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THE METABOLIC RESPONSE TO SALICYLATE

by

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SUMMARY

An investigation of the metabolic response which follows the administration of relatively large doses of sodium salicylate to the whole animal has shown that this response is characterised by a rapid but transient rise in oxygen consumption. Similarly, transient disturbances in electrolyte balance are associated with high plasma levels of the drug. When treatment with salicylate is continued for several days it is found that the effect on oxygen consumption is non-cumulative, and that there occurs a considerable body wastage which is not associated with a decreased food intake. Therefore salicylate administration produces a state of wasteful hypermetabolism.

Salicylate has been shown to uncouple mitochondrial oxidative phosphorylation *in vitro* and it has been suggested that this effect is the biochemical mechanism of action of the drug. In this study liver mitochondria were isolated from rats treated with large doses of salicylate until toxic effects were apparent. Although high plasma and intracellular levels of salicylate were detected in rats so treated, liver mitochondria isolated from these animals exhibited completely unimpaired efficiency of oxidative phosphorylation *in vitro* when compared to control experiments with liver mitochondria isolated from untreated rats.

Four hypotheses were postulated which may explain the apparent absence of effect of the *in vivo* administration of salicylate on mitochondrial oxidative phosphorylation.

Detailed examination of these hypotheses indicated that salicylate could penetrate isolated mitochondria at 0°C. Salicylate

Detailed examination of these hypotheses indicated that salicylate could penetrate isolated mitochondria at 0°C. Salicylate was not firmly bound to mitochondria under these conditions and was readily removed by a simple washing procedure. Irreversible damage was not observed in association with this procedure, as all mitochondrial functions subsequently examined were similar to those of untreated control mitochondria.

Sodium salicylate enhanced the ATPase activity of fresh mitochondria, but unlike dinitrophenol was without effect on the ATPase activity of aged mitochondrial preparations, even in the presence of Mg^{++}. This effect is explicable by an action of salicylate on the mitochondrial membrane, as the effect is only associated with direct contact between salicylate and mitochondria, and is not produced by subjecting the membrane to pretreatment with salicylate.

Subsequent experiments in vitro demonstrated a marked loss of K^{+} from isolated mitochondria in a sucrose medium containing sodium salicylate. This effect was not due to the sodium ion, nor could it be produced by the para-hydroxybenzoate radical. These observations were made at 0°C in the absence of substrates for mitochondrial respiration, and were compatible with a "primary" action of salicylate on mitochondrial membrane permeability.

Therefore an action of salicylate on metabolic rate is not obligatorily linked to a direct effect of salicylate upon the enzymes of mitochondrial oxidative phosphorylation in vivo, but may influence this process as a secondary effect.

The finding of significantly lowered "intracellular" K^+ concentrations following salicylate administration to rats supports this concept. The effect of salicylate on K^+ *in vivo* may be a cause rather than a consequence of decreased efficiency of mitochondrial oxidative phosphorylation. Mitochondria from treated rats are washed free of salicylate by the method of isolation and K^+ replacement can occur from the medium *in vitro*.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief this thesis does not contain any material previously published or written by another person except when due reference is made to such material in the text.

February, 1961.

PREFACE

The evidence reported in this thesis was obtained during an investigation of the metabolic response which follows the administration of salicylate to both man and animals. In an attempt to learn something of the biochemical mechanisms associated with this response, experiments have also been performed on subcellular particles isolated from livers of rats and examined *in vitro*.

The nature of the experimental work involved has thus been diverse. Because of this, each type of experimental investigation will be reported as a separate study which includes the relevant discussion. Although such a method of presentation leads to some repetition, it was felt that greater clarity could be so achieved. A resume of all material findings will also be included with the conclusions which are presented in the final chapter of this thesis.

The project was carried out in the Department of Medicine, University of Adelaide with the aid of a grant from the National Health and Medical Research Council of Australia, under the supervision of Dr. B.S. Hetzel of the Department of Medicine and Dr. E. Holdsworth of the Department of Biochemistry of the University of Adelaide.

CHAPTER I

INTRODUCTION

Part I: Historical Introduction

Part II: Metabolic response to Salicylate

A. General response to Salicylate

B. The role of endocrine glands in this response

C. Possible biochemical mechanisms associated with this response

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CHAPTER I

Part I

HISTORICAL INTRODUCTION

The history of the "salicylates" dates from the early nineteenth century when the French chemist, Lerous, isolated a "phenolic aromatic alcohol glucoside" from the bark of the white willow (*Sallix alba*).

This compound Lerous named *Salicin*, and in the year 1827 he ascribed to it the following structure:-

(cit. Petit, 1876)

The early work on derivatives of this compound, which are collectively known today as the "salicylates", was dominated by the French school of chemists who were most interested in natural products at that time. In 1838 Piria, reported that he had prepared from *Salicin* a free monocarboxylic phenolic acid ($C_6H_4OH.COOH$), which he named *Salicyl* (Piria, 1838) and later, in 1843, Cahours published an account of his preparation of the free acid from gaultheria oil (Cahours, 1843).

However, it was the German workers Kolbe and Lautemann who in 1860, accomplished the first synthesis of "salicylic acid" (Kolbe and Lautemann, 1860). This they did from phenol by a process which remained essentially unchanged for the next seventy-five years, until it was superceded by the process of Schmitt which is still used industrially today.

There has been some confusion concerning the first

pharmacological use of these compounds but perhaps the credit should go to Dr. T. J. Maclagen, an Edinburgh physician, who in the March issue of the Lancet in 1876, published a paper entitled, "The treatment of acute rheumatism by Salicin". (Maclagen, 1876). This report was based on the results of a successful two-year clinical trial of Salicin amongst those of his patients suffering from rheumatism, a common malady in Dr. Maclagen's locale.

As was the manner of those times, Dr. Maclagen rationalised his use of Salicin in the treatment of rheumatism in the following words:- "reasoning from the effect of quinine on 'intermittent fever', that nature seeming to produce the remedy under climatic conditions similar to those which give rise to the disease ... , it seemed to me that a remedy for that disease would most hopefully be looked for among those plants and trees whose favourite habitat presented conditions analogous to those under which the rheumatic miasma seemed most to prevail. A low-lying damp locality, with a cold rather than a warm climate, gives the conditions under which rheumatic fever is most readily produced. On reflection it seemed to me that plants whose haunts best correspond to such a description were those belonging to the natural order of the 'Saliciaceae', the various forms of willow. Among the 'Saliciaceae' therefore, I determined to search for a remedy of acute rheumatism. The bark of many species of willow contains a bitter principle called Salicin. This principle was exactly what I wanted; to it, therefore, I determined to have recourse."

Publication of Maclagen's article soon produced unusual supporting evidence for his claims. One Frederick Enser, Surgeon

to the Provincial Hospital in Port Elizabeth, South Africa, submitted to the editors of Lancet later that same year, an article entitled, "The willow as a remedy for acute rheumatism" (Enser, 1876), wherein he relates his experience with a Dutch Boer patient severely afflicted with rheumatic fever and, as he supposed, beyond his help. Seemingly amazingly this patient is cured by the use of a concoction obtained from an old Hottentot shepherd, which preparation Dr. Enser learns, is made from the "shoots of the willows which grow on the banks of the river". This concoction was the favourite tribal remedy for a malady known to the native Hottentots and local Boers as "Sinken Kors", a malady identified by Dr. Enser as rheumatic fever.

In the same year that Dr. Maclagan began his clinical trials, Kolbe, who had been responsible for the synthesis of salicylic acid fourteen years earlier, independently suggested (Kolbe, 1874) an antipyretic action for salicyl, on the basis of certain pharmacological resemblances to quinine.

This prompted Buss to experimentally examine this possibility and in the following year he reported (Buss, 1875) that he had observed an antipyretic action of salicyl which he believed to be "produced by a dilation of the peripheral vessels including those of the skin ... and this together with the accompanying sweating; at least in fever, suggests the probable mechanism of the antipyresis, that is increased heat dissipation." Buss did not extend his observations and failed to recognise the great therapeutic value of salicylates in the treatment of rheumatic fever.

After the publication of favourable reports on the use of salicylic acid in the treatment of rheumatic fever, by Broadbent in England (Broadbent, 1876) and Stricker and Reiss in Germany (1876), it was agreed by workers at that time that Maclagen should have the credit for these findings as he had introduced these agents into clinical medicine by the use of Salicin more than a year earlier.

By this time many people had taken an interest in the "salicylates" and reports on their action were numerous. Justi noted the rapidity of the onset of profuse sweating following salicyl administration, fifteen minutes in fever (Justi, 1876), and Goltdammer noted that this sweating could be so marked as to cause exhaustion. Like Buss (1875) these workers also felt that this sweating was of importance in the antipyretic action of the drug.

By now other actions of the drug were coming under notice. The previous year Furbringer had reported that a daily consumption of 1-2 grams of salicylic acid produced an acid urine, which was beneficial in cases of inflammation of the urinary passages (Furbringer, 1875).

The beneficial effect of sodium salicylate in the treatment of diabetes mellitus was mentioned by Ebstein in 1876 and his findings were confirmed two years later by Bartels (1878). These two workers were among the first to demonstrate a progressive diminution of glycosuria, polyuria and polydypsia concomitant with an increase in weight, strength and general improvement in the well-being of diabetic patients treated for prolonged periods with sodium salicylate in daily doses of 5 to 16 grams. However, the failure of salicylate to be of benefit in the advanced states of the disease and the frequent

development of "salicylism" were probably the principal reasons why this oral medication for diabetes never gained wide application, and was apparently abandoned long before the discovery of insulin.

Seemingly in conflict with these findings, in 1878 Chirone and Petricci reported that long-term experiments conducted on dogs and cats had shown that prolonged administration of "salicylate" led to emaciation and loss of body weight. A decade later Kumagawa (1888) demonstrated an increased protein breakdown following the very rapid intestinal adsorption of small doses of sodium salicylate.

Three years prior to Kumagawa's publication, reference to the therapeutic value of salicylate had been made in a report by Randolph and Dixon (1885). In a study of the cutaneous adsorption of salicyl, these investigators reported that in one patient examined, enough of the drug was adsorbed to relieve his symptoms of rheumatism.

Evidence for the rapid cutaneous adsorption of "salicylates" came from the finding by Linnoisier and Lannois in 1896, that they could detect salicyl in the urine of subjects, thirty minutes after the application of the ester methyl salicylate, to the skin.

The compound acetylsalicylic acid or "aspirin" † (C_6H_4O $(COCH)_3$.COOH) was introduced into clinical medicine by Wohlgemuth in 1899 and in the same year its pharmacological action was described by Dreser (1899).

By 1908 there was some evidence that there were interactions between the plasma proteins and salicylate;

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† The name "aspirin" appears to be coined from "spirsaure" the old German name for salicylic acid and the prefix 'a' indicating the "acetyl" derivative.

Jacoby (1908) suggested that salicylate in the blood seemed to be attached to these proteins "by a binding with the amino compounds" in a way not then understood. Today there is ample evidence that salicylate has a high affinity for binding with the plasma proteins (Smith, 1949) but the precise chemical nature of the linkage between these molecules is still unknown. Gastric absorption of the salicylates was examined in 1911 by Burrow who asserted that the soluble salts of salicylic acid were only slowly absorbed whilst the insoluble salts passed unchanged into the intestine. In the following year Levin stated that "judging from the quantity of salicyl in the blood, found when sodium salicylate is given intra-muscularly, it is absorbed much more readily in this manner than when given by mouth". (Levin, 1912).

The first suggestion of a mechanism by which salicylate relieved the symptoms of rheumatic fever was made by Oswald in 1910, in a paper entitled, "Salicylic acid increases the permeability of colloids for electrolytes".

Two years later P. J. Harzlick, working with excised loops of living intestine, confirmed the findings of Oswald (Harzlick, 1912) when he demonstrated an increased diffusion of both salicylate and other electrolytes which was related to the strength of salicylate employed. He stated that this phenomena "is suggestive as a possible basis of the absorption of effusions from joints in rheumatic fever under salicylate treatment". Fourteen years later Harzlick again suggested "that an increased permeability of the vessels with changes in local blood flow may facilitate the absorption of fluid, toxins and the products of inflammation from the joints". (Harzlick, 1926).

The initial studies on the metabolism and fate of salicylates also appeared about this time. The presence of salicyl ethereal sulphate, salicyl glycuronic acid and oxysalicylic acid in the urine, was demonstrated by Neuberg in 1911; it was also recognised that salicylates may be excreted unchanged in the urine.

Denis and Means showed in 1916 that following salicylate administration to two normal subjects there occurred a diuresis of nitrogen to the extent of 40% above normal, which was also accompanied by an increased phosphate excretion in the urine. However, in 1922 Oskaka asserted that nitrogen excretion was not a sole property of orthohydroxybenzoic acid as both the meta- and para- compounds produced an increased nitrogen excretion when given to rabbits.

Between 1919 and 1926, three groups of workers all reported that various derivatives of salicylic acid produced considerable acceleration of respiration and symptoms of "air hunger" accompanied by an increased pulse rate leading finally to respiratory failure. (cf. Harzlick, 1926).

Today the antipyretic, anti-inflammatory and analgesic properties of the salicylates are well known although intensive work on the pharmacology of these agents has failed to demonstrate their mechanism of action,which has remained controversial since 1885 when Latham published a paper entitled, "Why does salicylic acid cure rheumatism" (Latham, 1885). Despite this lack, a decade ago the production of these drugs was reported to exceed 6,000 tons per annum in the United States of America alone (News of Industry, 1949).

Current interest in the general effects of salicylate is still high; during 1960 there have been reports of at least six trials conducted in man of the recently introduced choline derivative of salicylate. Yet as recently as January of 1960 an editorial comment in the Pharmaceutical Journal stated that:- "Despite the use of salicylates for so many years and their relatively simple chemical structure very little is known about the exact mechanisms by which they produce their large variety of therapeutic and toxic effects". In man these latter effects are hyperpyrexia, respiratory alkalosis, metabolic acidosis, dehydration and ketosis (Annotations, British Medical Journal, 1960).

Part II

METABOLIC RESPONSE TO SALICYLATE

A. General response to Salicylate

It is well known that salicylates cause an increase in oxygen consumption and carbon dioxide production in man (Denis and Means, 1916) (Barbour and Devenis, 1919). This finding has also been reported in experimental animals by Singer (1901), Meade (1954) and Reid (1957).

Within the last decade intensive studies have been undertaken of the pattern of metabolic changes induced by salicylate in both man and experimental animals.

One method of investigation of these effects in the whole animal has been to measure the urinary constituents excreted whilst the subject is under the influence of the drug. In this manner Reid, Watson and Sproull (1950) demonstrated an increased loss of nitrogen and potassium, accompanied by a urinary "retention" of sodium and chloride, during the administration of salicylate to patients with rheumatic fever.

A rise in metabolic rate which was not associated with hyperventilation alone has been described by Cochrane (1952) in normal subjects following salicylate administration, and later this worker (Cochrane, 1954) demonstrated the appearance of similar changes in patients with rheumatic fever. In these latter subjects there was also a fall in body temperature, and the relief of symptoms in this condition was associated with the maintenance of a high level of oxygen consumption. The need for high plasma levels of salicylate in the "cure" of rheumatic fever has been stressed by Smith and Talbot (1950).

The fall in body temperature which follows the administration of salicylate in therapeutic dosage is thought to be in spite of increased heat production. The increased heat production is said to be counteracted by heat loss through sweating. However when the body water has been depleted beyond a critical level by sweating, further administration of salicylate cannot still cause heat loss in this way and hyperpyrexia results (Annotations, British Medical Journal, 1960). Associated hyperventilation and vomiting lead to further dehydration and the toxic hyperpyrexia becomes worse.

Reid, McDougall and Andrews (1957) have recently suggested that salicylate is a "primary metabolic stimulant" causing increased protein catabolism. As did Buss in 1875, these workers emphasised the importance of profuse sweating produced by salicylate as the main mechanism of its antipyretic action. If sweating failed to occur, hyperpyrexia again resulted, as has been readily observed in children with salicylate toxicity (Segar and Holliday, 1958).

Support for the belief of a "primary" action of salicylate comes from the studies of Tenny and Miller (1955). These workers demonstrated that decapitated or curarised dogs maintained in a total body respirator to keep ventilation constant, also showed the same increased oxygen consumption as the intact animal in response to salicylate administration.

Reid, McDougall and Andrews (1957) also suggested that the anti-rheumatic effect of salicylate was the result of loss of cell water arising from the loss of protein due to excess catabolism. One is again reminded of the speculations of both Oswald (1910) and Harzlick (1912).

B. The role of endocrine glands in the metabolic response to Salicylate

Not only has the possibility of a direct or "primary" stimulation of metabolism by salicylate been considered as the mechanism of action of this drug, but the possibility of an action of salicylate mediated through endocrine involvement has also received attention.

Cochrane, Watson and Reid (1950) have reported the occurrence of mild Cushing's syndrome due to the aspirin used in the treatment of rheumatic fever, and Hetzel and Hine (1951) have shown a significant relationship between plasma salicylate and the depletion of adrenal ascorbic acid in the rat which also suggests an effect of salicylate on the adrenal cortex. Eades and King (1953) have demonstrated an increase in blood adrenocorticotrophic hormone (ACTH) in rats following salicylate administration and Good, Done, Ely and Kelley (1959) have reported an increase in 17-hydroxycorticoids in guinea pigs under these conditions. This latter response was abolished by hypophysectomy and adrenalectomy.

The similarity between the electrolyte changes produced by salicylate and adrenal hormones has often been remarked upon (Hailmann, 1952) but there is as yet no positive evidence in man of an increase in adrenal cortical secretion following salicylate in therapeutic dosage.

A relationship of salicylate to the action of the thyroid gland has been suggested by the work of Alexander and Johnson (1956) who demonstrated a striking fall in the plasma cholesterol of patients with myxoedema when treated with salicylate. Although

thyroid activation plays no part in this effect it has been shown by work from this laboratory, that there is an effect of salicylate on the level of circulating thyroid hormone (Charnock, Good and Hetzel, 1959) (Hetzel, Good, Wellby and Charnock, 1960).

Recently there have been a number of reports indicating an effect of salicylate on the pituitary (Wolff and Austen, 1958). These observations suggest that the feed-back mechanism which controls the level of circulating thyroid hormone (Brown-Grant, 1957) is sensitive to salicylate in both normal and hyperthyroid states (Hetzel, Good, Wellby and Charnock, 1960).

A relationship between the action of salicylates and the pancreas in diabetes has been known for some time (Williamson, 1901). In recent years the effects of these drugs on carbohydrate metabolism generally has found new clinical interest, particularly since the similarity between the pharmacological effects of the corticosteroids and salicylates became apparent (Hailmann, 1952).

Both hyper- and hypoglycemic reactions to salicylates have been described. Hyperglyceamia has been noted in man mainly in instances of acute salicylate poisoning (Gross and Greenberg, 1948) (Schadt and Purnell, 1958). It has been produced experimentally in rats (Smith, 1955 b), mice (Sproull, 1954) and dogs (Barbour and Hettman, 1921). Hypoglyceamia has been observed clinically following the administration of large doses of acetylsalicylate by Reid, McDougall and Andrews (1957), and by Langeron, Michaux, Destombes and Paul (1950) following the administration of para-amino-salicylate.

Ingle and Meeks (1952) have demonstrated a decrease in hyperglyceamia and glycosuria following the parenteral administration of sodium salicylate to partially pancreatectomised rats and Smith, Meade and Bornstein (1952) have demonstrated beneficial effects of this drug in alloxan-diabetic rats.

Recently Hecht and Goldner (1959) have suggested that the hypoglycemic effect of salicylates is worthy of re-appraisal in the treatment of diabetes mellitus. Although these hormonal involvements will be referred to again, it is the possibility of a "primary" action of salicylate which is the major theme of the investigation to be reported here.

C. Possible biochemical mechanisms associated with the metabolic response to salicylate

Because of their great interest to biochemist and physician alike, the mechanisms underlying the striking stimulation of metabolism by salicylate have been investigated by a number of workers in recent years.

Much evidence for a "primary" action of salicylate has come from the study of isolated tissue and other similar experiments in vitro. This evidence generally suggests a "peripheral effect" of the drug, but does not exclude the possibility of an action upon the endocrine glands.

Although aspirin is so well known as an antipyretic, one of the more serious symptoms of large doses of salicylate is hyperpyrexia. This has been attributed to an increased heat production due to the "primary" stimulant effect of salicylates on body metabolism. The effect seems to be restricted to salicylic acid, acetylsalicylic acid and phenyl and methyl substituted derivatives.

Salicylamide when administered in equivalent dosage does not result in plasma levels equivalent to those found following the administration of acetylsalicylic acid (Buller, Muja and Carr, 1957) (Bavin, Macrae, Seymour and Waterhouse, 1952) (Seeburg, Hansen and Whitney, 1951). All these investigators believe that salicylamide is rapidly conjugated and excreted in this form, hence its apparent lack of both antipyretic and hypermetabolic effects.

In addition to the effect of salicylate on the oxygen consumption of the whole animal, Brody (1956) has claimed that the

oxygen consumption of tissues isolated from animals treated with salicylate in vivo is also increased when these tissues are examined in vitro. A similar action of salicylate on the oxygen consumption of tissues from normal animals when the drug is added in vitro has been reported by Sproull (1957) and Smith and Jeffrey (1956 a) (1956 b).

The investigations of these and other workers have shown that the rapid increase in oxygen consumption seen when salicylate is added to the isolated rat diaphragm, or slices of liver tissue, is accompanied by an equally rapid disappearance of glycogen as well as a reduction in the creatine phosphate and adenosine triphosphate content of these tissues (Lutwak-Mann, 1942) (Andrews, 1960).

In 1954 Mead had suggested that "the action of salicylate may resemble that of dinitrophenol" and the next year Smith (1955a) suggested that a possible mechanism for the salicylate depression of muscle and liver glycogen levels in the rat, was that salicylate interferes with oxidative phosphorylation reactions and inhibits the production of "high energy" phosphate bonds. The latter investigator also suggested that the increased lactic acid production and respiratory quotients found in isolated diaphragms of rats when incubated with salicylate may be an indication of substrate break-down to supply the energy needed for inefficient phosphorylation processes. A decreased glucose uptake by the diaphragm which Smith also described at that time was thought to be explicable on the same basis, if the first steps in glucose utilization involve phosphorylation.

As the liver is one of the main sites of heat production in the body (Fedorov and Shur, 1942) (Birnie and Grayson, 1952), and the heat formed there mainly arises during the oxidations carried out by the mitochondria, this and the hyperpyrexia seen to follow salicylate administration to the whole animal, has led biochemists to investigate the action of salicylate on isolated mitochondria. These preparations are particularly suitable for an examination of oxidative phosphorylation reactions. Examination of the in vitro effects of salicylate addition to isolated mitochondrial preparations from rat liver, as well as kidney and brain, have demonstrated that although there were differences in the response found with mitochondria isolated from various organs, in some cases there was a similar increase in the oxygen consumption to that found in respiring tissue slices. This increase was accompanied by a reduction in the concomitant esterification of inorganic phosphate in the reaction. That is the in vitro addition of salicylate to isolated mitochondria "uncouples" oxidative phosphorylation (Brody, 1956) (Penniall, Kalnitsky and Routh, 1956) (Penniall, 1958) (Jeffrey and Smith, 1959) ⸸

This concept of an "uncoupling" of mitochondrial oxidative phosphorylation appears to be compatible with many of the manifestations of salicylate action in the whole animal. For example the effect of aspirin therapy on the fasting blood sugar and glycosuria of patients with diabetes mellitus has been thought to

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⸸ The report of Jeffrey and Smith (1959) appeared during the progress of work presented in this thesis.

follow an uncoupling action of salicylate (Reid, 1958). Salicylate produces a number of similar effects on carbohydrate metabolism in the diabetic rat, for example reduction of glycosuria and hyperglycemia (Smith, Meade and Bornstein, 1952). In addition it has been shown to diminish glycogen synthesis in isolated rat liver slices (Smith, 1955 a) and diminish the incorporation of C^{14} labelled acetate into this compound (Smith, 1959).

Many workers have commented on the similarity of the effects of salicylate and 2.4-dinitrophenol when they are added to either respiring tissue slices or mitochondrial preparations (Sproull, 1957) (Reid, 1958) (Messer, 1958) (Smith, Meade and Bornstein, 1952). Recently Adams and Cobb (1958) have suggested that the similar anti-inflammatory activity of these compounds may be related to their capacity to uncouple oxidative phosphorylation, although these workers suggest that the biochemical sites of the uncoupling effect may differ.

One major difference between the action of these two agents *in vitro* is the great difference in concentration at which they will produce an uncoupling effect. Although experimental conditions have varied widely 2.4-dinitrophenol has been shown to exert an effect at molar concentrations a hundred times less than those at which salicylate will act.

In this investigation the effects of salicylate on oxygen consumption and associated phenomena have been re-investigated in the whole animal. An attempt has been made to relate these *in vivo*

changes to possible biochemical mechanisms which may be responsible for the *in vitro* action of the drug. These latter mechanisms have been studied in some detail, in particular the effect of salicylate on mitochondrial function has been examined. The results of these studies will be presented in the following chapters of this thesis.

CHAPTER II

METABOLIC RESPONSE TO SALICYLATE

Part I: The effects of Salicylate in man

Part II: The effects of Salicylate in rats

A. Electrolyte balance and body weight

B. Body weight

C. Metabolic rate

Part III: Conclusions

CHAPTER II

METABOLIC RESPONSE TO SALICYLATE

Part I

THE EFFECTS OF SALICYLATE IN MAN ǂ

Initially a study was made of the rapid metabolic response which followed the administration of a large single oral dose of sodium salicylate to healthy young male volunteers. This was compared to the metabolic response produced by a similar dosage of sodium para-hydroxybenzoate, which was used as a control for the sodium ion.

The metabolic response to salicylate was examined by observing possible changes in oxygen consumption and plasma and urine constituents for a period of five hours following the administration of salicylate. Recent Medical Research Council Trials (Empire Rheumatism Council, 1955, 1957) have disclosed a remarkable similarity between the anti-rheumatic effects of salicylate and those of either ACTH or cortisone.

It is possible to compare the response observed in this experiment with that seen to follow stimulation of the adrenal cortex (Hailman, 1952) (Hetzel, Williams and Lander, 1957), the thyroid (Hetzel, Charnock and Good, 1958), or a combination of both (Hetzel, Charnock, Lawrence and Wellby, 1960).

ǂ This study was performed in collaboration with Dr. B. S. Hetzel of the Department of Medicine, University of Adelaide. Our thanks are due to Dr. H. Lander for his help in collecting some of the blood samples and to Mr. B. F. Good for his assistance with the protein-bound iodine estimations.

Materials and Methods

These studies were performed on six healthy male medical student volunteers. The experiments were conducted between 8.00 a.m. and 5.00 p.m. on two separate days one week apart. The subjects were in the post-absorptive state, in addition they had maintained a similar food and fluid intake on the evening before each day of the experiment. Five grams of each salt was given by mouth, except for one subject weighing 213 lb. who received seven grams. Sodium salicylate was given on one day and sodium para-hydroxybenzoate on another. Three subjects received each treatment on each occasion. Both salts were given in solution suitably flavoured with aniseed. The drugs were taken after a one-hour preliminary control period, during which time the subjects' oxygen consumption was determined. Samples of urine and blood were collected ten minutes after the measurement of oxygen consumption was completed, and then at hourly intervals throughout the experiment. The subjects' oxygen consumption was determined again approximately ninety and two hundred and ten minutes after ingestion. The urine samples were acidified to pH 2 with hydrochloric acid and the volume of sample and of acid noted. Plasma was separated from oxalated whole blood by centrifuging at 3,000 x G for ten minutes.

The Jones apparatus was used to determine oxygen consumption - the time required by each subject to consume one litre of oxygen being calculated and the mean of two consecutive determinations taken. The results are expressed as cc./min./sq. metre of surface area.

Chemical methods:

Salicylate estimations were made on the plasma by the method of Trinder (1954). This method did not produce a colour reaction with para-hydroxybenzoate. The tests were performed on 0.5 ml. of plasma and the resultant colour read at 525 mu. in a spectrophotometer. Standards from 0 to 50 ug. were included with every run, although the curve obtained from standard quantities of salicylate is extremely reproducible and the colour is stable for many hours.

The recovery of salicylate added as the sodium salt to the plasma was found to be greater than 95%.

The method is simple and rapid and extremely suitable for the limits of accuracy required here. A further examination of some aspects of this method is discussed later (cf. Chapter VI, Part II).

Plasma cholesterol was originally determined by the direct method of Zlatkis, Zak and Boyle (1953) which relies upon the addition of a strong acid solution directly to the plasma. The colour developed in those tests which contained plasma was a different tint to that developed from standard solutions. Duplicate plasma samples produced readings which varied by ± 25 mgms.%. The colour density of all readings also varied considerably depending upon the temperature of the reaction, the amount of protein in the sample, and other unknown factors.

Later the extraction technique of Zak, Dickermann, White, Burnett and Cheney (1954) was employed in a re-examination of these plasma samples. The values obtained by this method were uniformly

lower, presumably due to the absence of protein in the final colour reaction and the variation in individual results somewhat reduced to ± 10 mgms.% although the difference in tint between the unknowns and the standard colour was still apparent.

As further investigation of plasma cholesterol levels was not envisaged, no further investigation of the method was undertaken. Standards between 0-50 ug. were included with every run and the colour developed at the same time as the unknowns. The colour was measured in a colorimeter using an Ilford filter No. 625.

A linear relationship existed between colour produced and cholesterol concentration at least up to 50 ug. cholesterol/ml. In these assays this was equivalent to a plasma level of 500 mgms.%.

Plasma and urine magnesium were determined by a modification of the titan yellow method of Orange and Rhein (1951). Polyvinyl alcohol (PVA) was used instead of gum ghatti, this increases the range of concentration of magnesium over which the calibration is linear. The standard solutions were treated with deproteinising reagents in the same manner as the test samples thus allowing for the effect of these reagents on the intensity of the colour developed. The PVA and titan yellow (TY) were mixed prior to addition so that a larger volume could be added to each test and the pipetting error thus reduced. The quantity of alkali added was controlled as accurately as possible. Potassium hydroxide (Merk, AR grade) was used as this was found to contain less Mg^{++} than any other reagent available. By comparison of the spectral characteristics of a reagent blank, an unknown Mg^{++} containing test reaction, and an aqueous solution of titan yellow it

was possible to select an optimum wave-length at which future tests should be measured. In this laboratory and using a Beckmann Model B spectrophotometer a wave-length of 560 mu. was selected as optimal.

A linear relationship existed between colour density and magnesium ion concentration up to 40 ug. Mg^{++}. Recovery of added Mg^{++} was between 100-103% within this range. The addition of calcium ions, as calcium chloride, did not influence the colour reaction until the ratio of Ca^{++}: Mg^{++} exceeded 100:1. These circumstances are unlikely in the biological fluids under examination here.

Serial determinations of acidified urine revealed no variation in the concentration of Mg^{++} which could be detected over a period of seven months, provided the pH of urine was maintained below pH 2.

A full set of standards, deproteinised and treated exactly as the unknowns, were included with every batch of samples for assay. The slope of the standard curve fluctuated with the ambient temperature. Maximal colour development occurred thirty minutes after the addition of alkali at room temperature. For this reason all measurements were made at this standard time after colour development.

Inorganic phosphorus was determined in both urine and plasma by the method of Taussky and Shorr (1953). This method has been in general use in these laboratories for several years and has been found to give excellent results. If the concentration of trichloracetic acid which is used to deproteinise the plasma and also provide some of the acidity for the final colour reaction is accurately controlled then results are reproducible on any one sample to less than

± 0.1 mgms.%. Instead of the method of calculation suggested by Taussky and Shorr, standard amounts of inorganic phosphorus up to 24 ug. and added as potassium dihydrogen phosphate, were assayed with every batch of samples.

The colour complex formed was stable for several hours and the slope of the standard curve very reproducible. This method differs from that of Fiske and Subbarow (1925) and Allen (1940) in that instead of complex reducing agents such as amino-naphol sulphonic acid and 2.4-diaminophenol hydrochloride it makes use of a reduction by ferrous sulphate of the phosphomolybdic acid formed during the analysis.

Plasma protein-bound iodine (PBI) was estimated by the alkaline ashing technique of Acland (1957). This method was thoroughly investigated before its use here and subsequent routine introduction to these and other associated laboratories (Charnock and Good, 1957). Each step of Acland's procedure had been critically examined and found to be satisfactory for this purpose. The temperature of the muffle furnace was checked by the introduction of a range of pure salts of known melting point as well as a pyrrometer. The temperature control was found to be critical and all samples were placed in a cold furnace and ashed for three hours after a temperature of 625°C ± 5°C was reached.

Strict attention was paid to the precision of the timing involved in the final decolorising reaction. All estimations of plasma PBI were performed at least in duplicate and rigid control was observed throughout. Control analyses on a pooled plasma sample

were included with every batch and occasional recoveries of added thyroxine were included throughout the period of estimations. These recoveries were hampered by the "impurity" of the thyroxine which was available. After initial estimations of the iodine content of "Glaxo" thyroxine, recoveries of over 90% organic iodine were obtained.

Direct addition of either sodium salicylate or sodium para-hydroxybenzoate to the plasma had no effect on the chemical determinations of PBI by this method.

Statistical analysis of the data obtained during analysis of these particular samples showed a standard error of only 0.18 ugms.% between batches, and a standard error of the mean of two duplicates of 0.12 ugms.%. In these circumstances a small variation between mean results of ± .25 ugms.% was of statistical significance at the five percent level.

All statistical analyses were performed by the staff of the Commonwealth Scientific and Industrial Research Organisation, Division of Mathematical Statistics, Adelaide.

Total nitrogen in the urine was determined by a wet digestion method (in the presence of a selenium catalyst) which had been developed by the analytical section of the Commonwealth Scientific and Industrial Research Organisation, Division of Biochemistry and General Nutrition, Adelaide. In this instance the digested sample was processed by Nesslerization and measurement of the colour developed.

Urine sodium and potassium were estimated by flame photometry (EEL) on aqueous dilutions of the acidified urine. Standards were estimated with every batch and were read between measurements of

unknown samples. Reasonable replication was obtainable if care was taken with the adjustment of the instrument.

Results

Clinical effects - Tinnitus, sweating and a feeling of unsteadiness and weakness were noted by the subjects within ninety minutes of ingestion of the sodium salicylate. No such effects were noted following the ingestion of sodium para-hydroxybenzoate. These symptoms usually diminished within three hours.

Effect on oxygen consumption - Mean increases of the order of 38% were apparent after ninety minutes in the subjects who received salicylate and this increase was still evident after a further two hours. No increase was observed following sodium para-hydroxybenzoate. This difference was significant at the five percent level. These results are given in detail in Table (1).

Plasma salicylate - After the first hour a mean plasma salicylate level of 33.1 mgms.% was found. This had increased to a mean level of 38.1 mgms.% by the end of the second hour. The mean plasma level now commenced to decline and had fallen to 32.1 mgms.% by the end of the period of sample collection. The plasma of subjects treated with sodium para-hydroxybenzoate did not give appreciable values of "salicylate" by the Trinder method of estimation. These results are given in detail in Table (2).

A relationship between the plasma level of salicylate and the rise in oxygen consumption is shown in Figure I.

Effect on plasma cholesterol - A significant fall ($P < .05$) in plasma cholesterol was observed two hours following salicylate ingestion, when estimated by the method of Zlatkis et al (1953). This method did not reveal any differences between the treatments at the end of the experimental period. However, when the measurements were repeated using the more precise method of Zak et al (1954), whilst a significant fall in plasma cholesterol had occurred by the second hour ($P < .02$) there was now apparent a significant decrease at the end of the experimental period four hours after ingestion of the drugs ($P < .05$).

These results are shown in detail in Tables (3A) and (3B). The presence of similar quantities of salicylate in the plasma has been shown not to affect the estimation of cholesterol by the method of Zak et al which was employed here (Alexander and Johnson, 1956).

Effect on plasma magnesium - There was a slight increase in plasma magnesium levels following both treatments. The mean level increased from 1.5 mgms.% before treatment to 1.7 mgms.% following the administration of salicylate; the level was further increased to 1.9 mgms.% under the influence of para-hydroxybenzoate. The effect of para-hydroxybenzoate was apparent within one hour, whilst that of salicylate was delayed until the second hour. The effects of either treatment were not apparent after the third hour. None of these changes reached statistical significance. These results are shown in Table (4).

Effect on plasma PBI - The initial levels of all six subjects were well within the normal range (3-8 ugms.%). There was a fall in plasma PBI two hours after the ingestion of sodium para-hydroxybenzoate

($P < 0.05$). There was an even greater fall at this time following ingestion of sodium salicylate. Comparison of the levels at this time revealed a significant difference in the fall achieved by either drug. ($P < 0.05$). The detailed results are given in Table (5).

<u>Effect on urine flow</u> - Increases in urine flow occurred for three hours following salicylate ingestion in four of the six subjects ($P < 0.1$ during the third hour).

<u>COMPARISON OF THE EFFECT OF SODIUM SALICYLATE AND SODIUM PARA-HYDROXYBENZOATE ON THE EXCRETION OF URINE CONSTITUENTS WITH TIME.</u>

<u>Sodium</u> - There was an increase in the rate of excretion of sodium after both treatments. The effect was much greater following the administration of para-hydroxybenzoate (but there was a trend to return to the pretreatment level by the fourth hour). On examination of the result following each treatment there was a significant decrease in the output of Na^+ during the second hour following the administration of salicylate ($P < .01$). The detailed results are given in Table (6).

<u>Potassium</u> - There was a slightly increased loss of potassium during the third hour following salicylate treatment ($P < .10$). Table (7) shows these results in detail.

<u>Inorganic Phosphorus</u> - There was a trend towards an increased loss of inorganic phosphorus over the initial level in the urine following either treatment. The effect was greater following the administration of para-hydroxybenzoate than salicylate. The difference found between treatments during the second hour following the administration of both drugs was found to be significant ($P < .01$). Table (8) shows these results in detail.

Magnesium - There was an increased loss of magnesium following the administration of para-hydroxybenzoate. This was a transient effect which had diminished by the fifth hour. There was a steady decline in the urinary excretion of magnesium throughout the whole period following the administration of salicylate. This effect had not diminished by the fifth hour. Comparison of the difference between the means following each treatment demonstrates that the decreased output seen to accompany the administration of salicylate reached statistical significance by the second hour ($P < .05$) and this was maintained until the end of the fourth hour. Table (9) shows these results in detail.

Total Nitrogen - Comparison between treatments showed only a transient fall in total nitrogen excretion following the administration of sodium salicylate when compared to the output following sodium para-hydroxybenzoate. This effect did not reach statistical significance.

Creatinine - No effect on creatinine excretion was observed.

A diagrammatic summary of the urine results is given in Figure II.

Discussion

The pattern of metabolic changes reported here following a single large oral dose of sodium salicylate to normal subjects is similar to that already described by Reid, Watson and Sproull (1950) in patients suffering from rheumatic fever and receiving salicylate therapy. It has not been previously reported following the

TABLE (1)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON OXYGEN CONSUMPTION IN MAN

[cc./min./sq.M.]

SUBJECT	SURFACE AREA	TREAT-MENT	TIME (minutes)		
			-30	+90	+210
A	1·93	S	119	178	145
		P	126	102	117
B	2·25	S	105	153	188
		P	116	135	103
C	1·77	S	121	179	134
		P	135	123	97
D	1·95	S	108	133	149
		P	111	127	115
E	1·93	S	104	125	131
		P	108	118	120
F	1·87	S	100	146	150
		P	124	122	114
Mean		S	110	152	150
		P	120	121	111

TABLE (2)

PLASMA SALICYLATE LEVELS IN MAN*

SUBJECT	†TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0	1	2	3	4
A	S	1·0	34·5	31·0	26·5	28·0
	P	-	1·0	1·3	2·3	1·8
B	S	1·5	37·0	34·5	50·0	38·0
	P	-	1·8	1·5	1·5	1·0
C	S	1·5	41·0	42·5	33·0	29·0
	P	-	2·5	2·3	1·8	1·8
D	S	1·5	24·0	-	33·0	32·5
	P	-	1·8	1·8	1·8	1·0
E	S	3·0	32·5	42·0	32·5	31·0
	P	1·0	1·1	1·2	1·1	-
F	S	2·3	30·5	38·0	37·5	33·0
	P	0·8	1·2	-	1·3	1·4
Mean	S	2·2	33·1	38·1	34·6	32·1

*Following the administration of a single oral dose of 5 gms. sodium salicylate or sodium para-hydroxy-benzoate to subjects A,C,D, E, and F. Subject B [weight 213 lb.] received 7 gms.

†S = sodium salicylate; P = sodium para-hydroxy-benzoate;
- not estimated.

FIGURE I

COMPARATIVE EFFECTS OF SODIUM SALICYLATE AND SODIUM PARA-HYDROXYBENZOATE ON OXYGEN CONSUMPTION IN MAN.

[Mean of six subjects]

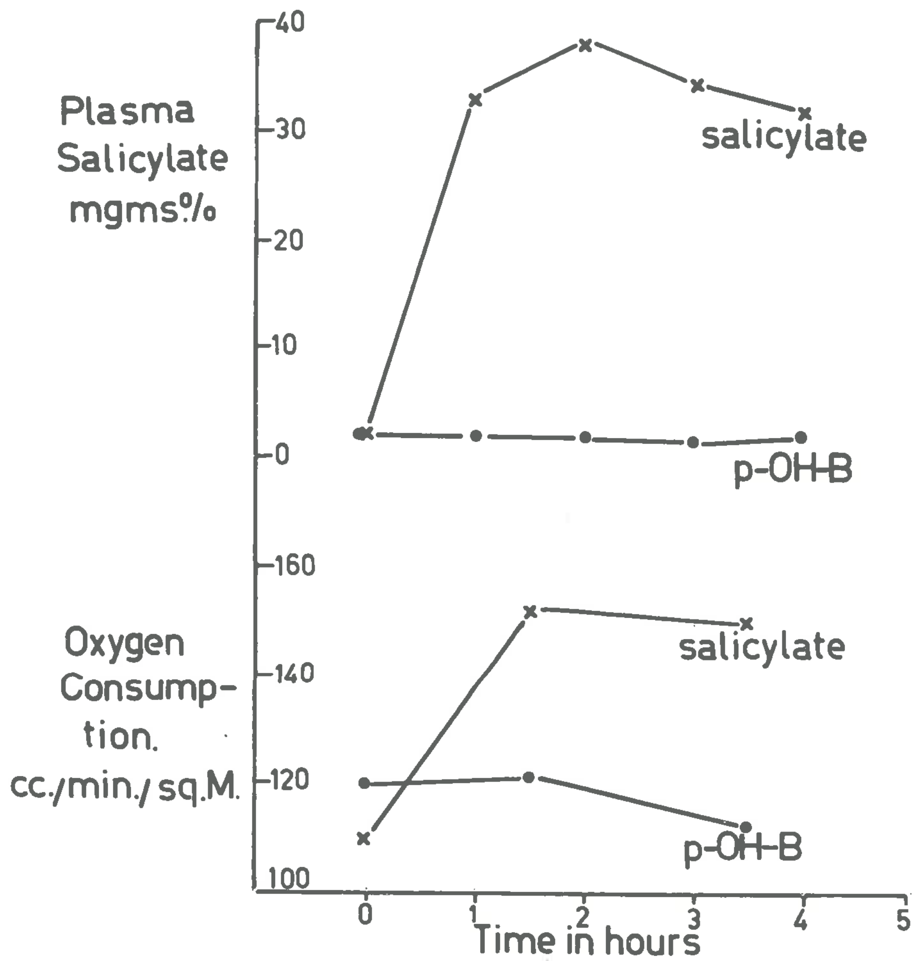


TABLE (3A)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON PLASMA CHOLESTEROL* IN MAN [mgms.%]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0	1	2	3	4
A	S	250	300	250	280	300
	P	320	280	260	260	270
B	S	260	260	230	250	320
	P	260	300	275	265	255
C	S	265	300	260	310	245
	P	290	275	310	270	310
D	S	290	270	260	260	260
	P	280	235	295	290	290
E	S	300	280	280	260	290
	P	340	305	310	300	220
F	S	275	260	250	250	230
	P	270	235	240	240	240
Mean	S	273	278	255	268	274
	P	293	272	280	270	264
Probability "P"		n.s.	n.s.	<·05	n.s.	n.s.

* Using the direct method of Zlatkis et al (1953)
"P" determined using "t" test
n.s.= not significant

TABLE (3B)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON PLASMA CHOLESTEROL * IN MAN [mgms.%]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS		
		0	2	4
A	S	211	185	218
	P	210	213	208
B	S	218	175	200
	P	218	213	210
C	S	275	218	298
	P	250	234	300
D	S	180	173	158
	P	180	168	180
E	S	250	225	188
	P	263	255	250
F	S	210	195	165
	P	185	200	175
Mean	S	224	195	205
	P	218	214	221
Probability "P"		n.s.	<·02	<·05

* Using the extraction method of Zak et al (1954)
"P" determined using "t" test
ns = not significant

TABLE (A)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON PLASMA MAGNESIUM IN MAN [mgms.%]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0	1	2	3	4
A	S	1·5	1·6	1·6	1·5	1·9
	P	1·2	2·0	1·3	1·6	1·4
B	S	1·6	1·4	1·5	1·7	2·1
	P	1·2	2·5	2·0	2·0	2·1
C	S	1·5	1·2	1·2	1·2	0·8
	P	1·7	1·3	1·5	1·1	1·1
D	S	1·5	1·6	2·1	1·3	1·4
	P	2·3	2·1	1·8	1·2	1·5
E	S	0·9	2·0	2·0	1·5	1·5
	P	1·7	1·7	1·7	1·2	0·9
F	S	1·7	1·7	2·0	1·5	1·0
	P	1·5	1·7	1·0	1·7	1·4
Mean	S	1·5	1·6	1·7	1·6	1·5
	P	1·6	1·9	1·6	1·6	1·6
Probability "P"		n.s.	n.s.	n.s.	n.s.	n.s.

"P" determined using "t" test. n.s. = not significant.

TABLE (5)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE ON PLASMA PROTEIN-BOUND IODINE IN MAN [µgms.%]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0	1	2	3	4
A	S	4·0	4·9	4·6	5·4	4·4
	P	4·6	4·6	4·5	4·1	4·5
B	S	5·1	4·3	3·2	4·0	6·6
	P	4·4	4·4	4·0	4·4	3·9
C	S	6·2	4·9	4·5	5·5	6·0
	P	6·3	5·7	5·7	5·0	6·1
D	S	4·4	4·2	3·5	3·0	3·5
	P	5·0	4·5	4·2	4·3	4·9
E	S	5·3	4·7	4·9	4·2	4·0
	P	6·5	5·7	5·4	5·7	5·9
F	S	5·4	4·2	4·7	5·2	4·7
	P	5·8	4·6	5·9	4·2	5·7
Mean	S	5·1	4·5	4·2	4·6	4·9
	P	5·4	4·9	5·0	4·6	5·2
Probability "P"		n.s.	n.s.	<·05	n.s.	n.s.

"P" determined using "t" test. n.s.= not significant.

TABLE (6)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON URINE SODIUM EXCRETION IN MAN [micro Equivs/min.]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0-1	1-2	2-3	3-4	4-5
A	S	43	44	42	23	33
	P	49	107	143	57	44
B	S	59	80	80	80	83
	P	62	185	354	327	246
C	S	83	353	346	420	362
	P	143	308	375	279	140
D	S	158	65	91	150	257
	P	142	218	330	292	254
E	S	59	470	130	510	127
	P	156	252	247	367	245
F	S	88	131	134	140	71
	P	102	765	362	274	294
Mean	S	82	190	137	221	156
	P	109	306	302	266	204
Probability "P"		ns	ns	$<\cdot 01$	ns	ns

"P" determined using "t" test. n.s.=not significant.

TABLE (7)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON URINE POTASSIUM EXCRETION IN MAN [micro Equivs/min]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0-1	1-2	2-3	3-4	4-5
A	S	39	49	61	45	40
	P	93	105	103	46	29
B	S	38	64	64	72	70
	P	28	73	85	63	44
C	S	10	56	61	62	54
	P	25	25	26	19	16
D	S	60	42	61	62	32
	P	54	56	42	36	37
E	S	13	127	38	164	52
	P	46	56	42	45	46
F	S	48	53	58	65	40
	P	21	78	35	37	43
Mean	S	35	65	53	78	48
	P	44	65	55	41	36
Probability "P"		n.s.	n.s.	n.s.	<·10	n.s.

"P" determined using "t" test. n.s. = not significant.

TABLE (8)

EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON URINE INORGANIC PHOSPHORUS IN MAN [mgms./min.]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0-1	1-2	2-3	3-4	4-5
A	S	·44	·39	·46	·48	·65
	P	·60	·55	·66	·36	·32
B	S	·48	·55	·55	·86	·99
	P	·29	·59	·79	·74	·58
C	S	·33	·39	·35	·32	·31
	P	·38	·48	·79	·64	·44
D	S	·52	·41	·36	·44	·46
	P	·43	·76	1·03	·99	·95
E	S	·33	·25	·28	·44	·54
	P	·14	·38	·84	·87	·79
F	S	·20	·18	·25	·26	·26
	P	·12	·11	·36	·46	·46
Mean	S	·38	·36	·38	·47	·53
	P	·33	·48	·74	·68	·59
Probability "P"		n.s.	n.s.	<·01	n.s.	n.s.

