REVIEW ARTICLE

SALICYLATES AND METABOLISM

By M. J. H. Smith, M.Pharm., Ph.D., F.R.I.C.

Reader in Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.5

The term "salicylates" is used collectively for a group of drugs which have in common the salicylate radical and the main members in current clinical use are sodium salicylate and acetylsalicylic acid.

The latter substance is almost universally known as "aspirin", a name which has gained widespread acceptance due to its distinctive sound, to its attractive brevity and to many years of pertinacious advertising[1]. The salicylates possess serious claims to scientific attention because despite their almost venerable antiquity as drugs 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. Apart from their well-known actions in small doses as antipyretics and analgesics they are widely used in the treatment of rheumatic disorders. When administered in adequate amounts they are capable of producing a striking change in the clinical picture of rheumatic fever and possess a smaller, but still definite, action in rheumatoid arthritis. Their beneficial effects seem to be concerned with alleviating the symptoms produced by the inflammatory processes which form a major part of the reaction of the body in rheumatic disease. The mechanism of this anti-inflammatory effect of salicylates is particularly obscure. The drugs may also produce a surprising number of toxic effects either in patients receiving medication or in cases of accidental overdosage and when taken for suicidal purposes. These include alterations in the acid-base balance in the blood, the occurrence of gastrointestinal haemorrhage and considerable disturbances of carbohydrate metabolism.

Early work on the salicylates was reviewed by Hanzlik in 1927[2] and a comprehensive monograph was compiled in 1948 by Gross and Greenberg[3]. Later articles dealing with various aspects of the pharmacology of the drugs have been published in 1949[4], 1953[5] and 1958[6]. In recent years an increasing amount of attention has been given to the effects of salicylates on metabolic processes in man, in experimental animals, in isolated tissues and in subcellular preparations. The purpose of the present article is to review this work and its implications with respect to some of the toxic actions of salicylates and to their effects in rheumatism.

Effective Concentrations of Salicylates

It is not always possible to compare directly the amounts of salicylate which produce therapeutic or toxic effects in man and those which cause pharmacological actions in experimental animals and biochemical changes in tissue preparations. This is because in clinical work the effective

dose of salicylate is often described in terms of the total amount of salicylate administered daily, whereas, in animals it is frequently reported as the amount of salicylate per unit of body-weight and with tissues it is stated as the molar concentration of salicylate present in the incubation medium.

The recent introduction of simple and reliable methods for the estimation of plasma salicylate concentrations has enabled more definite conclusions to be made about the relation of plasma salicylate levels to the relief of symptoms in rheumatism and to the occurrence of toxic symptoms. Thus it has become generally accepted that the establishment and maintenance of plasma salicylate concentrations of between 20 and 30 mg./100 ml. are desirable in the therapy of rheumatic fever. Toxic symptoms become apparent at plasma salicylate concentrations above 35 mg./100 ml. and their appearance seems to be directly related to the salicylate level attained in the blood[7]. Plasma salicylate concentrations up to 100 mg./100 ml. may be found in antemortem specimens in attempted suicide or accidental poisoning. Infants and children are more susceptible to the poisonous action of salicylate than are adults and convulsions and death may occur with plasma salicylate levels above 50 mg./100 ml. The increasing use and significance of plasma salicylate measurements also allows more direct comparisons to be made between the clinical and biochemical effects of the drugs.

The conventional method of expressing concentration with isolated tissues, homogenates and other cellular and subcellular preparations is in terms of the molarity of the salicylate in the appropriate incubation medium. Molar (M) salicylate ion is 137 g. of salicylate ion per litre and fractional molarites may be expressed in various ways, for example, 137 mg. /100 ml. may be described as M/100, 10^{-2}M or 10mM salicylate ion. Wherever possible in the present article, salicylate concentrations are expressed as mg. of salicylate ion per 100 ml. either in the plasma of man or experimental animals or in the incubation media of tissue preparations.

Effects on Metabolism

Body Temperature

It seems paradoxical that despite the well-known use of aspirin as an antipyretic, one of the more serious symptoms of large doses of salicylates is hyperpyrexia. A number of authors have stressed the prominence of a fevered state in salicylate poisoning, especially in children[8]. Segar and Holliday[9] reported that of 49 children with salicylate intoxication only 5 were afebrile on admission to hospital and that 13 had rectal temperatures of between 105° and 108° F. This hyperpyrexia was attributed to an increased heat production due to a stimulant effect of salicylate on body metabolism. An important contributory factor was considered to be the development of a state of water deficiency due firstly, to the loss of substantial amounts of sweat as a result of the peripheral mechanisms concerned with heat loss and secondly, because the fluid intake of the patients was inadequate to compensate for this

water loss. Thus both the defences against hyperpyrexia and also the antipyretic effect of salicylate itself, could not function efficiently when effective sweating was compromised by water deficiency.

Respiration

Oxygen consumption. It was clearly shown by Cochran[10] that full therapeutic doses of salicylates producing plasma salicylate levels of 18 to 50 mg./100 ml., caused a marked and progressive increase in oxygen consumption in normal subjects and in patients with acute rheumatic fever and subacute rheumatism. The relief of symptoms in the rheumatic patients was associated with the maintenance of the increased oxygen consumption. The isomers of salicylic acid, *m*- and *p*-hydroxybenzoic acids, which are devoid of antirheumatic effects did not significantly alter the oxygen consumption of convalescent patients. Similar results have been reported by Balogh and others[11] and Tenney and Miller[12] in man and in experimental animals. The latter authors concluded that the principal site of the increased oxygen consumption produced by salicylate in the dog was skeletal muscle, since the response to salicylate was observed in both the eviserated animal and the functionally hepatectomised preparation (Eck's fistula with hepatic artery ligation). Meade[13] has observed that salicylic acid produces a significant stimulation of oxygen uptake in the normal rat whereas benzoic acid, *m*- and *p*- hydroxybenzoic acids, and the 2,3-, 2,4-, 2,5-, 2,6- and 3,4-dihydroxybenzoic acids were inactive. Similar results were reported by Hall, Tomich and Woollet[14] who also found aspirin to be active and salicylamide without effect. Andrews[15] has recently found that the cresotic acids (3-, 4- and 5-methyl salicylate acids) are more powerful stimulants of oxygen consumption in the intact rat than salicylic acid and that 3-phenyl salicylic acid appears to be even more active. 3:5-Dihydroxybenzoic acid and salicyluric acid produced no stimulation of oxygen uptake.

