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REVIEW

Salicylate and enzymes

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Salicylate inhibits the activities of a number of cellular enzymes and in some instances the mechanisms of inhibition have been established (Smith, 1968a). Reported inhibitory actions of the salicylate ion on important enzyme systems *in vitro* are now reviewed and assessed in relation to the known clinical and toxic effects of the drug.

RELATION BETWEEN *in vitro* INHIBITIONS AND *in vivo* EFFECTS

The demonstration that salicylate, or indeed any drug, inhibits the activity of an enzyme *in vitro* is not sufficient evidence that one or more of the *in vivo* actions of the drug are due to such inhibition. The composition of *in vitro* incubation mixtures are varied over much wider ranges than those existing in body cells. The relative proportions of enzyme, substrates and cofactors and the pH and ionic constituents of the incubation media may bear little resemblance to physiological requirements. There may be foreign chemicals present either to activate the system or to trap reaction products. Such extreme and artificial conditions are frequently used to obtain an enzyme activity that can be measured conveniently. Any inhibition observed under *in vitro* conditions so different from those obtaining *in vivo* must obviously be interpreted with caution. Thus, *in vivo*, the concentration of the drug may vary with both tissue and time in the body whereas *in vitro* it remains effectively constant. The following factors must be taken into account when considering the possible relation between a particular enzyme inhibition and a clinical or toxic action of the salicylates.

Tissue concentrations of drug

If an interaction between salicylate and an enzyme is to be relevant to an *in vivo* effect then it must be elicited at drug concentrations equivalent to those occurring in the body tissues of man or experimental animals. It is not possible to predict accurately the tissue concentrations of salicylate from a knowledge of the exact size of the ingested dose since, in individual subjects, factors such as absorption from the gut and subsequent distribution among the various body fluids, organs and tissues are neither simple nor standard.

The total plasma salicylate concentrations observed during therapy or found in acute salicylate poisoning have been used as the basis for comparison (Smith, 1966). However, no allowance is made for the variable degree of binding of salicylate to the circulating proteins. The drug is largely bound to serum albumin in man and only a fraction is available to enter the body cells and subsequently determine the tissue concentrations. The accurate measurement of this fraction is not easy. The analytical methods, including equilibrium dialysis, ultrafiltration, gel filtration and frontal elution techniques, yield different results on the same sample. The ratio of protein-bound to free, i.e., unbound, salicylate varies with the total salicylate concentration, a greater proportion being bound at low than at high salicylate

concentrations (Davison & Smith, 1961). At a total plasma salicylate concentration of 0·1 mM, approximately 1% of the drug is in the free form and this increases to 10% at 1 mM, 25% at 3 mM and 30% at 5 mM. Table 1 indicates the relation between the dose of salicylate and the total and free plasma salicylate concentrations expected to occur in man. It is the plasma concentrations of the free salicylate that must be used to assess whether an *in vitro* effect of salicylate is of real or potential importance *in vivo*. However, there are some normal subjects and patients who may show higher plasma concentrations of free salicylate than would be predicted from Table 1. At least two types of binding sites are concerned in the interaction of salicylate and albumin and some human albumin molecules appear to possess fewer binding sites than others (Moran & Walker, 1968). There are also conditions such as infancy, acute infections and rheumatoid arthritis which are associated with hypoalbuminaemia.

Table 1. *Plasma salicylate concentrations in man.*

Situation	Dose		Maximum plasma salicylate concentration (mM)	
	Nature	mg/kg (70 kg man)	Total	Free*
Analgesia	Single dose 1 g aspirin	15	0·5	0·005
Therapy of rheumatoid arthritis	Divided doses 6–8 g sodium salicylate or aspirin/day	100	1·5–2·5	0·15–0·60
Acute poisoning	Single dose 30 g or more of aspirin	400 or more	3·0–10·0	1·0–5·0

* In this and the subsequent Table the term free salicylate refers to that fraction of the drug which is not bound to either circulating or tissue proteins.

The human and animal data indicate that for an *in vitro* action of salicylate as an inhibitor of an enzyme system to be relevant to its clinical effects it must occur in the range 0·005 to 0·6 mM and to be implicated as the basis of one or more of the major toxic symptoms it must be elicited at concentrations between 0·6 and 5 mM.

Mechanism of inhibition

All enzyme inhibitions are the result of an interaction between the inhibitor and some component of the enzyme system. Inhibitors are usually categorized as being either reversible or irreversible. Reversibility implies that equilibrium is set up between the enzyme system and inhibitor. Thus the enzyme activity of an inhibited system returns on merely removing the inhibitor by dialysis or similar means.

An important characteristic of reversible inhibitors is that a definite degree of inhibition, dependent on the concentration of the inhibitor, is usually reached quite rapidly. Thus the extent and duration of the inhibitory action *in vivo* will depend, at least in part, on the inhibitor concentrations attained and maintained with time. The total and unbound salicylate concentrations in several mouse tissues over a period of several hours after the injection of a single dose of the drug have been determined (Sturman, Dawkins & others, 1968; McArthur, Dawkins & Smith, 1970). Corresponding data for man are not available but an approximate guide to

the expected concentrations of unbound salicylate in the plasma after varying single doses of salicylate is given in Fig. 1. The maximum plasma concentrations of unbound drug are reached quite rapidly, within 2–4 h, even after oral administration and the persistence of the drug in the circulation is dose-dependent (Levy & Leonards, 1966). The apparent half-life of salicylate elimination is about 2–4 h with a small analgesic dose but increases to 15 to 30 h with large toxic doses of the drug, and will vary with dose in the intermediate range of doses. Chronic administration of the drug will cause fluctuating plasma concentrations depending on the size and spacing of the doses. In the treatment of rheumatoid arthritis (Table 1) the dosage regime is designed to maintain the concentrations of unbound salicylate in the plasma between 0·15 and 0·60 mM for several weeks.