"P" determined using "t" test n.s.= not significant

TABLE (9)

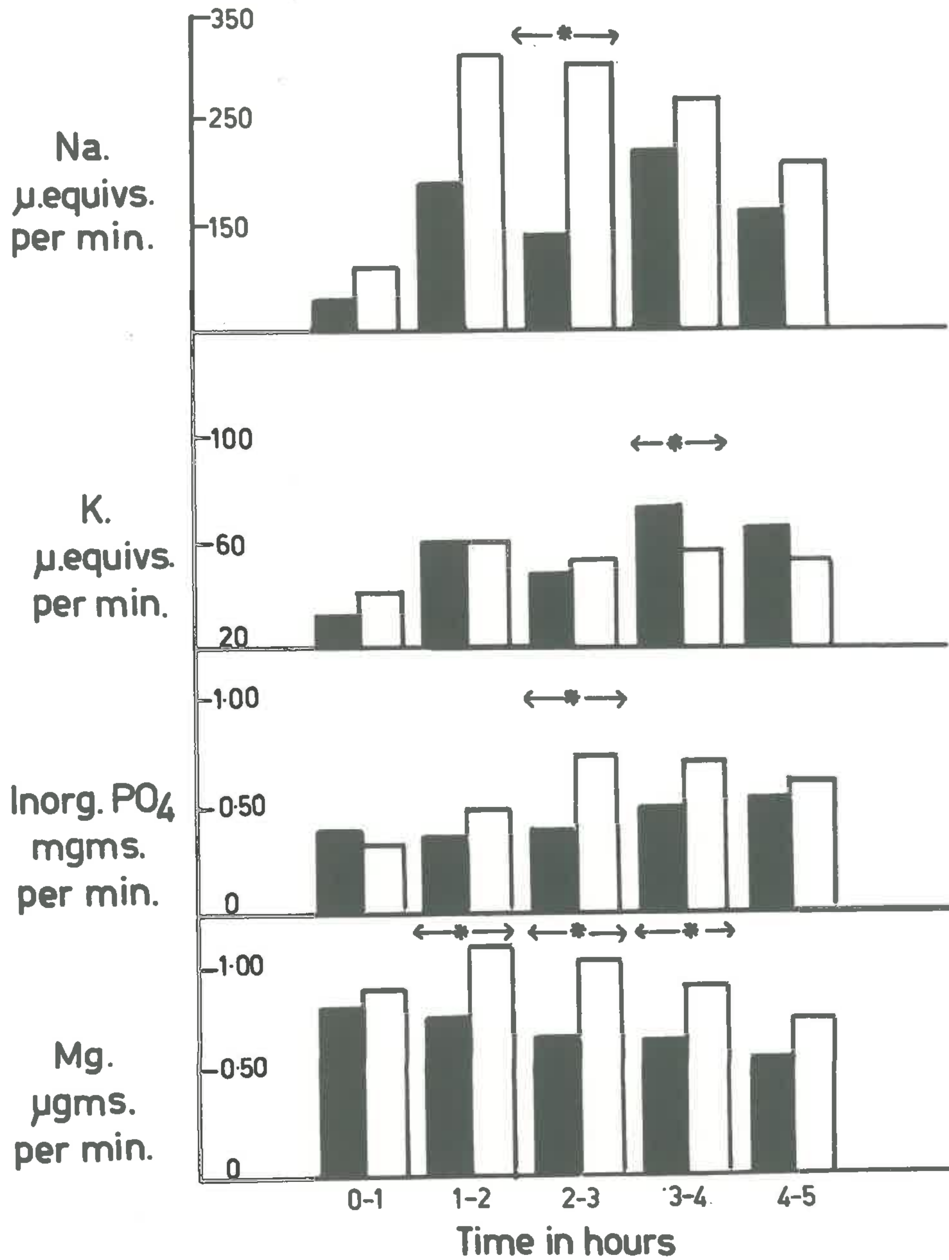
EFFECT OF SODIUM SALICYLATE (S) AND SODIUM PARA-HYDROXY-BENZOATE (P) ON URINE MAGNESIUM EXCRETION IN MAN [μgms/min.]

SUBJECT	TREATMENT	TIME AFTER ADMINISTRATION IN HOURS				
		0-1	1-2	2-3	3-4	4-5
A	S	64	64	61	54	60
	P	50	79	84	64	57
B	S	84	55	55	74	53
	P	101	144	127	134	116
C	S	42	59	51	51	50
	P	38	62	64	50	27
D	S	101	79	59	55	63
	P	89	137	134	118	119
E	S	104	101	83	75	53
	P	160	142	117	89	54
F	S	80	84	86	71	56
	P	98	119	101	94	58
Mean	S	79	74	66	63	56
	P	89	114	105	91	72
Probability "P"		n.s.	<·05	<·02	<·06	n.s.

"P" determined using "t" test. n.s.=not significant

FIGURE II

EXCRETION OF URINE CONSTITUENTS



salicylate para-hydroxybenzoate

Mean of at least five animals.

←✱→ = a significant difference between treatments.

administration of the drug to normal subjects. In addition to those changes reported here, Reid, Watson and Sproull also observed a pattern of increased nitrogen loss associated with the "retention" of sodium.

A rise in oxygen consumption was evident by ninety minutes and was still evident at two hundred and ten minutes after the ingestion of salicylate, but did not follow the ingestion of para-hydroxybenzoate. This manifestation of hypermetabolism was closely associated with the increased mean plasma salicylate level and this relationship is shown by Figure I.

There was a fall in plasma cholesterol under the influence of salicylate which was more pronounced at two hours than at four. This suggests not only a transient effect of salicylate upon plasma cholesterol, perhaps associated with maximal plasma concentration of the drug, but also the possibility of thyroid activation which is well known to decrease plasma cholesterol levels (Alexander and Johnson, 1956). The observations by Alexander and Johnson of an identical effect of salicylate on plasma cholesterol levels when given to patients with myxoedema makes stimulation of thyroid secretion by salicylate unlikely. When direct experimental evidence of thyroid stimulation was sought by an examination of the effect of salicylate on the circulating thyroid hormone of the plasma of normal subjects (by the estimation of protein-bound iodine), it was found that far from there being an increase in PBI, there was a significant fall within two hours in association with plasma salicylate levels in excess of 30 mgms.%. Since these observations were completed the report of Austen, Rubini, Meroney and Wolff (1958) has appeared, in which similar effects of salicylate on PBI were noted.

The decrease in PBI seen to follow the administration of sodium para-hydroxybenzoate may be related to the finding of Taurog, Chaikoff and Franklin (1943) who demonstrated an inhibitory action by this compound of some 50% on the conversion of inorganic iodide to either diiodotyrosine or thyroxine by thyroid tissue in vitro.

Work by myself and others in this laboratory has since extended this observation to other states of thyroid function. In addition possible mechanisms for this effect of salicylate are also under investigation (Hetzel, Good, Wellby and Charnock, 1960a, 1960b) (Good, Hetzel and Opit, 1960).

Preliminary studies of the rate of incorporation of I^{131} into thyroid tissue in vivo, by measurement of counts over the thyroid in normal and salicylate treated rats given five uc. of inorganic I^{131} indicate a marked reduction in the percentage of the dose taken up by those animals receiving salicylate (Charnock and Opit, 1959). This suggests that at least part of the inhibitory action of salicylate on thyroid function is due to a reduction in the rate of incorporation and possibly conversion of iodine by the thyroid to organic forms.

There were no significant changes in urine sodium, potassium, inorganic phosphorus or magnesium excretion during the first hour after administration of salicylate, and only magnesium was affected by the second hour. The effect of salicylate on oxygen consumption was maximal at this point. It was between the second and third hour that sodium and inorganic phosphorus excretion were affected. The effect of salicylate upon potassium was delayed until the third hour following ingestion. None of these effects persisted as there were no significant differences in the excretion of urine constituents between groups by the end of the fourth hour.

The effect of plasma cholesterol by this time was still significant but was declining, with a return of values towards the pretreatment level.

This can be taken as presumptive evidence that the action of salicylates upon these factors is essentially transient and presumably dependent upon relatively high plasma concentrations of the drug.

Although many of these electrolyte changes in urine closely resemble those shown to follow hydrocortisone administration in man (Hetzel, Williams and Lander, 1957) it is known that this agent does not increase metabolic rate (Hetzel, Charnock, Lawrence and Wellby, 1960).

The fall in plasma cholesterol therefore cannot be associated with an increased production of endogenous hydrocortisone compatible with the stimulation of the production of this agent from the adrenals.

As the possibility of thyroid activation has been found unlikely it would seem reasonable therefore to postulate that the decreased plasma cholesterol was a consequence of increased metabolism induced by a "primary" action of salicylate, although other explanations are possible (Hetzel, Charnock and Lander, 1959).

Throughout this study there has been a tacit assumption that the administration of sodium para-hydroxybenzoate is without inherant effect upon the parameters under observation. Certainly, this drug does not produce a rise in the oxygen consumption of the whole animal but it should be recalled that there was a significant decrease in the PBI within two hours of the ingestion of this compound.

Furthermore, examination of the urine data shown in Figure I suggests that this substance is not without effect on the excretion of magnesium and inorganic phosphorus. Perhaps these effects are due to the Na^+, but the administration of a sodium loading given as the salt of para-hydroxybenzoic acid results in a greater loss of Na^+ than that observed to follow its administration as the salt of salicylic acid. This latter finding may be related to a more rapid absorption of sodium para-hydroxybenzoate than salicylate.

Interpretation of the data obtained in this experiment with this in mind, does not seem to alter the conclusions reached in regard to an action of salicylate, as the pattern of sodium, potassium and phosphorus excretion described conforms to that reported in rheumatic fever patients repeatedly given large doses of salicylate.

Because of the nature of long term experiments, little evidence is available regarding the chronic effect of salicylate administration to man, and in particular to normal subjects. We have been able to perform limited observations on two hospitalised subjects, one a female diabetic aged 53 years, the other a young male (12 years) with rheumatic fever, both of whom received about 6.5 grams of salicylate as the calcium acetyl derivative per day. Findings on these subjects have been reported elsewhere and were in general agreement with the better controlled observations made over a shorter period. There was a consistent decrease in PBI and cholesterol levels during extended periods of salicylate administration (cf. Charnock, Good and Hetzel, 1959).

In the young male patient there was also found to be a decrease in the urine output of sodium and magnesium, associated with

an increased urine loss of potassium and inorganic phosphorus during treatment with salicylate.

The undesirable side-effects produced by this treatment - tinnitus, sweating and nausea, coupled with the difficulties of sample collection precluded the continuance of this type of study in man. Therefore, further observations were made in the rat following the prolonged administration of large doses of sodium salicylate. These experiments will be reported in the following Chapter (III) of this thesis.

CHAPTER II

Part II

THE EFFECTS OF SALICYLATE IN RATS

A. Electrolyte balance and body weight

Previous work had demonstrated an increase in oxygen consumption in man following administration of sodium salicylate. This effect was not reproduced by the administration of sodium para-hydroxybenzoate when given in equal dosage, and did not appear to be mediated through either thyroid or adrenal stimulation.

The experimental observations leading to these findings in man were made over a five-hour period. It was of interest to continue treatment with salicylate for longer periods, and to compare the possible effects of prolonged salicylate administration upon electrolyte balance with the acute response. Because of the difficulties of performing such observations in man, the rat was chosen for these studies.

Materials and Methods

In the first study, six adult male black and white rats were placed in pairs in metabolism cages so designed that the daily urine output could be collected free of faecal contamination. Initially, the animals were maintained for a period of thirteen days during which time the routine experimental conditions of daily weighing and injection of saline twice daily were instituted, so that a "base line" could be established.

Salicylate administration was commenced on the fourteenth day. This was given by intraperitoneal injection as the sodium salt adjusted to pH 7.4 in sterile water. The dosage was 30 mgms./100 gms. live body weight per day as two injections (10 mgms./100 gms. live body weight at 9.00 a.m. and 20 mgms./100 gms. live body weight at 6.00 p.m. each day). This dosage was selected because it was found to produce plasma levels of the drug comparable with therapeutic levels achieved in man (Smith and Talbot, 1950) (Seeburg, Hansen and Whitney, 1951). It is less than that employed by Smith (1959) in his investigation of the effects of salicylate on acetate metabolism. Although this dosage corresponds to about 20 gms. for a 70 kg. man, apparently the rat either excretes the drug more rapidly or does not absorb the same proportion of the dose as man. Rentsh, Bradley and Marsh (1959) do not consider that the concentration of salicylate in the plasma is "toxic" unless it exceeds 40 mgms.%.

One animal died during the third day of salicylate treatment without having previously demonstrated any clear symptoms of salicylate toxicity. Although the death of this animal may not have been related to the action of the drug, and the remaining five animals did not appear particularly distressed at this time, the dosage of salicylate was reduced to 10 mgms./100 gms. live body weight for the next twenty-four hours. After this time the full regime was recommenced and continued until the sixth day.

Throughout the whole nineteen days of observation and collection the temperature of the room in which the animals were housed was maintained between 70-80°F and the animals were supplied

with water and food (a casein bran mash) ad libitum exactly as were untreated colony rats.

The body weight of each animal, and the food and water intake of each pair of animals was recorded daily, as was the twenty-four hour urine output.

The urine samples were pooled and acidified prior to assay.

After six days of salicylate administration the animals were stunned and decapitated, some blood collected and pooled for assay. At this time a group of four untreated colony animals was also killed and their blood collected for assay.

The urine samples were assayed for sodium and potassium with the EEL flame photometer as before. Total nitrogen was estimated by Kjeldahl digestion followed by steam distillation in a modified Pregl apparatus and titration of the ammonia. The estimation of magnesium and inorganic phosphorus, whether in plasma or urine was by the methods previously employed in the observation of salicylate effects in man. Plasma salicylate was estimated by the method of Trinder (1954) whilst the plasma protein-bound iodine was again estimated by the method of Acland (1957) as a measure of the circulating thyroid hormone.

Where necessary the probability (P) was calculated by use of the "t" test.

Results

By the end of the sixth day of salicylate treatment, the five remaining animals exhibited an unusual degree of somnolence

and lassitude. Their fur was no longer sleek, their body temperature had apparently increased (they were hot to the touch) and there was a striking increase in their rate of respiration. There were no signs of tetany or convulsions which, in a series of preliminary experiments, had been shown to occur following the administration of lethal doses of salicylate.

The average body weight of each rat was 380 gms. ± 10 gms. when placed into the metabolism cages. This remained nearly constant during the initial "base line" period when the animals received twice daily injections of saline. There was a marked fall in body weight following the administration of sodium salicylate by intraperitoneal injection. After six days of treatment the mean decrease in body weight was 32 grams/rat. This fall in body weight was not initiated by a gross decrease in the total weight of food consumed per rat throughout this period. Although there was a tendency towards a decreased consumption soon after treatment with salicylate was commenced, the average food consumption per rat had returned to pretreatment levels by the fifth day. These results are shown diagrammatically in Figure III.

There was a wide fluctuation in the total daily water consumption of each pair of rats throughout the whole period of observation, with a small increase above the average immediately following the commencement of salicylate injection.

The mean daily urine volume per rat also varied considerably throughout the whole period of observation, but showed a trend towards increased output following salicylate administration. However this trend did not reach statistical significance ($P < 0.4$).

Examination of urine constituents

In contrast to the acute effect of salicylate seen in previous experiments in man, the urine sodium and potassium levels both appeared to fall slightly following salicylate treatment (e.g. the urine excretion of potassium ions was now 90% of the pretreatment level), but neither differed sufficiently from the average pretreatment levels to achieve statistical significance (Na^+, $P < .2$; K^+, $P < .4$).

The mean urine total nitrogen output did not differ significantly during the period of salicylate treatment but it should be noted that there was a marked increase above the average nitrogen output during the final day of the experiment.

The average daily output of urinary inorganic phosphorus fell to 83% of the average pretreatment level, but the difference in the means was again not significant ($P < .2$).

The most marked change was in urinary magnesium output. During the salicylate treatment the average daily output of this ion was only 47% of that of the pretreatment "control" period. Analysis of this data by difference of the means of the pretreatment and treated periods shows that the observed decrease in magnesium output which followed salicylate administration was highly significant at the 0.1% level ($P < .001$) (cf. Figure IV). These results are given in detail in Table (10).

Examination of plasma

Examination of plasma obtained from untreated rats taken at random from the colony, fed on essentially the same diet, and killed at the same time and in the same manner as the experimental salicylate

treated animals, gave a mean plasma salicylate level of less than 5 mgms.%. This value was attributed to a small degree of non-specificity in the method of assay. The mean plasma level of salicylate in the treated group was 28 mgms.% which must at least represent a concentration of 23 mgms.% salicylate radical in the plasma. This concentration is approximately 2 milli Molar. The blood samples were collected within four hours of the previous injection of salicylate on the last day of the experiment, and correspond only to a submaximal plasma concentration of the drug (Seeburg, Hansen and Whitney, 1951).

The protein-bound iodine (PBI) value of the pooled plasma of the salicylate treated group was only 1.7 ugms.% (five animals) compared to a value of 3.4 ugms.% which was determined for the untreated control group (four animals).

Plasma magnesium was found to be 2.1 mgms.% for the control animals and 2.9 mgms.% in the group treated with salicylate.

Discussion

In this experiment, salicylate again initiated a hypermetabolic response, shown by a weight loss, when given to the whole animal. The pattern of electrolyte changes produced here were not identical with those seen following the administration of a large single oral dose of sodium salicylate to man. There was no increase in sodium loss following salicylate administration in this instance, in fact there was a slight decrease in the rate of urine excretion. Inorganic phosphorus and potassium excretion were also slightly decreased.

FIGURE III

A METABOLIC RESPONSE TO SALICYLATE IN THE RAT

[Mean of five adult males]

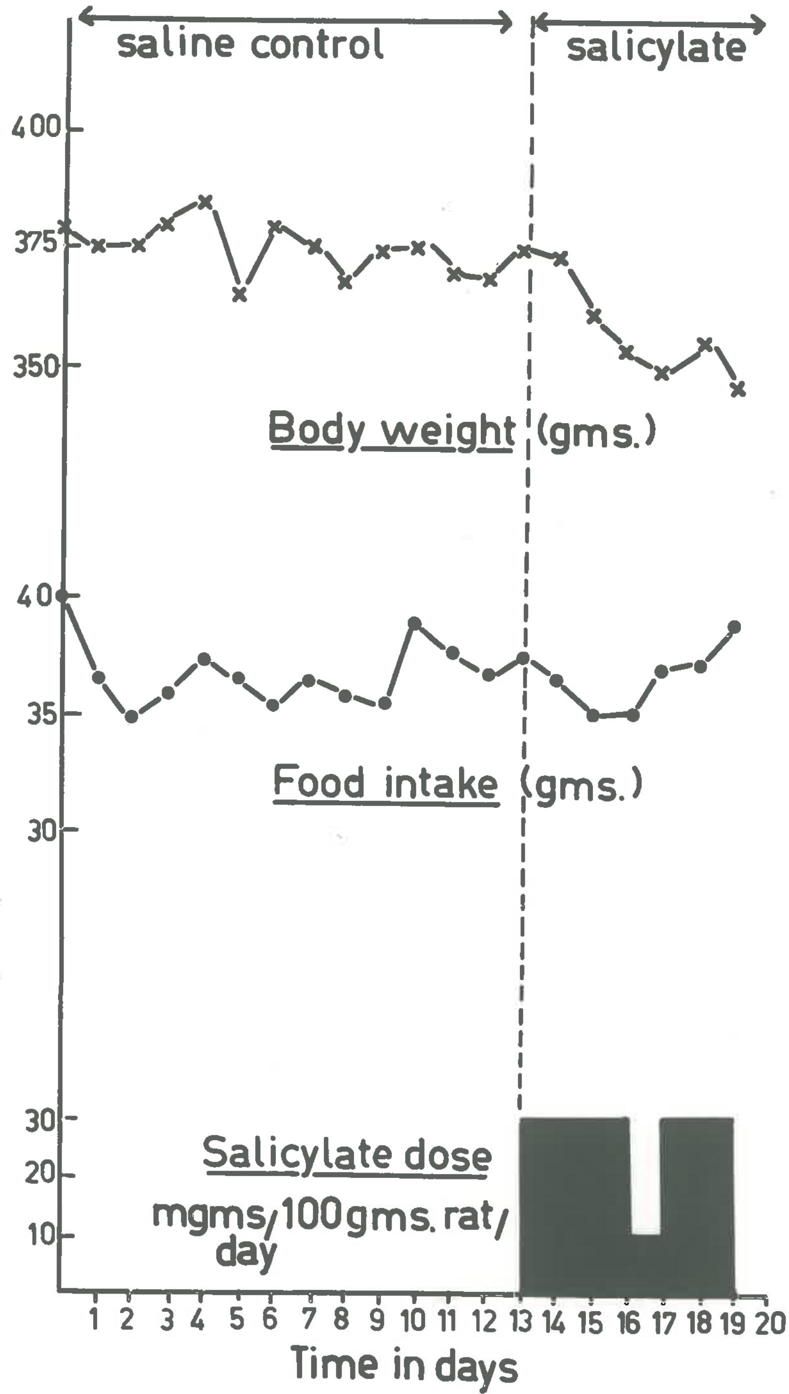


FIGURE IV

URINARY EXCRETION of MAGNESIUM in RATS given SODIUM SALICYLATE

(SIX ANIMALS)

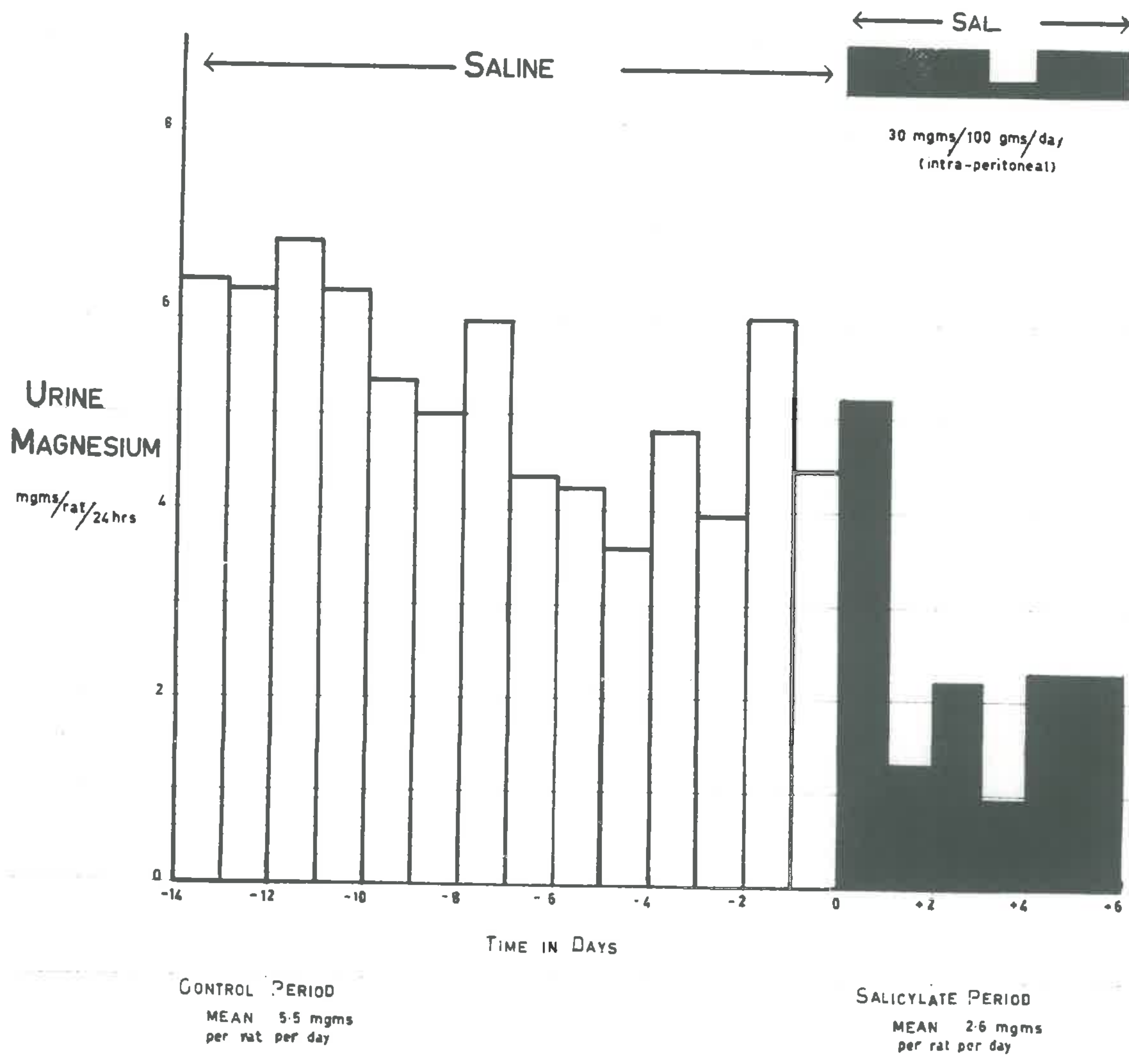


TABLE (10)

URINE EXCRETION OF SODIUM, POTASSIUM, MAGNESIUM, TOTAL NITROGEN AND INORGANIC PHOSPHORUS IN RATS BEFORE AND DURING THE INTRAPERITONEAL INJECTION OF 30 mgms. SODIUM SALICYLATE PER 100 gms BODY WEIGHT RAT PER DAY.

DAY	TREATMENT	NO. OF RATS	MEAN URINE VOLUME mls/rat/24 hrs.	Na.	K.	Mg.	Total N.	inorg. P.
				Concentration as mgms./rat/24 hours				
0	Sodium Chloride	6	13	41	58	6·8	312	42·9
1	"	"	15	41	61	6·6	267	37·5
2	"	"	12	33	52	7·7	264	36·0
3	"	"	16	54	59	6·6	276	33·6
4	"	"	11	51	46	5·7	239	30·8
5	"	"	10	36	40	5·0	239	27·0
6	"	"	13	39	51	6·0	218	27·2
7	"	"	13	33	44	4·7	202	24·0
8	"	"	11	32	45	4·5	220	27·6
9	"	"	9	31	39	3·2	180	19·8
10	"	"	13	44	52	5·7	260	28·6
11	"	"	10	34	39	3·9	206	25·0
12	"	"	16	41	53	6·1	237	30·2
13	"	"	13	34	46	4·9	224	27·4
	Mean		12·4	38·9	48·9	5·53	239	29·8
14	Sodium Salicylate	6	18	30	65	5·4	281	32·4
15	"	6	16	18	38	1·6	187	25·6
16	"	5	11	21	19	2·4	224	13·2
17	"	5	13	46	52	0·9	290	23·2
18	"	5	13	43	46	2·6	240	24·7
19	"	5	15	36	46	2·6	306	28·5
	Mean		14·3	32·3	44·3	2·58	255	24·6

Although there was a loss in body weight this was not reflected in a significant increase in daily urine volume or in increased total nitrogen content of that urine, although there seemed to be an upward trend in both these factors by the sixth day of treatment. The early loss of body mass may therefore be at the expense of non-protein material.

The only electrolyte disturbance to reach statistical significance was a striking renal retention of urine magnesium of more than 50% ($P < .001$). A similar finding was also noted in the short term experiments with man. As this change is not associated with a marked alteration in the daily food or water intake, it may therefore reflect a metabolic change initiated by salicylate. A rise in plasma magnesium was more pronounced in these experiments than in man, and suggests that this effect is a true "retention" of magnesium.

Although little is known of magnesium metabolism, Tapley (1956b) has reported an increase in Mg^{++} excretion following the administration of triiodothyronine and thyroxine to patients with myxoedema. Taken in conjunction with the decrease in plasma protein-bound iodine reported to follow salicylate administration both in this experiment and in previous studies in man, this constitutes further evidence for the improbability of salicylate acting via a stimulation of the thyroid gland.

A preliminary report by Alcock and MacIntyre (1960) suggests that the bivalent ions calcium and magnesium may be transported in the plasma by a common mechanism. Perhaps the alteration in plasma

magnesium levels which follow salicylate administration is associated with a disturbance in calcium balance.

The absence in this experiment of significant changes in the twenty-four hour urine pattern of excretion of sodium, potassium, nitrogen and inorganic phosphorus during six days of salicylate administration is not in accord with the view that salicylates act via a stimulation of the adrenal cortex (Hetzel and Hine, 1951). However, it is also at variance with the acute effects of salicylate administration to man which were reported earlier.

One possible explanation is that the hypermetabolic action of salicylate, and a possible associated disturbance of electrolyte balance, are both essentially transient effects dependent upon relatively high internal concentrations of the drug. Following the excretion or detoxication of this agent, compensation may occur via the homeostatic mechanisms. If this were so, then twenty-four hour urine samples may fail to reveal electrolyte changes, as it is probable they would be seen only over the period of action of the drug.

Clarification of the transient nature of the hypermetabolic response to salicylate was therefore sought in further experiments.

ADDENDUM

Experiments in man had suggested there was an increase in plasma magnesium levels following the administration of salicylate. Subsequent data obtained by studies with rats also suggested that this effect may be apparent. Therefore whenever possible in all future studies with rats plasma was obtained and assayed for this ion. Samples were collected over a period of about twelve months. Plasma was examined from twenty-three untreated rats and thirteen rats which had received salicylate. The mean plasma magnesium level of the untreated group was 2.9 mgms.% (range 2.1-3.3 mgms.%) whilst the mean plasma level of that group which had received salicylate was 3.7 mgms.% (range 2.9-4.6 mgms.%). These latter plasma magnesium levels were associated with plasma salicylate levels of between 23-82 mgms.% (mean 51 mgms.%). These results are given in detail below:-

Group	Plasma Constituent	Values	No.	Mean
Normal Rats	Mg^{++} mgms.%	2.1, 2.7, 2.3, 2.4, 3.0, 2.8, 3.0, 3.3, 3.0, 3.1, 2.6, 2.9, 3.0, 3.3, 3.2, 3.0, 3.1, 3.2, 3.3, 2.4, 2.9, 2.9, 2.8	23	2.9
Salicylate treated rats	Mg^{++} mgms.%	3.0, 3.8, 3.1, 3.8, 4.6, 4.2, 4.0, 4.5, 3.1, 3.2, 4.3, 3.9, 2.9	13	3.7
	Sal. mgms.%	39, 23, 52, 48, 82, 58, 65, 40, 33, 45, 56, 56, 75.	13	51

After calculating a common variance, comparison was made between the mean plasma Mg^{++} levels of these groups. There was a significant difference between the means of the treated and control groups ($P < .001$).

B. Body weight

The observation of increased rates of respiration, elevated body temperature and decreased body weight are usual manifestations of hypermetabolism. However, in the preceding experiment these effects were not accompanied by an increased loss of total nitrogen in the urine.

To check the loss of body weight following salicylate administration a simple experiment was performed utilizing three groups of four rats. One group received salicylate and was fed ad libitum (I). Another group received water as a "control" for the salicylate and was pair fed with that group which received salicylate (II). The remaining group (III) received no salicylate and was also fed ad libitum. The daily mean body weight of each group was determined and compared.

Materials and Methods

All animals were adult male black and white rats weighing over 340 grams. Salicylate was administered by gavage as the aqueous sodium salt. The dosage was equivalent to 30 mgms. salicylate radical per 100 gms. live body weight of rat/day.

The dosage was made up into small aqueous volume (2 mls.) without pH adjustment. It was given in two equal portions, morning and night, to rats in Group I. A weighed quantity of casein bran mash was supplied per day and the residue (if any) weighed the next morning. An amount of food equal to that consumed by the rats of Group I on the previous day, was then presented to the rats of Group II, who also received water by gavage in volumes equal to the dose of salicylate administered to animals in Group I. The animals

in Group III received no medication and were fed ad libitum.

All animals were marked for identification and weighed daily.

Salicylate administration was introduced on the morning of the fifteenth day and continued until after the morning dose on the twenty-fifth day. The observations were continued for another six days.

Results

The mean body weight of those animals receiving food ad libitum and no medication (Group III) remained virtually constant throughout the whole period of observation.

The mean body weight of those animals receiving salicylate (Group I) decreased by 50 gms./rat between the fifteenth and the twenty-fifth day during which time they received the drug. There was a rapid increase in the body weight of these animals after cessation of salicylate treatment, with a mean increase of 21 gms./rat on the following six days.

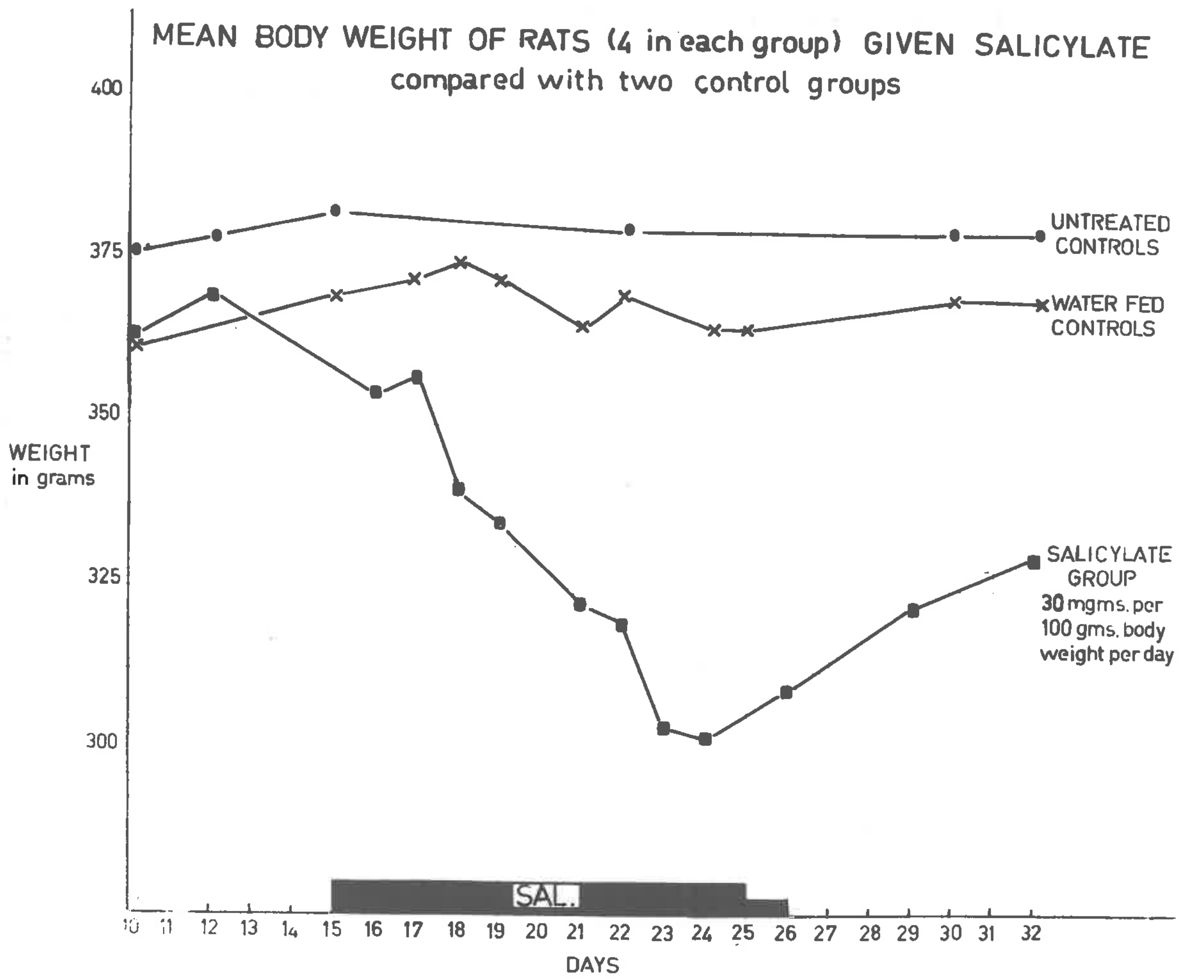
The animals of the remaining group which were pair fed with those receiving salicylate (Group II), fluctuated in mean body weight to some extent during the period when they were subjected to forced water feeding. However, there was no consistent change and only a mean decrease of a few grams was apparent after ten days of such treatment.

These results are shown diagrammatically in Figure V.

Discussion

The observations made here are regarded as good evidence of a hypermetabolic response to the chronic administration of sodium salicylate which is not associated with a decreased food intake. The finding described in the previous section of this thesis (subsection A)

FIGURE V



suggest that the intraperitoneal injection of sodium chloride does not produce a similar response. Thus the observed decrease in total body mass is probably a direct consequence of the administration of salicylate radical. More direct observations were now made of this hypermetabolic response.

C. Metabolic Rate

In view of the suggestion of the transient nature of the effect of salicylate upon electrolyte balance in rats, it was decided to examine the possibility of a similar effect of salicylate upon the oxygen consumption of rats. This was done by studying the time lag factor associated with the rise in oxygen consumption of rats receiving salicylate.

Materials and Methods

The measurement of oxygen consumption has long been associated with the measure of metabolic rate in both man and animals. There have been many different types of apparatus designed for the measurement of oxygen consumption in small laboratory animals such as the rat (Richards and Collison, 1928) (Gaddum, 1930) (Barbour and Trace, 1937) (Robbie and Leinfelder, 1945) (Maclagen and Sheahan, 1949).

Of these, the apparatus of Maclagen and Sheahan appeared the most suitable, but on examination it was found that calibration of the apparatus presented some difficulties. Furthermore, in a series of observations on twenty different rats before and one hour after the administration of salicylate, it was found that any differences which may have existed during these relatively short term observations were below the sensitivity of the manometer used to measure pressure changes.

This problem was overcome in an effective manner by replacing the usual manometer with a sensitive electronic recording device in the form of a multi-channel polygraph (Grass Instrument Co.) † This polygraph is equipped with attachments for the measurement of micro-pressure changes and when coupled to a vacuum desiccator containing a large mesh tray of soda-lime (Maclagan and Sheahan, 1949) provided an extremely sensitive apparatus for the measurement of oxygen consumption in the rat.

In fact the instrument had to be "damped" to the maximum extent, and the temperature of the desiccator chamber controlled to ± 0.1°C by immersion in a constant temperature bath (25°C), in order to remove variations produced by small changes in ambient temperature.

After the animal was placed in the desiccator, and the well-greased lid clamped into position, the whole chamber was immersed in the water bath to a level just below the lid.

The apparatus could then be flushed out and filled with oxygen enriched air by a system of taps and T-pieces. This procedure did not disturb the animal in any way. All animals including those heavily dosed with salicylate soon became accustomed to these surroundings and almost invariably became recumbent during the period of temperature equilibration (ten minutes) and remained thus throughout the period of observation, which was usually two or sometimes three consecutive five minute periods.

The apparatus could be exactly calibrated at any time after the animal was in position, by the careful withdrawal of a known

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† My thanks are due to Professor R. P. Jepson of the University Department of Surgery, Queen Elizabeth Hospital, Woodville, South Australia, for making the Grass polygraph available for these experiments.

volume of gas with a well-greased hypodermic syringe of good quality and calibration. The syringe was connected to the apparatus by fine pressure tubing supplied with the polygraph instrument. The sample of gas was then returned to the system.

With appropriate adjustment of the recording instrument the withdrawal of 10 cc. of gas from the system was made to equal about one fifth of the full-scale deflection of the apparatus.

When set into operation in conjunction with an accurate timing device, the whole apparatus would automatically measure and permanently record the rate of oxygen utilisation of the rat by means of a calibrated graph. At the end of a period of measurement, the recording apparatus could be reset and recalibrated, again without disturbing the animal in any way. The apparatus was exceptionally sensitive and the results obtained with any one animal were quite reproducible to within 0.1 cc/100 gm. rat/min.

The high cost of the recording apparatus precludes the suggestion that this method should be adopted generally.

As these observations were made over periods extending up to six days of salicylate administration, obviously the rats had to be fed throughout the experiment. However each animal was deprived of food for at least four hours prior to the measurement of its oxygen consumption. In most cases the animals had been placed in cages without food overnight and the measurements were made during the following morning.

Salicylate was again administered by gavage rather than injection and was given as an aqueous solution of the sodium salt (pH 8) in small volume. The dosage was the same as in the previous experiments.

Results

In the first of two experiments the effect of a large single dose of 30 mgms. of salicylate was observed for a period of six hours in a group of four young male rats weighing less than 200 grams. Each animal was examined on a different day. Observations of oxygen utilisation were made over consecutive periods of five minutes. Only when such consecutive measurements varied by more than 0.1 cc./100 gm. rat/min. was a third estimation made. In this case the average value of three estimations was then taken.

The mean oxygen consumption of these rats prior to treatment was 2.1 cc./100 gm. rat/min. This value had risen to a mean of 2.6 cc./100 gm. rat/min. within one hour of administration of a dose of salicylate. The oxygen consumption had declined from this maximum value to 2.4 cc./100 gm. rat/min. within two hours and had returned to the pretreatment level of 2.1 cc./100 gm. rat/min. within five hours.

These results are given in detail in Table (11).

In the second of these experiments sodium salicylate was administered (morning and night) to a group of four adult rats (weight about 300 grams) for a period of six days, in equally divided dosage at the rate of 30 mgms./100 gm. live body weight/day.

On the first day of the experiment the mean oxygen consumption of four rats prior to treatment was 1.4 cc./100 gm. rat/min. This value had increased to a mean of 1.9 cc./100 gm. rat/min. within one hour of the administration of salicylate. On the second and fourth days the measurements were taken four hours after the morning dose of salicylate, that is approximately mid-way between the daily doses of salicylate. On the second day the mean oxygen

TABLE (11)

EFFECT OF A SINGLE ORAL DOSE OF SODIUM SALICYLATE ON OXYGEN CONSUMPTION IN THE RAT

cc./100 gms. rat/minute

ANIMAL NO.	TIME AFTER TREATMENT IN HOURS			
	0	1	2	5
BB	2·1	2·6	2·4	1·8
RB	1·8	2·4	2·1	2·1
CF	2·5	3·0	2·6	2·4
TC	2·1	2·3	2·4	2·2
Mean	2·1	2·6	2·4	2·1

Each value represents the mean of at least two consecutive observations of 5 minutes each.

consumption was 1.7 cc./100 gm. rat/min. and on the fourth day it was 1.5 cc./100 gm. rat/min. These values are slightly higher than the pretreatment level on the first day of the experiment, but are not as high as that found within one hour of the initial dosage. No measurements were made on the third and fifth days but salicylate administration was continued until the morning of the sixth day.

One hour after the morning dose of salicylate on the sixth day the mean oxygen consumption was at the highest level found in this experiment - 2.3 cc./100 gm. rat/min. When these observations were repeated three hours later, and without further administration of salicylate, the mean oxygen consumption had decreased from this elevated level to a value of 1.7 cc./100 gm. rat/min. which was comparable with those levels found on the second and fourth days, also four hours after the administration of salicylate (see Table 12).

Discussion

The results obtained in the first experiment indicate that the hypermetabolic response found in the rat following a large single oral dose of salicylate is at its maximum about one hour after administration of the drug. This response had begun to wane by the second hour and was not apparent by the fifth hour.

When administration of the drug was continued at regular intervals for six days, the measurement of oxygen consumption did not reveal a cumulative effect on this manifestation of hypermetabolism, although the one hour response may have been heightened. Measurements on the second, fourth and sixth days of treatment consistently

TABLE (12)

EFFECT OF REPEATED ORAL ADMINISTRATION OF SODIUM SALICYLATE ON OXYGEN CONSUMPTION IN THE RAT.

cc./100 gms rat/minute

RAT NO.	DAY 1		DAY 2	DAY 4	DAY 6	
	no treatment.	+1 hr.	+4 hrs.	+4 hrs.	+1 hr.	+4 hrs.
L	1·7	2·2	1·7	1·8	2·4	1·8
M	1·3	2·0	1·7	1·3	2·5	1·6
N	1·3	1·8	1·9	1·3	2·1	1·7
O	1·5	1·7	1·5	1·6	2·1	1·9
Mean	1·4	1·9	1·7	1·5	2·3	1·7

Each value represents the mean of at least two consecutive observations of 5 minutes each.

demonstrated sub-maximal values when made four hours after the previous dose of salicylate.

This time sequence is suggestive evidence that an increase in oxygen consumption is only associated with high levels of salicylate (Hetzel, Charnock and Lander, 1959). Smith and Talbot (1950) have reported that the "cure of rheumatic fever" by salicylate depends not only on reaching a high plasma level of salicylate (40 mgms.%) but also on the maintenance of this level. A similar relationship between plasma salicylate and PBI levels has recently been shown by work from this laboratory (Hetzel, Good, Wellby and Charnock, 1960).

The difference in basal oxygen consumption between the two groups of rats studied is probably due to the difference in age and weight between the groups. The young animals used in the short term experiment (mean oxygen consumption before treatment 2.1 cc./100 gm. rat/min.) would possess a higher proportion of actively metabolising tissue in their lean body mass in comparison with the older, heavier animals used in the six day experiment. Undoubtedly a surface area measurement would have been more suitable than total body weight for a comparative study.

Nevertheless, these findings strongly suggest that the increase in oxygen consumption which has been shown to follow the administration of salicylate to man and animals, may be directly related to plasma levels of the drug, and is thus of a transient nature.

In these experiments an oral dose of 30 mgms. salicylate radical/100 grams live body weight of rat was found to exert an effect

on oxygen consumption in these animals for only a period of about four hours. This is quite consistent with the transient effect of salicylate which was observed on electrolyte balance. This latter effect was confined to an even shorter period and was only apparent during the second and third hours following salicylate administration.

CHAPTER II

Part III

CONCLUSIONS

The investigations of the metabolic response to salicylate in man and animals, which have been described in Parts I and II of this Chapter, indicate that an in vivo action of this drug is to promote a short term increase in the oxygen consumption of the whole animal. This is part of a hypermetabolic response which has associated with it an even more transient disturbance in electrolyte balance.

The whole effect appears to be geared to high plasma and presumably therefore high intracellular levels of the drug. The absence of a cumulative effect of salicylate on the oxygen consumption of the rat when the drug was given for six days, suggests that the mechanism of this reaction must be readily reversible.

Such findings, coupled with the known tolerance towards relatively large doses of this drug, suggest a biochemical mechanism of action which is either of a competitive or blocking nature, rather than of a specific chemical toxicity of this agent towards some particular metabolic step. This hypermetabolic reaction may be regarded as the whole animal response to an "uncoupling" action of salicylate on mitochondrial oxidative phosphorylation. Associated with this wasteful increase in metabolic rate was a disturbance in the electrolyte excretion pattern. This was characterised by a marked retention of magnesium ions and a slight transient effect on urine sodium concentration. A similar slight diuresis of K^+ and inorganic

phosphorus occurred as part of a rapid response, while there was some evidence that urine nitrogen was increased after some days of treatment. These changes in electrolyte excretion are probably significant and may be related either "post hoc" or "propter hoc" to an uncoupling action of salicylate.

Subsequent work to be presented in this thesis has been derived from a series of investigations both in vitro and in vivo which have been mainly directed towards clarification of possible biochemical mechanisms involved in this response. Particular attention has been paid to the effect of salicylate on the function of isolated rat liver mitochondria. These sub-cellular particles were examined because, as previously discussed in Chapter I of this thesis, there are reasons for believing that liver mitochondria may be a soft tissue site of this hypermetabolic response.

CHAPTER III

THE EFFECT OF SALICYLATE ON THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION PROCESSES IN ISOLATED RAT LIVER MITOCHONDRIA

Part I: A. Control experiments

B. The effect of salicylate addition to mitochondria *in vitro*

C. The properties of liver mitochondria isolated from rats treated with salicylate *in vivo*

Part II: Discussion, summary and conclusions. Four hypotheses proposed.

CHAPTER III

THE EFFECT OF SALICYLATE ON THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION PROCESSES IN ISOLATED RAT LIVER MITOCHONDRIA

INTRODUCTION

This section of the thesis is concerned with an examination of the uncoupling effect of salicylate on isolated rat liver mitochondria. To assess the physiological significance of salicylate induced "uncoupling", a study has been made of the properties of liver mitochondria isolated from rats treated with the drug *in vivo*. An examination of the effect of salicylate addition to mitochondria *in vitro* has also been conducted. An impairment of mitochondrial function detected by the former test can be attributed to an action of salicylate *in vivo*.

Similar comparative methods of examination have been employed by other workers to assess the physiological action of a variety of agents (Christie and Judah, 1954) (Aldridge and Cremer, 1955) (Vitale, Nakamura and Hegsted, 1957). Recently Riley and Deegan (1960) have also used this method of comparison to investigate disturbances associated with malarial infection.

Materials and Methods

Only young, actively growing, male, black and white rats weighing between 250 and 350 grams were used for this study. These animals were selected because Weinbach and Garbus (1956 and 1959) had demonstrated a decrease in the rates of phosphorylation of liver tissues of older rats. This decrease in phosphorylation was associated with a decreased tissue nucleotide content.

<u>Administration of salicylate to rats:</u>

Sodium salicylate was given by gavage to rats. The dosage was the same as that used in the previous study, that is 30 mgms. salicylate radical/100 gm. live body weight/day. The total dose was divided into two portions, one of 10 mgms. and the other 20 mgms./100 gm. body weight. The smaller dose was given at 9.00 a.m. and the larger dose at 6.00 p.m. each day. The animals were maintained on this regime until they exhibited signs of salicylate toxicity, for example gross body wastage and haemorrhage. These effects were usually apparent after one week of treatment.

The livers of these rats were utilised for the preparation of mitochondria from rats "<u>treated with salicylate in vivo</u>".

<u>Removal of rat livers:</u>

In early experiments the rats were killed by stunning and decapitation. Later, and only after comparison of the various test results, this procedure was replaced by the induction of light anaesthesia by an ether-air mixture. This was blown into a chamber under 5-10 lbs./sq. inch positive pressure. The rats were then placed on a portable table and held in position by limb clips. A gentle stream of ether-air was continuously passed around the animal's head. An abdominal incision was made, the viscera displaced and 5-8 mls. blood removed from the inferior vena cava into a heparinized syringe. The blood was transferred to a centrifuge tube and spun to separate the plasma, which was then used for various chemical assays. The liver was removed one lobe at a time, and the portions immediately immersed in ice cold 0.5 M sucrose contained in a tared vessel. The wet weight of the liver was obtained by difference.

Homogenising the liver:

The chilled liver was removed from the sucrose and cut into small pieces in the cold. The pieces were approximately 300 mgms. wet weight and were quickly replaced in ice-cold 0.5 M sucrose solution. The small pieces of liver were then separately homogenised. A Potter-Elvehjem homogeniser fitted with a Teflon plunger was used (clearance .002") under standard conditions of pestle speed and homogenisation time. Ten seconds homogenisation for each 300 mgms. portion was regularly employed. Although this procedure was conducted within a cold room regulated at 0-2°C, the apparatus was always surrounded by an ice-water cooling jacket. The final volume of the homogenate was such that 1 gm. of wet tissue was now dispersed through 5 mls. of fluid.

A non-ionic homogenising medium was used because it was desired to examine the effect of the later addition of ionic substances. After some preliminary experiments it was decided to employ a medium consisting of hypertonic sucrose (0.44 M) containing a complexing agent (0.001 M EDTA), adjusted to a pH of 6.8 by the addition of potassium hydroxide. Hypertonic sucrose and EDTA were employed because of the stabilising effect of these conditions on the mitochondrial membrane (cf. Chapter VIII, Part II). A pH of 6.8 was chosen in the belief that this value approximated more closely to that of the normal intracellular fluids, rather than pH 7.4 which is that of the plasma (Aldridge, 1958).

Differential centrifugation of the homogenate:

The principle employed was that described by Hogeboom, Schneider and Pallade (1948), but some variations in centrifugation

force were used. This was done because many subsequent workers with this preparative method have suggested modifications designed to obtain a better separation of the mitochondria from other liver fractions, in preference to a high yield of mitochondria.

