REVIEW ARTICLE

SALICYLATES AND METABOLISM

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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.

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RESEARCH PAPERS

OXIME THERAPY IN POISONING BY SIX ORGANOPHOSPHORUS INSECTICIDES IN THE RAT

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The effects of two oxime reactivators, PAM and TMB4, on poisoning by six organophosphate indirect cholinesterase inhibitors with different phosphorylating groups have been examined in the rat. Repeated oxime injections were beneficial in poisoning by parathion, parathion-methyl, phenkaptone and, to a lesser extent, diazinon, but ineffective with dimefox and dimethoate. PAM had no additional antidotal value against dimethoate poisoning when given with atropine. The findings are related to expected degrees of irreversibility of cholinesterase inhibition with different phosphorus compounds.

In recent years, considerable attention has been devoted to the treatment of organophosphate poisoning by specific cholinesterase reactivators, and a number of oximes have been found effective, particularly in conjunction with atropine. Until recently, attention was concentrated on pyridine-2-aldoxime methiodide (PAM), and work on this has recently been reviewed[1]. More recently, a number of other materials have been examined, and a further one, bis(pyridine-4-aldoxime) trimethylene dibromide (TMB4), found effective[1-9]. The greater effectiveness of TMB4 over PAM showed species variation[4,6,9]; TMB4 had the advantage of greater water solubility.

The mode of action of these oximes consists mainly of reactivation of inhibited cholinesterase by dephosphorylation[1,2,7-17], though there could occasionally be a slight additional benefit by direct reaction with the organophosphorus compound before inhibition occurs[1,11,13]. When used in combination with atropine, the benefit obtained was often far greater than expected from the individual effects of the materials alone[1,3,4,6,8-10,12,13,16,19]. Oxime reactivation of whole brain cholinesterase appears to take place *in vitro* as readily as that of other tissues, but *in vivo* the reactivation was relatively much slower or absent, suggesting that the oximes (for example, PAM and TMB4), possibly due to their ionic nature, would only slowly penetrate the blood-brain barrier to the nerve cells, though this may not necessarily apply to individual vital centres in the brain[1,9,15,16,19].

The reactivating efficiency of oximes varies with the nature of the enzyme-phosphorylating portion of the organophorphorus compound[20,21]; reactivation occurs most readily with diethyl phosphates such as TEPP and paraoxon, less readily with di-isopropyl phosphates such as DFP, and only to a negligible extent with the phosphoramide, schradan[1,8,9,13-16,18,22]. No reactivation occurs *in vitro* or *in vivo* with the dimethyl phosphates

demeton-methyl or endothion such as occurs with parathion; there is some reduction in mortality with endothion, but less with demeton-methyl[23]. Work on parathion[1,17,23–25] suggests that with this diethyl indirect inhibitor, from which the anticholinesterase oxidation product is formed only slowly in the liver, repeated doses of oxime are required to maintain the cholinesterase reactivation *in vivo*; this is consistent with the finding that the blood concentration of PAM injected into rats falls to an ineffective level after 1–2 hours[13,16]. *In vitro* tests have shown that oxime reactivation of inhibited cholinesterase only occurs when the enzyme is at the reversibly-phosphorylated stage, and not after the phosphorylation has become irreversible[1,16,26,27]. This change from reversibly- to irreversibly-phosphorylated enzyme takes place much more readily with phosphoramide or with dimethyl or diisopropyl phosphate inhibition than with diethyl phosphate inhibition[1,13,16,26–28], and a greater proportion of irreversible inhibition occurs if the direct inhibitor is released or formed slowly, or is relatively persistent in the body[28].

Most reported work on oxime reactivation has thus been with direct inhibitors of cholinesterase, while dimethyl phosphate inhibitors have similarly received little attention. The necessity for repeated injections against parathion poisoning seems likely to apply to most indirect inhibitors, where the active metabolite is formed comparatively slowly. On the evidence of comparative rates of change to irreversible phosphorylation, the oxime reactivation rate after dimethyl phosphate poisoning might be more comparable with those found with diisopropyl than diethyl phosphates, or may approach zero as suggested for demeton-methyl and endothion[23], and by reversibility data[28].

It thus seemed of interest and importance to explore the therapeutic activity of the two oximes at present of greatest interest, PAM and TMB4, against a series of common indirect inhibitors of various types, including dimethyl phosphates, to determine whether the inclusion of oxime reactivators in therapy of poisoning by these compounds should be recommended.

Experimental Methods

PAM was obtained from Messrs. L. Light & Company, and TMB4 kindly supplied by Dr. F. Hobbiger; both were administered as 5 per cent w/v aqueous solutions. Atropine sulphate was BP grade administered as a 3·48 per cent w/v aqueous solution. Dimethoate (*00*-dimethyl-*N*-methylcarbamoylmethyl phosphorodithioate) was used as a high purity sample prepared in these laboratories, or as impure technical material, administered as a 40 per cent w/v propylene glycol solution. Diazinon and phenkaptone were pure samples obtained from J. R. Geigy S.A. (Basle), administered undiluted. Dimefox was a pure sample prepared in these laboratories, administered as a 0·4 per cent w/v propylene glycol solution. Parathion was 98·5 per cent technical material, administered as a 0·4 per cent w/v solution in glycerol formal[29]. Parathion-methyl was a pure sample supplied by Dr. W. N. Aldridge, administered as a 3·2 per cent w/v solution in glycerol formal.

Rats were semi-adult (150–200 g.) animals of Wistar strain, maintained and fed under standard conditions. Female rats were used in most experiments (exceptions being referred to in the text). Administration techniques were orthodox, and, except where killing early, by decapitation, for tissue cholinesterase assay was required, the observation period was seven days.

In certain experiments, plasma, erythrocyte and whole brain cholinesterase activities were determined by a standard manometric method[30], and the results expressed as percentages of a series of normal values obtained in this laboratory on similar rats.

In all experiments, the organophosphate compound was given orally, with intraperitoneal injections of the oxime or atropine 15 minutes before and 4 hours after this, followed by a further subcutaneous oxime or atropine injection after 8 hours, as a "maintenance dose". Each dose of PAM was 100 mg./kg., and each dose of TMB4 25 mg./kg., these being the highest tolerated without toxic effect. Preliminary tests suggested a single dose intraperitoneal LD50 of TMB4 to the female rat of about 80 mg./kg., and of PAM about 400 mg./kg.; effects consisted of dyspnoea, convulsions and respiratory failure, as previously reported[8,9,31]. The dose of atropine sulphate used was 17·4 mg./kg.

Symptom intensities were assessed on a numerical basis, as follows. 1. Slight fibrillation. 2. Moderate fibrillation. 3. Severe toxic effects, animal mobile. 4. Severe toxic effects, animal prostrate.

Results

Approximate oral LD50 values to female rats were first obtained on the organophosphates, as follows.

Dimethoate, pure	600 mg./kg.
Dimethoate, technical	120 mg./kg. (male rats)
Parathion	3 mg./kg. (5 mg./kg. to male rats)
Parathion-methyl	16 mg./kg. (12 mg./kg. to male rats)
Diazinon	800 mg./kg.
Phenkaptone	50 mg./kg.
Dimefox	1·8 mg./kg.

The low toxicity of the diazinon and its very slow onset of effects, were noteworthy, and believed due to its unusually high purity[32].

Each of the organophosphates was then given orally, in doses slightly greater than the approximate LD50, to three groups of ten female rats. One of these groups served as control, the second received three injections of PAM, and the third three injections of TMB4, as detailed above. Mortalities and severity of effects were then recorded for seven days, and are summarised in Table I. There was initial narcosis with dimethoate, possibly less severe in the oxime-treated groups, and with phenkaptone in the groups receiving oxime only.

This experiment was then repeated using groups of six female rats and an organophosphate dose half the approximate LD50. Survivors were killed after 24 hours for plasma, erythrocyte and whole brain

cholinesterase assay. The results are summarised in Table II. A further similar experiment with a 50-hour observation period is summarised in Table III.

TABLE I

EFFECT OF OXIME THERAPY ON LETHAL ORGANOPHOSPHATE POISONING IN FEMALE RATS

Organophosphate and oral dosage	Therapy xl	Severity of effects*							Mortality (10 in group)						
		½ h	1 h	2 h	8 h	1 d	2 d	4 d	½h	1h	4h	8h	1d	2d	7d
Dimethoate (pure) (750 mg./kg.)	Nil	0	1	3–4	4	4	—	—	0	0	2	4	9	10	10
	PAM	0	0	3–4	4	4	—	—	0	0	0	0	5	10	10
	TMB4	0	0	3	4	4	—	—	0	0	0	1	1	10	10
Parathion (4 mg./kg.)	Nil	1–2	3–4	3–4	4	2	1–2	0	0	4	5	6	6	6	7
	PAM	0	1	1	1–2	0–1	0	0	0	0	0	0	0	1	1
	TMB4	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Parathion-methyl (20 mg./kg.)	Nil	3–4	4	3	1	1	0	0	4	9	9	9	9	9	9
	PAM	3–4	3–4	3	2	2–3	2–3	0	0	0	0	0	0	0	0
	TMB4	3–4	3–4	3–4	2	2–3	2–3	0–2	0	0	0	0	0	0	1
Diazinon (1000 mg./kg.)	Nil	0	0	0	1–2	3	4	0	0	0	0	0	0	4	9
	PAM	0	0	0	1–2	3	3–4	0–1	0	0	0	0	0	1	3
	TMB4	0	0	0	2–3	3–4	3	0	0	0	0	0	0	2	3
Phenkaptone (60 mg./kg.)	Nil	0	0	1–2	3–4	2–4	1–4	0	0	0	2	3	3	5	5
	PAM	0	0	0–2	2–4	1–2	1–2	0	0	0	0	0	0	0	0
	TMB4	0	0	1–3	3–4	1–2	0–1	0	1	1	1	1	1	1	1
Dimefox (2·2 mg./kg.)	Nil	0	0	0	3–4	2–3	1–2	0	0	0	0	4	7	7	8
	PAM	0	0	0	2–4	2–3	1–2	0	0	0	0	3	6	6	6
	TMB4	0	0	0	1–4	1–3	1–2	0–1	0	0	0	2	5	5	5

xl PAM dose 100 mg./kg. TMB4 25 mg./kg. Administered i.p. at 15 min. before and 4 hr. after, and s.c. at 8 hr. after organophosphate.

Code for toxic effects:
* 1. Slight fibrillation
2. Moderate fibrillation
3. Severe toxic effects, but animal mobile
4. Severe toxic effects, animal prostrate

TABLE II

EFFECT OF OXIME THERAPY ON SUB-LETHAL ORGANOPHOSPHATE POISONING AND CHOLINESTERASE INHIBITION IN FEMALE RATS

Organophosphate and oral dosage	Therapy	Severity of effects*						ChE at 24 hrs. Per cent normal		
		½ h	1 h	2 h	4 h	8 h	24 h	Erythrocyte	Plasma	Brain
Dimethoate (pure) (300 mg./kg.)	Nil	0	1	3	3–4	3	0–1	16	23	26
	PAM	0	0	2	3	3	2	20	19	20
	TMB4	0	0	2	3–4	3–4	1–3	15	28	24
Parathion (1·5 mg./kg.)	Nil	0	1–2	2	2	0–1	0	55	81	102
	PAM	0	0	0	0	0	0	73	83	107
	TMB4	0	0	0	0	0	0	78	95	96
Parathion-methyl (8 mg./kg.)	Nil	3–4	4	2–3	1–2	0–1	0	30	41	50
	PAM	2–4	2–4	2–3	1–2	0–2	0–1	34	41	42
	TMB4	1–3	2–4	2	1–2	0–2	0–1	23	28	43
Diazinon (400 mg./kg.)	Nil	0	0	0	0	0–1	2	9	5	18
	PAM	0	0	0	0	0–1	2–3	14	9	17
	TMB4	0	0	0	0	0–1	1–2	43	24	24
Phenkaptone (25 mg./kg.)	Nil	0	0	0	2–3	2–4	0–1	22	46	42
	PAM	0	0	0	1–2	1–2	0–1	61	58	37
	TMB4	0	0	0	1–2	2–3	0–1	50	40	40
Dimefox (0·9 mg./kg.)	Nil	0	0	0	0–1	1–2	0–2	26	33	98
	PAM	0	0	0	0–1	1–2	0–2	26	29	92
	TMB4	0	0	0	0	1–2	1–2	29	37	95

Code for toxic effects:
* 1. Slight fibrillation
2. Moderate fibrillation
3. Severe toxic effects, but animal mobile
4. Severe toxic effects, animal prostrate

Mortality at 24 hrs:—Parathion-methyl (untreated) 2/6
Phenkaptone (untreated) 1/6
Dimefox (treated TMB4) 1/6

TABLE III

EFFECT OF PAM THERAPY ON ORGANOPHOSPHATE POISONING AND CHOLINESTERASE INHIBITION IN MALE RATS

Organophosphate	Dose mg./kg.	Therapy	Effects of PAM on symptoms	Mortality 50 hour	ChE at 50 hours (per cent normal)		
					Erythrocyte	Plasma	Brain
Dimethoate (technical)	100	Nil	None detectable	1/6	23	42	28
		PAM		1/6	26	25	27
Parathion-methyl	12	Nil	Slower recovery	3/6	37	49	47
		PAM		0/6	38	54	28
Parathion	3	Nil	Obviously less severe; faster recovery	0/6	36	58	86
		PAM		1/6	56	90	89

A series of experiments was then performed to assess the effect of PAM on atropine therapy of dimethoate poisoning. Groups of ten male rats were given oral doses of pure or technical dimethoate, certain groups receiving three injections of atropine sulphate, or PAM, or both. Mortalities obtained are summarised in Table IV. PAM alone had little apparent

TABLE IV

EFFECT OF PAM AND ATROPINE THERAPY ON MORTALITY FROM ORAL DIMETHOATE POISONING IN FEMALE RATS

Dimethoate dose (mg./kg.) and grade	Therapy			
	Nil	PAM (100 mg./kg. × 3)	Atropine (17·4 mg./kg. × 3)	PAM + atropine
150 tech.	8/10	—	0/10	3/10
600 pure	5/10	8/10	0/10	3/10*
600 pure	5/10	6/10	0/10	2/10

* One of these deaths possibly not attributable to chemicals used.

effect; atropine gave slower development of effects and death, with almost complete control of secretions for 12 hours and considerably less weakness; atropine and PAM together gave an improvement similar to that with atropine alone, except that control of urinary incontinence appeared a little less lasting or effective.

