The Effect of Sodium Salicylate on the Ion and Water Content of Isolated Rat-Liver Mitochondria

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Previous investigations in this Laboratory (Hetzal, Charnock & Lander, 1959; Charnock, Lockett & Hetzel, 1962) have shown that the administration of therapeutic doses of salicylate drugs to both man and rats disturbs the normal electrolyte balance in addition to increasing the metabolic rate. The chronic administration of both sodium salicylate and potassium salicylate to rats produces a significant decrease in the intracellular K^+ ion concentration in the livers of these animals (Charnock, Opit & Hetzel, 1961). A similar effect of sodium salicylate on isolated rat diaphragm has been reported by Manchester, Randle & Smith (1958). These workers noted that an isomer of sodium salicylate, sodium *p*-hydroxybenzoate, failed to exert this effect just as it fails to exert a number of other effects associated with the action of the salicylate drugs both *in vivo* and *in vitro* (Hetzal *et al.* 1959; Randle & Smith, 1958*a, b*; Segal & Blair, 1959; Packer, Austen & Knoblock, 1959; Andrews, 1960).

The suggestion has been made that salicylates exert at least part of their metabolic effect *in vivo* by uncoupling oxidative-phosphorylation processes in mitochondria (Charnock, Opit & Hetzel, 1962; Smith, 1955; Smith, 1949). It was decided therefore to investigate a possible relationship between the uncoupling phenomenon and the disturbance of the electrolyte balance by comparing the effect of salicylate and *p*-hydroxybenzoate on the movement of Na^+ and K^+ ions in isolated rat-liver mitochondria. To dissociate the effect of active metabolism observations were made at 0°.

EXPERIMENTAL

Liver mitochondria were prepared from rats by a modification of the method of Hogeboom, Schneider & Pallade (1948). The fresh liver was initially homogenized in a solution of 0.44M-sucrose adjusted to pH 6.8 by the addition of potassium hydroxide. EDTA (1 mM) was also present in those experiments concerned with ion movement, but was sometimes absent (indicated in the text) in those experiments concerned with the water content of mitochondria. Sucrose (0.44M) without any additions was

used to wash all mitochondria once. Such sucrose solutions were used as similar concentrations inhibit spontaneous swelling and contraction of mitochondria (Lehninger, 1959), and confer stability to the K^+ ion-binding sites of mitochondria (Gamble, 1957).

In the ion-movement experiments washed mitochondrial pellets, each derived from approximately 1 g. of fresh liver, were resuspended to give a small final volume (usually 4 ml.) in 0.44M-sucrose to which sodium salicylate or sodium *p*-hydroxybenzoate or sodium chloride was added to give a final concentration of 5 mM, a concentration of salicylate previously shown to exert maximal uncoupling action on mitochondrial oxidative phosphorylation (Jeffrey & Smith, 1959; Charnock, Opit & Hetzel, 1962). The pH of all the various solutions was measured at 0° with a glass electrode and found to be 5.95–6.20, except for the solution containing sodium chloride which had a pH of 4.6. After the addition of mitochondria, the pH of the sucrose-sodium chloride mixture was 6.0, whereas with sucrose alone and the sucrose-*p*-hydroxybenzoate and the sucrose-salicylate mixtures the pH range was 7.0–7.2. After suspension for 10 min. in the various solutions at an air temperature of 0° the mitochondria were quickly harvested by centrifuging at 15 000*g* in preweighed tubes. The supernatant fraction was collected without disturbing the particulate phase and the pellets were drained for a few seconds by inversion of the tubes, the walls of which were then dried with absorbent tissue, and the wet weight of each pellet was quickly determined. The mitochondria were then resuspended in a small volume in electrolyte-free redistilled water. These suspensions and their corresponding supernatant fractions were then assayed for sodium and potassium by internal-standard flame photometry. Mitochondria were always prepared in batches of six pellets so that some from each batch could be suspended in 0.44M-sucrose solution without any additions, and thus serve as internal controls in individual experiments.

For the indirect examination of mitochondrial water content, about 200 mg. of freshly prepared once-washed mitochondria was resuspended in 10 ml. of 0.44M-sucrose. Sufficient of this suspension (usually about 0.01 ml.) was then added to 3 ml. of 0.3M-sucrose-1 mM-tris medium (Tapley, 1956) to give an initial extinction of 0.5–0.6 when read at 520 *mμ* in a Beckman DU spectrophotometer. The extinction measurements were then made at regular intervals for 30 min. at 23°. This method gives a reliable indication of intramitochondrial water content (Lehninger, 1959).

