both <u>in vivo</u> (see Figure 1, p. 44) and when added <u>in vitro</u> (see Figure 2, p. 48) the question arose as to whether this response was the result of sodium salicylate having an action on the cell surface or if the salicylate was acting on the respiratory enzymes within/

/within the cell, having first penetrated the membrane.

The oxygen consumption of leucocytes from the blood of two patients on salicylate therapy was studied, and the results (see Figure 1, p. 44) show that, with increasing concentration of salicylate, the oxygen uptake of the leucocytes increased. Prior to the determination of the oxygen consumption, almost the whole of the plasma (containing the administered salicylate) had been removed and the leucocytes were then suspended in a salicylate-free medium, and it was repeatedly observed that the oxygen uptake at all the (original) plasma salicylate concentrations was linear over a period of 2-3 hours, indicating that the salicylate administered therapeutically had reached its site of action. If the salicylate were exerting its effect at the surface of the cell, it would be expected that, as the cell was bathed in a salicylate-free medium, any salicylate would be washed off the surface of the membrane and the oxygen consumption of the cell system would steadily decrease. It is highly unlikely that the salicylate exerts any action on cell respiration if it is simply adsorbed on the outer surface of the cell, and hence the conclusion is that the active salicylate has penetrated the cell membrane and is exerting its effect on the respiratory enzymes within the cell protoplasm.

Each/

/Each cell requires a fixed amount of energy for its ordinary metabolic processes. Part of this energy is derived from glycolysis, of the rest of the necessary energy, the bulk comes from oxidative phosphorylation. Salicylate, like 2:4-dinitrophenol, is known to uncouple oxidative phosphorylation, i.e., for a fixed amount of energy, the cell requires more oxygen for oxidation within the tricarboxylic acid cycle - hence the increase in oxygen uptake on addition of limited amounts of salicylate to the system. The decrease in oxygen uptake when the salicylate concentration is increased beyond 40 mg. per 100 ml., and the later depression of oxygen consumption when the salicylate concentration is increased beyond about 70 mg. per 100 ml. (see Figure 2, p. 48) are presumable nothing more than toxic effects which might be brought about by any chemical agent.

The response of leucocytes from cases of chronic myeloid leukaemia (see Figure 8, p. 74) is quite different from that of normal leucocytes. There are several possible explanations of this difference. The cell membrane may be less permeable to salicylate ions; the metabolic processes of the myeloid cell may be less dependent on oxidative phosphorylation than the normal; or the metabolic processes of the myeloid cell may be less sensitive to the toxic action of high concentrations of/

/of salicylate. Of these possibilities, the first would seem to be the most probable since, at an external concentration of 160 mg. salicylate per 100 ml. of medium, the myeloid cell is still respiring at a greater rate than the control value, while with normal or lymphatic leukaemic cells, this concentration of salicylate causes a marked dpression of oxygen consumption.

Thyroid Stimulation of Oxygen Consumption.

It has been shown above (1) that exercise increases the oxygen consumption of leucocytes by more than 50% under the conditions selected, and that this increase is almost twice as great as the increase in metabolic rate of the subject under the same conditions, and (2) that addition to a leucocyte suspension of a metabolic stimulant such as sodium salicylate can increase the oxygen consumption of the leucocytes by more than 70%, while administration of salicylate in the normal therapeutic dose does not increase the metabolic rate of the patient by more than approximately 45% (Alexander and Johnson, 1958).

As further evidence of the correlation between leucocyte oxygen consumption and total body metabolism, we might collect together the data given in the text for/

/for the leucocyte oxygen consumption in hyperthyroid, euthyroid, and hypothyroid subjects.

Subjects.	Number of Cases.	Oxygen consumption. μ moles/hr./10^7 cells.	S.D.
Hyperthyroid. (Table IX).	14	0.185	0.037
Euthyroid. (Table III).	16	0.111	0.023
Euthyroid. (Table XI).	12	0.119	0.021
Hypothyroid. (Table X).	10	0.083	0.016

The rate of oxygen consumption agrees with the clinical state of the patient, and the individual variation is greatest in the hyperthyroid group, as would be expected, and least in the myxoedema group, as would also be expected. These figures also show that thyroid hormon has a direct effect on the oxygen consumption of circulating leucocytes.