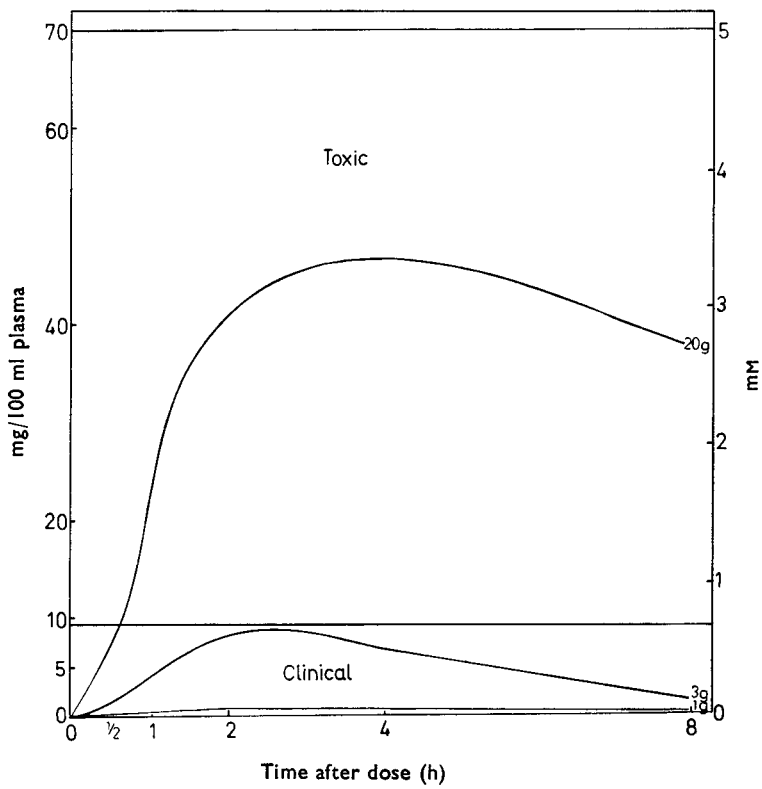

FIG. 1. Concentrations of free salicylate in human plasma after the oral administration of single doses of either 1, 3 or 20 g of sodium salicylate or aspirin.

If the inhibitor acts by a reversible competitive mechanism, involving a substrate or coenzyme, then the endogenous concentrations of the components of the enzyme system become of importance. For example, salicylate inhibits certain dehydrogenase enzymes *in vitro* by competing with the appropriate pyridine nucleotide coenzyme. The degree of inhibition achieved in any tissue *in vivo* will vary not only with the salicylate concentration but also with the coenzyme concentration in the tissue. Thus although the tissue concentration of drug may produce an initial inhibition of an enzyme activity *in vivo* this will progressively decrease with time even if the concentration of drug in the tissue is maintained. This effect will be superimposed on and enhance the diminishing degree of inhibition resulting from the elimination of the drug from the tissue.

Irreversible inhibition, in contrast to the reversible type, is characterized by a progressive increase with time ultimately reaching complete inhibition even with very dilute inhibitor, provided that the inhibitor is in excess of the amount of enzyme present (Dixon & Webb, 1964). A single dose of a drug, which is an irreversible inhibitor of an enzyme, will cause a gradual loss of the enzyme activity, the extent of which depends on the concentration of drug attained in the tissues and its rate of removal by elimination and biotransformation. The enzyme activity will return to normal at a rate depending on the rate of biosynthesis of new enzyme and the rates of synthesis of enzymes in mammalian tissues vary over a wide range (Schimke, 1969). The situation will be different if the drug is given in repeated, if smaller, doses. A balance will be set up between the rate of inactivation of the enzyme by the drug and the rate of synthesis of fresh enzyme leading to new levels of enzyme activity in the tissues. These levels may be either lower than the pretreatment values or higher if induction of the enzyme occurs.

Irreversible inhibitors form covalent bonds with enzymes. Carbamyl phosphate inactivates some enzymes by acylating the enzyme protein and it has been suggested that salicylates may act similarly (Grisolia, Santos & Mendelson, 1968). The term "chemotrophic regulation" has been used for such interactions of reagents with enzymes on sites other than the active centre and it has been assumed that such altered proteins are more susceptible to degradation by proteolytic enzymes. The formation of an initial complex between the inhibitor and the active site of an enzyme followed by the subsequent reaction of part of the inhibitor molecule and the enzyme protein to form a covalent bond compound has been proposed by Baker (1967). This type of irreversible inhibitor is described as "active-site-directed" and the chemical reactions between the inhibitor and enzyme protein include alkylation, acylation and phosphorylation. Baker, Lee & others (1960) suggested that certain substituted salicylates inhibit lactate and glutamate dehydrogenases by this mechanism although salicylate itself behaves as a strictly reversible competitive inhibitor.

