- Northrop, J. H. (1951). *J. gen. Physiol.* **34**, 715.
Postgate, J. R. (1954). *Biochem. J.* **56**, xi.
Postgate, J. R. (1955). *Résumés des Communications, 3rd Int. Congr. Biochem., Brussels*, 10-39.
Postgate, J. R. (1956). *J. gen. Microbiol.* **14**, 545.
Smith, L. (1954a). *Bact. Rev.* **18**, 106.
Smith, L. (1954b). *Arch. Biochem. Biophys.* **50**, 315.
Theorell, H. & Åkeson, A. (1941). *J. Amer. chem. Soc.* **63**, 1812.
Tissières, A. & Burris, R. H. (1956). *Biochim. biophys. Acta*, **20**, 436.
Tsou, C. L. (1952). *Biochem. J.* **50**, 493.
Vernon, L. P. (1953). *Arch. Biochem. Biophys.* **43**, 492.
Weichselbaum, J. E. (1946). *Amer. J. clin. Path. tech. Suppl.* **10**, 40.
Wiggert, W. P., Silverman, M., Utter, M. F. & Werkman, C. H. (1940). *Iowa St. Coll. J. Sci.* **14**, 179.

The Effects of Salicylate on Creatine Phosphate and Adenosine Triphosphate in the Isolated Rat Diaphragm

BY M. J. H. SMITH AND S. W. JEFFREY

Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5

(Received 31 May 1956)

Salicylate produces a number of effects on carbohydrate metabolism in isolated tissues, such as an increased oxygen consumption in mouse-liver slices (Sproull, 1954) and in rat-brain preparations (Fishgold, Field & Hall, 1951), and a decreased glycogen synthesis in rat-liver slices (Smith, 1955a). The effects of salicylate on the rat diaphragm *in vitro* include a rapid breakdown of glycogen, an accumulation of lactic acid and an increased oxygen uptake and respiratory quotient (Smith & Jeffrey, 1956). It was suggested that these changes were indications of increased substrate breakdown resulting from an interference with the formation of energy-rich phosphate bonds. The present paper is concerned with a study of the effects of salicylate on the levels of creatine phosphate, adenosine triphosphate (ATP) and inorganic phosphate in the isolated rat diaphragm.

METHODS

Preparation of the diaphragms. Male rats of the Wistar strain (wt. 100-120 g.) were used. They were kept without food for 24 hr. before being killed by decapitation. The diaphragms were removed and treated as described previously (Smith & Jeffrey, 1956). Two hemidiaphragms from the same animal were used in each Warburg flask and were incubated at 37° for 15-60 min. under an atmosphere of O_2 .

Incubation media. The phosphate-saline solution of Stadie & Zapp (1947) was used initially, because it had been employed in the earlier experiments (Smith & Jeffrey, 1956) concerned with the effects of salicylate on glycogen breakdown and oxygen uptake. However, after incubation in this medium the diaphragms were frequently observed to contract during transference to the freezing mixture before extraction of the phosphate compounds. The salt solution of Sacks & Sinex (1952), in which such contractions did not occur, was therefore used in the later experiments. Glucose was added to both media to give a final concentration of 0.01M, and the sodium salicylate was added from the side arm of the flasks after temperature equilibration to give final salicylate concentrations ranging from 0.1 to 5 mM.

The pH was 7.4 for the salt solution of Stadie & Zapp and 7.0 for the Sacks & Sinex solution, and it did not alter significantly during the course of the experiment.

Methods of analysis. After incubation the diaphragms were removed, blotted on filter paper, and immediately frozen in a mixture of ether and solid CO_2 in a stainless-steel crucible. The subsequent extraction procedures were performed in a room maintained at 2-5°. The frozen tissue was powdered with a stainless-steel pestle and mortar, previously cooled with the freezing mixture, and transferred to a centrifuge tube containing 2 ml. of 10% (w/v) trichloroacetic acid. The mixture was thoroughly stirred for 1 min., centrifuged, the supernatant removed, and the residue re-extracted with 2 ml. of 5% (w/v) trichloroacetic acid. The combined supernatants were neutralized to phenolphthalein with 5N-NaOH and the volume was made up to 10 ml. with water. The total extraction time for each diaphragm was 5-6 min. Inorganic phosphate and creatine phosphate (as acid-molybdate-labile phosphate) were estimated in the trichloroacetic acid extract by the method of Ennor & Stocken (1948). This method is not specific for creatine phosphate and may include small amounts of hexose phosphates and other unidentified compounds (Ennor & Rosenberg, 1952). Total acid-labile phosphate was estimated in the trichloroacetic acid extract after hydrolysis in N-HCl for 10 min. and was assumed to consist mainly of creatine phosphate and ATP. The values observed for the phosphate compounds in the control diaphragms showed good agreement with those reported by other workers using more precise analytical techniques (Sacks & Sinex, 1952).