Brody[16] reported that if a rat is given 600 mg./kg. body weight of sodium salicylate intraperitoneally and slices are subsequently prepared from various organs then the oxygen consumption of liver and diaphragm, but not of kidney, exceeds that of corresponding control slices. With isolated tissues incubated in the presence of salicylate, an increased rate of oxygen consumption is produced by salicylate concentrations up to approximately 50 mg./100 ml. and a depression of the oxygen uptake is observed at higher salicylate concentrations. Sproull[17] showed that 5 to 40 mg./100 ml. salicylate caused a significant increase of the oxygen uptake of slices from mouse liver and rat brain. Above 40 mg./100 ml. salicylate the oxygen uptake values fell and at a salicylate concentration of 70 mg./100 ml. became less than those of the corresponding control slices. Similar results have been observed with rat liver or brain preparations in the presence of pyruvate[18] and citrate[19]. Fishgold, Field and Hall[20] found that 1 to 9 mg./100 ml. sodium salicylate stimulated the oxygen uptake of slices of rat cerebral cortex and that higher salicylate concentrations caused an initial increase in the oxygen consumption followed by a progressive fall. They also reported that aspirin, but not

sodium salicylate, increased the oxygen consumption of rat liver slices and Lutwak-Mann[21] was also unable to find an appreciable effect of 140 mg./100 ml. of sodium salicylate on rat liver slices and extracts. 14 to 70 mg./100 ml. salicylate produces an initial increase in the oxygen uptake of the isolated sacs of rat small intestine, 7 to 14 mg./100 ml. salicylate had no effect and 70 mg./100 ml. salicylate caused a depression of oxygen consumption[22]. The stimulating effect of salicylate on oxygen consumption is not observed when homogenates are used instead of tissue slices and an intact cellular structure appears to be necessary for the effect to be evident. Thus Kaplan, Kennedy and Davis[23] found that salicylate inhibited the oxidation of citrate by homogenates of rat liver and kidney, and Penniall, Kalnitsky and Routh[24] reported no definite stimulation of oxygen uptake by salicylate in rat brain homogenates.

The stimulating action of salicylate on the oxygen uptake of isolated tissues shows that this must be a peripheral effect of the drug. The salicylate concentrations which elicit this response in isolated tissues are very similar to those observed in the plasma of man and experimental animals showing an increased oxygen uptake after salicylate administration. The effect appears to be restricted to salicylic acid, acetylsalicylic acid and ring substituted methyl and phenyl salicylic acids.

Christensen[25] has recently observed that under *in vitro* conditions 10 to 50 mg./100 ml. salicylate causes a release of thyroxine from its combination with plasma proteins. He suggested that the increased oxygen consumption observed during salicylate administration in man is mediated by an increase in the concentration of free circulating thyroxine. This mechanism cannot explain the stimulant effect of salicylate on the oxygen uptake of isolated tissues. In addition, Alexander and Johnson[26] reported that the calorigenic response of euthyroid or myxoedemic patients to thyroid hormones differed markedly from the response to salicylates and could find no evidence of interaction between salicylates and thyroid homrones *in vivo*.

CO_2 production. There is a voluminous literature dealing with the effects of salicylate in man on the respiratory excretion of CO_2 and the production of changes in the acid-base balance in the blood. Most authors agree that respiratory stimulation and hyperventilation are important actions of salicylates but the mechanism of these effects has been a source of controversy. Recent work has provided evidence in favour of the view that the hyperventilation caused by salicylate is caused by a direct stimulation of the respiratory centre rather than being secondary to the development of a transient period of acidosis which serves as the initial respiratory stimulant. However the respiratory response to salicylate in the intact dog appears to be a summation of both a direct central stimulation and an increased metabolic production of CO_2[12]. The increased respiratory excretion of $^{14}CO_2$ in the salicylated rat after the injection of acetate-2-^{14}C[27] may also indicate an effect of salicylate in augmenting the production of CO_2 in the tissues. An increase in the respiratory quotient has also been observed in the isolated rat diaphragm incubated with salicylate[28].

An increased CO_2 production together with an enhanced uptake of oxygen are therefore important peripheral effects of salicylate on the tissues.

Protein Metabolism

The literature published before 1948 on the effects of salicylate on the excretion of non-protein nitrogenous substances, other than uric acid, was reviewed by Gross and Greenberg[3], who stated that the data was so variable as to justify no conclusions. Later work in animals has indicated more definite actions of salicylate on protein metabolism. Manchester, Randle and Smith[29] have recently shown that 69 mg./100 ml. salicylate ion significantly reduces the incorporation of ^{14}C from labelled glycine, glutamic acid and lysine into the protein of isolated rat diaphragm. Winters and Morrill[30] found that rats injected with 75 mg./100 g. weight of salicylate showed a significant negative nitrogen balance. Reid, Watson and Sproull[31] have produced evidence that in patients with rheumatic fever exhibiting plasma salicylate levels from 20 to 70 mg./100 ml., there was a reduction of total plasma protein content and negative nitrogen balances. It is not clear if these latter effects are solely explicable in terms of decreased protein synthesis, as occurs in the isolated rat diaphragm, or whether an increased rate of protein breakdown is also involved. However, the therapeutic evaluation of salicylate in rheumatism should obviously consider this effect on the depletion of the metabolic nitrogen stores.