The loss of activity of enzymes *in vitro* in the presence of high concentrations (>50 mM) of salicylate and other aromatic anions may be due to denaturation. This is associated with changes in the solubility and other similar properties of the enzyme protein and will be grossly evident if the proteins precipitate. Denaturation implies that a spatial change, i.e., a modification of either the secondary, tertiary or quaternary structure of the enzyme has occurred (Joly, 1965) leading to loss of activity. Inhibition is assessed and classified by kinetic measurements and in some instances, such as that of chemotrophic regulation, the distinction between the two terms is more apparent than real.

Intracellular reserve of enzyme

A major difference between *in vitro* and *in vivo* studies on enzyme inhibition is that in living cells an enzyme normally functions as one step in a chain. Its variation in activity in the cell is governed not only by the varying intracellular concentration of its substrates, which are, in turn regulated by the actions of other enzyme systems, but also by its potential catalytic activity. Some enzymes, termed equilibrium enzymes (Krebs, 1969), normally function at only a fraction of their maximum capacity and their potential activity is far in excess of the flux rate of their substrates. This excess enables them to establish near-equilibrium between starting materials and end products not only when the flux rate of the material varies widely, but also when a substantial proportion of the enzyme has been inhibited. For example, a 90% inhibition of fumarase is not likely to affect the flux rate of metabolic intermediates through the tricarboxylic acid cycle. On the other hand there are enzymes, the non-equilibrium enzymes, that normally function at near their maximum capacities

and any variation in these capacities is liable to affect the metabolic pathways in which they catalyse individual steps. Such enzymes are potentially rate-limiting and inhibition of their activities *in vivo* is of much more significance. The interpretation of the possible relation between *in vitro* inhibition and *in vivo* effects should therefore, take into account whether the enzyme being studied does or does not establish near-equilibrium in the tissues.

Intracellular compartmentation of enzymes

Enzymes are not distributed homogeneously throughout a cell but tend to be concentrated, rather than exclusively localized, in certain compartments or subcellular structures. The *in vivo* effects of drugs must therefore depend on the relative ease with which they penetrate the various cellular and intracellular membranes. A drug which is a potent inhibitor of an enzyme *in vitro* may be inactive in the whole animal because it is unable to reach the enzyme in body cells. However, a drug may affect the intercompartmental barriers between enzymes and substrates and thus change metabolism without any direct effect on the enzymes involved. This is an accepted mechanism for the stabilization of lysosomal membranes by cortisol and chloroquin both *in vitro* and in living cells (Weissmann, 1967). A similar mode of action of salicylate as an anti-inflammatory agent has been suggested (Duthie, 1963) but recent work (Harford & Smith, 1970) showed that the only effect of the drug in concentrations above 1 mM is to cause an increased release of the enzymes *in vitro*.

ENZYME SYSTEMS INHIBITED BY SALICYLATE *in vitro*

In this section the reported effects of salicylate on enzyme systems are reviewed. Emphasis is placed on those inhibitions that may be related to the known metabolic, pharmacological, clinical and toxic effects of the drug. Some degree of selection has therefore been inevitable. Whenever possible an account of the discovery of each effect is followed by a description of the mechanism of inhibition and a discussion of its possible relevance to known *in vivo* actions of salicylate.

Oxidative phosphorylation reactions

The discovery of this important effect of salicylate on cellular metabolic processes has followed a time-honoured and traditional approach to the study of drug action. It was observed that administration of the drug to man (Cochran, 1952) caused a marked and progressive increase in the oxygen consumption. This effect was searched for in systems of decreasing organization and complexity until the subcellular level was reached. Salicylate was found to uncouple oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and it appeared that the drug interfered with one or more of the sequence of enzymes involved in the phosphorylation of ADP which occurs subsequent to the electron transport chain. However, the exact site of action has not been unequivocally defined. This is not an unknown situation with uncoupling reagents principally because the mechanism of oxidative phosphorylation itself remains to be established (Boyer, 1968). It has been suggested that salicylate acts by increasing the permeability of the mitochondrial membrane to ATP causing an enhanced rate of its destruction by ATP-ase (Charnock & Opit, 1962). However, it has been shown that salicylate uncouples in mitochondrial fragments, i.e., in the absence of an intact mitochondrial membrane (Miyahara & Karler, 1965). The observation of Penniall (1958) that the terminal phosphorylation, associated with the oxidation of cytochrome c, is the most sensitive to the uncoupling action of salicylate is also consistent with a direct mechanism of action involving the phosphorylation enzymes.

There is no doubt that salicylate at concentrations in the range 0·2 to 2·0 mM, which is within that observed in the unbound form in the plasma of patients either receiving therapy or with acute poisoning (Table 1), uncouples oxidative phosphorylation reactions in mitochondrial suspensions (Brody, 1956; Packer, Austen & Knoblock, 1959; Jeffrey & Smith, 1959). Furthermore it has been established that this action is reversible (Charnock, Opit & Hetzel, 1962; Whitehouse, 1963). It has also been possible to show that the drug in concentrations of 0·1 mM and above decreases the content of ATP and creatine phosphate and increases the concentrations of inorganic phosphate in isolated rat muscle preparations (Smith & Jeffrey, 1956).