To remove cellular debris and red cells, the tissue homogenate was centrifuged in the cold at 800 x g for twenty minutes. The supernatant was decanted and re-centrifuged in a high speed refrigerated centrifuge at 5,000 x g for twenty minutes at 0°C to separate the mitochondrial pellet. Supernatant fluid was then removed without disturbing the pellet, which was subsequently re-suspended in a "washing medium" of ice-cold 0.44 M sucrose.

This suspension was then centrifuged again at 0°C for ten minutes at 10,000 x g. The supernatant fluid was removed, again without disturbing the mitochondrial pellet, which was finally re-suspended in a volume of a "suspending medium" (0.25 M sucrose) numerically equal in mls. to the original weight of wet liver in gms. The mitochondria derived from 1 gm. wet weight of liver tissue were now suspended in a volume of 1 ml. The entire preparation was conducted with as little delay as possible between each step, and routinely occupied some ninety to one hundred and twenty minutes. The final suspension was maintained at 0°C in an ice bath, and was always utilized within a few minutes of preparation.

<u>Measurement of oxidative phosphorylation:</u>

The test vessels contained 1 ml. of a "basic" medium which consisted of 50 u.mols. inorganic phosphorus as phosphate buffer at pH 6.8; 24 u.mols. potassium chloride; 2.5 u.mols. sodium adenosine

triphosphate (ATP); 30 u.mols. glucose and 30 u.mols. of potassium fluoride. To this mixture was added 25 u.mols. of substrate, which was either the potassium salts of α -keto-glutaric, β -hydroxybutaric or succinic acids. The pH of all these salts was adjusted to between 6.5 and 7.0 with potassium hydroxide. When potassium α-keto-glutarate was the substrate, 25 u.mols. of the potassium salt of malonic acid (pH 6.8) was also added to the mixture in order to inhibit succinic dehydrogenase activity and ensure the "one step" nature of the reaction. Varying amounts of magnesium ion, as $MgSo_4$, and in some experiments sodium salicylate were also added to the test medium contained in a Warburg cup (the actual concentrations employed are shown with the individual experimental results). 0.5 ml. portions of the ice-cold suspension of mitochondria were added last to the outer compartment of the vessels.

The phosphate acceptor system was either 0.2 ml. hexokinase in 1% glucose solution (25-30 units of "Sigma" Grade III) or 15-35 u.mols. aqueous adenosine diphosphate (ADP) and was placed in the side arm of each vessel, except in those experiments where no "phosphate trap" was supplied in order to examine the effects of salicylate in an "acceptor-deficient" system. The final volume of the reaction mixture was adjusted with electrolyte free water to not more than 3 mls. The lip of the centre well of the vessels had been greased, and the well itself contained a strip of fluted filter paper which protruded above the edge of the well. To this compartment was added 0.3 ml. of 5 M potassium hydroxide solution which was thus spread over a large surface area and served to trap any carbon dioxide evolved during the reaction.

The flasks were then incubated with shaking at 30°C, the gas phase being air. After a five minute equilibration period the vessels were quickly removed from the bath and the contents of the side-arm then tipped into the main compartment of the vessel and swirled to mix thoroughly with the other constituents. All vessels were then returned to the bath and re-equilibrated for three minutes during which time the stoppers and joints were "ground in".

Zero-time reaction flasks were then removed from the bath and the reaction "stopped" by the addition of 0.2 ml. of 10 N H_2SO_4 which was added via the side-arm. These flasks provided the measure of the "zero-time" inorganic phosphorus content of the reactions.

The manometer taps on the remaining flasks were closed at the same time as the zero-time flasks were removed from the bath, and the oxygen uptake in the "reaction-time" vessels was then measured for a known time. Readings were taken at regular intervals and these provided data for rate studies. After about thirty minutes the vessels were removed from the bath and the reaction rapidly stopped, again by the addition of 0.2 ml. 10 N H_2SO_4.

Inorganic phosphorus was assayed in the Warburg cup contents by the method of Taussky and Shorr (1953). Dilution was necessary to bring an aliquot into the range of the method. Comparison of the values between zero and reaction time vessels gave a measure of the esterification of inorganic phosphorus which had occurred during the reaction. This is regarded as a measure of "high energy" phosphate bond formation in this system.

The result is expressed as a phosphorus to oxygen ratio (P/O); the units are ug.atoms. Comparison of experimental results to theoretical values for each substrate measures the efficiency of phosphorylation.

CHAPTER III

Part I

A. CONTROL EXPERIMENTS

These experiments were performed on mitochondria isolated from the livers of untreated rats. Salicylate was not added *in vitro*. This type of "control" was used with every batch of mitochondria prepared. It enabled a check to be made of the efficiency of oxidative phosphorylation of each batch of mitochondria, and also supplied "base-line" data for comparison with that obtained in other series.

Results

The mean P/O ratio obtained in thirty experiments with α-keto-glutarate as substrate was 2.6. In sixteen experiments with β-hydroxybutyrate as substrate the mean was 1.6 and in thirteen experiments with succinic acid as substrate the mean was 1.4. These results are in agreement with those of other workers (Penniall, Kalnitsky and Routh, 1956) (Jeffrey and Smith, 1959) and approach the maximum theoretical values of 4, 3 and 2 for each of these substrates respectively (Hunter, 1951)(Copenhaver and Lardy, 1951).

These P/O ratios and the range of phosphorus and oxygen values obtained are shown in detail in Table (13).

The relatively wide range of values obtained with all three substrates is mainly attributed to variations in the weight of mitochondria used per vessel. This varied from batch to batch of mitochondria. The efficiency of oxidative phosphorylation of individual preparations also varied. No explanation is offered for this phenomenon. However all preparations were "coupled".

TABLE (13)

NORMAL RATS

The P/O ratios of normal rat liver mitochondria utilising three substrates

SUBSTRATE	NUMBER OF EXPERIMENTS	P UPTAKE		O UTILISATION		P/O RATIOS	
		µgm. atoms	range	µgm. atoms	range	P/O	range
α-Keto-Glutarate	30	12·6	2·7–29·0	4·9	1·2–10·2	2·6	1·6–3·2
β-Hydroxy-Butyrate	16	4·4	0·9–8·1	2·7	1·3–3·7	1·6	0·6–2·8
Succinate	13	7·8	1·8–18·5	5·4	2·2–9·9	1·4	0·7–1·9

The reaction mixture was as described in the text [p.76] with the addition of mM Mg^{++} as magnesium sulphate.

B. THE EFFECT OF SALICYLATE ADDITION IN VITRO TO LIVER MITOCHONDRIA ISOLATED FROM UNTREATED RATS

The usual concentration of sodium salicylate employed in these reactions was 5 mM. This concentration was known to be one commonly achieved in the plasma of man, and had been shown in earlier experiments to be associated with a hypermetabolic response in the whole animal. There is no evidence to suggest that salicylates are actively concentrated in any specific mammalian tissue (Smith, 1949). Therefore it was considered unreasonable to examine higher concentrations of the drug although maximal effects were sought.

Results

The effect of the *in vitro* addition of 5 mM sodium salicylate on the P/O ratio of untreated rat liver mitochondria, suggest that when succinate or β-hydroxybutyrate are the substrates, the phosphorylations associated with the oxidations of these acids were completely inhibited. That is, these systems were completely uncoupled although oxygen utilisation still occurred. These results are shown in detail in Table (14).

When α-keto-glutarate was the substrate in these systems, the phosphate uptakes were markedly reduced, but not completely inhibited as with succinate and β-hydroxybutyrate. The resultant P/O ratios were less than unity in all but one experiment.

In a smaller series of experiments using α-keto-glutarate as the substrate, the effect of a lower concentration of salicylate (5×10^{-4}M) was examined. Here the average phosphate uptake was increased from a value of 3.4 ug.atoms, which had been observed when

TABLE (14)

EFFECT OF SALICYLATE *in vitro*

The effect of the *in vitro* addition of sodium salicylate on the efficiency of oxidative phosphorylation of normal rat liver mitochondria utilising three substrates

SUBSTRATE	CONCENTRATION OF SALICYLATE ADDED	NUMBER OF EXPERIMENTS	P UPTAKE		O UTILISATION		P/O RATIOS	
			µgm.atoms	range	µgm.atoms	range	P/O	range
α-Keto-Glutarate	5×10^{-3}M	10	3·4	1·6 - 5·7	4·0	1·9 - 5·7	0·8	0·5 - 1·2
"	5×10^{-4}M	4	5·3	4·9 - 5·6	3·3	2·9 - 3·7	1·6	1·5 - 1·7
β-Hydroxy-Butyrate	5×10^{-3}M	10	Nil		3·5	2·9 - 5·0	-	
Succinate	5×10^{-3}M	8	Nil		2·9	2·5 - 3·7	-	

The reaction mixture was as described in the text [p.76] with the addition of mM Mg^{++} as magnesium sulphate. Salicylate was added as the sodium salt.

5 mM sodium salicylate was added to the system to a value of 5.3 ug. atoms. The P/O ratio whilst now greater than unity was still reduced below that of the control experiments (P/O found with 5×10^{-4} M salicylate = 1.6 and P/O without salicylate = 2.5).

C. THE PROPERTIES OF LIVER MITOCHONDRIA ISOLATED FROM RATS TREATED WITH SALICYLATE IN VIVO

An examination was made of the oxidative phosphorylation ability of liver mitochondria isolated from rats treated with sodium salicylate *in vivo* for periods of up to eight days. The P/O ratios obtained in this series were then compared with those obtained in subsections A and B of this investigation.

The same conditions of isolation, incubation and the same substrates were used in all three series.

Results

Comparison of the P/O ratios revealed that mitochondria which had been isolated from the livers of rats treated with sodium salicylate *in vivo* had completely undiminished powers of oxidative phosphorylation.

The mean plasma salicylate level of treated rats was 50 mgms.% which is equal to 3.6 mM. The range of plasma salicylate values was from 20-80 mgms.%. The results obtained in this series are shown in Table (15); comparison between the series is shown in Table (16).

TABLE (15)

RATS FED SALICYLATE

The P/O ratios of liver mitochondria from rats fed salicylate

SUBSTRATE	NUMBER OF EXPERIMENTS	P UPTAKE		O UTILISATION		P/O RATIOS	
		μgm.atoms	range	μgm.atoms	range	P/O	range
α-Keto-Glutarate	18	16·5	8·4-25·3	5·3	2·9-10·1	3·0	2·3-3·8
β-Hydroxy-Butyrate	10	7·6	3·3-13·2	4·2	2·7-6·0	1·7	1·2-2·3
Succinate	12	5·1	2·0-10·3	7·5	3·2-12·8	1·6	1·2-2·2

The reaction mixture was as described in the text [p 76] with the addition of mM Mg^{++} as magnesium sulphate.

TABLE (16)

NORMAL AND SALICYLATE FED RATS

Comparison of P/O ratios of liver mitochondria from normal and salicylate treated rats. The average values for both phosphate uptake and oxygen utilisation are given in addition to the P/O ratios obtained with three substrates

SUBSTRATE	NORMAL			SALICYLATE FED		
	P	O	P/O	P	O	P/O
α-Keto-Glutarate	12·8	4·8	2·6	16·5	5·6	3·0
β-Hydroxy-Butyrate	4·4	2·7	1·6	7·6	4·2	1·7
Succinate	7·8	8·4	1·4	7·5	5·1	1·6

Animals fed salicylate by gavage (30 mgms./100 gms. live body weight/day) for from three to eight days. All animals exhibited clinical signs of salicylate toxicity. The reaction mixture is as described in Table [Normal Rats] and Table [Rats fed Salicylate]

CHAPTER III

Part II

DISCUSSION

The results of the control experiments (Subsection A) are in agreement with those reported in the literature and are in reasonable accordance with the concept that the one step aerobic oxidations of α-keto-glutarate, β-hydroxybutyrate and succinate are associated with maximal P/O ratios of 4, 3 and 2 respectively.

The average P/O value obtained for each of the substrates examined here was: α-keto-glutarate, 2.6; β-hydroxybutyrate, 1.6; succinate, 1.4. These values are considered to be reasonable experimental results and indicate that the mitochondrial preparations examined here functioned with an acceptable degree of efficiency.

The observations of Brody (1956) and Penniall (1958), and the more recent report of Jeffrey and Smith (1959), indicate that the *in vitro* addition of salicylate to mitochondrial preparations similar to those used in this investigation, results in the complete uncoupling of the phosphorylations associated with the oxidation of either β-hydroxybutyrate or succinate. This has been attributed by the latter authors to a total inhibition of all the phosphorylations of the respiratory chain.

In addition it was also suggested by Jeffrey and Smith (1959) that the remaining salicylate insensitive phosphorylation associated with the oxidation of α-keto-glutarate ($P/O > 0 < 1$) was that at the substrate level, (Hunter, 1951) rather than those of the respiratory

chain which were again inhibited, for example in the case of β-hydroxybutyrate and succinate. These findings have been confirmed here (Subsection B) and there is no evidence to doubt the conclusions of these workers.

The principal criteria in assessing the possible physiological significance of such _in vitro_ results should rest where possible in a demonstration of _in vivo_ "uncoupled" mitochondria derived from salicylate treated animals. This was attempted in the experiments reported in Subsection C.

It was found that mitochondria prepared in the usual way from rats heavily dosed with salicylate for periods of up to eight days, still carried out oxidative phosphorylation with undiminished efficiency when examined under identical test conditions to those of the control experiments. In fact the average P/O ratios determined with all three substrates employed were consistently, if only slightly, increased over the control levels (see Table 16). This was not due to a simple increase in phosphorylation. When either α-keto-glutarate or β-hydroxybutyrate were the substrates the degree of phosphorylation was increased over control levels. Although the associated oxygen uptake figures were also increased, the P/O ratios were slightly higher than the controls. When succinate was the substrate the degree of phosphorylation observed was virtually the same in either group. However, mitochondria isolated from rats treated with salicylate exhibited a decreased oxygen consumption in this instance which again resulted in an increased P/O ratio.

To examine this observation in greater detail it was realised that consideration must be given to the possibility of batch to

batch variation in the yields of mitochondria used in these experiments. This variation was chiefly governed by two factors:- the wet weight of liver used to produce the initial homogenate, and the volume of that homogenate. Also the time of each run varied slightly (25-35 minutes) as in practice all Warburg vessels could not be removed from the bath and stopped at precisely the same instant in any one run. The oxygen uptake data from these experiments was therefore recalculated to allow for these variables. Although the actual mitochondrial yields were not known it was possible to calculate the weight (in grams) of wet liver per ml. of the original homogenate from which each batch of mitochondria was derived. As the appropriate dilutions for each step of each preparation were also known, an estimate of the original weight of liver contributing mitochondria to each test vessel could be obtained.

It was known that the actual yield of mitochondria per gram wet liver was reasonably constant (even when normal and salicylate fed rats were compare) for any given method of preparation (cf. Tables 42 and 44). Hence it was considered that this derived value would be a reasonable correction to apply in these calculations. The results, expressed as the average ug.atoms oxygen/gm. wet weight liver/min. are shown in Table (17). No consistent overall trend is apparent, apart from a uniform decrease in the rates of oxygen uptake of mitochondria to which salicylate was added _in vitro_, when compared to the control level. The administration of salicylate to rats _in vivo_ also produced a decrease in the rates of respiration found with two substrates (α-keto-glutarate and succinate) while a small increase was observed with β-hydroxybutyrate.

TABLE (17)

RATES OF OXYGEN UTILISATION

The average rates of mitochondrial oxygen utilisation with three substrates. Results expressed as μgm.atoms oxygen per the mitochondria derived from 1 gm. wet weight of liver per minute

SUBSTRATE	NORMAL RATS	SALICYLATE TREATED		
		IN VITRO	IN VIVO	IN PREPARATION AT 0°C
α-Keto-Glutarate	0·56	0·30	0·43	0·35
β-Hydroxy-Butyrate	0·25	0·20	0·34	0·30
Succinate	0·53	0·18	0·38	0·28

Comparison is made between normal and various salicylate treated groups. The reaction mixture was as described previously.

This finding was surprising in view of the effects of salicylate on the respiration rate of the whole animal and the claims of other workers of the stimulatory effect of added salicylate on the oxygen consumption of isolated tissues, such as brain slices. Another detailed study of this phenomenon was that of Sproull (1957) whose work with mouse brain slices also showed that the effect of salicylate on oxygen consumption varied with the substrate utilised. A stimulation of oxygen consumption in these preparations could only be observed when either glucose or pyruvate, and not when α-keto-glutarate or succinate, were the substrates. Indeed with these latter Kreb's cycle intermediates as substrates, Sproull found that there was a slight decrease in the rates of oxygen consumption in the presence of salicylate, a result in agreement with the data presented in Table (17).

Clearly, any uncoupling reaction which does not result in the increased respiration of isolated mitochondria, but lowers the efficiency of phosphorylation, would necessitate an increased respiration by the whole cell in order to provide the cell with the same amount of energy for its various activities. This may be the explanation of the stimulatory action of salicylate on the respiration of the whole animal, which was not apparent upon detailed examination of isolated mitochondria.

SUMMARY AND CONCLUSIONS

It has been shown by the results presented in this section of the thesis that "control"preparations of isolated rat liver mitochondria metabolised a variety of substrates with a degree of

efficiency (P/O) which reasonably approached theoretical values. The *in vitro* addition of salicylate at a concentration of 5×10^{-3} M to these preparations completely inhibited the phosphorylations associated with the oxidation of both β-hydroxybutyrate and succinate and appreciably reduced those associated with the oxidation of α-keto-glutarate.

When an effort was made to put these latter observations on a comparative basis, the reduction in P/O ratio found in these circumstances could not be attributed to a simple increase in the oxygen uptake of these preparations. The enzymes of respiration were therefore not stimulated by the *in vitro* addition of salicylate at this concentration.

With the three substrates examined it was found that in conjunction with the decrease in phosphorylation there was a concomitant decrease in oxygen uptake. The actual P/O ratios were much reduced.

To examine the possible physiological significance of the uncoupling effect of the *in vitro* addition of sodium salicylate, rat liver mitochondria were isolated from rats heavily dosed with salicylate. It was demonstrated that under the test conditions employed, there was no diminution in the mitochondria's ability to efficiently oxidise all substrates presented, nor was there any consistent variation found between the respiration rate (oxygen uptake rates) of these preparations and the untreated controls. An obvious interpretation of this finding is that the *in vitro* addition of salicylate to normal mitochondria results in an artifactual uncoupling response which has no physiological significance.

In view of the effects which follow the administration of salicylate to the whole animal, described by myself and others, it was felt imperative to consider a number of alternative hypotheses which could be offered to explain the apparent discrepancy between the "in vitro" and "in vivo" results presented here.

Amongst these alternatives the following four hypotheses appeared to be of sufficient merit to warrant further investigation. These are as follows:-

1. That the in vitro effect of salicylate on mitochondrial oxidative phosphorylation is caused by an inhibitory effect of salicylate on the phosphate acceptor system employed in these experiments.

2. That mitochondria isolated from the liver of rats treated with salicylate were lacking in or deprived of an essential co-factor for oxidative phosphorylation in vivo, which was made available again in the in vitro test system.

3. That salicylate produces a direct but readily reversible effect on the mitochondrial enzymes associated with the functions of oxidative phosphorylation, which may be spontaneously overcome if the drug were leached out or washed off mitochondria during their isolation from the livers of rats given salicylate in vivo.

4. That salicylate may affect the permeability of the mitochondrial membrane to normal intracellular substrates, ions, or co-factors required for oxidative phosphorylation. Again this may be a reversible effect which could be overcome by either the mitochondrial membrane or its environment being washed free of salicylate during the preparative stages.

The results of an examination of these four hypotheses will be presented and discussed in subsequent chapters of this thesis.

CHAPTER IV

AN EXAMINATION OF THE FIRST HYPOTHESIS

"That the *in vitro* effect of salicylate on mitochondrial oxidative phosphorylation is caused by an inhibitory effect of salicylate on the phosphate acceptor system employed in these experiments."

CHAPTER IV

AN EXAMINATION OF THE FIRST HYPOTHESIS

The hypothesis:

"That the *in vitro* effect of salicylate on mitochondrial oxidative phosphorylation is caused by an inhibitory effect of salicylate on the phosphate acceptor system employed in these experiments."

Introduction

An examination of this hypothesis, which is the first of four which have been proposed to explain the apparent discrepancy between the *in vitro* and *in vivo* effects of salicylate on oxidative phosphorylation, has been made by a study of systems deficient in phosphate acceptor, and in others in which ADP was utilised as the phosphate acceptor instead of the usual hexokinase-glucose reaction.

Within the actively metabolising cell, it is probable that the rate of ATP production is geared to the requirements of the cell, so that a build-up of ATP does not occur under normal conditions, in accordance with a dynamic concept of cellular function and the control of metabolic processes as described by Krebs (1957). This is not the situation in a "static" test system such as that employed in the measurement of mitochondrial oxidative phosphorylation. It has been shown that a high concentration of ATP is of itself inhibitory to this process *in vitro*, and it may well be that the ATP:ADP ratio within the living mitochondrion is one of the natural controlling mechanisms of cellular energy production.

It was first demonstrated by Lardy and Wellman (1952) that

to attain maximal experimental rates of respiration with mitochondrial preparations *in vitro*, it was necessary to remove or "trap" the ATP formed in this reaction.

These workers introduced the use of the "hexokinase-glucose trap". This system transfers the terminal phosphate of adenosine triphosphate (ATP) which is produced by oxidative phosphorylation, to glucose. This latter compound now becomes available as the initial phosphate acceptor substance and the reaction proceeds at maximum rates *in vitro*. In one sense this "trap" drives the reaction. Hexokinase is not naturally located within mitochondria, but is a "cytoplasmic" constituent of the cell (Lardy, 1951). *In situ*, adenosine triphosphate must be utilised within, or diffused out of mitochondria for reaction with other intracellular systems. For both these reasons the hexokinase-glucose system may not be "physiological".

If salicylate were exerting its *in vitro* effect solely on the hexokinase-glucose reaction it may account for the variation observed between the *in vitro* uncoupling action of salicylate when added to oxidative phosphorylation test systems, and the apparent lack of effect of salicylate on mitochondria prepared from rats heavily dosed with this drug *in vivo*.

This hypothesis has also been considered by Brody (1956) who, in an examination of this concept, made use of the so-called "acceptor-deficient" system, i.e. a system without the *in vitro* addition of any phosphate trapping mechanism. The rationale for this approach is considered below.

As the oxidations and phosphorylations of this reaction are "coupled" then the rate of oxidation must be influenced by the rate of

removal of end-product, that is ATP; which in turn must be influenced by the presence or absence of either an acceptor or transferring mechanism.

Hence, when no other factors are operating the rate of oxygen uptake in an acceptor deficient system should be less than in an acceptor saturated system. Furthermore, the rate in an acceptor deficient system plus an uncoupling agent (for example, salicylate) should exceed that of the acceptor deficient system alone, and may approximate the rate of an acceptor saturated system without uncoupler, because the uncoupling agent removes the requirement for an acceptor.

Brody found that the rate of oxygen uptake by mitochondria in the presence of 4×10^{-4} M sodium salicylate, but without phosphate acceptor system, was greater than in a system without salicylate but with hexokinase and glucose. The oxygen uptake in both these systems was greater than in a "control" without salicylate and phosphate acceptor system.

Although Brody did not actually say so, one infers from his presentation of the results that an increase in the rate of oxygen uptake in the presence of salicylate and without acceptor substance, to a rate equal to that of the preparation with acceptor and without salicylate, was presumptive evidence of uncoupled mitochondria without interference with the trapping system per se.

This concept is further supported by the recent finding of Jeffrey and Smith (1959) that salicylate (5 mM) stimulates the rate of oxygen consumption of rat liver (in the presence of α-keto-glutarate) in an ATP deficient system to near the level found with a similar system having optimum amounts of ATP, although like Brody, these authors also make no comment in this regard.

This whole concept presumably relies on a low level of mitochondrial ATPase activity so that when no acceptor substance is present the rate of hydrolysis of ATP to ADP is limiting to the uptake of oxygen, since it has been shown that ADP is an important rate limiting substance of the Krebs cycle as it operates in mammalian mitochondria (cf. Lardy and Wellman, 1952).

Materials and Methods

Mitochondria were prepared from untreated rats exactly as described for previous investigations of oxidative phosphorylation. The basic test system was also identical to that used previously, and the substrate was α-keto-glutarate. This substrate was selected for its high number of maximum phosphorylations.

The systems differed only in the "phosphate acceptor" mechanism supplied. In some cases hexokinase-glucose was added as a control, in other systems no "trapping" mechanism was provided. In further experiments ADP was added at either of two levels as a phosphate accepting compound. These different systems will be indicated with the appropriate experimental results.

The physical conditions under which measurements were made were identical with those previously described (Chapter III).

The rates of oxygen uptake were determined by taking manometer readings at regular intervals. Inorganic phosphorus measurements were made on the Warburg cup contents exactly as described previously and the P/O ratios were calculated where possible.

Results and Discussion

In the first of these experiments comparison was made between the rates of oxygen uptake observed in control systems containing hexokinase and glucose, and other systems in which no phosphate acceptor mechanism was provided.

In these experiments there was no real difference between the rates of oxygen uptake by either of these systems. This similarity was apparent during thirty minutes incubation. These results are shown in Figure VI. Where the P/O ratio could be measured, that is in those systems containing hexokinase and glucose, this ratio attained reasonable experimental values (2.7; 3.1 and 2.9). Therefore the mitochondria were "coupled" although there was negligible phosphate uptake in the absence of an effective phosphate "trap".

This finding can be correlated with the observations appearing later in this thesis, of the levels of mitochondrial ATPase activity which were found in normal mitochondrial preparations concomitantly shown to be "coupled". Although the "basic" or inherant levels of mitochondrial ATPase activity are low, it is also known that the level of ATPase activity of fresh mitochondria, is markedly affected by changes in temperature (Myers and Slater, 1957a) and the osmolarity of the medium in which the estimations are made (Potter and Recknagel, 1951) as well as the ionic composition and pH of that medium (Swanson, 1956).

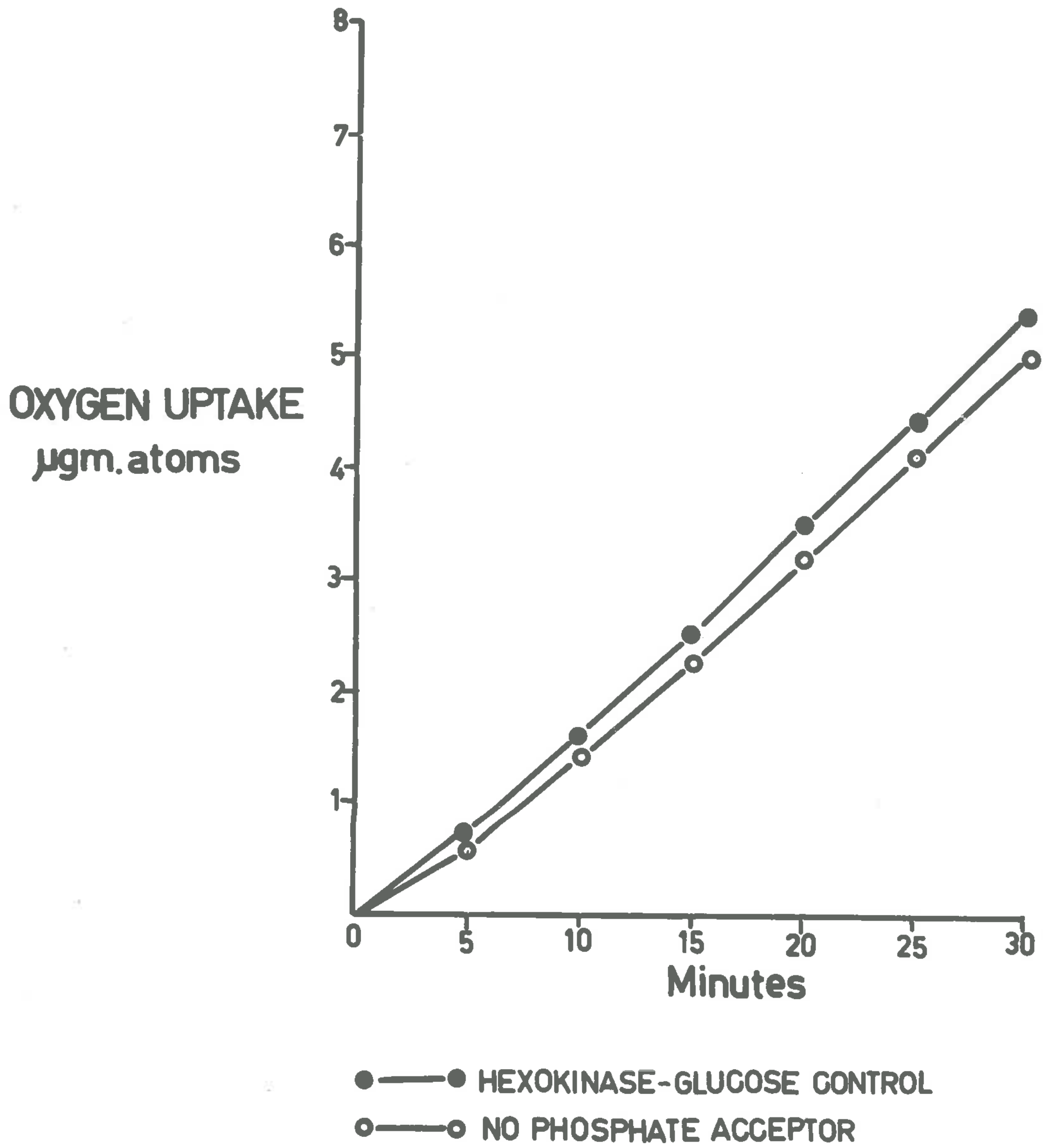
It seems reasonable therefore to expect a "stimulated level" of ATPase activity by the mitochondria in test systems lacking in effective phosphate acceptor mechanisms, rather than the much lower "basic" levels of activity of which they may be capable. It is equally reasonable to expect that sufficient phosphate acceptor could

FIGURE VI

RATES OF OXYGEN UPTAKE

(Mean of 3 experiments)

(Substrate-α-keto-glutarate)



be produced via ATPase activity to permit a significant rate of respiration under these conditions.

The results obtained in these particular experiments agree with Slater's conception of the significance of mitochondrial ATPase activity in oxidative phosphorylation (Hulsman and Slater, 1957). Furthermore, this data indicated that information relating to the effect of salicylate addition to these systems would not be forthcoming from this type of experiment. Here the rate limiting requirements of ADP were obviously being met by "fresh" mitochondrial ATPase activity without the need for fortification by the action of salicylate.

Therefore, rather than pursue this line of approach further, it was decided to attempt to replace the "hexokinase-glucose" trap with another acceptor system - preferably one which more closely resembles the situation _in vivo_. To this end, adenosine diphosphate (ADP) was employed in a second series of experiments, although it was appreciated that adenylate kinase activity, taken in conjunction with mitochondrial ATPase activity would render the measurement of appreciable P/O ratios unlikely (see Lardy and Wellman, 1952).

In this small series of experiments in which α-keto-glutarate was used as a substrate, acceptable P/O ratios were again obtained when "hexokinase-glucose" was added as the "trap". When this "trap" was replaced by ADP (6 mM), the rate of oxygen uptake declined sharply and the P/O ratios determined were very low ($\simeq$ 0.6). When 5 mM salicylate was added to the system utilising ADP as the "trap", phosphorylation was completely inhibited, and the rate of oxygen uptake was slightly reduced from that found with ADP alone. Later, in an attempt to exclude the possibility of an inadequate concentration gradient of ADP, the quantity of ADP present was increased from 6 mM to 14 mM/test vessel.

The rates of oxygen uptake determined with and without salicylate in the system, again indicate the absence of an enhancing effect by this agent on oxygen uptake rates. Once more phosphorylation was inhibited by 5 mM salicylate.

The phosphate utilization found in the experiments which utilized ADP as the phosphate acceptor substance were too low for comparison with "control" experiments after salicylate addition *in vitro*.

These results, which are shown in Figure VII, suggest that salicylate can inhibit phosphorylation in a test system independent of the hexokinase-glucose reaction.

The recent evidence of Bellamy and Bartley (1960) relating to the relative impermeability of mitochondria to ADP, and the need for the exceedingly high concentration of this substance in their investigation (some three times the highest concentration employed here) may explain the lack of phosphate "trapping" effect in those experiments where ADP was used to accept phosphate.

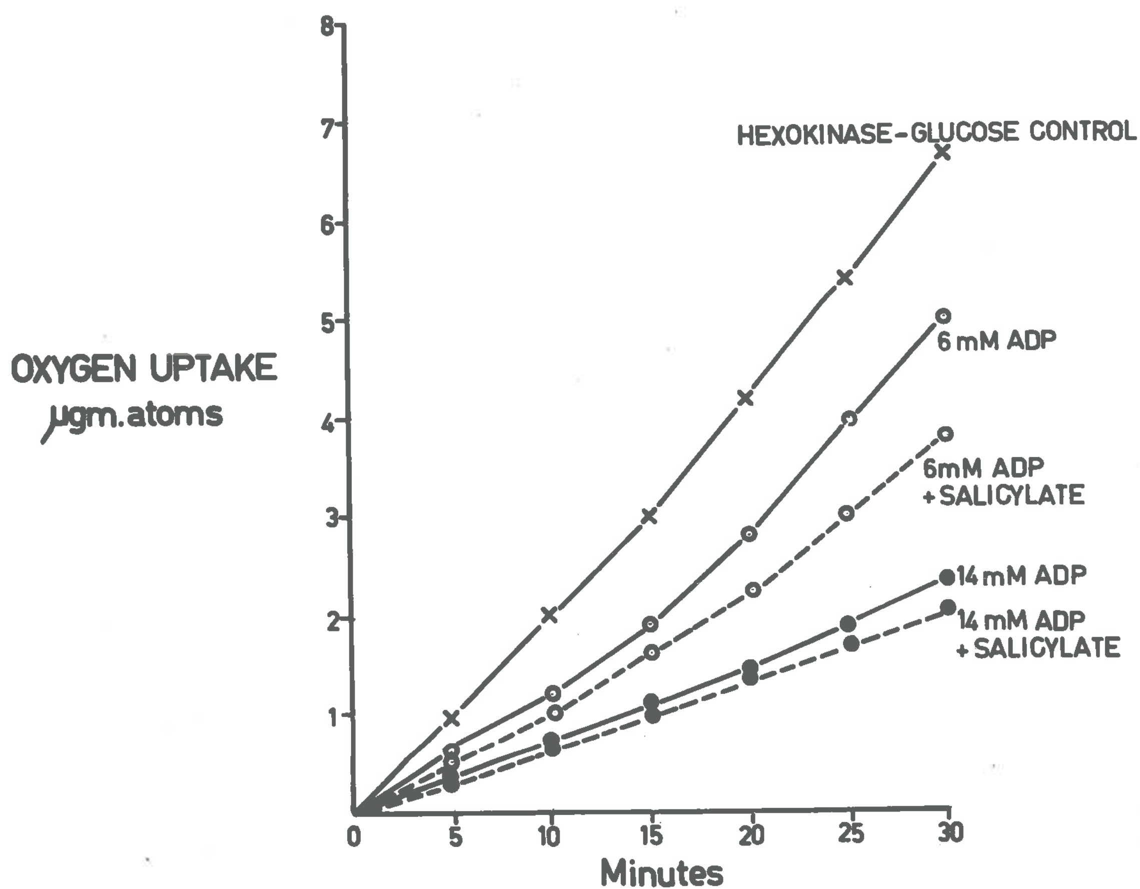
Another possible mode of action of salicylate on the hexokinase-glucose system is suggested from the whole animal experiments, where it was shown that there were marked changes in Mg^{++} balance. These changes may have been the result of a "binding" of magnesium following the administration of large doses of salicylate. A simple mole to mole chelation is quite probable at physiological pH values, and salicylate may "bind" Mg^{++} *in vitro* as well as in the whole animal.

Such an interaction may produce an effect on the hexokinase-glucose reaction in vitro as Mg^{++} is an essential co-factor. This suggestion is examined more appropriately in the following Chapter.

FIGURE VII

RATES OF OXYGEN UPTAKE

(Mean of 3 experiments)
(Substrate-α-keto-glutarate)



The evidence presented in this Chapter suggests that the inhibitory effect of salicylate on mitochondrial oxidative phosphorylation *in vitro* is not due to a direct action of salicylate on the hexokinase-glucose reaction. As the evidence is not unequivocal, such an action cannot be completely excluded although it was thought to be improbable. Therefore, other explanations were examined in an effort to explain the apparent discrepancy between the *in vitro* and *in vivo* effects of salicylate on mitochondrial oxidative phosphorylation. One of these hypotheses is examined in the following Chapter.

CHAPTER V

AN EXAMINATION OF THE SECOND HYPOTHESIS

"That mitochondria isolated from the liver of rats treated with salicylate were lacking in or deprived of an essential co-factor for oxidative phosphorylation *in vivo*, which was made available again in the *in vitro* test system."

Part I: The role of Mg^{++}

Part II: The role of K^{+}

Part III: General conclusions

CHAPTER V

AN EXAMINATION OF THE SECOND HYPOTHESIS

INTRODUCTION

The work reported in this Chapter has been concerned with an examination of the second of the four hypotheses which have been proposed to explain the apparent discrepancy between the *in vitro* and *in vivo* effects of salicylate on oxidative phosphorylation.

The hypothesis:

"That mitochondria isolated from the liver of rats treated with salicylate were lacking in or deprived of an essential co-factor for oxidative phosphorylation *in vivo*, which was made available again in the *in vitro* test system."

By definition, this hypothesis must exclude such intra-mitochondrial co-enzymes as cytochrome C, the pyridine nucleotides, or any other substance not present in the *in vitro* test system, although both these former substances have been shown to "leak" from isolated mitochondria (Lehninger, 1951) (Birt and Bartley, 1960). From the nature of the components of the "test system" it is apparent that the proposed substance (or substances) must either be ionic, or present in very low concentration as a contaminant of the reagents involved. The two principal ionic components of this test system are potassium and magnesium, which of course are the two principal intracellular electrolytes of normal mammalian tissue (Hastings, 1940).

The possible involvement of either of these ions in relation to this hypothesis will be discussed separately. The effect of calcium ions was not examined as these ions are not a component of the *in vitro* test

system. Evidence will also be presented later which suggests that this bivalent cation does not normally occur in the intracellular fluid of tissue cells *in vivo*.

CHAPTER V

Part I

THE ROLE OF Mg^{++} IN RELATION TO THE SECOND HYPOTHESIS

Introduction

In studies with the whole animal it was shown that there were changes in Mg^{++} balance following the administration of large doses of sodium salicylate. These changes were increased plasma levels in conjunction with decreased urinary excretion of this ion. Both these effects could be ascribed to an interference in the normal transport mechanism of the bivalent cation, possibly by a "binding" of these ions to the salicylate radical. Such a reaction is predictable on physico-chemical evidence, as carboxyphenols are known to be suitable for chelation reactions with a number of bivalent metal ions including Mg^{++}.

Therefore, experiments were performed to ascertain whether the inhibitory effect of salicylate on mitochondrial oxidative phosphorylation could be overcome by the presence of "excess" Mg^{++}. In an effort to maintain some correlation with physiological conditions, sodium salicylate was used at a concentration of 5 mM. This corresponds to the highest plasma levels of the drug likely to be encountered in man (about 70 mgms.%). This level is toxic in man.

A normal intracellular concentration of magnesium ions has been reported to be about 5 mM (Watchorn and McCance, 1937) (Hastings, 1940). If salicylate and magnesium interact chemically to form a mole to mole chelate complex (which is likely from physico-chemical considerations) then the provision of Mg^{++} at a concentration of 10^{-2} M should enable sufficient "free" Mg^{++} to be available for mitochondrial phosphorylation to take place.

Materials and Methods

Mitochondria were isolated from untreated rats by the procedure described on page 75. The test system for this reaction and the conditions of measurement were also the same as those described previously except that in some vessels Mg^{++} were added to give a final concentration of 1×10^{-2} M. This is twice the usual concentration (5 mM) and approaches the limit of solubility of this ion in the systems employed here. Sodium salicylate (5 mM) was also added to some of the vessels. The oxygen uptake and the inorganic phosphorus utilisation were determined exactly as before. The substrates presented to the mitochondria were α-keto-glutarate, β-hydroxybutyrate and succinate.

Results

When 5 mM sodium salicylate was added to systems containing 5 mM Mg^{++} and either β-hydroxybutyrate or succinate then, as was described previously, phosphorylation was completely abolished. If the substrate was α-keto-glutarate however, then the phosphorylations were not completely abolished but were uniformly reduced to a level interpreted as "substrate level phosphorylation" only. In those systems without salicylate addition the P/O ratios achieved indicated that the mitochondria were "coupled". All these results are in agreement with those reported earlier.

When both salicylate (5 mM) and "excess" Mg^{++} (1×10^{-2} M) were added together, this higher concentration of Mg^{++} failed in every instance to overcome the inhibitory effect of salicylate on mitochondrial phosphorylation reactions. The Mg^{++} was added to the reaction vessels prior to the addition of salicylate. These latter results are shown in Table (18).

TABLE (18)

SALICYLATE WITH EXCESS MAGNESIUM *in vitro*

The effect of excess magnesium and the *in vitro* addition of salicylate on the P/O ratios of normal rat liver mitochondria utilising three substrates

SUBSTRATE	NUMBER OF EXPERIMENTS	P UPTAKE µgm. atoms	O UTILISATION µgm. atoms	P/O RATIOS
α-Keto-Glutarate	5	2·5	3·9	0·7
β-Hydroxy-Butyrate	6	* -ve	2·9	-
Succinate	4	* -ve	3·2	-

* -ve indicates small phosphate release.

Reaction mixture as described previously with the addition of 1×10^{-2} molar magnesium (as magnesium sulphate) and 5×10^{-3} molar salicylate (as sodium salicylate)

In a small series of experiments, the results of which are not given in Table (18), the final molar concentration of Mg^{++} was raised to the absolute limit of its solubility in these systems. This upper limit was a final concentration of 2×10^{-2} M Mg^{++}, added as magnesium sulphate. In two experiments in which α-keto-glutarate was the substrate, and two experiments in which β-hydroxybutyrate was the substrate, this elevated level of Mg^{++} again failed to overcome the inhibitory effect of 5 mM sodium salicylate on mitochondrial phosphorylation reactions.

Discussion

Magnesium ions have been shown to be an essential co-factor in ATP catalysed reactions generally (Lardy, 1951) (Green and Ingraham, 1958) (Lowenstein, 1958). When Mg^{++} are added to mitochondrial suspensions they facilitate the "coupling" of oxidation and phosphorylation (Chance and Williams, 1956) and their omission may result in a rapid increase in the rate of oxygen uptake after a few minutes incubation (Baltscheffsky, 1957). Vitale, Nakamura and Hegsted, (1957) have shown that Mg^{++} is necessary for maximal oxidative phosphorylation in intact mitochondria and Wadkins and Lehninger (1958) found that a requirement for Mg^{++} was still present in digitonin subfragment preparations of mitochondria.

These latter effects may be a demonstration of the apparent need of Mg^{++} for the oxidation of reduced diphosphopyridinenucleotide ($DPNH^{+}$) by the mitochondrial respiratory chain, a reaction essential for the oxidative phosphorylation of many substrates. The demonstration by Green (1959) that Mg^{++} are required for the being of DPN^{+} by

mitochondria, may explain this concept, which is also in accordance with the view that magnesium ions are required for the binding of some mitochondrial component essential for oxidative phosphorylation (Kielley and Bronk, 1957) (Lennane and Ziegler, 1958).

If an interaction of salicylate and magnesium occurred then indirect effects may also arise with the pyruvic decarboxylase reaction (Green, 1952) _in vivo_, or with the hexokinase-glucose reaction (Dickens, 1951) _in vitro_, as a requirement for magnesium ions has been demonstrated for both these systems. A defect in the pyruvic decarboxylase reaction may alter the rate of supply of substrates for the operation of the Krebs cycle _in vivo_, whilst an effect on the hexokinase-glucose reaction would clearly change the rate of phosphorylation _in vitro_.

The work of Vitale, Nakamura and Hegsted (1957) strongly suggests that there is a requirement for Mg^{++} for the efficient function of oxidative phosphorylation processes _in vivo_. These workers demonstrated that there was an _in vivo_ "uncoupling" of rat heart mitochondria consequent upon an induced dietary deficiency of magnesium. However, it appears from this work that neither liver nor kidney mitochondria are affected to the same extent as heart mitochondria.

Thus there is ample evidence to suggest that a competitive utilisation or binding of magnesium ions by salicylate could lead to marked effects on mitochondrial oxidative phosphorylation both _in vivo_ and _in vitro_.

The possibility of reversing the "uncoupling" effect of salicylate by provision of an "excess" of magnesium ions _in vitro_ had been investigated previously by two groups of workers. The results of these studies are conflicting.

In 1954 Kaplan, Kennedy and Davis demonstrated the inhibition of Krebs cycle oxidations in kidney and liver homogenates in the presence of 6.7×10^{-3} M sodium salicylate. When the concentration of Mg^{++} was increased to a concentration of 1.9×10^{-2} M in the reaction mixture, the inhibition of α-keto-glutarate oxidation by salicylate was reversed, whereas the oxidation of succinate appeared to be independent of Mg^{++} as the salicylate induced inhibition was not affected.

Another group of workers investigated the effect of "excess" Mg^{++} on the inhibitory effects of uncoupling agents when added to rat brain mitochondria preparations. When pyruvate (plus malate) was the substrate for these systems, 2 mM sodium salicylate stimulated the oxygen uptake and produced some 60% uncoupling when compared to control preparations without salicylate addition. The concentration of magnesium ions in these experiments was 3 mM. When the magnesium concentration of the reaction mixture was increased eight-fold (2.4×10^{-2} M) there was no observable alteration in either the rate of oxidation nor the incorporation of inorganic phosphate in the presence of salicylate (Brody, 1956). Mg^{++} also failed to reverse the inhibition of oxidative phosphorylation produced by 2.4-dinitrophenol (Brody and Bain, 1954).

However, these two groups of workers employed different material as their enzyme source. Kaplan, Kennedy and Davis used whole tissue homogenate whereas Brody and Bain used isolated rat brain mitochondria. There were also differences between the substrates and test systems employed, which may account for the conflicting evidence obtained in these studies. Therefore, it was felt advisable to re-examine the possibility of a reversal by Mg^{++} of the uncoupling effect of salicylate _in vitro_, in the test system for oxidative

phosphorylation used previously in this work. Thus rat liver mitochondria were used as the enzyme source, and either α-keto-glutarate, β-hydroxybutyrate or succinate as the substrates.

Under these particular conditions the provision of "excess" Mg^{++} (as magnesium sulphate) failed to reverse the uncoupling effect of 5 mM sodium salicylate, irrespective of the substrate being utilised by these liver mitochondria. This observation is in agreement with the findings of Brody (1956) who also used isolated mitochondria as the enzyme source in similar investigations.

The failure of Mg^{++} to overcome the inhibitory effect of salicylate on mitochondrial oxidative phosphorylation suggests that the absence of an inhibitory effect when these particles are prepared from rats treated with salicylate *in vivo*, is not due to a salicylate induced chemical interference in the availability of intracellular magnesium ions. Further evidence supporting an absence of an effect of salicylate on another aspect of Mg^{++} dependent mitochondrial function (ATPase activity) is presented later in this thesis.

CHAPTER V

Part II

THE ROLE OF K^+ IN RELATION TO THE SECOND HYPOTHESIS

Introduction

There was some evidence from the whole animal studies reported earlier, that the administration of large doses of salicylate resulted in a transient alteration in potassium balance *in vivo*. In man there was an increased loss of urinary potassium which was accompanied by a slight fall in the plasma level of this ion.

Neither of these changes is as marked as those associated with the retention of magnesium under these conditions. Furthermore, the effect of salicylate on potassium balance is transient whereas that on magnesium balance was much more persistent (cf. Chapter II).

Whether this change in electrolyte distribution in the whole animal can be specifically related to intracellular enzyme dysfunction *in vivo* and in particular to mitochondrial oxidative phosphorylation is at present unknown. Over the years there has been considerable discussion of the mechanism of intracellular potassium concentration *in vivo* (Fenn, 1940) (Spiegelman and Reiner, 1942) and the dynamic nature of this process has often been stressed. Without doubt, energy production within the cell is in some way geared to the maintenance of a high level of intracellular potassium ions against an extracellular concentration gradient of approximately 1:25.

Whilst a requirement for potassium ions is well established as a co-factor in many enzyme systems (Lardy and Ziegler, 1945) (Livoff and Ionescu, 1947) (Nossal, 1951) particularly the synthesis of carbohydrate by liver slices (Buchanan, Hastings and Nesbett, 1949), a specific

requirement for K^+ has not yet been clearly established in the processes of mitochondrial oxidative phosphorylation.

In 1943 Boyer, Lardy and Phillips presented some evidence for a dependence of tissue phosphorylations upon the presence of K^+. Using a minced rat muscle preparation and creatine as a phosphate acceptor, in a system in which sodium hydroxide was used to neutralise all acidic components, no esterification of inorganic phosphate occurred although pyruvate oxidation proceeded rapidly. The addition of 0.2 M potassium chloride to the reaction mixture slowed down respiration slightly and phosphorus was now "fixed" in large amounts.

Additional evidence implicating potassium in oxidative phosphorylation, particularly in reference to the inhibitory effect of salicylate is found in the observations of Kaplan, Kennedy and Davis (1954). These workers noted a marked difference in the degree of inhibition produced by salicylate on succinate oxidation, when kidney homogenates were made in either isotonic potassium chloride or in a water media. The effect of salicylate on the water homogenates was pronounced, whilst those homogenates prepared in potassium chloride showed but slight inhibition in the presence of salicylate. These observations were made despite the fact that the final test system for the measurement of oxidation contained approximately equal concentrations of potassium, - 33 u.mols. K^+/ml. with the water preparations, and a concentration of 42 u.mols. K^+/ml. with those homogenates prepared in a potassium chloride medium.

Less direct evidence is seen in the results of Aebi and Meyer (1951) and Aebi and Abelin (1953) who worked with normal and hyperthyroid rat tissue. These investigators have shown that although isolated liver

slices from untreated rats swell considerably in a medium of low potassium content, similar slices from hyperthyroid rats lose K^+ to the suspending medium much more readily than do the "normal" slices. Later Tapley (1956) commented on this work and its relationship to his own investigation of the swelling of isolated rat liver mitochondria. Tapley stated that "such a loss of potassium could be a primary consequence, and that uncoupling of phosphorylation if it occurs at all in vivo (in hyperthyroidism), may be a secondary manifestation" of this loss.

