SUMMARY

1. Rat-liver slices have been incubated with $[1-^{14}\text{C}]$ glucose and with $[6-^{14}\text{C}]$ glucose, and the yields of $^{14}\text{CO}_2$ from these two substrates have been compared in normal, alloxan-diabetic, insulin-treated diabetic and thyroxine-treated animals. Livers from the same animals have been used for determining the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and of phosphoglucose isomerase. In addition, glucose 6-phosphatase activity has been determined in the control and hyperthyroid-rat livers.

2. In diabetes the formation of $^{14}\text{CO}_2$ from $[6-^{14}\text{C}]$ glucose is significantly reduced, whereas that from $[1-^{14}\text{C}]$ glucose is only very slightly reduced. This indicates a decrease in the relative proportion of glucose catabolized via glycolysis.

3. Treatment of diabetic rats with insulin partially reverses the changes mentioned above by increasing the $^{14}\text{CO}_2$ production from both $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose, with an accompanying fall in the quotient (yield of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glucose)/(yield of $^{14}\text{CO}_2$ from $[6-^{14}\text{C}]$ glucose). The levels of activity of glucose 6-phosphate and 6-phosphogluconate dehydrogenases are also increased, glucose 6-phosphate dehydrogenase activity being significantly higher than that of normal liver.

4. After thyroxine treatment $^{14}\text{CO}_2$ production from $[6-^{14}\text{C}]$ glucose is significantly increased, whereas that from $[1-^{14}\text{C}]$ glucose is only very

slightly increased, indicating greater participation of the glycolytic pathway.

5. Glucose 6-phosphatase activity is significantly higher in the livers of thyroxine-treated rats.

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The Effects of Salicylate on Oxygen Consumption and Carbohydrate Metabolism in the Isolated Rat Diaphragm

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Salicylate produces a number of effects on carbohydrate metabolism in the normal rat, such as depletion of liver and muscle glycogen (Lutwak-Mann, 1942; Winters & Morrill, 1955), an initial hyperglycaemia (Smith, 1955*a*) and an inhibition of the actions of adrenal corticosteroids in producing glycosuria and hyperglycaemia (Smith, 1952). The depletion of glycogen and antagonism of the effects of adrenal cortical extracts have also been observed with rat-liver slices (Smith, 1955*b*). Salicylate decreases the glycosuria and hyperglycaemia in the diabetic rat (Ingle, 1950; Smith, Meade & Bornstein, 1952) and produces hypoglycaemia in adrenalectomized and hypophysectomized animals (Smith, 1955*a*; Comulada, Carlo & Smith, 1953).

An increased consumption of oxygen after the administration of salicylate occurs in man (Cochran, 1952) and the rat (Meade, 1954), and in isolated tissues such as mouse-liver slices (Sproull, 1954) and rat-brain preparations (Fishgold, Field & Hall, 1951) incubated with salicylate. The possible relation between this increased oxygen consumption and the effects of salicylate on carbohydrate metabolism is of some interest, particularly because of the suggestion (Meade, 1954) that the action of salicylate may resemble that of the dinitrophenols, which interfere with oxidative phosphorylation processes (Loomis & Lipmann, 1948). The present work is concerned with a study of the effects of salicylate on the isolated rat diaphragm, which

provides a convenient system for the estimation of oxygen uptake, glycogen content, lactic acid accumulation and glucose uptake. A preliminary account of the work has already been communicated to the Biochemical Society (Smith & Jeffrey, 1956).

METHODS

Preparation of the diaphragms. Male rats of the Wistar strain (wt. 100–120 g.) were used, and unless otherwise stated were fasted for 24 hr. before killing by decapitation. The diaphragms were excised according to the directions of Stadie & Zapp (1947), spread on filter paper and divided into two or four pieces. These were immediately transferred to 5 ml. of phosphate-saline (Stadie & Zapp, 1947), maintained at 2° and after any remaining connective tissue had been removed the pieces were weighed on a torsion balance and placed in the appropriate medium in Warburg flasks. When O₂ uptake, respiratory quotient, glycogen and lactic acid were to be measured, quarter-diaphragms (40–50 mg.) were employed. In the glucose uptake experiments two hemidiaphragms from separate rats were placed in the control flask and the corresponding pair of hemidiaphragms in the flask containing salicylate. The flasks were attached to manometers and gassed with O₂ or N₂ for 5 min.; yellow phosphorus was used in the centre wells in the anaerobic experiments. The flasks were incubated at 25° or 37°. In the O₂-uptake experiments 10 min. equilibration was allowed before the first reading.