Fat Metabolism

The available data on the possible effects of salicylate on fat metabolism are very scanty. The development of ketosis and ketonuria[9] in salicylate poisoning, especially with children, indicates an increased rate of fat catabolism. It has also been shown that the administration of salicylate to rats, producing plasma salicylate levels of about 70 mg./100 ml., will reduce lipogenesis in the liver[27].

Carbohydrate Metabolism

Hyperglycaemia and glycosuria. A number of authors have reported either elevated blood sugar concentrations or glycosuria or both in patients receiving salicylates. Morris and Graham[32] found that rheumatic children receiving salicylates had fasting blood sugar increased above normal and Cochran, Watson and Reid[33] reported glycosuria and a diminished glucose tolerance in a rheumatic fever patient given 5 g. of aspirin per day. Hyperglycaemia after the administration of salicylate also occurs in experimental animals. Barbour and Herrman[34] found that aspirin causes hyperglycaemia in rabbits. Sproull[35] observed that salicylate induced raised blood sugar levels in female mice but not in males. However, the hyperglycaemic response to salicylate in the rat is irrespective of sex or previous fasting but if the rats were adrenal-demedullated before salicylate administration then the hyperglycaemic phase

largely disappeared[36]. It was concluded that salicylate in a normal animal caused stimulation of the adrenal medulla and the secreted adrenaline produced an increased rate of hepatic glycogenolysis and hence hyperglycaemia. Glycosuria would be expected to occur when the renal threshold for the sugar is exceeded.

The adrenaline-induced hyperglycaemia caused by salicylate in the intact rat is a transient phenomenon lasting a few hours only. This mechanism cannot explain the hyperglycaemia persisting for 3 to 5 days, reported to occur in salicylate-intoxicated children by Segar and Holliday[9]. Such a prolonged effect of salicylate on blood sugar concentration may be related to stimulation of the adrenal cortex because significantly elevated plasma steroid levels have been observed in patients with salicylate intoxication[37]. It has also been reported that salicylates cause an apparent acceleration of glucose absorption from the small intestine in the intact rat[38] but they do not influence the rate of glucose absorption from either perfused[39] or ligatured[40] intestinal loops and completely inhibit the active transport of glucose and fluid across the intestinal wall of isolated sacs of rat small-intestine[22]. It is therefore unlikely that an increased intestinal absorption of glucose contributes to the prolonged hyperglycaemia observed in salicylate poisoning in children.

Hypoglycaemia. While salicylates may produce a hyperglycaemic effect in rheumatic patients and in normal animals, they elicit a quite different response in diabetic patients and in animal preparations with endocrine imbalance. Before the advent of insulin there were numerous reports in the clinical literature that salicylates were useful in the management of diabetic patients because they reduced glycosuria. This decreased glycosuria follows a marked lowering of the elevated blood sugar levels present in the patients with diabetes mellitus[41,42]. Earlier work with animals had revealed similar effects of salicylates on the hyperglycaemia and glycosuria of rats or rabbits made diabetic by partial pancreatectomy[43], alloxan[44], cortisone[45] and adrenaline[46]. A striking hypoglycaemic effect is produced by salicylates in the bilaterally adrenalectomised[36] and the hypophysectomised rat[47].

The mechanism of this hypoglycaemic effect of salicylates may be attributed, at least in part, to an increased entry of glucose into the cells from the extracellular fluid. Randle and his co-workers[29,48] have shown that salicylates increase the glucose uptake of rat diaphragm muscle incubated in a bicarbonate medium but do not increase the accumulation of free glucose within the tissue. These observations suggest that salicylates may reduce the hyperglycaemia and glycosuria in diabetic animals and man by promoting the uptake of glucose by skeletal muscle.

Liver glycogen. In the intact animal salicylates cause a rapid and severe depletion of liver glycogen. This has been shown to occur in the rabbit[49], the rat[21] and the mouse[35], and may be largely due to adrenal medullary stimulation. However, a diminished synthesis of liver glycogen is also concerned since Edelmann, Bogner and Steele[50] and Feeney, Carlo and Smith[47] have reported that the liver glycogen deposition, which

occurs in fasted intact and adrenalectomised rats given glucose, is reduced by salicylates.

The administration of salicylate also inhibits the deposition of glycogen induced by cortisone[45], hydrocortisone[30] and stilboestrol[51] in the adrenalectomised and intact rat. It has also been demonstrated that the incorporation of ^{14}C into the liver glycogen of fasted rats after the intraperitoneal injection of acetate-2-^{14}C is completely inhibited by salicylate[27]. Additional evidence in support of the view that salicylates interfere with glycogen synthesis in the liver is provided by their effects on liver slices incubated under aerobic conditions. When such slices are incubated in a medium containing a high potassium : low sodium ratio there is an increased rate of glycogenesis from substrates such as glucose[52]. Chiu and Needham[53] have also shown that the addition of adrenal cortical extract to liver slices incubated in a high sodium : low potassium medium increases the formation of new glycogen in the slices. The addition of salicylates inhibits both effects, that is, the increased glycogenesis induced by the high potassium medium and by the adrenal extract[54].

The liver glycogen depletion produced by salicylates in the intact animal is therefore caused by two mechanisms; first, an increase in glycogeneolysis due to adrenal medullary stimulation and second, a decrease in the rate of glycogen synthesis from carbohydrate precursors such as glucose, and also from glyconeogenesis, that is, from small molecules such as the two carbon fragments originating from protein breakdown.