However, the direct demonstration of an uncoupling action cannot be accomplished in the whole animal. The administration of salicylate, in doses of 250 to 750 mg/kg body weight, in the rat does not reduce either the normal ATP concentrations in the liver and paw or the elevated ATP concentrations in paws rendered oedematous by pretreatment with dextran (Slater & Sawyer, 1966; Kalbhen, Domenjoz & Ehlers, 1967; Slater & Delaney, 1970). Some authors have reported morphological changes in liver mitochondria in animals given chronic doses of salicylate (Gutowaka-Grzegorczyk & Kalczak, 1968) but others have not observed changes in the liver mitochondria characteristic of uncoupling after single doses of the drug (Bullock, Delaney & others, 1970). The most probable explanation is that, unlike isolated mitochondria and rat diaphragm muscle, the whole animal can maintain ATP levels in the presence of salicylate. Under normal physiological conditions the processes of catabolism are not proceeding at a maximum rate and can show a compensatory increase if the efficiency of oxidative phosphorylation is impaired. Greater amounts of substrate are sacrificed to supply energy and to maintain ATP levels. In addition ATP may be formed by anaerobic phosphorylation reactions at the substrate level which are not affected by uncoupling reagents. This has been demonstrated in an *in vitro* system by Kalbhen & Domenjoz (1969) who showed that when neoplastic mast cells, which rely on glycolysis for most of their energy production, are incubated with salicylate, their level of ATP increases although the efficiency of ATP production by the respiratory chain is reduced.

A reduction in ATP levels in body tissues, resulting from a direct action of the drug on the phosphorylation of ADP, cannot therefore be the mechanism by which salicylate exerts either its clinical anti-inflammatory effects in rheumatoid patients (Whitehouse, 1968) or its hypoglycaemic action in patients with diabetes mellitus (Manchester, Randle & Smith, 1958). The effects of an uncoupling action due to salicylate which become evident either in experimental animals or in man are those that are caused by the compensatory increase in body catabolism. Included here are the increased oxygen consumption, the depletion of liver glycogen, the increased production of heat at the tissue level and the increased metabolic production of carbon dioxide. The increased production of heat is responsible for the dangerous hyperpyrexia which is a prominent symptom of salicylate poisoning in infants (Segar & Holliday, 1958). The enhanced rate of oxidation of both endogenous and exogenous substrates leads to augmented production of CO_2 which as a pure action can produce respiratory acidosis but more often either exaggerates or antagonizes other effects of toxic amounts of salicylate on acid-base balance (Winters, 1963).

Dehydrogenases

The oxygen uptake of isolated tissue preparations shows a biphasic response to exposure to increasing salicylate concentrations. An initial stimulation, due to the uncoupling effect of the drug, is succeeded by a marked depression of the oxygen consumption (Smith, 1958). The latter response suggested that respiratory enzymes,

less sensitive to the drug than the oxidative phosphorylation reactions, were being inhibited by the higher salicylate concentrations. Studies on the oxidation of intermediary metabolites by tissue homogenates (Kaplan, Kennedy & Davis, 1954) and the transfer of radioactivity from labelled metabolites in mitochondria (Bryant, Smith & Hines, 1963) indicated that 5–7 mM salicylate inhibits dehydrogenase enzymes in the tricarboxylic acid cycle. Later work (Baker, Lee & others, 1960; Bargoni, 1964; Hines & Smith, 1964; Smith & Sturman, 1967; Grisolia, Mendelson & Diederich, 1969) showed that the drug inhibits a wide variety of pyridine nucleotide-dependent dehydrogenases.

Euler & Ahlstrom (1943) reported that salicylate inhibited the activity of lactate dehydrogenase and that the inhibition could be reversed by the addition of coenzyme. This general mechanism also applies to the other dehydrogenase enzymes. Later work (Dawkins, Gould & others, 1967) has established kinetic constants for selected enzymes. It has been suggested (Grisolia & others, 1968) that several dehydrogenases are inhibited by salicylate by chemotrophic regulation (see p. 732), and the topic has produced an atmosphere of brisk controversy (Smith, 1968b; Grisolia, 1968). The most serious objection is that in order to demonstrate the existence of such an effect it is necessary to incubate dehydrogenase enzyme preparations for prolonged periods in the presence of salicylate concentrations which, if the kinetic constants are correct, should either largely or completely inhibit the enzyme activity. The inhibitory action of salicylate on glutamate dehydrogenase *in vitro* does not involve competition with the pyridine nucleotide coenzyme, NAD, (Gould, Huggins & Smith, 1963) but may be caused by the drug producing a dissociation of the enzyme molecule into subunits (Villela & Calcagnotto, 1970).

The pyridine nucleotide-dependent dehydrogenases are not very sensitive to inhibition by salicylate *in vitro*, the inhibitor constants ranging from 1·5 mM for liver alcohol dehydrogenase to 70 mM for the glucose-6-phosphate dehydrogenase of human red cells. Other oxidase enzymes, including succinate dehydrogenase and the L-amino acid oxidases, require high concentrations of the drug (>10 mM) to interfere significantly with their activities *in vitro* (Hines, Bryant & Smith, 1963). The only *in vivo* effects of salicylate that could be related to an inhibition of dehydrogenases would be those that occur after the administration of large toxic doses of the drug. The depressed oxygen consumption, which occurs terminally in animals given lethal amounts of salicylate, belongs to this category. A further example may be the accumulation of organic acids, including malate and citrate, in the plasma of patients with acute salicylate intoxication (Dienst & Greer, 1967). The principal disturbance in acid-base balance in the human infant (Done, 1963) and in some adults (Brown & Proudfoot, 1969) poisoned with salicylate, is a metabolic acidosis caused by the accumulation of organic acid anions in the plasma. The organic acid anions may accumulate because of other actions of the drug (see p. 736) but an inhibition of cellular dehydrogenases may be a contributory factor.