Incubation media. Two salt solutions were used: (a) phosphate medium of Stadie & Zapp (1947); (b) a modified Ringer solution containing 9.0 g. of NaCl, 0.46 g. of KCl, 0.211 g. of KH₂PO₄, 0.382 g. of MgSO₄·7H₂O and 0.26 g. of NaHCO₃ in 1 l. of distilled water; 90 ml. was mixed with 10 ml. of phosphate buffer (8 parts of 0.4 M-Na₂HPO₄ and 2 parts of 0.4 M-KH₂PO₄, pH 7.4) for the aerobic experiments, and 97 ml. was mixed with 3 ml. of the phosphate buffer for the anaerobic experiments. The lower phosphate concentration (0.012 M) was used in the anaerobic work to decrease the rate of glycogen breakdown; it was found that with the higher phosphate concentration (0.04 M) the glycogen disappeared within a few min. from the control diaphragms. Glucose was added to both media to give a final concentration of 0.01 M and the salicylate was added from the side arm of the Warburg flasks after temperature equilibration. Salicylate (5 × 10⁻³ M, 69 mg./100 ml.) was

chosen as a suitable initial concentration because plasma salicylate concentrations of 50–70 mg./100 ml. were found in rats at the time when maximum liver glycogen depletion occurred after the injection of sodium salicylate. In all cases the pH was initially 7.4 and did not alter significantly during the course of the experiments.

Methods of analysis. Glycogen was estimated according to the directions of Walaas & Walaas (1950); the glucose in the final stage of the procedure and also the glucose in the media were measured by the method of Nelson (1944). The glucose uptake was calculated as the amount of glucose which disappeared from the medium during incubation. Portions of diaphragm for glycogen analysis were placed in 0.3 ml. of 30% (w/v) aqueous KOH immediately after excision and at the end of the incubations the pieces of diaphragm were removed from the flasks, blotted and treated in a similar manner. Lactic acid (medium + tissue) was determined by the method of Barker & Summerson (1941), all the values being corrected for the amounts present initially in the tissue. The respiratory quotient was measured by Warburg's direct method and expressed as the overall value.

RESULTS

Effect of salicylate on glycogen and lactic acid of diaphragms incubated aerobically. The results in Table 1 show the effect of 5 × 10⁻³ M salicylate on the glycogen content and lactic acid production of rat diaphragms incubated for 30, 60 and 120 min. at 37° under O₂. The control diaphragms showed a significant net synthesis of glycogen at 120 min. (P = 0.01) and incubation with salicylate caused significant depletions of the glycogen at 30, 60 and 120 min. A progressive accumulation of lactic acid occurred in the control diaphragms, which was significantly increased by salicylate at 30 and 60 min.; at 120 min., however, the lactic acid of the control diaphragms was usually higher than in the diaphragms incubated with salicylate, although the difference was not significant.

Table 2 shows that the effect on glycogen breakdown was diminished by decreasing the concentration of salicylate and no significant effect was detected after an incubation of the diaphragms for 60 min. with 10⁻⁴ M salicylate.

Table 1. *Effect of 5 × 10⁻³ M salicylate on glycogen and lactic acid of rat diaphragms incubated aerobically*

Quarter-diaphragms were incubated at 37° in 2 ml. of phosphate medium containing 0.01 M glucose under an atmosphere of O₂. Mean values ± s.e. and ranges are given for the results, which are expressed as mg./g. of wet wt.

Time of incubation (min.)	...	Glycogen				Lactic acid		
		0	30	60	120	30	60	120
No. of experiments	...	13	7	13	6	5	7	6
Control		1.46 ± 0.54	1.40 ± 0.47	2.07 ± 0.70	2.38 ± 0.48	1.30 ± 0.27	2.10 ± 0.22	2.86 ± 0.71
Range		0.53–2.13	0.72–2.21	0.64–3.10	1.52–3.52	1.04–1.64	1.80–2.39	1.95–3.91
Salicylate		—	0.81 ± 0.45	0.51 ± 0.38	0.16 ± 0.12	1.79 ± 0.21	2.32 ± 0.18	2.50 ± 0.66
Range		—	0.30–1.76	0.12–1.49	0.08–0.37	1.51–2.07	2.07–2.68	1.82–3.61

Statistical significance of differences between results of control and salicylate groups. Glycogen. 30 min.: $t = 2.3758$, $P = 0.05$; 60 min.: $t = 4.2305$, $P < 0.001$; 120 min.: $t = 7.8932$, $P < 0.001$. Lactic acid. 30 min.: $t = 3.2071$, $P = 0.02$; 60 min.: $t = 2.2776$, $P = 0.05$; 120 min.: $t = 2.0353$, $P = 0.1$.