In one test (see Table IV), some toxic effects were noted due to administration of PAM and atropine sulphate together; this anomalous result may have arisen from the use of an aged batch of PAM for this test, and did not occur when the test was repeated with newer material. Simultaneous administration of 17·4 mg./kg. atropine intraperitoneally reduced the intraperitoneal LD50 of PAM from about 400 to about 150 mg./kg.

DISCUSSION

Examination of Tables I–IV showed that oxime therapy alone was of no apparent value in poisoning by dimethoate or dimefox. With diazinon there was some reduction of mortality, together with reduced erythrocyte and plasma cholinesterase inhibition particularly with TMB4. With phenkaptone there was reduction in mortality and (particularly with PAM) of symptom severity, with reduced erythrocyte cholinesterase

inhibition. The narcosis occurring with oxime therapy of phenkaptone poisoning cannot at present be explained. With parathion there was marked reduction of mortality and symptom severity, accompanied by reduced erythrocyte and plasma cholinesterase inhibition. With parathon-methyl there was marked reduction in mortality but not in symptom severity, recovery being delayed, with no apparent reduction in cholinesterase inhibition.

These results are largely consistent with observations on reversibility of inhibition previously reported[13,16,26–28]. Dimefox is a phosphoramide similar to schradan, whose inhibition appears almost entirely irreversible[1,13,24,25], so that the lack of benefit from oxime therapy is not unexpected. Parathion, diazinon and phenkaptone are diethyl phosphates, inhibition by whose active metabolites would become irreversible only relatively slowly[26,27]. Cholinesterase reactivation and beneficial effects found after oxime therapy in poisoning by these compounds were therefore to be expected. The lower effectiveness of oxime therapy with diazinon than with parathion and phenkaptone may be connected with the much slower onset and development of toxic effects with this compound, suggesting slower formation and greater persistence of the anticholinesterase metabolite, with a greater degree of irreversible inhibition[28]. With the dimethyl phosphates dimethoate and parathion-methyl the governing factor is almost certainly the rate of production and persistence of the active metabolite, controlling the amount of irreversible inhibition produced[28]. With parathion-methyl, onset and recovery were rapid, suggesting a relatively small proportion of irreversible inhibition compared with dimethoate. This is consistent with the early beneficial effect on mortality, and with the later absence of significant cholinesterase reactivation after the remaining inhibition had had time to become irreversible. The delayed recovery after oxime therapy with parathion-methyl can probably be explained on a similar basis. With dimethoate, on the other hand, onset and recovery were much slower, and so the active metabolites were apparently produced much more slowly and were more persistent, inhibition thus being almost entirely irreversible[28], and oxime therapy therefore of no benefit. This is thus a more extreme but similar effect to that with demeton-methyl, where oxime therapy was of relatively little benefit[23].

The finding that oxime therapy alone was of no apparent benefit in dimethoate poisoning then raised the question of whether it increased the value of atropine therapy, as occurs with many compounds[1,3,4,6,8,9,10,12,13,16,18,19,23]. The results summarised in Table IV suggested that it did not. It thus appears likely that synergism between oximes and atropine, as well as reactivation by oximes, depends on the enzyme inhibition being reversible, as expected. There was, in fact, a suggestion from the experiments of Table IV that administration of PAM in addition to atropine in dimethoate poisoning in the rat may possibly have been deleterious under these conditions, giving slightly higher mortality and less complete control of urinary incontinence. However, Dr. J. M. Barnes has seen no similar effect in parallel tests where no oxime therapy was given until toxic effects

had developed. In other reported work on parathion and endothion there was some indication of a slight increase in mortality of atropinised poisoned mice when the total PAM dose was increased from 50 to 100 mg./kg.[23]. Such findings may possibly be connected with the belief that low concentrations of PAM may potentiate the action of acetylcholine at nerve endings, and so possibly increase the toxic effects produced by acetylcholine accumulation[33]. In addition, there was some indication of an increase in the toxicity of PAM in the presence of atropine, but the effects were readily distinguished from those of anticholinesterase poisoning. This change is opposite from that found in the rat with P2S[31].

With the phosphorus compounds used in this investigation with rats, there was very little difference between the beneficial effects of TMB4 and of PAM at four times the TMB4 dosage. The only apparent differences were that TMB4 gave more cholinesterase reactivation with diazinon and possibly less reduction of symptom intensity with phenkaptone. TMB4 has the advantage of greater water solubility than PAM, but is more toxic. The absence of any whole brain cholinesterase reactivation with either oxime is consistent with previous results[1,9,15,16,19].

It is clear from the present study and other work that the oximes present a potentially valuable addition to the use of atropine in the treatment of poisoning by organophosphorus insecticides. It is equally clear that their therapeutic value is likely to vary considerably according to differences in the chemical structure and consequent enzyme-phosphorylating properties of organophosphates. Although with some compounds the therapeutic value of oximes may be nil, and deliberate prophylactic injection to animals may even cause adverse effects with some compounds, there appears to be no contraindication to the therapeutic use of PAM, for example, in emergency treatment of any case of human poisoning by organophosphates. There is, of course, no way of prophesying whether the results obtained in the rat are likely to apply to man, but it is known that very satisfactory results follow the use of PAM in human poisoning by parathion and parathion-methyl (Sumitomo, personal communication).

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THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART II. FACTORS AFFECTING THE ACTION OF MERCURIC CHLORIDE

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Increase in the peptone concentration of the medium causes an increase in the amount of mercuric chloride necessary for bacteriostasis whilst an increase in the sodium chloride content decreases it. The age of the organisms is a factor likely to produce variability in the results and the use of a stored suspension of organisms is suitable for reducing this. Suspensions prepared from plate cultures are less satisfactory than those from slope cultures. The number of viable cells in inocula affects the bacteriostatic concentration of mercuric chloride, an increased concentration of bacteriostat being required with an increased number of organisms. The addition of heat-killed cells appears to make no appreciable difference to the bacteriostatic activity of mercuric chloride.

IN Part I[1] the antibacterial activity of mercuric chloride against *E. coli* I was investigated. The relations between mercuric chloride, the medium and the inoculum have now been investigated.

EXPERIMENTAL

Peptone Concentration in the Medium

A series of peptone waters were prepared containing 1·0 per cent of sodium chloride and 4, 3, 2, 1·5, 1 or 0·5 per cent of peptone. A single batch of Oxoid peptone was used and media were prepared and sterilised under the same conditions. The bacteriostatic value of mercuric chloride against *E coli* I was determined by the liquid dilution method[2] with each of the six samples of peptone water simultaneously and with replication.

Plots relating the concentration of mercuric chloride necessary for bacteriostasis with the peptone concentration of the medium were linear, increased peptone concentration being accompanied by an increase in the amount of mercuric chloride required. The line did not pass through the origin but, at a point corresonding to a peptone concentration of nil, cut the mercuric chloride concentration axis at a value of about 5 μM.

Sodium Chloride Concentration in the Medium

The bacteriostatic value of mercuric chloride against the test organism was determined similarly using a series of peptone waters containing 2·0

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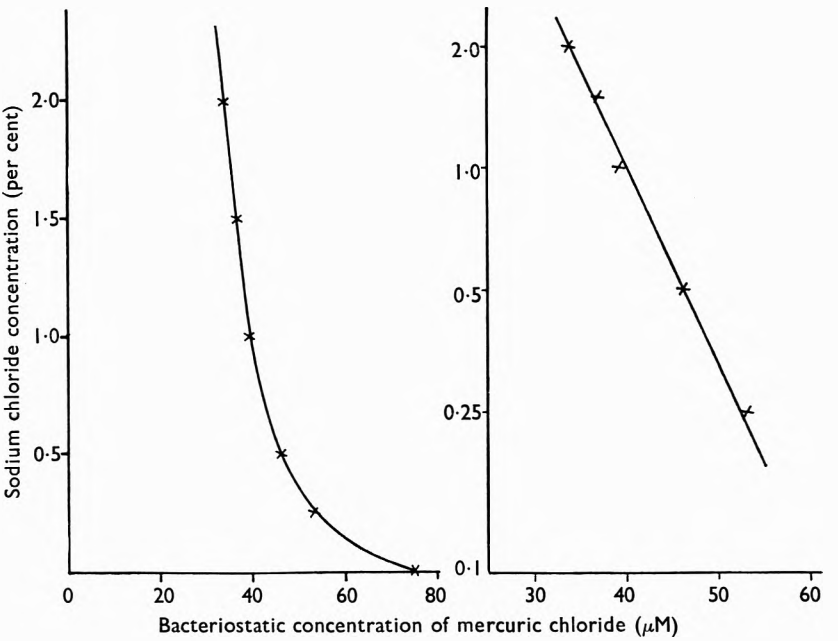
per cent of peptone and 4, 3, 2, 1, 0·5 or 0 per cent of sodium chloride. Figures 1 and 2 show typical results.



FIGS. 1 and 2. Effect of sodium chloride concentration on the bacteriostatic value of mercuric chloride toward *E. coli* I.

Age of the Organisms in the Inoculum

The bacteriostatic value of mercuric chloride was determined by the liquid dilution method using, as inocula, unwashed suspensions prepared from slope cultures of *E. coli* I incubated for varying periods and a stored suspension[3] prepared from 24-hour slope cultures. The number of viable organisms in each inoculum whilst not identical was approximately constant. The results are shown in Table I from which it can be seen

TABLE I

INFLUENCE OF THE AGE OF THE CULTURE OF *E. coli* I UPON THE BACTERIOSTATIC ACTIVITY OF MERCURIC CHLORIDE

Inoculum age (hours)	Bacteriostatic concentration of $HgCl_2$ μM	Inoculum age (hours)	Bacteriostatic concentration of $HgCl_2$ μM	Storage (days)	Bacteriostatic concentration of $HgCl_2$ μM
19	57	24	58	0	60
20·5	57	"	60	1	60
23·5	60	"	55	2	60
24·5	60	"	57	3	60
25	57	"	65	4	60
28	83	"	55	5 (Stored suspension)	60

that with the exception of the inoculum prepared from a 28-hour culture, the bacteriostatic values all fall within the range of 55 to 65 μM.

Further experiments showed that stored suspensions of *E. coli* I maintained their resistance to mercuric chloride for at least one week and such suspensions were used for the remainder of the work recorded in this series of papers, except where otherwise stated. Fresh suspensions were prepared at the beginning of each week and were stored at 4° when not in use.

It was noted that suspensions prepared from plate cultures[1] did not maintain their resistance as did those prepared from slope cultures, and typical results for the bacteriostatic value of mercuric chloride against a suspension of *E. coli* I prepared from a plate culture are shown in Table II.

TABLE II

BACTERIOSTATIC VALUE OF MERCURIC CHLORIDE AGAINST A STORED SUSPENSION OF *E. coli* I PREPARED FROM A PLATE CULTURE

Storage (days)		0		2	3	4	6
Bacterostatic concentration of $HgCl_2$ as μM solution	Liquid method	75	85	90	95	100	100
	Solid method	150	175	150	175	200	200

Effect of Inoculum Size

Preliminary experiments indicated that there was some relation between the number of organisms in the inoculum and the concentration of mercuric chloride necessary for bacteriostasis, an increased concentration of bacteriostat being required with increased number of cells in the inoculum. Table III shows typical results.

TABLE III

EFFECT OF NUMBER OF CELLS IN THE INOCULUM ON THE BACTERIOSTATIC CONCENTRATION OF MERCURIC CHLORIDE

Expt.	No. of viable cells in inoculum	Bacteriostatic concn. of $HgCl_2$ μM
A	2×10^7	50
	2×10^6	50
	2×10^5	45
	2×10^4	40
B	4×10^7	65
	4×10^6	60
	4×10^5	57
	4×10^4	47
	4×10^3	43

Effect of the Addition of Dead Cells

From the above results it is apparent that the presence of a small number of dead cells in the inoculum would make little or no difference to the concentration of mercuric chloride necessary for bacteriostasis. It was decided therefore to investigate the possible effects of large numbers of dead cells.

A suspension of *E. coli* I was prepared and divided into four portions, one of which was used as the living cell suspension whilst the other three

portions were heat-treated, in different ways, to kill the cells. The heat-treatments used and the results of microscopical examination of the killed cells are recorded in Table IV. The heated suspensions were tested for sterility and no growth occurred in any test.

TABLE IV

METHODS OF KILLING SUSPENSIONS OF *E. coli* I, AND MICROSCOPICAL EXAMINATION OF THE KILLED CELLS

Method of killing	Microscopical examination
Heating at 60° for 1 hour	Rods, with no signs of disruption and very little clumping
Heating at 98 to 100° for 20 minutes	Many of the cells appeared normal but some clumps were present and some cells showed signs of disruption
Heating in an autoclave at 115 to 116° for 10 minutes	Few intact cells present, some cells were visibly swollen and many aggregates of disrupted cells were present

The bacteriostatic value of mercuric chloride against *E. coli* I was determined by the liquid dilution method in two sets of experiments: With inocula containing a constant total number of cells, but varying in the ratio of living and dead cells, and with inocula containing a constant number of living cells and varying numbers of dead cells in the system. Control determinations without added dead cells were performed simultaneously. The suspensions used as inocula were prepared by serial dilution of the living cell suspension with killed suspension or, for the control determinations, with sterile water.

TABLE V

EFFECT OF THE PRESENCE OF HEAT-KILLED CELLS (60°/1 HOUR) ON THE BACTERIOSTATIC ACTIVITY OF MERCURIC CHLORIDE AGAINST *E. coli* I

Number of living cells in inoculum	Percentage of killed cells in inoculum	Bacteriostatic concentration of $HgCl_2$ (as micromolar solution) in		Ratio of B'static concentrations PRESENCE : ABSENCE of killed cells
		ABSENCE of killed cells	PRESENCE of killed cells	
$3{\cdot}5 \times 10^7$	0	78	—	—
" 10^6	90	72	68	0·94
" 10^5	99	60	56	0·93
" 10^4	99·9	46	46	1
" 10^3	99·99	40	40	1
" 10^2	99·999	30	34	1·13
$3{\cdot}6 \times 10^7$	0	80	—	—
" 10^6	90	70	70	1
" 10^5	99	67	67	1
" 10^4	99·9	50	53	1·06
" 10^3	99·99	43	53	1·23
$4{\cdot}1 \times 10^7$	0	83	—	—
" 10^4	99·9	52	47	0·90
" 10^3	99·99	38	43	1·13
" 10^2	99·999	31	32	1·03
$4{\cdot}16 \times 10^7$	0	83	—	—
" 10^4	99·9	48	47	0·98
" 10^3	99·99	36	35	0·97
" 10^2	99·999	30	30	1

Typical results for these two series of experiments are recorded in Tables V and VI respectively. It should be noted that in both Tables the "number" of killed cells is a nominal value based on the count before

heat treatment. The actual number of intact killed cells will be much lower, especially in the autoclaved suspension where microscopical examination showed only a few intact cells (Table IV).