Aminotransferases

Radioactive glutamate accumulates in tissue preparations utilizing labelled pyruvate in the presence of salicylate, suggesting that the drug interferes with the further metabolism of the amino-acid (Huggins, Smith & Moses, 1961). The most important quantitative pathway for glutamate metabolism in animal tissues is that controlled by aminotransferase enzymes and it was shown subsequently (Steggle, Huggins & Smith, 1961; Yoshida, Metcoff & Kaiser, 1961; Gould & Smith, 1965a) that salicylate inhibits the activity of a number of aminotransferase activities in rat serum and in rat isolated tissues.

The mechanism of inhibition is reversible and involves competition with the amino- and 2-oxoacid substrates of the enzymes (Gould, Dawkins & others, 1966). Some of the enzymes, e.g., alanine aminotransferase are relatively sensitive to the drug, the K_I for 2-oxoglutarate being 0·6 mM, whereas others resemble the pyridine-nucleotide dehydrogenases.

The intraperitoneal injection of single doses of salicylate, in the range 150–600 mg/kg, has been observed to cause changes in the pattern and pool sizes of mouse liver amino-acids consistent with the drug differentially inhibiting aminotransferase activities (Dawkins & Smith, 1971). On the other hand, the acute or chronic administration of the drug either to man or to experimental animals increases the activities of alanine and aspartate aminotransferases in the serum and tissues and of tyrosine-2-oxoglutarate aminotransferase in the liver (Manso, Taranta & Nydick, 1956; Janota, Wincey & others, 1960; Singer & Mason, 1965; Vaughan, Steele & Korty, 1969). These findings bear little, if any, relevance to the clinical actions of salicylate but a disturbance of amino-acid interconversions caused by large amounts of salicylate may contribute to the amino-aciduria observed in acute salicylate poisoning in man (Elliot & Murdaugh, 1962; Andrews, Bruton & de Baare, 1962; Ben-Ishay, 1964) and in the rat (Berry & Guest, 1963).

Decarboxylases

The actions of salicylate on the enzymes that decarboxylate amino-acids have been studied in attempts to explain the mechanism of the convulsions that occur in acute salicylate intoxication in children and the anti-inflammatory effects of the drug. Glutamate decarboxylase was investigated because alterations in the ratio between glutamate and γ-aminobutyrate in various regions of the central nervous system may induce convulsions. However, the enzyme from bacteria and in homogenates of rat brain is only inhibited by high concentrations (>10 mM) of the drug (Gould & Smith, 1965b; McArthur & Smith, 1969).

The mammalian histidine decarboxylases are more sensitive to the drug *in vitro*. These may be subdivided into the specific enzyme, which acts on histidine only, and the non-specific L-amino-acid decarboxylase which is also capable of decarboxylating L-5-hydroxytryptophan and L-dihydroxyphenylalanine. Salicylate inhibits both types of enzyme activity *in vitro*, the specific enzyme by a mechanism involving competition with the pyridoxal phosphate coenzyme, the K_I being 2·7 mM, and the nonspecific enzyme by competing with the amino-acid substrates, the K_I being 1·5 mM (Skidmore & Whitehouse, 1966a; 1967).

One important implication of these findings is that salicylate could inhibit the biosynthesis of histamine *in vivo* (Whitehouse, 1968) and this could be the mechanism of its clinical anti-inflammatory actions. Schayer (1965) has argued that newly synthesized histamine is the major mediator of inflammatory responses and has observed raised levels of histidine decarboxylase activity during inflammation. It seems to be generally accepted that histamine is the initial pharmacological mediator of the vascular changes associated with inflammatory reactions but its action is transient and the participation of other mediators is necessary to maintain and extend inflammation (Spector & Willoughby, 1968). This does not exclude the possibility that salicylate could modify inflammatory reactions by interfering with the biogenesis of histamine in the tissues. It must also be remembered that the experimental work on tissue mediators of inflammation is virtually restricted to acute inflammatory responses induced by a bewildering variety of stimuli and may bear little, if any, relevance to the chronic inflammatory reactions characteristic of human rheumatic disorders. However, the evidence that salicylate is an anti-histamine due to its inhibitory action on the histidine decarboxylases is not

convincing. In favour is the observation that the oral administration of 4 g of aspirin per day halves the urinary excretion of histamine in patients with rheumatism and in normal subjects (Sjaastad & Sjaastad, 1965). However, the administration of 400 mg/kg of salicylate to the rat increased the levels of the enzyme in gastric tissue by several fold (Petillo, Gulbenkian & Tabachnick, 1969). Again the administration of salicylate to some patients with chronic urticaria exacerbates the condition. This effect is dependent on the size of the salicylate dose and seems to be caused by an increased rate of formation and release of histamine (Hamrin, 1957).

Aminoacyl-tRNA synthetases

The discovery of the uncoupling effect of salicylate on oxidative phosphorylation reactions stimulated several studies of the possible actions of the drug on biosynthetic reactions requiring ATP. One of these was the incorporation of amino-acids into the protein of isolated tissues. It was shown that salicylate, in high concentrations, 10–15 mM, inhibits the *in vitro* incorporation of radioactive amino-acids into the protein of rat costal cartilage (Bellamy, Huggins & Smith, 1963) and into the epithelial glycoproteins of sheep mucosal scrapings (Kent & Allen, 1968). Protein biosynthesis in the rat isolated diaphragm is more sensitive to the drug and salicylate concentrations from 0·5 to 5 mM interfere with the incorporation of a variety of amino-acids into protein (Manchester & others, 1958; Dawkins, Gould & Smith, 1966). The next steps were to show that the effect occurred in cell-free preparations, at salicylate concentrations of 0·3 mM and above, and that it was independent of the uncoupling action (Dawkins, Gould & Smith, 1966; Reunanen, Hanninen & Hartiala, 1967).

