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EFFECTS OF ACETYLSALICYLATE ON GLUCONEOGENESIS IN ISOLATED RAT KIDNEY TUBULES

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Abstract—The effect of acetylsalicylate on gluconeogenesis in isolated rat kidney tubules was investigated. It was found that acetylsalicylate, at a concentration of 2 mM, inhibited the formation of glucose from several potentially glucogenic substrates including 2-oxoglutarate, succinate and D-fructose. Addition of butyrate to the incubation medium partially reversed or, in the case of oxoglutarate, eliminated the inhibition. Acetylsalicylate also caused an increase in the respiratory rate of isolated tubules and brought about a decrease in the intracellular ATP level. The effects of acetylsalicylate on gluconeogenesis and respiration were almost identical to the effects of 2,4-dinitrophenol on these processes. It is proposed that, in isolated rat kidney tubules, the fall in the intracellular level of ATP, caused by acetylsalicylate, is a consequence of the uncoupling of respiratory chain phosphorylation. The decreased rate of gluconeogenesis is considered to be a response to the lowered ATP level.

Salicylates have been shown to affect carbohydrate metabolism in mammals, normally causing a fall in the liver glycogen content but having effects on the blood glucose level which vary according to the nutritional and endocrine status of the body [1–10]. With normal, fed rats the blood glucose level is usually raised by salicylates owing to increased hepatic glycogenolysis and/or decreased glycogenesis [1–4]. The same response has been observed in dogs [5]. In contrast, with adrenalectomized, diabetic or fasted rats, in which hepatic glycogen is usually depleted, the blood glucose level is lowered by salicylates [1, 2, 6, 7]. A similar effect has been recorded in human subjects suffering from diabetes mellitus [8–10]. This hypoglycaemic action could be mediated through either an inhibition of gluconeogenesis or a stimulation of glucose utilization. Evidence supporting the former possibility includes the observation that certain enzymes of the gluconeogenic pathway are inactivated or inhibited by salicylates at high concentrations [11, 12]. Also it has been found that ingestion of acetylsalicylate by rats over a 5-week period leads to decreases in the activities of two important gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase [13]. However, this long-term decline in enzymic activity is unlikely to account for the rapid fall in the blood glucose level brought about by the administration of a single dose of salicylate [1, 2, 6].

The present study demonstrates that acetylsalicylate inhibits gluconeogenesis in isolated rat kidney tubules. The inhibition appears to be due to the uncoupling of respiratory chain phosphorylation by acetylsalicylate. The data lend weight to the suggestion that the uncoupling activity of salicylates might have important metabolic consequences [14, 15].

MATERIALS AND METHODS

Chemicals. Hyaluronidase, collagenase, glucose oxidase, peroxidase, hexokinase, glucose-6-phosphate dehydrogenase, NADP, ATP, L-malic acid and D-glucose-6-phosphate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; sodium succinate, 2-oxoglutaric acid, L-glutamic acid, sodium *n*-butyrate and acetylsalicylic acid were from BDH Ltd., Poole, Dorset, U.K.; 2,4-dinitrophenol was from Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K.; xylitol was from E. Merck AG Darmstadt, Germany; D-fructose and glycerol were from Ajax Chemicals Ltd., Sydney, Australia; bovine serum albumin powder (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. All other reagents were A.R. grade.

Tubule preparations. Small fragments of kidney tubules were isolated by the enzymatic disruption of kidney slices from adult albino rats (Wistar strain) as described previously [16].

Incubations. Isolated tubules (3–8 mg of cell protein) were incubated in 2 ml of medium containing the following ions (mEq/l.), Na 148, K 4·4, Mg 1·1, Ca 2·7, Cl 147, SO_4 1·1, PO_4 8, buffered at pH 6·8, with substrates and inhibitors present at the concentrations indicated in the text. Incubations were carried out at 37° in conventional Warburg manometry vessels fitted with stoppers and shaken at 120 oscillations/min. The gas phase was air and the centre well of each flask contained 0·2 ml of 2 M–NaOH to absorb metabolically produced CO_2. All systems were preincubated for 8 min prior to the addition of glucogenic substrates and inhibitors to the tubule suspensions. At the end of the incubation period 0·2 ml of 3 M perchloric acid was added to each flask. The acidified mixture was transferred to a centrifuge tube and the precipitate removed by centrifugation.