Table 2. *Effect of decreasing concentrations of salicylate on glycogen of rat diaphragms incubated aerobically*

Quarter-diaphragms were incubated aerobically for 60 min. at 37° in 2 ml. of phosphate medium containing 0.01M glucose. Mean values $\pm$ s.e. and ranges given for results, which are expressed as mg. of glycogen/g. wet wt.

Concn. of salicylate (M)	No. of experiments	Glycogen		Comparison of control and salicylate glycogen	
		Control	Salicylate	<i>t</i>	<i>P</i>
5×10^{-3}	13	2.07 $\pm$ 0.70 (0.64–3.10)	0.51 $\pm$ 0.38 (0.12–1.49)	4.2305	<0.001
1×10^{-3}	6	2.53 $\pm$ 0.61 (1.49–2.92)	1.58 $\pm$ 0.65 (0.84–2.39)	2.6087	0.05
5×10^{-4}	6	2.40 $\pm$ 0.27 (1.87–2.60)	1.60 $\pm$ 0.34 (1.09–1.84)	4.3937	0.01
1×10^{-4}	6	1.76 $\pm$ 0.58 (0.64–2.26)	1.70 $\pm$ 0.57 (0.57–2.20)	—	—

Table 3. *Effect of 5×10^{-3} M salicylate on glycogen and lactic acid of rat diaphragms incubated aerobically*

Quarter-diaphragms from non-fasting rats were incubated for 15 min. at 25° in 2 ml. of phosphate-Ringer (0.04M phosphate) containing 0.01M glucose under an atmosphere of O₂. Mean values (10 expts.) $\pm$ s.e. and ranges are given for the results, which are expressed as mg./g. wet wt.

Glycogen					Lactic acid	
Initial	Control	Salicylate	Initial – Control (A)	Initial – Salicylate (B)	Control (C)	Salicylate (D)
3.08 $\pm$ 0.80 (1.83–4.40)	2.46 $\pm$ 0.89 (1.54–4.31)	1.73 $\pm$ 0.61 (1.19–3.00)	0.62 $\pm$ 0.46 (0.10–1.42)	1.35 $\pm$ 0.43 (0.47–2.00)	0.30 $\pm$ 0.13 (0.10–0.54)	0.59 $\pm$ 0.33 (0.29–1.00)

Statistical significance of differences between results. For groups A and B: $t=4.2131$, $P=<0.001$; for groups C and D: $t=3.3896$, $P=0.01$.

Table 4. *Effect of 5×10^{-3} M salicylate on glycogen content and lactic acid production of rat diaphragms incubated anaerobically*

Quarter-diaphragms from non-fasting rats were incubated for 10 min. at 25° in 2 ml. of phosphate-Ringer (0.012M phosphate) containing 0.01M glucose under an atmosphere of N₂. Mean values $\pm$ s.e. and ranges are given for the results, which are expressed as mg./g. wet wt.

Glycogen						Lactic acid		
No. of experiments	Initial	Control	Salicylate	Initial – Control (A)	Initial – Salicylate (B)	No. of experiments	Control (C)	Salicylate (D)
5	3.27 ± 0.70 (2.66–4.25)	2.17 ± 0.74 (1.27–3.10)	1.56 ± 0.61 (0.90–2.35)	1.09 ± 0.31 (0.84–1.59)	1.70 ± 0.31 (1.18–1.96)	7	2.10 ± 0.20 (1.94–2.52)	2.63 ± 0.32 (2.12–3.08)

Statistical significance of differences between results. For groups A and B: $t=3.1506$, $P=0.02$; for groups C and D: $t=3.7527$, $P=0.01$.

Table 5. *Effect of 5×10^{-3} M salicylate on glucose uptake of rat diaphragms incubated aerobically*

Two hemidiaphragms were incubated in 2 ml. of phosphate buffer containing 0.01M glucose for 60 or 120 min. at 37°. Results given as means $\pm$ s.e. and ranges, expressed as mg./g. wet wt.

60 min.			120 min.		
No. of experiments	Control (A)	Salicylate (B)	No. of experiments	Control (C)	Salicylate (D)
9	5.24 $\pm$ 1.10 (3.03–6.95)	5.25 $\pm$ 0.80 (4.20–5.85)	11	8.94 $\pm$ 1.18 (7.00–10.30)	6.86 $\pm$ 1.33 (4.40–8.50)

Statistical significance of difference between C and D: $t=5.7322$, $P=<0.001$.

When diaphragms from rats which were not fasted before the experiments were incubated at 37°, a rapid disappearance of glycogen occurred in the control diaphragms. Experiments were therefore performed at 25° to reduce the rate of glycogen breakdown, and the addition of 5×10^{-3} M salicylate caused significant increases in glycogen breakdown and lactic acid production after 15 min. incubation (Table 3).