TABLE VI

EFFECT OF KILLED CELLS IN THE SYSTEM ON THE BACTERIOSTATIC CONCENTRATION OF MERCURIC CHLORIDE AGAINST *E. coli* I

Number of live cells in inoculum	"Number" of killed cells in the system					Temperature at which cells killed
	0	$9{\cdot}3\times10^{7}$	$1{\cdot}86\times10^{8}$	$4{\cdot}65\times10^{8}$	$9{\cdot}3\times10^{8}$	
$9{\cdot}3\times10^{6}$	72	78 (1·08)	76 (1·06)	78 (1·08)	71 (0·99)	60°
,,	72	72 (1)	76 (1·06)	78 (1·08)	78 (1·08)	98–100°
,,	72	73 (1·01)	80 (1·11)	79 (1·10)	79 (1·10)	115–116°
$1{\cdot}1\times10^{7}$	79	86 (1·09)	80 (1·01)	74 (0·94)	76 (0·96)	60°
,,	79	75 (0·95)	75 (0·95)	78 (0·99)	82 (1·04)	98–100°
,,	79	72 (0·91)	73 (0·92)	71 (0·90)	79 (1)	115–116°

Bacteriostatic concentration expressed as μM mercuric chloride solution.

Figures in parentheses = $\frac{\text{B'static concentration in presence of dead cells}}{\text{,, ,, ,, absence ,, ,, ,,}}$

DISCUSSION

That the amount of nitrogenous material present in a medium affects the apparent efficiency of mercuric chloride as an antibacterial agent was shown by Baumgartner and Wallace[5]. In bactericidal tests they demonstrated that an increase of the nitrogenous material above that in ordinary broth increased the death time, whilst a decrease in content decreased it; that is, with more peptone in the recovery medium, either more mercuric chloride or a longer exposure time were necessary to produce the same effect.

Under the experimental conditions used, a linear relation existed between the concentration of peptone in the medium and the amount of mercuric chloride necessary for bacteriostasis. Two explanations are advanced to account for these results. Firstly, the increased peptone content may merely serve as a physical protectant for the organisms or, secondly, combination of mercuric chloride with the peptone would be more marked with higher peptone concentrations and this would reduce the antibacterial efficiency of the mercuric salt. Comparison of the behaviour of mercuric chloride with that of other mercury compounds (e.g. an organomercurial salt) with an extended range of peptone concentrations might shed some light on the nature and mechanism of this phenomenon.

It is not known whether the linear relation would hold over a wider range of peptone concentrations or whether it would prove to be exponential. Much smaller concentrations of peptone could be used as

it has been reported that 0·0125 per cent will support the growth of *E. coli*[5].

Paul and Prall[6] were among the first to report the diminution in activity of mercuric chloride in the presence of sodium chloride, this being attributed to decreased dissociation of the mercuric salt. Krahé[7] considered a complex was formed which decreased the concentration of mercuric ions and also decreased the solubility of mercuric chloride in lipids. The formation of complex anions by mercuric chloride is well known, but Gay and others[8] regarded the complex formed with sodium chloride as undissociated. Some workers[9,10] however considered the addition of sodium chloride increased the antibacterial activity of mercuric chloride. Italian workers[10] assumed the complex formed was more active than mercuric chloride alone.

The shape of the graph (Fig. 1) resembles half of a parabola having its vertex at a point corresponding to a mercuric chloride concentration of 75 μM and a sodium chloride content of zero. If however it was half of a true parabola the curve would extend to infinity. It is reasonable to assume that the sodium chloride itself would be inibitory at some concentration and hence the bacteriostatic activity of mercuric chloride under these conditions would be zero and the curve could not extend to infinity. Plotted on a logarithmic axis, the relation is linear (Fig. 2). Extrapolation of this line back to the abscissa gives a value for the sodium chloride content of about 55 to 60 per cent. As the solubility of sodium chloride in water is only about 1 in 3, it is not possible to prepare media having such high sodium chloride contents. If the experiments were repeated with a range of concentrations from 0 to 30 per cent it is believed that the true shape of Figure 1 would be a sigmoid.

From the results however it appears that an increase in the sodium chloride content of the medium increases the bacteriostatic activity of mercuric chloride against *E. coli* I. The presence of 0·25 per cent of sodium chloride greatly enhances the bacteriostatic activity when compared with that in its absence, but the effect of further increasing the sodium chloride concentration up to 2 per cent causes a less marked increase in the bacteriostatic activity of the mercuric salt.

The following explanations are advanced to account for these observations. The increased bacteriostatic efficiency of mercuric chloride in the presence of sodium chloride may be due to the formation of complex anions (e.g. $HgCl_4^{--}$) as mercuric compounds form such complex anions with chlorides of the alkali metals[11]. It is well known that when solutions of two electrolytes having a common ion are mixed, the dissociation of each is diminished. As the bacteriostatic activity of mercuric chloride is increased in the presence of sodium chloride, the theory that the activity of mercuric salts is dependent upon the free mercuric ions in solution[12] appears to be contra-indicated. The apparent increased efficiency of mercuric chloride in the presence of sodium chloride may not be connected with the mercurial compound *per se* but may be due to osmotic effects on the organism, caused by the sodium chloride and rendering the cells more susceptible to the action of mercuric chloride. It is unlikely however

that the relatively low sodium chloride concentrations used in these experiments would exert much influence on the cells by way of simple osmotic pressure effects. A possibly more valid explanation lies in the fact that the cell surfaces of bacteria possess a net negative charge at physiological pH[13]. As the concentration of monovalent cations increases this potential is decreased, although neither monovalent nor divalent cations of the alkaline earth metals can reverse the charge on the bacterial surface[14]. It is believed that with a decreased surface potential the bacteria would be more sensitive to mercuric chloride, and thus the surface potential effect of sodium chloride might potentiate the antibacterial action of mercuric chloride.

Hotchkiss[15] observed that *E. coli* grew in peptone water containing 1·0 per cent of sodium chloride but was inhibited by 2·0 per cent. In our experiments, the organism grew equally well, as determined by turbidity and the time necessary to give a strong indole reaction, in all concentrations of sodium chloride tested, in its absence and in all peptone concentrations tested. Matsuyama[16] reported the growth of *E. coli* in 1 per cent peptone solution was most favourable with sodium chloride concentrations between about 0·58 and 1·75 per cent; increasing inhibition was observed above 1·75 per cent with complete inhibition at about 17·5 per cent.

Table I shows the reduction in variation in bacteriostatic evaluations when a stored suspension from a slope culture is used as the inoculum. Such results support previous work[3].

From the results in Table II it is seen that the sensitivity of a suspension of *E. coli* I from a plate culture to mercuric chloride decreased on storage. A rapid decrease in the viable counts on such suspensions on storage has been shown to occur[17] and it is concluded that suspensions prepared from plate cultures are not as satisfactory as those derived from slope cultures.

A possible explanation of the phenomenon is that in plate cultures, the ratio of surface to depth of the medium is considerably increased over that in a slope culture. Brewer[18] noted an increased resistance to phenol was shown by *Staph. aureus* when the surface area of the medium, on which it was cultured, was increased.

At the outset of this investigation it was hoped to establish a relation between the number of organisms in the inoculum and the bacteriostatic concentration of mercuric chloride, from which conclusions might have been reached about the mechanism of the antibacterial action of mercuric chloride. This correlation was not found although there was a trend for an increase in the number of organisms to be paralleled by an increase in the bacteriostatic concentration of mercuric chloride (Table III). Plots relating the number of organisms in the inoculum with the bacteriostatic concentration of mercuric chloride showed a pronounced scatter. The poor reproducibility of replicate results obscures any relationship, but in a few experiments an approximately linear relation was found between the logarithm of the number of cells in the inoculum and the bacteriostatic concentration of mercuric chloride. During their studies of *Aerobacter aerogenes*, Poole and Hinshelwood[19] demonstrated that for a

given inoculum size there was a critical concentration of mercuric chloride above which no growth occurred, and, conversely, for a given concentration of mercuric chloride there was a critical inoculum size below which no growth occurred.

It was believed that any effect of adding killed cells to a mercuric chloride-bacteria reaction mixture would be one towards increasing the concentration of bacteriostat required, for the following reasons. The dead cells would offer a large competitive surface area for adsorption of the bacteriostat; a similar adsorptive capacity between heat-killed and live cells of *E. coli* has been demonstrated[20]. The dead cells might contribute cell constituents and essential nutrients to the system which could afford protection to the living cells either by adsorption onto the cell surface or by promoting subsequent recovery and growth of cells damaged by the bacteriostat. The dead cells would contribute a large number of sulphydryl groups to the system, if these groups were unaffected by the conditions necessary to kill the cells.

From the results in Table V it is seen that the presence of dead cells in the inoculum makes little difference to the concentration of mercuric chloride necessary to produce bacteriostasis of the living cells. The high value of 1·23, for the ratio of the bacteriostatic concentrations in the presence and absence of dead cells, occurring in the 11th line of the Table appears suspiciously large but, statistically, the means from which this ratio was calculated were not significantly different ($P = 0{\cdot}95$).

From an analysis of variance of the results recorded in Table VI there is no evidence to suggest that either the different heat treatments used in killing the cells or the different concentrations of dead cells in the systems made any significant difference to the amount of mercuric chloride required to produce bacteriostasis of the living cells of *E. coli* I.

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COMPARATIVE TOXICITY STUDIES ON TEN ANTIBIOTICS IN CURRENT USE

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The acute toxicities of ten currently used antibiotics have been compared on two strains of mouse by four different routes.

On several occasions we have needed to know the toxicity of one or other of the antibiotics currently in clinical use. Information on the subject is widely scattered and therefore hard to collect, though this difficulty was largely met by Spector's publication[1], which deals with some 340 antibiotics, including all of those in which we were interested.

Study of its contents revealed clearly what we had already good grounds for suspecting, namely, that exactly to compare the toxicities of different antibiotics, and especially of the dozen or so commercially available, was not possible on the basis of available information. Often insufficient details were given in the original papers of the type (and strain) of animal used, and seldom, if ever, were simultaneous comparisons, by identical routes, made on more than two substances. We therefore decided to conduct a more strictly controlled study of antibiotic toxicity than we believe previously to have been made or at any rate to have been recorded in the literature. We present here the results of this study, which began in February 1956 and was concluded in September of that year, except for a small supplementary test in August 1958.

We are fully aware that relative toxicities by the same route and on the same strain of the same species of laboratory animal do not necessarily indicate the relative toxicities to any other species of animal, including man. Nevertheless, there is a wealth of experience to confirm the view that such studies, which can at worst supply negative evidence, may provide positive leads to therapeutic practice.

Materials and Methods

Antibiotics

Ten antibiotics were studied. Full details of them, with batch or code number when available, are given in Appendix I.

Animals

In our experiments we used two different strains of highly inbred mice, both males and females. The original experiments involved the use of male albino mice (A26 strain)* and female fawn mice (GFF strain)*.

* Some details of these strains are to be found in the *Catalogue of Uniform Strains of Laboratory Animals maintained in Great Britain* (Laboratory Animals Bureau, 1959).

When marked differences in toxicity appeared between the two strains, further experiments were made on male mice of the fawn strain and female mice of the albino strain. All the results are included in Table I, which gives the median lethal doses for the ten antibiotics. All mice weighed between 14 and 22 g. at the beginning of the experiment. The LD50 values were calculated by the method of de Beer[2] from the number of deaths occurring within seven days of treatment.

Methods of Administration

The different antibiotics were administered in graded doses to groups of ten mice by one of four different routes—intravenous, intraperitoneal, subcutaneous and oral. The intravenous injections were made at the rate of approximately 1 ml. per 30 seconds into the tail veins. All antibiotics were administered as aqueous solutions or suspensions, but in some instances, as indicated in Table I, a small amount of gum tragacanth was added to aid suspension. All solutions or suspensions were prepared immediately before use.

The range of doses was chosen so as to lie on each side of the expected LD50 when an approximate value for this was available: otherwise a probe test was undertaken to supply such a value.

Results

Penicillin remains the least toxic of all currently used antibiotics, by either intraperitoneal or intravenous route. Streptomycin, neomycin and penicillin have one-eighth to one-quarter the oral toxicity of the other seven substances tested. Polymyxin appeared to be the most toxic by any route.

Generally the differences in toxicity to the two strains of mice were small; in the few instances when marked differences occurred, it was between strains rather than between sexes.

For the most part it seems to us that the figures speak for themselves. It may, however, be pointed out that the subcutaneous toxicity of tetracycline hydrochloride is greatly decreased when it is administered as a suspension neutralised with sodium hydroxide. At pH 1·8 tetracycline hydrochloride produces severe necrosis at the site of injection, but at neutral pH it produces none; the necrosis is almost certainly due to the relatively high acidity of the unneutralised solution.