RESULTS

The results in Table 1 show the effect of 5 mM salicylate on the levels of inorganic phosphate, creatine phosphate and ATP in rat diaphragms incubated aerobically for 15, 30 and 60 min. at 37° in the medium of Stadie & Zapp (1947), and Table 2 gives the results of similar experiments in the medium of Sacks & Sinex (1952). In all the following tables the significances of the differences between the means of the control and salicylate groups have

Table 1. *Effect of 5 mM salicylate on inorganic phosphate, creatine phosphate and ATP of diaphragms incubated aerobically in the medium of Stadie & Zapp (1947)*Results are expressed as mg. of P/100 g. wet wt. (Mean values $\pm$ S.D.).

Time (min.) ... No. of expts.	Inorganic P			Creatine phosphate			ATP		
	15 3	30 6	60 6	15 3	30 6	60 6	15 3	30 6	60 6
Control	63.9 $\pm$ 7.6	61.5 $\pm$ 5.5	65.5 $\pm$ 8.5	22.2 $\pm$ 3.8	21.5 $\pm$ 4.7	17.2 $\pm$ 9.1	30.4 $\pm$ 4.5	38.9 $\pm$ 8.9	30.5 $\pm$ 2.4
Salicylate	81.0 $\pm$ 4.0	77.7 $\pm$ 4.1	82.3 $\pm$ 5.3	5.0 $\pm$ 4.1	3.6 $\pm$ 2.4	4.4 $\pm$ 3.2	15.4 $\pm$ 5.6	6.4 $\pm$ 7.5	11.8 $\pm$ 8.5
P	0.02	0.001	0.01	0.01	0.001	0.01	0.02	0.001	0.001

Table 2. *Effect of 5 mM salicylate on inorganic phosphate, creatine phosphate and ATP of diaphragms incubated aerobically in the medium of Sacks & Sinex (1952)*Results are expressed as mg. of P/100 g. wet wt. (Mean values $\pm$ S.D.).

Time (min.) ... No. of expts.	Inorganic P			Creatine phosphate			ATP		
	15 8	30 12	60 11	15 8	30 12	60 11	15 8	30 6	60 8
Control	23.2 $\pm$ 3.7	25.5 $\pm$ 3.7	25.7 $\pm$ 4.0	22.6 $\pm$ 3.4	19.1 $\pm$ 3.6	18.4 $\pm$ 4.8	27.9 $\pm$ 4.4	20.9 $\pm$ 3.8	24.3 $\pm$ 3.4
Salicylate	51.6 $\pm$ 3.6	41.8 $\pm$ 4.2	36.4 $\pm$ 4.6	1.5 $\pm$ 2.5	3.5 $\pm$ 2.9	3.8 $\pm$ 2.0	14.3 $\pm$ 6.0	3.9 $\pm$ 2.9	7.6 $\pm$ 2.7

Statistical comparison of the results of the control and salicylate groups showed that $P < 0.001$ in every case.Table 3. *Effect of decreasing concentrations of salicylate on inorganic phosphate, creatine phosphate and ATP of diaphragms*Diaphragms were incubated aerobically for 60 min. in the medium of Sacks & Sinex (1952). Results are expressed as mg. of P/100 g. wet wt. (Mean values $\pm$ S.D.).

Concn. of salicylate (mM)	No. of experiments	Inorganic phosphate	Creatine phosphate	ATP
Control	8	25.7 $\pm$ 4.0	18.4 $\pm$ 4.8	24.3 $\pm$ 3.4
5	8	36.4 $\pm$ 4.6 $P = 0.001$	3.8 $\pm$ 2.0 $P = 0.001$	7.6 $\pm$ 2.7 $P = 0.001$
1	7	35.0 $\pm$ 7.5 $P = 0.01$	4.9 $\pm$ 2.6 $P = 0.001$	7.8 $\pm$ 4.3 $P = 0.001$
0.5	6	31.5 $\pm$ 1.9 $P = 0.01$	14.2 $\pm$ 3.7 $P = 0.05$	16.6 $\pm$ 3.4 $P = 0.001$
0.1	7	27.4 $\pm$ 2.4 $P = 0.3$	13.7 $\pm$ 2.1 $P = 0.02$	16.5 $\pm$ 2.5 $P = 0.001$

been analysed by Student's *t* test and values of *P* are included.