Effect of salicylate on glycogen and lactic acid of diaphragms incubated anaerobically. With diaphragms from animals not fasted before the experiment, incubation with 5×10^{-3} M salicylate under anaerobic conditions caused significant increases in glycogen breakdown and in lactic acid production in 10 min. at 25° as shown in Table 4.

Effect of salicylate on glucose uptake and respiratory quotient of diaphragms. The results in Table 5 show that 5×10^{-3} M salicylate caused a significant decrease in the uptake of glucose by the diaphragms at 120 min. but not at 60 min.

Table 6. *Effect of 5×10^{-3} M salicylate on respiratory quotient of diaphragms*

Quarter-diaphragms in 4 ml. of phosphate medium containing 0.01 M glucose at 37° under atmosphere of O₂.

Time (min.)	Respiratory quotient	
	Control	Salicylate
30	0.86	1.02
	0.87	1.00
60	0.93	0.98
	0.90	0.99
	0.91	0.99
	0.94	0.99
120	0.83	0.93
	0.85	0.92

The respiratory quotient of the control diaphragms measured after 30, 60 and 120 min. varied from 0.83 to 0.94, whereas in the presence of 5×10^{-3} M salicylate it was increased to values of between 0.92 and 1.02 as shown in Table 6.

The results in Table 6 show that the respiratory quotient in the presence of salicylate fell during incubation, the difference between the control diaphragms and those incubated with salicylate being most marked at 30 min.

Effect of salicylate on O₂ uptake of diaphragms. The effects of different concentrations of salicylate on the O₂ uptake of the diaphragms are shown in Fig. 1.

Salicylate (5×10^{-3} M) always produced a marked increase in the rate of O₂ uptake of the diaphragms for the first 30–60 min. of the experiment, but this subsequently declined and respiration had almost entirely ceased at 2 hr. Salicylate (10^{-3} M) usually caused a larger and more prolonged increase in O₂ uptake than 5×10^{-3} M salicylate, but in about one-quarter of the experiments a smaller although more sustained increase was produced by the lower salicylate concentration. When the diaphragms were incubated with 5×10^{-4} M or 10^{-4} M salicylate there was usually an initial lag period before an increase in O₂ uptake became apparent.

DISCUSSION

The results show that salicylate causes an increased breakdown of glycogen in the rat diaphragm under aerobic conditions whether a net synthesis or breakdown of glycogen occurs in control diaphragms. The oxygen uptake, lactic acid production and respiratory quotient were also increased, and the glucose uptake was decreased. A marked depletion of the glycogen content of the diaphragms could be detected within 30 min., whereas a significant reduction in glucose uptake was observed after 2 hr. but not after 1 hr. It is therefore unlikely that the primary effect of salicylate was to diminish the uptake of glucose by the diaphragms, thus causing a decreased glycogen content.

With increasing time of incubation under aerobic conditions with 5×10^{-3} M salicylate the glycogen progressively diminished and had almost completely disappeared at 2 hr. (Table 1). The increased oxygen uptake observed with salicylate during the first 60 min. of the experiment had declined with an almost complete cessation of respiration at 2 hr. (Fig. 1), at which time the differences between the lactic acid and respiratory quotients of the control diaphragms and those incubated with salicylate had also diminished (Tables 1 and 6). Salicylate also caused increased breakdown of glycogen and lactic acid production in the absence of oxygen.

A possible mechanism for these effects is that salicylate interferes with oxidative phosphorylation

Fig. 1. Effect of increasing concentrations of salicylate on O₂ uptake of diaphragms incubated at 37° in phosphate medium. O, Control; ●, 5×10^{-3} M; △, 10^{-4} M; ▲, 10^{-3} M.

reactions and inhibits the production of high-energy phosphate bonds. The depletion of glycogen and the increased lactic acid and respiratory quotient found in the diaphragms incubated with salicylate may be indications of substrate breakdown to supply energy needed for increasingly inefficient phosphorylation processes. The decreased glucose uptake is explicable on the same basis if the first step in glucose utilization involves phosphorylation. In order to obtain further information concerning a possible action of salicylates on the formation of high-energy phosphate bonds the effect of salicylate on the levels of creatine phosphate and adenosine triphosphate in the isolated rat diaphragm is being studied.

SUMMARY

1. The effects of salicylate on the glycogen content, lactic acid production, glucose uptake, oxygen consumption and respiratory quotient of the isolated rat diaphragm have been investigated.

2. Salicylate caused an increased glycogen breakdown and lactic acid accumulation under both aerobic and anaerobic conditions, an increased oxygen uptake and respiratory quotient and a decreased glucose uptake of the diaphragms.

3. Possible mechanisms of these changes are discussed.

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