Discussion

Certain other differences seem to us to call for comment and in some instances to raise questions of interest, to most of which, however, we do not know the answers. For example, toxicity by the intraperitoneal route is, as might have been expected, shown to have been almost always less than by the intravenous route, but the difference seems doubtfully significant for chlortetracycline and, curiously, is reversed for bacitracin. On taking the means for the two strain-sex groups of mice, it is seen that intravenous injections of chloramphenicol, neomycin and streptomycin were 6 or more times as toxic, at the LD50 level, as intraperitoneal

TABLE I

ACUTE SYSTEMIC TOXICITIES OF TEN ANTIBIOTICS IN CURRENT USE

(Mouse LD50 values in mg./kg. per body weight)

Antibiotic	Intravenous		Intraperitoneal		Subcutaneous		Oral			
	A2G(M)	GFF(F)	A2G(M)	GFF(F)	A2G(M)	GFF(F)	A2G(M)	A2G(F)	GFF(M)	GFF(F)
Bacitracin	356 (2·5 per cent solution)	313	219 (2·5 per cent solution)	300	538 (5 per cent solution)	660	3375 (15 per cent solution)	—	—	3375
Chloramphenicol	180 (2 per cent suspension[T])	210	1225 (10 per cent suspension[T])	1300	2300 (10 per cent suspension[T])	2585	3150	2600	5500	5300 (20 per cent suspension[T])
Chlortetracycline hydrochloride	102 (0·75 per cent solution)	108	128 (0·75 per cent solution)	168	5500 (25 per cent suspension)	8250	3350 (20 per cent suspension)	—	—	4200
Erythromycin	120 (0·5 per cent suspension)	138	655 (2·5 per cent suspension)	720	4700 (20 per cent suspension)	6800	4050	3500	5800	7100 (20 per cent suspension[T])
Neomycin sulphate	32 (0·2 per cent solution)	33	213 (per cent solution)	180	240 (1 per cent solution)	310	14,500 (50 per cent solution)	—	—	14,000
Oxytetracycline hydrochloride	154 (0·75 per cent solution)	189	285 (1·5 per cent solution)	420	243 (1·5 per cent solution)	330	3600 (20 per cent solution)	—	—	4400
Sodium benzylpenicillin	1800 (15 per cent solution)	1913	3880 (20 per cent solution)	4120	6000 (50 per cent solution)	8000	12,750	13,000	15,750	16,500 (50 per cent solution)
Polymyxin B sulphate	5 (0·03 per cent solution)	5	24 (0·1 per cent solution)	24	84 (0·5 per cent solution)	103	713 (5 per cent solution)	—	—	1050
Streptomycin sulphate	85 (0·75 per cent solution)	111	610 (5 per cent solution)	575	500 (5 per cent solution)	550	15,550	16,125	30,000	30,000 (75 per cent solution)
Tetracycline hydrochloride	145 (1 per cent solution pH 2·6)	170	390 (1·5 per cent solution pH 2·6)	495	1125 (15 per cent partial suspension pH 1·8)	1950	3375 (15 per cent partial suspension pH 1·8)	—	—	3550
	80 (0·5 per cent partial suspension[N])	75	230 (2 per cent partial suspension[N])	440	8640 (24 per cent partial suspension[NT])	8880	Not tested			

A2G(M)—Male mice of A2G strain GFF(M)—Male mice of GFF strain
A2G(F) —Female mice of A2G strain GFF(F) —Female mice of GFF strain
T —suspension aided by trace of gum tragacanth
N—neutralised with NAOH

injections, the corresponding ratios falling for erythromycin, polymyxin, tetracycline, penicillin, oxytetracycline and chlortetracycline, in that order, to the reversed ratio for bacitracin.

Strain differences unconfounded with sex differences can only be established from our results for four of the antibiotics tested by the oral route. The GFF mice were markedly less susceptible than the A2G mice to the oral toxic effects of chloramphenicol, erythromycin, benzylpenicillin, and streptomycin. The confounded strain-sex differences by the oral route showed the GFF females to be somewhat less susceptible than the A2G males to chlortetracycline, oxytetracycline and polymyxin, but about equally susceptible to bacitracin, neomycin, and tetracycline.

The confounded strain-sex differences by any of the parenteral routes were never marked; they tended to be greatest by the subcutaneous and least by the intravenous route. Almost always the GFF females were less sensitive than the A2G males when there was any apparently significant difference at all. Intraperitoneally the females of strain GFF seemed more sensitive than the males of strain A2G only to neomycin and streptomycin, the difference being so small, however, as possibly to have been fortuitous. This is also true of bacitracin only for the intravenous route, all the eight other substances being either equally lethal, or non-lethal, to both groups of mice (chlortetracycline, neomycin, polymyxin) or slightly less lethal to the GFF females (chloramphenicol, erythromycin, oxytetracycline, benzylpenicillin, streptomycin, tetracycline) after intravenous injection. By the subcutaneous route toxicities were either the same for both strain-sex groups or slightly to distinctly greater for the A2G males. The evidence of the oral tests, the only ones permitting a distinction between strain and sex effects, shows the difference between strains to have been much greater than the difference between sexes for four of the antibiotics, namely, chloramphenicol, erythromycin, benzylpenicillin and streptomycin, though for benzylpenicillin the difference was small, giving ratios of 1·24 and 1·27 for males and females respectively.

There is increasing evidence in the literature of interstrain differences between the responses of animals of the same species to the same stimulus or stress. Examples of this are differences between the two strains used by us in susceptibility to infection by *Bordetella pertussis* and *Mycobacterium tuberculosis* seen by our colleague, Dr. Ungar. At a recent symposium it was reported that mice of different strains showed an enormous difference in susceptibility to the toxic action of histamine hydrochloride[3]. These are only three of many examples that might be cited. The same phenomenon, though less marked, has appeared again in the course of the work reported here. Nor is its occurrence an occasion for surprise. "Pure lines" of mice were originally produced for the use of cancer investigators because of the wish to take advantage of differences in susceptibility to spontaneous or implanted tumours. It is hardly surprising that these differences, largely or entirely genetic, manifest themselves also in other types of investigation. The existence of these intraspecific differences between animals of nominally the same species makes it all the more desirable that investigators should invariably give the fullest possible

details, by mention or by reference, not only of the species, but also of the strain, of animal used in any work they report.

Acknowledgements

We are most grateful to the pharmaceutical manufacturers who made us generous gifts of their antibiotics and supplied relevant information about batch numbers or other indications of identity.

Appendix I

The preparations used.

1. Bacitracin (production batch BAC–0001, 76 U/mg., Glaxo Laboratories).
2. Chloramphenicol (Chloromycetin, batches LT 831A, LS 366M and LU 181A, Parke Davis and Co.).
3. Chlortetracycline hydrochloride (Aureomycin, batch 7–8281, Lederle Laboratories).
4. Erythromycin (Erythrocin, batches 21057, 900 μg./g. and 24170, Abbot Laboratories).
5. Neomycin sulphate (production batch 3PB/2/47, 600 U/mg., Glaxo Laboratories).
6. Oxytetracycline hydrochloride (Terramycin, Pfizer).
7. Sodium benzylpenicillin (production material, 1680 U/mg. and 1670 U/mg.. Glaxo Laboratories).
8. Polymyxin B sulphate (Aerosporin, batch AN 49960, 4949 U/mg., Burroughs Wellcome).
9. Streptomycin sulphate (production batches B-1333 and SSU 2353, 740 U/mg.. Glaxo Laboratories).
10. Tetracycline hydrochloride (Tetracyn, 967 U/mg., 968 U/mg. and 965 U/mg.. Pfizer.

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DELAYED VOMITING INDUCED IN DOGS BY INTRAMUSCULAR DIGOXIN

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Conditions necessary to the use of intramuscular digoxin as a challenge of delayed digitalis vomiting were studied in dogs. The ED50 ± SE was 0·0566 ± 0·00405 and the ED84 ± SE was 0·070 ± 0·004 mg./kg. Intramuscular injection of the ED84 was followed by an emetic syndrome which began in 2 to 3 hours, consisted of 5 to 10 vomiting spells and 50 to 100 retching spells, and stopped at 8 to 10 hours after injection. The ED84 produced some bradycardia, anorexia and diarrhoea. Repetition of the ED84 at intervals less, but not greater, than 4 days produced a more marked vomiting syndrome. The emetic syndrome was the same in male as in female dogs, the same during the night as during the day, and the same at all seasons of the year. The onset of vomiting was delayed and the number of vomiting and retching spells reduced when food was given at any time from 12 to 16 hours before, to 1 to 2 hours after, injection of the ED84. Excessive (72-hour) starvation lessened the vomiting syndrome, probably due to excitement. The vomiting syndrome was less marked when recorded by observers who were particularly friendly with the dogs, outside the period of recording, apparently due simply to the nearness of such persons.

The pharmacological testing of new agents for anti-emetic activity requires screening in animals, usually dogs, against a series of emetic challenges[1]. The list of emetics includes[2] apomorphine hydrochloride, morphine sulphate, hydergine, copper sulphate, lanatoside C, and sometimes vertical swing[3]. The lanatoside C is usually given intravenously to produce vomiting in about 10 minutes[2] or intraperitoneally to produce vomiting in about 50 minutes.

Gold and others[4] have reported that when lanatoside C is given orally to cats, vomiting begins at intervals of from 4 to 8 hours (range 19 to 695 minutes). Borison[5] found that ablation of the emetic chemoreceptor trigger zone prevented the early type of vomiting from intravenous cardiac glycosides in over 90 per cent of cats and the late or delayed type of vomiting from parenteral or oral glycosides in less than 50 per cent of cats. The difference may be calculated to be significant at $P<0{\cdot}001$. Borison and Wang[6] conclude that cardiac glycosides may produce vomiting, "by acting at more than one receptor site".

Since the purpose of an anti-emetic screening programme is to challenge suitable animals against as many as possible of the known types of vomiting, it seemed desirable to include delayed vomiting induced by cardiac glycosides in such a programme. Digoxin was selected in our laboratory as the challenging agent for delayed digitalis vomiting. It was found in preliminary trials that a suitable dose of digoxin given

intramuscularly would produce in dogs a vomiting syndrome beginning in 2 to 3 hours and ending in 9 to 10 hours after injection. A systematic study was then made of factors which might affect this vomiting syndrome. This work is reported below.

METHOD

The experiments were performed upon mongrel dogs of both sexes and 6 to 12 kg. body weight. The animals were housed in Wahmann LC93 dog cages, one dog per cage. They were fed Purina fox chow checkers, meat, bread, milk and water *ad libitum* and given supplementary Decavitamin Capsules, U.S.P. XV. They were observed for a period of at least one month to assure good health before being challenged with digoxin. Some dogs have a more pronounced vomiting syndrome than others to emetic challenges. In arranging a group for any study, an attempt was made to obtained a cross section or an "average" group in so far as susceptibility to vomiting was concerned.

In each dog there were recorded time in minutes to onset of vomiting, the number of vomiting and the number of retching spells, the duration of the vomiting syndrome, and the general clinical signs. The technique was that of Boyd and Boyd[7].

Digoxin was used in the form of Digoxin Injection, B.P. 1958, and was obtained from Burroughs Wellcome and Company (Canada) Limited of Montreal.

RESULTS

FOURTEEN dogs were given at weekly intervals increasing doses of digoxin intramuscularly. They were arbitrarily given no food from 16 hours before injection to 8 hours after injection. Pertinent data are summarised in Table I. The median emetic dose $\pm$ standard error (ED50 $\pm$ SE) was 0·0566 $\pm$ 0·00405 and the ED84 $\pm$ SE was 0·070 $\pm$ 0·004 mg./kg. From this experiment a dose of 0·07 mg./kg. intramuscularly was selected as the challenging emetic dose of digoxin. This dose is approximately one-tenth the intramuscular median lethal dose in the guinea pig per kg. weight[8].

TABLE I

DOSE-RESPONSE DATA UPON 14 DOGS

Dose mg./kg. i.m.	Incidence of vomiting per cent	Interval to onset minutes mean $\pm$ SE	Number of vomiting spells mean $\pm$ SE	Number of retching spells mean $\pm$ SE
0·03	0	—	0 $\pm$ 0	0 $\pm$ 0
0·04	0	—	0 $\pm$ 0	0 $\pm$ 0
0·05	43	193 $\pm$ 8·3	0·93 $\pm$ 0·32	7·0 $\pm$ 3·1
0·06	71	197 $\pm$ 16·7	2·64 $\pm$ 0·59	28·7 $\pm$ 7·1
0·07	86	158 $\pm$ 13·4	4·85 $\pm$ 0·86	43·4 $\pm$ 12·4
0·08	93	156 $\pm$ 14·7	5·29 $\pm$ 0·99	57·1 $\pm$ 13·1

The challenging dose of 0·07 mg./kg. produced a bradycardia which reached a maximal average of 22 per cent fewer beats per minute at 2 hours after injection. Cardiac rate returned to normal at 24 hours (Table II). During the first day the dogs ate less than usual, in spite

of the long fast, and lost some weight (Table II). A diarrhoea appeared during the second day. From the 3rd day on, the animals appeared clinically normal.

TABLE II

THE EFFECT OF THE CHALLENGING DOSE OF DIGOXIN ON HEART RATE, BODY WEIGHT, AND FOOD INTAKE

Interval days	Heart rate per minute mean ± SE	Weight kg. mean ± SE	Food intake g. chow/kg./24 hr. mean ± SE
0 (before)	87·4 ± 6·8	9·13 ± 0·56	26·7 ± 2·6
1 (after)	89·5 ± 7·2	8·99 ± 0·55	12·6 ± 2·2
3 (after)	81·5 ± 5·8	9·31 ± 0·62	29·2 ± 2·3
5 (after)	85·3 ± 6·6	9·16 ± 0·60	22·8 ± 3·4
7 (after)	88·3 ± 6·1	9·22 ± 0·56	22·1 ± 2·8

The minimal necessary interval of rest between injections of challenging doses was determined in 12 dogs. They were given no food for 16 hours and injected intramuscularly with digoxin in a dose of 0·07 mg./kg. at intervals of 6, 4 and 2 days on days 0, 6, 10 and 12 respectively. The vomiting syndromes on days 0, 6 and 10 were insignificantly different from each other. The vomiting syndrome on the 12th day had a significantly ($P<0·001$) greater number of vomiting and retching spells. Presumably enough of the challenging emetic dose of digoxin was present at 2 days to augment the effect of a further challenging dose given at that time. The results indicated that an interval of 4 to 7 days should be allowed between emetic challenges.

Influence of sex of the animal was determined upon 14 male and 18 female dogs. The syndrome produced by the challenging emetic was the same in male as in female dogs.

Influence of the time of day that digoxin was injected was determined upon 16 dogs. They were given digoxin at 8·00 a.m. and the syndrome measured during the hours of daylight on one occasion. On the second trial, they were given digoxin at 4.00 p.m. and the syndrome followed until past midnight. The vomiting syndrome in one group did not differ significantly from that in the second group.

Influence of season was measured in 48 dogs, given 0·07 mg./kg. of digoxin intramuscularly in October, 1957, and in January, May and July, 1958. The vomiting syndrome was the same on all four occasions.