Respiration studies. The oxygen consumption of isolated kidney tubules at 37° was followed polarographically using a Clarke-type Rank Oxygen Electrode (obtained from Rank Bros., Bottisham, Cambs., U.K.). The ionic composition of the 2-ml system was identical to that used in the incubations described above. Succinate (5 mM) and butyrate (5 mM) were present as substrates.

Table 1. Effect of acetylsalicylate (2 mM) on the formation of glucose from various substrates in isolated kidney tubules incubated in the presence of butyrate (5 mM)

Substrate (5 mM)	Glucose formed (nmoles/20 min per mg protein) Control	+2 mM acetylsalicylate
None	4·1 ± 0·7 (5)	2·6 ± 0·2 (5)
Xylitol	11·7 ± 1·6 (3)	8·3 ± 0·8 (3)
L-Glutamate	11·7 ± 1·2 (4)	10·9 ± 1·3 (4)
2-Oxoglutarate	28·7 ± 1·1 (8)	37·1 ± 1·2 (8)
Glycerol	31·8 ± 3·1 (4)	23·3 ± 2·4 (4)
Succinate	48·3 ± 1·7 (8)	30·0 ± 1·8 (8)
L-Malate	49·4 ± 5·2 (3)	27·3 ± 2·9 (3)
D-Fructose	102·1 ± 4·5 (9)	69·0 ± 3·3 (9)
D-Glucose-6-phosphate	97·8 ± 3·3 (4)	85·8 ± 3·3 (4)

Tubules (3–8 mg of protein) were incubated for 20 min at 37° in 2 ml of buffered medium, pH 6·8, with or without 2 mM acetylsalicylate, and containing both butyrate (5 mM) and a glucogenic substrate (5 mM). The results are given as means ± S.E.M. and the number of observations with independent preparations of tubules is given in parentheses.

Analyses. Glucose was determined in samples of acidified supernatant fluid by means of the glucose oxidase method [17].

For ATP measurements, the acidified supernatant was neutralized with KOH and the ATP was assayed by a fluorimetric enzyme method [18], using a Zeiss PMQ II Spectrophotometer with fluorimeter attachment.

Protein was determined by the method of Lowry *et al.* [19] with a solution of bovine serum albumin as the reference standard.

RESULTS

The data presented in Table 1 indicate that, in isolated kidney tubules, the conversion of succinate, malate, fructose or glucose-6-phosphate to glucose was significantly inhibited by 2 mM acetylsalicylate ($P < 0·05$). A similar trend emerged with glycerol or xylitol as substrate ($0·05 < P < 0·1$). In contrast, the formation of glucose from oxoglutarate was stimulated by acetylsalicylate ($P < 0·001$). In all cases, butyrate (5 mM) was present in order to serve as a respiratory energy source and thus spare the glucogenic substrate from this metabolic role. It is possibly significant that acetylsalicylate inhibited gluconeogenesis from succinate and fructose to a similar degree, despite the fact that these two substrates enter the gluconeogenic pathway at different levels. Because of this similarity, the metabolism of these substrates and that of oxoglutarate, which responds differently to acetylsalicylate, was subjected to a more thorough investigation.

The results in Fig. 1 are those of experiments in which the concentration of substrate was varied while the acetylsalicylate concentration was maintained at a constant level. The inhibitory effect of 2 mM acetylsalicylate on glucose formation from either fructose (Fig. 1a) or succinate (Fig. 1b) remained almost constant throughout the range of substrate concentrations used. With oxoglutarate as substrate (Fig. 1c) both 2 and 4 mM acetylsalicylate inhibited glucose production at low concentrations of oxoglutarate but stimulated gluconeogenesis at higher substrate concentrations.

