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An Evaluation of the Effect of Salicylate on Oxidative Phosphorylation in Rat-Liver Mitochondria

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The antipyretic, anti-inflammatory and analgesic properties of the salicylates are well known, although intensive investigation of the pharmacology of these agents has failed to demonstrate their mechanism of action. An uncoupling action of salicylate on oxidative phosphorylation processes in isolated mitochondria *in vitro* has been well documented (Brody, 1956; Penniall, 1958; Jeffery & Smith, 1959); this action appears to be compatible with many of the effects of salicylate on the whole animal (Hetzel, Charnock & Lander, 1959; Charnock, Opit & Hetzel, 1961), and has been proposed as the physiological mechanism of action of the drug (Smith, 1955; Reid, 1958).

During a study of the effect of salicylate on mitochondrial adenosine-triphosphatase activity (Charnock & Opit, 1962), it was found that mitochondria with unchanged properties of oxidative phosphorylation and adenosine-triphosphatase activity could be isolated from rats poisoned with salicylate *in vivo*.

Therefore an assessment of the physiological significance of salicylate-induced uncoupling of mitochondrial oxidative phosphorylation seemed warranted. The results of an examination of both this effect and of the permeability of mitochondria to salicylate *in vitro* and *in vivo* are reported here.

EXPERIMENTAL

Oxidative phosphorylation. Young actively growing male laboratory rats weighing 200–250 g. were used, to avoid the decrease in phosphorylation rates associated with liver tissue of older animals (Weinbach & Garbus, 1956, 1959). Sodium salicylate (30 mg. of salicylate radical/100 g. body weight/day) was given by stomach tube until symptoms of salicylate poisoning, e.g. hyperventilation, body wastage and haemorrhage, were evident. One-third of the dose was given at 9.00 a.m. each day, and the remainder 10 hr. later.

Liver tissue was homogenized in 0·44M-sucrose, containing EDTA (1 mM), adjusted to pH 6·8 with potassium hydroxide.

Mitochondria were isolated from this mixture by the method of Hogeboom, Schneider & Pallade (1948). The temperature was maintained below 1° at all stages in the preparation. The mitochondria were washed once with 0·44M-sucrose and the pellets were finally suspended in ice-cold 0·25M-sucrose. Mitochondria derived from 0·5 g. of wet liver were added last to each test vessel, which contained (final vol. 3 ml.): 50 μmoles of inorganic phosphorus as phosphate buffer, pH 6·8; 24 μmoles of potassium chloride; 30 μmoles of magnesium sulphate; 2·5 μmoles of ATP (sodium salt) (Sigma Chemical Co.); 30 μmoles of glucose; 30 μmoles of potassium fluoride; 25 μmoles of an aqueous soln. of α-oxoglutaric acid, β-hydroxybutyric acid or succinic acid as substrate. The free acids were neutralized to pH 6·8 with potassium hydroxide. Potassium malonate (pH 6·8) (25 μmoles) was added to inhibit succinoxidase activity when α-oxoglutarate was the substrate. The phosphate-acceptor system, which was tipped from the side arm of the vessels after temperature equilibration, was 0·2 ml. of 1% (w/v) glucose containing 30 units of hexokinase (Sigma Type III). The centre well of each Warburg vessel contained 0·2 ml. of 5M-potassium hydroxide. After incubation for 30 min. at 30° in air, the reaction was stopped by the addition of 0·2 ml. of 10N-sulphuric acid. The esterification of inorganic phosphorus was determined indirectly by the loss of inorganic phosphorus from the medium, with the method of Fiske & Subbarow (1925) as modified by Taussky & Shorr (1953).

When the effect of the addition of sodium salicylate *in vitro* was examined, a final concentration of 5 mM was

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used. This concentration was selected because similar concentrations of salicylate have been demonstrated in the plasma and intracellular fluid of the liver of rats poisoned with salicylate *in vivo* (Charnock *et al.* 1961); also, Jeffery & Smith (1959) have shown that 5 mM-sodium salicylate produces maximal 'uncoupling' effects *in vitro*.

Permeability of mitochondria to salicylate in vitro. Mitochondrial pellets were suspended in a known volume (usually 4 ml.) of 0·44M-sucrose with or without sodium salicylate (5 mM) for 10–20 min. The mitochondria were then rapidly sedimented by centrifuging at 10 000*g* for 5 min. The mitochondrial pellets were freed from suspending medium by removal of the supernatant fluid by Pasteur pipette and then rapid inversion of the centrifuge tubes; after a few seconds' draining the sides of the tubes were dried with absorbent tissue. All procedures were conducted at 0°. Some of the drained mitochondrial pellets were used for the gravimetric determination of their fluid content by drying to constant weight at 105°; others were immediately resuspended in water and assayed for salicylate; and some were resuspended in a small volume of ice-cold 0·25M-sucrose and examined for oxidative phosphorylation in the system already described. In some cases mitochondria originally suspended in medium containing salicylate were washed once in sucrose medium, sedimented by centrifuging and resuspended in water, and their salicylate content was then determined. Salicylate was estimated by the method of Trinder (1954). The molar concentration of salicylate in the total fluid space of the mitochondria was calculated from the salicylate content (in μg.) and the fluid space of the pellets, derived (in ml.) from the wet weight and percentage water content of the mitochondria.