Salicylate (5 mM) caused significant reductions of creatine phosphate and ATP and a concomitant increase in the inorganic phosphate at 15, 30 and 60 min. in both media. The creatine phosphate disappeared more rapidly than the ATP as shown by the results at 15 min.

Table 3 shows that these effects of salicylate were diminished by decreasing the concentration of salicylate, although significant reductions of the creatine phosphate and ATP in the diaphragms were observed after an incubation for 60 min. with 0.1 mM salicylate.

DISCUSSION

The present results show that salicylate caused significant reductions in the levels of energy-rich phosphate bond compounds in the rat diaphragm. Creatine phosphate almost completely disappeared after incubation for 15 min. and ATP was reduced to about half the corresponding control value at 15 min. and to a constant low level with longer incubation periods.

One of the possible mechanisms to explain these changes is that salicylate interferes with the formation of ATP and thus causes an increased breakdown of creatine phosphate by the Lohmann reaction. The

Table 4. Comparison of the effect of dinitrophenol and salicylate on the rat diaphragm

	Dinitrophenol	Salicylate
O ₂ uptake	Increased by 5×10^{-5} M. Increase at 10^{-4} M followed by inhibition ^{a-c}	Increased by 10^{-4} M to 5×10^{-3} M. Increase at 5×10^{-3} M followed by inhibition ^a
Glycogen breakdown	Increased by 5×10^{-5} M to 10^{-4} M under aerobic conditions ^{b, d, e}	Increased by 5×10^{-4} M to 5×10^{-3} M under aerobic conditions ^a
Glucose uptake	Decreased by 5×10^{-5} M to 10^{-4} M at 1 hr. ^b	Decreased by 5×10^{-3} M at 2 hr. ^a
CO ₂ production	Increased production of ¹⁴ CO ₂ from [¹⁴ C]glucose by 5×10^{-5} M ^b	Increased R.Q. by 5×10^{-3} M ^a
Creatine phosphate and ATP concn.	Decreased by 2×10^{-5} M to 10^{-4} M ^{c, f, g}	Decreased by 10^{-4} M to 5×10^{-3} M ⁱ

^a Beloff-Chain *et al.* (1953). ^b Villee, Deane & Hastings (1949). ^c Sacks & Sinex (1952). ^d Pierce & Field (1949). ^e Stadie, Haugaard & Marsh (1951). ^f Barnes, Duff & Threlfall (1955). ^g Ottaway (1955). ^h Smith & Jeffrey (1956). ⁱ Present work.

increased oxygen uptake of the diaphragms incubated with salicylate (Smith & Jeffrey, 1956) suggests that the impairment of production of energy-rich phosphate bond compounds may result from an interference with oxidative phosphorylation reactions rather than with either creatine phosphokinase or glycolysis. Some support for this view is provided by the close similarity between the effects of salicylate and 2,4-dinitrophenol which is known to uncouple oxidative phosphorylation reactions. Both substances increase oxygen consumption in man (Cochran, 1952; Tainter & Cutting, 1933), in the rat (Meade, 1954), and in isolated tissues (Sproull, 1954), and they have very similar actions on the rat diaphragm (Table 4).

The rapid breakdown of glycogen and the increased accumulation of lactic acid and increased respiratory quotient observed in diaphragms incubated with 5 mM salicylate (Smith & Jeffrey, 1956) may be evidence of substrate breakdown to provide energy for inefficient phosphorylation mechanisms. The decreased glucose uptake which was also found is explicable on the same basis if the first step in glucose utilization involves phosphorylation.

The concept that salicylate may cause a defect in the energy production and hence impair the synthetic efficiency of the tissues may provide an explanation of some of the effects of the substance on carbohydrate metabolism in animals. The injection of sodium salicylate, producing plasma salicylate concentrations of the order of 5 mM, causes a marked lowering of the blood-glucose concentration of the fasted alloxan-diabetic rat (Smith, Meade & Bornstein, 1952). In this animal preparation the fasting blood glucose must be derived from synthetic reactions, the increased liver glucose 6-phosphatase activity present (Langdon & Weakley, 1955) producing hyperglycaemia rather than liver-glycogen deposition. The hypoglycaemic effect of salicylate may therefore be due to defective glucose synthesis resulting from an interference with the