The mechanism of the inhibitory action of salicylate on protein biosynthesis *in vitro* involves a differential action of the drug on the formation of aminoacyl-tRNAs (Burleigh & Smith, 1970). The most sensitive aminoacyl-tRNA synthetases are those that incorporate glutamate, aspartate and histidine, and the drug shows a simple competitive inhibition with respect to the amino-acid substrate.

It requires high toxic doses of the drug to interfere with protein biosynthesis in the adult animal. Thus the intraperitoneal injection of 600 mg/kg significantly inhibits the incorporation of radioactive amino-acids into liver proteins of the mouse (Dawkins, McArthur & Smith, 1971). Young animals exhibiting active growth are more sensitive to the drug. The immature rat shows a decreased rate of weight grain and skeletal growth when given an injection of either sodium salicylate or aspirin between 250 and 300 mg/kg (Limbeck & others, 1966) and young chicks react similarly (Nakaue, Weber & Reid, 1967; Thomas, Nakaue & Reid, 1967).

The relative insensitivity of general protein synthesis *in vivo* does not exclude the possibility that the formation of specific proteins may be preferentially inhibited by the drug. Amongst these may be the immunoglobulins. Ambrose (1966) has shown that salicylate inhibits secondary antibody responses *in vitro* although the drug is only considered to possess weak immunosuppressive activity in the whole animal (Griswold & Uyeki, 1969). The biosynthesis of collagen in granulation tissue induced by carrageenan in the rat is inhibited by salicylate *in vivo* whereas the formation of non-collagen protein is not altered (Fukuhara & Tsurufuji, 1969). Other experimental work with collagen in the intact rat (Trnavska, Trnavsky & Kuhn, 1968) suggests that the administration of 300 mg/kg of salicylate increases the turnover of the protein, implying more rapid rates of both synthesis and breakdown. Salicylate inhibits collagen biosynthesis in tissue culture preparations and in cell-free systems (Rokosova-Cmuchalova & Bentley, 1968) but the mechanism is concerned, at least in part, with the capacity of the drug to chelate ferrous ions (Nakagawa & Bentley, 1969).

Proteases

Proteolytic enzymes may be involved in inflammatory reactions at several points. They may act on protein precursors of tissue mediators causing the release of polypeptides. The later stages of rheumatoid joint disease may involve the disruption of intra-articular lysosomes causing the release of hydrolase enzymes, including the proteolytic cathepsins, which degrade the protein-polysaccharide complexes of cartilage. The lysosomal enzymes may also degrade auto-immune bodies and the tissue debris resulting from a continuing inflammatory process.

Studies with general proteolytic enzymes, such as trypsin and chymotrypsin, have not provided evidence that salicylate is an effective inhibitor. The K_I for chymotrypsin is 9·0 mM (Skidmore & Whitehouse, 1966b) and the inhibitory effect of the drug on trypsin-induced casein breakdown is not impressive (Morsdorf, 1965). The autoproteolytic process that occurs in fresh homogenates of rat paw tissue is more sensitive to salicylate but it requires 5 mM drug to produce an approximately 50% inhibition (Morsdorf, 1965). It was claimed that kallikrein, the enzyme that releases kinins from circulating globulins, was inhibited by salicylate *in vitro* (Northover & Subramanian, 1961) but this has been disproved by Hebborn & Shaw (1963), Davies, Holman & others (1966) and Bertelli, Donati & Marek (1969). Some of the chrondrolytic enzymes are inhibited by 1 to 2 mM salicylate (Simmons & Chrisman, 1965) both *in vivo* and *in vitro*. No information appears to be available about the possible inhibitory effects of the drug on individual lysosomal proteases and such data must await the further purification and characterization of these materials.

Ribonucleic acid polymerases

The reports (Warkany & Takacs, 1959; Larsson, Ericson & Bostrom, 1963) that the administration of salicylate to pregnant rats caused several congenital malformations of the litters carried to full term stimulated interest in the effects of the drug on nucleic acids. One finding (Kazakova & Chebotar, 1969) is that sodium salicylate induces conformational changes in DNA prepared from rat liver nuclei and mitochondria. A second interaction of the drug and nucleic acids is its inhibitory effect on rat liver DNA and RNA polymerase activities *in vitro* (Janakidevi & Smith, 1969). The DNA polymerase activity was more sensitive to the drug being inhibited by salicylate concentrations of 1 mM. However, incubation of rat foetuses with 2 mM salicylate revealed that RNA synthesis, assessed by measurement of the polymerase activity and the incorporation of labelled orotic acid, was inhibited whereas DNA synthesis, followed by the incorporation of radioactive thymidine, was not (Janakidevi & Smith, 1970a). Later work showed that only the RNA polymerase stimulated by ammonium sulphate and manganese ions was sensitive to salicylate (Janakidevi & Smith, 1970b). This enzyme is thought to be responsible for the biosynthesis of certain RNA species, including messenger RNA, and the administration of 400 mg/kg of salicylate to adult mice preferentially interfered with the formation of rapidly labelled RNA (Janakidevi & Smith, 1970c). Salicylate also inhibits the incorporation of radioactive uridine into the nucleic acids of human peripheral lymphocytes (Packman, Esterly & Peterson, 1969).