This correlation is again shown by the effect of treatment on the oxygen consumption of leucocytes in cases of hyperthyroidism and of myxoedema (see Figure 4, p. 58, and Table XII, p. 59). Since the hyperthyroid cases were treated with ^{131}I, and the myxoedema cases were treated with l-thyroxine, the changes in the oxygen consumptions of the leucocytes must/

/must have been due to the changes in the concentration of the circulating hormone in the patients' blood.

There is much evidence that an intact cell structure is an essential preliminary to the study of hormone action. The effect of TRIAC has been examined both in the intact human subject, and simultaneously, on the isolated leucocytes of the same subject in cases of myxoedema. The considerable difficulties in obtaining suitable subjects have already been mentioned (see p. 61). Only two patients have been obtained so far on whom this experiment could be safely performed, and the response of these two patients was quite different (see Figures 5 and 6, pp. 63 and 65). The first showed an immediate response to TRIAC by an increase in both the leucocyte respiration rate and the total body metabolic rate. The second showed a delayed increase in leucocyte respiration and an irregular increase the total body metabolic rate. This difference cannot be resolved without a further supply of suitable patients. The discrepancy is not necessarily due to experimental error, for similar irregularities have been described in animal work.

Várnai (1957) studied the effect of TRIAC injection in rats. Using hypophysectomised animals, intravenous injection of a few microgram was invariably followed/

/followed by a marked rise in oxygen consumption and in body temperature; using a normal intact animal, such changes were never observed; with thyroidectomised rats approximately half responded by a marked increase in oxygen consumption and in body temperature, the others did not. Donhoffer, Varnai and Szeibert-Horvath (1958) confirmed these results obtained with hypophysectomised rats and showed that both effects were completely abolished by the prior administration of 1 mg. of cortisone. They concluded that depressed adrenal cortical function was a necessary factor in the production of an increase in oxygen consumption and in body temperature following injection of TRIAC. In this work, they also observed that (1) no immediate response (in terms of increased oxygen consumption and of increased body temperature) could be obtained in hypophysectomised rats adequately treated with ACTH; (2) this immediate response to TRIAC injection could be elicited within 16 hours of removal of the pituitary gland; and (3) the immediate response in the hypophysectomised rat is not abolished by elevating the reduced metabolic rate to normal or supra-normal levels by prior administration of l-thyroxine.

The different responses in the two patients studied in this work, may possibly be related to adrenal function although there was no clinical evidence of cortical adrenal/

/adrenal hypofunction in the case of the patient who responded to TRIAC injection. It is not suggested that the discrepancy can be explained in terms of results obtained by experiments on animals.

It is known that about 60% of cases of primary myxoedema are extinct Hashimoto's disease. In Hashimoto's disease, the lymphocytes produce an antibody to the thyroid hormone. Perhaps the leucocytes from such a patient react differently from leucocytes from a true primary myxoedema to TRIAC circulating in the blood stream. Further cases, suitable for investigation, are required before any conclusion can be drawn.

The Effects of Triiodothyroacetic acid (TRIAC).

Since the thyroid hormones and salicylate are both metabolically active substituted phenols, Du Toit (1952) suggested that both might act by uncoupling oxidative phosphorylation in the same way as do certain other substituted phenols such as 2:4-dinitrophenol and various halogenated phenols. In the previous year, Martius and Hess (1951) had shown that when l-thyroxine was added *in vitro* to normal mitochondrial preparations, the esterification of AMP and ADP to ATP was reduced; and/

/and Maley and Landy (1953) and Hoch and Lipmann (1954) both showed that thyroxine and triiodothyronine depressed the P:O ratio in normal respiring mitochondrial preparations. In such systems, Landy and Maley (1954) provided evidence that one of the actions of 2:4-dinitrophenol was to increase the activity of ATP-ase, and that thyroxine and triiodothyronine had similar effects. However, the uncoupling of oxidative phosphorylation cannot be accounted for solely in terms of ATP-ase activation, and attempts to identify the specific locus at which the thyroid hormone uncouples oxidative phosphorylation have not yet been successful.