What might be called the personality of the observer was found to be a factor influencing the intensity of the vomiting syndrome in the dogs. Four different observers recorded the vomiting syndrome on repeated occasions in 14 dogs given a challenging dose of digoxin. Each observer was well known to the dogs from frequent previous recordings. The observation itself was carried out in the same manner by each observer who sat at some distance from the cages and did not speak to or in any way handle the animals after injecting digoxin. The vomiting syndrome was ($P<0·001$) less as observed by two persons than by the two other persons. The only apparent difference was that the two former observers were what might be called friendly with dogs while the two latter observers paid little attention to the dogs apart from the actual

experiment. Because of this, each factor was worked out entirely by at least one observer and results of one observer were not otherwise compared with results of a second.

The time of feeding influenced the severity of the vomiting syndrome. When this was discovered, it was systematically investigated by removing food from dogs at 48 hours before injection of a challenging dose of digoxin. This made the animals hungry and when offered a standard meal at the intervals noted in Table III, they ate it quickly. As shown by data summarised in Table III, the onset of vomiting was delayed and the number of vomiting and retching spells reduced when food was given any time from 12 to 16 hours before to 1 to 2 hours after injection of digoxin. These results suggest that some substance produced during digestion of food, possibly by the liver, decreases the sensitivity of the medullary areas concerned to the emetic action of digoxin.

TABLE III

THE VOMITING SYNDROME AS AFFECTED BY TIME OF FEEDING

Time of feeding-hr. after or before digoxin	Incidence of vomiting per cent	Minutes to onset mean ± SE	Number of vomiting spells mean ± SE	Number of retching spells mean ± SE
10 (after)	98	146 ± 6	7·6 ± 0·6	68 ± 8
2 (after)	92	175 ± 13*	7·3 ± 1·7	57 ± 16
1 (after)	50**	212 ± 15**	4·2 ± 0·5**	20 ± 9**
0	60**	216 ± 9**	3·3 ± 0·6**	13 ± 3**
1 (before)	67*	223 ± 8**	3·1 ± 0·9**	21 ± 8**
2 (before)	78	198 ± 18**	4·1 ± 1·6*	9 ± 4**
4 (before)	62**	212 ± 9**	2·6 ± 0·6**	15 ± 8**
6 (before)	56*	168 ± 20	1·0 ± 0·4**	8 ± 4**
8 (before)	80	201 ± 12**	2·4 ± 1·3**	13 ± 8**
12 (before)	86	190 ± 25	3·0 ± 0·9**	19 ± 10**
16 (before)	86	158 ± 13	4·9 ± 0·9*	43 ± 12
24 (before)	100	156 ± 23	7·3 ± 1·2	99 ± 18
48 (before)	100	150 ± 12	9·4 ± 1·1	75 ± 18
72 (before)	90	171 ± 5	2·6 ± 0·5**	30 ± 6**

* Probability (P) that mean difference from controls (10 after) equals zero ≤ 0.05.
** Probability (P) that mean difference from controls (10 after) equal zero ≤ 0.01.

The results indicated that a maximal emetic response to digoxin is obtained by giving the dogs no food between 16 to 48 hours before injection of the drug and the time that vomiting ceases. These conditions with a dose of 0·07 mg./kg. intramuscularly of digoxin caused vomiting to start during the 3rd hour, to reach and maintain a peak during the 3rd to 7th hours, and to gradually lessen to zero during the 8th to 10th hours.

When dogs were starved for 72 hours before giving digoxin, the vomiting syndrome was less marked than when starved for 48 hours (see Table III). After the injection of digoxin, the 72-hour-starved dogs, normally placid and readily handled, became excited, restless, irritable and occasionally vicious.

DISCUSSION

From the results described above, certain conditions have been established for the use of digoxin intramuscularly as a delayed digitalis emetic challenge in dogs. The dogs should be starved for 16 to 48 hours before injection. Food must be withheld further until at least 2 hours (and

is given most conveniently 10 hours) after injection of digoxin. The intramuscular ED84 of digoxin is 0·07 mg./kg. The vomiting syndrome begins during the 3rd hour and lasts to the 9th or 10th hour after injection. The syndrome consists of 5 to 10 vomiting spells and 50 to 100 retching spells. It is accompanied by bradycardia, some anorexia at the end of the syndrome, and some diarrhoea the next day. The dogs appear normal on the 3rd day but should not be used again for digoxin-challenge until at least the 4th day.

The sex of the dog, the season of the year, and the time of day digoxin is injected have no influence upon the vomiting syndrome. The vomiting syndrome is reduced if the attention of the dogs is diverted by the nearness of a recorder who is friendly with them. The vomiting syndrome is also reduced when the dogs become restless and irritable from prolonged (3 day) starvation.

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STUDIES ON A NEW CELLULASE PREPARATION FROM PENICILLIUM

I. Method of Determining Enzymatic Activity

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The viscosimetric method described is suitable for the determination of enzymatic activity when the substrate is a polyelectrolyte or a polymer to which Staudinger's law is not applicable. It has been tested experimentally for determining the activity of cellulase isolated from Penicillium, with carboxymethylcellulose as substrate.

Recording of the enzymatic activity of cellulase preparations has been based largely on two methods. The first method is determination of the amount of reducing sugar formed per unit of time, and the second is determination of the rate at which the specific viscosity of an appropriate substrate changes under the action of the enzyme. The former method, among others, has been employed by Levinson and Reese[1]; the viscosimetric method by Levinson and Reese[1], Tracey[2], and Thomas[3], all of whom used carboxymethylcellulose as substrate. In viscosimetric determinations of the activity of cellulolytic enzymes, other cellulose derivatives too have been used, such as ethylhydroxyethylcellulose by Sandegren and others[4], and methylcellulose by Menziani and others[5] and Desarmenien and others[6]. The viscosimetric method is extremely sensitive during the initial phase of enzymatic decomposition. It was therefore employed exclusively in the present investigation, since it was desirable to complete the determinations while the concentrations of both enzyme and substrate could be considered constant.

Theoretical

Hultin[7] introduced the term $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)_\tau$ as a criterion of the enzyme concentration c_A. In this expression $(\eta_{sp})\tau$ signifies the specific viscosity of the enzyme-substrate solution at the time τ. Under certain given conditions it was shown that

$$c_A = C_1 \cdot c_S^2 \cdot \frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right) \qquad (1)$$

where c_S equals the substrate concentration and C_1 a proportionality constant. In deriving the above equation the following assumptions were made. (i) All substrate molecules have the same initial molecular weight, which is assumed to be high compared with the average molecular weight, M, after enzymatic digestion. (ii) Staudinger's law is assumed to be valid; that is the intrinsic viscosity, $[\eta]$ of the substrate is proportional to

its average molecular weight, M. (iii) The reduced viscosity of the substrate solution is independent of the concentration (c_s),

$$\text{i.e., } \frac{\eta_{sp}}{c} = [\eta]$$

But, the above assumptions are true only of very special combinations of polymer (substrate) and solvent. Assumptions (ii) and (iii) are not valid for sodium carboxymethylcellulose the substrate usually employed in the determination of the activity of cellulase preparations. Thus, no linear curve emerges when $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)$ is plotted against the enzyme concentration (Fig. 1). Nor is $\left(\frac{1}{\eta_{sp}}\right)_\tau$ a linear function of the reaction time as it would be if Hultin's formula were valid (Fig. 2). It is accordingly essential, when using carboxymethylcellulose to replace the above assumptions by more realistic ones. 1. For the relevant range of molecular weights the relation of the intrinsic viscosity to the number average molecular weight can be expressed by the equation

$$[\eta] = C_m(M)^a \quad .. \quad .. \quad .. \quad (2)$$

This relation was first derived by Flory for a monodisperse polymer but was later found experimentally to be valid for a large number of true polydisperse polymers. 2. The reduced viscosity of the substrate solution is a linear function of the concentration, i.e. $\frac{\eta_{sp}}{c_s} = [\eta](1 + kc_s)$.

3. The quantity, $\frac{d}{d\tau}\left(\frac{c_s}{M}\right)$ which is proportional to the number of split bonds per unit time, is given by the equation

$$\frac{d}{d\tau}\left(\frac{c_s}{M}\right) = C_A c_A (c_s)^\beta \quad .. \quad .. \quad .. \quad (3)$$

where c_s and β are constants. The significance of the exponent, β, will be discussed later. From these three assumptions it is possible to deduce the following formula,

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \frac{C_A}{(C_m)^{1/a}} \frac{c_A}{(1 + kc_s)^{1/a} . c_s^{1/a+1-\beta}} \quad .. \quad .. \quad (4)$$

The value of the exponent, a, depends upon the degree of interaction between the polymer segments and the molecules of the solvent. According to Flory the following equation is valid for a freely rotating, linear polymer having no interaction with the solvent:

$$[\eta] = \Phi\left[\frac{R_0^2}{M}\right]^{3/2} M^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

where R_0 and M are, respectively, the hydrodynamic radius and the molecular weight of the polymer, and Φ is a universal constant. It can be shown that for a freely rotating polymer the relation $\frac{R_0^2}{M}$ is constant in a homologous series of polymers.

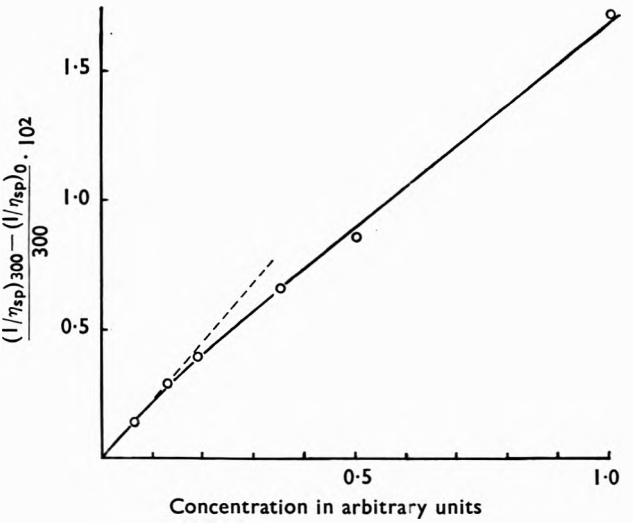

FIG. 1. The derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)$, as a function of the enzyme concentration expressed in arbitrary units. The derivative was determined as quotient $\frac{(1/\eta_{sp})_{300}-(1/\eta_{sp})_0}{300}\cdot 10^2$

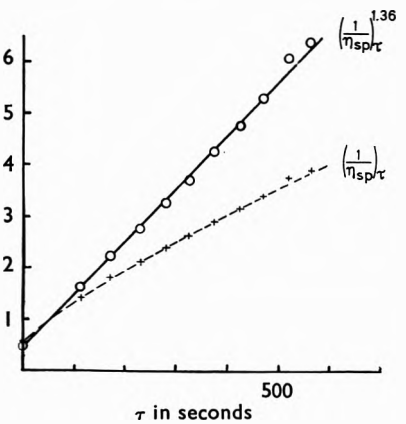

FIG. 2. Broken line, $\left(\frac{1}{\eta_{sp}}\right)$, as a function of the reaction time, τ. Continuous line, $\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$, as a function of the reaction time, τ.

With interaction between the polymer segments and the solvent the polymer will expand, with the consequent enlargement of its hydrodynamic radius R. Flory has deduced, by thermodynamic treatment of this effect, the following relation between the polymer's degree of expansion, $\alpha = \frac{R}{R_0}$ and its molecular weight

$$\alpha^5 - \alpha^3 = C_T M^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where C_T is a constant if all measurements are made at the same temperature.

The dependence of α upon the molecular weight can be represented quite satisfactorily, by an exponential expression, $\alpha^3 \sim M^{a'}$, in a considerable range of molecular weights. If $R = R_0\alpha$ is substituted for R_0 in equation (5), we obtain equation (2), where $a = \frac{1}{2} + a'$.

If the polymer is a polyelectrolyte, the electrostatic forces between equal charges in the polymer chain will attempt to extend the chain. In this instance α will be a function of the ionic strength of the solution, S, and the number of electronic charges per polymer segment, i.

Although the polyelectrolyte molecule in its greatly elongated state in a solution of very low ionic strength has eluded theoretical treatment, one plausible theory has been propounded which is applicable to the less elongated polymer chain existing in the presence of salts. This theory is but an extension of the one outlined above in the case of interaction between uncharged molecules. Instead of equation (6) we obtain the relation:

$$\alpha^5 - \alpha^3 = C_T\sqrt{\left(1 + \frac{Bi^2}{S}\right)^2 M} \quad .. \quad .. \quad (7)$$

Analogously with Flory's treatment of uncharged molecules, we approximate the dependence of α^3 upon $M\left(1 + \frac{Bi^2}{S}\right)^2$ with an exponential expression, $\alpha^3 \sim \left[M\left(1 + \frac{Bi^2}{S}\right)^2\right]^{a'}$: This approximation may be considered satisfactory if the new variable, $M\left(1 + \frac{Bi^2}{S}\right)^2$, is confined to the same range as the previous variable, M, above. Equations (2) and (4) must now be replaced by the expressions:

$$[\eta] = C_m M^{1/2+a'}\left(1 + \frac{Bi^2}{S}\right)^{2a'} \quad .. \quad .. \quad .. \quad (8)$$

and

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \frac{C_A}{(C_m)^{1/a}} \frac{1}{(1 + kc_s)^{1/a} c_s^{1/a+1-\beta}} \frac{1}{(1 + Bi^2/S)^{2a'/a}} c_A \quad (9)$$

This expression, which replaces Hultin's formula in equation (1) where Staudinger's law is not applicable to the substrate, constitutes the basis of the method used in this investigation to determine enzymatic activity. For given conditions of substrate concentration and ionic strength equation (9) reduces to:

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \text{constant} \times c_A \quad .. \quad .. \quad .. \quad (9a)$$

For details of the hydrodynamic and thermodynamic principles on which the theory is founded, the reader is referred to an up-to-date monograph on macromolecular chemistry; for instance, Flory, P. J., *Principles of Polymer Chemistry*, Cornell Press 1953, Chapters 10, 12 and 14.