The mechanism by which salicylate inhibits nucleic acid polymerase activities is unknown and it will be necessary to solubilize and purify the enzymes as a preliminary step in the investigations. The drug differs from other inhibitors such as actinomycin D and aflatoxin B_1 and resembles α-amanitin in only inhibiting the Mn^{2+}: ammonium sulphate-activated RNA polymerase (Stirpe & Fiume, 1967).

If salicylate inhibits the biosynthesis of messenger RNA *in vivo* this may be involved with its teratogenic effects in rodents. However, salicylate while frequently quoted as a teratogen in animals is not considered to be so in man (Palmer, 1969). The

main argument is the shape of the dose-response curve showing that malformation occurs over a very narrow range and embryopathic activity occurs at dosages where a maternally toxic effect is evident. Thus the only predicted chance of a human foetal malformation would be in circumstances such as an attempted suicide using a large toxic dose of salicylate by a woman 4–5 weeks pregnant who recovered without suffering a miscarriage. This may be a somewhat oversanguine conclusion since Richards (1969) considers that the results of a retrospective epidemiological study of malformations in human pregnancy showed that the taking of salicylate preparations in the first trimester is associated with significant increases in abnormalities of the central nervous system and the alimentary tract of the foetus.

Prostaglandin biosynthesis

It was shown by Piper & Vane (1969) that among the substances released during anaphylaxis in guinea-pig lung was an unidentified material (RCS) which contracted rabbit aorta. The release of this substance was blocked by salicylates. It was subsequently found that arachidonic acid, one of the precursors of prostaglandins, also released RCS from perfused lungs. Although RCS has not been isolated and its chemical nature is unknown, attention was directed to the effects of salicylates on prostaglandin production. Three different biological systems were used, cell-free homogenates of guinea-pig lung incubated with arachidonic acid (Vane, 1971), isolated perfused dog spleen stimulated by adrenaline (Ferreira, Moncada & Vane, 1971) and human blood platelets exposed to thrombin (Smith & Willis, 1971). In each instance the stimulated production of prostaglandins was inhibited by 0·005–0·2 mM aspirin but sodium salicylate was either much less potent or without action. The effect was interpreted as inhibition of the biosynthesis of prostaglandins since the tissues could be stimulated to release more prostaglandins than they contain. However, it will be necessary to perform metabolic studies on the incorporation of labelled precursors into the various prostaglandins, to demonstrate the inhibitory action of aspirin on an isolated enzyme preparation such as prostaglandin synthetase (Takeguchi, Kohno & Sih, 1971) and to exclude an action of the drug on prostaglandin breakdown before this mechanism is proven.

The relevance of this interesting and important effect of aspirin *in vitro* to the therapeutic and other actions of the drug is a matter for speculation (Collier, 1971). It will be necessary to show that the prostaglandins are mediators of pain, fever and inflammation in man. At present there is little acceptable evidence that the prostaglandins are concerned in either pathological or experimental pain. They elevate body temperature when injected in the cerebral ventricles of cats (Milton & Wendlandt, 1971) and are found in increased amounts in acute inflammatory exudates in animals (Willis, 1969). In man, it has been shown that they are present in blister fluid (Angaard, Arturson & Jansson, 1970) and accumulate in the interstitial fluid of the skin of patients with allergic contact dermatitis (Greaves, Sondergaard & McDonald-Gilson, 1971) but this is not proof that they have any role as mediators in acute inflammatory reactions. Their possible occurrence in the chronic inflammation characteristic of rheumatoid arthritis has not been studied.

There is no reason to differentiate between salicylate and acetylsalicylate with respect to either experimental (Wilhelmi, Gydnia & Ziel, 1968) or clinical anti-inflammatory (Woodbury, 1965) actions since they are equally effective. The relative lack of activity of sodium salicylate as an inhibitor of prostaglandin production cannot be reconciled with a mechanism relating an interference with the biosynthesis of prostaglandins in the tissues to clinical antirheumatic actions.

This mechanism may be of more significance in situations in which aspirin has been shown to cause different effects to salicylate. These include analgesia (Lim, 1966),

bradykinin-induced bronchoconstriction (Berry & Collier, 1964), aggregation of blood platelets (O'Brien, 1968), thurfyl nicotinate erythema (Truelove & Duthie, 1959) and hypersensitivity reactions (Samter & Beers, 1967). Clearly, the acetylsalicylate ion may act *in vivo* as an independent chemical entity and its interactions with tissue components, including the generating system for prostaglandins, may differ from those of its immediate metabolite, salicylate.

Other interactions with enzyme systems

The inhibitory effects of salicylate *in vitro* and *in vivo* on the biosynthesis of mucopolysaccharide sulphates have been described by Bostrom, Whitehouse and their colleagues (Whitehouse, 1965). The processes are affected by 2 mM drug *in vitro* and after the administration of 100 mg/kg of salicylate in whole animals. The mechanism has been explained in terms of the uncoupling action of salicylate but many enzymes are involved in mucopolysaccharide synthesis and it is likely that salicylate exerts multiple inhibitory effects (Lee & Spencer, 1969). Salicylate inhibits xanthine oxidase activity *in vitro* and *in vivo* (Bergel & Bray, 1959; Mitidieri & Affonso, 1959) and the biosynthesis of uric acid in perfused liver (Ramia, Boyal & Calvet, 1966). This action may be partially responsible for the beneficial effects of large doses (over 5 g per day) of salicylate in gout.