Lardy and Wellman (1953) have demonstrated an effect of K^+ on mitochondrial ATPase and recently Lowenstein (1960) has demonstrated a role for potassium ions in a non-enzymatic transphosphorylation from ATP to orthophosphate. This or similar reactions may possibly be the site of a requirement for K^+ in the mechanism of mitochondrial oxidative phosphorylation, should such a requirement actually exist.

An effort was made to assess the effect of K^+ on mitochondrial oxidative phosphorylation by studying the efficiency of this reaction when mitochondria isolated from untreated rats were incubated in a media containing graded concentrations of potassium (from 20 milli moles/L. to 170 milli moles/L). This medium was as free as possible from other univalent metal cations as these may be able to replace, at least partially, any action of potassium (Lardy 1951) (Lowenstein 1960).

Materials and Methods

"Control" reaction mixture (A)

This mixture contained potassium dihydrogen phosphate (50 milli equivalents K^+/litre) and was identical with that previously used to examine the effect of salicylate upon mitochondrial oxidative phosphorylation. Further potassium ions were added with the substrate, which was either the potassium salt of α-keto-glutaric acid or succinic acid (25 u.mols.) When α-keto-gluterate was the substrate a further 25 u.mols of potassium malonate was also added.

"PVP" reaction mixture (B)

The basic medium in this case was an aqueous solution of poly-vinyl-pyrrolidone (7% PVP) which supplied suitable osmotic conditions for the mitochondria (Novikoff, 1956). Dilute phosphoric acid was added to this solution to give a final concentration of 50 u.mols/ml. of inorganic phosphorus. Initially this mixture was strongly acid, and was therefore adjusted to pH 7 by the slow addition of solid tris hydroxy-methylaminomethane (TRIS) buffer. The final concentration of TRIS which was required was about 1%.

Mg^{++} as magnesium sulphate, glucose and adenosine-triphosphate (ATP) were added so that the final concentration of these substances in the mixture was the same as that in the control medium (A). The ATP was added as its sodium salt and this was the only deliberate addition of Na^+ to the mixture. The final concentration of Na^+ was low being only one milli equivalent/litre.

No K^+ was found in this medium when it was assayed by flame photometry. The substrate (α-keto-glutaric acid or succinic acid) was

added as the potassium salt (25 u.mols.) Malonic acid was not added as "one step" reactions were not imperative.

"Glycylglycine" reaction mixture (C)

This medium did not contain PVP or K^+. 1% glycylglycine and 1% Tris buffer solution was adjusted to pH 6.9 by the addition of dilute phosphoric acid. Analysis of the final mixture for inorganic phosphorus by the method of Taussky and Shorr (1953) demonstrated that its concentration was equal to 42 u.mols P/ml. This level was suitable for this investigation. 25 u.mols of potassium α-keto-glutarate or succinate were again added to this mixture. Malonic acid was not added.

Measurement of oxidative phosphorylation

0.5 mls. of mitochondria suspended in 0.25 M sucrose and prepared from the livers of untreated rats by the method described before (p.75) were added last to Warburg vessels which already contained a CO_2 "trap", hexokinase-glucose in the side arm, and 1 ml. of reaction mixture A, B or C. Each vessel also contained up to 0.5 ml. of 0.5 M sucrose.

Various quantities of K^+ to give concentrations between 20-170 milli equivalents/litre were added to reaction mixtures B and C, on some occasions. This ion was added as potassium chloride dissolved in 0.5 M sucrose.

Some mitochondria from each batch prepared were examined in reaction mixture (A) as a "control" for these experiments.

The pH of the "complete" mixture plus mitochondria was always between pH 6.8 and pH 7.4, although the volume of these systems varied slightly. The conditions of incubation and methods of measuring P/O ratio were identical with those described previously (cf. p.75).

Results

Control experiments conducted in medium A demonstrated that the mitochondria examined here were always "coupled". The concentration of K^+ in these particular reactions was between 30-40 milli equivalents/litre. Although K^+ were added to medium B (the PVP mixture) to give concentrations up to 170 milli equivalents/litre, phosphorylation never occurred under these conditions. The relatively large oxygen uptakes associated with these "uncoupled" reactions declined with increasing concentrations of potassium ions (c.f. Boyer, Lardy and Phillips 1943).

Later when the glycylglycine-tris reaction mixture (C) was employed the rates of oxygen uptake were found to decline sharply when the concentration of K^+ exceeded 50 milli equivalents/litre. In this medium some phosphorylation was associated with the oxidation of α-keto-glutaric acid although the efficiency of this reaction was always low (mean P/O ratio of four experiments =1.0). A strict dependence of phosphorylation upon K^+ could not be demonstrated as the phosphorylations observed were only associated with concentrations of K^+ below 50 milli moles/litre. Above this level of K^+, phosphorylation completely ceased and the rates of oxygen utilisation also declined sharply. Another observation associated with this finding was the physical appearance of the mitochondria after incubation in these systems. The usual turbid appearance of the mitochondrial suspensions were strikingly clarified after incubation in reaction mixtures containing more than 50 milli equivalents K^+/litre. The results of a typical experiment using medium (C) are given in Table (19).

TABLE (19)

K^+ AND OXIDATIVE PHOSPHORYLATION

Expt. No.13. Substrate α-keto-glutarate

Concentration of K^+ in mEquivs./ L.	µl.Ox./ 30 mins.	µ.atoms Ox.	µ.atoms P.	P/O
20	48	4·5	5·5	1·2
50	45	3·7	4·5	0·8
80	19	1·8	0	—
110	18	1·7	0	—

Glycylglycine-Tris reaction medium.

K^+ ions added as potassium chloride.

Each value represents duplicate analyses.

Discussion

In an effort to produce a medium with a variable potassium ion content, yet similar inorganic phosphorus levels and buffering power an alternative source of K^+ to potassium dihydrogen phosphate had to be found. This variation from the usual medium for mitochondrial oxidative phosphorylation resulted in the construction of two media, neither of which was able to support efficient phosphorylation in the presence of either α-keto-glutarate or succinate as substrates.

Another major consideration was the desire to not replace K^+ with other univalent cations, particularly Na^+, as had been done by Boyer et al (1943). Although some Na^+ was added in conjunction with ATP, the concentration of this ion was only one milli equivalent/litre, which was negligible in this study. These restrictions initially led to the construction of a poly-vinyl-pyrrolidone containing medium (B). Although oxidation of succinate and α-keto-glutarate proceeded rapidly in this medium, it failed to support phosphorylation at all levels of added K^+ up to a concentration of 170 milli equivalents K^+/litre.

As the buffering power of this medium (B) may have been inadequate, another medium was constructed in which the PVP was replaced by 1% glycylglycine. In this medium (C) a small degree of phosphorylation was observed with α-keto-glutarate as the substrate. However this reaction was only evident at the lower concentrations of K^+ ($\ll$50 milli equivalents/litre); at higher concentrations phosphorylation ceased. Whether this observation demonstrates an antagonism of K^+ towards

mitochondrial phosphorylation cannot be said at present, because no control was provided for the presence of chloride ions in these reactions. Other explanations may also exist.

In both mediums (C) and (B) there was a decline in the rates of oxygen uptake as the concentration of K^+ increased. This suggests that the effect of K^+ on oxidation reactions may not be obligatorily linked to the ability of mitochondria to carry out phosphorylation reactions.

The change in the physical appearance of the mitochondrial suspensions after incubation in reaction mixtures containing concentrations of potassium chloride above 50 milli equivalents/litre is perhaps suggestive of a physical effect rather than a purely chemical effect of this salt. No definite evidence is available from these experiments.

It had been intended to determine the efficiency of oxidative phosphorylation of liver mitochondria isolated from rats treated with salicylate *in vivo* and examined in a K^+ "deficient" medium *in vitro*.

The results of these preliminary studies did not provide sufficient grounds to warrant an investigation of the possibility of a relationship between K^+ and salicylate upon mitochondrial oxidative phosphorylation. Before such a project could yield conclusive evidence it would be necessary to provide mitochondria isolated from untreated rats with a reaction mixture which would support efficient oxidative phosphorylation in the presence of variable concentrations of added K^+. Neither of the two media specifically devised for this purpose proved satisfactory for this purpose. Further investigation of this aspect of the project is under investigation.

CHAPTER V

Part III

GENERAL CONCLUSIONS

The evidence presented in Part I of this Chapter of the thesis indicated that the inhibitory effect of salicylate on mitochondrial oxidative phosphorylation could not be overcome by the provision of an excess quantity of Mg^{++}. This finding suggests that the uncoupling action of salicylate is not directly related to an interaction between the salicylate radical and magnesium ions.

Part II of this Chapter has been concerned with the possible relationship of K^+ to the <u>in vitro</u> uncoupling action of salicylate. The experiments performed can only be regarded as being of a preliminary nature, and are not satisfactory at this stage.

The addition of 170 milli equivalents of K^+/litre would not initiate mitochondrial phosphorylation reactions when added to media devoid of appreciable amounts of other univalent cations. Concentrations of potassium ions equal to or less than 50 milli equivalents K^+/litre did support inefficient phosphorylation in a glycylglycine containing medium.

Concentrations of potassium ions above 50 milli equivalents K^+/litre inhibited mitochondrial phosphorylation. The effect of equal concentrations of chloride ions in these systems is unknown.

This scant evidence of the effect of K^+ on mitochondrial oxidative phosphorylation does not constitute a denial of the conditions postulated in the hypothesis under examination here.

The possibility of K^+, which is the major intracellular cation, being involved in the action of salicylate on mitochondrial function is referred to in subsequent sections of this thesis.

CHAPTER VI

AN EXAMINATION OF THE PERMEABILITY OF LIVER CELLS AND MITOCHONDRIA TO SALICYLATE

Part I: Permeability of the cell to salicylate

Part II: A consideration of the metabolic fate of salicylate

Part III: Permeability of the mitochondria to salicylate

CHAPTER VI

AN EXAMINATION OF THE PERMEABILITY OF LIVER CELLS AND MITOCHONDRIA TO SALICYLATE

Introduction

Four hypotheses have been proposed to explain the apparent difference between the *in vitro* and *in vivo* action of salicylate on mitochondrial oxidative phosphorylation. Of these, two have already been examined. The remaining hypotheses (3) and (4) are as follows:-

(3) That salicylate produces a direct but readily reversible effect on the mitochondrial enzymes associated with the functions of oxidative phosphorylation, which may be spontaneously overcome if the drug were either leached out or washed off mitochondria during their isolation from the livers of rats given salicylate *in vivo*.

(4) That salicylate may affect the permeability of the mitochondrial membrane to normal intracellular substrates, ions, or co-factors required for oxidative phosphorylation. Again this may be a reversible effect which could be overcome by either the mitochondrial membrane or its environment being washed free of salicylate during the preparative stages.

Both these hypotheses are related in that as a mutual corollary, it is necessary that salicylate must pass through the cell membrane and enter into the "cell-sap" *in vivo*. Therefore prior to an examination of either of these hypotheses, it was first necessary to demonstrate the presence of salicylate within the cell; and if present to determine the concentration of this drug in the liver cells of animals exhibiting signs of salicylate toxicity.

CHAPTER VI

Part I

THE PERMEABILITY OF THE CELL TO SALICYLATE

Seeburg, Hansen and Whitney (1951) have demonstrated that maximum plasma and "liver" levels were reached in ten minutes, when salicylic acid was given orally to rats in a dosage of .4 gm./kgm. In a study reported in Chapter II of this thesis it has been shown that maximum plasma levels were reached within thirty minutes following the administration of calcium acetyl salicylate to man. This and other evidence (cf. Buller, Muja and Carr, 1957) (Levin, 1912) suggests that the salicylate radical is rapidly absorbed from the gastrointestinal tract. In addition, Smith, Gleason, Stoll and Orgorzalek (1946) have affirmed that salicylates are not concentrated in any particular body locus, but that such organs as the liver, kidneys and lungs contain similar concentrations as the serum after the administration of this drug.

To examine this belief a study was made of the salicylate content of various tissue fractions prepared from the livers of rats treated with salicylate *in vivo*.

Materials and Methods

Male black and white rats of approximately equal age and body weight, were fed by gavage a dosage of 30 mgms. salicylate radical/ 100 gm. body weight/day, as an aqueous solution of the sodium salt without any pH adjustment (pH 8-9). The dosage was equally divided into three portions given throughout the day (at 9.00 a.m., 5.00 p.m. and 11.00 p.m. daily). Treatment was continued until clear symptoms of salicylate toxicity were evident, e.g. hyperpyrexia and haemorrhage.

This usually occurred by the eighth day.

In order to attain near maximal levels of the drug, the rats were used within thirty minutes of receiving the final dose of salicylate. The rats were lightly anaesthetised and a 5 ml. blood sample removed from the inferior vena cava, following an abdominal incision and displacement of the viscera. The blood was collected into a heparinised syringe and the plasma separated by centrifugation.

A portion of the now partially blanched liver, weighing between two and three grams, was excised and blotted dry with filter paper. The accurate weight of this sample was determined as quickly as possible. This portion was homogenised at 0°C into a minimum volume of isotonic sucrose (about 4 mls.), by the method previously described (p. 75). The final volume of the homogenate was noted. A suitable aliquot of this preparation, termed the "whole homogenate" was then assayed for salicylate as soon after preparation as possible.

Another small portion of liver, about 1 gm. was also removed, blotted and weighed in a tared vessel. This was dried to constant weight in an air oven at 105°C (about forty-eight hours) and re-weighed. By comparison of the "wet" and "dry" weights so determined, a measure of the "total organ water" could be obtained.

The remainder of the liver was removed and chilled in ice cold 0.5 M sucrose. It was then subjected to the usual procedures of homogenisation and centrifugation employed to separate mitochondria from cytoplasm. Some of the mitochondrial pellets so obtained were utilised for wet and dry weight determinations, whilst the remainder were re-suspended to a known volume in distilled water and assayed for salicylate.

Salicylate was estimated on the plasma, "whole homogenate" and mitochondrial fractions by the method of Trinder (1954). Standards were incorporated with every batch of material for assay.

<u>Calculation of results:</u>

When due allowance was made for the dilutions involved in the preparation of the various fraction it was possible to derive values for the concentration of salicylate in "whole tissue", "cytoplasm" and "mitochondria".

No correction was applied to the "cytoplasm" value to allow for the contribution from extracellular fluids which may have contaminated this fraction. The amount of salicylate determined as ugms./gm. wet tissue was converted to ugms./ml. of "cytoplasm" by use of the percentage water content obtained from the wet and dry weights of the liver. In the same way the "intramitochondrial" concentration of salicylate was expressed as ugms./ml. of total intramitochondrial water.

Results and Discussion

Measurable quantities of salicylate were detectable in the "whole homogenate" fraction of the livers of rats given salicylate <u>in vivo</u>. When the values obtained in this fraction were compared to the plasma level obtained from individual animals it was apparent that the concentration of the drug appears to be in near equilibrium in these phases. Only in one animal was the concentration of salicylate in the "whole homogenate" considerably less than in the plasma, and the value determined here still exceeded 50% of the plasma value. This finding is in agreement with the recent observations of Wolff and Austen (1958).

When the whole liver was partitioned into fractions and these were assayed for salicylate, almost the entire salicylate concentration

could be accounted for within the "cytoplasm" fraction. In some cases the concentration calculated in this fraction exceeded that of the "whole homogenate". Other work presented elsewhere in this thesis (Chapter IX) has shown that the degree of extracellular contamination of intracellular fluid (determined by the "chloride" space method) never exceeds 30%. When this correction is applied to the "cytoplasm" values obtained here, the "corrected" levels are still sufficiently high to demonstrate an appreciable level of true "intracellular" salicylate. Thus the cell membrane must be permeable to the salicylate radical. These results are given in detail in Table (20).

As it was felt that this point was now established, no further efforts were made to increase the "recovery" of the salicylate estimations.

Little or no salicylate was found in any of the mitochondrial fractions despite the appearance of this radical in the intracellular fluid (see Table 20). However, this finding cannot be taken as proof of a near total absence of salicylate from the mitochondria. The sensitivity of the assay method employed here was such that it would not detect an absolute concentration of salicylate below 10 ugms./ml. From a knowledge of the average wet weight and the water content of the mitochondrial yields (approximately 300 mgms. wet mitochondria/gm. wet liver, and 70% water respectively) it was calculated that a level of 10 ugms./ml. could represent an intramitochondrial concentration of 3×10^{-4} M (if all salicylate were distributed evenly throughout the total intramitochondrial fluid space).

The method of preparation of the cell fractions examined in these experiments results in a dilution of the intracellular fluid

TABLE (20)

SALICYLATE CONCENTRATIONS in vivo

The concentration of salicylate in the plasma and various liver fractions of rats given this drug in vivo.

EXPERIMENT NO.	DAYS OF SALICYLATE TREATMENT	SALICYLATE CONCENTRATION					MITOCHONDRIAL %H_2O
		Plasma	"Whole Homogenate"	"Whole homogenate treated with β-Glucuronidase	"Cytoplasm"	"Mitochondria"	
3 - 12	8	4·7 mM	2·6 mM	3·0 mM	-	-	71
4 - 12	8	2·9 mM	2·4 mM	2·8 mM	-	-	71
5 - 12	8	* -	1·5 mM	1·8 mM	-	-	70
8 - 12	8	2·4 mM	2·5 mM	-	-	-	70
11 - 3	14	-	1·4 mM	1·6 mM	-	-	72
30 - 3	3	3·3 mM	2·5 mM	2·9 mM	2·5 mM	trace	-
1 - 4	3	4·1 mM	3·5 mM	4·1 mM	2·8 mM	trace	-
13 - 4	2	4·1 mM	3·6 mM	4·2 mM	3·3 mM	0	-
22 - 4	4	5·5 mM	3·8 mM	4·5 mM	4·0 mM	0	-
21 - 6	8	-	1·9 mM	2·3 mM	-	-	70
19 - 7	8	2·6 mM	2·0 mM	2 0 mM	0·8 mM	trace	-

* - signifies not done.
The conditions for estimation of each fraction are described in the text.

during the initial homogenisation. This dilution is about five-fold, and can thus produce an appreciable concentration gradient effect on intramitochondrial solutes including salicylate, if present in a labile form. This effect would be greatly accentuated when the mitochondrial pellet was subsequently "washed" in sucrose alone.

Such a procedure may well result in the dilution or loss of "intramitochondrial" salicylate to a level below the sensitivity of the method of assay employed here. Thus it is possible that despite the absence of appreciable salicylate, the mitochondria may have contained a relatively high concentration of the drug *in vivo*, which would not be detected by these assays.

The permeability of mitochondria to salicylate is discussed further in Part II of this Chapter.

CHAPTER VI

Part II

A CONSIDERATION OF THE METABOLIC FATE OF SALICYLATE

Introduction

It has been demonstrated that animals fed large doses of any of the salicylate drugs, excrete in the urine an amount of the unmetabolised substance somewhat in proportion to the dose administered. In addition, however, the urine has been shown to contain the glycine conjugate, salicyluric acid (orthohydroxy-hippuric acid). It is believed that this product is formed in the liver and in other tissues (Baldoni, 1914). Another metabolite of salicylic acid found in the urine under these circumstances is the corresponding dihydroxy acid, gentisic acid (2.5-dihydroxybenzoic acid) (Kapp and Coburn, 1942). There appears also to be a moiety in the liver combined with glucuronic acid (salicyl glucuronide) (cf. Wolff and Austen, 1958).

It may be then, that the method of salicylate determination used here (Trinder, 1954) which is based on the reaction of ferric salts with phenolic hydroxyl groups (Brodie, Udenfriend and Coburn, 1944) fails to estimate some of the metabolic products of salicylate which could be present within the cell fluid. This possibility was examined by treatment of the whole homogenate prior to assay with a number of chemical and enzymatic procedures.

Materials and Methods

The dihydroxy metabolite of salicylate (gentisic acid) was found to be susceptible to assay by the method of Trinder. Standard quantities

of this substance (0-50 ug.) produced a curve indistinguishable from that constructed with ortho-hydroxybenzoic acid. It should be recalled that it has been established previously that para-hydroxybenzoate does not give a colour reaction with this method (Hetzel, Charnock and Lander, 1959).

Alkaline hydrolysis of rat liver homogenate from rats fed salicylate produced a chromogen reaction which interfered with the Trinder method of estimation. Gentle acid hydrolysis, using the acidity of the colour reagent (0.12 N hydrochloric acid) itself and heating for thirty minutes in a boiling water bath produced an increase in colour intensity. However, a nearly comparable rise was found when standard salicylate solutions were similarly treated, and therefore this increment could not be attributed to the formation of additional material in the "whole liver homogenate" which now estimated as salicylate. Much stronger hydrolysis conditions caused a digestion of the tissue and as in the case of alkaline hydrolysis, chromogen was formed which interfered with the Trinder method of estimation.

To examine the possibility of there being a glucuronide present, samples of the "whole homogenate" fraction from rats treated with salicylate _in vivo_ (as previously described) were incubated at 37°C for one hour with a preparation of Sigma bacterial β-glucuronidase. This preparation was chosen in preference to mammalian β-glucuronidase as the former has a pH optima between 6 and 7 (Sigma Bulletin, No. 105, 1958) compared to that of pH 4.3 for the mammalian enzyme (Bernfeld and Fishman, 1953). One drop of chloroform was added to each incubation tube as this is believed to enhance the enzyme activity appreciably (Sigma Special Bulletin, 1959).

Control estimations were performed on identical aliquots of "whole liver homogenate" incubated in the same manner but without β-glucuronidase addition.

About 5,000 enzyme units were added as 0.2 ml. of aqueous solution per incubation vessel.

Results and Discussion

Treatment of the whole homogenate fraction by β-glucuronidase produced a rise in the salicylate concentration which was detectable by the Trinder method employed here. This indicates that not only does this method not estimate the glucuronide of salicylate, but that the actual amount of salicylate within the organ is probably even closer to the plasma level than the "cytoplasm" levels indicate. As stated earlier, an effort was made to estimate the salicylate present in both alkaline and acid hydrolysates of some of these liver homogenates, but this proved to be unsuccessful as the colour of the hydrolysate so formed interfered strongly with the method.

The effect of β-glucuronidase treatment of the "whole tissue" fraction is shown in Table (20).

CHAPTER VI

Part III

THE PERMEABILITY OF THE MITOCHONDRIA TO SALICYLATE

Introduction

In Part I of this Chapter reference was made to the possibility that the salicylate radical may penetrate the mitochondrial membrane. When this possibility was examined by a study of the salicylate content of mitochondria isolated from the livers of rats given salicylate *in vivo* no appreciable "intramitochondrial" salicylate could be detected by the method of preparation and assay employed.

To study the possibility of an *in vitro* mechanism of action of salicylate which requires a binding of this radical to intramitochondrial enzymes, e.g. the third hypothesis, it is necessary to establish a method of preparation of mitochondria which subjects these particles to a concentration of salicylate equal to that found in the liver "cytoplasm" of rats given this drug *in vivo*.

Therefore it was obligatory to construct a system whereby the mitochondria were in contact with an external medium containing salicylate at a concentration of about 4 mM. Mitochondria so prepared could be examined both in relation to the degree of efficiency at which they carried out oxidative phosphorylation in addition to an examination of the degree of penetration of this radical into the internal fluids of these particles.

The results of a study of this latter effect are given here.

Materials and Methods

During the preliminary homogenisation and isolation procedures the dilution factor of 5 to 1 was again produced; usually the next step

of the procedure is the "washing" of the mitochondria to free them from "cytoplasmic" contamination. To this end the pellet is usually re-suspended at 0°C in 0.44 M sucrose and quickly centrifuged again at this temperature. Some pellets were treated in this way as a "control" but others were re-suspended at 0°C in a known volume of "washing" medium consisting of 0.44 M sucrose plus sodium salicylate. The concentration of the drug approximated the "intracellular" levels which had been previously determined to result from the administration of salicylate to rats in vivo. In these experiments 5 mM sodium salicylate was incorporated into the "washing" medium. Mitochondria were permitted to remain suspended in one of these "washing" media for from ten to twenty minutes at 0°C. They were then rapidly separated from the medium by centrifuging at 10,000 x g for ten minutes at 0°C.

This procedure of contact between mitochondria and a "washing" medium which sometimes contained 5 mM salicylate has been termed the "soaking" procedure.

After the mitochondrial pellets were separated by centrifugation, the supernatant phases were collected, free of mitochondria, by removal with a Pasteur pipette. The centrifuge tubes plus mitochondrial pellets were then inverted for a short period (about seven seconds) and the sides of the tubes wiped dry with absorbent tissues. This latter process "drained" the wet mitochondria free of remaining "washing" medium.

Some pellets were then re-suspended to a small volume (about 1 ml.) in distilled water and this suspension was assayed for salicylate. Replicate mitochondrial pellets obtained from the same liver homogenate were used to obtain the water content of each batch of mitochondria following "soaking" in either control or salicylate containing "washing"

media. The gravimetric method described on page 126 was used for these estimations. Salicylate was determined in all cases by the method of Trinder (1954).

Results and Discussion

The water content of the mitochondria varied between batches from 76.6% to 81.5% depending upon the nature of the "washing" medium and the time of contact between mitochondria and the medium. The variation between pellets within a batch, and subjected to a single treatment, was very small (± 0.5%) by comparison. A similar study is referred to later in this thesis.

By estimating the salicylate content of the "washing" media before and after the "soaking" procedure, as well as the salicylate content in the re-suspended pellet fraction, it was possible to obtain some measure of the degree of penetration of the mitochondria by salicylate *in vitro*.

In some cases a "duplicate" pellet was not estimated for salicylate after the "soaking" procedure, but was re-suspended again at $0^{o}C$ in 0.44 M sucrose without salicylate and then quickly centrifuged once more at $0^{o}C$. That is, the mitochondria were given an additional sucrose "wash". The supernatant fluid, which under these conditions could have gained salicylate only from the mitochondrial pellet, was assayed for its salicylate content. The mitochondrial pellets obtained after this second "wash" were "drained" and re-suspended in distilled water as described previously. The salicylate content of these mitochondria was also estimated. Data obtained from this type of experiment gives further information relating to the permeability of mitochondria to salicylate in addition to a measure of the lability of "intramitochondrial"

salicylate.

The detailed results obtained in this study are given in Table (21). It is clear that the salicylate radical is taken up by the mitochondria when suspended in a sucrose medium containing the sodium salt of the drug. This reaction takes place at 0°C.

If it is assumed that the salicylate determined in the mitochondrial fraction is actually within the mitochondria, and that this salicylate is distributed throughout the "total" intramitochondrial water space, then by use of the determined water content of the pellet, the value for salicylate can be expressed as a concentration in the intramitochondrial fluid. The work of Werkheiser and Bartley (1957) makes it extremely unlikely that the total water space of mitochondria would be available as a distribution space for the salicylate radical and therefore this assumption would mean that the calculated salicylate concentration would probably be less than the actual intramitochondrial salicylate concentration.

When these calculations are made, it was found that the level of salicylate in the mitochondrial fractions is approximately equal to the extramitochondrial concentration. Equilibrium appears to be attained within ten minutes. The level in the mitochondrial fraction increased above that of the "washing" medium in some cases. As the whole process was carried out at 0°C, this effect is more likely to be one of adsorption rather than an active concentration of salicylate within mitochondria.

Subsequent rapid "washing" of the mitochondria in a sucrose medium results in a sharp drop in the "intramitochondrial" concentration of the drug, with the appearance of salicylate in the washing fluid.

TABLE (21)

THE PERMEABILITY OF MITOCHONDRIA TO SALICYLATE AT 0°C

Results expressed as the milli-molar concentration of salicylate in the separate fractions

Experiment No.		6/7	8/7	12/7	14/7	21/7	26/7	29/7
Time of "soak" in minutes		10	15	15	20	20	20	20
Wet weight mitochondria in gms.		0·15	0·41	0·26	0·23	0·22	0·21	0·32
Fraction								
1	medium before soak	5·1	5·4	5·4	5·0	5·0	4·8	5·1
2	medium after soak	-	4·8	4·8	3·8	4·5	4·5	4·5
3	mitochondria after soak	5·0	4·1	4·1	5·4	5·6	11·5	10·3
4	medium after wash	-	0·6	0·3	0·4	0·25	-	-
5	mitochondria after wash	-	0·9	not detectable	1·2	0·8	-	-

Although it has been known for a long time that salicylates are bound to the plasma proteins (Jacoby, 1908) (Smith, 1949) this result suggests that "intramitochondrial" salicylate is labile under these conditions. If the binding of the salicylate radical to proteins is an energy requiring process, then the low temperature and lack of substrate and other factors - the conditions under which these experiments were conducted - may account for the observed lability of this radical.

This finding suggests that unlike nicotinamide, which is another mitochondrial enzyme inhibitor, salicylate is not firmly bound to an enzyme surface and therefore is not retained by "washed" preparations of mitochondria (Zatman, Kaplan and Colwick, 1953) (Zatman, Kaplan, Colwick and Ciotti, 1954).

In a consideration of a similar study of the permeability of mitochondria to a number of metabolites at 0°C, Amoore (1958) introduced the following concept to distinguish between the phenomena of adsorption to and permeability of mitochondrial membranes: "... the distribution of solutes between the mitochondria was expressed as the ratio of the internal and external concentrations. When this ratio was greater than unity ... it was taken to represent the degree of adsorption of solutes by the mitochondria."

The same principle was adopted in the evaluation of the data presented in Table (21). That is, where the "intramitochondrial" salicylate concentration exceeded that of the external medium; it was taken to indicate that adsorption as well as penetration had occurred. It was realised that this type of data cannot exclude the possibility of a degree of adsorption at any level of salicylate determined in the "mitochondrial" fraction but it was also felt that it was improbable

that adsorption alone could account for the values obtained.

When the data obtained from experiments in which mitochondria were previously soaked in a salicylate containing medium, and then given a subsequent wash in sucrose without salicylate, is considered in conjunction with that discussed above, it was felt that these experiments had produced strong evidence that mitochondria are permeable to salicylate at 0°C. Since it has been demonstrated that there is a concentration of salicylate in the "cytoplasm" of rats given salicylate *in vivo*, which is approximately equal to that of the plasma of those rats, then it would seem reasonable to anticipate that at least some of this salicylate could penetrate the mitochondrial membrane *in vivo*. Equilibrium may be reached at a level similar to that of the plasma.

Unless salicylate is fixed within mitochondria by an active process not apparent at 0°C, then during the course of a normal type of preparation, mitochondria derived from the livers of rats given salicylate *in vivo* would loose part if not all of the labile "intramitochondrial" salicylate. This loss would be brought about by the change in concentration gradients between the mitochondria and their experimental environment, which is very favourable towards a "leakage" of labile mitochondrial constituents.

With the information provided by the experiments reported in this Chapter of the thesis, it was now possible to proceed to an examination of the remaining hypotheses which had been proposed earlier, as possible explanations of the apparent difference in the action of salicylate on mitochondrial oxidative phosphorylation *in vitro* compared with that seen when salicylate is given to the whole animal.

CHAPTER VII

AN EXAMINATION OF THE THIRD HYPOTHESIS

"That salicylate produces a direct but readily reversible effect on the mitochondrial enzymes associated with the functions of oxidative phosphorylation, which may be spontaneously overcome if the drug were leached out or washed off mitochondria during their isolation from the livers of rats treated with salicylate *in vivo*."

Part I: The effects of salicylate on mitochondrial ATPase activity

Part II: A re-examination of the effects of salicylate on mitochondrial oxidative phosphorylation

Part III: Conclusions

CHAPTER VII

AN EXAMINATION OF THE THIRD HYPOTHESIS

Introduction

The third hypothesis states:-

"That salicylate produces a direct but readily reversible effect on the mitochondrial enzymes associated with the functions of oxidative phosphorylation, which may be spontaneously overcome if the drug were leached out or washed off mitochondria during their isolation from the livers of rats treated with salicylate *in vivo*."

At least some of the queries raised by this hypothesis have been answered in the preceding section, where it was shown that not only were rat liver mitochondria permeable to salicylate, but that at 0°C the "intramitochondrial" salicylate was not firmly bound to mitochondria and could be easily removed from these particles by "washing" them in a small volume of 0.44 M sucrose.

Although salicylate is known to have a high affinity for binding with plasma proteins (Smith, 1949), this radical is not bound firmly to mitochondria at 0°C. It is reasonable to suppose that many of the enzymes of the multi-step processes collectively termed "oxidative phosphorylation" are of a protein nature although they have not been identified as yet. If salicylate can become bound to these moieties in a loose fashion, and easily removed by a change in the concentration gradient of this radical between mitochondria and their environment, then this may be the *in vivo* mechanism by which salicylates exert a transient effect upon the rates of oxygen consumption of the whole animal.

In Chapter VI of this thesis it was shown that salicylate penetrates the liver cell membrane of rats treated with this drug _in vivo_. The "cytoplasmic" concentration of this agent under these conditions was found to be approximately equal to that of the plasma of the animal. In these circumstances the mitochondrial membrane is surrounded by an environment containing salicylate at a range of concentrations between 1 mM and 5 mM. There is presumptive evidence that salicylate can also penetrate the mitochondrial membrane _in vivo_ and enter into the fluid matrix of these sub-cellular organelles, again to a concentration in approximate equilibrium with that of the plasma and "cytoplasm". Should this occur it is reasonable to suppose that salicylate may become attached to "intramitochondrial" enzymes and affect their function.

Therefore, in relation to the third hypothesis stated above the questions requiring answer were:-

(1) Does "intramitochondrial" salicylate affect mitochondrial enzyme function, particularly those associated with the reactions of oxidative phosphorylation; and

(2) Would the subsequent removal of salicylate from mitochondria restore that function to normal?

In order to study these possibilities two very closely related aspects of mitochondrial enzyme function were examined. The first of these was the phosphate to adenosine triphosphate exchange reaction of isolated mitochondria. Recently Falcone (1959) had suggested that this reaction may be the "site" of the _in vitro_ uncoupling effect of salicylate on mitochondrial oxidative phosphorylation. Secondly, and only after the first study was completed, it was decided to re-examine the _in vitro_ effect of salicylate on the "complete" reaction of oxidative phosphorylation in mitochondria isolated from untreated rats.

Both these examinations were carried out in an effort to determine the effect of "intramitochondrial " salicylate. Therefore, it was necessary to prepare mitochondria from untreated rats and subject them to contact with salicylate by the "soaking" method previously described.

The results of these investigations will be reported separately.

CHAPTER VII

Part I

THE EFFECT OF SALICYLATE ON MITOCHONDRIAL ATPase ACTIVITY

Introduction

It has often been suggested that the *in vitro* hydrolysis of added ATP by mitochondria, is brought about by a mechanism which is the reversal of the reactions responsible for the synthesis of ATP during the process of oxidative phosphorylation of substrates by mitochondria (Hunter, 1951) (Lardy and Wellman, 1953). This process is known as mitochondrial ATPase activity, and may be considered to be a disordered portion of the "complete" mechanism of oxidative phosphorylation.

Recent work on the mechanism of oxidative phosphorylation by Lehninger, Wadkins and Remmert (1958) suggests that the following steps are involved in this "complete" reaction:-

(1) Carrier (red) + Acceptor (ox.) + X $\rightarrow$ Carrier (ox.) $\sim$ X + Acceptor (red)

(2) Carrier (ox.) $\sim$ X + Pi $\rightleftharpoons$ Carrier (ox.) + P $\sim$ X

(3) P $\sim$ X + E $\rightleftharpoons$ P $\sim$ E + X

(4) P $\sim$ E + ADP $\rightleftharpoons$ ATP + E

"Carrier" and "acceptor" are two consecutive members of the electron transport chain, E and X are as yet unknown intermediates that can exist in either a normal or high energy state, Pi and P are inorganic and organic phosphate respectively.

The recent studies of Bronk (1960) make it appear likely that it is the combined steps (2), (3) and (4) which are responsible for mitochondrial ATPase activity.

During "respiratory chain" phosphorylation the complex

reactions (1 $\rightarrow$ 4) may occur at three separate points, and therefore the mechanisms of ATPase activity may also occur at three separate points. It is possible that these ATPase systems may be distinguishable one from the other by biochemical differences.

It has been suspected for some time that mitochondrial ATPase activity does not represent either a simple hydrolytic enzyme or even a single enzyme (Potter, Siekevitz and Simonson, 1953). Recently the studies of Myers and Slater (1957a, 1957b) have indicated that mitochondrial ATPase activity has four separate pH optima, and these workers have suggested that this phenomena could represent four different enzyme systems, each of which they have shown to be relatively specific for ATP.

The "respiratory chain" enzymes as well as the "coupling enzymes" of oxidative phosphorylation are present in the mitochondrial membranes. Indeed they make up a large part of its substance (Lehninger, Wadkins, Cooper, Devlin and Gamble, 1958). Therefore the enzymes responsible for ATPase activity should also be present within the mitochondrial membrane. The enzyme activity and the physical properties of these membranes may therefore be related, and it is quite conceivable that a change in enzyme activity could be reflected in an alteration in the mechanical properties of the membrane.

It was thought reasonable therefore to examine the possibility of an effect of salicylate on mitochondrial ATPase at these four pH optima (described by Myers and Slater) rather than at a single "physiological pH" (see Potter, 1954), particularly as Myers and Slater had found that the action of at least one "uncoupling" agent

(2.4 dinitrophenol) on mitochondrial ATPase was not uniform throughout the whole pH range.

In addition it was noted that several workers had related the increased activity of the so-called "latent ATPase" of mitochondria to disruptive changes in the structure of these particles (Witter, Watson and Cottone, 1955). Kielley and Kielley (1951) have claimed that carefully isolated liver mitochondria exhibit minimal hydrolytic activity towards ATP. It is well known that this activity increases during either the ageing process or in conjunction with such procedures as freezing and thawing, that is, procedures known to promote the physical break-down of mitochondria. This is the reason for the designation "latent" which is applied to mitochondrial ATPase activity. In addition, the ATPase activity of aged mitochondria shows an increased dependence on the availability of magnesium ions (Klemperer, 1957).

A system was set up to examine the effect of salicylate and Mg^{++} on mitochondrial ATPase activity, both in fresh and aged mitochondrial preparations and at the four pH optima described by Myers and Slater (1957a)(1957b).

It was anticipated that this study would not only provide information relative to the postulates contained in the third of the hypotheses under examination in this work, but may also provide information regarding - (a) the mode of uncoupling of oxidative phosphorylation by salicylate *in vitro*; (b) a possible effect of salicylate on mitochondrial integrity; and (c) a possible relationship between salicylate and Mg^{++}.

Materials and Methods

Mitochondria were prepared for purity rather than yield from

the livers of both untreated and salicylate treated rats. These mitochondria were isolated by both the usual and the salicylate "soaking" methods described previously. Extreme care was taken at all stages of the methods to ensure that the temperature of the various fractions did not rise above 1°C, and that the mitochondria were used immediately the final suspension was obtained. Each batch of fresh mitochondria were examined with at least one substrate for their degree of efficiency of oxidative phosphorylation in a test system known to support this reaction (cf. p. 76). This was done to exclude the possibility of a degree of mitochondrial "damage" incurred during the preparation of these particles which would be incompatible with a functional mitochondrial phosphorylating mechanism.

To provide a further control for the effects of "soaking" mitochondria in a medium containing sodium salicylate, duplicate mitochondrial pellets were "soaked" by re-suspension in a medium of 0.44 M sucrose without salicylate, at 0°C for exactly the same period of contact as those in the presence of salicylate also at 0°C. After the "soaking" period these mitochondria were quickly re-sedimented and the pellet freed from the supernatant fluid by draining the tubes in the manner previously described. Thereafter the mitochondria were re-suspended to a final volume of 0.25 M sucrose so that the mitochondria derived from one gram of wet liver tissue were now dispersed through 10 mls.

A representative value for the mitochondrial "yield" per gm. of wet liver tissue was obtained by weighing at least one pellet from each batch of mitochondria prepared. The percentage dry weight of the mitochondria was determined gravimetrically on these samples after drying

the pellet to constant weight at 105°C.

Some of each batch of "fresh" mitochondrial suspension was set aside for "ageing". After a number of preliminary experiments standard conditions of ageing were adopted. These were eighteen hours at 4°C. This procedure was easy to control and although it did not produce maximum effects, it also did not lead to complete loss of activity as was sometimes found by repeated freezing and thawing of a mitochondrial suspension. This aged preparation was subsequently examined in precisely the same manner as the fresh preparations.

The basic reaction mixture for these tests was an aqueous solution of .075 M potassium chloride, .05 M sucrose and .002 M sodium adenosine triphosphate (ATP). This mixture was diluted five-fold with a Tris-acetic acid buffer mixture whose pH had been adjusted to either pH 6.3, 7.4, 8.5 or 9.4. The final mixtures were re-adjusted to these pH values, if necessary, by the addition of dilute hydrochloric acid or potassium hydroxide. A glass electrode was used to make the pH adjustments.

Two mls. of these reaction mixtures were added to a series of vessels. Magnesium ions (as $MgCl_2$) and sodium salicylate were added either alone or in combination in volumes of 0.1 ml. to some of the vessels. The final concentration of these substances was 1 mM Mg^{++} and 5 mM sodium salicylate.

To these mixtures was added 0.1 ml. aliquots of the final suspensions of either fresh or aged mitochondria. This aliquot is equivalent to approximately 0.6 mgms. dry weight of mitochondria. This "enzyme" concentration is well within the range of linear

relationship known to exist between mitochondrial dry weight/ml. of solution and "enzyme" activity. This phenomenon was first recognised by Kielley and Kielley (1951) and later this observation was placed on a quantitative basis by Myers and Slater (1957a). This relationship is shown in Figure VIII, where enzyme activity has been plotted against mitochondrial weight. The data for Figure VIII was taken from the published work of Myers and Slater (1957a).

The mitochondrial preparations were then incubated at 23°C for thirty minutes, while being subjected to continuous mixing through gentle rotation of the vessels.† Some vessels were removed from the apparatus and the reaction in these vessels stopped by the addition of two mls. of 10% trichloracetic acid (TCA) as soon as possible after the addition of the mitochondria. These tests served as zero-time measurements of ATP break-down. At the end of the incubation period the reaction in the remaining vessels was stopped by the addition of TCA in the same way as in the zero-time vessels. These latter samples gave a measure of the reaction time hydrolysis of ATP by mitochondrial ATPase activity of fresh or aged preparations at four pH values, unsupported or fortified by either or both Mg^{++} and sodium salicylate.

The contents of all tubes were transferred to centrifuge tubes, spun in the cold to sediment the denatured mitochondrial residue, and an aliquot of the supernatant taken for inorganic phosphorus estimation by the method of Taussky and Shorr (1953).

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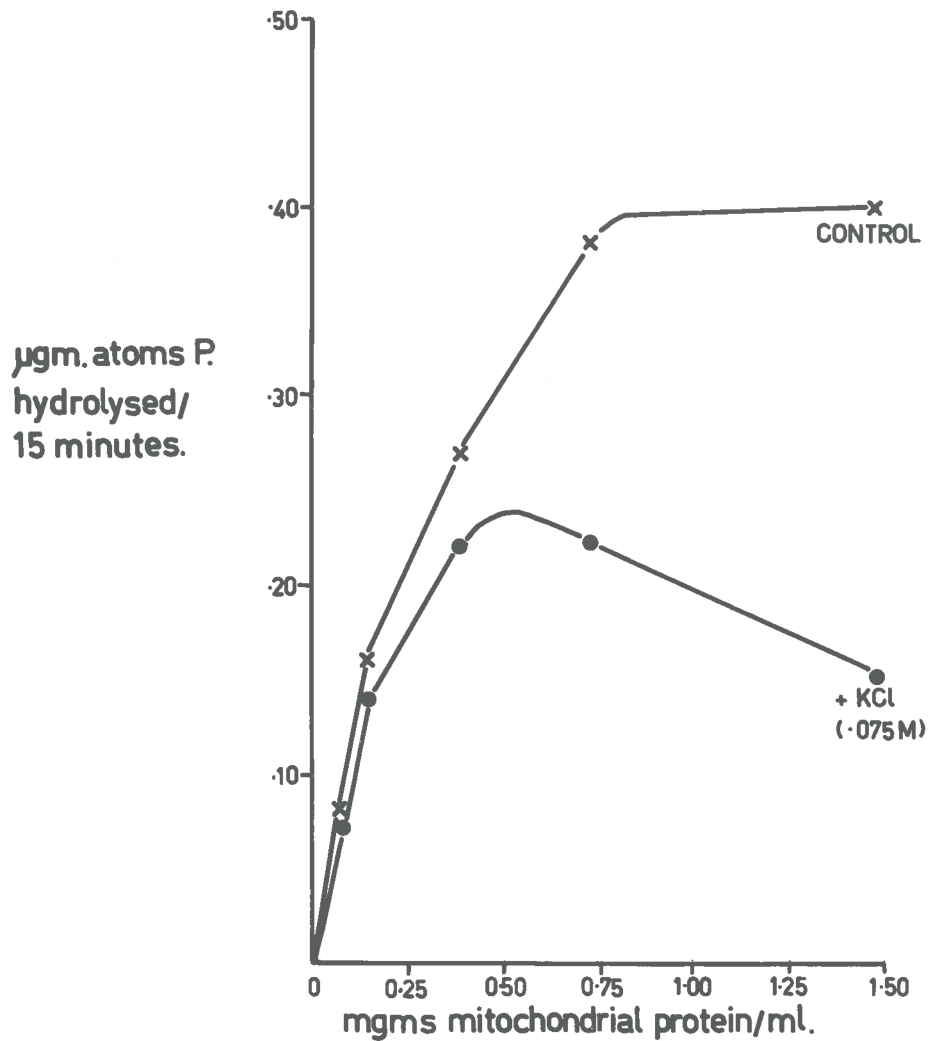
† My thanks are due to Dr. L. J. Opit of the Department of Surgery, University of Adelaide, for use of this apparatus which he designed.

FIGURE VIII

THE RELATIONSHIP OF ENZYME ACTIVITY TO MITOCHONDRIAL WEIGHT



(From the data of Myers, D.K. and Slater, E.C., Biochem. J., 1957, 67, 558)

An increase in the inorganic phosphorus content of a reaction vessel compared to a zero-time vessel at the same pH, was taken as a measure of the ATPase activity of the mitochondria in any particular test system.

The results are expressed as ug. atoms of inorganic phosphorus (Pi)/mg. dry weight of mitochondria/hour. These values were calculated after subtraction of the respective zero-time values from the reaction-time values and correction for both the time of hydrolysis and the dry weight of mitochondria added per 0.1 ml. of final mitochondrial suspension.

Results

The level of ATPase activity determined in those systems without addition of Mg^{++} or sodium salicylate will be termed the "basic activity". The shape of the curve formed by joining the activities measured under similar conditions but at four different pH values will be termed the "pattern" of activity.

The results obtained in this study were of the same order of activity as those subsequently described by Aldridge and Parker (1960). These values are considerably less than those reported by many other workers, e.g. Kielley and Kielley (1951) and Myers and Slater (1957). The temperature at which these observations were made is an important difference, as this factor has been shown by Myers and Slater (1957a) to greatly influence the ATPase activity of fresh mitochondria.

Although direct comparison is not possible between the activities found in these experiments and those reported by others, as the tests were performed under widely different conditions and the mitochondria were prepared by different methods of isolation, an approximation of some of the various mitochondrial ATPase activities

reported at pH 7.4 has been made in a number of instances.

This comparison, with some of the pertinent experimental details and the bibliography is given below:-

COMPARATIVE MITOCHONDRIAL ATPase ACTIVITIES

Reference	Enzyme Source	Temp.	Co-factors	Result	ugm. atoms Pi/ hour/mg. dry protein
(1)	Rat liver	38°C	-	9.0 u.mols. P/10 mins./ mgm. N.	513
(2)	Mouse liver	28°C	-	0.6 u.mols. P/10 mins./ mgm. N.	34
(3)	Rat liver	20°C	-	4.4 u.mols. P/hour/ mgm. protein	6.7
(4)	Rat liver	25°C	+ Mg	3.2 u.mols. P/10 mins./ 0.12 mgm. N.	1,500
(5)	Rat liver	37°C	+ Barbiturate	3 ugm. atoms P/hour/ mgm. protein	4.6
(6)	Rat liver	28°C	-	1.6 u.mgm. atoms P/ 15 mins./mgm. N.	61
(7)	Rat liver	23°C	-	0.76 ugm. atoms P/hour/ mgm. dry weight mitochondria	0.8

(1) Schneider, W.C., J.Biol.Chem. (1950), 183, 264.
(2) Kielley, W.W. and Kielley, R.K., J.Biol.Chem. (1951) 191, 485.
(3) Myers, D.K. and Slater, E.C., Biochem.J. (1957) 67, 558.
(4) Dawkins, R.F., Biochem.J. (1960) 76, 411.
(5) Aldridge, W.N. and Parker, B.H., Biochem.J. (1960) 76, 47.
(6) Riley, M.V. and Deegan, T., Biochem.J. (1960) 76, 41.
(7) Charnock, J.S. (1961) This work.

To calculate a conversion factor for these various experimental results it was assumed that the mitochondria contained 66% protein in the dry matter, and that the mitochondrial protein contained 16% nitrogen (cf. Birt and Bartley, 1960).

Both Kielley and Kielley (1951) and Myers and Slater (1957a) had accepted their respective results as representative of "low" basic ATPase activity.

In addition to obtaining "comparative" values lower than both these groups, in these experiments every batch of mitochondria used for ATPase estimations was also checked for its oxidative phosphorylation ability with at least one citric acid cycle intermediate as a substrate. Sometimes β-hydroxybutyric acid was also used as a substrate. The results of these experiments are given in Table (22). From the values reported here it is apparent that each batch of mitochondria were capable of carrying out oxidative phosphorylation at an experimentally acceptable degree of efficiency.

As reported in a previous section of this thesis, mitochondria derived from the livers of rats treated with salicylate _in vivo_, again produced P/O ratios similar to those measured with mitochondria from untreated rats. When α-keto-glutarate was the substrate the P/O ratios obtained in these experiments approximate more closely to the theoretical maximum value of four than any other P/O ratios obtained in the whole of this project. It is felt that this could be attributed to the extreme care taken in the preparation of the mitochondria for this investigation.

Because of these findings of "low" basic ATPase activity in conjunction with acceptable P/O ratios for every batch, it was considered that the preparations were satisfactory in that artifactual results produced by disruptive changes in the mitochondria were improbable.