Permeability of the liver to salicylate in vivo. Portions of the livers of rats poisoned with salicylate were disrupted in the minimal volume of 0·44M-sucrose medium to yield a concentrated fraction designated 'whole homogenate'. A sample of this fraction was immediately assayed for free salicylate (Trinder, 1954) and chloride content (Schales & Schales, 1941). The remainder of the concentrated homogenate was diluted with 2 vol. of sucrose medium and separated by differential centrifuging into fractions designated 'intracellular fluid' and 'mitochondrial fraction'. The mitochondria were washed once in 0·44M-sucrose, as for the mitochondria used for oxidative-phosphorylation experiments, and then both these fractions were assayed for their salicylate content.

Plasma was prepared from blood collected from these animals immediately before hepatectomy. Plasma salicylate was estimated by the method of Trinder (1954) and chloride by the method of Schales & Schales (1941). Determination of the 'chloride space' in these animals (Widdowson & Southgate, 1959; Charnock *et al.* 1961) allowed the extracellular contribution of salicylate to the intracellular fractions of the liver to be calculated.

RESULTS

Oxidative phosphorylation. The addition of 5 mM-sodium salicylate to the test systems *in vitro* completely inhibited the phosphorylation associated with the oxidation of both succinate and β-hydroxybutyrate, and markedly reduced that associated with the oxidation of α-oxoglutarate (Table 1). The mean P:O ratio obtained in ten such experiments with α-oxoglutarate in the presence of salicylate was below unity. These results are in agreement with those of Jeffery & Smith (1959).

Table 1 also gives the P:O ratios found in 40 experiments with mitochondria from the livers of rats poisoned with salicylate and examined in test systems identical with those of the control experiments. No decrease in the efficiency of oxidative phosphorylation could be detected with any of the three substrates examined under these conditions. The mean P:O ratios were uniformly higher than those of 59 control experiments, but the differences were not statistically significant ($P > 0.05$) (Student's t test). Hence the mitochondria from salicylate-poisoned animals were 'coupled' to an extent equal to that of those from untreated animals, even though the plasma concentration of salicylate from the former rats was 2–5 mM.

In further experiments, mitochondria from untreated rats were resuspended for 10–20 min. at 0° in sucrose medium with or without sodium salicylate (5 mM) and then isolated again, and their efficiency of oxidative phosphorylation was determined. This procedure ensured that some mito-

Table 1. *Efficiency of oxidative phosphorylation of rat-liver mitochondria utilizing three substrates*

The reaction medium and incubation conditions are described in the text. The amount of inorganic phosphate esterified and oxygen utilized are signified by P and O respectively. Figures shown are the arithmetic means.

Treatment	Substrate	No. of experiments	P (μg.atoms)	O (μg.atoms)	P:O ratio
None	α-Oxoglutarate	30	12·6	4·9	2·6
	Succinate	13	7·8	5·4	1·4
	β-Hydroxybutyrate	16	4·4	2·7	1·6
Salicylate *in vitro*	α-Oxoglutarate	10	3·4	4·0	0·8
	Succinate	8	0	3·3	—
	β-Hydroxybutyrate	10	0	2·9	—
Salicylate *in vivo*	α-Oxoglutarate	18	16·5	5·3	3·1
	Succinate	12	7·5	5·1	1·6
	β-Hydroxybutyrate	10	7·6	4·2	1·8

chondria were exposed to sodium salicylate at a concentration shown to uncouple oxidative phosphorylation under other conditions.

Suspension of the mitochondria in sucrose solution had little effect on the efficiency of mitochondrial oxidative phosphorylation, the P:O ratios being similar to those found in previous control series (Table 2). Suspension of mitochondria in a sucrose solution containing salicylate depressed the P:O ratios slightly (data from ten experiments), but the differences were not statistically different ($P > 0.05$) [rank T test (White, 1952)]. The concentration of salicylate in duplicate mitochondrial pellets after suspension in medium containing salicylate ranged from 4·8 to 11·0 mM.

Permeability of isolated mitochondria to sodium salicylate at 0°. The concentration of salicylate was estimated in a number of particulate and non-particulate fractions obtained by a procedure in which liver mitochondria (washed once) from untreated rats were suspended at 0° in a sucrose medium containing sodium salicylate (5 mM). The salicylate content of the mitochondrial fraction reached equilibrium with that of the suspending medium in 10 min., and in some experiments of longer duration exceeded the concentration of the medium (Table 3). Salicylate was not bound firmly to mitochondria under these conditions, for 78–95 % of it could be removed by one rapid wash in salicylate-free sucrose medium.