In this work, triiodothyroacetic acid in vitro was found to have no significant effect on the rate of oxygen uptake of normal leucocytes (Table XV , p. 68) or of leucocytes isolated from cases of chronic lymphatic leukaemia (Table XX, p. 81). On the other hand, addition of triiodothyroacetic acid in vitro causes a significant increase in the rate of oxygen consumption of leucocytes isolated from cases of chronic myeloid leukaemia (Table XIX, p. 79). In all three cases, the results are clear-cut and there are sufficient data to justify this conclusion.

Although the experiment has been attempted on several occasions recorded in the literature, no one has/

/has ever been able to demonstrate an *in vitro* effect on theoxygen consumption of normal cells or tissues by addition of thyroxine, triiodothyronine or triiodothyroacetic acid to the system (Klemperer (1955) and several others). This work adds the normal leucocyte to the other types of cells which have been studied. Either the cells of normal tissues are "saturated" with thyroid hormone, or, in isolated systems, the cell membranes are impermeable to the various thyroid hormones which have been tried out.

Lymphocytes isolated from cases of chronic lymphatic leukaemia do not respond *in vitro* to the addition of TRIAC. This is in keeping with the findings of Barker (1955) who examined the effect of thyroid hormone *in vitro* on various tissues isolated from thyroidectomised rats. He found that lymph nodes were among the tissues which did not respond to TRIAC (see p. 23). Since these thyroid hormones are thought to be carboxylic acids, Barker suggested that the explanation was to be found in the fact that lymph nodes, and lymphocytes as well, are very rich in decarboxylase and that the thyroid hormones were inactivated by this enzyme.

Cells of the myeloid series isolated from cases of chronic myeloid leukaemia do respond *in vitro* to the addition of TRIAC. Now, normal polymorphonuclear leucocytes/

/leucocytes are also formed in bone marrow and are of the same series as the myeloid cells, hence the in vitro response of myeloid cells to TRIAC represents a metabolic abnormality. It may be recalled at this point that Heimberg et al (1955) showed that ascites tumour cells also responded in vitro to the addition of TRIAC by increased oxygen consumption rate and an increased rate of glycolysis (see p. 22).

The failure of the lymphocytic leukaemic cell to respond to the addition of TRIAC in vitro is of no immediate practical importance since, apart from the negative response, it belongs to a different series of leucocyte from the normal polymorph and the myeloid leukaemic cell. The response of the myeloid leukaemic cell to TRIAC in vitro is slight, but significant, and since the normal leucocyte population does not respond under these circumstances, one must conclude that the metabolism of the myeloid leukaemic cell has become deranged, and there is a certain amount of evidence that this metabolic derangement may be due to a slight hormonal imbalance.

Certain differences between the normal leucocyte population (which belongs mainly to the granulocytic series) of the blood, and leucocytes from cases of chronic myeloid leukaemia have already been recorded in the literature. Valentine, Follette and Lawrence (1953)/

/(1953) found that the normal granulocyte has almost twice as much glycogen per cell as the chronic myeloid leukaemic cell, and if the former is regarded as normal, the latter must have an abnormally low glycogen content. It is a known fact that in cortisone deficiency, e.g., in Addison's disease, the cell glycogen content is also abnormally low. It is therefore possible that the chronic myeloid leukaemic cell is deficient in cortisone or some closely related glucocorticoid hormone.

Valentine and Beck (1951) have shown that the normal granulocytic leucocyte has five times as much alkaline phosphatase activity as the chronic myeloid leukaemic cell, and it has been shown that the administration in vivo of A.C.T.H., cortisone, or hydrocortisone can induce a 3- or 4-fold increase in the alkaline phosphatase activity of the normal granulocytic leucocyte. This would also indicate that the chronic myeloid leukaemic cell is deficient in one or more of the steroid adrenal hormones. There is no record in the literature of the effect of A.C.T.H., or any of the adrenal cortical hormones on the alkaline phosphatase activity of the leucocytes from the blood of patients with chronic myeloid leukaemia.

A third difference is the one recorded here, namely, that the leucocytes from cases of chronic myeloid leukaemia show/

/show a significant response to the addition of TRIAC *in vitro* whereas the normal leucocyte does not.

The work by Varnai and his co-workers referred to above (p. 96) has shown that the TRIAC effect in hypophysectomised rats could be completely abolished by prior administration of cortisone.