TABLE (22)

P/O RATIOS OF MITOCHONDRIA CONCOMITANTLY EXAMINED FOR ATP-ase ACTIVITY

	SUBSTRATE	THE AVERAGE P/O RATIOS OBTAINED WITH EACH BATCH OF MITOCHONDRIA			
UNTREATED RATS	α-Keto-Glutarate	2·8	3·1	3·1	-
	β-Hydroxy-Butyrate	1·3	1·4	1·2	1·2
	Succinate	1·8	1·9	0·9	1·4
RATS FED SALICYLATE	α-Keto-Glutarate	2·6	3·2	3·5	3·8
	β-Hydroxy-Butyrate	1·2	1·5	0·8	1·2
	Succinate	1·7	1·8	1·3	1·6

The P/O ratios were determined in identical test systems to those described in the section on oxidative phosphorylation

The effect of pH on the ATPase activity of rat liver mitochondria prepared in the usual way and in the absence of Mg^{++} or sodium salicylate

The basic activity of -

(a) **Fresh mitochondrial preparations**

The basic activity determined at pH 6.3 was the least active, that at pH 7.4 and pH 8.5 were uniformly greater, whilst the activity at pH 9.4 was maximal. This finding is in agreement with that of Myers and Slater (1957).

(b) **Aged mitochondrial preparations**

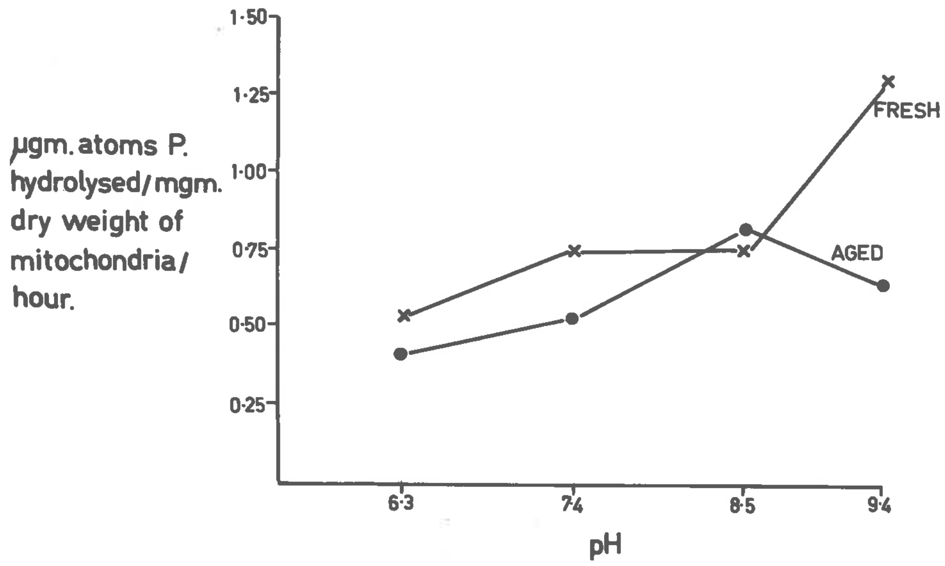
The effect of ageing the mitochondria for eighteen hours at 4°C was to decrease the activity of the "enzymes" at pH 6.3, 7.4 and 9.4. This decrease in activity was greatest at higher pH values. The pH 8.5 "enzyme" was resistant to ageing, and occasionally ATPase activity at this pH was slightly greater than that seen in fresh preparations at pH 8.5

These findings are shown diagrammatically in Figure IX which gives the mean values obtained from five separate experiments.

FIGURE IX

THE EFFECT OF pH ON THE DETERMINATION OF MITOCHONDRIAL ATP-ase ACTIVITY AT 23°C

Activity without added co-factors (Basic) (Mean of 5 experiments)



The effect of 5 mM sodium salicylate on the ATPase activity of rat liver mitochondria

(a) Fresh preparations

The effect varied with pH. Salicylate "stimulated" † the activity of the pH 6.3 enzyme to 180% of the basic level, that of the pH 7.4 and 8.5 enzymes to approximately 100%, i.e. the degree of stimulation decreased with increasing pH. At pH 9.4 there was no enhancement of enzyme activity, and within the limits of the method, there may have been slight inhibition.

(b) Aged preparations

The levels of activity obtained in the presence of salicylate differed by a small amount from those in the absence of salicylate and were within the range of experimental error. It was considered that salicylate had virtually no effect on any of the pH distinguished ATPases of aged mitochondria.

This contrasts with the effects of salicylate seen with fresh mitochondria wherein a stimulation was observed at pH 6.3, 7.4 and 8.5 and perhaps a slight inhibition at pH 9.4. This data is given in Tables (23) and (24) and the patterns of ATPase activity are shown, as a mean of five experiments in Figure XX.

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† The degree of stimulation was calculated as follows:-

$$\frac{(\text{level plus co-factor}) - (\text{level without co-factor})}{(\text{level without co-factor})} \times 100 = \%\ \text{stimulation}$$

TABLE (23)

THE EFFECTS OF SALICYLATE AND MAGNESIUM ON FRESH MITOCHONDRIAL ATP-ase

Activity	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Basic	·53	·76	·76	1·33
+ magnesium 1×10^{-3}M	1·82 [+260%]	1·85 [+140%]	1·93 [+150%]	2·45 [+85%]
+ salicylate 5×10^{-3}M	1·46 [+180%]	1·45 [+90%]	1·56 [+105%]	1·03 [-20%]
+ magnesium 1×10^{-3}M + salicylate 5×10^{-3}M	2·08 [+290%]	2·38 [+210%]	2·33 [+210%]	1·63 [+20%]

The results, which are the average of five experiments, are expressed as µgm. atoms P/ mg dry weight mitochondria/hour; and the percentage stimulation. Incubation temperature was 23°C

TABLE (24)

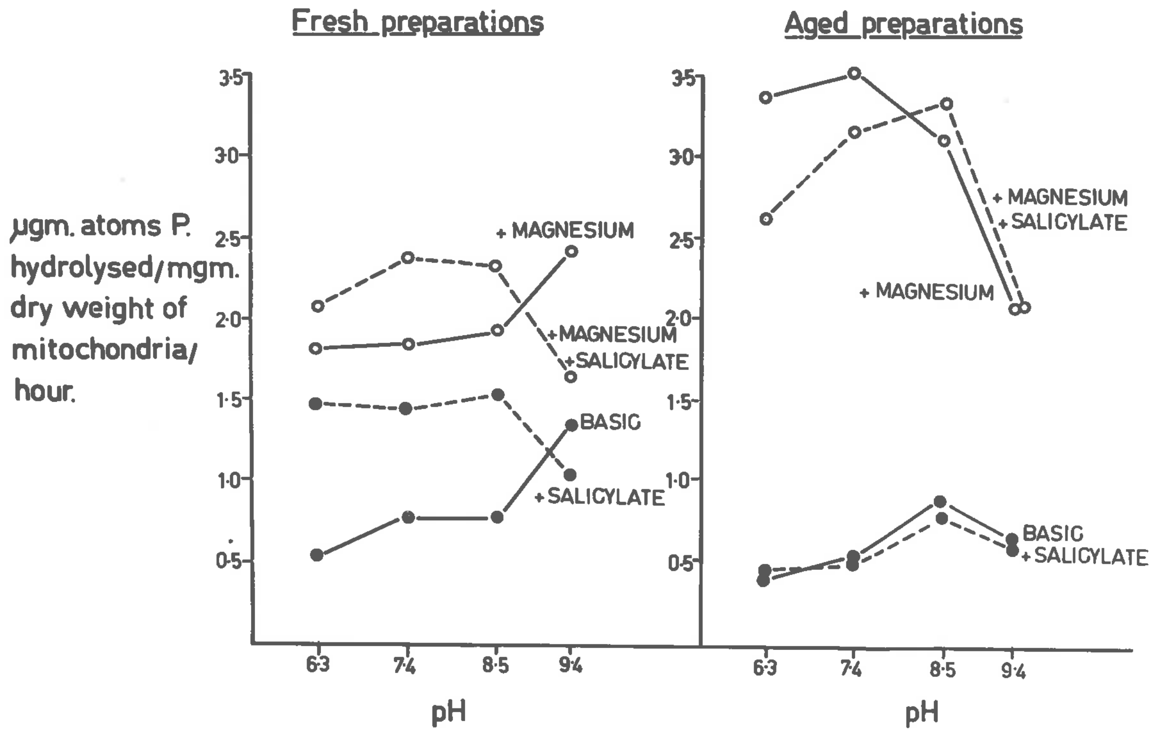
THE EFFECTS OF SALICYLATE AND MAGNESIUM ON AGED MITOCHONDRIAL ATP-ase

Activity	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Basic	·42	·54	·87	·65
+ magnesium 1×10^{-3} M	3·39 [+710%]	3·54 [+555%]	3·13 [+260%]	2·09 [+220%]
+ salicylate 5×10^{-3} M	·46 –	·51 –	·78 –	·60 –
+ magnesium 1×10^{-3} M + salicylate 5×10^{-3} M	2·62 [+525%]	3·16 [+470%]	3·34 [+300%]	2·06 [+220%]

The results, which are the average of five experiments, are expressed as µgm. atoms P/ mg. dry weight mitochondria/hour; and the percentage stimulation. Incubation temperature was 23°C

FIGURE X

COMPARISON OF THE ATP-ase ACTIVITY PATTERNS OF FRESH AND AGED MITOCHONDRIA



Each point represents the mean of five experiments.

The effect of 1 mM Mg^{++} on the ATPase activity of rat liver mitochondria

(a) Fresh preparations

Irrespective of the initial basic levels of ATPase activity, which have been shown to vary with pH, the addition of 1 mM Mg^{++} to the medium produced a stimulation in activity at all pH values examined. The greatest effect was 260% at pH 6.3, this increase fell with increasing pH to approximately 150% at pH 7.4 and pH 8.5 with the lowest degree of stimulation at pH 9.4, where the level was only increased by 8% over the basic value.

(b) Aged preparations

The degree of stimulation achieved by 1 mM Mg^{++} with aged preparations was much greater than in the case of fresh preparations. The values obtained were 710% for the pH 6.3 enzyme, 560% for the pH 7.4 enzyme, 260% for the pH 8.5 enzyme and 220% for the pH 9.4 enzyme. Similarly to fresh preparations the degree of stimulation by magnesium falls with increasing pH.

These effects demonstrate that aged mitochondria have an increased dependence upon magnesium for ATPase activity, when compared with fresh preparations. This finding has previously been reported by other workers (Potter, Siekevitz and Simonson, 1953) who used only a limited pH range; and has now been confirmed at all the pH values examined here.

This data is also given in Tables (23) and (24) and the pattern of ATPase activity shown as a mean of five experiments in Figure X.

The effect of the simultaneous addition of 5 mM sodium salicylate and 1 mM Mg^{++} to the ATPase activity of rat liver mitochondria

(a) **Fresh preparations**

Magnesium ions increased the ATPase activity in the presence of sodium salicylate, that is the combined effect of these agents was greater than the effect of either agent alone.

This enhanced activity was still apparent at pH 9.4 where the effect of sodium salicylate alone was to slightly decrease the ATPase activity of these preparations, whilst that of Mg^{++} was to stimulate, i.e. Mg^{++} plus sodium salicylate gave a value of activity intermediate between that found in the presence of Mg^{++} or salicylate alone. At pH 6.3, 7.4 and 8.5 the levels of activity were greater than when either Mg^{++} or sodium salicylate were added alone.

(b) **Aged preparations**

At pH 6.3 and 7.4 the stimulation observed with Mg^{++} was reduced in the presence of sodium salicylate, i.e. although sodium salicylate had no marked effect per se when added to aged mitochondria, it was antagonistic to the effect of Mg^{++} at pH 6.3 and 7.4.

However, this effect was not apparent at either pH 8.5 or pH 9.4 where the effect of Mg^{++} appeared to be only slightly changed by the presence of sodium salicylate. This differs from the effect seen with fresh preparations.

This data is also given in Tables (23) and (24) and the pattern of ATPase activity shown as a mean of five experiments in Figure X.

The effect of dinitrophenol on the ATPase activity of rat liver mitochondria

One experiment was performed to determine the effect of 2.4 dinitrophenol (DNP) on fresh and aged mitochondrial preparations, and to compare the result with that obtained by Myers and Slater (1957a) (1957b).

It had been reported by these workers that only the pH 6.3, 7.4 and 8.5 characterised enzymes of fresh mitochondria were stimulated by DNP and that the pH 9.4 enzyme was "scarcely affected". Here it was found that 10^{-4} M DNP (without the presence of Mg^{++}) had its greatest effect with fresh preparations on the pH 6.3 enzyme (960%), considerable effect on the pH 7.4 and pH 8.5 enzymes (410% and 130% respectively) and that there was some effect with the pH 9.4 enzyme (80%).

It is not possible to say from the report of Myers and Slater what the amount of stimulation was at pH 9.4 with this agent, but in the experiments reported here the effect of 10^{-4} M DNP is not entirely negligible, particularly in a consideration of the uncoupling mechanism of DNP on oxidative phosphorylation.

The decreased stimulation of ATPase activity by 10^{-4} M DNP, when added to aged mitochondrial preparations , compared to the effect of this agent on fresh mitochondrial preparations, is in accordance with the views of Hunter (1951), Lardy and Wellman (1953) and Potter, Siekevitz and Simonson (1953). Presumably this effect is related to the well known increased requirement for Mg^{++} by the ATPase enzymes of aged mitochondria. Cooper (1958a) has suggested that this latter effect is brought about by a loss of Mg^{++} from mitochondria on ageing, and that there is no activation by DNP until Mg^{++} is replaced.

However in these experiments there was considerable activation by DNP of aged mitochondrial ATPase at pH 7.4, 8.5 and 9.4 without the addition of Mg^{++}. The effect was greatest at pH 8.5. As the dissociation of DNP would decrease with increasing pH, the results obtained here do not support the view that it is the undissociated phenol which is the ATPase activator (de Deken, 1955) (Cooper, 1958a).

It has been shown that 5 mM sodium salicylate had no effect upon aged mitochondrial ATPase activity. The effects of 10^{-4} M DNP were quantitatively greater than those found when sodium salicylate was added to both fresh and aged mitochondrial preparations.

These results are given in Table (25).

TABLE (25)

THE EFFECTS OF 10^{-4}M D.N.P. ON MITOCHONDRIAL ATP-ase

	Activity	pH 6·3	pH 7·4	pH 8·5	pH 9·4
FRESH	Basic	·46	·92	1·82	2·96
	+ 10^{-4} D.N.P.	4·86 [+960%]	4·64 [+405%]	4·17 [+130%]	5·47 [+85%]
AGED	Basic	·15	·05	1·01	·15
	+ 10^{-4} D.N.P.	·15	·81 [+150%]	3·60 [+260%]	1·11 [+640%]

Results expressed as μgm.atoms P/mg. dry weight mitochondria/hour and the percentage stimulation. Incubation temperature was 23°C.

Examination of ATPase activity of liver mitochondria from rats treated with sodium salicylate *in vivo*

Rats were again given sodium salicylate by gavage, in the dosage described previously and for periods of not less than seven days. All animals exhibited considerable weight loss and hyperpyrexia, both typical signs of salicylate toxicity. All animals had an appreciable intracellular salicylate level of 2-3 mM.

Fresh and aged preparations

The "pattern" of basic ATPase activity determined at pH 6.3, 7.4, 8.5 and 9.4 for both fresh and aged mitochondria prepared from the livers of rats treated with sodium salicylate *in vivo*, were similar to those found on examination of the appropriate preparations made from the livers of untreated rats. However, the quantitative value for these ATPase activities were reduced from the levels found in preparations from untreated rats. The degree of stimulation or inhibition produced by the addition of 1mM Mg^{++} or 5 mM sodium salicylate either alone or in combination, was of the same order as that found with the liver mitochondria from untreated rats. These results are shown in Tables (26) and (27).

TABLE (26)

THE EFFECTS OF MAGNESIUM AND SALICYLATE ON LIVER MITOCHONDRIAL ATP-ase ACTIVITY OF RATS TREATED WITH SALICYLATE in vivo

FRESH PREPARATIONS

Activity	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Basic	·30	·38	·45	·67
+ magnesium 1×10^{-3}M	1·10	1·45	1·45	1·52
+ salicylate 5×10^{-3}M	·55	·73	·68	·52
+ magnesium 1×10^{-3}M + salicylate 5×10^{-3}M	1·02	1·58	1·68	1·30

The results, which are the average of two experiments, are expressed as µgm. atoms P/mg. dry weight mitochondria/hour. Incubation temperature was 23°C [cf. Table]

TABLE (27)

THE EFFECTS OF MAGNESIUM AND SALICYLATE ON LIVER MITOCHONDRIAL ATP-ase ACTIVITY OF RATS TREATED WITH SALICYLATE *in vivo*

AGED PREPARATIONS

Activity	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Basic	·12	·20	·30	·33
+magnesium 1×10^{-3} M	2·00	2·73	2·47	1·83
+ salicylate 5×10^{-3} M	·10	·15	·30	·25
+magnesium 1×10^{-3}M +salicylate 5×10^{-3}M	1·50	2·65	2·45	1·63

The results, which are the average of two experiments, are expressed as µgm.atoms P/mg dry weight mitochondria/hour. Incubation temperature was 23°C [cf. Table 24]

The effect of various modifications in the method of preparation of rat liver mitochondria upon their ATPase activity

1. Control

To control these experiments a portion of each batch of mitochondria which had been sedimented in the usual way, was not subjected to any variation in preparative method, and was immediately tested for ATPase activity exactly as described in the preceding sections. The "patterns" of basic activity found in these experiments corresponded to that shown previously for both fresh and aged mitochondria (cf. Figure IX).

2. The effect of soaking the mitochondria for 15 minutes in 0.44 M sucrose at 0°C prior to the assay of ATPase activity

(a) Fresh preparations

The activity at pH 6.3 was unchanged, that at pH 7.4 and 8.5 slightly increased whilst that at pH 9.4 was increased to the greatest extent. Hence this "soaking" procedure generally enhanced basic ATPase activity, which however still conformed to the pattern shown by fresh mitochondria.

(b) Aged preparations

The usual "pattern" seen with a normal but "aged" preparation of mitochondria was reproduced, i.e. pre-soaking the mitochondria in sucrose appeared to have no appreciable effect on the basic ATPase activity of "aged" mitochondrial preparations. Such a procedure only increased the duration of the ageing process (eighteen hours) by a few minutes.

3. The effect of soaking the mitochondria for 15 minutes in 0.44 M sucrose plus 5 mM sodium salicylate at 0°C prior to the assay of ATPase activity

Fresh and aged preparations

The usual "pattern" of both fresh and aged activity was found with these mitochondria, that is the patterns were the same as those found with either control preparations or those soaked in sucrose alone. Hence the pre-treatment of mitochondria by soaking in a sucrose medium containing 5 mM sodium salicylate at 0°C did not have a significant effect on basic mitochondrial ATPase activity. The results are given in detail in Tables (28) and (29) and the "patterns" of activity are shown in Figure XI.

TABLE (28)

THE EFFECT OF SOAKING PROCEDURES ON MITOCHONDRIAL ATPase ACTIVITY

Comparison of mitochondrial ATPase activity after various soaking procedures for 15 minutes at 0°C.

FRESH ACTIVITY

TREATMENT	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Unsoaked control (Basic activity)	0·55	1·04	1·30	2·32
Sucrose soaked	0·61 (+12%)	1·22 (+25%)	1·83 (+41%)	3·54 (+53%)
Sucrose + salicylate	0·67 (+24%)	0·74 (-39%)	1·77 (+36%)	2·99 (+29%)

Results expressed as µgm.atoms P released/ mgm.dry weight mitochondria/hour; and as the % change from the levels of basic activity (mean of 2 experiments)

TABLE (29)

THE EFFECT OF SOAKING PROCEDURES ON MITOCHONDRIAL ATPase ACTIVITY

Comparison of mitochondrial ATPase activity after various soaking procedures for 15 minutes at 0°C.

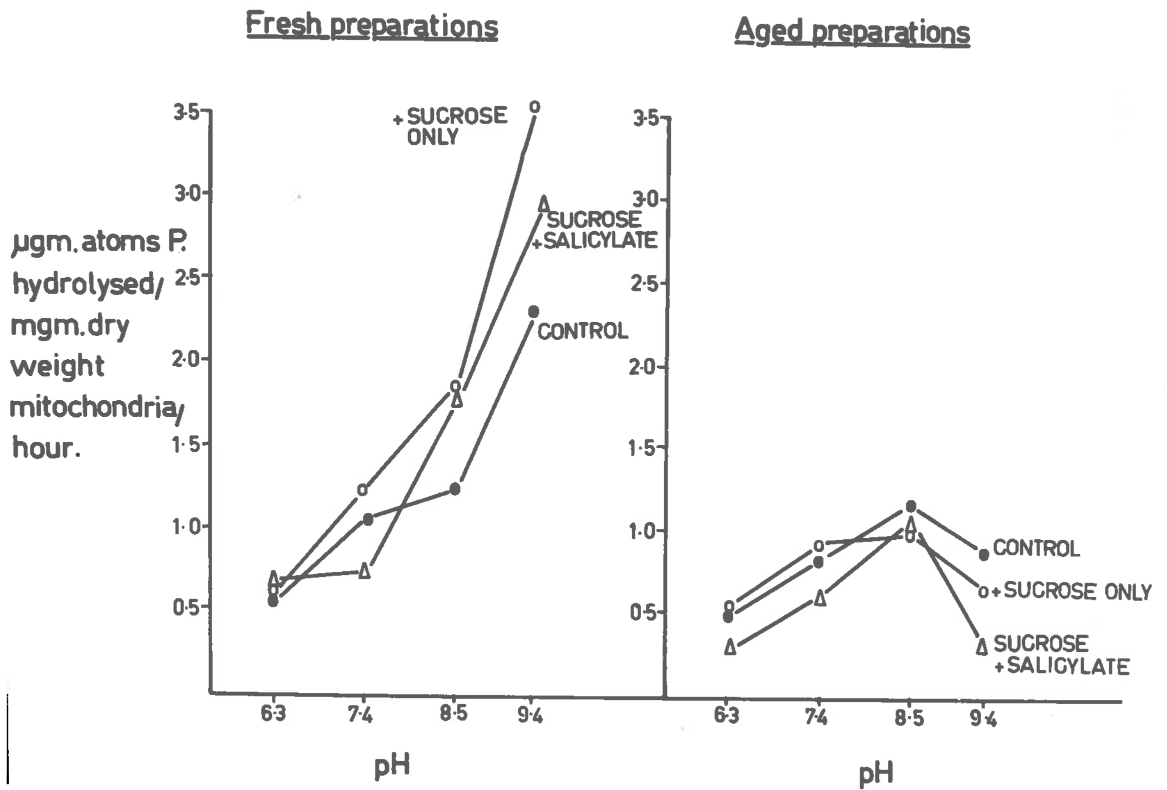
AGED ACTIVITY

TREATMENT	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Unsoaked control (Basic activity)	0·48	0·81	1·16	0·88
Sucrose soaked	0.53	0·90	1·04	0·66
Sucrose + salicylate	0·38	0·66	1·11	0·42

Results expressed as µgm.atoms P released/mgm.dry weight mitochondria/hour. (mean of 2 experiments)

FIGURE XI

EFFECT OF "SOAKING" ON ATP-ase ACTIVITY



The effect of pre-treatment by a soaking procedure on the release of ATPase activity from the mitochondria into the suspending fluid

Method

The supernatants from the soaking procedures described above in the preceding section were again collected. They were then centrifuged at 22,000 x g for thirty minutes to sediment any particulate matter collected with these fractions.

The pH of each suspending fluid was determined before use by means of a glass electrode and found to be:-

Sucrose only - pH 5.95

Sucrose + 5 mM sodium salicylate - pH 6.15

Any differences in the ATPase activity of these fractions is unlikely to have been caused by this small variation in pH.

The ATPase activity was determined in the following soluble enzyme fractions:-

(a) The sucrose only "soaking" medium either alone, or with the subsequent addition of sodium salicylate or sodium salicylate plus Mg^{++}.

(b) The sucrose plus sodium salicylate "soaking" medium either alone or with the subsequent addition of Mg^{++}.

The dry weight of the mitochondria, and the volumes of "soaking" media involved were known. One ml. aliquots of these fluids were added to the usual test system described on p. 148 (instead of 0.1 ml. of mitochondrial suspension) and the "soluble" ATPase activity determined by the procedure already described.

The detailed results are given in Tables (30) and (31) and the activity patterns are shown in Figures XII and XIII.

The "pattern" of soluble ATPase activity found in the medium containing sucrose alone was that of aged mitochondria, this was confirmed by the relative lack of effect of 5 mM sodium salicylate on the basic activity "pattern". There were small quantitative differences in ATPase activity in the presence of sodium salicylate but the qualitative characteristics of the "pattern" were unchanged and were those previously found with aged particulate mitochondrial preparations (compare Figure XIII to Figure X). When the "soluble" enzyme activity was measured in the presence of both salicylate and magnesium, the "pattern" of activity found again resembled that of an aged rather then a fresh particulate fraction.

There was considerably more activity present in the supernatant containing only sucrose than in the supernatant fluid containing sucrose and sodium salicylate. This result was irrespective of the pH at which the activity was measured (cf. Figure XIII). Far from enhancing the loss of ATPase enzyme activity from mitochondria, salicylate soaking pre-treatment reduced this loss.

In an attempt to quantitatively evaluate these "soluble" enzyme activities with those of a particulate system, the values of activity found in these experiments have been calculated on the basis of the activity in the volume of supernatant fluid which had contained the equivalent of one milligram dry weight of mitochondria. These comparative values are given in Tables (30) and (31).

It is apparent therefore that the actual loss of activity by the mitochondria into the suspending fluids, either in sucrose alone or when supplemented with sodium salicylate, is only a very small proportion of the total activity of the particulate fraction. Furthermore, the pH characteristics of such "soluble" ATPase activity are clearly those of "aged" mitochondria and not "fresh" particulate preparations.

TABLE (30)

"SOLUBLE" ATPase ENZYME ACTIVITY

TREATMENT	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Basic Activity	·035	·052	·092	·068
+Sodium Salicylate (5 mM)	·052	·077	·093	·060

The activity was determined in the supernatant fluid (soluble or non-particulate activity) following the soaking of normal rat liver mitochondria in 0·44 M sucrose for 15 minutes at 0°C.

The results are expressed as μgm.atoms P hydrolysed per hour by the volume of supernatant fluid which had contained the equivalent of one mgm. dry weight of mitochondria.

TABLE (31)

"SOLUBLE" ATPase ACTIVITY

Comparison of the soluble ATPase activity which was determined in the supernatant fluids with and without salicylate during the suspension of normal rat liver mitochondria for 15 minutes at 0°C.

TREATMENT	pH 6·3	pH 7·4	pH 8·5	pH 9·4
Sucrose medium + Sal. + Mg.	·013	·018	·020	·016
+ Sucrose Sal. medium + Mg.	·004	·006	·008	·006

Mg^{++} as $MgCl_2$ was added to both supernatants to give a concentration of 10^{-2} M in the assay system. 5mM salicylate was added to the supernatant fraction without this agent to give a concentration identical to that which had contained salicylate during the soaking phase.

Results expressed as μgm atoms P hydrolysed per hour by the volume of supernatant fluid which had contained the equivalent of one mgm. dry weight of mitochondria.

FIGURE XII

"SOLUBLE" ATP-ase ACTIVITY

[in sucrose supernatants]

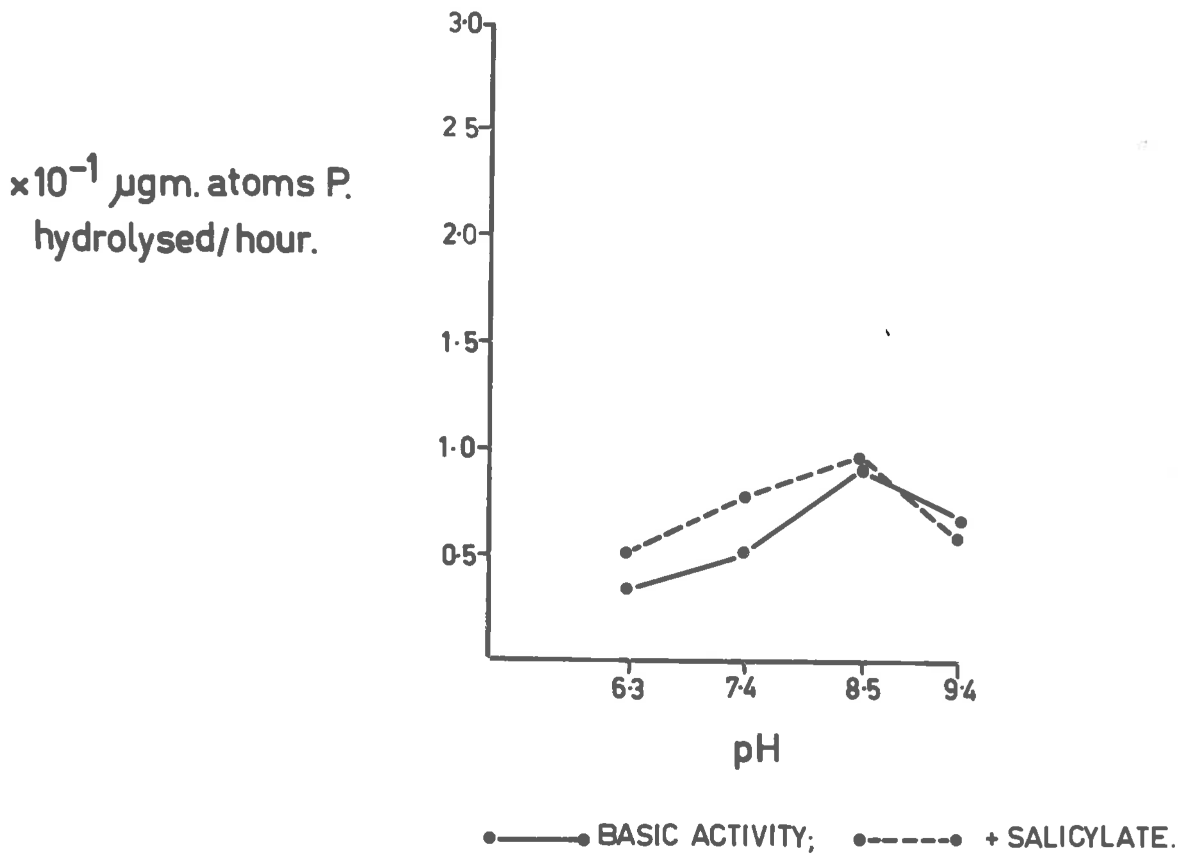
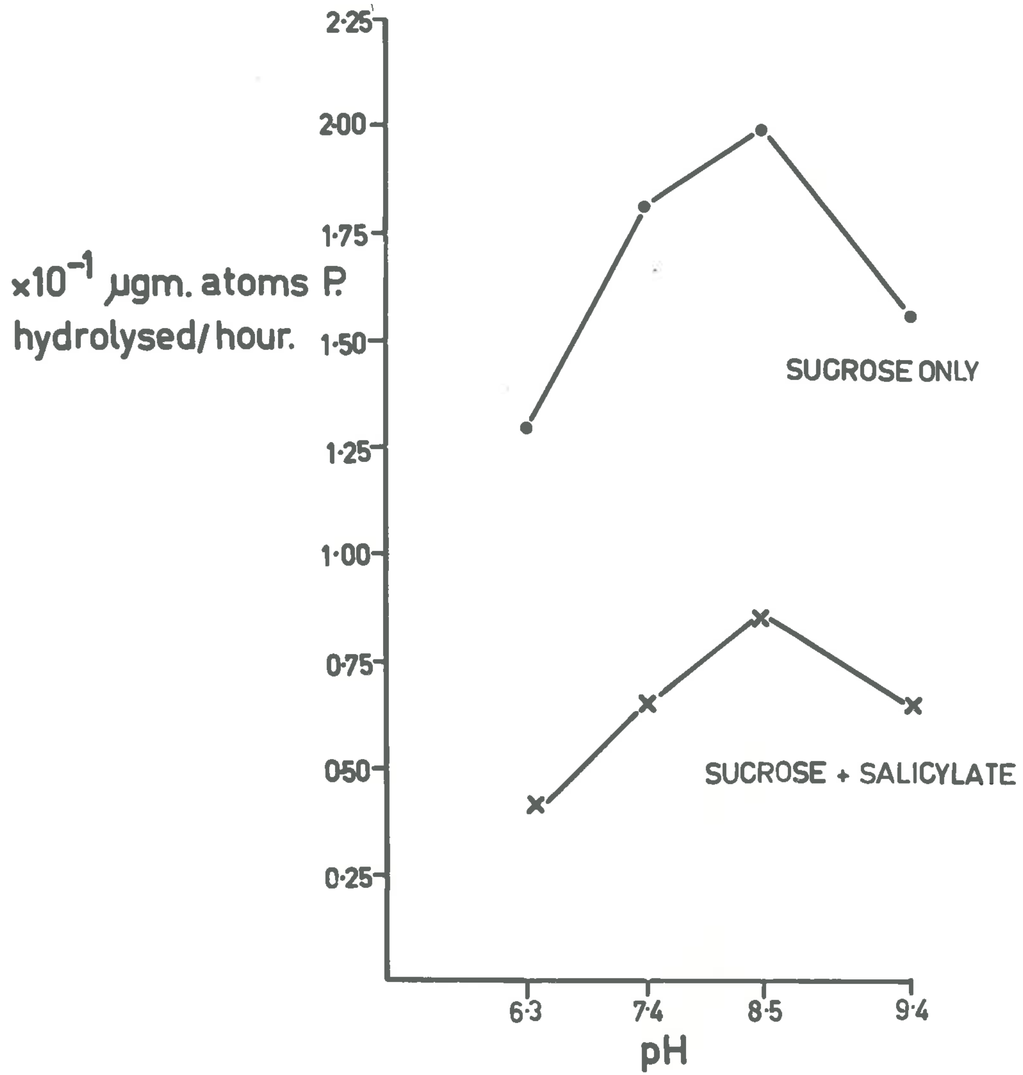


FIGURE XIII

"SOLUBLE" ATP-ase ACTIVITY

[in the presence of salicylate and magnesium]



Discussion

The concept of a low level of inherant or basic ATPase activity associated with carefully prepared mitochondria has been confirmed here. This parameter has often been used in assessing the relative "intactness" of mitochondria. It is apparent from this work, and the findings of others, that such factors as the temperature, osmolarity, ionic composition and pH of the test medium are all capable of exerting considerable effect upon the degree of ATPase activity measured. Not only does this make direct comparison of reported enzyme activities difficult, but it detracts considerably from the usefulness of the criteria of "intactness".

However, it has been clearly established that there are large changes in the activity of mitochondrial ATPase following such procedures as freezing and thawing, incubation at elevated temperatures, or even prolonged standing of mitochondria without substrate at or below room temperature. It is probable that only such "disruptive" procedures as these lead to really damaged mitochondria, irrespective of their fresh ATPase activity; as it is only after such procedures that an increased dependence upon Mg^{++} can be demonstrated prior to complete activation. It is the appearance of this phenomenon following disruptive changes, which has led to the concept of the so-called "latency" of mitochondrial ATPase activity.

The conversion of this enzyme from a latent to an active state is not fully understood at present; in 1953 Lardy and Wellman suggested that this process may either involve the liberation of bound (inactive) enzyme into an unbound (or active) state, or alternatively this process

may result from the partial denaturation of the phosphate transferring enzymes with the result that they can now react with water instead of some specific organic acceptors.

From the observations made in this study relating to mitochondrial membrane function and in conjunction with the mitochondrial double membrane concept of Werkheiser and Bartley (1957) it is felt that another type of explanation of ATPase activity can be offered, particularly with respect to the action of salicylate on the "enzyme" activity.

The electron microscope studies of Witter and his colleauges (Witter, Watson and Cottone, 1955) and the work of Lehninger's group (Lehninger, Wadkins and Remmert, 1958) support the belief that mitochondrial ATPase activity is localised within the membrane of these particles. Because the normal metabolising mitochondrion is probably in a constantly recurring cycle of swelling and contraction (Lehninger, 1960) associated with normal alterations in the permeability of their membranes, it is conceivable that mitochondrial ATPase activity would also be variable, and alter in accord with a changing membrane state, of which it is an integral part. Such a concept is supported by the recent reports of Bellamy and Bartley (1960) and Lehninger (1960).

As a consequence of such a relationship, it is suggested that an increased permeability of the mitochondrial membrane towards ATP could allow increased contact between enzyme and its substrate, and hence influence the rate of hydrolysis of ATP. The results of this study of the effects of salicylate on mitochondrial ATPase activity are thought to be in accord with this general view.

Although there is no direct evidence available from this work the results of this study could support the postulate of Myers and Slater (1957) that there exist in mitochondria four separate enzyme systems having ATPase activity, all of which are characterised by specific pH optima. However, other explanations of this type of data may be possible (cf. Cooper, 1958a). Although a pH effect on the substrate (ATP) can not be excluded by this data, these systems interact with Mg^{++}, sodium salicylate and dinitrophenol in a variable way which seems to preclude an effect solely dependent upon pH. If the evidence of Myers and Slater is accepted, then these systems have relatively specific ATPase activity and are not of a more general phosphatase nature.

The effect of addition of 5 mM sodium salicylate to the medium varies widely, and is dependent upon the condition of the mitochondria. There is a marked effect when the mitochondria are fresh; an effect which is found to be totally absent after the mitochondrial preparations have been aged. In such aged preparations it is probable that a substantial amount of disruption has occurred, particularly of the mitochondrial membrane, leading not only to an increase in ATPase activity, but to an increase in the activity of many other so-called "latent" mitochondrial enzyme systems (Bendall and de Duve, 1960).

These observations suggest that the enhancing effect of sodium salicylate on mitochondrial ATPase activity requires an "intact" membrane which is found only in fresh preparations; and is presumably associated with a change in the permeability of that membrane.

The increase in ATPase activity of aged mitochondria which is found after the addition of Mg^{++} is well known. The levels of this Mg^{++} supplemented activity have often been reported to be greater than those

observed with corresponding fresh preparations in the presence of the same concentration of Mg^{++}. The results in the present study confirm and extend these findings to all four pH levels examined, particularly those at pH 7.4 and 8.5. Cooper (1958a)(1958b) has said that he believes this effect to be due to a loss of "bound" Mg^{++} from the mitochondria, a loss associated with ageing. When this factor is overcome by the establishment of a high external concentration of this ion *in vitro*, the level of activity is enhanced. It is hard to see how this mechanism alone would lead to levels of activity in aged mitochondria which are greater than those found in corresponding fresh preparations. However, if the permeability of the membrane is also increased so that the substrate for this reaction (ATP) can now react more readily with the enzymes, then the observation of not only restored but enhanced activity may be explained.

An examination of the effect of sodium salicylate on the activity "pattern" of "soluble" ATPase activity which is released into the medium by soaking the mitochondria, suggests that this pattern resembles that of aged mitochondria rather than fresh preparations. As this "soluble" enzyme activity must clearly be without membrane control, these findings lend support to a concept of absence of such membrane effects with aged preparations, and a salicylate-membrane interaction as the mode of salicylate effect on fresh preparations.

The activity patterns obtained with both fresh and aged mitochondria in the presence of salicylate and Mg^{++} suggests an independence of action of these agents compatible with two different sites of enzyme activation. This is in accord with the postulate just made that salicylate activation requires an "intact" membrane whereas

that produced by Mg^{++} does not. Cooper concluded from his study of Mg^{++} and dinitrophenol activated ATPases, that these agents acted on "two distinct" enzyme systems (Cooper, 1958a).

In Chapter V of this thesis it was suggested, but not verified, that there existed an antagonism between Mg^{++} and the salicylate radical. The experimental results obtained in this phase of the project, also do not support a concept of chemical antagonism between these agents.

Although it must be speculative as yet, a common explanation for the findings discussed above, is one which embodies the mitochondrial membrane as the site of the effect of salicylate on mitochondrial ATPase activity. The basic "pattern" of ATPase activity which was observed at four different levels of pH with fresh mitochondrial preparations, is thought to primarily represent a change in membrane permeability with pH.

The absence of an enhancement of fresh ATPase activity by salicylate at pH 9.4 may suggest that the activating substance is the undissociated phenol. In a manner analogous to that of dinitrophenol, (De Deken, 1955) the concentration of undissociated salicylic acid would decrease with increasing pH. However at pH 7.4 for example, the concentration of the undissociated complex would be very low with the 5 mM solutions of sodium salicylate used in these experiments. As considerable activation was produced by salicylate at pH 7.4 and pH 8.5 it seems more likely therefore that the changes in activation found with changing pH reflect an effect on the enzymes systems, rather than on the activating agent. The different effects of salicylate at pH 9.4 may be associated with a separate localisation of this enzyme and may not be related to a membrane effect. This possibility is referred to later.

The enhancing action of salicylate on fresh mitochondrial ATPase activity requires the presence of salicylate per se, for it cannot be produced simply by subjecting the membrane to pre-treatment with salicylate. Furthermore, the effects are not due to a loss of the actual enzyme from the mitochondria with its subsequent appearance in a non-particulate form, as is found when mitochondria are treated with phospholipase (Petrushka, Quastel and Scholefield, 1959) or lecithinase (Kielley and Meyerhof, 1950).

The response of the ATPase activity of "aged" mitochondria and of the "soluble" enzyme or enzymes to a change in pH, could represent the pH characteristics of the "true enzyme system" freed of any superimposed permeability effects. The failure of salicylate to either stimulate the activity or alter the response pattern of "aged" mitochondria adds further weight to the view that salicylate per se has no direct effect on the ATPase enzyme or enzymes, but rather changes the permeability of the membrane to ATP and hence varies the rate of entry of substrate to the enzyme surface. The work of Bartley and Davies (1954) has shown that in other circumstances _in vitro_ the mitochondrial membrane is relatively impermeable to ATP.

Before this theory of membrane permeability can be accepted however, it would be necessary to exclude an effect of pH upon the substrate (ATP) with consequent changes in its ease of passage through the mitochondrial membrane. Such experiments are currently in progress in these laboratories. Preliminary observations indicate that a simple effect of pH on the ionic species of ATP (and magnesium) will not account for the patterns of activity found here (Opit and Charnock, 1961).

As mentioned previously, the action of Mg^{++} on mitochondrial ATPase activity seems to differ from that of salicylate, and may be directly associated with the chemical reactions of ATP hydrolysis and the enzyme systems of the "respiratory chain" rather than with the permeability of the mitochondrial membrane. Nevertheless Mg^{++} has often been implicated in the maintenance of mitochondrial structure (Tapley, 1956a) and function (Vitale, Nakamura and Hegsted, 1957). In addition this ion has been repeatedly shown to either prevent or reverse the swelling produced by a wide variety of agents added to mitochondria in vitro (See Chapter VIII, Part II of this thesis). Recently Lehninger (1959c) has demonstrated that in conjunction with ATP, magnesium ions are involved in the expulsion of water from metabolically active mitochondria. Perhaps this ion fulfils the role of a "link" between ATP and enzyme surface. Clearly a full understanding of the role of Mg^{++} in relation to the function of mitochondrial membranes would greatly facilitate an explanation of its role as an activator of these ATPase enzyme systems.

Recently Falcone (1959) has asserted that the Pi-ATP exchange reaction is the "site" of the uncoupling action of salicylate on oxidative phosphorylation. Hulsman and Slater (1957) have claimed that there are three pH optima associated with the three step oxidative phosphorylation of glutamate by "intact" guinea pig heart mitochondria. These three optima were at pH 6.4, 7.4 and 8.5. There were only two such optima found on examination of the two step reaction associated with the oxidation of succinate (pH 6.4 and 7.4) and only one (pH 6.4) when ascorbate was the substrate.

These workers have since associated these optima with the

three corresponding optima found for ATPase activity, and have suggested that these observations are aspects of the same three reactions which are thought to be the three phosphorylating sites of the respiratory chain (Slater, 1958).

Therefore, and only by inference, it may be assumed that the ATPase enzyme system having a pH optima at pH 9.4 is not associated with the respiratory chain. It is possible that this system may have a different "intramitochondrial" loci from the other ATPase enzyme systems and it may be the reaction which is associated with the "substrate level" phosphorylation accompanying the coupled oxidation of α-keto-glutaric acid. It is this pH 9.4 characterised ATPase reaction of fresh mitochondrial preparations which is least stimulated by dinitrophenol, and is unchanged or perhaps slightly inhibited by salicylate.

However, the evidence of Hulsman and Slater was derived from a curve relating the efficiency of oxidative phosphorylation (P/O) to the pH of the reaction and these peaks have not been found when β-hydroxybutyrate was the substrate for rat liver mitochondria (Cooper and Lehninger, 1956) (Chance, 1956) (Chance and Conrad, 1959). The concept of Hulsman and Slater has been criticised on this and other grounds (Aldridge and Parker, 1960). Therefore it would seem unwise to conclude at this stage that the salicylate enhancement of ATPase activity at pH 6.3, 7.4 and 8.5, in conjunction with the absence of an effect at pH 9.4, is the mechanism by which salicylate inhibits the phosphorylations associated with the "coupled" <u>in vitro</u> oxidation of β-hydroxybutyrate and succinate, whilst there remains one salicylate insensitive step in the coupled oxidation of α-keto-glutarate, although this is an attractive hypothesis.

Before this postulate could be accepted as the mechanism of the *in vitro* uncoupling effect of salicylate on mitochondrial oxidative phosphorylation, it will be necessary to separate these enzyme systems from each other and examine the action of salicylate upon each, under the conditions employed for the measurement of oxidative phosphorylation *in vitro*.

When the ATPase activity of mitochondria prepared from the livers of rats treated with salicylate *in vivo* was examined, it was found that the "patterns" of activity were similar to, but reduced from the mean of those found with mitochondria isolated from the livers of untreated rats. That is, the activity was qualitatively similar but quantitatively different. This reduced activity may be associated with the loss of an unknown co-factor for ATPase activity, which loss may be consequent upon high intracellular salicylate concentrations *in vivo* for periods of up to seven days.

These results are in accordance with the finding of "coupled" mitochondria when these organelles were obtained from the livers of rats treated with salicylate *in vivo* and examined for their efficiency of oxidative phosphorylation *in vitro* (cf. Chapter III, Part I.C of this thesis). This finding is in contrast to the effect seen when salicylate addition is made to ATPase test systems *in vitro*.

Whilst this particular investigation may have explained the mechanism by which salicylate uncouples oxidative phosphorylation *in vitro*, the physiological significance of this effect is still in doubt. Again an apparent discrepancy exists between the action of salicylate upon an enzymic process *in vitro* and *in vivo*.

However, it has been shown in Chapter VI of this thesis that

under certain conditions salicylate may readily pass into or out of mitochondria. It has been suggested that salicylate can be lost from mitochondria during their isolation from the livers of rats treated with the drug *in vivo*. Therefore, the effect of salicylate upon oxidative phosphorylation *in vitro* was re-examined by using mitochondria derived from untreated rats, but which were subjected to pre-treatment by "soaking" in a salicylate containing medium during their preparation. The results of this investigation are presented in the following section of this thesis.

CHAPTER VII

Part II

A RE-EXAMINATION OF THE EFFECT OF SALICYLATE UPON MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

Introduction

In this section, a second aspect of the effect of "intramitochondrial" salicylate upon mitochondrial enzyme function was examined. Once more the "complete" system of oxidative phosphorylation exhibited by isolated rat liver mitochondria was employed. In these studies the mitochondria were derived from the livers of untreated rats but were then subsequently "soaked" in a sucrose medium containing 5 mM sodium salicylate. The effect of this pre-treatment was compared to that known to accompany the direct addition of 5 mM sodium salicylate to reaction mixtures *in vitro* (cf. Chapter III, Part I.B of this thesis).

Materials and Methods

All mitochondria were prepared from the livers of male black and white rats weighing between 250 and 300 gms. These rats were comparable to those used in the previous study of the effects of salicylate on oxidative phosphorylation. The method of isolation of the mitochondria was similar to those described in Part III of Chapter VI of this thesis. That is all mitochondria were subjected to a "soaking" procedure. Duplicate mitochondrial pellets from each batch were re-suspended at 0°C for exactly the same time in a known volume of media composed of either 0.44 M sucrose or 0.44 M sucrose plus 5 mM sodium salicylate.

After the "soaking" period all the mitochondria were quickly re-sedimented and carefully drained free of any remaining supernatant fluid by the procedure described on p. 135. All the mitochondrial pellets were then re-suspended to a small volume (usually 1.5 mls./ pellet) in ice-cold 0.25 M sucrose. 0.5 ml. aliquots of these mitochondrial suspensions were then added to Warburg vessels which contained complete reaction mixtures previously shown to support oxidative phosphorylation. The conditions of measurement of oxygen uptake and inorganic phosphate utilisation were identical with those described in the previous study of these phenomena (cf. p. 76).

Some mitochondrial pellets which had been "soaked" in the salicylate containing sucrose medium were weighed and used for either the determination of their salicylate content or their water content, again by methods described previously in Part I of Chapter VI of this thesis.

Results and Discussion

Although there was an overall decrease in the mean P/O ratios obtained when mitochondria "pre-treated" with 5 mM sodium salicylate utilised α-keto-glutarate, succinate or β-hydroxybutyrate as substrates in these systems, there was little difference in the efficiency with which these preparations carried out coupled phosphorylation. This finding was irrespective of the presence of 5 mM sodium salicylate in the "soaking" medium.

These results are shown in Table (32). This general decrease in P/O ratio may be a reflection of the increased time required to

TABLE (32)

SALICYLATE SOAKED MITOCHONDRIA

The P/O ratios of normal rat liver mitochondria after soaking at 0°C in 0·44M sucrose, with and without various concentrations of salicylate

EXPERIMENT NO.	SUBSTRATE	P/O WITHOUT SALICYLATE	P/O WITH SALICYLATE	MOLAR CONCENTRATION OF SALICYLATE IN MITOCHONDRIAL PELLETS
1	α-Keto-Glutarate	2·8 2·9	2·1 2·4	5×10^{-3} M
2	"	2·2 2·6	2·2 2·2	$4{\cdot}8 \times 10^{-3}$ M
3	"	1·6 2·4	1·9 1·2	1×10^{-2} M
4	Succinate	1·7 1·4	0·9 1·1	$1{\cdot}1 \times 10^{-2}$ M
5	β-Hydroxy-Butyrate	1·2 1·0	0·8 0·7	$5{\cdot}1 \times 10^{-3}$ M

The reaction mixture was as described in the text [p. 76] with the addition of mM Mg^{++} as magnesium sulphate.

prepare mitochondria by a method embodying a "soaking" phase, which probably increases the "leakage" of intramitochondrial nucleotides and other factors from mitochondria (Lehninger, 1951 and 1960).