The changes in glucose formation that occurred when the concentration of substrate was held constant at 5 mM while that of acetylsalicylate was varied are shown in Fig. 2. With fructose or succinate (Figs. 2a and 2b respectively) it was found that the degree of inhibition of gluconeogenesis increased with increasing acetylsalicylate concentration, the inhibition being greater when butyrate was omitted from the incubation medium. When oxoglutarate was the substrate (Fig. 2c) the effect of acetylsalicylate differed markedly according to whether butyrate was present or not. With butyrate present, the formation of glucose was significantly stimulated by acetylsalicylate at concentrations between 1 and 3 mM ($P < 0·01$). In the absence of butyrate, acetylsalicylate above 2 mM exerted a significant inhibition ($P < 0·01$). It should be noted that with no acetylsalicylate present the formation of glucose from oxoglutarate was greater in the absence of butyrate than in its presence ($P < 0·002$).

As salicylates are known to uncouple respiratory chain phosphorylation in isolated mitochondria, it appeared possible that such an action might underlie the effect of acetylsalicylate on gluconeogenesis. In order to investigate this possibility, the effect of 2,4-dinitrophenol (DNP) on glucose synthesis was studied and compared with the effect of acetylsalicylate. Figure 3 indicates that DNP, over an appropriate range of concentrations, influences glucose formation from both succinate (Fig. 3a) and oxoglutarate (Fig. 3b) in an almost identical fashion to acetylsalicylate (cf. Fig. 2). These results point to the uncoupling action of acetylsalicylate as the fundamental cause of its observed effect on gluconeogenesis.

In order to determine more directly whether acetylsalicylate was acting as an uncoupler, its effect on the respiratory activity of kidney tubules was studied. In Fig. 4 are reproduced polarograph traces portraying the consumption of oxygen by tubules supplied with succinate and butyrate as respiratory substrates. Curve (a) shows that the addition of 10 μM DNP to respiring tubules brought about a marked increase in the rate of oxygen consumption. Increases of lesser magnitude were caused by the addition of 2 or 4 mM acetylsalicylate (curves (b) and (c) respectively). Addi-

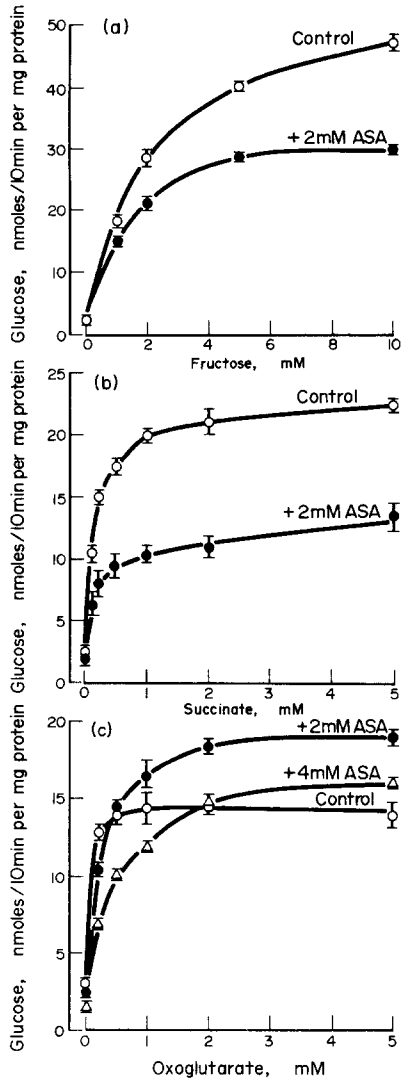

Fig. 1. Effect of acetylsalicylate on glucose formation in isolated kidney tubules. Tubules were incubated for 10 min at 37° in 2 ml of buffered medium, pH 6·8, containing 5 mM butyrate and with (a) D-fructose, (b) succinate or (c) oxoglutarate as the glucogenic substrate. Acetylsalicylate (ASA) was present as indicated. Each point represents the mean value ± S.E.M. from 3 or 4 separate experiments.

tion of 2 mM acetylsalicylate subsequent to 10 μM DNP had no further effect on the rate of oxygen utilization (curve (d)) but 4 mM acetylsalicylate lowered the DNP-stimulated rate (curve (e)), an effect possibly attributable to an inhibition of the respiratory chain by acetylsalicylate at the higher concentration.