Yet another effect of salicylates on liver glycogen deposition is found in the intact rat which is being fed a solid diet during the experiment. When salicylates were injected into rats, immediately after feeding with the usual cube diet, there is an initial impairment of liver glycogen deposition for a period up to 12 hours followed by an increased deposition of glycogen after 24 hours. Studies of the action of sodium salicylate on the passage of a barium meal in the rat have revealed a 12 hour delay in the rate of gastric emptying[55]. It has not been established if this action of salicylate is either spastic or paralytic but it explains both the initial diminution and subsequent increase in liver glycogen deposition after a solid meal in this species.

Muscle glycogen. Although Feeney and others[47] reported that aspirin and sodium salicylate produced no effect on muscle glycogen in the intact rat, Winters and Morrill[30] found that sodium salicylate caused a considerable fall in muscle glycogen. A decreased muscle glycogen content after salicylate administration also occurs in hypophysectomised rats[47]. It has also been shown that there is a decrease in the amount of glycogen in the isolated rat diaphragm muscle incubated in the presence of salicylates[28].

Metabolic intermediates. Dell'Aquilla and Angarano[41] found that the administration of salicylates together with glucose, increased the blood pyruvate levels in both normal subjects and diabetic patients. Lutwak-Mann[21] reported no significant changes in either blood pyruvate or lactate concentrations in normal or B_1 deficient rats given salicylate. However,

an increased production of lactic acid has been reported to occur in the isolated rat diaphragm incubated aerobically in the presence of 69 mg./100 ml. salicylate[28]. This effect was not observed in homogenates from rat brain[24].

The phosphate compounds in the isolated rat diaphragm show striking changes in the presence of salicylate[56]. The content of inorganic phosphate is increased and the amounts of creatine phosphate and adenosine triphosphate (ATP) are severely reduced. Significant effects on these phosphate compounds are observed with salicylate concentrates as low as 1·4 mg./100 ml. The inorganic phosphate uptake of homogenates of rat brain is also decreased in the presence of salicylate[24].

Oxidative Phosphorylation

An important feature of normal cellular metabolism is the conversion of the energy derived from substrate oxidation to that contained in the pyrophosphate bonds of compounds such as ATP. The energy is stored in these high energy phosphate bonds and is subsequently used for such functions as muscular and osmotic work and synthetic reactions. The oxygen consumption and the phosphorylation, that is, the production of high-energy phosphate bond compounds, are therefore interdependent phenomena and the enzymic mechanisms concerned are described collectively as oxidative phosphorylation processes. A number of substances are known to interfere with these processes by inhibiting the formation of the high-energy phosphate bond compounds without reducing the oxygen consumption of the system. This dissociation of oxidation from phosphorylation is termed "uncoupling". The possible relation between uncoupling and drug action has been reviewed by Brody[57] and his article includes a description of the methods available for the study of uncoupling actions. The most convenient *in vitro* system for this purpose is a respiring suspension of mitochondria. It is known that this subcellular fraction, which can easily be separated from disrupted cell preparations by differential centrifugation procedures, contains a major fraction of the metabolic activity of the cell, including the enzyme systems responsible for the synthesis of high-energy phosphate compounds during the aerobic oxidation of a number of substrates. The overall efficiency of this oxidative phosphorylation mechanism is expressed as the P : O ratio, which is defined as the ratio of the moles of inorganic phosphate converted to high-energy phosphate bond compounds, per atom of oxygen consumed. It is assessed by measuring both the disappearance of inorganic phosphate from and also the oxygen uptake of the preparation. The P : O ratio is generally accepted as an accurate measure of the ability of the *in vitro* system to synthesis high-energy phosphate bonds under aerobic conditions.

It has been shown by several workers that salicylates must be included among the agents which uncouple oxidative phosphorylation processes in respiring mitochondrial preparations. Brody[16] has reported that sodium salicylate and sodium acetylsalicylate concentrations above 3 mg./100 ml. decrease the P : O ratios of liver and kidney mitochondrial

preparations oxidising succinate, glutamate, α-ketoglutarate and pyruvate plus malate. 30 mg./100 ml. sodium salicylate decreased the oxygen consumption of the preparations by about 20 per cent but completely inhibited the uptake of inorganic phosphate (P : O = O). The inhibition of phosphorylation was less pronounced in brain mitochondria. Similar results have been reported by Penniall, Kalnitsky and Routh[24], Bosund[58] and Jeffrey and Smith[59] using mitochondria prepared from rat brain and liver. Brody[16] has also shown that only sodium salicylate, aspirin, methyl salicylate and 2,3-dihydroxybenzoate possessed uncoupling activity while benzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, salicylamide and 4-amino-2-hydroxybenzoate were inactive. Another method by which the uncoupling action of a substance may be detected is by the use of the so-called "acceptor-deficient" system. It is known that certain uncoupling agents will stimulate the respiratory rate in mitochondrial preparations deficient in phosphate acceptors, such as ADP. Packer, Austen and Knoblock[60] used a polarographic method for measuring the oxygen consumption of rat heart mitochondria oxidising α-ketoglutarate in the presence of a phosphate-acceptor deficient system. They found that salicylate, acetylsalicylate and thiosalicylate produced significant stimulations of oxygen consumption in this system whereas *m*- and *p*- hydroxybenzoates, 2,4-; 2,5-; 2,6-; 3,5-dihydroxybenzoates, salicylaldehyde, salicylamide, methyl salicylate, salicyl ureate and 5-sulphosalicylic acid were inactive.