This demonstration of active phosphorylation by mitochondria which had been pre-treated with salicylate in vitro is again a positive finding similar to that of "coupled" mitochondria isolated from rats treated with salicylate in vivo. This demonstrates that the contact of mitochondria with 5 mM sodium salicylate immediately prior to the measurement of oxidative phosphorylation in vitro, has no significant effect on the efficiency of this process.

It was of interest to calculate the maximum amount of salicylate radical that was introduced into the final test system via the mitochondrial pellets which had been "soaked" in media containing 5 mM sodium salicylate, because salicylate introduced in this way may behave as a direct in vitro addition of the drug to the test system.

The concentration of salicylate was assayed in a mitochondrial pellet suspension from every batch pre-treated with the drug. The maximum quantity encountered in these experiments was 280 ug./pellet. In this particular experiment, three other similar pellets which had also been subjected to the same "soaking" treatment were used in the study of oxidative phosphorylation. These three pellets had been finally re-suspended to a total volume of 5 mls. in 0.25 M sucrose. 0.5 ml. aliquots of this suspension were added to each test vessel. It was known that the volume of fluid plus mitochondria in the test vessels was 2.5 mls. The concentration of salicylate that would be produced in these systems if it were equally distributed throughout the mixture

was calculated to be 2.4×10^{-4} M. Similar calculations were performed for each experiment, but this value (2.4×10^{-4}) was the maximum obtained. No direct observations were made of the effect upon mitochondrial oxidative phosphorylation of the *in vitro* addition of this concentration of salicylate but in a previous study (p. 81) it had been shown that this concentration of salicylate had some effect on the P/O ratios. Jeffrey and Smith (1959) reported that concentrations of salicylate of this order were the minimum levels at which mitochondrial oxidative phosphorylation was affected.

Therefore, it seems reasonable to disregard the possibility of sufficient salicylate being introduced into these test systems via a 0.5 ml. aliquot of the mitochondrial suspension to duplicate the marked effect of a direct *in vitro* addition of the drug. It is highly probable that the concentration of "intramitochondrial" salicylate is markedly decreased once the mitochondria are dispersed through the reaction mixture for the measurement of oxidative phosphorylation, as previous studies have suggested that salicylate is readily lost from mitochondria.

To achieve a higher concentration of salicylate within mitochondria by this "soaking" method would require concentrations of salicylate in the "soaking" medium far above those likely to be encountered *in vivo*. In these particular experiments, this method gave a calculated intramitochondrial salicylate concentration ranging from 4.8×10^{-3} M to 1.1×10^{-2} M. Although the possibility of surface adsorption cannot be excluded, and if operative, would decrease this calculated concentration, the probability of salicylate being dispersed through less than the total water space of mitochondria would tend to

increase the calculated value. These factors have been discussed previously. In addition the provision of higher "intramitochondrial" salicylate concentrations would almost certainly introduce sufficient salicylate to the final test medium to duplicate a direct _in vitro_ addition of this drug. If this happened much of the intention of this particular study would be lost.

Although the acceptance of this conclusion precluded further study of this aspect of the investigation, it had already shown that any action of salicylate on mitochondrial oxidative phosphorylation _in vitro_ as well as _in vivo_, can only be associated with the physical presence of relatively high levels of the drug in the immediate mitochondrial environment.

Contact between mitochondria and 5 mM sodium salicylate _in vitro_ did not produce any apparent irreversible changes in the function of mitochondrial enzymes associated with oxidative phosphorylation, and thus it is unlikely that irreversible effects would result _in vivo_ from temporarily elevated levels of salicylate in the cell "cytoplasm". This is in accordance with the transient nature of the hypermetabolic response produced by salicylate in the whole animal.

CHAPTER VII

Part III

CONCLUSIONS

In Parts I and II of this Chapter two closely related aspects of mitochondrial enzyme function were examined in order to assess the possibility of an effect of the binding of salicylate to intramitochondrial enzyme systems associated with the functions of oxidative phosphorylation.

Within the limits of the experimental methods employed, it appears that the presence of relatively high concentrations of salicylate "within" the mitochondria immediately prior to their addition to the respective test systems studied, did not lead to significant alteration of normal enzyme function.

However, the possibility that the salicylate was entirely adsorbed to the mitochondrial membrane and hence "extramitochondrial" cannot be excluded although it seems rather improbable. On the other hand, it is felt that there is stronger evidence supporting the claim that salicylate can be lost from within the mitochondria as readily as it can enter. Sufficient dilution of the so-called "intramitochondrial" concentration of the drug may take place during the experimental preparation of mitochondria from different sources to reduce the concentration of salicylate below a level which would disturb mitochondrial enzyme function.

The results of the study of mitochondrial ATPase activity strongly imply a primary action of salicylate on mitochondrial membrane permeabiliby. This is an extramitochondrial rather than an intramitochondrial effect, and thus does not fall within the concepts defined by the third hypothesis.

This concept of a primary action of salicylate on mitochondrial membrane permeability has been related in a speculative manner to a mechanism by which salicylate inhibits three of the four stages of the coupled phosphorylations associated with the efficient utilisation of α-keto-gluteric acid by mitochondria.

Salicylate does not irreversibly affect mitochondrial ATPase activity nor does it cause a release of these systems from mitochondria, but must of itself be in physical contact with the mitochondrial membrane for enhanced activity to occur. Perhaps the complete answer to the apparent differences in the response of ATPase activity to salicylate and Mg^{++}, is that the pH may effect the penetration or ionisation of Mg^{++}, ATP^{++}, and the permeability of the mitochondrial membrane whilst salicylate effects only the permeability of the mitochondrial membrane. The pattern of ATPase activity seen with fresh mitochondrial preparations would be the sum of these phenomena.

These changes were all readily reversible by the subsequent dilution of the salicylate concentration. This may provide a simple explanation for the non-cumulative, transient effect of salicylate on the oxygen consumption of the rat (cf. Chapter II, Part II.C).

Further examination of the action of salicylate upon mitochondrial membrane permeability leads directly to an examination of the fourth of the hypotheses proposed earlier. The results of this investigation are presented in the next Chapter.

CHAPTER VIII

AN EXAMINATION OF THE FOURTH HYPOTHESIS

"That salicylate may effect the permeability of the mitochondrial membrane to normal intracellular substrates, ions or co-factors required for oxidative phosphorylation".

Part I: The effect of salicylate on the water content of mitochondria

A. The effect of salicylate *in vivo*.

B. The effect of salicylate *in vitro*.

Part II: The effect of salicylate on the processes of "swelling and contraction" of isolated rat liver mitochondria *in vitro*.

Part III: The effect of salicylate on electrolyte exchange in isolated rat liver mitochondria at $0^{o}C$.

Part IV: Conclusions.

CHAPTER VIII

AN EXAMINATION OF THE FOURTH HYPOTHESIS

A number of distinct methods of approach were employed to investigate this hypothesis. The results of these investigations will be presented as separate studies.

Part I

THE EFFECT OF SALICYLATE ON THE WATER CONTENT OF MITOCHONDRIA

A. The effect of salicylate in vivo

Introduction

It has long been established that one of the changes associated with an alteration in the permeability of a cellular membrane, is a change in the total water content of the cell (Claude, 1946). Recently this concept has been examined in relation to the mitochondrial membrane and the finding confirmed for the sub-cellular particles (Price, Fonnesu and Davies, 1956) (Lehninger, 1959).

As a direct gravimetric estimation of water content was employed by these investigators, it was decided to use this relatively simple technique in an examination of the water content of mitochondria obtained from untreated rats and those treated with salicylate *in vivo*.

Materials and Methods

Rats were treated with either sodium or potassium salicylate *in vivo*. The drug was given by gavage in the usual dosage. Liver mitochondria were prepared by the method described previously (p.75).

The mitochondrial pellet was obtained in a tared glass high-speed centrifuge tube and the supernatant fluid removed as completely as possible (cf. 135).

The wet weight of the pellet was then quickly obtained by difference, loss by evaporation being negligible during this operation. The mitochondrial pellet was then dried at 105°C to constant weight after which time the tubes plus mitochondria were cooled to room temperature in a dessicator and weighed again. The dry weight of the mitochondria was obtained by difference. The percentage water content was calculated from this data. The mitochondria were always prepared from a 1:10 homogenate of whole liver in 0.44 M sucrose without the addition of either chelating or buffering agents. The washing medium was also o.44 M sucrose without further additions.

Results

The average values determined for the mitochondrial yields/gm. wet weight of liver were not greatly different in either group of rats. However, there was a small increase in the average mitochondrial water content in the group treated with salicylate (71.6% instead of 70.8%). These results which are not significant ($P > 5\%$) are given in detail in Table (33).

Discussion

These results could not be taken to confirm an action of salicylate on mitochondrial water content *in vivo*. However, it has been shown by Lehninger (1960) that the passive, i.e. non-metabolically controlled, osmotic adjustments by mitochondria to their environment may be extremely rapid, often of the order of seconds rather than minutes. Hence it is possible that any variations in mitochondrial

TABLE (33)

THE in vivo EFFECT OF SALICYLATE ON THE WATER CONTENT AND MITOCHONDRIAL YIELD OF RAT LIVER

GROUP	SALT GIVEN	DAYS OF TREATMENT	PLASMA SALICYLATE CONCENTRATION	MITOCHONDRIA	
				% H_2O content	yield wet weight/ gm wet liver
Normal:					
22 - 10	None	-	-	69·9	·3304
22 - 10	"	-	-	68·0	·2200
18 - 11	"	-	-	71·1	·3480
20 - 11	"	-	-	72·5	·3170
24 - 11	"	-	-	70·4	·2955
26 - 11	"	-	-	73·0	·3280
				average 70·8	average ·306
Salicylate:					
8 - 10	Potassium	5	36	69·4	·2390
8 - 10	"	"	48	68·2	·2300
9 - 10	"	"	60	68·6	·2440
10 - 10	"	"	48	71·9	·2440
3 - 12	sodium	6	57	74·7	·3940
4 - 12	"	"	51	73·8	·4000
5 - 12	"	"	32	73·0	·3863
8 - 12	"	"	52	70·6	·3180
10 - 3	"	14	38	73·8	·2690
11 - 3	"	14	42	71·6	·2320
				average 71·6	average ·296

water content which may exist *in vivo* would be lost or diminished during the separation of the mitochondria from the cell. Furthermore, the mitochondria from any source would come into osmotic equilibrium with their *in vitro* environment (which in this instance was 0.44 M sucrose). Hence the determined water contents may be merely a reflection of this phenomenon.

Because of this belief, the effect of salicylate on mitochondrial water content *in vitro* was examined. The results of this latter investigation will be presented in the following sections.

B. The effect of salicylate on mitochondrial water content in vitro

Introduction

If an *in vivo* effect of salicylate on mitochondrial water content were being diminished or abolished during the preparation and isolation of these particles, then differences in water content may be apparent if mitochondria were initially isolated from untreated rats and then subsequently suspended in media of similar osmolarity, only one of which contained salicylate.

Materials and Methods

Replicate mitochondrial pellets were prepared as before. After they had been rendered as free as possible of other cellular components they were re-suspended into a small volume, usually 4 mls. per pellet, of a medium which was either 0.44 M sucrose without any additions or 0.44 M sucrose plus 5 mM sodium salicylate. The time of re-suspension, known as the "soaking" phase, was from ten to twenty-five minutes during which period the temperature was maintained at 0°C.

After this procedure the mitochondria were rapidly re-sedimented by centrifuging at 12,000 x g for ten minutes at 0°C, and the pellet so formed drained free of supernatant material as described in Part A. above. The percentage water content of the mitochondrial pellets was then determined gravimetrically.

Results

The presence of sodium salicylate in the medium during the "soaking" phase increased the average water content of the mitochondria from a control value of 79.5% to a value of 81.8%. The effect of sodium salicylate became more apparent with an increase in the length of the "soaking" period, but did not reach statistical significance.

These results are shown in Table (34).

Discussion

The difference between the water content of "untreated" mitochondria in Parts A. and B. of this study is a reflection of the increased time of contact between the medium and the mitochondria in Part B. of this experiment. The data obtained in this *in vitro* study suggest that the observation of a small increase in mitochondrial water content under the influence of salicylate *in vivo* (Part A. of this section), may have been a reflection of an *in vivo* effect. This *in vivo* effect was probably diminished during the isolation of the mitochondria. As the salicylate induced increase in water content *in vitro*, appears to increase with time, the data also imply that this increase is not simply an adjustment by the mitochondria to their osmotic environment. The difference in osmolarity between the two

TABLE (34)

THE in vitro EFFECT OF SODIUM SALICYLATE ON THE WATER CONTENT OF RAT LIVER MITOCHONDRIA AT 0°C

NUMBER OF EXPERIMENTS	SOAKING TIME IN MINUTES	PERCENTAGE WATER CONTENT OF MITOCHONDRIA	
		In 0·44 M Sucrose	In 0·44 M Sucrose + 0·005 M Sodium Salicylate
1	10	81·5	81·8
2	10	80·5	80·6
3	15	78·0	78·0
4	15	80·9	80·3
5	20	76·6	78·8
6	20	78·1	80·1
7	25	81·1	84·0
	Average	79·5	81·8

media used here must be extremely slight because of the low concentration of the sodium salicylate.

Possibly this action of sodium salicylate reflects a change in permeability of the mitochondrial membrane; an effect which is either promoting the entry of sucrose or sodium salicylate ions into the mitochondria, or the loss of some factor from the mitochondria to the medium which results in a change in water distribution. These differences in mitochondrial water content were not large, either in vivo or in vitro, and can only be taken as an indication of a change in mitochondrial permeability. Furthermore, no control was provided for the effect of sodium ions.

Also it is difficult to relate observations made in such static non-metabolising test systems in vitro to possible conditions prevailing in an actively metabolising cell in situ. These difficulties are increased when it is recalled that recent work by Lehninger, Ray and Schneider (1959) has shown that sucrose itself has an inhibitory effect upon the "spontaneous" swelling processes of rat liver mitochondria.

However, these observations prompted further investigation of the effect of salicylate upon mitochondrial water content by use of other methods. The first of these is discussed in Part II of this Chapter.

CHAPTER VIII

Part II

THE EFFECT OF SALICYLATE ON THE PROCESSES OF SWELLING AND CONTRACTION OF ISOLATED RAT LIVER MITOCHONDRIA IN VITRO

Introduction

Since thyroxine and other compounds known to increase metabolic rate in the whole animal, also uncouple oxidative phosphorylation and cause mitochondria to swell *in vitro*, it was thought of interest to examine the effect of salicylate on the "swelling" and "contraction" of isolated rat liver mitochondria. Although not all of the uncoupling agents so far examined have been active in this regard, it has been suggested by Lehninger and his group that such activity is the physiological basis for the action of the thyroid hormone.

These phenomena were investigated with mitochondria isolated from normal rat liver and with mitochondria isolated from the livers of rats treated with salicylate *in vivo*. As it has now been firmly established that a decrease in the optical density of a mitochondrial suspension measured at a fixed wave-length in a spectrophotometer, corresponds to a gain in total mitochondrial fluid content (Lehninger, 1959b), the results of this investigation would also relate to the previous observations on mitochondrial water content which were made in Part I of this Chapter.

<u>Preliminary consideration of the significance of data obtained by this method.</u>

Since the demonstration by Harris (1943) that the mitochondria of intact cells were sensitive to changes in osmolarity, and the work of Claude (1946) which showed that isolated heart sarcosomes swell in hypotonic sucrose, many workers have used a change in the optical density of a mitochondrial suspension as an index of the water content, morphological stability and the permeability of mitochondria (Cleland, 1952) (Raaflaub, 1953). Lehninger has now clearly established that a "swelling" of mitochondria corresponds to a gain in total intramitochondrial fluid content (Lehninger, 1959b).

Several attempts have been made to determine whether a relationship exists between the so-called "swelling" and "contraction" phenomena and a functional property of mitochondria, and in particular their ability to carry out oxidative phosphorylation (Harman and Feigelson, 1952 a, 1952 b) (Price, Fonnesu and Davies, 1956). Conflicting results and incomplete knowledge of the mechanisms of either process have rendered the evaluation of such work difficult. In fact many workers have concluded that these observations were of no physiological significance (Slater, 1959).

However, within the last few years much more information has been gained relating to the mechanism of oxidative phosphorylation and the "swelling" and "contraction" processes. These latter phenomena have now been shown to be highly complex interactions of many effects. Before proceeding to an examination of experimental data, a discussion of the present state of knowledge of the possible mechanisms involved in the processes of mitochondrial "swelling" and "contraction" will be presented.

(a) Mitochondrial volume expansion - "swelling"

Since the recent independent reports of Chappell and Greville (1958) and Hunter, Levy, Fink, Schutz, Guerra and Hurlvitz (1959), it has become possible to recognise the existence of two distinct phases during the process of mitochondrial swelling.

The first and most rapid phase is the prompt adjustment (which may be either swelling or shrinking) of the mitochondrial volume to the osmotic pressure of the medium, and to the nature of the external solutes. This adjustment is normally complete in a matter of seconds. As it does not require the presence of an oxidisable substrate, it has therefore been termed "passive". These changes are superimposed on and may obscure those of the "active" or second phase. This latter phase is much slower than the first, it is non-osmotic in nature and is enzymatically controlled specifically by the action or condition of the mitochondrial "respiratory chains" responsible for electron transport and coupled ATP production. It is this phase which represents true mitochondrial "swelling" and whilst a low intramitochondrial ATP content may increase the susceptibility of these particles to swelling (Brenner-Holzack and Raaflaub, 1954) (Ernster and Lindberg, 1958), this process cannot occur unless some respiration, albeit of endogenous substrate, is taking place. In addition this swelling is completely abolished by anaerobiosis or such respiratory inhibitors as cyanide or the barbiturate sodium amytal. It is significant that these "respiratory chains" have been shown to be present in the mitochondrial membrane (Tapley and Cooper, 1956).

Evidence that different intracellular conditions _in vivo_ can effect the spontaneous swelling phenomena of mitochondria comes from the work of Tapley (1956a) who demonstrated that liver mitochondria from

hyperthyroid rats had a much higher spontaneous swelling rate than did liver mitochondria from normal rats. Mitochondria from hypothyroid rats were, by comparison, very resistant to spontaneous swelling. When these findings are considered in conjunction with the electron microscope evidence that the mitochondria of hyperthyroid tissues are distinctly swollen and show diffuse cristae (Schutz, Low, Ernster and Sjörstrand, 1957) this becomes some of the most convincing evidence for the physiological significance of mitochondrial swelling in vivo.

(b) Mitochondrial contraction

Like the swelling phenomenon, this process can also be either "passive" or "active".

The passive phase of contraction is that observed to follow the addition of high molecular weight substances such as polyvinyl pyrrolidone, to already swollen mitochondrial preparations. The response found in these circumstances is identical with that which would be apparent in any structure, animate or inanimate, which was surrounded by a membrane impermeable to such large molecules. This process is not affected by respiratory inhibitors.

On the other hand, Price, Fonnesu and Davies (1956) and Beyer, Ernster, Low and Beyer (1955) have presented evidence for an active phase of the contraction process of mitochondria. They clearly demonstrated that swollen mitochondria could be induced to contract if respiration were instituted. Such observations are best made in the absence of sucrose as this substance itself inhibits both mitochondrial swelling and contraction as well as other mitochondrial functions (Potter and Recknagel, 1951) (Lehninger, 1959b).

There is some evidence that the active phase of mitochondrial

contraction specifically requires ATP in vitro as other nucleotide triphosphates and ADP are inactive (Lehninger, 1959a) (Keller and Lotspeich, 1959), except that ADP may have some activity through the action of mitochondrial adenylate kinase (Lehninger, 1959c). Previously Fonnesu and Davies (1956) had demonstrated that in their experiments adenosine monophosphate (AMP) was more beneficial than either ADP or ATP. However, the conditions of incubation used by these latter workers may account for this observation as there was considerable liberation of inorganic phosphorus from both ADP and ATP, and this substance is known to cause swelling (Price, Fonnesu and Davies, 1956).

At least one aspect of mitochondrial contraction is exceptionally stable and has been shown to be independent of an intact functioning respiratory chain and "phosphate" coupling mechanism; unlike the active swelling process it can proceed maximally in the presence of cyanide, and is not inhibited by dinitrophenol.

Recently, Lehninger and Gotterer (1960) have demonstrated that the contractile process requires the presence of a soluble protein which can escape from swollen mitochondria. This protein will restore activity to swollen mitochondria if replaced via the addition of the medium in which the original swelling took place. During this process ATP is hydrolysed at the rate of about one mole to several hundred moles of water extruded and ATP hydrolysis ceases when contraction stops.

The majority of the remarks concerning mitochondrial contraction are based on the reports of Lehninger's group, who worked almost exclusively with thyroxine swollen mitochondria. It is the opinion of Lehninger that the reversal of thyroxine induced swelling has somewhat different properties and requirements from that induced by

other agents. This reasoning is employed by Lehninger to differentiate the "stable" cyanide insensitive contraction process from the respiration induced, dinitrophenol sensitive, reversal of mitochondrial swelling observed by Price, Fonnesu and Davies (1956).

These latter workers have shown that respiring mitochondria maintain an initially low water content when incubated in an ionic medium with sodium succinate as substrate. If ATP is absent from the medium, respiration continues essentially unchanged but the mitochondria take up water. If swelling has not proceeded too far, the subsequent addition of ATP can cause the extrusion of water. This "active" contraction process is coincident with the resumption of oxidative phosphorylation and can be abolished by 10^{-4} M dinitrophenol.

Thus it can be seen that whilst the rapid phases of this swelling and contraction phenomena are non-metabolically controlled "passive" processes reflecting merely osmotic adjustments to the external medium, there are at least two distinct processes responsible for metabolic swelling and contraction, both of which have different enzymatic components, and one is not the simple reversal of the other. These processes may be morphologically as well as enzymatically distinct. Recent findings on the permeability of the mitochondrial membranes and compartmentalisation of intramitochondrial water (Lehninger, Ray and Schneider, 1959) (Tedeschi and Harris, 1955) suggest that the "outer" of the "two" mitochondrial membranes is freely permeable to most of the solutes so far examined, and that the "inner membrane" is more likely to be the site of the swelling and contraction phenomena. Presumably it is the "inner membrane" which is the site of the respiratory chain enzyme assemblies. Arguing from the electron microscope findings of Palade (1956), Lehninger has recently suggested that a reversible "unpleating"

and "repleating" of the invaginations of this inner membrane, i.e. the mitochondrial cristae, may be responsible for these effects.

Diagrammatic presentation of the various phases of these processes is given in Figure XIV. The results of an exploration of the foregoing concepts, with particular reference to the action of salicylate on these processes, will now be presented.

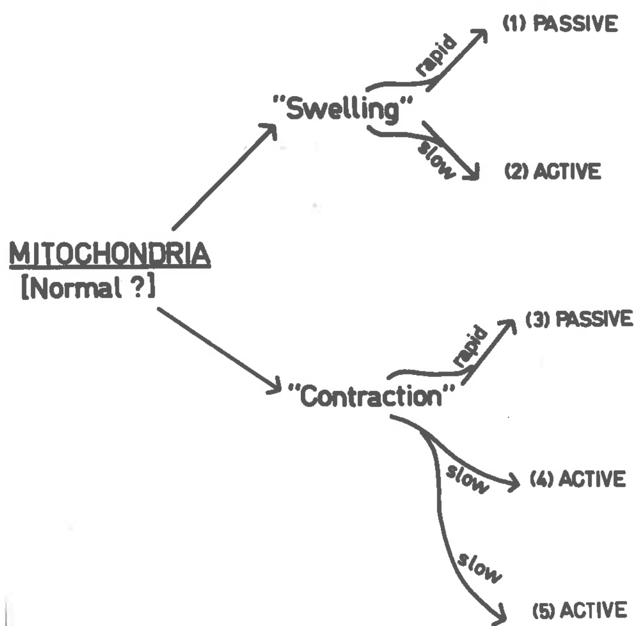
Materials and Methods

Only young, actively growing male black and white rats weighing approximately 200 gms. were used for this study. These animals were selected because Weinbach and Garbus (1956, 1959) had demonstrated that there is a decrease in the rates of phosphorylation of liver tissues of older animals which was associated with a decreased nucleotide content of those tissues, and Ernster and Lindberg (1958) had suggested that a lowered ATP content of mitochondria increased the tendency of the isolated particles towards "spontaneous" swelling.

Those animals treated with salicylate *in vivo*, received by gavage the same dosage as that employed for the study of the effects of salicylate on mitochondrial oxidative phosphorylation (30 mgms. salicylate radical/day/100 gm. body weight). Treatment was maintained until the animals exhibited unmistakable signs of salicylate toxicity, which was usually after the fourth day.

The method used for the preparation of the mitochondria was similar to that described for an examination of mitochondrial oxidative phosphorylation (p. 75). However, in this study the initial homogenising medium was either 0.44 M sucrose or 0.25 M sucrose, which sometimes contained 1 mM EDTA. When this chelating agent was present, the pH of the mixture was adjusted with potassium hydroxide to pH 6.8.

FIGURE XIV



(1) Purely osmotic. Not inhibited by anaerobiosis or cyanide.

(2) Non-osmotic, requires respiration (oxygen uptake) and hence must have substrate and a functioning electron transport chain. Inhibited by anaerobiosis and cyanide.

(3) Swelling reversed by high molecular weight substances. Not affected by inhibition.

(4) Requires ATP and a soluble protein. Very stable. May be a consequence of respiration although no effect of cyanide. Is not the reversal of (2). Is suppressed by sucrose.

(5) Requires ATP and hence oxidative phosphorylation, abolished by DNP.

The washing and final suspending solutions were always without further additions. The actual solutions employed are indicated with each set of experimental results.

The mitochondria derived from one gram wet weight of rat liver were suspended in a volume of 10 mls. of sucrose, and less than 0.1 ml. of this suspension was added to 3 ml. aliquots of the test medium contained in the spectrophotometer cuvettes. The actual amount of mitochondrial suspension was varied so that the initial optical density of the test system could be adjusted to about 0.5.

The test medium for the observation of what were primarily osmotic effects was a mixture of 0.3 molar sucrose and 0.02 molar tris hydroxymethylaminomethane (TRIS) buffer, adjusted to pH 7.4 with hydrochloric acid (cf. Tapley, 1956a). The medium used for the observation of metabolic or "active" phases of this phenomena had both to contain a substrate and be ionic in nature, because high concentrations of sucrose have been shown to inhibit these processes (Lehninger, 1959b). It was possible to adapt (by a total volume change) the reaction mixture previously used for the measurement of oxidative phosphorylation into one suitable for these measurements.

This mixture contained 100 u.mols. of phosphate buffer, 48 u.mols. potassium chloride, 5 u.mols. ATP as the sodium salt, 60 u.mols. glucose, 60 u.mols. potassium fluoride and 25 u.mols. of substrate. To these were added 30 units of hexokinase (Sigma grade III). Mg^{++} (as the chloride), and salicylate (as either the sodium or potassium salt) were added to the concentrations indicated in the data given with individual experiments.

The pH of this mixture was adjusted to pH 6.8 with potassium

hydroxide and the final volume adjusted to 3 mls. by the addition of 0.5 mls. of 0.25 M sucrose. After dilution into the total volume of this system, this amount of sucrose gave a final concentration of only 0.05 M. This concentration was known to have practically no effect on the swelling and contraction of mitochondria in a potassium chloride - tris medium at pH 7.4 (Lehninger, 1959b). To this mixture was added a similar quantity of the mitochondrial suspension as that used in the "passive" experiments described immediately above.

It was realised that this mixture contained many factors which could affect the swelling of mitochondria in diverse ways, nevertheless it was comparable to that medium in which salicylate was known to uncouple oxidative phosphorylation in vitro.

The major difference between these and the oxidative phosphorylation test systems, is that there is a vast dilution of the mitochondrial concentration in these experiments when compared to those for the measurement of oxidative phosphorylation.

Optical density readings were taken as soon as possible after the addition of the mitochondria to the test medium, and thence serially at short intervals for the next twenty to thirty minutes. In a few experiments the observations were continued for forty minutes. The measurement of optical density changes were made in a Beckman model DU spectrophotometer at 520 mu. and at a controlled room temperature of 23°C.

The addition of "swelling" agents such as salicylate were made to the test systems in minimal volumes (usually 0.1 ml.) at various times throughout the period of observation. This information is shown with the data of individual experiments. When necessary any volume change was controlled by the addition of the same volume of solvent (water) to the reference and "control" test vessels.

Results

Preliminary observations

(a) The effect of a change in the pH of the observation medium

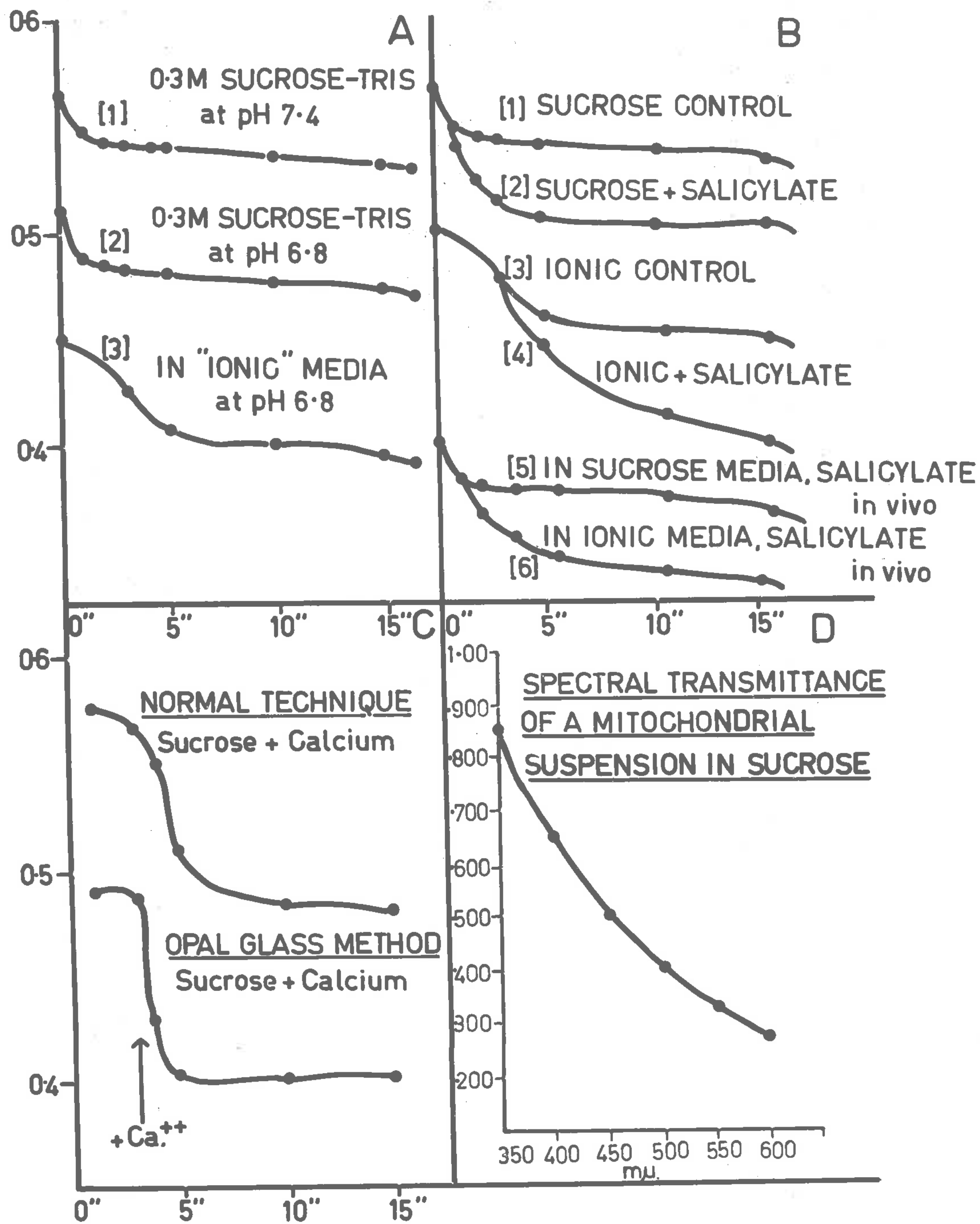
There is some evidence that the pH of intracellular fluid *in vivo* is more acid than the plasma (Chambers and Fell, 1931) (Hill and Kupalov, 1930) (Caldwell, 1954), although the usual pH for these experiments is that of the plasma, i.e. pH 7.4. Here the effect of lowering the pH of the medium to pH 6.8 was determined while all other conditions remained constant. The sucrose - tris medium of Tapley (1956) which is isotonic with intracellular fluid (Krebs and Henseleit, 1932) (Conway and McCormack, 1953) was employed in each case.

By comparison of curves (1) and (2) in Figure XV (A) it can be seen that a change of pH from pH 7.4 to pH 6.8 had no effect upon the "passive" or spontaneous swelling of isolated liver mitochondria from normal rats, when all other factors were constant.

(b) The effect of varying the composition of the observation medium

Many workers have made the plea that experiments on mitochondria which are designed to relate to the physiology and biochemistry of the whole animal, should be conducted in an environment which resembles as closely as possible the cell fluid itself (Harrington, 1957) (Aldridge, 1957). Therefore, comparison was made between some properties of rat liver mitochondria examined in an "unphysiological" sucrose medium and in an "ionic" medium containing potassium and magnesium ions, which are the two major intracellular electrolytes, in the concentrations that are believed to exist in the cell fluid (Hastings, 1940) (Manery, 1954) (Charnock, 1960). The medium contained 0.1 M potassium chloride, 0.01 M magnesium sulphate and

FIGURE XV



ALL TISSUES HOMOGENISED IN 0·44M SUCROSE + mM E.D.T.A.

0.014 M potassium dihydrogen phosphate. The pH was adjusted to pH 6.8 with potassium hydroxide.

The results indicate that where salicylate promotes swelling of mitochondria in a sucrose media, then the same effect is apparent in an "ionic" medium, cf. Figure XV (B), curves (1) to (4) inclusive.

In addition mitochondria from rats treated with salicylate *in vivo* behave in a manner analogous to that of mitochondria isolated by the same procedure from untreated animals, when both were examined in either a sucrose or an ionic media. Compare curves (5) and (6) with the "control" curves (1) and (3) in B. of Figure XV.

(c) Measurement of transmitted or refracted light

The work of Shibata (1958) suggested that the absorption spectra of a translucent mitochondrial suspension may be "sharpened" if an opal plate were interposed between the detector side of the sample and the reference cells of a spectrophotometer. Such a technique is designed to change the geometry of the incident light measured by the photo cell and ensure that the contribution due to refracted light from surfaces within the suspension is minimised (Procedure A, Shibata, 1959).

Although the fall in optical density which is produced by the addition of Ca^{++} to a mitochondrial suspension appears sharper when measured by the opal glass technique, than when measured by the usual method, the difference did not appear to warrant the adoption of Shibata's method in subsequent experiments. These results are given in Figure XV,C.

(d) The wave-length at which measurements were made

Since the original s tudy of Cleland (1952) many workers have

used his spectrophotometric method which measures the rate of change in optical density of a mitochondrial suspension. A wave-length of 520 mu. has often been used but from time to time there have been reports of observations made at different wave-lengths (Price, Fonnesu and Davies, 1956) (Gayet, 1958) (Chari-Bitron and Avi-Dor, 1959).

Therefore an examination was made of the absorption spectrum of a suspension of mitochondria in a sucrose medium over a restricted range of wave-lengths, in order to determine the possibility of there existing some particular spectral characteristic or optima which may be of value in these studies. Figure XV, D. indicates that the spectral curve so obtained did not possess any maxima or minima characteristics, and hence measurement of this system at any wave-length between 350-600 mu. would be satisfactory.

The effect of salicylate on the processes of swelling and contraction of liver mitochondria isolated from untreated rats.

The mitochondria were always examined in a sucrose-tris system without added substrate, but the method of preparation of the mitochondria varied.

(a) When the method of preparation of the mitochondria was to homogenise whole tissue at 0°C. into an initial medium of 0.44 M sucrose which also contained .001 M EDTA and the pH was adjusted to pH 6.8 with potassium hydroxide.

When mitochondria were prepared in this way and then examined in a system without substrate they were quite "stable". The optical density of the suspension, which usually undergoes an initial change within the first few minutes of observation, then remained constant or very nearly so throughout the remainder of the observation period which

was approximately twenty-five minutes.

The mitochondria were capable of a response to a change in the composition of the medium. This was demonstrated by the addition of varying concentrations of the bivalent cations calcium and magnesium to the system. These ions were added as their chloride salts. As is well known, the addition of Ca^{++} at concentrations above 0.5 mM, to mitochondria suspended in a sucrose medium, produced a rapid and progressive fall in the optical density of the mitochondrial suspension, i.e. the mitochondria became "swollen". The addition of Mg^{++} at slightly higher concentrations (1 mM and above) would either inhibit the progressive swelling produced by salicylate and some other agents, or when added alone Mg^{++} could initiate mitochondrial contraction.

Both the sodium and potassium salts of salicylic acid were found to promote the swelling of isolated mitochondria when added to this system.

These results are shown in Figure XVI.

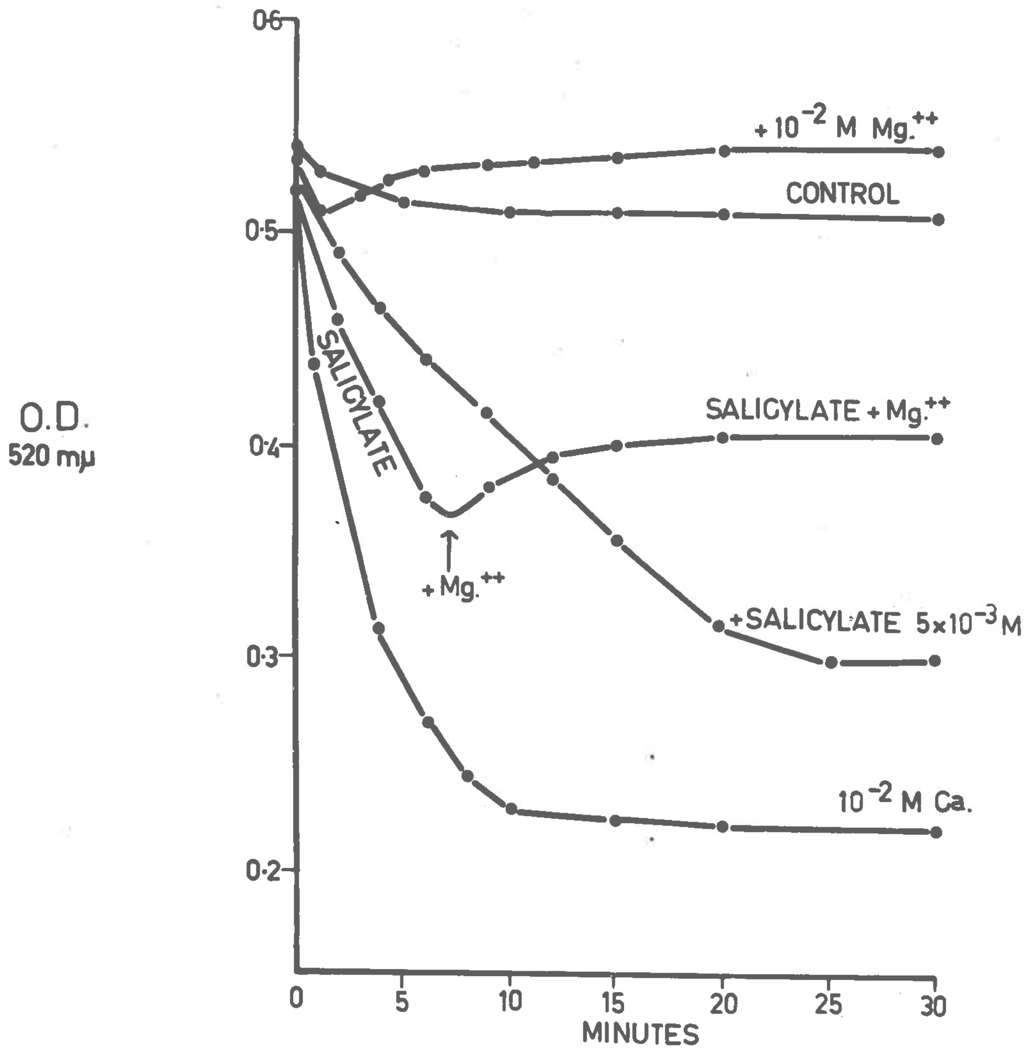
(b) When the method of preparation of the mitochondria was to homogenise whole tissue at 0°C into an initial medium of 0.44 M or 0.25 M sucrose, which did not contain EDTA nor was the pH adjusted.

When mitochondria were prepared by this method and then examined in an identical system to that described in (a) above, they clearly exhibited the phenomenon of "spontaneous" progressive swelling. That is, there was a continuous decrease in the optical density of the mitochondrial suspension. This effect differs markedly from the "stability" of control suspensions found when the tissue was originally homogenised in the presence of EDTA.

Another difference noted in the behaviour of these mitochondria is that the addition of either sodium or potassium salicylate

FIGURE XVI

HOMOGENATE PREPARED IN SUCROSE WITH E.D.T.A.



THE BEHAVIOUR OF MITOCHONDRIA DERIVED FROM THE LIVER OF NORMAL RATS. THE ORIGINAL TISSUE HOMOGENATE WAS PREPARED IN 0·44 M SUCROSE + ·001 M E.D.T.A. pH ADJUSTED TO 6·8 WITH KOH. OBSERVATION MEDIUM 0·3 M SUCROSE + 0·02 M TRIS (pH 7·4)

to give a concentration of 5×10^{-3} M, which has been shown to uncouple oxidative phosphorylation <u>in vitro</u> now inhibited the "spontaneous" swelling seen in the controls. This effect is unlike the swelling effect of salicylate which is seen with mitochondria which were prepared in the presence of EDTA. A similar finding has been reported by Jeffrey and Smith (1959).

The spontaneous swelling of "controls" prepared without the protection of EDTA in the original homogenate could be inhibited by magnesium ions at a concentration of 10 mM, but was not affected by 0.1 mM Mg^{++}.

These results are shown in the graphs (A) and (B) of Figure XVII .

<u>The swelling and contraction properties of liver mitochondria isolated from rats treated with salicylate in vivo.</u>

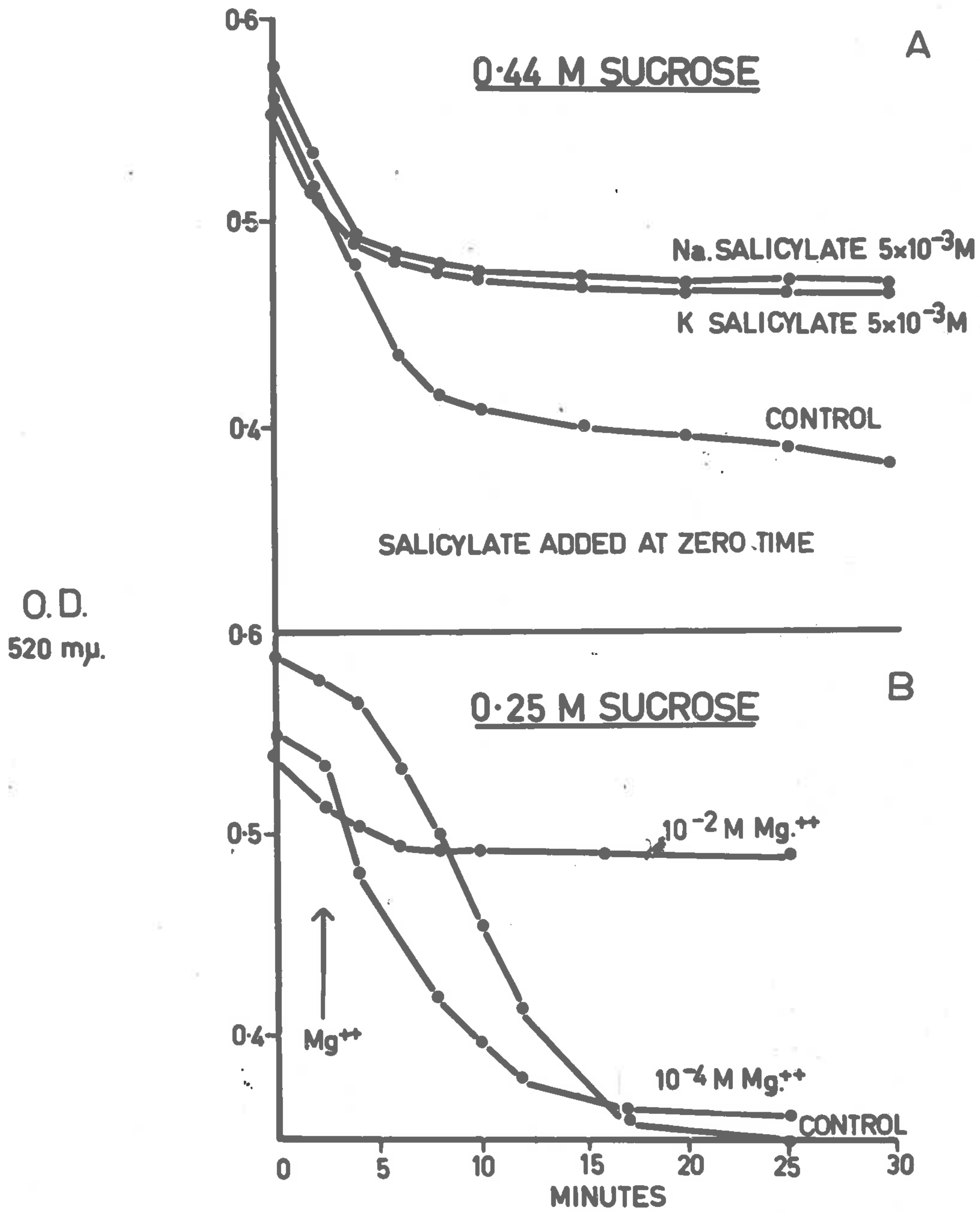
The mitochondria were always examined in a sucrose-tris system without added substrate, but again there were variations in the method of preparation of these particles.

(a) <u>When the method of preparation of the mitochondria was to homogenise whole tissue at 0°C into an initial medium of 0.44 M sucrose which also contained .001 M EDTA and the pH was adjusted to pH 6.8 with potassium hydroxide.</u>

The control systems, that is those systems without the addition of swelling agents or "co-factors" were very "stable" under these conditions. This observation is analogous to that of similar control systems containing mitochondria isolated from untreated rats, when the method of their preparation and examination was identical.

FIGURE XVII

HOMOGENATE PREPARED IN SUCROSE WITHOUT E.D.T.A.



OBSERVATION MEDIUM 0·3M SUCROSE + 0·02M TRIS (pH 7·4)

Magnesium ions were again able to promote a contraction of the mitochondria.

These results are shown in graph (A) of Figure XVIII.

(b) When the method of preparation of the mitochondria was to homogenise whole tissue at 0°C into an initial medium of 0.44 M sucrose which did not contain EDTA nor was the pH adjusted.

The control systems were very unstable under these circumstances, as were similar systems containing mitochondria from untreated animals. These systems clearly exhibited the phenomenon of "spontaneous progressive swelling".

Here increasing concentrations of Mg^{++} were able to at first inhibit "spontaneous" swelling, and then at higher concentrations were able to promote the contraction of mitochondria.

These results are shown in graph (B) of Figure XVIII.

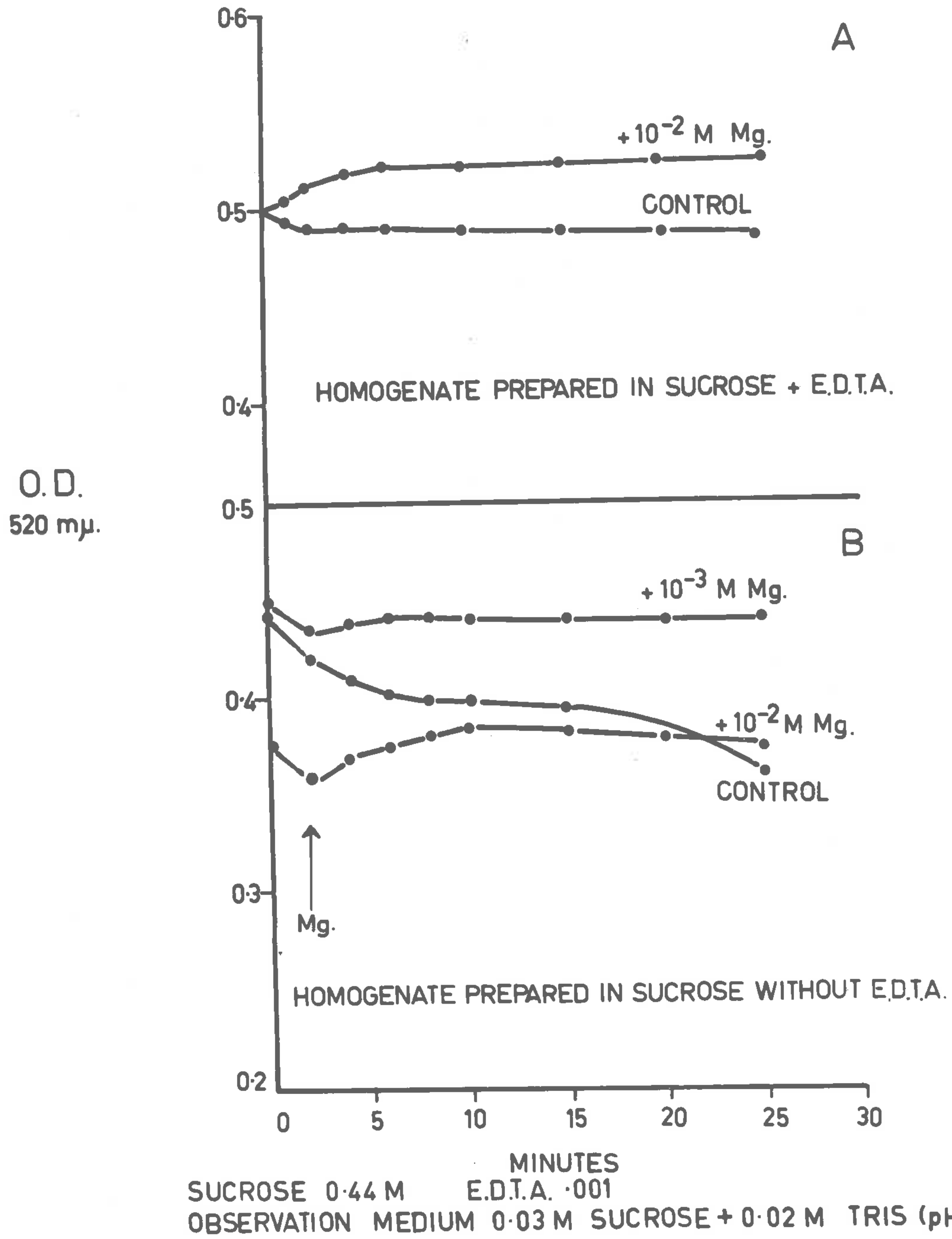
The swelling and contraction properties of mitochondria obtained from specimens of human liver ǂ taken at operation

The size of the individual samples available, and the infrequency of obtaining such samples, precluded a large scale investigation of their properties.