Because the uncoupling of respiratory chain phosphorylation might be expected to result in a decrease in the rate of intramitochondrial ATP production, the effect of acetylsalicylate on the level of ATP in isolated tubules was examined. The results presented in Fig. 5 show that 2 mM acetylsalicylate significantly lowered the ATP level in tubules incubated without added substrate ($P < 0.01$) or with succinate or oxoglutarate as the sole exogenous substrate ($P < 0.05$). A smaller decrease, reproducible though not statistically significant, was brought about by acetylsalicylate when butyrate was added together with succinate or oxoglutarate.

DISCUSSION

Salicylate-induced hypoglycaemia in mammals could be caused by a decrease in glucose synthesis, an increase in the uptake and utilization of glucose by the tissues, or an increase in the loss of glucose in the urine. The last of these seems unlikely to occur [8] and, of the remaining alternatives, the first is the more firmly supported by existing experimental evidence including that in the present report.

Two distinct actions of salicylates could conceivably be responsible for a decrease in glucose synthesis. The first of these is the inhibition, under certain conditions, of some NAD^+-dependent enzymes including glyceraldehyde-3-phosphate dehydrogenase [11], lactate dehydrogenase [12, 20] and dehydrogenases of

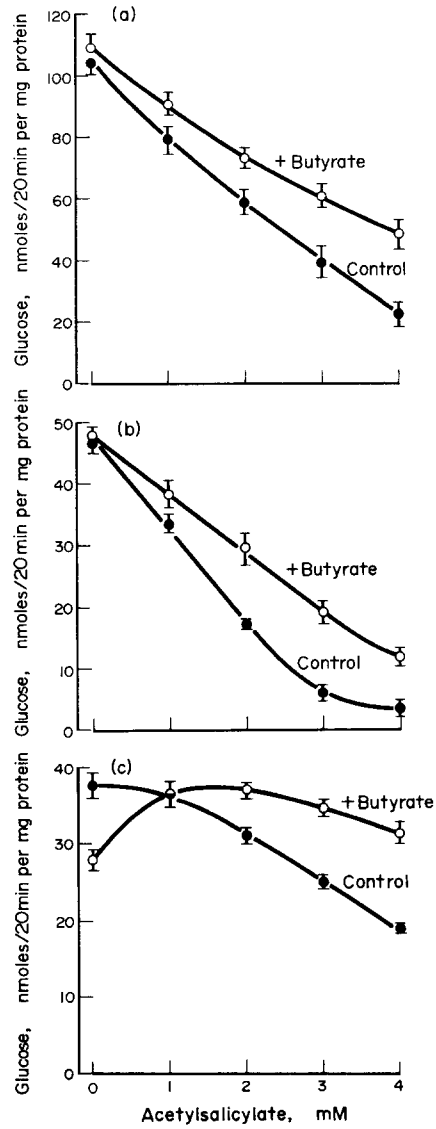

Fig. 2. Effect of increasing concentration of acetylsalicylate on glucose formation in isolated kidney tubules. Tubules were incubated for 20 min at 37° in 2 ml of buffered medium, pH 6·8, containing (a) 5 mM D-fructose, (b) 5 mM succinate or (c) 5 mM oxoglutarate as the glucogenic substrate, and with acetylsalicylate at concentrations up to 4 mM. Butyrate (5 mM) was present as indicated. Each point represents the mean value ± S.E.M. from between 3 and 6 separate experiments.