The similarities between the uncoupling action of salicylates and that of other known uncoupling agents have also been studied. Brody[16], Penniall[61], Jeffrey and Smith[59] and Packer, Austen and Knoblock[60] have shown that salicylates will stimulate the respiration of mitochondria in a phosphate deficient or acceptor-deficient *in vitro* system, that their action is not dependent on magnesium and that they will inhibit the spontaneous swelling of mitochondria which occurs in hypotonic media. Salicylates therefore belong to the general type of uncoupling agents exemplified by the dinitrophenols rather than to the second type of uncoupling substances, including thyroxine and the tetracycline antibiotics, which have opposite effects on the above system.

Lehninger[62] has shown that the dinitrophenols, which inhibit the swelling of mitochondria in 0·3M sucrose solution, also uncouple oxidative phosphorylation reactions in mitochondrial fragments prepared by treating mitochondria with digitonin. These mitochondrial fragments are of much smaller weight than the original mitochondria and although they still contain the enzyme systems concerned with oxidative phosphorylation they lack the mitochondrial membrane. It therefore seems possible that the uncoupling action of the dinitrophenols is due to a direct effect on the intra-mitochondrial enzyme systems whereas the uncoupling action of thyroxine, which does not inhibit mitochondrial swelling and has no effect on the sub-mitochondrial particles prepared with digitonin, may be secondary to some structural change induced in the mitochondrial membrane. The inhibition of swelling of mitochondria caused by salicylates suggests that they may also be acting primarily on the

intramitochondrial enzyme systems rather than on the mitochondrial membrane.

Oxidative phosphorylation reactions essentially involve the oxidation of a reduced pyridine nucleotide which is formed initially by the transfer of hydrogen from a substrate molecule to that of a pyridine nucleotide. The subsequent oxidation involves a chain of intermediate hydrogen and electron carriers arranged in series and terminating in molecular oxygen. The main types of these intermediate carriers are successively the flavoproteins and cytochromes. For the basic reaction, that is, the oxidation of reduced pyridine nucleotide, the P:O ratio is 3, so that three high-energy phosphate bonds are formed for every atom of oxygen consumed. It is probable that one high-energy phosphate bond is formed at each of the three component reactions, that is, reduced pyridine nucleotide to flavoprotein, flavoprotein to cytochrome and cytochrome to oxygen. Most substrates, for example, malate and β-hydroxybutyrate, which are oxidised by *in vitro* mitochondrial preparations, donate hydrogen atoms directly to the pyridine nucleotide and therefore yield P:O ratios of 3. Exceptions are succinate (P:O = 2) which donates hydrogen to a flavoprotein and α-ketoglutarate (P:O = 4). The latter substance reacts with a pyridine nucleotide and coenzyme A to give a reduced pyridine nucleotide, which is subsequently oxidised to yield three high-energy phosphate bonds, and also succinyl coenzyme A which can undergo a further reaction to yield an additional high-energy bond compound. This second reaction does not involve oxygen and is known as an anaerobic phosphorylation at substrate level. Penniall[61] and Jeffrey and Smith[59] have shown that only the aerobic phosphorylations accompanying the oxidation of α-ketoglutarate by mitochondrial suspensions are sensitive to salicylate and that the anaerobic phosphorylation is unaffected.

The phosphorylations associated with the entire respiratory chain, that is, the three high-energy phosphate bonds produced by the oxidation of a reduced pyridine nucleotide, are uncoupled by salicylate since a P:O ratio of 0 is obtained during the oxidation of β-hydroxybutyrate. However, Penniall[61] has reported that 4 to 6×10^{-4}M salicylic acid eliminated the phosphorylation accompanying Fe^{++} cytochrome *c* oxidation in mitochondrial preparations while such levels had no effect on the oxidation of β-hydroxybutyrate in the presence of $K_3Fe(CN)_6$. These observations suggest that the terminal phosphorylation step concerned with the oxidation of cytochrome *c*, may be more susceptible to the uncoupling action of salicylate.

Salicylate inhibits phosphorylation but not oxidation in mitochondrial preparations which indicates that it acts on the sequence of phosphorylation reactions which occur subsequent to the electron transport chain. The individual steps in this sequence have not been clearly defined and both phosphorylated and non-phosphorylated high-energy intermediates have been postulated. The site of action of salicylate in these systems is a major problem for future research. The various mechanisms which have been formulated to explain the mode of action of 2,4-dinitrophenol may also be applied to that of salicylate. These mechanisms have been

thoroughly discussed by Boyer[63], who favours the view that dinitrophenol acts by causing the breakdown of a non-phosphorylated intermediate which arises from direct interaction with the electron transport components. A strict analogy between the modes of action of dinitrophenol and salicylate is however questionable, since Falcone[64] has observed that the two substances do not have identical effects on the ^{32}P-ATP exchange reaction and on ATPase activity in mitochondria. It is unlikely that dinitrophenol uncouples through the formation and spontaneous breakdown of a dinitrophenyl phosphate[63] but a similar mechanism may account for the effects of salicylate. The formation of a salicoyl phosphate and its intramolecular rearrangement to salicyl phosphate followed by hydrolysis, is a possible mechanism by which salicylate could act as a "high-energy phosphate acceptor" and hence uncouple. This hypothesis could be tested experimentally by preparing the two salicylate phosphate compounds and studying both their interconversion and also their effects on the ^{32}P and ^{18}O exchange reactions in mitochondria[65].