The mitochondria were examined at 520 mu. in a sucrose-tris medium (pH 7.4) without added substrate.

ǂ I am greatly indebted to Dr. L. J. Opit of the University Department of Surgery, Queen Elizabeth Hospital, for his aid in the collection and examination of these samples. A wedge of human liver was taken at operation and immediately placed in a vacuum flask containing ice-cold 0.44 M sucrose plus .001 M EDTA (pH adjusted to 6.8 with potassium hydroxide. As soon as possible the liver sample was sliced into thin

FIGURE XVIII



SUCROSE 0·44 M E.D.T.A. ·001
OBSERVATION MEDIUM 0·03 M SUCROSE + 0·02 M TRIS (pH 7·4)

The optical density of a suspension of human liver mitochondria without the addition of further agents was found to remain steady for thirty minutes, in a manner similar to that of rat liver mitochondria prepared and examined under the same conditions.

When 10^{-2} M Ca^{++} was added (as the chloride) to a suspension of human liver mitochondria, there was a decrease in the optical density of that suspension. The same effect was observed when 5 mM sodium salicylate replaced the calcium chloride.

Mg^{++}, added as 10^{-2}M magnesium sulphate, promoted contraction of these mitochondria, whereas the addition of 5 mM sodium para-hydroxybenzoate to a suspension of these mitochondria appeared to exert little effect. This latter substance is an isomer of sodium salicylate and served to control the effect of the sodium ion.

In general, isolated human liver mitochondria appeared to possess properties of swelling and contraction analogous to those of isolated rat liver mitochondria when prepared and examined in the same way.

These results are shown in Figure XIX.

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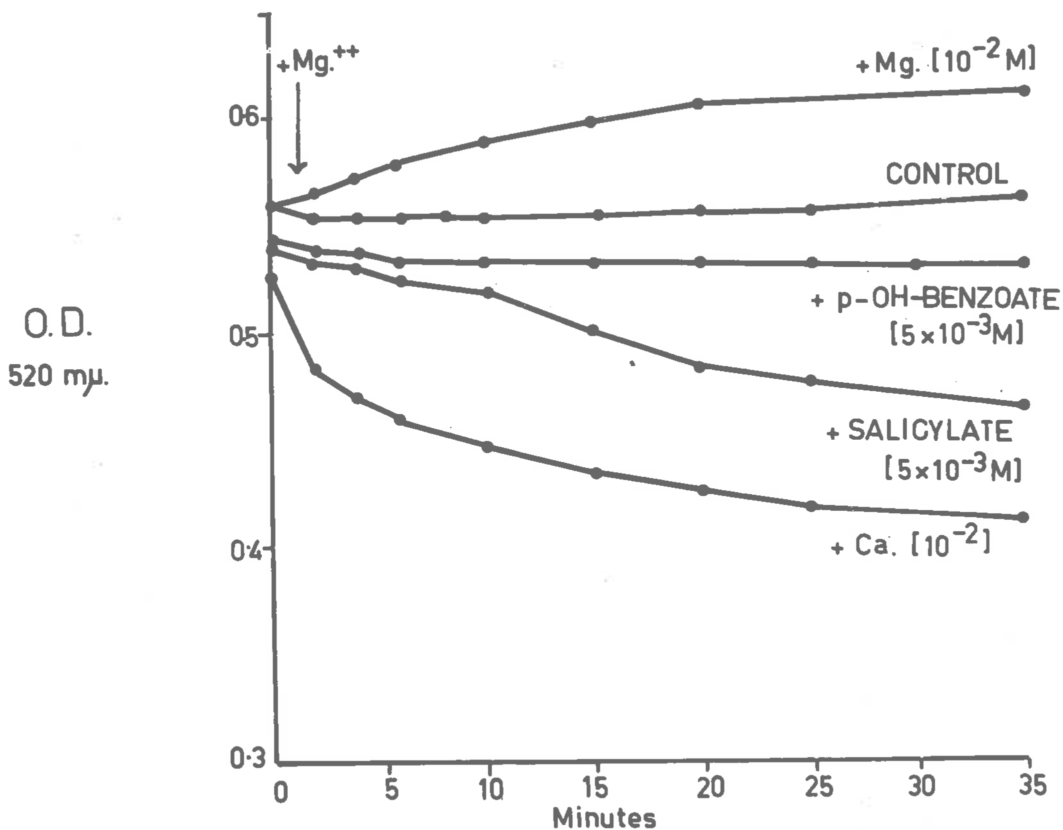
pieces with a scalpel. This was done because human liver is very fibrous by comparison with rat liver. It was then homogenised and the mitochondria prepared by centrifugation in the usual way. The homogenising medium was the same as the cooling fluid in the vacuum flask. The temperature was maintained between 0-2°C for all procedures.

FIGURE XIX

HUMAN LIVER MITOCHONDRIA



PROPERTIES OF HUMAN LIVER MITOCHONDRIA; THE ORIGINAL HOMOGENATE WAS PREPARED IN SUCROSE + E.D.T.A. OBSERVATION MEDIUM 0·3M + 0·02M TRIS (pH 7·4)

The swelling and contraction properties of mitochondria isolated from the liver of untreated rats and examined in an ionic medium in the presence of an oxidisable substrate.

The mitochondria were always prepared by homogenising the whole tissue into a medium of 0.44 M sucrose which contained .001 M EDTA which was adjusted to pH 6.8 by the addition of potassium hydroxide.

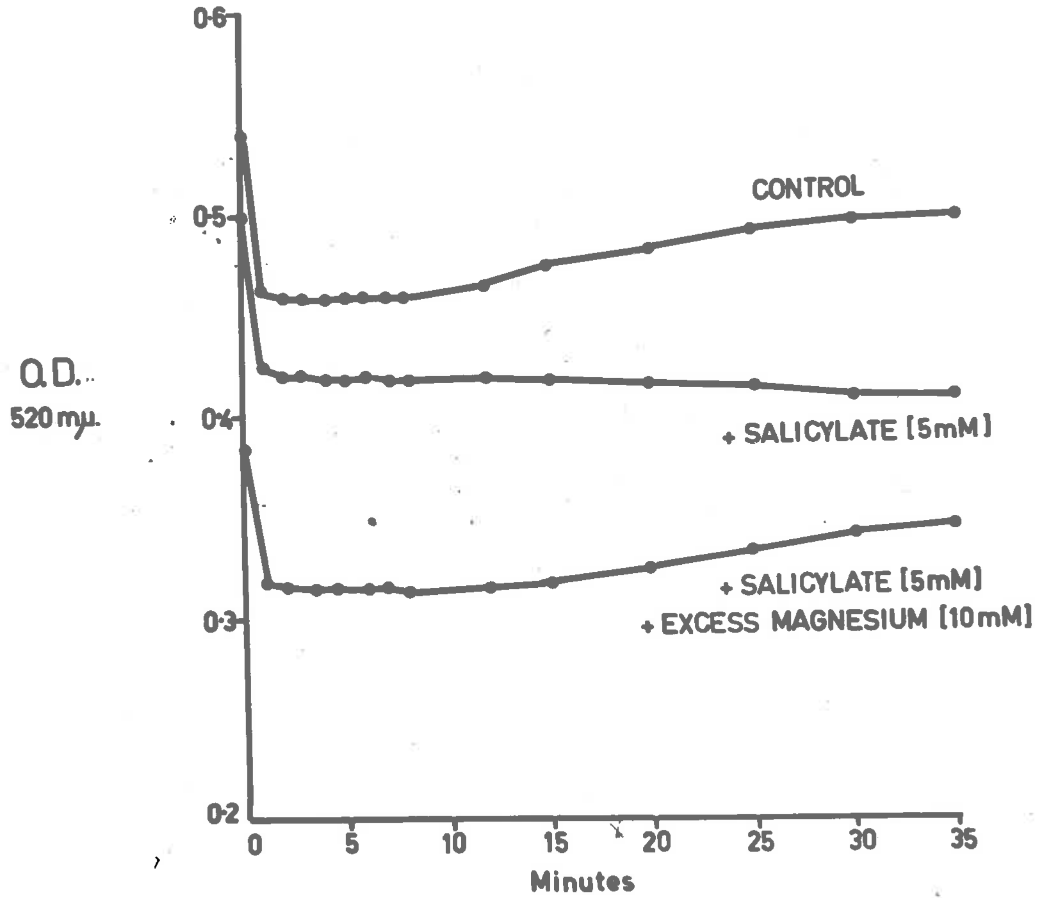
When mitochondria from untreated rats in this ionic system, there was an immediate decrease in optical density from an initial level which was measured as soon as possible after the addition of the mitochondria. This decrease was apparent until the two minute reading. After this time the optical density of the suspension began to increase. This phenomenon continued for at least twenty-five minutes. After this time the optical density of the system was often equal to that of the zero-time value.

In the presence of all three substrates examined (α-keto-glutarate, succinate, and β-hydroxybutyrate) the initial effect was a swelling of the mitochondria. This swelling effect was only of short duration. It was then followed by a progressive contraction of the mitochondria, which continued throughout the remainder of the period of observation.

When sodium salicylate was added to the system in a concentration known to uncouple oxidative phosphorylation (5×10^{-3} M), the initial rapid decrease in optical density was still evident. Following this change, the optical density of the mitochondrial suspension remained constant at the decreased level for the remainder of the period of observation. When the concentration of Mg^{++} was increased from 5 mM to 10^{-2}M, the pattern of response in the presence of salicylate became identical to that seen without salicylate. A phase of contraction again followed an initial decrease in optical density. (See Figure XX).

FIGURE XX

BEHAVIOUR OF "RESPIRING" MITOCHONDRIA



PROPERTIES OF ISOLATED MITOCHONDRIA FROM UNTREATED RATS. MITOCHONDRIA SUSPENDED IN IONIC-GLUCOSE MEDIUM CONTAINING SUBSTRATE [SUCCINATE], ATP AND HEXOKINASE.

Discussion and Conclusions

It should be recalled that this investigation was initiated in an effort to explain the apparent discrepancy between the *in vivo* and *in vitro* effects of salicylate on mitochondrial enzyme function. Particular reference was made to the question of whether salicylate could affect the permeability of the mitochondrial membrane, that is the fourth of the hypotheses proposed to explain this discrepancy.

During this study of the swelling and contraction properties of isolated rat and human liver mitochondria, it became apparent that many of the effects observed were dependent upon the presence or absence of EDTA in the homogenising medium used in the initial stages of the isolation procedure. The presence of EDTA imparted stability to mitochondria, whereas its absence produced mitochondria having a rapid rate of spontaneous swelling. One explanation for this finding may come from the particular properties of EDTA which are said to have a "direct stabilising effect on the mitochondrial membrane" (Hunter, Levy, Fink, Schutz, Guerra and Hurlvitz, 1959). Clearly such an action of EDTA must persist throughout the subsequent washing, re-suspension, and observation of the mitochondria. This explanation suggests that EDTA itself must be firmly bound to the mitochondrial membrane, and be carried through subsequent procedures in this way.

A more probable explanation is that during the homogenisation procedure a swelling agent may be liberated from the disrupted tissue and brought into contact with the mitochondria, perhaps for example, the calcium ions of the extracellular fluids which always contaminate these preparations. In the presence of EDTA, this swelling agent could be complexed and thus rendered inactive. This reasoning implies that the hypothetical swelling agent produces irreversible mitochondrial

membrane changes during the homogenisation procedure. Either these changes are not corrected by the subsequent removal or dilution of this agent during the remainder of the preparation, or in a manner similar to that postulated for EDTA above, this agent itself must be bound to the mitochondrial membrane sufficiently firmly to resist removal through the remaining stages of the preparation. In this way it could exert an effect in the final test system.

Salicylate, which itself is capable of strong chelation with bivalent cations was able in some conditions, to produce a marked stabilising effect on mitochondria which had been prepared without the protection of this complexing agent. This effect was shown in graph A. of Figure XVII, where mitochondria prepared in a medium without EDTA were subject to progressive swelling. On the addition of either sodium or potassium salicylate the degree of swelling was markedly inhibited. A similar finding has recently been reported by Jeffrey and Smith (1959).

A further possibility which should be considered in this regard is that some intramitochondrial factor, which is normally inhibitory to the swelling process, is lost from mitochondria prepared during the homogenisation of whole tissue in the absence of EDTA. Lester and Hatefi (1958) have demonstrated a decreased leakage of "co-enzymes" from mitochondria in the presence of EDTA when incubated at 30°C in 0.25 M sucrose.

Obviously these various explanations can only be speculative at this stage as definitive evidence is not available from the data of these experiments.

The mitochondrial swelling observed when either salicylate or calcium ions are added to some test systems appears to be a too prolonged process to merely be a passive adjustment of the mitochondria to the

osmolarity of their environment. A leakage of mitochondrial solutes under these circumstances may lead to a "progressive" or spontaneous swelling which could be unrelated to the type of metabolic or respiratory controlled swelling described by other workers (Chappell and Greville, 1958) (Hunter et al, 1959).

If "respiratory" controlled swelling occurs in these experiments, it must be related to the oxidation of endogenous substrate, as none was added to the test systems. There seems little doubt that strict anaerobiosis can protect mitochondria from virtually every agent known to promote swelling, even when the mitochondria are prepared under a wide variety of conditions. (Hunter, Davis and Carbat, 1956) (Lehninger, 1957) (Lehninger and Schneider, 1958). This observation clearly supports the concept of a dependence of at least one type of swelling upon mitochondrial respiration. However, in the experiments reported here there can also be little doubt that the effect of salicylate on isolated mitochondria varied with the method of preparation of the mitochondria.

If control systems of mitochondria were found to be "stable" when examined spectrophotometrically, then salicylate could promote swelling. Alternatively when mitochondrial systems were already "spontaneously unstable", then the addition of salicylate to the system could inhibit this process. An explanation for some of these observations has already been discussed.

The swelling effect of salicylate on previously "stable" mitochondria is similar to the effect of a number of other agents which also uncouple mitochondrial oxidative phosphorylation; for example thyroxine and some of its analogues and also phlorizin (Keller and

Lotspeich, 1959). In addition to the observations made here, Jeffrey and Smith (1959) have recently reported that salicylate could inhibit "progressive" swelling of mitochondria. These investigators felt that this observation placed salicylate along with dinitrophenol, in that group of uncoupling agents which had been shown to prevent the spontaneous swelling of mitochondria in hypotonic sucrose - an effect which differentiated this agent from those of the other major group of uncoupling agents which promote swelling in these circumstances. Such a scheme of classification was introduced by Lehninger (1956).

As already pointed out the results of this investigation strongly suggest that the method of preparation of the mitochondria must also be considered in any assessment of the effect of salicylate upon mitochondrial swelling. This probably applies to all other agents as well. Therefore, before a scheme of classification can serve any real purpose in this regard, the basic mechanisms of the effect must first be clarified. For instance, before an attempt can be made to interpret the experimental findings of salicylate effects observed here, it would be necessary to know the answers to many questions. For example, is the swelling of stable mitochondria by salicylate an osmotic or a respiratory effect? What causes spontaneous swelling when EDTA is absent during mitochondrial preparations? How does salicylate inhibit this process? The answer to any of these questions is not available at the present time.

The protective or inhibitory action of magnesium ions on mitochondrial swelling may be related to the role of this bivalent metal in "respiratory and associated processes" (Hunter et al, 1959). Alternatively, it has often been suggested that this ion may prevent

swelling through some structural effect on the mitochondrial membrane itself, either by occupying a specific site required for structural integrity (Tapley, 1956) (Fonnesu and Davies, 1956) or by complexing with some groups in the membrane (Siekevitz, Low, Ernster and Lindberg, 1958).

If the processes of swelling and contraction of mitochondria are independent, not only enzymatically, but even morphologically, this view could lead to a situation in which both processes were taking place simultaneously. Under these circumstances the end result, as seen by relatively short term spectrophotometric observation, would merely be a quantitative measure of the overall effects of these opposing actions. This speculation demands a mitochondrion with a compartmentalised water space, one compartment susceptible to osmotic penetration and the other varying with the metabolic production or extrusion of water. Such a model has recently been proposed by Lehninger (1960) who suggested that the function of the "outer membrane" is confined to rapid passive osmotic adjustments.

In an attempt to examine the physiological significance of a possible swelling of mitochondria by salicylate, an examination was made of the mitochondria isolated from rats treated with relatively large doses of salicylate <u>in vivo</u>. The systems used were identical to those previously employed and the properties of these mitochondria were compared to those of mitochondria isolated from untreated animals. Similarly to the findings in experiments relating to both mitochondrial oxidative phosphorylation and ATPase activity, once more an <u>in vitro</u> test system failed to reveal inherent differences between the properties of mitochondria isolated from both groups of animals.

This evidence could support the view that the salicylate effects which are demonstrated *in vitro* are of no physiological significance, or it could equally well suggest that the effect of salicylate is readily reversible, and apparently is unlike the action of thyroxine which has been shown to persist when examined under similar conditions (Tapley, 1956).

An examination of the spectrophotometric properties of mitochondria isolated from human liver, demonstrated that these mitochondria appeared to respond to the addition of salicylate and other agents in a manner analogous to rat liver mitochondria prepared under the same conditions. The scant evidence in the literature of human liver mitochondria, also suggests that whilst the rates of some enzymatic processes may differ, these mitochondria carry out such processes as respiration, oxidative phosphorylation and nucleotide synthesis in a manner apparently analogous to those from the rat (Waterlow, 1953) (Griffiths and Rees, 1957) (Frei and Ryser, 1958).

Whilst the evidence which was derived from the experiments described in much of Part II of this Chapter was of interest, it was felt that because the mechanisms involved were not yet clear, an interpretation could only be extremely speculative. However, when an ionic medium which contained an oxidisable substrate was employed in these studies, the results were more meaningful.

When mitochondria were suspended in this ionic medium, the initial effect was a rapid fall in the optical density of the mitochondria. This can be readily explained as a rapid adjustment by the mitochondria to the osmolarity of the medium. Presumably there was a relatively large uptake of fluid by the mitochondria under these conditions with a consequent decrease in the optical density of the mitochondrial suspension.

This initial phase is followed by one of contraction which is of longer duration. The evidence of Lehninger (1959 a, b, and c; 1960) suggests that this phenomenon is an active expulsion of water from the mitochondria. This process specifically requires ATP and may be directly, if not obligatorily, related to oxidative phosphorylation.

An attractive explanation for the *in vitro* effect of salicylate on the fluid content of respiring mitochondria is that in the absence of salicylate, respiration and oxidative phosphorylation proceed concurrently. Under these circumstances sufficient ATP is synthesised to initiate the unknown steps leading to the expulsion of intramitochondrial water. In the presence of salicylate, these processes are dissociated and electron transport phosphorylation is abolished. Insufficient ATP is now produced and mitochondrial contraction ceases. This explanation can be correlated with the evidence presented in an earlier section relating to the *in vitro* effect of salicylate on mitochondrial oxidative phosphorylation.

In the absence of other than presumptive evidence that the mitochondria were actually respiring in these test systems, and indeed the oxygen gradient here could not be entirely favourable, one cannot assume that the explanation just proposed has been unequivocally established.

Any study by this method of the effect of an agent upon the water content of isolated mitochondria must be clouded at this time, by the lack of precise evidence relating to the mechanisms involved. It has been established that these processes are complex and can be influenced by a wide variety of agents and conditions. Whilst an attractive explanation can be offered for the effect of salicylate on

the water content of respiring mitochondria, it is felt that a conservative attitude should be adopted towards such experimental observations.

However, one observation which has been firmly established by the work described here is that salicylate is able to influence the water content of mitochondria *in vitro*. The mechanism of this action is not known and the physiological significance of this finding is uncertain.

Both Parts I and II of this examination of the fourth hypothesis have been concerned with the effect of salicylate on the transport of water across the mitochondrial membrane. In Part I, this effect was examined gravimetrically and in Part II, the change in optical density of mitochondrial suspensions was employed.

Neither of these investigations produced really conclusive results. No irrefutable evidence was found for an effect of salicylate upon mitochondrial permeability by an examination of mitochondrial water content. The lack of basic knowledge of the mechanisms involved in the swelling and contraction of mitochondria rendered controversial the interpretation of the data in Part II of this work. However, it was felt that an effect of salicylate on mitochondrial membrane permeability remained probable. In an effort to produce more definite evidence a further study of this possibility was made by examination of the transport of mitochondrial electrolytes under the influence of salicylate. The results of an investigation of this phenomenon are presented in Part III of this Chapter of the thesis.

CHAPTER VIII

Part III

THE EFFECT OF SALICYLATE ON ELECTROLYTE EXCHANGE IN ISOLATED MITOCHONDRIA AT 0°C

Introduction

The previous studies in this section suggested that useful evidence for a possible effect of salicylate on the mitochondrial membrane might be forthcoming by an examination of electrolyte gradients under the influence of salicylate.

Similar studies by other workers on the distribution of solutes in mitochondrial systems *in vitro*, had shown that freshly isolated rat liver mitochondria were partially permeable to a variety of solutes including sugars, nucleotides and certain electrolytes (Spector, 1953) (Stanbury and Mudge, 1943) (Werkheiser and Bartley, 1957, 1958) (Amoore and Bartley, 1958) (Bartley and Amoore, 1958) (Amoore, 1958).

In this investigation a study was made primarily of the loss of potassium ions from isolated rat liver mitochondria and the influence of salicylate upon this phenomenon. The test system was similar to that employed for the studies of water exchange previously reported (p. 201).

When such observations are carried out at 0°C and in the absence of substrate, then the possibility of metabolic or "active" transport by the mitochondrion is minimised. Any effects observed should therefore represent passive changes in membrane permeability. Potassium was selected as the ion for examination because it is in high concentration within the cell (Hastings, 1940) and hence could

well be the most important cation within mitochondria. In addition previous work reported in this thesis had suggested that a transient disturbance in the distribution of this ion may follow the administration of salicylate *in vivo*.

Materials and Methods

Only untreated colony animals were used in this study. The detailed method for the preparation of rat liver mitochondria has already been described (p. 75) in the section relating to oxidative phosphorylation. The homogenising medium was 0.44 M sucrose + .001 M EDTA (pH 6.8).

As soon as the pellet was obtained free of cellular contamination it was re-suspended as quickly as possible into a known volume of suspending fluid. Usually the mitochondria derived from one gram wet weight of whole liver were re-suspended into a volume of 4 mls. The suspending fluid was 0.44 M sucrose either without further additions, or on other occasions containing either sodium salicylate, sodium para-hydroxybenzoate or sodium chloride where indicated. All these substances were added to give a final concentration of 5 mM.

This concentration of salicylate was chosen as previous studies reported in this thesis had shown it to be a level exerting a marked effect both *in vivo* and *in vitro*.

The pH of each of these suspending media was checked with a glass electrode and found to be:-

(1) 0.44 M sucrose, pH 5.95

(2) 0.44 M sucrose + 5 mM sodium salicylate, pH 6.15

(3) 0.44 M sucrose + 5 mM sodium para-hydroxybenzoate, pH 6.2

(4) 0.44 M sucrose + 5 mM sodium chloride, pH 4.6

A hypertonic (0.44 M sucrose) medium was employed for both the isolation and re-suspension of the mitochondria, rather than isotonic (0.33 M) or hypotonic (0.25 M) sucrose as has sometimes been used by other workers. This was chosen because it has recently been established that such hypertonic concentrations of sucrose are inhibitory to a number of mitochondrial functions *in vitro*, including "spontaneous swelling and contraction of mitochondria" (Lehninger, 1959b) and oxidative phosphorylation (Cooper and Lehninger, 1957 a, 1957 b).

It seems reasonable therefore to assume from the findings of these workers and those described in the preceding section of this work (Chapter VIII, Part II), that mitochondria prepared and re-suspended in a sucrose medium of less tonicity than that used here may well become "swollen". It was hoped that the use of this higher tonicity of sucrose would keep to a minimum the leakage of endogenous solutes which occur from mitochondria during their preparation and storage (Amoore and Bartley, 1958).

Sodium para-hydroxybenzoate and sodium chloride were used to control the effect of the sodium ion, whilst the isomer of salicylate also provided a radical of comparable molecular size to that of salicylate itself. Furthermore, it had already been demonstrated that unlike salicylate, para-hydroxybenzoate was without hypermetabolic effect when administered to man (cf. Hetzel, Charnock and Lander, 1959), that it failed to exert a number of actions *in vitro* known to be associated with salicylate (Randle and Smith, 1958) (Segal and Blair, 1959) (Andrew, 1960) and did not uncouple mitochondrial oxidative phosphorylation at least in comparable concentrations with that employed with salicylate (Packer, Austen and Knoblock, 1959).

Following their re-suspension, the mitochondria remained in contact with the various suspending media for ten minutes at an air temperature of 0°C.

The mitochondria were then completely sedimented from the medium by centrifuging at 12,000 x g for ten minutes at 0°C. In preliminary experiments this sedimentation process had been found to be satisfactory for this purpose.

An aliquot of the supernatant fluid was removed by Pasteur pipette without disturbing the mitochondrial pellet, which was then drained by inversion of the centrifuge tube until it was as free as possible of supernatant fluid. The sides of the tubes were wiped with absorbant tissue and the wet pellet so obtained was immediately weighed to give a measure of the mitochondrial yield. Some of the pellets were then re-suspended to a known volume in electrolyte free water, and the resulting suspension assayed for sodium and potassium by flame photometry. The supernatant fluids were similarly assayed, and where appropriate salicylate was determined on both the pellet and supernatant fractions by the method of Trinder (1954). In addition each batch of each suspending medium was also assayed for sodium and potassium before use.

The volume of the suspending fluid was recorded, as was the wet weight of each pellet of mitochondria. The results could therefore be expressed as equivalents (or moles) of electrolyte ion per unit weight of wet mitochondria and hence comparison made between treatments.

Although the dry weights, and hence the moisture content of duplicate mitochondrial pellets were only determined occasionally throughout this study, the results obtained were always found to be 80% ± 2%. This was in agreement with the values previously reported

in Table (34) when mitochondria remained in contact with a similar medium for the same length of time. The constancy of this result rendered further examination of this parameter unnecessary.

A value of 80% was therefore uniformly adopted for the conversion of an electrolyte concentration per unit weight of wet mitochondria, to a concentration per unit volume of "intramitochondrial" water. However, it was realised that the total water space of mitochondria was unlikely to be available as a distribution space for these solutes (Werkheiser and Bartley, 1957). It was assumed therefore that the space available to potassium and sodium remained a constant proportion of the total water content of the mitochondria in these experiments, and would therefore also be constant under the present conditions. This assumption renders the derived value for the "intramitochondrial" concentration of potassium and sodium ions low in an absolute quantitative sense, but should do little to invalidate any change induced by salicylate or other substances, relative to the control situation.

An increase in concentration of an ion in the suspending fluid was taken to represent a loss of that ion from the mitochondria, whilst a decrease in value below the level of the reagent blank, indicated an uptake of that ion by the mitochondria. Either process must be "passive" under the conditions of these tests.

The mitochondria were always prepared in batches of at least six pellets, so that some mitochondria from each batch could always be suspended in 0.44 M sucrose without any additions and thus serve as internal controls in every experiment.

Results

The loss of salicylate ion from the medium, its penetration into or adsorption by mitochondria have already been described in detail (p. 134) particularly with reference to the concept of "intramitochondrial" solutes proposed by Bartley and Amoore (1958).

POTASSIUM AND SODIUM EXCHANGE

The effect of suspending the mitochondria in 0.44 M sucrose without any additions

Neither sodium nor potassium ions could be detected in any of the "control" suspending fluids when assayed prior to the addition of mitochondria. After ten minutes contact with such media at 0°C there was a measurable "leak" of both potassium and sodium ions from the mitochondria into the suspending fluid. The ratio of these ions was usually about 2:1. The average loss of potassium ion was 12.6 micro equivalents per gram of wet mitochondria, whilst the average loss of sodium ion was 6.4 micro equivalents per gram of wet mitochondria.

These results are shown in Table (35).

When the concentration of potassium and sodium ions were determined in the "intramitochondrial" water by assay and calculation as described, mitochondria treated in this manner were found to contain an average concentration of K^+ of 16.9 milli equivalents/litre whilst the value of Na^+ was 9.3 milli equivalents/litre.

These results are shown in Table (39).

Traces of inorganic phosphorus could be found in the supernatant fluid of these experiments following treatment of the mitochondria when assayed by the method of Taussky and Shorr (1953). Magnesium ions were not detectable under these circumstances by the method of Orange and Rhein (1951).

<u>The effect of suspending the mitochondria in 0.44 M sucrose plus 5 mM sodium salicylate.</u>

The suspending medium did not contain any potassium ions when assayed prior to the addition of the mitochondria, but in addition to the salicylate ion, there were always sodium ions present at a concentration of approximately 5 mM. Any Na^+ transfer has been corrected for the appropriate reagent concentration of this ion.

The presence of 5 mM sodium salicylate in the medium greatly enhanced the passive loss of K^+ from mitochondria. The average value found was 20.9 milli equivalents lost per gram of wet mitochondria. This is an increase of 66% over that of the controls treated with 0.44 M sucrose alone. When the increased loss of K^+ produced by the addition of sodium salicylate to the medium was compared to the loss produced by the medium without salicylate, by comparison of the means, there was a highly significant difference between treatments ($P < .001$). The loss of K^+ to the medium was not accompanied by a commensurate increase in mitochondrial Na^+. The actual variations in the concentration of sodium ion from the reagent blank values were so small, that after allowance for a small experimental error ($\pm$ 0.5 mM) they could be regarded as negligible. The consistent loss of Na^+ seen from mitochondria soaked in sucrose alone, was prevented by the presence of sodium salicylate in the medium. These results are shown in Table (36).

The loss of K^+ into the suspending fluid under these circumstances was reflected by a much lower concentration of this ion in the "intramitochondrial" water. After treatment with salicylate the level was 10 milli equivalents K^+/litre, when compared to 16.9 milli equivalents K^+/litre in those mitochondria treated with 0.44 M sucrose alone. There was only a slight variation in the "intramitochondrial"

sodium ion concentration which was now 8.7 milli equivalents Na^+/litre. These results are shown in Table (39).

The effect of suspending the mitochondria in 0.44 M sucrose and 5 mM sodium para-hydroxybenzoate.

Like the previous mixture, the suspending medium did not contain any potassium ion when assayed prior to the addition of the mitochondria, but in addition to the para-hydroxybenzoate ion, there was always sodium ion present at a concentration of approximately 5 mM.

Unlike the action of salicylate, the presence of sodium para-hydroxybenzoate in the medium did not affect the leakage of potassium ion from mitochondria to suspending fluid; the average value for the loss of this ion under these conditions was unaltered from that found when 0.44 M sucrose alone was the medium - 12.6 milli equivalents K^+ lost /gm. wet mitochondria. The presence of approximately 5 milli equivalents of Na^+ per litre in the suspending fluid again prevented the loss of this ion as seen with mitochondria soaked in 0.44 M sucrose alone. There was a small but consistent uptake of Na^+ by mitochondria under these circumstances.

The decreased loss of K^+ when compared to the salicylate treated group was reflected in a much higher concentration of potassium in the "intramitochondrial" water. The value found under these conditions was 17.8 milli equivalents K^+/litre. Both these values are considerably greater than that seen to accompany salicylate treatment, i.e. 10 milli equivalents K^+/litre.

The concentration of these electrolytes in the suspending medium after the soaking period is shown in Table (37) and that in the "intramitochondrial" water are shown in Table (39).

The effect of suspending the mitochondria in 0.44 M sucrose plus 5 mM sodium chloride.

When assayed prior to the addition of the mitochondria the suspending medium again did not contain any K^+.

In a small number of experiments the action of sodium chloride in the suspending medium appeared to be similar to that of sodium para-hydroxybenzoate rather than sodium salicylate, although the loss of potassium ion from the mitochondria was slightly greater (14.4 milli equivalents K^+/gm. wet mitochondria) than in either the presence of sodium para-hydroxybenzoate or sucrose controls (12.6 milli equivalents K^+/gm. wet mitochondria) but was still considerably less than in the presence of sodium salicylate (20.9 milli equivalents K^+/gm. wet mitochondria).

Sodium chloride did not promote the loss of sodium ion from the mitochondria as did sucrose alone, but neither did it promote the entry of this ion into the mitochondria under these circumstances. The values for the concentration of sodium and potassium ions in the suspending medium are shown in Table (38) whilst those for the "intramitochondrial" water are again given in Table (39).

The use of sodium chloride as a "control" was not persisted with as the pH of this solution was considerably below that of the other media employed, and in addition, it was felt that the free chloride ions of this solution may exert an unknown effect.

TABLE (35)

MITOCHONDRIAL ELECTROLYTES in vitro

The effect of "soaking" mitochondria in 0·44 M Sucrose for 10 minutes at 0° C.

EXPT. NO.	WET WEIGHT OF MITOCHOND-RIAL PELLET (mgms.)	VOLUME OF MEDIUM (mls.)	CONCENTRATION IN MEDIUM *		MITOCHONDRIA	
			Δ K. mEq/l.	Δ Na. mEq/l.	K. LOST μEq/gm wet mitochondria	Na. LOST μEq/gm wet mitochondria
6	166	4	+0·60	+0·55	14·5	13·2
8	239	5	+0·75	+0·45	15·6	9·4
13	308	4	+0·90	+0·55	11·6	7·1
14	297	4	+0·90	+0·40	12·1	5·4
15	291	4	+0·80	+0·40	11·0	5·5
16	311	4	+0·85	+0·40	10·9	5·1
28	331	4	+1·00	+0·40	12·1	4·8
29	244	5	+0·55	+0·25	11·3	5·1
30	201	3	+1·20	+0·55	21·2	8·2
31	300	4	+0·82	+0·40	10·9	5·3
32	289	4	+0·80	+0·40	11·0	5·5
33	284	4	+0·82	+0·30	11·4	4·2
34	302	4	+0·81	+0·32	10·8	4·3
				Average	12·6	6·4

* These values were determined after the "soaking" procedure and have been corrected for the control values prior to the addition of the mitochondria to the medium.

An uptake by the mitochondria is shown by a -ve value.
A loss from the mitochondria is shown by a +ve value.

TABLE (36)

SALICYLATE AND THE MITOCHONDRIAL ELECTROLYTES in vitro

The effect of "soaking" mitochondria in 0·44 M Sucrose plus salicylate for 10 minutes at 0°C

EXPT. NO.	WET WEIGHT OF MITOCHONDRIAL PELLET (mgms.)	VOLUME OF MEDIUM (mls.)	CONCENTRATION IN MEDIUM *		MITOCHONDRIA	
			Δ K. mEq/l.	Δ Na. mEq/l.	K. LOST µEq/gm wet mitochondria	Na. GAINED µEq/gm wet mitochondria
1	411	4	+1·4	-0·2	13·6	0·4
2	260	4	+1·0	-0·3	15·4	0·5
3	266	4	+1·0	-0·3	15·0	0·5
4	201	4	+1·6	-0·4	31·8	0·8
5	244	4	+1·3	0	21·3	0
6	272	4	+1·6	0	23·5	0
7	147	4	+1·0	0	27·2	0
8	143	4	+1·0	-0·1	28·0	-
9	314	4	+1·4	0	17·8	0
10	310	4	+1·2	0	15·5	0
				Average	20·9	0·2

* Values determined and corrected as for Table

An uptake by the mitochondria is shown by a -ve value.
A loss from the mitochondria is shown by a +ve value.

TABLE (37)

PARA-HYDROXY-BENZOATE AND MITOCHONDRIAL ELECTROLYTES in vitro

The effect of "soaking" mitochondria in 0·44 M Sucrose plus 5 mM sodium para-hydroxy-benzoate for 10 minutes at 0°C.

EXPT. NO.	WET WEIGHT OF MITOCHONDRIAL PELLET (mgms.)	VOLUME OF MEDIUM (mls.)	CONCENTRATION IN MEDIUM *		MITOCHONDRIA	
			Δ K mEq/l.	Δ Na mEq/l.	K. LOST mEq/gm wet mitochondria	Na. GAINED mEq/gm wet mitochondria
31	295	4	+0·95	-0·50	12·9	0·7
32	258	4	+0·80	-0·50	12·4	0·8
33	274	4	+0·90	-0·50	13·1	0·7
34	259	4	+0·80	-0·50	12·3	0·8
35	256	4	+0·80	-0·45	12·5	0·7
				Average	12·6	0·7

*Values determined and corrected as for Table

An uptake by the mitochondria is shown by a -ve value.
A loss from the mitochondria is shown by a +ve value.

TABLE (38)

SODIUM CHLORIDE AND MITOCHONDRIAL ELECTROLYTES in vitro

The effect of "soaking" mitochondria in 0·44 M Sucrose plus 5mM sodium chloride for 10 minutes at 0 °C.

EXPT. NO.	WET WEIGHT OF MITOCHONDRIAL PELLET (mgms.)	VOLUME OF MEDIUM (mls.)	CONCENTRATION IN MEDIUM *		MITOCHONDRIA	
			Δ K. mEq/l.	Δ Na. mEq/l.	K. LOST µEq/gm.wet mitochondria	Na. GAINED µEq/gm.wet mitochondria
17	304	4	+1·0	-0·2	13·2	0
18	244	5	+0·75	-0·1	15·6	0
19	229	4	+0·8	0	14·4	0
				Average	14·4	0

* Values determined and corrected as for Table

An uptake by the mitochondria is shown by a -ve value.

A loss from the mitochondria is shown by a +ve value.

TABLE (39)

COMPARATIVE EFFECT OF SODIUM SALICYLATE, SODIUM PARA-HYDROXY-BENZOATE AND SODIUM CHLORIDE ON THE DISTRIBUTION OF MITOCHONDRIAL POTASSIUM AND SODIUM AT 0°C

TREATMENT*	NO. OF EXPTS.	pH OF MEDIUM	ELECTROLYTE FLUX µEq/gm. wet mitochondria		CONCENTRATION OF ELECTROLYTES RETAINED BY MITOCHONDRIA µEq/l "intramitochondrial water"	
			K. LOST	Na. GAINED	K.	Na.
Control [0·44M sucrose]	13	5·95	12·6	0	16·9	9·3
*Sodium Salicylate [5 mM]	10	6·15	20·9	0·2	10·0	8·7
*Sodium para-hydroxy-benzoate [5mM]	5	6·20	12·6	0·7	17·8	7·9
*Sodium Chloride [5mM]	3	4·6	14·4	0	16·6	8·6

*10 minutes at 0°C.

Discussion

The evidence produced in this section demonstrated an enhancing action of sodium salicylate on the "leakage" of potassium from isolated mitochondria at 0°C. This action was not reproduced by either sodium para-hydroxybenzoate or sodium chloride, which strongly suggests that it is an effect specifically attributable to the salicylate ion.

The loss of potassium ions from the mitochondria was not accompanied by a commensurate gain in sodium ions, although the presence of 5 mM sodium in the medium could prevent the loss of this ion from the mitochondria despite an apparently greater intramitochondrial concentration of Na^+ (approximately 9 mM). Therefore, this action of salicylate does not promote a simple mole for mole or charge for charge re-arrangement of univalent ions within the mitochondria, consequent to attaining equilibrium with the environment. Rather salicylate would seem to affect the binding capacity of mitochondria for potassium in a selective manner.

This effect was observed at 0°C, in mitochondrial suspensions without added substrate, and under conditions in which the availability of oxygen for mitochondrial respiration would be far from optimal. It seems unlikely therefore that this effect of salicylate could be metabolic in origin, but rather it must be "passive" in nature. This means that the increased loss of K^+ which has been observed from tissues (Manchester, Randle and Smith, 1958) and mitochondria under the influence of salicylate may not be due to an uncoupling of oxidative phosphorylation and subsequent loss of high energy phosphate, but could either precede or accompany these changes.

Workheiser and Bartley (1957) and Berger (1957) have

independently reported a similar action for dinitrophenol on "bound" mitochondrial potassium which also occurred at 0°C, and in a concentration which at higher temperatures is known to uncouple oxidative phosphorylation.

To reiterate, the loss of potassium ions from mitochondria may therefore be the cause of uncoupling rather than its consequence. This suggestion recalls the opinion of Tapley (1956a) who reported a somewhat similar increased loss of potassium from slices of hyperthyroid rat liver to the suspending medium, when compared to normal controls. This worker also felt that the loss of potassium may be a "primary consequence" and that any associated enzymatic disturbances "may be a secondary manifestation" of this loss.

Similarly to the mitochondrial effect seen here, sodium para-hydroxybenzoate did not promote the loss of potassium from isolated rat diaphragm in vitro when added in the same concentration as sodium salicylate (Manchester, Randle and Smith, 1958) and as already mentioned above this compound is not an uncoupling agent. The fact that Gamicidin S, another powerful uncoupling agent, has also been shown to produce a decrease in mitochondrial potassium (Berger, 1957) seems to support the view that there is a definite relationship between this effect and the uncoupling ability of these compounds.

CHAPTER VIII

Part IV

CONCLUSIONS

All the work reported in Chapter VIII of this thesis has been concerned with the possibility of salicylate exerting an effect on the permeability of the mitochondrial membrane. In Parts I and II of this Chapter only suggestive evidence was obtained for such an effect of salicylate. The evidence presented in Part III was much stronger. Here there was a definite effect of salicylate which was compatible with a change in mitochondrial membrane permeability. When this combined evidence is considered it would seem reasonable to suggest that at least one mode of action of salicylate is to promote passive changes in the permeability of the mitochondrial membrane. *In vitro* these changes lead to a loss of intramitochondrial K^+ and possibly an increase in the total water content of the mitochondria.

In addition it was suggested that this effect of salicylate was not obligatorily associated with active metabolic processes and may even precede any enzymatic disturbances which occur. This observation is offered in support of a concept that the *in vitro* action of salicylate on mitochondrial oxidative phosphorylation is mediated through a primary effect of this drug on mitochondrial membrane permeability, that is, the fourth hypothesis proposed earlier.

Unfortunately direct supporting evidence for this hypothesis was not obtained when an attempt was made to study the effect of the concentration of potassium ions upon mitochondrial oxidative phosphorylation (cf. Chapter V, Part II). A major obstacle encountered in this latter investigation was that the provision of either a zero or

low potassium medium which was fundamental to this study, required either a high content of sodium ions (Boyer, Lardy and Phillips, 1943) or in their absence a medium so unsuitable for oxidative phosphorylation that little coupled phosphorylation could be observed, even with potassium replacement.

The suggestion that salicylate effects the mitochondrial membrane is also in accord with the deductions made from a study of the action of the effects of this drug on mitochondrial ATPase activity (Chapter VII, Part I).

However, the observation of an increased loss of K^+ from isolated mitochondria at 0^oC under the influence of salicylate was made under conditions which were most unfavourable for a retention of this ion. For example there was a highly artificial concentration gradient between the mitochondria and their environment with respect to this ion. In addition there was probably a complete absence of active metabolism by the mitochondria.

Therefore it was of great interest to examine the possibility of such an effect of salicylate occurring in the whole animal, particularly as this effect of salicylate may not be confined to the mitochondrial membrane but may also apply to that of the whole cell.

The results of such studies, performed on the rat, are reported in the following Chapter.

CHAPTER IX

THE EFFECT OF SALICYLATE ON ELECTROLYTE DISTRIBUTION IN VIVO

CHAPTER IX

THE EFFECT OF SALICYLATE ON ELECTROLYTE DISTRIBUTION IN VIVO

Introduction

The results of an examination of the metabolic response of the whole animal to salicylate demonstrated that some changes in the normal pattern of urinary electrolyte excretion follow the administration of relatively large doses of this drug. In addition, investigations *in vitro* had suggested that a primary consequence of relatively high intracellular salicylate concentrations is an alteration in the permeability of the mitochondrial membrane. If membrane permeability is altered *in vivo*, where perhaps the whole cell and not only the mitochondria are affected, the result of such an action of salicylate could be an alteration in the distribution between extracellular and intracellular electrolytes. However, if the mitochondria are specifically the target of this effect of salicylate *in vivo*, then a major change may be a shift in the normal intracellular distribution of electrolytes between compartments of the cell.

To examine these possibilities, not only is plasma and whole tissue data necessary, but some method of examination of intracellular distribution is also required. Few such studies have been reported, and information relating to the distribution of electrolytes within the cell, and particularly the electrolyte content of mitochondria, is extremely meagre. MacFarlane and Spencer (1953), Stanbury and Mudge (1953), Bartley and Davies (1954) and Werkheiser and Bartley (1957) have all demonstrated concentration gradients between mitochondria and the medium for several ions. In at least one instance the gradients were dependent on metabolism for their maintenance (Bartley and Davies, 1954).

All these workers showed considerable interest in potassium which is the major intracellular cation, but much less interest in magnesium which is second only to potassium with respect to concentrations within the cell. Magnesium is of great importance to the metabolism of living tissue.

Although much of the early work on whole tissue electrolytes was performed by direct chemical assay of acid extracts of ashed muscle and liver (Watchorn and McCance, 1937) there has been a trend in recent years to examine tissues prepared in other ways (Baldwin, Robinson, Zierler and Lilienthal, 1952). One such method which appears to be gaining in favour, is total tissue homogenisation followed by chemical assay of acid or aqueous extracts (Vignos and Lefkowitz, 1959) (du Ruisseau and Mori, 1959). An advantage of this latter method is that it may be used to provide fractions of the cell, for example a partition of cellular material into "soluble" and "particulate" components.

The results presented here were obtained during an investigation of the effects of salicylate upon intracellular electrolyte distribution in the rat when the drug was administered *in vivo*. The method of preparation used was one of whole tissue homogenisation followed by differential centrifugation of the homogenate to provide various sub-cellular fractions.

Materials and Methods

The animals used in this study were adult male black and white rats weighing over 300 grams. These animals were supplied with food and water ad libitum throughout the experiment. All animals were weighed daily. The treatments which were either salicylate or water which was given as a control, were administered by gavage. The dosage of drug

was 30 mgms. salicylate radical/100 gm. body weight/day, and this was given in an aqueous solution of small volume (4 mls.). The dose was equally divided into two portions and given morning and evening, and the water control was given at the same time. Salicylate was given as either the potassium or sodium salt without pH adjustment.

Water was given as the control, rather than sodium chloride or salts of para-hydroxybenzoic acid because of the additional chloride load imposed by the former and the belief, which is based on previous work, that the administration of sodium para-hydroxybenzoate may not be without effect upon electrolyte balance in man (cf. Chapter II, Part I).

The whole investigation was conducted over a period of about four months, throughout which time untreated animals were occasionally killed. This randomisation was introduced in order to spread the control observations throughout the whole experimental period and thus reduce the possibility of chance environmental or dietary factors influencing the result.

After a period of treatment of between four to fourteen days (indicated in each experiment), during which time the rats that received salicylate for more than eight days decreased in body weight by at least twenty grams, the animals were anaesthetised with the ether-oxygen mixture already described elsewhere in this thesis (cf. p. 74). An abdominal incision was made, the viscera displaced and blood collected into a herparinised syringe from the inferior vena cava. This procedure was always performed within thirty minutes after the previous administration of salicylate or water. It was necessary to collect at least 8 mls. of blood from each rat because of the number of plasma assays required on each sample. Plasma was separated by centrifuging at 3,000 r.p.m. for ten minutes. Sodium, potassium, magnesium,

inorganic phosphorus, chloride, calcium and salicylate were determined in plasma obtained from control and salicylate treated rats.

After the blood sample had been collected the whole liver was removed with a pair of small scissors, and freed of large ducts or vessels. The tissue was weighed and then quickly immersed into a small volume of 0.5 M ice-cold sucrose. This procedure washed the tissue as free as possible of contaminating fluids including blood. The liver portions were quickly removed from the sucrose, drained and cut into small pieces in the cold, preparatory to homogenisation into a medium of 0.44 M sucrose which did not contain any additions. The following procedure was essentially similar to that described earlier (p. 75) except that the final volume of the homogenate was such that one gram of wet tissue was dispersed into 10 mls. of medium. A small portion of peripheral liver tissue was excluded from this process, blotted dry, weighed, and utilised for the gravimetric determination of total organ water by drying to constant weight at 105°C. The final volumes and all aliquot sizes of each succeeding step of the preparative procedure were measured and recorded for later calculations.

The mitochondria derived from 6 gms. of wet liver were finally re-suspended to a volume of 15 mls. in 0.44 M sucrose. A representative sample of mitochondria derived from a calculated wet weight of whole liver, was drained as free as possible of supernatant fluid and any "fluffy layer" and weighed. These samples were also dried to constant weight at 105°C and thus gave a measure of the mitochondrial water content as well as the yield of mitochondria per gram of wet tissue.

This method produced the following fractions:-

<u>Fraction (1)</u>: "Whole tissue homogenate"

<u>Fraction (2)</u>: Fraction (1) less whole cells, large cell debris, nuclear fragments and erythrocytes.

<u>Fraction (3)</u>: A sucrose dilution of the "soluble" cell components, termed the "cytoplasmic" fraction.

<u>Fraction (4)</u>: A sucrose suspension of the "particulate" cell components, termed the "mitochondrial" fraction.

Each of these fractions were prepared in the cold (0-20°C).

Chemical methods

Sodium and potassium were determined by flame photometry (EEL). A reference standard which was internally compensated with Na^+ was employed in the estimation of plasma potassium, but not for any of the cellular fractions.

Salicylate was determined, where appropriate, by the method of Trinder (1954), magnesium by the method of Orange and Rhein (1951), phosphate by the method of Taussky and Shorr (1953), and calcium by a method employing calcein as an indicator in ultraviolet light (Hermann, 1958).

Magnesium and phosphorus were assayed in trichloracetic acid extracts of the various cell fractions. It was found that the results obtained for these substances varied. This variation depended on whether the extracts were prepared before or after storage of the sucrose dilutions at -25°C. On storage the values for magnesium decreased, whilst those for inorganic phosphorus increased. The concentration of these ions reported in Series I of this work, were determined in acid extracts which were prepared after various periods of storage at -25°C

and a number of freezing and thawing cycles. The values given in Series II were determined in acid extracts made immediately the various tissue fractions were prepared, except in the case of two animals (Nos. 9 and 10).

Total organ and intramitochondrial water were determined gravimetrically by drying weighed samples of wet liver and mitochondria to constant weight at 105°C. This usually took from twenty-four to forty-eight hours.

Chloride was estimated by the method of Schales and Schales (1941).