Experimental

Changes in viscosity of the carboxymethylcellulose inoculated with cellulase were measured with an Ostwald viscometer at 37°. The outflow time of water was 22 seconds. The substrate was two parts 0·4 per cent aqueous solution of sodium carboxymethylcellulose ("medium viscosity" type; substitution 0·89; polymerization 150; from Hercules Powders, U.S.A.), one part 0·2 mol. sodium acetate buffer at pH 4·1 ± 0·1 (measured in the reaction mixture), and one part of an aqueous solution of the cellulase previously purified by centrifugation. The pH is the optimum one[8]. Owing to the thixotropic character of carboxymethylcellulose solutions it is essential to avoid excessive agitation of the solution when dissolving the polymer. Furthermore, the solution must not be kept longer than one day. A stop-watch was started on inoculation of the carboxymethylcellulose solution with cellulase; a second stop-watch measured the outflow time, t_τ, in the viscometer at intervals. At the start of each determination of the outflow time, the time, $t_{1\tau}$, was read. This value, $t_{1\tau}$, plus half the outflow time in the viscometer, t_τ, was considered equivalent to the reaction time of the enzyme, $\tau = t_{1\tau} + \frac{t_\tau}{2}$. To preclude deactivation of the enzyme during measurement reaction times of only 100 to 500 seconds were used. The relative viscosity, $\frac{t_\tau}{t_{aq,}}$ was not allowed to fall below 1·25.

The best way, theoretically, to determine the value of $\frac{1}{a}$ in equation (9) is to measure simultaneously the intrinsic viscosity, $[\eta]$, and the number average molecular weight of carboxymethylcellulose solutions after varying degrees of enzymatic breakdown, then to use relation (2). Since this would have been a laborious procedure, we proceeded instead on the basis of equation (9a). This equation may be written,

$$\log\left(\frac{1}{\eta_{sp}}\right) = C + \frac{1}{(1/a - 1)}\left[\log c_A - \log\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)\right] \quad .. \quad (10)$$

where C is a constant and c_A, as before, the enzyme concentration, measured this time with an arbitrary unit. Equation (10) presupposes that the carboxymethylcellulose concentration, the electronic charge, i, per polymer segment and the ionic strength, S, are kept constant. (For the composition of the reaction mixture, see above.) The quantity $\frac{1}{(1/a - 1)}$ was determined graphically by plotting

$$\log \tfrac{1}{2}\left[\frac{1}{(\eta_{sp})_{\tau = 300}} - \frac{1}{(\eta_{sp})_{\tau = 0}}\right] \text{ against } \left[\log c_A - \log \frac{(1/\eta_{sp})_{\tau = 300} - (1/\eta_{sp})_{\tau = 0}}{300}\right]$$

The value of $\frac{1}{a}$ was found to be 1·36. Quotient $\frac{d/d\tau(1/\eta_{sp})^{1\cdot36}}{c_A}$, as will be

seen from Table I, is independent of the enzyme concentration, c_A, in conformity with the desiderata of equation (9). According to this equation, $\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ also must be a linear function of the reaction time, τ, provided the latter is so short that the enzyme concentration may be considered constant. Figure 2 shows that this requirement too is satisfied for $\frac{1}{a} = 1{\cdot}36$.

TABLE I

ENZYME CONCENTRATION CALCULATED BY MEANS OF EQUATION (9) AND EQUATION (1) RESPECTIVELY

Weighed amount of enzyme in arbitrary unit c_A	$\frac{d/d\tau(1/\eta_{sp})^{1\cdot36}}{c_A}100$	$\frac{d/d\tau(1/\eta_{sp})}{c_A}100$
1·000	3·05	1·73
0·500	2·87	1·72
0·353	2·88	1·87
0·187	3·02	2·08
0·130	3·05	2·25
0·066	3·13	2·56

The derivative was determined as quotient $\frac{(1/\eta_{sp})_{\tau=300} - (1/\eta_{sp})_{\tau=0}}{300}$

In establishing the value of β in equation (9) the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ was determined for a series of different carboxymethylcellulose concentrations but with the usual composition of the acetate buffer and at a fixed enzyme concentration. If we ignore the influence of carboxymethylcellulose itself on the total ionic strength of the solution and on the charge density, i, equation (9) is reduced to

$$(1 + kc_s)^{1.36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36} = C_1(c_s)^{-2.36+\beta} \quad .. \quad .. \quad (11)$$

where C_1 is a constant under the given experimental conditions. The exponent β was determined graphically by plotting

$\log\left[(1 + kc_s)^{1.36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}\right]$ against $\log(c_s)$.

The value was found to be 0·17. For experimental data, see Table II.

TABLE II

INFLUENCE OF THE SUBSTRATE CONCENTRATION, C_S, ON DERIVATIVE $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ AT A CONSTANT ENZYME CONCENTRATION

Carboxymethylcellulose concentration in the viscometer	$c_s^{2\cdot36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$	$c^{2\cdot36}(1 + kc_s)^{1\cdot36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$
0·40	0·015	0·029
0·30	0·014	0·025
0·20	0·014	0·024
0·15	0·014	0·024
0·10	0·015	0·026

The ionic strength, S, of a solution of carboxymethylcellulose has a substantial influence upon the viscosity, and hence also upon the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$. Thus the addition, of NaCl to yield a 0·2N solution will reduce the value of $(\eta_{sp})^{1.36}$ to 50 per cent of the corresponding value obtained when using an ordinary sodium acetate buffer, coincident with an increase of $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ by 60 to 70 per cent. For other examples, see Figure 3. An approximate correction for changes in the ionic strength can be obtained if the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ is multiplied by $\left(\eta_{sp}\right)^{1/a}_{\tau=0}$.

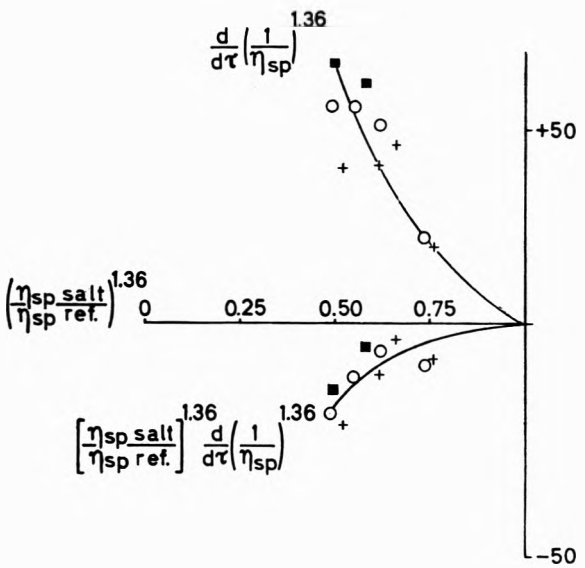

FIG. 3. The upper part of the diagram shows the per cent increase of the derivative, $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ in the presence of salts compared with the corresponding expression for the pure sodium acetate buffer. The lower part indicates the per cent decrease of $\left(\eta_{sp}\right)^{1.36}_{\tau=0}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ in the presence of salts by comparison with the corresponding expression for the pure sodium acetate buffer.

+	Na_2SO_4	0·2	0·1	0·05	0·02 N
⊙	NaCl	0·2	0·1	0·05	0·02 N
■	$CaCl_2$			0·05	0·02 N

This follows from equations (8) and (9). Experiments with known additions of salt to enzyme-substrate mixtures show that this correction yields an enzyme concentration which is too low. This may be attributable to the approximations used in deriving equations (8) and (9), though the possibility of actual deactivation of the enzyme in the presence of salt cannot be ruled out. A more detailed study of this problem was beyond the scope of our investigation. Since the specific viscosity of a solution

of carboxymethylcellulose is dependent not only upon the polymer's molecular weight, which in turn is a function of the number of split glucoside bonds but also upon the amount of dissociation as well as the ionic strength of the solution, quantitative determination of cellulase by the carboxymethylcellulose method requires a solution the composition of which is accurately specified.

Definition of Cellulase Unit

The unit for cellulase was defined arbitrarily by assuming that an enzyme solution contained one cellulase unit per ml. if a mixture composed of one part of that solution, two parts of a 0·4 per cent solution of carboxymethylcellulose ("medium viscosity" type; degree of substitution 0·89; degree of polymerization 150; from Hercules Powders, USA), and one part of 0·2 mol. sodium acetate buffer at pH 4·10 $\pm$ 0·1 was such that $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ = 0·01 when the reaction temperature was 37·0°. In other words: $10^2 \frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ = the number of cellulase units per ml. of enzyme solution.

Discussion of the Carboxymethylcellulose Method for Determining Enzymatic Activity of Cellulase

Under the experimental conditions defined in the foregoing, the value of the exponent, β, in equation (3) is 0·17. This means that the rate at which the enzyme splits glucoside bonds in carboxymethylcellulose is proportional to the product of the enzyme concentration and the substrate concentration raised to 0·17. According to Michaeli the enzymatic breakdown is preceded by chemical binding of a complex compound formed by the enzyme (A) and the substrate (s). The enzyme-substrate compound (As) subsequently disintegrates again through a monomolecular reaction in free enzyme and split substrate. As the enzyme-substrate compound is in stoichiometric equilibrium with enzyme and substrate, the following equation is obtained:

$$\frac{c_{AS}}{c_S(c_A - c_{AS})} = K_{AS} \quad .. \quad .. \quad .. \quad (12)$$

and

$$\frac{d}{d\tau}\left(\frac{c_S}{M}\right) = k_{AS}c_{AS} \quad .. \quad .. \quad .. \quad .. \quad (13)$$

or

$$\frac{d}{d\tau}\left(\frac{c_S}{M}\right) = k_{AS}\,K_{AS}\,\frac{c_A c_S}{(1 + K_{AS}c_S)} \quad .. \quad .. \quad (14)$$

where K_{AS} and k_{AS} are equilibrium and velocity constants respectively, and c_{AS} equals the concentration of enzyme-substrate compound. If the equilibrium constant has a high value, that is, the equilibrium between enzyme and substrate favours the formation of the enzyme-substrate compound, equation (14) can be approximated to equation (3). The observed value of 0·17 for β may thus be taken to imply that at pH 4·10 the cellulase and

carboxymethylcellulose form a stable complex compound. The explanation of the very pronounced maximum action of cellulase upon carboxymethylcellulose at pH 4·10 may well be (see part II), therefore, that at that pH both the enzyme molecules and the carboxymethylcellulose molecules are slightly ionized, though with opposite charges. At higher pH both enzyme and substrate are negatively charged, making formation of a complex compound difficult or impossible. Since the charges and hence the tendency towards formation of complex compounds are not identical for all substrates, one and the same cellulase may naturally have its maximum activity at different pH for different substrates. If the enzyme concentration is high compared with the free surface area of the substrate as it often is in the enzymatic decomposition of large particles, for example of vegetables, the effectiveness of the cellulase will be substantially influenced by its stability, as well as by the pH of the medium and the diffusion rate of enzyme and breakdown products.

In determination of the exponent $\frac{1}{a}$ in equation (9) it was assumed that a relation existed between the intrinsic viscosity and the number average molecular weight of the digested carboxymethylcellulose substrate which can be expressed by the exponential equation (2).

This expression is, as mentioned above, a satisfactory approximation only if the molecular weights employed are within a limited range, that is digestion is not allowed to proceed too far. When determining the value for $\frac{1}{a}$, moreover, the effect of the enzyme itself upon the total ionic strength of the solution, and hence upon the specific viscosity of carboxymethylcellulose, was not taken into account. Since both the extent of digestion and the enzyme concentration accordingly influenced the experimentally determined value of exponent $\frac{1}{a}$, the best results will be obtained, with the carboxymethylcellulose method, if enzyme concentration and reaction times are within the ranges used for establishing the exponent value. When applying the carboxymethylcellulose method for determination of the enzymatic activity of unknown or unpurified preparations, it is essential to bear in mind that the viscosity will be affected if salts are present. When the cellulase preparation contains salts, it must be purified before its enzymatic activity is determined; or if the effect of the salts on the viscosity is known, a correction for it must be made in accordance with what is said under the experimental section.

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STUDIES ON A NEW CELLULASE PREPARATION FROM PENICILLIUM

II. Properties and Action upon Different Substrates

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The properties of a new cellulase preparation from *Penicillium* were studied. In particular, its action upon some vegetable substrates was investigated under varying experimental conditions, and the possibility of its containing at least two enzymes is discussed. Comparative studies were made with an enzyme preparation from *Aspergillus oryzae*.

Oral administration of digestive enzymes has been used in the treatment of pancreatic insufficiency and fermentative dyspepsia. Most therapeutic preparations contain trypsin, lipase and amylase from pancreas, but some contain mold enzymes, primarily amylase and cellulase.

According to Grassmann and Rubenbauer[1] cellulase breaks down vegetable cell walls and thus facilitates digestion. They used enzymes isolated from *Aspergillus oryzae*, and measured their cellulolytic activity against the insoluble substrates hydrocellulose and lichenin. Others have employed soluble cellulose derivatives as substrates.

The soluble cellulose derivatives are hydrolysed more rapidly by cellulases than is cellulose under similar experimental conditions. This is due, in the view of Karrer[2] and Walseth[3], to the crystalline state of the substrate; though the phenomenon has also led to the postulation of a multi-enzyme mechanism in the decomposition of cellulose. According to Reese and others[4,5] a cellulase preparation from *A. oryzae* contains both an enzyme that alters the physical state of cellulose fibres and liberates polymer chains, and one which catalyses the hydrolysis of the latter to reducing sugar. The last-named enzyme is assumed to break down carboxymethylcellulose also.

Other investigations lend support to the view that several enzymes are responsible for the effect of cellulase preparations. Jermyn[6] showed, following paper chromatographic separation, that impure *A. oryzae* preparations have no fewer than eight components which act upon carboxymethylcellulose. Reese and Gilligan[7] found, with a chromatographic technique, three components in *Myrothecium* filtrate, while Miller and Blum[8] observed in the same product at least eight components with a cellulolytic action.

Heiwinkel, Lindvall and Reizenstein[9] reported that heterogenous cellulases are not identical in their actions. Vegetables treated with identical amounts of cellulase, as measured by a viscometric method[10], were digested both more rapidly and more extensively by the *Penicillium* preparation than by a commercial *Aspergillus* preparation.

Vegetable decomposition produced by a cellulase preparation from *A. oryzae* was studied in detail by Silberschmidt[11]. He found macroscopically that the enzyme digested cucumber, kohl-rabi, common radish, and turnip radish. Microchemical methods revealed that the sensitivity of vegetable substrates to cellulose-colouring substances was lost earlier than that to pectin-colouring subtances. These changes proceeded parallel with the lytic action on the cell walls. Decomposition of vegetables was studied also by Holden[12] using tobacco leaves as substrate and various types of enzyme systems.