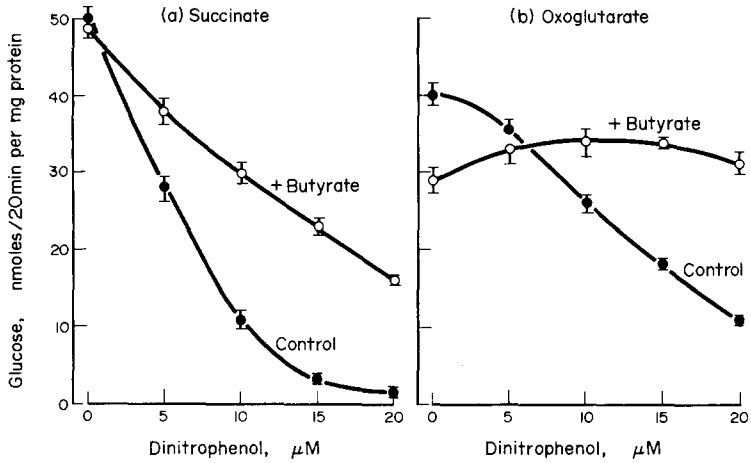

Fig. 3. Effect of increasing concentration of 2,4-dinitrophenol on glucose formation in isolated kidney tubules. Tubules were incubated for 20 min at 37° in 2 ml of buffered medium, pH 6·8, containing (a) 5 mM succinate or (b) 5 mM oxoglutarate as the glucogenic substrate, and with dinitrophenol at concentrations up to 20 μM. Butyrate (5 mM) was present as indicated. Each point represents the mean value ± S.E.M. from 3 or 4 separate experiments.

the tricarboxylic acid cycle [12, 21]. If such an inhibition were to occur in the intact kidney cortex cell its capacity for gluconeogenesis, an ATP-requiring and electron-dependent process, would be impaired since the supply of electrons for respiratory chain phosphorylation and reductive syntheses would be decreased. The second relevant action of salicylates is the uncoupling of respiratory chain phosphorylation [22–29] which would also lead to a decrease in the intramitochondrial formation of ATP. Thus, the fall in the concentration of ATP in isolated kidney tubules incubated with acetylsalicylate, an effect similar to those observed in isolated rat diaphragm in the presence of salicylate [14] and in the kidney cortex of salicylate-treated dogs [5], could result from either action. However, the stimulation by acetylsalicylate of the oxygen consumption of kidney tubules is inconsistent with an inhibition of the dehydrogenases of the tricarboxylic acid cycle; rather, it indicates an uncoupling effect particularly since dinitrophenol, a recognised uncoupler, also stimulates respiration in these cells.

A drop in the intracellular concentration of ATP, resulting from a lower rate of ATP synthesis, could lead to a decrease in the rate of reactions that require ATP, or GTP, as a phosphate donor. In the formation of glucose from succinate, malate or oxoglutarate such reactions are catalysed by phosphoenolpyruvate carboxykinase and phosphoglycerate kinase. With fructose, which enters the main gluconeogenic pathway at the level of the triose phosphates, ATP is

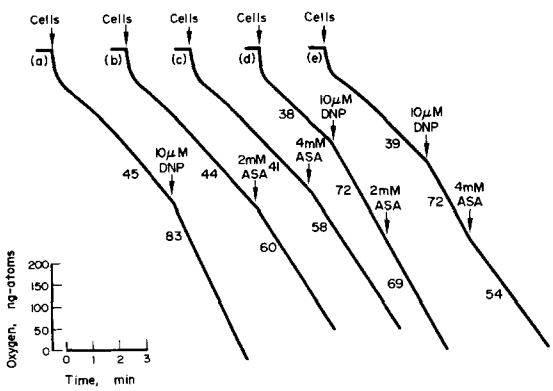

Fig. 4. Effects of acetylsalicylate and 2,4-dinitrophenol on the respiratory rate of isolated kidney tubules. Tubules (1·6 mg of protein) were incubated in 2 ml of buffered medium, pH 6·8, in the reaction chamber of an oxygen electrode assembly maintained at 37°. Succinate (5 mM) and butyrate (5 mM) were present as substrates. Additions (25–50 μl) of acetylsalicylate (ASA) and 2,4-dinitrophenol (DNP) were made at the points indicated by arrows on each curve, giving the final concentrations shown adjacent to the arrows. The numbers alongside each curve represent the steady rate of oxygen consumption (ng-atoms/min per mg protein) before and after each addition.