formation of energy-rich phosphate bonds. A similar explanation may apply to the hypoglycaemic action of salicylate observed in diabetic patients (Dibenedetto Dell'Aquila & Angarano, 1954) and in adrenalectomized rats (Smith, 1955b) and hypophysectomized rats (Comulato, Carlo & Smith, 1953). Although an increased rate of glycogenolysis due to release of adrenaline may be an important factor (Smith, 1955b) in the depletion of liver glycogen caused by salicylate in the normal rat (Lutwak-Mann, 1942), impairment of glycogenesis due to an inadequate production of energy-rich phosphate bonds may also be concerned, particularly as salicylate diminished glycogen synthesis in isolated rat-liver slices (Smith, 1955a). The antagonism between salicylate and adrenal-cortical extracts and steroids in the deposition of liver glycogen in the fasting rat (Winters & Morrill, 1955) and the adrenalectomized rat (Smith, 1952), and in rat-liver slices (Smith, 1955a), is also explicable on the same basis if the glycogenesis, normally induced by the adrenal-cortical compounds, is inhibited by a failure of formation of energy-rich phosphate bonds in the presence of salicylate.

To obtain further information about the possible action of salicylate on oxidative-phosphorylation processes it is proposed to study the effect of salicylate on the oxygen consumption and uptake of inorganic phosphate of mitochondrial preparations.

SUMMARY

1. The effects of salicylate on the amounts of creatine phosphate, adenosine triphosphate (ATP) and inorganic phosphate in the isolated rat diaphragm have been investigated.

2. Salicylate caused decreased levels of creatine phosphate and ATP and increased the inorganic phosphate of the diaphragms.

3. A possible mechanism and some implications of these changes are discussed.

We wish to thank the Diabetic Association and the Board of Governors of King's College Hospital for grants towards the cost of the work. The Warburg tissue respirometer was purchased by a grant from the Central Research Fund of the University of London.

Note added in proof. Since this paper was accepted for publication Penniall (1956) and Brody (1956) have shown that 2 mM salicylate uncouples oxidative phosphorylation in preparations of rat-brain, -kidney and -liver mitochondria.

REFERENCES

- Barnes, J. M., Duff, J. I. & Threlfall, C. J. (1955). *J. Physiol.* **130**, 585.
- Beloff-Chain, A., Cantanzaro, R., Chain, E. B., Longinotti, L., Masi, I. & Rossi, F. B. (1953). *R.C. Accad. Lincei*, **14**, 740.
- Brody, T. M. (1956). *J. Pharmacol.* **117**, 39.
- Cochran, J. B. (1952). *Brit. med. J.* **2**, 964.
- Comulato, G. M., Carlo, P. & Smith, P. K. (1953). *Fed. Proc.* **12**, 313.
- Dibenedetto Dell'Aquila, M. & Angarano, D. (1954). *Folia endocrin., Pisa*, **7**, 5.
- Ennor, A. H. & Rosenberg, H. (1952). *Biochem. J.* **51**, 606.
- Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 549.
- Fishgold, J. T., Field, J. & Hall, V. E. (1951). *Amer. J. Physiol.* **164**, 727.
- Langdon, R. G. & Weakley, D. R. (1955). *J. biol. Chem.* **214**, 167.
- Lutwak-Mann, C. (1942). *Biochem. J.* **36**, 706.
- Meade, B. W. (1954). *Ann. Rheum. Dis.* **13**, 60.
- Ottaway, J. H. (1955). *Biochem. J.* **61**, 441.
- Penniall, R. (1956). *Fed. Proc.* **15**, 608.
- Pierce, F. T. & Field, J. (1949). *J. biol. Chem.* **180**, 895.
- Sacks, J. & Sinex, F. M. (1952). *Arch. Biochem. Biophys.* **39**, 205.
- Smith, M. J. H. (1952). *Biochem. J.* **52**, 649.
- Smith, M. J. H. (1955a). *Biochem. J.* **59**, 52.
- Smith, M. J. H. (1955b). *Brit. J. Pharmacol.* **10**, 110.
- Smith, M. J. H. & Jeffrey, S. W. (1956). *Biochem. J.* **63**, 524.
- Smith, M. J. H., Meade, B. W. & Bornstein, J. (1952). *Biochem. J.* **51**, 18.
- Sproull, D. H. (1954). *Brit. J. Pharmacol.* **9**, 262.
- Stadie, W. C., Haugaard, N. & Marsh, J. B. (1951). *J. biol. Chem.* **188**, 173.
- Stadie, W. C. & Zapp, J. A. (1947). *J. biol. Chem.* **170**, 55.
- Tainter, M. L. & Cutting, W. C. (1933). *J. Pharmacol.* **49**, 187.
- Villee, C. A., Deane, H. W. & Hastings, A. B. (1949). *J. cell. comp. Physiol.* **34**, 159.
- Winters, R. W. & Morrill, M. F. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 409.