The investigation herein reported is concerned with a new cellulase preparation. Special attention has been given to its action on carboxymethylcellulose and on various vegetables. The preparation is identical with that used by Heiwinkel and others[9] in their studies on gastrointestinal cellulolytic activity after oral administration in man, and was produced microbiologically from a special strain of *Penicillium notatum*. It was purified to an activity of 5 units per milligram, measured by the viscometric method of Eriksson and Lindvall.[10]

The actions of the *Penicillium* cellulase were also compared in some respects with those of various preparations from *Aspergillus*. The designations and nature of the preparations used are listed in Table I.

TABLE I

Preparation	Formula	pH optimum	Cellulase units per tablet
A	Conc. e cult. penicill. spec.	4·15	200
B	Conc. e cult. penicill. spec. Pancreatin Diastase	4·15	135
C	Extr. aspergill. oryzae	3·85	155
D	Extr. aspergill. oryzae Pancreatin.	3·85	75
E	Extr. mycel. aspergill. Papainum pur. Cystein. hydrochl.	3·85	45
F	Extr. mycel aspergill. spec. diff. pur. sicc. Extr. gl. pancreat. pur. sicc. Papainum pur. Cystein. hydrochl. Faex sicc.	3·85	30
G	Aspergill. oryzae sicc. dep. Extr. pancreat. Extr. fellis bovis	3·85	12

Experiments and Results

pH Optimum for Enzymatic Activity

The pH optimums for enzymes from *Aspergillus* and *Penicillium* in the decomposition of carboxymethylcellulose were determined viscometrically with the use of McIlvaine's buffer or 0·05 mole acetate buffer at different pH. Cellulase from *Penicillium* has its optimal activity at pH 4·15, and enzymes from various commercial *Aspergillus* preparations (according to the makers' formula, *A. oryzae*) at a somewhat lower pH of 3·85.

The pH optimums for the different enzymes are sharply defined, although there is relatively high activity even at pH 5 to 6, especially with the *Penicillium* preparation.

The cellulolytic activity of the medicinal forms (tablets) of the various preparations was measured at the respective pH optimums, using the viscometric method devised by Eriksson and Lindvall[10]. The results are in Table I. Some of the *Aspergillus* preparations showed fluctuating values, but only the highest in each instance is tabulated.

pH Optimum for Thermal Stability

Solutions of the enzyme preparations were made by extraction with water. After adjustment to different pH values by addition of known amounts of hydrochloric acid or sodium hydroxide, the specimens were exposed at 37° for 30 minutes. After neutralisation, the cellulase activity was determined at the pH optimums, that is 4·15 for *Penicillium* cellulase and 3·85 for *Aspergillus* cellulase. The results are presented in Figure 1, which shows optimal stability at pH 5 to 7 for the *Penicillium*, and at pH 4·0 to 6·5 for the *Aspergillus* preparation.

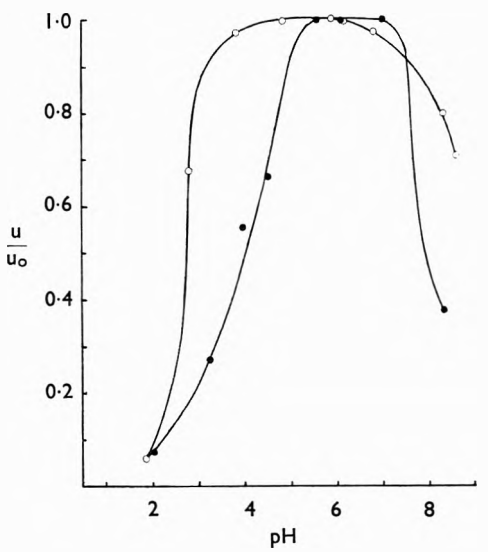

FIG. 1. Persisting activity of the *Penicillium* (preparation A) and *Aspergillus* (preparation C) enzymes after 30 minutes' treatment at 37° and at different pH.

○——○ Aspergillus enzyme.
●——● Penicillium enzyme.

Temperature Optimum for Enzymatic Activity

The enzymatic activity at temperatures between 30 and 65° was determined with carboxymethylcellulose as the substrate. The reaction time was 5 minutes at these temperatures. For determinations at 32 and 40°, both substrate and enzyme solution were heated to the requisite degree

before being combined. For determinations at higher temperatures the substrate had been heated so as to produce the required temperature when combined with enzyme solution of room temperature.

Enzymatic digestion of the carboxymethylcellulose was subsequently terminated by adding mercury acetate in an amount to give a concentration of 0·001 mole, which inactivates the enzyme completely (*cf.* Grassmann and others)[13]. The viscosity of the reaction mixture was then measured at 37°, and the activity calculated by the method of Eriksson and Lindvall[10]. The results are in Figure 2B, from which it will be seen that the enzyme had its optimal activity at 50 to 55°.

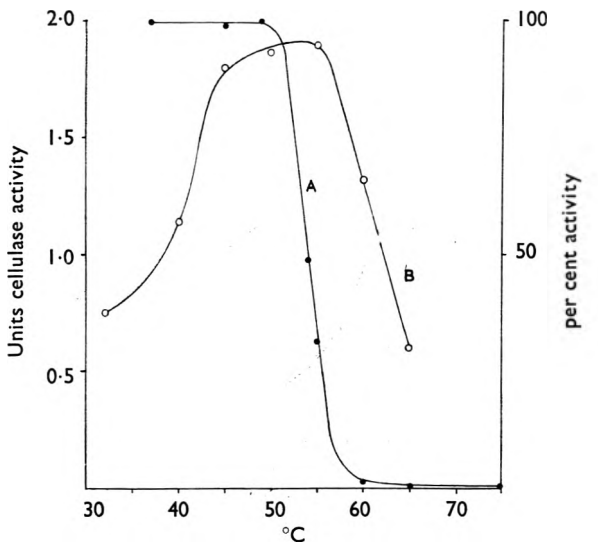

FIG. 2A. Persisting activity of the *Penicillium* enzyme after 30 minutes' heating to different temperatures. B. Enzymatic action of the *Penicillium* preparation upon carboxymethylcellulose at different temperatures.

Thermal Inactivation of the Enzyme

An aqueous solution of the *Penicillium* enzyme was treated in a thermostat at various temperatures for 30 minutes. The solution was then rapidly cooled to 37° and the activity determined viscometrically. The results, which are presented in Figure 2A, show that under these conditions the enzyme is totally inactivated at temperatures exceeding 60°.

The rate of deactivation was measured by heating solutions of the *Penicillium* enzyme to 50, 55, 60 and 65° for varying periods up to 30 minutes. After rapid cooling, the persisting activity was determined in the usual manner. Figure 3A shows the results. The values after 5 minutes' deactivation must be considered unreliable, since temperature equilibrium does not occur instantaneously.

Relatively high enzymatic activity could also be shown to be present at pH 5 to 6, especially with the *Penicillium* enzyme. This suggests the

presence of two enzymes with different pH optima. To find out if the thermal sensitivity corroborated such an assumption, a solution of the enzyme was exposed to temperatures of 55 and 60° for varying periods, after which its activity was determined at different pH values. The results (Fig. 3B) do indeed point to the existence of two cellulolytic enzymes of differing thermal stability, one of which (optimum at pH 4·15) appears to be more sensitive to heat than the other, which has its optimum at approximately pH 6.

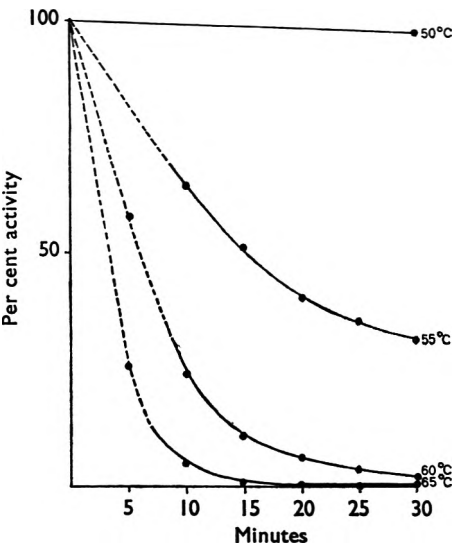

FIG 3A. Persisting activity of the *Penicillium* enzyme after thermal treatment for varying periods at 50, 55, 60 and 65°.

Action of the Enzyme on Different Substrates

Heiwinkel and others[9] found, in regard to digestion of vegetables, a substantial difference per viscometric cellulase unit between the cellulase used in this study and preparations from *Aspergillus*. Their experiments were made at pH 4·2.

With the aim of establishing whether the decomposition of vegetables was dependent on pH, potato cubes measuring 5 mm. per side were digested by 0·05, 0·5 and 5 viscometric enzyme units per ml. from *Penicillium* and *Aspergillus* respectively. Twenty-five g. of potato cubes were shaken in 100 ml. McIlvaine's buffer at pH ranging from 3·0 to 7·5, diluted with an equal volume of aqueous solution of cellulase. The duration of treatment was 2 hours and the temperature 37°. Undigested cubes were filtered off with a Büchner funnel of Jena Duran glass having a filter 5·5 cm. in diameter, with 0·5 by 3 mm. perforations. After filtration, the fragments collected on the filter were weighed. The results are shown in Figure 4, from which it is evident that digestion of potato was

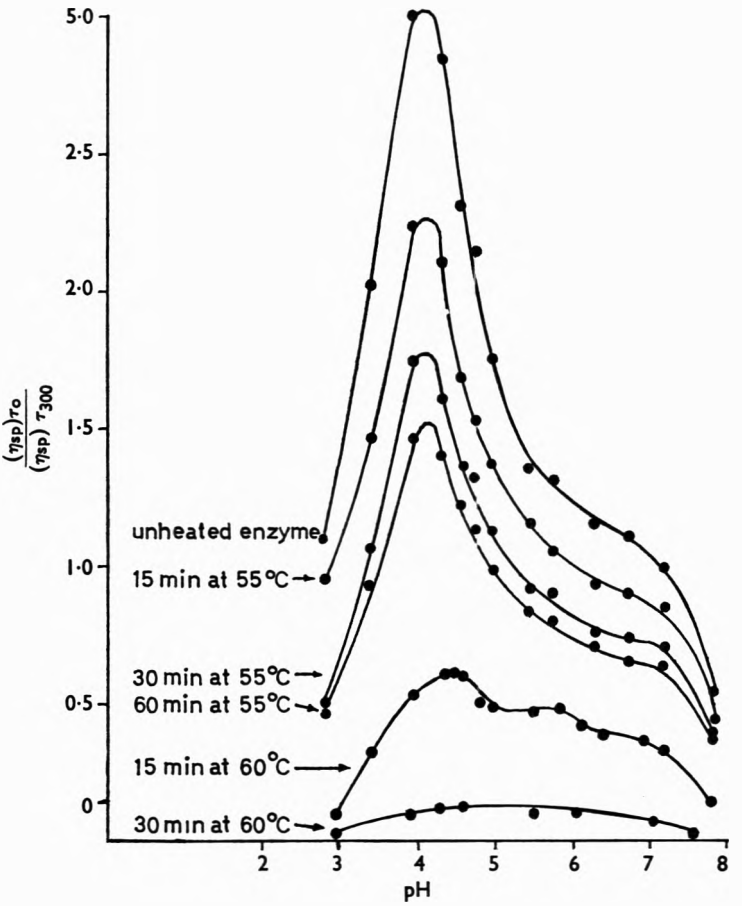

FIG. 3B. Activity of the *Penicillium* preparation measured at different pH after heating for varying periods.

less with *Aspergillus* than with *Penicillium* enzyme, not only at pH 4·2, but throughout the pH range tested.

The *Penicillium* preparation, as mentioned in the foregoing, seems to contain more than one enzyme. The above experiments with potato lend weight to this supposition. To secure further evidence a number of other vegetables were digested at pH values in the range of 3 to 8. Twenty-five to 30 g. of finely chopped carrot, white cabbage, apple and cauliflower were each treated in the same way as potato. The digestion was determined after 3 hours' treatment with 25 enzyme units per ml. It appears evident from Figure 5 that the preparation contains at least two enzymes, one with its optimum at approximately pH 6 and the other at approximately 4·5. In conjunction with the experiments on white cabbage, digestion of that substrate by the *Aspergillus* enzyme was subjected to comparative study. Here too, a substantial difference was noted between enzyme preparations from *Aspergillus* and from *Penicillium*.

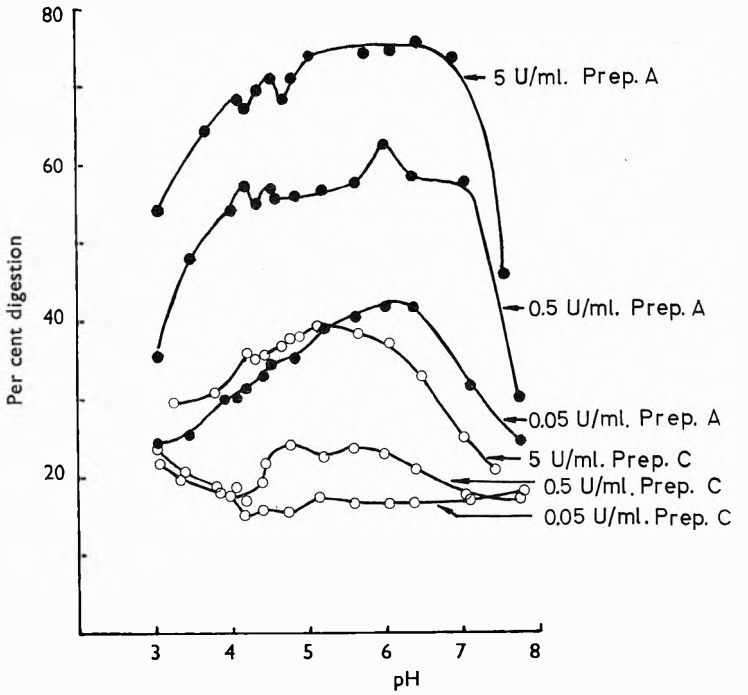

FIG. 4. Degree of digestion of potato after shaking for 2 hours in solutions of varying enzyme concentrations from *Penicillium* (preparation A) and *Aspergillus* (preparation C) at different pH and at 37°.