All of these results lead to the conclusion that the leucocytes in cases of chronic myeloid leukaemia may be deficient in a specific corticoid hormone. The precise nature of this chemical is not known, but presumably it is a glucocorticoid with =O at C11, and it is hoped to pursue this research along these lines.

Ecclesiastes, Chap. 1, v. 18.

SUMMARY.

1. Normal human leucocytes, taken from patients under basal conditions respire at a rate of 0.111 ± 0.023 μ moles per hour per 10^7 cells.

2. Moderate exercise by the blood donor, prior to venepuncture results in an average increase in oxygen consumption of the leucocytes of 54% over the control level.

3. The oxygen consumption of leucocytes taken from patients on salicylate therapy, increases with increasing salicylate concentration between 20-60 mg. per 100 ml. plasma. Between 0-20 mg. salicylate per 100 ml. plasma, there is no increase in oxygen consumption.

4. In vitro, normal human leucocytes show a response to sodium salicylate. Between 0-40 mg. sodium salicylate per 100 ml. medium, the oxygen consumption increases with increasing salicylate concentration, reaching a maximum at 40 mg. per 100 ml. Between 40-70 mg. per 100 ml. medium, the respiration decreases and from 70-160 mg. per 100 ml. medium, the oxygen consumption is depressed to values lower than normal.

5./

/5. There is a direct relationship between the oxygen uptake of leucocytes and B.M.R. of the patient. Thirty-six patients with different degrees of thyroid activity were studied. The leucocytes from hyperthyroid patients had an oxygen uptake of 0.185 ± 0.037 μ moles per hour per 10^7 cells. Leucocytes from euthyroids had an oxygen uptake of 0.119 ± 0.021 μ moles per hour per 10^7 cells. The leucocytes from myxoedematous patients had an oxygen uptake of 0.083 ± 0.016 μ moles per hour per 10^7 cells.

6. The oxygen consumption of leucocytes from three cases of myxoedema and three cases of hyperthyroidism was studied before and after therapy. In the three cases of myxoedema, the oxygen consumption of the leucocytes increased with thyroxine therapy. In the three cases of hyperthyroidism the oxygen consumption of the leucocytes decreased with ^{131}I therapy.

7. An injection of 5 mg. TRIAC was given to two myxoedematous patients and the oxygen uptake of the patients' leucocytes was determined at fixed intervals after injection. The leucocytes from the first patient gave a significant increase in/

/in oxygen consumption at 30 minutes. At 2 hours the oxygen consumption had increased by more than 100%. The second patient gave no significant increase in oxygen consumption until 3 hours after injection. At that time, there was an increase in oxygen uptake of the leucocytes of 50%.

8. There was no increase in oxygen uptake of leucocytes from normal patients with TRIAC in the range 0-50 ug. per 100 ml. medium.

9. Leucocytes from eight cases of Chronic Myeloid Leukaemia (Myeloid cells 96% of the total) had an oxygen uptake of 0.126 $\pm$ 0.039 μ moles per hour per 10^7 cells. Leucocytes from eight cases of Chronic Lymphatic Leukaemia (lymphocytes:- 94%) had an oxygen uptake of 0.160 $\pm$ 0.032 μ moles per hour per 10^7 cells. It is not considered that these values are significantly different either from each other, or from the values obtained for normal human leucocytes.

10. Salicylate was added <u>in vitro</u> to leucocytes from eight cases of Chronic Myeloid Leukaemia and eight cases of Chronic Lymphatic Leukaemia. The response of the two types of leukaemic leucocytes to salicylate was different.

Salicylate/

/Salicylate stimulates myeloid cells to a much smaller extent than normal cells, maximum stimulation is not reached until a concentration of 80 mg. sodium salicylate per 100 ml. medium. At a concentration of 160 mg. salicylate per 100 ml., the myeloid cells are still respiring at a rate which is greater than normal. The stimulation of oxygen uptake with lymphatic leukaemia leucocytes is small but definite, reaching a maximum at 20 mg. per 100 ml. medium. Between 20-70 mg. per 100 ml. medium, the oxygen uptake is depressed. Between 70-160 mg. the oxygen uptake is less than the control. Inhibition of oxygen uptake with lymphatic leukaemic leucocytes is much less than with normal leucocytes.