All chemicals were of the highest purity AnalaR grade, and none of the sucrose-salt solutions contained detectable K^+ ions before the addition of the mitochondria; Na^+ ions could not be detected in the 0.44M-sucrose solution.

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Representative samples of the mitochondrial pellets were used for the gravimetric determination of their fluid content by drying to constant weight at 105°. The concentration of solutes dispersed in the total fluid space of the mitochondrial pellet could then be calculated (Charnock, Opit & Hetzel, 1962). In these calculations no allowance was made for any extra-particulate fluid component.

RESULTS

Movement of sodium and potassium ions

Effect of 0.44 M-sucrose. Neither K^+ nor Na^+ ions were present in the suspending solutions before the experiments, but both were detected after the immersion of washed rat-liver mitochondria for 10 min. at 0° and their subsequent isolation by high-speed centrifuging at 0°. The mean loss of K^+ ions from mitochondria exceeded that of Na^+ ions by about 2:1 (12.6 μ equiv. of K^+ ion/g. of wet mitochondria; 6.4 μ equiv. of Na^+ ion/g. of wet mitochondria). The mean concentrations of these ions remaining in the mitochondria after this treatment were 16.9 μ equiv. of K^+ ion/ml. and 9.3 μ equiv. of Na^+ ion/ml. of 'intramitochondrial fluid' (Table 1).

Effect of 5 mM-sodium salicylate. The immersion of mitochondria in a 0.44 M-sucrose medium containing sodium salicylate (5 mM) increased the mean loss of K^+ ions by nearly 60 % to 20.9 μ equiv. of K^+ ion/g. of wet mitochondria ($P < 0.001$). Salicylate also prevented the loss of Na^+ ions. The mean residual mitochondrial K^+ ion concentration was decreased to 10.0 μ equiv. of K^+ ion/ml. of intramitochondrial fluid but the concentration of Na^+ ions was little changed (8.7 μ equiv. of Na^+ ion/ml.) from that found after immersion in 0.44 M-sucrose alone (Table 1).

Effect of 5 mM-sodium p-hydroxybenzoate. The presence of this isomer of sodium salicylate in the suspending solution did not increase the loss of K^+

ions from mitochondria, which was unchanged from that of the control experiments (12.4 μ equiv. of K^+ ion/g. of wet mitochondria), but did prevent the loss of Na^+ ions from the mitochondria. The mean concentrations of K^+ and Na^+ ions remaining in the mitochondria after this treatment were 17.8 μ equiv. of K^+ ion/ml. and 7.9 μ equiv. of Na^+ ion/ml. of intramitochondrial fluid (Table 1). These values were similar to those of the control experiments.

Effect of 5 mM-sodium chloride. The presence of 5 mM-sodium chloride in the suspending solution produced a 14 % increase in the loss of K^+ ions from mitochondria (14.4 μ equiv./g. of wet mitochondria) when compared with the control experiments. The loss of Na^+ ions was again prevented. The concentrations of ions retained by the mitochondria after this treatment were 16.6 μ equiv. of K^+ ion/ml. and 8.6 μ equiv. of Na^+ ion/ml. of intramitochondrial fluid. These values are similar to those of the control (Table 1).

Mitochondrial water content

Liver mitochondria from untreated rats can exhibit different spectrophotometric properties, dependent on the presence or absence of the complexing agent EDTA in the solution in which the fresh liver was homogenized (Fig. 1). When the mitochondrial suspension was prepared in the presence of EDTA the extinction, and hence the water content, remained nearly constant for 30 min. (Fig. 1A); these mitochondria were termed 'stable'. When prepared in the absence of EDTA, the extinction of the mitochondrial suspension declined markedly in 30 min. (Fig. 1B), i.e. these mitochondria spontaneously increased in water content and were termed 'unstable'. This EDTA-influenced difference in spectrophotometric behaviour was also apparent in the response of these

Table 1. *Changes in potassium and sodium ions in rat-liver mitochondria at 0° after soaking for 10 min. in various solutions*

The experimental conditions are given in the text. -, Loss from mitochondria; +, gain to mitochondria.