Enzymes

Salicylates have been observed to affect the activities of various enzymes either *in vitro* or *in vivo*. Segal and Blair[66] have shown that when rat diaphragm muscle is incubated with 30 mg./100 ml. salicylate there is a susbstantial decrease in phosphorylase activity. However, salicylates were found to have no effect on the activity of crystalline rabbit muscle phosphorylase and it was concluded that salicylates either stimulated the PR enzyme, which converts active phosphorylase (phosphorylase *a*) to the inactive form (phosphorylase *b*), or inhibited the activating enzyme, which carries out the reverse reaction. The latter explanation is the more likely since the activating enzyme is dependent on ATP, the production of which would be diminished as a result of the uncoupling action of salicylate on oxidative phosphorylation. Lutwak-Mann[21] found that milk and liver xanthine oxidase were inhibited by salicylate *in vitro* and Bergel and Bray[67] have utilised this property for the stabilisation of xanthine oxidase by adding salicylate during the purification procedure for the enzyme. Mitidieri and Affonso[68] reported that the administration of salicylate to intact rats, producing blood salicylate concentrations of about 30 mg./100 ml., caused a decrease of xanthine oxidase activity in the liver and increased enzyme activity in the blood. This result was interpreted as suggestive of damage to the liver tissue caused by the salicylate leading to an increased passage of the enzyme into the blood. A similar effect has been described by Manso, Taranta and Nydich[69], who found that serum transaminase levels were elevated in more than 50 per cent of children receiving aspirin or sodium salicylate. 137 mg./100 ml. salicylate has no effect on hexokinase, cytochrome oxidase, DPNH oxidase and DPNH-cytochrome *c* reductase[61], but lower concentrations have been reported to inhibit α-ketoglutaric dehydrogenase and succinic dehydrogenase[23]. The reported action of salicylate on hyaluronidase and its implications in rheumatism have been discussed critically[5].

Conclusions

It is evident that salicylates produce a number of effects on metabolism in animals, isolated tissues and sub-cellular preparations. The interrelation of these effects and their possible relevance to the known toxic and therapeutic actions of the drugs pose intriguing questions. In recent years pharmacologists have been increasingly concerned with the effects of drugs on cellular metabolism and it has been suggested that the actions of a drug might be explained by its ability to influence the functions of either an enzyme or of multi-enzyme systems. This position has now been reached with salicylates because of the demonstration that they will uncouple oxidative phosphorylation processes in respiring mitochondrial preparations. The possible relation of this action of salicylates on important metabolic processes in the cell to the other effects of the drug therefore merits serious consideration.

The relation of uncoupling to other effects of salicylates. The main changes which should result from an uncoupling action are a diminished synthesis of high-energy phosphate bond compounds, such as ATP and creatine phosphate, an increased accumulation of inorganic phosphate but no depression of the oxygen consumption. These effects have been observed to occur in the rat diaphragm incubated with salicylate[56]. The uncoupling effect observed with salicylate in mitochondrial suspensions is therefore also evident in the more highly organised tissue. At present no data is available about the possible depletion of tissue nucleotides in intact animals given salicylates.

The reduced synthesis of ATP caused by uncoupling action may in turn produce several widespread effects on tissue metabolism. These include firstly, a decreased production of large molecules, such as glycogen and proteins, because high-energy phosphate bonds are necessary at various intermediate stages in their synthesis; secondly, an interference with the selective permeability of cellular membranes because the movement of many substances across such membranes depends on the supply of ATP; thirdly, an enhanced breakdown of existing substrates in the tissues because of the increasing inefficient phosphorylating mechanisms and fourthly, an increased heat production because the energy normally used for the conversion of orthophosphate to the high-energy pyrophosphate bonds of ATP will be liberated in the form of heat.

These secondary changes in metabolism could explain several of the metabolic and toxic effects of salicylate observed in isolated tissues, experimental animals and man. The increased oxygen consumption and CO_2 production found to occur in normal subjects, rheumatic patients, animals and tissue preparations are evidence of increased substrate oxidation. The hyperpyrexia observed in animals after brain stem transection[70] and which is a frequent symptom of salicylate intoxication in children, is explicable in terms of an increased heat production. Diminished protein synthesis arising from an interference with ATP production could explain the failure of incorporation of ^{14}C from labelled amino acids into the protein of rat diaphragm in the presence of salicylate[48] and the negative nitrogen balances induced by salicylates in

experimental animals and man[30,31]. The antagonism between salicylate and adrenocortical extracts and steroids in the deposition of liver glycogen in the fasting rat[30], in the adrenalectomised rat[45] and in rat-liver slices[54] is also explicable on the same basis if the glyconeogenesis, normally induced by the adrenalcortical compounds, is inhibited by a failure of formation of high-energy phosphate bonds in the presence of salicylate.

A similar argument may be applied to the decreased glycogen synthesis which occurs in isolated liver slices and in intact and adrenalectomised animals in the presence of salicylate. Diminished carbohydrate synthesis may also be concerned in the hypoglycaemic effect of salicylates in the alloxan-diabetic rat. In this animal preparation the fasting blood glucose must be maintained by synthetic reactions, the increased liver glucose-6-phosphatase activity present leading to hyperglycaemia rather than to deposition of liver glycogen. Experimental support for this mechanism is provided by the demonstration that salicylate inhibits the incorporation of ^{14}C from acetate-2-^{14}C into liver glycogen of the normal rat[27]. However, another factor may be concerned in the hypoglycaemic effect of salicylate in diabetic animals and man because salicylate increases the glucose uptake of isolated rat diaphragm. Thus salicylate may increase the entry of glucose from the blood into the tissues by inhibiting an ATP-dependent process, which normally acts as a restraint to glucose uptake[48]. The hypoglycaemic effect of salicylate in experimental diabetic animals and in human diabetics appears to be due to a combination of increased glucose entry into the cells and decreased intracellular synthesis of carbohydrate, both processes being primarily affected by diminished ATP production in the presence of salicylates.