11. The <u>in vitro</u> effect of TRIAC (0-50 µg. per 100 ml. medium) was studied with leucocytes from eight cases of Chronic Myeloid Leukaemia and eight cases of Chronic Lymphatic Leukaemia. Myeloid leukaemic leucocytes responded to TRIAC by increase in oxygen consumption. Lymphatic leukaemic leucocytes gave no response.

BIBLIOGRAPHY.

Barker,S.B. (1955). Proc.Soc.Exp.Biol.N.Y. 90, 109.

Barrow,E.S.G. & Harrop,G Jr. (1929). J.Biol.Chem. 84, 89.

Beck,W.S. & Valentine,W.N. (1952). Cancer Res. 12, 818.

Beck, W.S. & Valentine,W.N. (1952).Cancer Res. 12, 823.

Beck,W.S. & Valentine,W.N. (1953). Cancer Res. 13, 309.

Bird,R., Clements,J., & Becker,L. (1951). Cancer 4, 1009.

Britton,S.W. & Silvette,H. (1934). Am.J.Physiol. 107. 190.

Brody,T.M. (1956). J.Pharmacol. 117, 39.

Bruce,H.M., Pitt-Rivers,R., & Sloviter,H.A. (1954). J.Endocrinol. 10, 340.

Buckley,E.S., Powell,M., & Gibson,J.G. (1950). J.Lab.clin. Med. 36, 29.

Campbell,E.W., Small,W.J., & Dameshek,W. (1956). J.Lab.clin. Med. 47, 835.

Cochran,J.B. (1952). Brit.med.J. 2, 964.

Cochran,J.B. (1954). Brit.med.J. 1, 733.

Cohn,M. (1953). J.biol.Chem. 201, 735.

Cooper,E.H. & Fitzgerald,M.G. (1958). Biochem.J. 68, 5p.

Cutting,W.C., Rytand,D.A. & Tainter, M.L. (1934). J.clin.Invest. 13, 547.

Denis,W. & Means,J.H. (1916). J. Pharmacol. 8, 273.

Dixon,M. (1943). Manomentric Methods, Cambridge University Press.

Donhoffer, Sz., Varnai,I. & Szeibert-Horvath, E. (1958). Nature 181, 346.

Du Toit,C.H. (1952). Phosphorus Metabolism, John Hopkins Press, Baltimore, Vol. 11, 597.

Goldberg,R.C., Wolff,J & Greep,R.O. (1957). Endocrinology. 60, 38.

Grafe,E. (1911). Deutsch.Arch.f.Klin.Med. 102, 406.

Gross,J. & Pitt-Rivers,R. (1952). Lancet 1, 439, 593, 1044.

Gross,J., Pitt-Rivers,R. & Thibault,O. (1953). C.R.Soc.Biol.Paris. 147, 75.

Hall,V.E. & Furth,J. (1942). Cancer Res. 2, 411.

Harington,C.R. (1945). Proc.roy.Soc. 132, 223.

Harington,C.R. & Pitt-Rivers,R. (1952). Biochem.J. 50, 438.

Harris,G.W. & Woods,J.W. (1957). Ciba Foundation Colloquium on Endocrinology Vol. 10. London.

Hartman,J.D. (1952). Proc.Soc.exp.Biol.N.Y. 79, 3.

Heimberg,M., Park,J.H., Isaacs,A. & Pitt-Rivers,R. (1955). Endocrinology 57, 756.

Hoch,F.C. & Lipmann,F. (1954). Proc.nat.Acad.Sci. 40, 909.

Hunter,F.E. (1951). Phosphorus Metabolism Vol. 1, John Hopkins Press, Baltimore.

Ingle,D.J. (1950). Proc.Soc.exp.Biol.N.Y. 75, 673.

Keible,E. & Spitzy,K.H. (1951). Arch.exp.Path. 213, 162.

Kempner,W. (1939). J.clin.Invest. 18, 291.

Klemperer,H.G. (1955). Biochem.J. 60, 122.

Krebs,H.A. (1950). Metabolism and Function Ed by Nachmansohn, Elsevier, London.

La Chaze,A. & Thibault,O (1952). C.R.Soc.Biol.Paris. 146, 50.

Lardy,H.A. & Maley,G.F. (1954). Recent Progress in Hormone Research. 10, 129.

Lardy,H.A. & Wellman,H. (1952). J.Biol.Chem. 195, 215.