Treatment of mitochondria	No. of expts.	Pellet water content (ml./100 g. of mitochondria)		Electrolyte taken up or extruded (μ equiv./g. of wet mitochondria)		Residual mitochondrial ionic concentration (μ equiv./ml. of intramitochondrial fluid)	
		Before soaking	After soaking	K^+ ion	Na^+ ion	K^+ ion	Na^+ ion
0.44 M-Sucrose alone	13	71.3 $\pm$ 2.0	72.3 $\pm$ 2.0	-12.6 $\pm$ 3.1	-6.4 $\pm$ 2.5	16.9	9.3
5 mM-Sodium salicylate + 0.44 M-sucrose	10		71.7 $\pm$ 2.2	-20.9 $\pm$ 6.4	+0.2 $\pm$ 0.87	10.0	8.7
5 mM-Sodium p-hydroxybenzoate + 0.44 M-sucrose	5		71.6 $\pm$ 2.2	-12.4 $\pm$ 0.84	+0.7 $\pm$ 0.55	17.8	7.9
5 mM-Sodium chloride + 0.44 M-sucrose	3		72.3 $\pm$ 1.3	-14.4 $\pm$ 0.92	0	16.6	8.6

Fig. 1. Effect of salicylate on the water content of rat-liver mitochondria at 23°. The isolation and test system of mitochondria are described in the text. A, Fresh tissue homogenized in 0.44M-sucrose containing EDTA (1 mM). B, Fresh tissue homogenized in 0.44M-sucrose alone. All mitochondria were washed in 0.44M-sucrose without addition. ●, No addition; ▲, 5 mM-sodium salicylate; ■, 5 mM-sodium *p*-hydroxybenzoate; □, 10 mM-magnesium chloride; ○, 5 mM-sodium salicylate, then 10 mM-magnesium chloride added after 10 min.

differently prepared mitochondria to the subsequent addition of 5 mM-sodium salicylate. This agent initiated the swelling of previously stable mitochondria and inhibited the spontaneous swelling of previously unstable mitochondria. Sodium *p*-hydroxybenzoate (5 mM) did not affect the behaviour of either stable or unstable mitochondria. The salicylate-induced swelling of mitochondria can be abolished by the addition of excess of Mg^{2+} ion (Fig. 1A).

Mitochondria were also prepared from three samples of fresh human liver taken at operation. Although the difficulties in obtaining sufficient material prevented a full investigation of their properties, no apparent differences could be found in the response of human-liver mitochondria to salicylate when compared with that of rat-liver mitochondria, prepared in the presence or absence of EDTA.

In contrast with these studies carried out at 23° gravimetric studies on intramitochondrial water content at 0° revealed only insignificant changes after soaking in the various media (Table 1).

DISCUSSION

Isolated mitochondria from a variety of sources have been reported to actively accumulate K^+ and Na^+ ions and maintain relatively high internal concentrations of these ions against concentration

gradients (Macfarlane & Spencer, 1953; Bartley & Davies, 1952; Price, Fonnesu & Davies, 1956). Ulrich (1961) has confirmed a potassium gradient but suggested that there was no evidence for active extrusion of sodium.

The present experiments were carried out at 0° in the absence of added oxidizable substrate or cofactors and with no attempt to oxygenate the system. Under these conditions, active transport would be minimal and any movement of ions would not be influenced by metabolic effects.

The presence of sodium salicylate (5 mM) in the suspending solution markedly increased the loss of endogenous K^+ ions from mitochondria compared with that observed in the presence of 5 mM-sodium *p*-hydroxybenzoate or in control experiments with 0.44M-sucrose alone. Ulrich (1961) has reported that the leakage of K^+ ions from kidney mitochondria was increased at acid pH. In the present studies such a factor can be excluded by the fact that there was little difference in pH between sucrose alone and the sucrose-salicylate medium. The effect of salicylate cannot be attributed to an exchange of Na^+ for K^+ ions, because although Na^+ ions prevented a decrease of the mitochondrial Na^+ ion concentration they did not compensate for the loss of K^+ ions. The finding suggests that salicylates may directly affect the bound fraction of mitochondrial K^+ ions described by Stanbury & Mudge (1953).

The demonstration of this effect of salicylate on the mitochondrial K^+ ion content at 0° is in agreement with a previous suggestion that salicylates act at the mitochondrial membrane (Charnock & Opit, 1962). This could be a direct effect not dependent on uncoupling oxidative-phosphorylation processes leading to a breakdown in a metabolically maintained K^+ ion gradient (Manchester *et al.* 1958). As suggested by Tapley (1956) these changes may precede such an uncoupling effect. Both Werkheiser & Bartley (1957) and Berger (1957) have independently reported a similar effect of 2,4-dinitrophenol at 0°, in concentrations which at higher temperatures were shown to uncouple mitochondrial oxidative phosphorylation.