Calculation of results

"Chloride Space"

The estimation of the "chloride space" was employed to give a measure of the degree of extracellular contamination of the intracellular fluids, particularly that of the "cytoplasmic" material of fraction (2).

The following formula was employed -

$$\text{Extracellular fluid volume} = \frac{\text{"Cytoplasmic" chloride}}{\text{Plasma chloride}} \times \frac{\text{Total organ water}}{1}$$

$$\text{Intracellular fluid volume} = \text{Total organ water} - \text{Extracellular fluid volume}$$

The corrected or "true" intracellular fluid volume was expressed as a percentage of the total organ water.

Electrolyte concentrations

(a) In the cytoplasm

Once the previous factors were obtained for each preparation, and the plasma and "cytoplasmic" levels of any particular constituent determined, then the concentration of that ion in the "true" intracellular fluid could be calculated by the following formula:-

$$\frac{\left[(I_{cyt.} - I_{blk.}) \times F\right] - \left[I_{pl.} \times W_{ex.}\right]}{W_{int.}}$$

where, $I_{cyt.}$ = the concentration of the electrolyte in the "cytoplasm" after correction for dilution during preparation.

$I_{blk.}$ = the concentration of the electrolyte in the reagent blank.

$I_{pl.}$ = the concentration of the electrolyte in the plasma.

$W_{ex.}$ = the calculated extracellular fluid volume for a particular preparation.

$W_{int.}$ = the calculated intracellular fluid volume for a particular preparation.

F = the volume of cytoplasm equal to one gram of wet tissue.

After allowance for the atomic weight of each substance, its concentration in the cytoplasm could be expressed as milli moles/litre of intracellular fluid, corrected for extracellular contamination.

(b) In the mitochondria

Both the wet weight and the percentage water content of each batch of mitochondria were determined. With this information and the concentration of the electrolyte in the mitochondrial suspension it is possible to calculate the concentration of solute dispersed through the

total mitochondrial water space.

The findings of Werkheiser and Bartley (1957) make it extremely unlikely that all this space is available to these solutes. However, the "space" available to each electrolyte should remain constant under constant conditions. Comparison between values may therefore be possible by this method of investigation.

Results

The extracellular phase:

The plasma is thought to be in equilibrium with the extracellular fluids and is often taken to be representative of this phase (Maurier, 1923). Gamble (1942) has shown that "the only large item of difference between the blood plasma and interstitial fluid is the relatively small quantity of the non-diffusible component, protein, in the interstitial fluid". Values of plasma components which were obtained by assay were considered in their own right, and in addition they were also used to "correct" the values determined for the respective "cytoplasmic" concentrations by the calculation already described. The results of this investigation are given in Table (40) where the mean concentration of the constituents is shown as milli moles/litre. The range of values encountered, the number of animals in each group, and the treatments administered (either the potassium or the sodium salts of salicylic acid) are also shown in this Table.

Examination of the plasma data obtained from control rats (Group I on Table 40) indicates that the values obtained in this study are very similar to those reported by du Ruisseau and Mori (1959).

TABLE (40)

RAT PLASMA CONSTITUENTS

Concentrations expressed as milli moles/litre

CONSTITUENT	GROUP I Control Rats (12 animals)		GROUP II Sodium Salicylate (10 animals)		GROUP III Potassium Salicylate (4 animals)	
	Mean	Range	Mean	Range	Mean	Range
Sodium	146	[122-158]	141	[124-150]	147	[136-163]
Potassium	4·3	[3·3-5·4]	4·8	[3·4-6·0]	6·7	[4·2-9·0]
Magnesium	1·2	[1·0-1·4]	1·5	[1·2-1·9]	1·6	[1·3-1·9]
Phosphorus (inorganic)	2·6	[1·9-2·9]	2·9	[2·5-3·7]	2·8	[2·2-3·2]
Chloride	103	[98-118]	105	[95-109]	108	[101-120]
Calcium	2·9	[2·5-3·2]	2·3	[2·0-2·6]	2·2	[2·0-2·6]
Salicylate	0	-	4·0	[2·5-4·7]	3·7	[1·7-6·0]

Dosage: 30 mgms of "salicylate ion"/100 gms body weight/day. Given by gavage in aqueous solution. Control group fed an equal volume of water.

Mean plasma sodium levels were not greatly altered from control levels by the administration of salicylate. This lack of effect was apparent even when there was a sodium loading, as was the case when sodium salicylate was administered (Group II). However, when the drug was given as the potassium salt, there was an increase in plasma potassium in that Group (III) to 6.7 milli moles/litre from the control level of 4.5 milli moles/litre.

Salicylate treatment elevated the plasma magnesium level from 1.2 to 1.5 and 1.6 milli moles/litre in Groups II and III respectively. Although this increase is small, it was a consistent finding and there was very little "overlap" into the range found in rats not given salicylate. This observation, although not significant itself, agrees with the statistically significant finding previously reported in the whole animal studies (p. 58). In addition the plasma calcium levels were simultaneously decreased by salicylate treatment. The mean plasma Ca^{++} of the two treated groups (2.3 and 2.2 milli moles/litre) were reduced below the level of the control group (2.9 milli moles/litre). Although the degree of "overlap" of the ranges determined in Groups II and III when compared to the control group (I) was small, this finding did not reach statistical significance.

There was a slight increase in the mean plasma inorganic phosphorus levels in the groups receiving salicylate, but there was considerable "overlap" of the ranges in each group and the finding was not significant.

The mean plasma chloride level found in all three groups of rats examined in this study were not appreciably different. The range encountered in each group was quite large, this was due in every instance to a small number of determinations falling outside the usual scatter of

values encountered within each group.

There were no appreciable concentrations of salicylate in the plasma of the control rats when assayed by the method of Trinder (1954). The levels found in the plasma of all treated animals, within thirty minutes of administration of the drug, were comparable to those therapeutically induced in man, and those known to be associated with a hypermetabolic response in the rat. (cf. Table 40).

The intracellular phase

General remarks:- This phase is represented by fraction (2) which was obtained by the preparative method previously described. In this study fraction (2) was further partitioned into the "soluble" cell components represented by fraction (3), and the mitochondrial phase represented by fraction (4). The results obtained from each of these latter fractions will be presented separately. These results are presented as Series I and II, and both series include their own controls. Series I and II are separated from each other both because of differences in the salt of salicylate administered to the "treated" animals within each group, and because of differences in the treatment of material for the assay of Mg^{++} and inorganic phosphorus content. Potassium salicylate was given to some animals in Series I, and sodium salicylate was given to some animals in Series II. In this way the effect of sodium ion loading was controlled in those experiments in which sodium salicylate was administered.

The differences in treatment of material for assay of Mg^{++} and inorganic phosphorus will be described in detail subsequently.

The plasma values obtained from each animal are not shown in the following Tables, but were used to calculate the "intracellular" concentrations given by the method described previously.

SERIES I:

The effect of potassium salicylate

This group consisted of six animals, four of which received potassium salicylate for either four or five days. Magnesium and inorganic phosphorus assays were performed on trichloracetic acid extracts of material which had been stored at $-25^{o}C$ for about ten days.

The total organ water content of all animals in this group, irrespective of treatment, was 70% $\pm$ 1.5%. The mean extracellular water "contaminating" the intracellular fluids was calculated to be 16% $\pm$ 1%. This value also did not change when salicylate was administered to the rats.

"Cytoplasmic" electrolytes and other constituents

Sodium:

The administration of potassium salicylate produced an increase in the mean sodium content of this fraction from 0.7 milli moles/litre to 8.9 milli moles/litre. However, there was great variation within the range of values found in the treated group, which were 0, 1.9, 10.2 and 23.4 milli moles Na^+/litre respectively.

Potassium:

The mean level of potassium within this fraction decreased when the animal was treated with salicylate *in vivo*, despite the fact that there was potassium loading in this instance and an increased plasma potassium level was found (see Table 40). Although the mean potassium level had decreased from 99 milli moles K^+/litre to 84 milli moles K^+/litre following salicylate treatment, there was again a wide scatter of values within the treated group. Only one of these treated animals (rat No. 4) had a cytoplasmic potassium level similar to those determined in the control group. When these "cytoplasmic" potassium values were

examined statistically by obtaining a common variance and determining the significance of the variation of the means, the result was not significant ($P < .3$). This lack of statistical significance may be due to the small sample size of the control group.

Magnesium, calcium and phosphorus

There was no change in the mean concentration of magnesium ions found in this fraction following treatment with salicylate to the whole animal, and calcium was not detectable by the method of Hermann (1958) in any of the fractions obtained from this group. The mean level of inorganic phosphorus was 33 milli moles/litre in the control animals and 20 milli moles/litre in the treated animals with no "overlap" of values between the ranges.

"Intracellular" salicylate estimations were not performed on this series, but the plasma salicylate levels found in animals 3, 4, 5, and 6 were 23, 52, 48 and 82 mgms.% respectively.

These results are shown in Table (41).

The mitochondrial phase

There was a decrease in the mean wet weight yield of mitochondria per gram of whole liver following the administration of potassium salicylate to these rats *in vivo*. However, the range of values in the control group was very variable. This decrease in mean yield was not accompanied by an increase in total water content of the mitochondria derived from salicylate treated animals. This data is given in Table (42).

Following the administration of potassium salicylate the mean concentration of "intramitochondrial" sodium ions increased from 8.6 to 16.6 milli moles/litre. As was found with the "cytoplasm"

TABLE (41)

EFFECT OF POTASSIUM SALICYLATE ON INTRACELLULAR ELECTROLYTE CONCENTRATIONS

Series I - "Cytoplasm"

RAT NO.	TREATMENT	DAYS OF TREATMENT	CONCENTRATION IN MILLI M/L INTRAMITOCHONDRIAL WATER					
			Na.	K	Mg.	P inorganic	Ca.	Salicylate*
1	Control	5	1·4	97	5·4	29·2	0	-
2	"	5	0	100	6·4	35·6	0	-
Average			0·7	99	5·9	33	0	-
3	Potassium Salicylate	5	1·9	73	7·2	25·5	0	-
4	"	4	0	101	5·8	16·0	0	-
5	"	5	10·2	92	5·9	17·9	0	-
6	"	4	23·4	69	5·7	21·2	0	-
Average			8·9	84	6·2	20	0	-

* "Intracellular" salicylate concentrations not estimated. Plasma salicylate levels of rats nos. 3,4,5,6 were 23, 52, 48 and 82 mgms % respectively.

Dosage: 30 mgms. salicylate as the potassium salt/100 grams body weight/day.

TABLE (42)

EFFECT OF POTASSIUM SALICYLATE ON INTRACELLULAR ELECTROLYTE CONCENTRATIONS

Series I - Mitochondria

RAT NO.	TREATMENT	DAYS OF TREAT-MENT	YIELD* IN MGMS.	% WATER CONTENT	CONCENTRATION IN MILLI M/L INTRAMITOCHONDRIAL WATER			
					Na.	K.	Mg.	P. inorganic
1	Control	5	330	69·9	6·5	16·2	4·7	10·5
2	"	5	220	68·0	10·7	26·8	7·7	18·6
Average			275	68·9	8·6	21·5	6·2	14·6
3	Potassium Salicylate	5	239	69·4	8·2	22·2	8·4	4·4
4	"	4	230	68·2	15·1	21·6	7·6	5·9
5	"	5	244	68·6	0	31·8	8·2	6·6
6	"	4	244	71·9	43·0	19·2	6·3	7·6
Average			239	69·5	16·6	23·7	7·6	6·1

* Yield. The wet weight of mitochondria derived from one gram of wet liver by the standard procedure.

All treatment administered by gavage.

Dosage 30 mgms. salicylate as the potassium salt/100 grams body weight/day.

fractions prepared from these animals the range of values was again wide (0 to 43 milli moles Na^+/litre). The animal with the highest cytoplasmic concentration of Na^+ (rat No. 6) also had the highest intramitochondrial concentration of this ion. Intramitochondrial potassium and magnesium ion concentrations were also variable but the mean values of the treated and control animals were quite similar.

The level of inorganic phosphorus found in the treated group decreased strikingly from a mean of 14.6 milli moles/litre in the controls to 6.1 milli moles/litre following the administration of potassium salicylate *in vivo*. There was no "overlap" between the ranges encountered.

This data is also shown in Table (42).

SERIES II

The effect of sodium salicylate

This group consisted of ten animals. Rats No. 1 to 4 were fed water as a control group, whilst rats No. 5 to 8 received sodium salicylate for from five to eight days. The treatment with sodium salicylate was continued for fourteen days with animals No. 9 and 10. These latter animals were the only two out of the group of six which survived this treatment for this length of time. The homogenising and isolation medium was again 0.44 M sucrose without additions. Magnesium and inorganic phosphorus assays were performed on protein free filtrates of the various fractions which were made as soon as the material was prepared. In most cases the chemical estimations were carried out after the acid extracts had been stored at -25°C overnight. However, in two cases (animals No. 9 and 10) where the rats received salicylate for fourteen days, the acid extracts were stored at -25°C for much longer.

The mean total organ water (70%) and the mean calculated extracellular compartment size (16%) of the "cytoplasmic" fractions of the control group were not greatly changed by treatment of the animal with sodium salicylate for periods of up to eight days. After treatment the mean values were (70%) and (14%) respectively. However, when treatment was continued for fourteen days the mean total organ water content had increased to 74% while the mean extracellular water compartment size of the "cytoplasm" fraction had decreased to 6.5% with no "overlap" of values into the control range. This represents a decrease of about 35% in the extracellular water space of these animals compared to the control animals.

"Cytoplasmic" electrolytes and other constituents

Sodium:

The relatively short term administration of sodium salicylate resulted in a small increase in the mean Na^+ content of this fraction from a level of 4.2 milli moles/litre in the controls to 6.1 milli moles/litre in the treated group. When salicylate treatment was continued for fourteen days the mean level increased further to 14.1 milli moles Na^+/litre. However, the range of values encountered was again variable (0 to 14.8 milli moles/litre). When the sodium content of the whole tissue homogenage (fraction 1) was estimated in this group, the Na^+ content of this fraction was found to be much more consistent than in the "cytoplasm" fraction. Means of 16, 18 and 17.5 milli moles Na^+/litre were determined for the control, "short term" and "long term" administration of salicylate respectively. In addition the range of values was much more restricted (12 - 19 milli moles Na^+/litre).

Potassium:

The mean level of "cytoplasmic" K^+ decreased when sodium salicylate was administered to rats in vivo, in the same manner as that found previously when potassium salicylate was given. The mean level of this ion in the control group was 119 milli moles/litre, and that following relatively short term administration was 91 milli moles/litre. Administration of sodium salicylate for fourteen days did not decrease the level of this ion further as the mean value of "cytoplasmic" K^+ for these animals was 94 milli moles/litre. Although the individual values were again variable similar to those found following the administration of potassium salicylate, only one of the control values (animal No. 4) had a concentration of this ion which was within the range of values found in those animals which had received salicylate treatment.

Statistical examination of this data by the method used for the examination of the "cytoplasmic" potassium levels of Series I, demonstrated a significant difference ($P < .05$) between the mean level of the control group and the mean of all animals treated with sodium salicylate.

Magnesium:

There was a progressive fall in the mean concentration of Mg^{++} in this fraction following the administration of sodium salicylate. The mean control level was 7.3 milli moles/litre which declined to 6.2 milli moles/litre with the shorter period of treatment. The level of this ion had further fallen to 3.5 milli moles/litre by the fourteenth day.

Phosphorus:

The effect of sodium salicylate administration on the concentration of inorganic phosphorus in this fraction was not consistent. Relatively short term administration produced a small decrease from a mean control level of 23 milli moles/litre to a mean value of 19 milli moles/litre. The mean value found after fourteen days treatment was 33 milli moles/litre. However, this result, as well as the marked decrease found in the magnesium ion content of these two samples, following prolonged treatment, may not reflect the real comparative concentrations of these substances, as in this instance the protein free filtrates of these particular fractions had been stored at $-25^{\circ}C$ for some time.

"Cytoplasmic" calcium and salicylate levels were only assayed in the control and the relatively short term salicylate treated animals. As in Series I where animals were treated with potassium salicylate, no

calcium was detectable in any of the fractions examined. Salicylate could not be detected in the control group, but the mean salicylate level of the treated group was 4.2 milli moles/litre.

The details of these results are shown in Table (43).

Mitochondrial phase

Unlike the effect seen to follow the administration of potassium salicylate to rats *in vivo*, in this Series there was an increase in the mean yield of mitochondria per gram of wet liver following the short term administration of the drug. However, there was a marked decrease in the mitochondrial yield when sodium salicylate administration was continued for fourteen days. The water contents of all the mitochondria examined in this particular series were slightly higher than were those found in the study where potassium salicylate was used. The differences between the mean level of the control group and both the treated groups were only small.

The individual "intramitochondrial" sodium ion concentrations were again variable, although the range of values was not as great as that encountered in the previous study. The mean value of the control group was 4.7 milli moles Na^+/litre, that of the "short term" group 5.5 milli moles Na^+/litre whilst the group which received sodium salicylate for fourteem days had a mean level of 7.2 milli moles Na^+/ litre.

The potassium ion content of this fraction was also variable and no trend was apparent following the administration of sodium salicylate. This was brought about by two of the six treated animals (Nos. 8 and 10) having mitochondrial potassium concentrations greater than any of the control values, whilst the remaining four animals had

TABLE (43)

EFFECT OF SODIUM SALICYLATE ON INTRACELLULAR ELECTROLYTE CONCENTRATIONS

Series II - "Whole tissue" and "Cytoplasm"

ANIMAL NO.	TREATMENT	DAYS OF TREAT-MENT	% TOTAL ORGAN WATER	% EXTRA-CELLULAR WATER	WHOLE TISSUE Na.	CONCENTRATION AS MILLI M/L "INTRACELLULAR" WATER				
						Na.	K.	Mg.	inorganic P.	Salicylate
1	Control	5	69·9	16	16	0·5	106	7·0	17·2	0
2	"	5	70·8	25	12	0	146	7·2	25·8	0
3	"	6	68·7	12	16	6·6	128	7·8	24·6	0
4	"	8	70·4	12	18	9·7	95	7·1	25·0	0
	Average		70·0	16	16	4·2	119	7·3	23	0
5	Sodium Salicylate	5	70·9	16	18	3·7	93	7·2	23·0	5·0
6	"	5	71·1	17	19	3·9	83	7·3	24·8	4·1
7	"	6	70·0	11	16	8·5	77	5·2	14·1	3·1
8	"	8	69·3	12	18	8·4	111	4·9	12·3	4·4
	Average		70·3	14	18	6·1	91	6·2	19	4·2
9	Sodium Salicylate	14	76·0	3	16	13·5	94	2·4	34·0	-
10	"	14	72·0	10	19	14·8	95	4·6	33·0	-
	Average		74·0	6·5	17·5	14·1	94	3·5	33	

*Whole tissue Na concentration as µmols./gm. wet weight liver.

- = not done.

All treatments administered by gavage.

Dosage: 30 mgms. salicylate as the sodium salt/100 gms body weight/day.

values below the lowest value found in the control group. The mean level of K^+ in the control group was 16.2 milli moles/litre, that of the "short term" salicylate treated group 14.3 milli moles/litre, whilst that of the group having more prolonged administration was 18.7 milli moles/litre.

The range and the mean "intramitochondrial" magnesium ion content did not change when sodium salicylate was administered for up to eight days. The mean value in the control group was 5.7 (range 5.1 - 6.9) milli moles Mg^{++}/litre whilst the mean value of the treated group was 6.2 (range 4.6-7.2) milli moles Mg^{++}/litre. "Intramitochondrial" Mg^{++} was not detectable in either of the samples derived from the animals which had been treated for fourteen days. However, the acid extracts used for these assays and for inorganic phosphorus, had been subjected to storage at -25°C.

There was no marked difference between the range of inorganic phosphorus values found when the control group was compared to the values determined in those animals which had received sodium salicylate for up to eight days. The mean values of these groups were 5.2 and 4.2 milli moles P/litre respectively. However, the values were much increased in those animals which had received salicylate for fourteen days, but here the acid extracts had been stored at - 25°C for some time.

These results are shown in detail in Table (44).

TABLE (44)

EFFECT OF SODIUM SALICYLATE ON INTRACELLULAR ELECTROLYTE CONCENTRATIONS

Series II- Mitochondria

ANIMAL NO.	TREATMENT	DAYS OF TREAT-MENT	YIELD * IN mgms.	% WATER CONTENT	CONCENTRATION AS MILLI M/L "INTRAMITOCHONDRIAL" WATER			
					Na.	K.	Mg.	inorganic P.
1	Control	5	348	71·7	7·4	17·4	5·1	4·2
2	"	5	317	72·5	2·7	16·5	6·9	5·2
3	"	6	296	70·4	5·8	14·5	5·3	5·2
4	"	8	328	73·0	2·7	16·6	5·7	6·2
	Average		322	71·9	4·7	16·2	5·7	5.2
5	Sodium Salicylate	5	394	74·7	4·2	13·0	4·6	3·8
6	"	5	400	73·8	8·1	10·8	6·6	5·2
7	"	6	386	73·0	6·9	13·1	6·5	3·4
8	"	8	318	70·6	2·8	20·2	7·2	4·3
	Average		375	73·0	5·5	14·3	6·2	4·2
9	Sodium Salicylate	14	269	73·8	6·1	12·3	0	9·2
10	"	14	232	71·6	8·3	25·0	0	15·2
	Average		250	72·7	7·2	18·7	?	12·2

*Yield: the wet weight of mitochondria derived from one gram of wet liver by the standard procedure.

All treatments administered by gavage.

Dosage: 30 mgms. salicylate as the sodium salt/100 gms. body weight/day.

Discussion

A purposeful discussion of the data obtained in this study is hampered by the sample size as well as inherent difficulties in both the method of preparation of the various fractions and other factors which influenced some of the chemical assays or the calculation of the results. All these factors contributed to the variability of the results, not only within a group but also between the two series. Therefore it is proposed to examine these problems in some detail as they are material to any interpretation of the data.

Some of the objections to the use of a method involving tissue homogenisation followed by differential centrifugation into various fractions at $0^{\circ}C$ are:-

(a) the degree of extracellular contamination of the intracellular phase;

(b) artifactual changes produced in distribution between phases consequent to cellular disruption;

(c) the lability of gradients _in vivo_, which may be lost in preparation of sub-cellular fractions.

These objections will be considered separately:-

(a) In any work of this nature there is always some degree of "contamination" of intracellular fluids by the extracellular phase, as suitable techniques for examination of tissues from experimental animals without any transfer of fluids from one compartment to another are extremely difficult. Because of this, much experimental data has been presented as "whole tissue" electrolyte concentrations (Watchorn and McCance, 1937). The method used here does not appear to suffer from this defect to an extent greater than other methods in use, and does provide an opportunity for correction of the results by means of an

estimation of the chloride space, which is thought to give a reasonable measure of the extracellular water in tissue preparations (Fenn, 1936) (Dickinson and Widdowson, 1960).

Utilisation of this concept is possible because most workers today agree with the original observations of Maurier (1938), Manery and Hastings (1939) and Traux (1939) which suggested that all the chloride of the liver is outside the hepatic cells and its concentration is the same as that of the plasma. Hence by estimation of the plasma and cell fraction chloride one can calculate the proportion of "extracellular" contamination of the various fractions. However, one further aspect of this problem must also be considered. Because of the nature and number of assays required on the plasma (which is regarded as representative of the extracellular phase) it was necessary to collect a relatively large blood sample of at least 8 mls. from each animal. Although exsanguination is known to cause a movement of interstitial fluid into the vascular system many previous investigations of this type, particularly those concerned with the electrolyte content of skeletal tissue, have been made after the animal has lost a considerable proportion of its total blood volume (Harrison and Darrow, 1938) (Darrow, Harrison and Taffel, 1939). In their recent publication Mounib and Evans (1960) also suggest that under conditions of extensive blood loss there is a movement of fluid from the intracellular to the extracellular phase. The report of Widdowson and Southgate (1959) which appeared during the progress of this investigation also supports this view, although these latter workers found the rat to be the least affected of all the species they investigated. Widdowson and Southgate concluded that "if the chloride space is only used to calculate the composition of the intracellular compartment it probably will not make

much difference to the conclusion reached whether the animal (rat) had bled or not."

It is not possible to say to what extent if any the results presented here have been influenced by these movements, but presumably any bias introduced will always be in the same direction in both control and salicylate treated animals.

(b) Despite the variation of the electrolyte content of the "cytoplasm" within groups and between series, the results are generally of the same order as those found by other workers using different methods of preparation. It seems reasonable therefore to assume that no marked changes in distribution have occurred between the extracellular and intracellular phases of the tissue when the cells are disrupted.

(c) In 1954, Bartley and Davies recognised that changes in the mitochondrial content of labile compounds may occur very rapidly. Because of this observation Werkheiser and Bartley (1957) have developed a method for "fixing" some of the labile constituents of mitochondria concurrently with the centrifugal sedimentation of the mitochondrial pellet. However, the method is extremely involved and does introduce factors which interfere with later chemical assays. In addition this method could not be used in its present form to maintain gradients within the cell cytoplasm during homogenisation.

For these reasons it was decided to prepare the sub-cellular fractions, particularly the "mitochondrial phase" by the usual type of procedure (Schneider, 1948) and not to adopt the "fixative" technique of Werkheiser and Bartley (1957). However from the results of investigations carried out in vitro, which have been previously

reported in this thesis it is clear that there was an opportunity for some losses of mitochondrial solutes during the isolation procedure (cf. Table 35). The washing phase of this process was carried out as quickly as possible so that the "leakage" of intramitochondrial solutes would be kept to a minimum, but due to the favourable gradients produced, some leakage still occurred. Examination of the washing fluids after this procedure revealed the presence of potassium and sodium ions and inorganic phosphorus. Magnesium ions could not be detected. Werkheiser and Bartley (1957) also demonstrated that the composition of the medium employed for the isolation of the mitochondria could greatly affect the water and solute content and "sucrose space" of isolated mitochondria. However, if a simple medium were employed throughout a series of observations then the results should be influenced in a similar way, and whilst not correct in an absolute quantitative sense, may be comparable in a qualitative manner which would be suitable for the aims of this investigation.

Bartley and Davies (1954) have demonstrated that at least some cellular and intracellular ion gradients are maintained by active metabolic processes. In addition, Fenn (1940) and Spiegelman and Reiner (1942) have discussed the "dynamic" nature of the maintenance of high intracellular potassium concentrations. However there was evidence from the previously reported studies of the effect of salicylate on mitochondrial electrolytes _in vitro_, that an action of salicylate _in vivo_ would not be lost by a preparative method carried out at low temperature (0-2°C).

The time at which the trichloracetic acid extracts of the various cell fractions were made, and their length of storage at -25°C,

altered the results obtained when this material was assayed for inorganic phosphorus and Mg^{++} content. The values for inorganic phosphorus were most affected by variations in the method, and substantial increases were found when tissue fractions were stored for long periods. Presumably this was brought about by the break-down of organic phosphate complexes, such as adenosine triphosphate, which would not normally contribute to the estimation of inorganic phosphorus by the method of Taussky and Shorr (1953). This effect was still apparent when the acid filtrates themselves were frozen and thawed several times.

Recent findings in this laboratory suggest that there is an effect upon plasma magnesium levels associated with the storage of biological material at -25°C when this material contains protein. In a study of plasma magnesium concentrations in a group of one hundred hospital patients with varying thyroid status, it was found that the plasma magnesium levels of this ion declined on prolonged storage of the sample (Charnock and Lockett, 1960), when the plasma was assayed by the method of Orange and Rhein (1951). A similar effect may be partially responsible for the absence of this ion in two acid extracts of mitochondria which had been stored for some weeks at -25°C. This result is shown in Table (44), animals No. 9 and 10.

It is now possible to proceed to an examination of the experimental data obtained in this particular investigation. Consideration will be given to the points discussed above. The following observations are considered to be the essential findings of this work. Statistical analysis was only invoked where it was desired to confirm a finding.

The extracellular component

This compartment is represented by the plasma fraction. It was found that there was an increase in plasma K^+ when potassium salicylate was given in vivo. If this were an effect of the salicylate ion resulting in the loss of potassium from the cells then it would also be evident when sodium salicylate was given. However such an effect was not evident and therefore the increased plasma K^+ level which followed the administration of potassium salicylate is thought to be simply the result of potassium loading.

The only positive changes found in this compartment were the small but constant increases in plasma magnesium levels and the corresponding fall in plasma calcium levels. There can be little doubt that under the influence of salicylate the plasma Mg^{++} level increases quickly (cf. p. 58). In previous studies in man and rats it had been shown that there was a marked fall in the urine output of this ion following the administration of salicylate to the whole animal. This suggests a "true" retention of magnesium under the influence of this drug. Although there is no data on the urine calcium levels in this thesis it is well known that extracellular calcium bears an inverse relationship to magnesium (Tufts and Greenberg, 1938 a, 1938 b) (Moore, Orent and McCollum, 1936) (Hegsted, Vitale and McGrath, 1956). This is born out by the finding here of a small but definite depression of the calcium level of the plasma. Such a relationship may be associated with a common vascular transport mechanism for these bivalent cations (Alcock and MacIntyre, 1960).

However, it is felt that if the retention of Mg^{++} were a "purposeful" retention by the whole animal, a change would also be seen

in the intracellular Mg^{++} concentrations. This intracellular change was not evident with the methods used here. One explanation which could be offered for this finding is that if renal re-absorption of salicylate ions occur as a magnesium chelate complex, then there may be an obligatory magnesium re-absorption. No evidence is available in this regard from this work.

It is well known that intravenous magnesium sulphate is both an analgesic and an antipyretic agent. In 1928, Winter and Barbour demonstrated a "reduced toxicity of salicylate by a protective antagonism with magnesium salts". It is possible that the retention of magnesium ions is an explanation of the antipyretic property of salicylate.

Water distribution

The administration of salicylate to rats for periods of up to eight days, and in a dosage sufficient to produce a hypermetabolic response, did not produce a change in either the total organ water content of the liver or the distribution of water between the compartments examined in this study. However, continued administration of the drug for periods of up to fourteen days resulted in considerable body wastage by the animals. Only two rats survived out of a group of six which received this treatment. Examination of the data obtained from these two animals suggests a decrease in the extracellular water compartment with an overall increase in the total organ water. This may be regarded as presumptive evidence of intracellular overhydration. This may be a non-specific phenomenon which occurred in association with a marked reduction in the dietary intake of these rats which followed this prolonged administration of salicylate *in vivo*.

The intracellular compartment

The preliminary discussion has suggested the difficulties involved in drawing firm conclusions from the data obtained in these experiments. Because of the restricted sample size, variability of the results, and lack of knowledge of the distribution of values of electrolyte concentrations under "steady-state" conditions, statistical analyses were only occasionally invoked in an examination of this data.

No consistent changes were apparent when the mitochondrial electrolyte data was examined. The problem of artificial concentration gradients introduced during the isolation of these particles, and the rapidity with which mitochondrial solutes come into equilibrium with their environment (Bartley and Davies, 1954), suggests that little value can be attached to these results.

The variation seen in mitochondrial inorganic phosphorus content is thought to be the result of the break-down of labile phosphate complexes during the storage of material for assay. There were no consistent changes in the yield of mitochondria obtained from treated rats when compared to the control animals, and the water content of these particles was not influenced by administration of the drug in vivo, when judged by the results obtained here. Limitations imposed by the method of isolation of the mitochondria would probably preclude the observation of an effect of salicylate on mitochondrial water content even if it were in force in vivo.

"Cytoplasmic" electrolytes

A change in intracellular magnesium ion concentration was not found although salicylate administration does produce a decrease in urinary output and elevation in the plasma concentration of this ion.

However, it is known from the work of others how difficult it is to produce states of magnesium deficiency in experimental animals, as the large skeletal reserve of this ion provides a mechanism which "damps" rapid changes in soft tissue magnesium content (Watchorn and McCance, 1937) (Blaxter, Rook and MacDonald, 1954) (Duckworth and Wernock, 1942) (MacIntyre and Davidson, 1958).

When consideration is given to all the technical problems already discussed, it still seems reasonable to comment on three findings in this compartment. These are the extreme variation in the concentration of "intracellular" sodium ions, the salicylate induced fall in "intracellular" potassium ions and the changes in inorganic phosphorus seen between the various sub-groups.

"Cytoplasmic" sodium

In a discussion of cellular electrolytes E. J. Conway (1957) has suggested that the cell does not specifically exclude the entry of sodium ions but rather actively expels them after entry. Acceptance of this hypothesis leads to a situation in which there is a variable and rapid exchange of sodium between extracellular and intracellular compartments. Whilst the total organ Na^+ may remain fairly constant, it is reasonable to expect large variations in the quantity of sodium ion found in the extracellular phase at any one time. Such a range of values was first observed in the "cytoplasm" of those animals grouped together as Series I. Later when the assays were performed on Series II material, the sodium ion concentration of the whole tissue homogenate was determined in addition to its concentration in the "cytoplasm" fraction. In the former material the sodium ion concentration was quite steady and varied about a mean of 16.8 milli

moles/litre by only about ± 5 milli moles/litre. Examination of the "cytoplasm" sodium ion concentration again showed great variation between 0 to 14.8 milli moles/litre. Under these conditions the "steady-state" concentration of intracellular sodium is hard to define. This observation suggests that there is no obvious relationship between "whole tissue" sodium concentrations and the "true intracellular" sodium concentrations at any particular moment.

"Cytoplasmic" potassium

In these small series of animals there was a decrease in the mean intracellular K^+ concentration following the administration of salicylate. This effect was evident both when salicylate was given as the sodium or potassium salt. The large range of values within sub-groups, and the consequent large standard deviation calculated for these groups rendered the statistical analysis of this data of decreased significance. However, within Series II only two values obtained from treated animals entered into the range of values obtained with the controls, and no value exceeded the mean of the control group -

Series II "controls" 119 ± 14.6 milli moles K^+/litre

Series II "treated" 91 ± 14.3 milli moles K^+/litre.

Application of the "t" test revealed a significant difference between the means of these two groups, $P < .05$.

In Series I, where some animals were given potassium salicylate, a somewhat similar situation is found. Of the four animals which received the drug only one value (animal No. 4) exceeded the mean of the control group which only contained two animals. However, two of the four treated animals (Nos. 3 and 6) had

"cytoplasmic" K^+ values of 73 and 69 milli moles/litre. These two levels are considerably below the mean control value of 99 milli moles K^+/litre found in this Series (I). Statistical analysis of the data from this Series showed no significant decrease in cytoplasmic potassium following administration of salicylate ($P < .3$). This statistical result is almost certainly due to the small sample size of the control group. Therefore it is considered that these results are more meaningful than statistical analysis would indicate, particularly when it is remembered that the treated animals received a K^+ load because potassium salicylate was administered.

As the total number of measurements is small and the variation within the combined series quite large, analysis of this data by the "Rank t-test" (White, 1952) seemed appropriate, as it is not necessary to assume normal distributions with equal variance to derive this test. Analysis by this method demonstrated a significant difference (5%) between the "cytoplasmic" K^+ concentrations of the salicylate treated and combined control groups. Therefore the possibility of the decrease in "cytoplasmic" K^+ being due to an action of sodium ions (in Series II) is very slight.

This data could be explained by at least two possible mechanisms. Firstly the loss of K^+ may be a secondary consequence of disorders of energy producing mechanisms within the cell, for example a primary effect of salicylate on the production of "high energy" phosphate bonds required to maintain the potassium gradient. Alternatively the loss of potassium ions themselves may be the cause of intracellular metabolic disorders which lead to an uncoupling of oxidative phosphorylation.

It is impossible from this work to supply an answer to either of these hypotheses. However, the marked decrease in intramitochondrial potassium concentration under the influence of salicylate at 0°C, conditions under which uncoupling of oxidative phosphorylation could not be in operation, did suggest the possibility that this phenomenon was a cause and not an effect.

"Cytoplasmic" inorganic phosphorus

Because of the possibility of the break-down of organic phosphate complexes contributing to the assay of inorganic phosphorus, interpretation of these results is difficult. However the conditions of assay were such that the higher inorganic phosphorus concentration of the two control animals in Series I may be a reflection of a higher total phosphate concentration in the control group than in those animals treated with salicylate. In Series II (animals No. 1 to 8 only) there was some evidence again suggesting a decrease in phosphorus concentration following the administration of salicylate. These latter assays were performed under circumstances in which the break-down of organic phosphate complexes would be minimal. The remaining two assays of this series (animals No. 9 and 10) were not performed under these conditions as the material for assay had been stored at -25°C for several weeks. Here the inorganic phosphorus values found were uniformly much higher than the remainder of the series. This finding is probably due to the break-down of organic phosphate complexes on storage.

One speculative interpretation of this data is that while there may be little change in the total phosphate content of

the "cytoplasm" there is less readily hydrolysable phosphate and a fall in inorganic phosphate in the "cytoplasm" of salicylate treated rats, than is present in the "cytoplasm" of untreated rats. A similar pattern of results was found on examination of the mitochondrial inorganic phosphorus concentrations determined in both Series I and II of this study.

Previously a similar finding has been reported by two other groups of workers who demonstrated that there was a fall in inorganic phosphorus associated with a fall in "tissue" potassium when the active phosphorylation of glucose is inhibited both in vivo and in vitro (Kaplan, Franks and Friedgood, 1945) (Kamininja, Willebrands, Groen and Blickman, 1950).

Summarising these findings of an effect of salicylate upon electrolyte distribution in vivo, good evidence was found for an extracellular retention of magnesium ion and somewhat less evidence for a loss of potassium ions from the cell. Certainly there was a loss of potassium ions in eight of the ten "cytoplasm" fractions obtained from salicylate treated rats. The possibility that this effect may have been present in vivo but not detected in the mitochondria in vitro has been discussed. In addition the fall in intracellular K^+ may have been accompanied by a fall in the concentration of inorganic phosphorus within the "intracellular" phase.

CHAPTER X

RECAPITULATION AND CONCLUSIONS

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An investigation of the metabolic response which follows the administration of salicylate to the whole animal was reported in Chapter II of this thesis. This study demonstrated that the administration of a large single dose of sodium salicylate to man produced a considerable increase in oxygen consumption. This effect was not evident when sodium para-hydroxybenzoate was given in the same dosage. A decrease in the level of plasma cholesterol and a transient disturbance in electrolyte excretion were associated with this phenomenon. In rats the effect of repeated administration of large doses of sodium salicylate were again characterised by an increase in oxygen consumption and associated disturbances in electrolyte balance. The increase in oxygen consumption was found to be a non-cumulative effect which was operative for only a few hours after administration of the drug, even when treatment was continued for six days. A marked retention of Mg^{++} was the most consistent of the electrolyte changes. Rats which had been treated with salicylate for six days also exhibited a considerable decrease in total body mass. Thus the increase in oxygen consumption did not appear to be related to a productive stimulation of metabolism.

This "wasteful" increase in the oxygen consumption of the whole animal was therefore thought to be consistent with the view that salicylate "uncouples" oxidative phosphorylation reactions _in vivo_. An _in vitro_ uncoupling action of salicylate had already been demonstrated by several other investigators who examined this phenomenon under varying laboratory conditions and with mitochondria which had

been isolated from a number of different tissues. It was unlikely that the effect of salicylate was brought about by a specific action of this drug upon the enzymes of mitochondrial respiration as Sproull had shown that in the presence of salicylate in vitro the rate of oxygen uptake by isolated mitochondria was reduced when several Krebs cycle intermediates were the substrate.

The transient nature of the metabolic response to salicylate administration in whole animals and the "tolerance" to relatively large doses of the drug in clinical medicine, suggest that the biochemical mechanism of action of salicylate is readily reversible. This possibility has been examined here. In addition an investigation has been made of a possible relationship between the transient disturbances in electrolyte balance seen in vivo, to the mechanism of action of the drug.

Evidence has been produced to confirm the in vitro uncoupling effect of salicylate upon mitochondria isolated from the livers of untreated rats. However, when mitochondria were isolated from the livers of rats treated with large doses of salicylate in vivo, and then examined under identical test conditions to those isolated from untreated rats but without the direct in vitro addition of salicylate to the test system, no inherent defect was evident in their ability to carry out oxidative phosphorylation reactions. This absence of effect was supported by the finding that "pre-soaking" mitochondria by suspension in a medium containing sodium salicylate at a concentration equivalent to high plasma levels of the drug failed to uncouple oxidative phosphorylation, when these sub-cellular organelles were subsequently examined in vitro. The suggestion that salicylate

did not primarily act upon the enzymes of mitochondrial respiration was supported by the finding that _in vitro_ the oxygen uptake of isolated mitochondrial preparations utilising either α-keto-glutarate, succinate or β-hydroxybutyrate as substrates were not increased by the addition of sodium salicylate.

It was established that the "cell sap" of the livers of salicylate treated rats could contain a concentration of salicylate radical approximately equal to that of the plasma of these rats. Evidence was also found that salicylate could penetrate mitochondria _in vitro_ and its concentration here attain rapid equilibrium with that of the mitochondrial environment. At 0°C this "intramitochondrial" salicylate was not firmly bound by mitochondria and could be readily removed by a single "wash" _in vitro_.

Four hypotheses were proposed whereby the apparent absence of an effect on isolated mitochondria of salicylate administered _in vivo_ may be compatible with an "uncoupling" action of the drug in the whole animal.

The first of these hypotheses was concerned with the possibility that the _in vitro_ action of salicylate was confined to an inhibition of the phosphate acceptor moiety of the _in vitro_ test system. The hexokinase-glucose reaction is usually employed for this purpose. Under _in vitro_ conditions this reaction may be regarded as "unphysiological" as it creates a continual and competitive demand for ATP, rather than the variable demand which must be encountered within the cell. Hence it could be claimed that in this manner the hexokinase-glucose reaction serves to "drive" the reaction. Unequivocal evidence was not obtained from an examination of test systems in which

the usual phosphate acceptor reaction was absent or replaced by ADP, although some results were obtained which suggested that the inhibitory effect of salicylate on phosphorylation reactions was still evident in test systems independent of the hexokinase-glucose reaction.

The second hypothesis which was proposed, suggested that the mitochondria of rat liver were lacking in an essential co-factor necessary for oxidative phosphorylation _in vivo_. By definition this lack of co-factor was overcome by placing isolated mitochondria in a test system which would support efficient oxidative phosphorylation _in vitro_. This hypothesis therefore excludes such co-factors as DPN and cytochrome C, as these agents were not present in the _in vitro_ test system. Adenosine nucleotide may be a factor to be considered, but no evidence for the loss of this substance from the mitochondria of rats treated with salicylate _in vivo_ is presented in this thesis. The role of magnesium and potassium ions was examined in relation to this hypothesis. The inhibitory _in vitro_ effect of salicylate upon oxidative phosphorylation reactions could not be overcome by the addition of "excess" magnesium, and therefore a simple complexing of Mg^{++} by salicylate did not appear to be the cause of the _in vitro_ uncoupling effect of salicylate. Subsequent work did not show either a decreased intracellular concentration or loss of Mg^{++} from the mitochondria of rats treated with salicylate _in vivo_. An examination of a possible relationship between K^{+} and the _in vitro_ effect of salicylate on oxidative phosphorylation reactions was hampered by the necessity to substitute the usual _in vitro_ test medium, containing potassium dihydrogen phosphate, with another of equal inorganic phosphorus content and buffering power but without K^{+} or other

univalent cations. All media devised for this purpose failed to support efficient phosphorylation even in the presence of K^+ when these ions were added as the chloride salt. This experimental difficulty precluded an examination of liver mitochondria isolated from rats treated with salicylate _in vivo_. In the light of subsequent evidence, the importance of such an examination is increased in an evaluation of the mechanism by which salicylate uncouples oxidative phosphorylation reactions.

The third hypothesis which was examined was related to the possibility of a direct but readily reversible action of salicylate upon the enzymes of mitochondrial oxidative phosphorylation. Earlier experiments had suggested that the enzymes of phosphorylation rather than those of respiration were inhibited by salicylate _in vitro_. As these enzymes have not yet been identified any specific examination of this particular phase of the complex reactions of oxidative phosphorylation is difficult. The effect of salicylate on mitochondrial ATPase was examined in relation to this hypothesis as it is believed that this enzymic reaction of mitochondria is not a simple hydrolysis of ATP but a summation of the terminal steps of phosphorylation, that is ATPase activity was related to the ATP-inorganic phosphorus exchange reaction. The studies of Myers and Slater had suggested that there are four separate ATPases of mitochondria which are characterised by separate pH optima. These enzyme systems are influenced by the presence or absence of Mg^{++} and are acted upon to different extents by dinitrophenol, which is a powerful uncoupling agent. There is also a difference between the ATPase activity of fresh and aged mitochondrial preparations.

An examination was undertaken to determine the relative significance of these factors in relation to the stimulation of "latent" ATPase activity, and the effect of salicylate on this process.

This study demonstrated definite, but separate "patterns" of response to salicylate addition by fresh and aged mitochondrial preparations relative to four pH values. The activity of aged mitochondrial ATPase activity was greatly dependent upon the presence of Mg^{++}, whereas fresh preparations exhibited considerable ATPase activity in the absence of Mg^{++}. Salicylate considerably enhanced the activity of fresh mitochondrial ATPase activity at three of the four pH values examined, but failed to influence the ATPase activity of aged mitochondrial preparations at any of the pH levels studied.

To exert ATPase activity <u>in vitro</u> mitochondria must be permeable to ATP which is supplied by the addition of this substance to their environment. Presumably the permeability of mitochondria is enhanced by membrane damage associated with the ageing process. It was found that salicylate had no effect on these enzyme systems per se for no enhancement of activity was seen when salicylate was added either to aged mitochondrial preparations or to the "soluble" ATPase activity of a mitochondrial free system. When the mitochondrial membrane was "intact", salicylate would enhance ATPase activity presumably by increasing the availability of substrate (ATP) to enzyme surface. This evidence suggests that the action of salicylate on this aspect of mitochondrial enzyme function is indirect and is primarily concerned with an alteration in membrane permeability. This suggestion is supported by the evidence of others who have shown that ATPase activity is localised in the mitochondrial membrane and

that fresh mitochondria normally have a low permeability to ATP.

A further speculation was made from this evidence suggesting an explanation of the one salicylate insensitive step in the four stage phosphorylation associated with the oxidation of α-keto-glutarate by isolated mitochondria in vitro.

A detailed examination of the possibility that salicylate acts on the permeability of the mitochondrial membrane led directly to an examination of the fourth hypothesis, in which the possibility of a change in the permeability of the mitochondrial membrane was explored by a number of different methods. Both gravimetric and spectrophotometric examinations of the effect of salicylate on mitochondrial water content failed to yield conclusive evidence. This hypothesis was further examined by an in vitro study of the effect of sodium salicylate on the distribution of K^+ between mitochondria and their experimental environment at 0°C.

Clear evidence was obtained that the presence of the salicylate radical in the medium greatly enhanced the loss of K^+ from isolated mitochondria at 0°C. This loss was not apparent when either sodium para-hydroxybenzoate or sodium chloride replaced sodium salicylate in the medium. As this loss of K^+ occurred at 0°C this observation suggests that the effect is not a disorder of a metabolic process and hence is not an obligatory consequence of an action of salicylate on mitochondrial oxidative phosphorylation. The possibility exists that it may be a cause rather than a consequence of an action of salicylate on mitochondrial enzyme function.

In order to examine the concept of a "primary" effect of salicylate on membrane permeability in vivo, a study was made of the

distribution of electrolytes following the administration of salicylate to the whole rat. Not only was the distribution of electrolytes between the extracellular and intracellular phases of rat liver tissue examined, but the distribution between the "soluble" and particulate components of the cells was also studied.

Technical problems associated with the maintenance of concentration gradients _in vitro_ prevented a fruitful examination of the mitochondrial phase of these sub-cellular fractions, and hampered the assay of Mg^{++} and inorganic phosphorus.

Examination of the "cytoplasmic" fractions obtained from the livers of salicylate treated rats disclosed a significant fall in the concentration of intracellular K^{+}. This effect was apparent even when the potassium salt of salicylic acid was administered to rats. The loss of K^{+} from this fraction was not accompanied by a compensating increase in the concentration of Na^{+}.

Examination of the plasma of salicylate treated rats showed an increase in the concentration of Mg^{++}. This finding confirmed a previous finding where the increase in plasma Mg^{++} of salicylate treated rats was accompanied by a striking decrease in the urinary excretion of this ion. These findings suggested that salicylate produced a true retention of this ion. However, examination of the intracellular fractions did not reveal any decrease in the concentration of Mg^{++}. Therefore this retention is only extracellular although it was felt that the large skeletal reserves of Mg^{++} may prevent a depletion of the intracellular concentration of this ion.

The decreased intracellular concentration of K^{+} in liver samples prepared from rats treated with salicylate _in vivo_ and

the increased loss of K^+ from isolated mitochondria at 0^oC in the presence of sodium salicylate _in vitro_ suggest a physiological mechanism whereby salicylate could interfere with mitochondrial oxidative phosphorylation _in vivo_. A primary action of salicylate which changes the permeability of biological membranes including that of the mitochondria with a resultant depletion of intracellular potassium ions is the type of effect which is compatible with transient metabolic effects in the whole animal. While the plasma and intracellular levels of salicylate are high the effect of this drug is evident. When the concentration of the drug falls _in vivo_, a re-distribution of K^+ may occur and normal enzymic function could be restored, as the presence of salicylate in high concentration within the cell does not produce irreversible damage.

This concept could also explain the finding of relatively unimpaired efficiency in oxidative phosphorylation reactions when rat liver mitochondria isolated from rats treated with salicylate _in vivo_ were examined _in vitro_. It could be assumed that these mitochondria were inefficiently carrying out oxidative phosphorylation reactions _in vivo_ in an environment which contained salicylate and was depleted in K^+. Under these conditions the permeability of the mitochondrial membrane may be enhanced. During the isolation of these particles they are washed free of salicylate, and when incubated in a medium _in vitro_ which contained relatively high concentrations of K^+ it is quite conceivable that the intramitochondrial depletion of this ion is rapidly restored. As no irreversible damage is produced by contact between salicylate and mitochondria _in vivo_ or _in vitro_ it is suggested that normal mitochondrial oxidative phosphorylation

efficiency is restored.

A direct effect of reduced environmental K^+ on mitochondrial oxidative phosphorylation *in vitro* could not be demonstrated during the experimental work reported here. Further investigation of this point is necessary before the effects of a depletion of intracellular K^+ by salicylate *in vivo* can be established. This will require the production of a test system which will support efficient mitochondrial oxidative phosphorylation when the *in vitro* addition of K^+ is the only variable factor.

Supporting evidence for this concept may also come from a study of the metabolic response to salicylate administration to the whole animal when maintained on a K^+ deficient diet.

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