Lerman,J. & Pitt-Rivers,R. (1955). J.clin.Endocrin.Metab. 15, 653.

Lester,D., Lolli,G., & Greenberg,L.A. (1946). J.Pharmacol. 87, 329.

Lutwak-Mann,C. (1942). Biochem.J. 36, 706.

MacLeod,J. & Rhoads,C. (1939). Proc.Soc.exp.Biol.N.Y. 41, 268.

Magne,H., Mayer,A., & Plantefol,L. (1933). Ann.Physiol. 8, 1.

Maley,G.F. & Lardy,H.A. (1955). J.Bio.Chem. 215, 377.

Manchester,K.L., Randle,P.J. & Howard-Smith,G. (1958). Brit.med.J. 1, 1028.

Martius,C. & Hess,B. (1951). Arch.Biochem. 33, 486.

McKinney,G., Rundles,R.W. & Martin,S. (1952). Fed.Proc. 11, 257.

Meade,B.W. (1954).Ann.rheum.Dis. 13, 60.

Means,J.H. (1948). The Thyroid & its Diseases 2nd ed. (Philadelphia). Lipincott & Co. 535.

Moloney,W.C. & Lange,R.D. (1954). Blood. 2, 663.

Packer,L. (1957). Arch.Biochem. 70, 290.

Papper,S., Burrows,B.A., Ingbar,S.H., Sisson,J.H. & Ross,J.F. (1952). New England J.Med. 247, 897.

Penniall,R. (1956). Fed.Proc. 15, 608.

Pitt-Rivers,R. (1953). Lancet 1, 234.

Ponder,E. & MacLeod,J. (1936). J.gen.Physiol. 20, 267.

Ponder,E. & MacLeod,J. (1938). Amer.J.Physiol. 123, 420.

Potter,V.R. & Recknagel,R.O. (1951). Phosphorus Metabolism Vol.1. John Hopkins Press, Baltimore.

Rapoport,S. & Guest,G.M. (1945). J.clin.Invest. 24, 759.

Reid,J. (1950). Brit.J.exp.Path. 31, 65.

Reid,J., Macdougall,A.L. & Andrews,M.M. (1957). Brit.med.J. 2, 1071.

Reid,J., Watson,R.D. & Sproull,D.H. (1950). Quart.J.Med. 19, 1.

Roche,J., Michel,R. & Jouan,P. (1957). Ciba Foundation Colloquium on Endocrinology, Vol. 10.

Simkins,S. (1937). J.Amer.med.Ass. 108, 2110 & 2193.

Smith,M.J.H. (1952) Biochem.J. 52, 649.

Smith,M.J.H. & Jeffrey,S.W. (1956) Biochem.J. 63, 524.

Smith,M.J.H., Meade,B.W. & Bornstein (1952). Biochem.J. 51, 18.

Soffer,L.J. & Wintrobe,M.M. (1932). J.clin.Invest. 11, 661.

Tenney,S.M. & Miller,R.M. (1955). Amer.J.Med. 19, 498.

Thibault,O. & Pitt-Rivers,R. (1955a). Lancet 1, 285.

Thibault,O. & Pitt-Rivers,R. (1955b). C.R.Soc.Biol.Paris. 149, 880.

Thomson,W.O., Thomson,P.K., Taylor,S.G. & Dickie,L.F.N. (1934). Arch.int.Med. 54, 888.

Trinder,P. (1954). Biochem.J. 57, 301.

Valentine,W.N. (1956). Progress in Haematology Vol. 1.

Valentine,W.N., Follette,J.H. & Lawrence,J.S. (1953). J.clin.Invest. 32, 231.

Varnai,I. (1957). Comm. 23rd. Ann.Meet.Hung.Physiol.Soc. 56.

Victor,J. & Potter,J. (1938). Brit.J.exp.Path. 19, 227.

Wagner,R. & Reinstein,S. (1950). Arch.Biochem. 29, 260.

Warburg,O. (1930). The Metabolism of Tumours. London Constable & Co., Ltd.

Winkler,A.W., Laietes,P.H., Robbins,C.L. & Man,E.B. (1943). J.clin.Invest. 22, 535.

Winters,R.W. & Morrill,M.F. (1955). Proc.Soc.exp.Biol.N.Y. 88, 409.