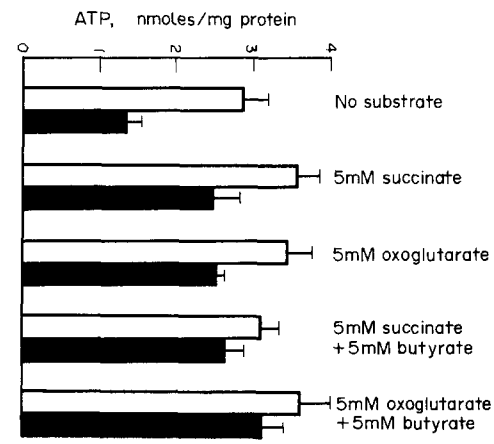

Fig. 5. Effect of acetylsalicylate (2 mM) on ATP levels in isolated kidney tubules. Tubules (6–10 mg of protein) were incubated for 20 min at 37° in 2 ml of buffered medium, pH 6·8, with or without 2 mM acetylsalicylate and containing substrates as indicated. Each bar represents the mean value + S.E.M. from 4 or 5 separate experiments: □ control; ■ + acetylsalicylate.

required for the reactions catalysed by ketohexokinase and triokinase. Additionally, or alternatively, the fall in the ATP level, if accompanied by rises in the ADP and AMP levels, should lead to the negative allosteric modulation of fructose diphosphatase, a gluconeogenic enzyme, and the positive allosteric modulation of phosphofructokinase, a glycolytic enzyme, thereby decreasing the net rate of glucose formation. The stimulation of glycolysis by salicylate in the brain [30] and several types of cultured cell [31] might be due to this regulatory phenomenon. A positive correlation between the rate of glucose production and the intracellular ATP concentration has also been observed in other studies with rat kidney cortex [32, 33].

It may be noted that salicylates also inhibit glycogenesis [3], protein synthesis [34], lipogenesis [35] and glutamine synthesis [36], all of which are ATP-requiring processes. The inhibition has, in some cases, been attributed to the uncoupling of respiratory chain phosphorylation as have the salicylate-induced increases in the metabolic rate [8] and the oxygen consumption [37] of human subjects.

The smaller inhibition of gluconeogenesis by acetylsalicylate that occurred when butyrate was added with either succinate or fructose might be explicable in terms of the 'sparing' of these substrates as respiratory fuels especially in the uncoupled condition when the rate of respiration was increased. In addition there was an indication that acetylsalicylate had less effect on the intracellular ATP level when butyrate was present.

A second important action of butyrate was evident with oxoglutarate as the glucogenic substrate. In the absence of acetylsalicylate, butyrate caused a decrease in the rate of formation of glucose from oxoglutarate. This might be explained by butyrate oxidation lowering the intramitochondrial NAD^+/NADH ratio and thereby decreasing the rate of conversion of oxoglutarate to succinate. A rise in the intramitochondrial NAD^+/NADH ratio, resulting from either the omission of butyrate or the addition of low concentrations of uncouplers, could be responsible for the observed rise in glucose production under such conditions provided that sufficient cytoplasmic NADH was available for the conversion of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate.

In the absence of butyrate the formation of glucose from oxoglutarate was less susceptible to inhibition by acetylsalicylate or dinitrophenol than was glucose formation from succinate. It is possible that in the presence of uncouplers the metabolism of oxoglutarate might help to maintain a higher intracellular nucleoside triphosphate concentration than the metabolism of succinate because of the substrate-level phosphorylation of GDP that accompanies the conversion of oxoglutarate to succinate. The data on ATP levels in tubules are equivocal on this point though in isolated mitochondria the phosphorylation associated with oxoglutarate oxidation is less completely uncoupled by salicylate than that associated with succinate oxidation [25, 28].

In conclusion it is proposed that the inhibition of renal gluconeogenesis, *in vitro*, by acetylsalicylate is the direct consequence of a fall in the intracellular ATP level following the uncoupling of respiratory chain phosphorylation. The results are consistent with the concept that salicylates induce hypoglycaemia in mammals by inhibiting gluconeogenesis [38] though the possible contribution of an increased rate of glycolysis should not be overlooked.

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