The stabilizing effect of EDTA on the spectrophotometric properties of isolated mitochondria (Hunter *et al.* 1959) was confirmed, as was the salicylate-induced inhibition of spontaneous swelling described by Jeffrey & Smith (1959). Salicylate also promoted passive swelling in stable preparations of mitochondria prepared in the presence of EDTA. However, the gravimetric estimation of water content of the mitochondrial pellets soaked at 0° showed no change. It was considered that this absence of swelling at 0° may be explained by two factors. The first factor is the increased osmolarity of the medium used at 0° compared with that used

for the spectrophotometric study. The second factor results from the marked decrease of electron transport occurring at 0° since such electron transport seems to be necessary for non-osmotic mitochondrial swelling (Hunter *et al.* 1959).

In the light of all these findings it appears likely that there is a complex interaction between salicylate, EDTA and magnesium which affects the permeability of a mitochondrial membrane. Such membrane effects may be quite distinct from changes in oxidative phosphorylation.

SUMMARY

1. Sodium salicylate (5 mM) (but not sodium *p*-hydroxybenzoate or sodium chloride) increases by 60% the loss of K⁺ ions from washed rat-liver mitochondria suspended in 0.44M-sucrose for 10 min. at 0°.

2. Salicylate also induced swelling of rat-liver mitochondria prepared in the presence of EDTA.

3. The conditions of the experiments minimize a metabolic effect of salicylate. It is suggested that these effects of salicylate are primarily on the mitochondrial membrane.

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REFERENCES

- Andrews, M. M. (1960). *Biochem. J.* **75**, 298.
Bartley, W. & Davies, R. E. (1952). *Biochem. J.* **52**, xx.
Berger, M. (1957). *Biochim. biophys. Acta*, **23**, 504.

- Charnock, J. S., Lockett, R. & Hetzel, B. S. (1962). *Nature, Lond.*, (in the Press).
Charnock, J. S. & Opit, L. J. (1962). *Biochem. J.* **83**, 596.
Charnock, J. S., Opit, L. J. & Hetzel, B. S. (1961). *Metabolism*, **10**, 874.
Charnock, J. S., Opit, L. J. & Hetzel, B. S. (1962). *Biochem. J.* **83**, 602.
Gamble, J. L., jun. (1957). *J. biol. Chem.* **228**, 955.
Hetzel, B. S., Charnock, J. S. & Lander, H. (1959). *Metabolism*, **8**, 205.
Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Hunter, F. E., Levy, J. F., Fink, J., Schutz, B., Guerra, F. & Hurlvitz, A. (1959). *J. biol. Chem.* **234**, 2176.
Jeffrey, S. W. & Smith, M. J. H. (1959). *Biochem. J.* **72**, 462.
Lehninger, A. L. (1959). *J. biol. Chem.* **234**, 2187.
Macfarlane, M. G. & Spencer, A. G. (1953). *Biochem. J.* **54**, 569.
Manchester, K. L., Randle, P. J. & Smith, G. H. (1958). *Brit. med. J.* **i**, 1028.
Packer, L., Austen, F. K. & Knoblock, E. C. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 239.
Price, C. A., Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 754.
Randle, P. J. & Smith, G. H. (1958a). *Biochem. J.* **70**, 490.
Randle, P. J. & Smith, G. H. (1958b). *Biochem. J.* **70**, 501.
Segal, S. & Blair, A. (1959). *Nature, Lond.*, **183**, 1609.
Smith, M. J. H. (1955). *Biochem. J.* **59**, 52.
Smith, P. K. (1949). *Pharmacol. Rev.* **1**, 353.
Stanbury, S. W. & Mudge, G. H. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 675.
Tapley, D. F. (1956). *J. biol. Chem.* **222**, 325.
Ulrich, F. (1961). *Biochem. J.* **80**, 532.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

Biochem. J. (1962) **85**, 193

Carbohydrate Sulphatases of Marine Molluscs

ASSAY OF GLYCOSULPHATASES

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Sulphatases catalyse the reaction:

and methods of assay of such enzymes may be based upon measurement of unchanged substrate (I), desulphated residue (II), inorganic sulphate (III) or change of pH (IV). When (I) is a sugar sulphate and therefore (II) is the corresponding free sugar, the hydrolytic enzyme is usually described as a glycosulphatase. The assay of enzymes

of the sulphatase type has been discussed by Dodgson & Spencer (1957).

Soda (1936) and his co-workers, in the investigation of the glycosulphatases of Japanese molluscs, used methods of assay which included the indirect determination of unhydrolysed substrate (by gravimetric estimations of inorganic sulphate liberated on acid hydrolysis) and the measurement of liberated inorganic sulphate by potentiometric titration or nephelometrically. These methods are not capable of a high degree of accuracy unless