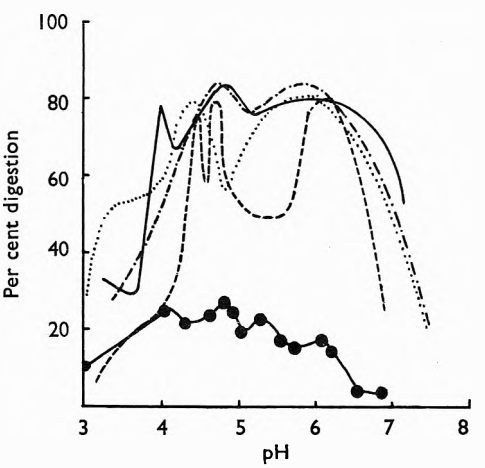

FIG. 5. Degree of digestion of some vegetables after shaking in solution containing 25 units of the *Penicillium* enzyme per ml. for 3 hours at 37°; also, for cauliflower, in a solution of the *Aspergillus* enzyme (preparation C) under identical experimental conditions.

——— Apple. — — Cauliflower.
— · — Carrot. . . . White cabbage.
●—● Prep. C.

Discussion

According to our results the cellulase preparation from *Penicillium* contains at least two enzymes with the ability to digest vegetables. These enzymes have not only disparate pH optima, but also differ in their thermal sensitivity.

As regards the action upon vegetables, the pH optimum diverges from that obtained with carboxymethylcellulose as substrate. The question whether this difference in pH activity is due to the presence of several enzymes which are substrate-specific, or to the physical state of the different substrates like solubility, charge, or crystallinity, will be the subject of a future investigation. The cellulase prepration from *Penicillium* differs in many respects from the *A. oryzae* preparation. With carboxymethylcellulose as substrate, the two have somewhat disparate pH optimums: moreover, the *Penicillium* cellulase shows a better action at pH 5 to 6 than the *Aspergillus* preparations that we studied. There are certain discrepancies also in pH stability. The principal difference, however, is in the digestion of vegetables, in which respect the *Penicillium* cellulase is considerably more active. When tested on potato, for instance, it has, in a concentration of 0·05 viscometric units per ml. approximately the same activity as the *Aspergillus* cellulase in a concentration of 5 viscometric units per ml. Similar findings were recorded by Heiwinkel and others[9], using other vegetables. The cause of these discrepancies between the *Penicillium* and *Aspergillus* preparations is not clear, but probably it is to be sought in differing enzyme systems in the preparations. The purity is possibly of some significance.

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ON THE FLUORIMETRIC DETERMINATION OF ADRENALINE AND NORADRENALINE

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A modification of the fluorimetric method for the determination of adrenaline and noradrenaline in pharmaceutical solutions is described. In this method sulphite present is destroyed with iodine, the added excess of which is reduced with arsenous acid, a manipulation which does not affect the fluorescence obtainable.

Adrenaline and noradrenaline in low concentrations are most suitably determined by the fluorimetric method[1,2]. In pharmaceutical solutions, sulphite is usually present as a stabiliser. This introduces a difficulty in the determination of the catechols as these cannot be quantitatively transformed into the corresponding adrenochromes by potassium ferricyanide or by manganese dioxide if the sulphite present is not destroyed before the oxidation of the catechols.

Adrenaline, however, can be determined *ad modum* Ehrlén[3], even in the presence of sulphite by measuring the transient fluorescence obtained by oxidation with air in strongly alkaline solution. In this procedure there seems to be no interaction between the adrenochrome formed and the sulphite. A possible explanation of this phenomenon is that this reaction is performed at such a high pH that all sulphite is present as SO_3^{--} being non-reactive, while the oxidation with MnO_2 or $Fe(CN)_6^{---}$ is performed in neutral or slightly acid solution, where the sulphite is present partly as HSO_3^- which is reactive forming a bisulphite compound with the adrenochromes.

Noradrenaline cannot effectively be determined by the method of Ehrlén[3] as the sensitivity is much lower than for adrenaline. It is, therefore, necessary first to oxidise noradrenaline with ferricyanide or MnO_2 to noradrenochrome and then to transform it with sodium hydroxide to the fluorescent adrenolutine. This procedure may sometimes be preferable in the determination of adrenaline as a higher fluorescent energy is obtained.

As mentioned before, the sulphite present has to be destroyed in such a way that the fluorescence obtained is not affected. Several methods described are based on the separation of the catechols from the sulphite or on the destruction of the latter. The separation methods are tedious and not well suited to routine work. The destruction of the sulphite ions is usually brought about by oxidation with iodine in an acid solution such that the reaction between iodine and the catechols proceeds very slowly. The excess of iodine added has to be removed in order not to suppress the fluorescence later on. This can be done by addition of thiosulphate, an excess of which, however, also has a strong quenching influence on the fluorescence. The influence of thiosulphate is rather peculiar; added to a solution of the adrenochrome it has no influence on

the fluorescence but if added before the oxidation of the catechol a concentration of 10^{-5} equiv./1. thiosulphate diminishes the fluorescence by about 40 per cent.

Because of the difficulties in obtaining reproducible results in this way, the use of the very small excess of iodine which is obtained when starch is used as an indicator for reaching the equivalent point in the oxidation of the sulphite has been proposed[4]. The best result with this method is reached when MnO_2 is used as oxidant for the catechols as MnO_2 seems to diminish the influence of the free iodine on the fluorescence. Nevertheless in our opinion it is difficult to obtain reproducible results this way even in careful work and a method, where the influence on the fluorescence is still smaller, therefore, seems to be highly desirable. In this paper a method is described where the excess of iodine is destroyed with arsenious acid before the oxidation of the catechols.

Experimental

Reagents

Iodine solution containing 12 g. of iodine and 20 g. of potassium iodide in 1 l. of water.

Arsenious acid solution containing 2 g. of As_2O_3 in 1 l. of water.

Phosphate buffer pH 7 made up from one part of M/15 potassium dihydrogenphosphate and two parts of M/15 disodium hydrogenphosphate.

Manganese dioxide (Baker A.R.) purified according to Crawford and Law[5].

Ascorbic acid solution 0·1 *per cent* in boiled and cooled water.

Sodium hydroxide 1·1 *M*. If the sample also contains a local anaesthetic, this can be kept in solution if the NaOH-solution is made up in 50 per cent ethanol.

As *fluorescence standard* a solution of quinidine sulphate in 0·005 M sulphuric acid is suitable. A suitable fluorimeter is the Photovolt filter fluorimeter Model 540, equipped with a photomultiplier as detector, using a primary filter with maximum transmission at 365 mμ and a secondary filter transmitting maximally over 505 mμ.

Method

Into a 15 ml. volumetric flask is pipetted an aliquot of the sample containing about 25 to 50 μg. noradrenaline or adrenaline. 2 ml. of 0·1N hydrochloric acid and 2 drops of starch indicator are added. Iodine solution is added drop by drop with shaking until a blue colour develops. Then 0·5 ml. of arsenous acid solution is added and the mixture diluted to the mark with water. After mixing, 5·0 ml. is pipetted into 5·0 ml. buffer solution in a centrifuge tube. When mixing the blue colour should disappear completely but the pH of the resulting solution should not be less than 6·5 to 6·7. About 100 mg. of MnO_2 is added to the tube and this is closed with a rubber stopper and shaken for about 90 seconds. The mixture is then centrifuged 1 to 2 minutes at 3000 r.p.m. 5 ml. of ascorbic acid solution is pipetted into a 25-ml. measuring flask. To this solution 5·0 ml. of the clear solution from the centrifuge tube is

added and with mixing immediately diluted to the mark with sodium hydroxide solution. The whole procedure from the addition of MnO_2 should not exceed 4 to 5 minutes. If a suitable manganese dioxide is used a perfectly clear solution is obtained after centrifugation making filtration unnecessary.

The solution is pipetted into the fluorescence measuring cell and the fluorescence continuously measured. A slightly pronounced fluorescence maximum appears about 5 minutes after the addition of the sodium hydroxide and the maximum reading is recorded.

The background fluoresence is determined by adding the ascorbic acid solution when the sodium hydroxide and the adrenochrome solution have reacted for at least fifteen minutes. The background fluorescence can usually be neglected.

A standard graph is constructed from standard adrenaline or noradrenaline solutions containing all the ingredients in the unknown solution.

Discussion

The method described is essentially similar to the methods which Crawford and Law[5] and Lund[2] used on biological samples. The reason why MnO_2 was chosen as oxidant instead of ferricyanide is that ferricyanide in itself a strong fluorescence quencher could be a possible source of errors in the determination.

It was shown that standard graphs of good linearity could be obtained for adrenaline as well as for noradrenaline. The reproducibility was tested for both substances, by calculation of the standard deviation. For the method applied to pure solutions of adrenaline and noradrenaline without introducing iodine or arsenous acid, SD = $\pm$1·9 was found for adrenaline (n = 5) and SD = $\pm$1·2 for noradrenaline (n = 9). When sulphite (50 mg./ml.) was added to the test solution the corresponding figures was $\pm$1·5 (n = 6) and $\pm$0·6 (n = 6). In all these experiments the instrument showed about 75 scale deflections. In a separate series the standard deviation was determined in ten experiments each with varied sulphite and noradrenaline content.

Sulphite mg./100 ml. ..	0	50	100
Noradrenaline μg. ..	10·0	14·1	5·03
Scale deflections, mean ..	52	72	27
$\pm$ SD	1·7	1·1	0·8

It was also shown that the final concentration of arsenous acid could be increased several times without influencing the results.

This method cannot be applied to pharmaceutical combinations containing procaine and noradrenaline. In such solutions it is necessary to separate the catechol before determination[6].

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LETTER TO THE EDITOR

Effects of the Spleen Cardio-active Factor on Thyrotoxic Heart Damage

SIR,—One of the effects of experimentally-induced thyrotoxicosis on the heart is the depletion of heart concentrations of adenosine nucleotides, and of creatine and glycogen[1], a depletion which may be partially prevented by treatment with adenosine triphosphate[1,2]. As the capacity of the heart to accomplish its work depends on the efficiency of the chemical reactions which lead to the final contraction process, any change that may occur in the concentration of the substances mentioned and the resulting effect on contractile efficiency must be of importance.

We therefore investigated whether the cardio-active factor present in the spleen[3,4], the pharmacological properties of which have recently been studied[5], exerted any influence on concentrations of heart components in rats poisoned by DL-thyroxine.

The thyroxine was given by intramuscular injection, in doses of 0·2 mg./kg./day for ten days into rats of 120 to 150 g. Over the same period some of these animals also received intraperitoneal injections of freeze-dried acetonic spleen extract, while others were treated with a cardiotonic factor extractable from the liver, also known as "Zuelzer's hormone"[6]. Lastly a group of animals received intraperitoneal injections of a heart extract of almost identical composition and pharmacological effects as those recently reported by Conway[7].

At the end of the test period, the animals were killed and their hearts assayed for adenosine nucleotides[8], creatine[9], and glycogen[10], soluble and insoluble in trichloroacetic acid.

TABLE I

EFFECTS EXERTED BY SPLEEN AND OTHER EXTRACTS ON HEART COMPOSITION (AVERAGE VALUES ± STANDARD ERROR)

No. of Rats	Treatment and dose (mg./kg.)	P_{APP+} (μg./g.)	Glycogen (mg./100 g.)		Creatine (mg./g.)
			TCA^{++}–Sol.	TCA–Insol.	
10	Normal	349 ± 12	155·2 ± 7·0	172·9 ± 8·4	4·26 ± 0·19
10	Control	133 ± 7	118·6 ± 5·8	81·4 ± 3·9	2·71 ± 0·12
10	Spleen Principle, 2·5	286 ± 11	149·0 ± 7·5	142·1 ± 8·2	3·88 ± 0·19
8	Liver Principle, 2·5	162 ± 9	137·5 ± 6·6	104·7 ± 5·2	3·19 ± 0·14
8	Heart Extract, 2·5	257 ± 11	152·9 ± 8·1	129·5 ± 7·6	3·64 ± 0·16

(+) P adenosine polyphosphates. (++) Trichloroacetic acid.

Findings, set out in Table I, demonstrate the considerable protective efficiency of spleen extract, the effect of which appears to be slightly stronger than that exerted by heart extract. But the cardio-active liver extract under these experimental conditions, appears to possess a very mild protective effect.

As the nature of the factor (or factors) present in the spleen and heart is unknown, it is impossible to specify the mechanism of the effect they exert; however, in the light of the first findings of work still in hand, it is reasonable to suppose that spleen extract effect is exerted, at least partially, through a greater retention in heart tissues of Mg^{++} the heavy consumption of which in thyroxine poisoning is well known.

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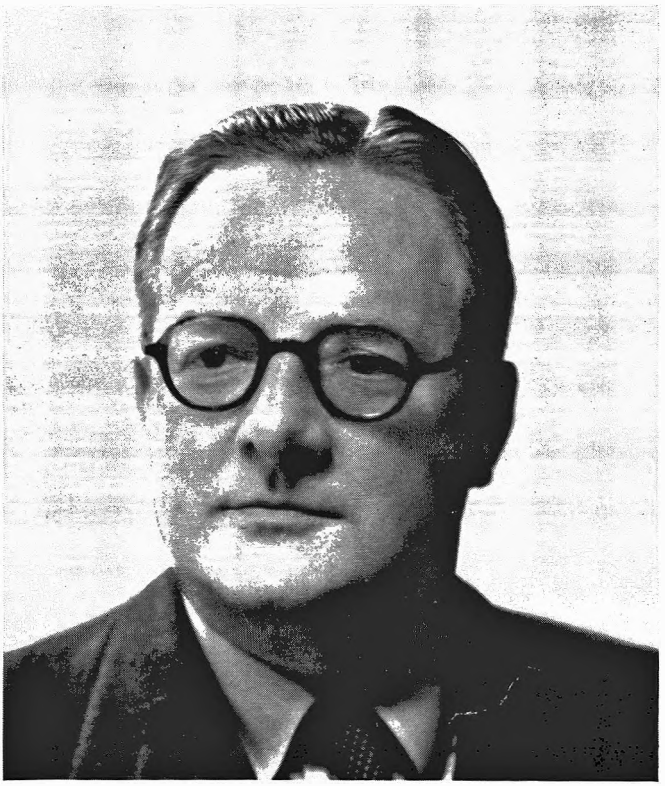

H. TREVES BROWN

Chairman, 1959