SUMMARY AND CONCLUSIONS

The work of the last two decades has shown that salicylate is a versatile inhibitor of many important enzyme activities *in vitro*. These include oxidative-phosphorylation reactions, dehydrogenases, decarboxylases, aminotransferases, aminoacyl-tRNA synthetases and nucleic acid polymerases. In some instances the mechanisms of inhibition have been established and resemble each other in being reversible and competitive with either a substrate or a coenzyme.

With many enzyme systems concentrations of greater than 5 mM are required to demonstrate an appreciable interference with the measured activities *in vitro*. Such interactions can bear little relevance to *in vivo* actions of the drug even in extreme cases of acute salicylate poisoning. However, there are some *in vitro* inhibitory effects of salicylates that occur within the concentration range (0·6 to 5·0 mM) of free salicylate observed either in the plasma of poisoned human subjects or in the tissues of animals given high toxic doses of the drug. The inter-relation between these *in vitro* inhibitions, the effects observed after the administration of doses of salicylate giving equivalent concentrations of salicylates in the tissues of whole animals, and known toxic symptoms in man, are indicated in Table 2.

It is therefore possible to state a reasonable case for the proposition that several of the major toxic symptoms in salicylate intoxication are caused by the drug interfering with the activities of cellular enzyme systems. This is not so with respect to the anti-inflammatory action of the drug. The only two interactions with enzyme systems *in vitro* that occur in the range 0·15 to 0·60 mM (see Table 1) are the uncoupling effect and the inhibition of protein biosynthesis. An interference with cellular energy-yielding reactions due to uncoupling could theoretically affect many aspects of the inflammatory reaction (Whitehouse, 1968) but no convincing evidence relating uncoupling potency to anti-inflammatory activity has been obtained. Salicylate inhibits general protein biosynthesis in the mouse in toxic amounts only, and the possibility that it preferentially interferes in man with the biosynthesis of specific proteins which either initiate or maintain inflammation is purely speculative. It could be concluded that the biochemical investigation of the salicylates has failed to elucidate the mechanism(s) of their anti-inflammatory and antirheumatic actions.

Table 2. *Relation between inhibitory effects of salicylate on isolated enzyme systems and toxic actions in man.*

In vitro		*In vivo*	
Enzyme system	Inhibitory salicylate concentration (mM)	Effects in animals and man	Toxic action in man
Oxidative phosphorylation	0·2	Increased O_2 consumption Liver glycogen depletion Enhanced heat production Increased metabolic formation of CO_2	 Hyperpyrexia Respiratory acidosis
Dehydrogenases	1·5–10·0	Decreased O_2 consumption Accumulation of organic acid anions in tissues	 Metabolic acidosis
Aminotransferases	0·6–10·0	Changed patterns and pool sizes of tissue amino-acids	Amino-aciduria
RNA polymerases	2·0	Impaired biosynthesis of messenger RNA	Teratogenicity

Such a statement is both premature and over-simplified. The prematurity stems from the fact that only a comparatively limited number of interactions of enzymes and the drugs have been described and studied. Many enzymes, more intimately concerned with the inflammatory processes await either discovery or characterization. The enzyme systems responsible for the biosynthesis of prostaglandins and other mediators of inflammation should be considered in this connection. The anti-rheumatic properties of salicylate may be mediated by effects on such enzymes rather than on general cellular reactions catalysed by the ubiquitous dehydrogenases or aminotransferases. Secondly, it is equally possible that the drug may act at several sites rather than specifically interfere with a single enzyme system. The overall anti-inflammatory effect may be due to multiple interactions with several components; biochemical, pharmacological and immunological, of the chronic inflammatory response. Thus either uncoupling or a selective interference with protein biosynthesis need not be the only mechanism but may play a supporting role to other effects of salicylate. These may include the release of small biologically active molecules from their binding sites on circulating and tissue proteins because it has been shown that the drug will displace tryptophan (McArthur & Dawkins, 1969), long-chain fatty acid anions (Dawkins, McArthur & Smith, 1970) and pyridoxal-5-phosphate (Dempsey & Christensen, 1962) from human and bovine serum proteins.

It has been demonstrated that the clinically useful antirheumatic drugs displace protein-bound tryptophan and L-phenylalanyl-L-phenylalanine from human serum *in vitro* (McArthur, Dawkins & Smith, 1971). This action is not shared by other drugs which lack clinical anti-inflammatory actions but which bind to the serum proteins and are administered to man over similar periods of time (Smith, Dawkins & McArthur, 1971). In a comparison with normal subjects the percentage of tryptophan bound to serum proteins is significantly reduced in patients with rheumatoid arthritis receiving therapy with antirheumatic drugs (McArthur, Dawkins & others, 1971). This also occurs in pregnancy and jaundice, two conditions which are associated with an increased incidence of remissions in rheumatoid arthritis. It has been proposed by these workers that human serum contains peptide-like substances in protein-bound and free forms and that the latter protect susceptible tissues against the effects of chronic inflammatory processes. In patients with

rheumatoid arthritis the anti-inflammatory peptides are bound to an abnormal extent to the circulating proteins and the antirheumatic drugs act by redressing the bound:free ratio to that in the normal subject.

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