The relation of uncoupling to the anti-inflammatory effects of salicylate in rheumatism is more speculative. A possible approach is through studies of the effects of salicylates on the movement of fluid and electrolytes from the cells to the extracellular fluid. Reid, Watson and Sproull in 1950[31] produced evidence that various fluid shifts occurred in patients with rheumatic fever either during spontaneous remissions or during treatment with salicylates. These fluid movements consisted of an initial transfer of water and electrolytes from the cells to the extracellular fluid followed by a diuresis and it was suggested that such shifts of fluid from swollen joints, etc., could be associated with the relief of pain and other symptoms in acute rheumatism. Copeman and Pugh[72] induced similar fluid movements in rheumatic patients by the administration of hypertonic solutions and stated that the clinical effects produced were strikingly similar to those obtained with salicylate therapy. Waltner, Csernovszky and Kelemen[73] have also shown that salicylates will cause a diuresis and an increased renal excretion of electrolytes in the rat.

The movement of water and electrolytes from the intracellular to the extracellular fluid will be mainly influenced by two factors, the selective permeability of the cellular membranes and the amounts of the intracellular large colloidal molecules. Both these factors could be significantly altered by an uncoupling agent. The transport of electrolytes and water across cell membranes are active processes dependent on energy supply

and hence on the maintenance of ATP production. Thus the uncoupling action of salicylate may induce new equilibria leading to the net loss of water and electrolytes from the tissues. This effect should be accentuated by the diminished synthesis and increased breakdown of the large intracellular molecules, such as protein, resulting from inhibition of ATP production because the consequent diminution of the intracellular osmotic pressure will also lead to loss of fluid and electrolytes from the tissues. The anti-inflammatory action of salicylate in rheumatism may therefore be initiated by an uncoupling effect of the substance at the cellular level and mediated by fluid shifts between the cells and extracellular fluid.

There are, however, many dangers in applying the results obtained with subcellular preparations or isolated enzymes to explain effects obtained on more biologically complex systems. Indeed, in this context, it is probably true that the simpler the experimental system then the more difficult will be the interpretation. The presence of membranes around cells and subcellular particles, the metabolic interdependence of cellular constituents, the organisation of various tissues in slices and the existence of neuro-endocrine and nervous mechanisms in intact animals are factors which cannot be ignored. It would be a gross oversimplification to attempt to explain all the varied effects of salicylates solely on the basis of their uncoupling action on oxidative phosphorylation reactions. Most drugs act at more than one site and the overall pattern of their pharmacological, clinical and toxic actions is a complex mixture of superimposed effects on several different systems. Thus the adrenal medullary stimulation and delayed rate of gastric emptying observed in salicylated animals and the occurrence of tinnitus, deafness and hyperventilation in salicylism in man cannot be directly related to an uncoupling action. Despite these reservations, the discovery that salicylates possess this fundamental action on cellular metabolism is an important advance particularly because it provides a rational explanation for many of the metabolic and toxic effects of the drugs.

Further research should be concerned with the effects of uncoupling on membrane permeability, particularly with respect to fluid and electrolytes. The alterations in the composition and distribution of body fluids and intracellular components in animals and patients given therapeutic amounts of salicylates should be confirmed and extended. The older methodology in this field was necessarily of an indirect nature but application of recent techniques such as the use of tritium labelled water for total body water determination[74], the measurement of blood volume using ^{32}P-labelled red cells[75] and the helium dilution method[76] for body volume and density estimations could provide accurate and additional data. The use of ^{14}C-labelled metabolic intermediates, such as glucose, acetate and bicarbonate, in conjunction with chromatographic and radioautographic techniques to study the metabolic patterns in isolated tissues could establish if the major metabolic effects of salicylates result solely from an uncoupling action or whether the drugs primarily affect other enzyme systems. The metabolic effects of other anti-rheumatic drugs should be investigated. It is unlikely that such chemically diverse

substances as salicylates, butazolidine, steroids and antimalarials produce their beneficial effects by the same mechanism and the investigation of their modes of action may throw some much needed light on the pathogenesis of the rheumatic process.

References

1. Rouché, *The Incurable Wound*, Gollancz, London, 1958.
2. Hanzlik, *Medicine Monographs*, Vol. 9, Williams & Wilkins, Baltimore, 1927.
3. Gross and Greenberg, *The Salicylates*, Hillhouse Press, New Haven, 1948.
4. Smith, *Pharmacol. Rev.*, 1949, **1**, 353.
5. Smith, *J. Pharm. Pharmacol.*, 1953, **5**, 81.
6. Smith, *Pharmacology in Medicine*, edit. Drill, Chapter 20, McGraw Hill Book Co., New York, 1958.
7. Graham and Parker, *Quart. J. Med.*, 1948, **17**, 153.
8. Erganian, Forbes and Case, *J. Pediat.*, 1947, **30**, 129; Barnett, Powers, Benward and Hartmann, *ibid.*, 1942, **21**, 214.
9. Segar and Holliday, *New Engl. J. Med.*, 1958, **259**, 1191.
10. Cochran, *Brit. med. J.*, 1952, **2**, 964.
11. Balogh, Donhoffer, Mestyan, Pap and Toth, *Arch. exp. Path. Pharmak.*, 1952, **214**, 299.
12. Tenney and Miller, *Amer. J. Med.*, 1955, **19**, 498.
13. Meade, *Ann. Rheum. Dis.*, 1954, **13**, 60.
14. Hall, Tomich and Woolett, *Brit. med. J.*, 1954, **2**, 99.
15. Andrews, *Brit. J. Pharmacol.*, 1958, **13**, 419.
16. Brody, *J. Pharmacol.*, 1956, **117**, 39.
17. Sproull, *Brit. J. Pharmacol.*, 1954, **9**, 262.
18. Patel and Heim, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 251.
19. Alwall, *Acta med. scand.*, 1939, **102**, 390.
20. Fishgold, Field and Hall, *Amer. J. Physiol.*, 1951, **164**, 727.
21. Lutwak-Mann, *Biochem. J.*, 1942, **36**, 706.
22. Smith, *Amer. J. Physiol.*, 1958, **193**, 29.
23. Kaplan, Kennedy and Davis, *Arch. Biochem. Biophys.*, 1954, **51**, 47.
24. Penniall, Kalnitsky and Routh, *ibid.*, 1956, **64**, 390.
25. Christensen, *Nature, Lond.*, 1959, **183**, 1190.
26. Alexander and Johnson, *Clin. Sci.*, 1958, **17**, 377.
27. Smith, *J. biol. Chem.*, 1959, **234**, 144.
28. Smith and Jeffrey, *Biochem. J.*, 1956, **63**, 524.
29. Manchester, Randle and Smith, *Brit. med. J.*, 1958, **1**, 1028.
30. Winters and Morrill, *Proc. Soc. exp. Biol., N.Y.*, 1955, **88**, 409.
31. Reid, Watson and Sproull, *Quart. J. Med.*, 1950, **19**, 1.
32. Morris and Graham, *Arch. Dis. Child.*, 1931, **6**, 273.
33. Cochran, Watson and Reid, *Brit. med. J.*, 1950, **2**, 1411.
34. Barbour and Hermann, *J. Pharmacol.*, 1921, **18**, 165.
35. Sproull, *Brit. J. Pharmacol.*, 1954, **9**, 121.
36. Smith, *ibid.*, 1955, **10**, 110.
37. Done, Ely and Kelley, *J. Pediat.*, 1954, **44**, 153.
38. Smith, *Biochim. Biophys. Acta*, 1954, **14**, 241.
39. Escribano and Ponz, *Rev. espan. fisiol.*, 1955, **11**, 153.
40. Smith, *Biochemical Pharmacology*, In the press.
41. Dell'Aquila and Angarano, *Folia endocrin., Pisa*, 1954, **7**, 5.
42. Reid, Macdougall and Andrews, *Brit. med. J.*, 1957, **2**, 1071; Reid and Lightbody, *ibid.*, 1959, **1**, 897.
43. Ingle, *Proc. Soc. exp. Biol., N.Y.*, 1950, **75**, 673.
44. Smith, Meade and Bornstein, *Biochem. J.*, 1952, **51**, 18.
45. Smith, *ibid.*, 1952, **52**, 649.
46. Starkenstein, *Z. exp. Path. Ther.*, 1912, **10**, 78.
47. Feeney, Carlo and Smith, *J. Pharmacol.*, 1955, **114**, 299.
48. Randle and Smith, *Biochem. J.*, 1958, **70**, 490; 501.
49. Jackson, *J. Path. Bact.*, 1948, **60**, 587.
50. Edelmann, Bogner and Steele, *Fed. Proc.*, 1954, **13**, 39.
51. Teague and Barnett, 20th International Physiological Congress, Brussels, 1956, Abs., p. 880.
52. Hastings, Teng, Nesbett and Sinex, *J. biol. Chem.*, 1952, **194**, 69.

53. Chiu and Needham, *Biochem. J.*, 1950, **46**, 114.
54. Smith, *ibid.*, 1955, **59**, 52.
55. Smith and Irving, *Brit. J. Radiol.*, 1955, **28**, 39.
56. Smith and Jeffrey, *Biochem. J.*, 1956, **64**, 589.
57. Brody, *Pharmacol. Rev.*, 1955, **7**, 335.
58. Bosund, *Acta chem. scand.*, 1957, **11**, 541.
59. Jeffrey and Smith, *Biochem. J.*, 1959, **72**, 462.
60. Packer, Austen and Knoblock, *Proc. Soc. exp. Biol., N.Y.*, 1959, **100**, 239.
61. Penniall, *Biochim. Biophys. Acta*, 1958, **30**, 247.
62. Lehninger, *Enzymes; Units of Biological Structure and Function*, edit. Gaebler, Academic Press, New York, 1956, p. 217.
63. Boyer, Proceedings of the International Symposium on Enzyme Chemistry, Tokyo-Kyoto, Pergamon Press, London, 1958, p. 301.
64. Falcone, *J. Clin. Invest.*, 1959, **30**, 1002.
65. Boyer, Luchsinger and Falcone, *J. biol. Chem.*, 1956, **223**, 405.
66. Segal and Blair, *Nature, Lond.*, 1959, **183**, 1609.
67. Bergel and Bray, *ibid.*, 1956, **178**, 88.
68. Mitidieri and Affonso, *ibid.*, 1959, **183**, 471.
69. Manso, Taranta and Nydich, *Proc. Soc. exp. Biol., N.Y.*, 1956, **93**, 84.
70. Isenschmid, *Arch. exp. Path. Pharmak.*, 1913, **75**, 10.
71. Langdon and Weakley, *J. biol. Chem.*, 1955, **214**, 167.
72. Copeman and Pugh, *Lancet*, 1950, **2**, 675.
73. Waltner, Csernovszky and Kelemen, *Biochemical Pharmacology*, 1959, **2**, 50.
74. Prentice, Siri, Berlin, Hyde, Parsons, Joiner and Lawrence, *J. clin. Invest.*, 1952, **31**, 412.
75. Berlin, Huff, Van Dyke and Hennessy, *Proc. Soc. exp. Biol., N.Y.*, 1949, **71**, 176.
76. Siri, *Advances Biol. and Physics*, Vol. 4, Academic Press, New York, 1956, p. 239.