Permeability of rat liver to salicylate in vivo. Table 4 shows the concentration of salicylate both in the plasma and various liver fractions of salicylate-poisoned rats. In six of the seven preparations examined the concentration of free salicylate in the whole homogenate was greater than 75 % of that in the plasma of those animals; in the remaining animal (A) the concentration was 55 % of that of the plasma. A mitochondria-free fraction equivalent to the intracellular fluid contents of the liver cell also had a salicylate concentration at least 80 % of that in the whole homogenate (in four of five samples examined). No appreciable salicylate was detected in any of the mitochondrial fractions examined, nor was there any evidence of a concentration of salicylate within the liver greater than that in the plasma.

Table 2. *Oxidative phosphorylation of rat-liver mitochondria after suspension at* 0° *in* 0·44 M-*sucrose with and without sodium salicylate* (5 mM)

Reaction medium and incubation conditions were as described in Table 1. The method of suspension and isolation is described in the text. Each value is the mean of at least four separate determinations.

Treatment	Time of suspension (min.)	Concn. of mitochondrial salicylate* (mM)	Substrate	P:O ratio
Without salicylate	10	0	α-Oxoglutarate	2·9
	15	0	α-Oxoglutarate	2·4
	20	0	α-Oxoglutarate	2·0
	15	0	Succinate	1·5
	10	0	β-Hydroxybutyrate	1·1
With salicylate	10	5·0	α-Oxoglutarate	2·3
	15	4·8	α-Oxoglutarate	2·2
	20	10·0	α-Oxoglutarate	1·5
	15	11·1	Succinate	1·0
	10	5·1	β-Hydroxybutyrate	0·8

* See Table 3 also.

Table 3. *Permeability of rat-liver mitochondria to salicylate at* 0°

For experimental conditions see text. Varying the wet wt. of mitochondria/experiment (150–410 mg.) and the volume of suspending solution (3–5 ml.) was without effect. The average water content of mitochondria isolated from salicylate-containing medium was 81·8%; after a further wash in salicylate-free medium it was 79·5%. —, Not done.

Mean wt. of mitochondria (mg.)	No. of estimations	Time of expt. (min.)	Concn. of salicylate (mM) In medium: Before treatment	In medium: After treatment	In mitochondria: Before wash	In mitochondria: After wash	Salicylate removed by wash (%)
280	8	10	5·2±0·30	4·8±0·21	4·5±0·11	0·8±0·12	82
260	6	15	5·4±0	4·8±0·09	4·1±0·02	0	>95
225	10	20	5·0±0	4·2±0·41	5·5±0·35	1·2±0·08	78
265	8	20	4·9±0·15	4·5±0·32	10·7±1·22	—	—

Table 4. *Permeability of rat liver to salicylate* in vivo

For preparation of liver fractions see text. The mitochondria were washed once, as for the mitochondria used for oxidative-phosphorylation experiments. —, Not done.

Rat	Duration of administration of salicylate (days)	Concn. of salicylate (mM)			
		In plasma	In whole homogenate	In intracellular fluid	In mitochondria
A	8	4·7	2·6	—	—
M	8	2·9	2·4	—	—
N	3	3·3	2·5	2·5	Trace
O	3	4·1	3·5	2·9	Trace
P	2	4·1	3·6	3·3	0
R	4	5·5	4·5	4·0	0
S	8	2·6	2·0	0·8	Trace

DISCUSSION

The results confirm the finding by Jeffery & Smith (1959) of three salicylate-sensitive phosphorylations associated with the oxidation of succinate, β-hydroxybutyrate and α-oxoglutarate by rat-liver mitochondria *in vitro*. In addition there is one further (salicylate-insensitive) phosphorylation associated with the oxidation of α-oxoglutarate. In this respect salicylate resembles 2,4-dinitrophenol, another mitochondrial uncoupling agent to which its action has often been compared (Meade, 1954; Sproull, 1954; Adams & Cobb, 1958).

To assess the possible physiological significance of these findings salicylate was administered to rats *in vivo*, as the principal criteria in an examination of this type should be, where possible, the isolation of biologically uncoupled mitochondria from treated rats. However, mitochondria from salicylate-poisoned rats had similar P:O ratios to those from untreated rats, a finding in agreement with a study which also showed unaltered patterns of adenosine-triphosphatase activity (Charnock & Opit, 1962).

One possible interpretation of these findings is that the uncoupling effect of salicylate on respiring mitochondria *in vitro* is an artifact and has no physiological significance.

However, other explanations for the apparent absence of an effect of salicylate on mitochondrial oxidative phosphorylation *in vivo* are possible. As the concentration of salicylate in the livers of salicylate-poisoned rats is only slightly less than that in the plasma of such animals (Charnock *et al.* 1961) under conditions of salicylate poisoning *in vivo*, mitochondria are in contact with 2–5 mM-salicylate.

However, if salicylate is not firmly bound to mitochondria, as it is to the plasma proteins (Smith, 1949), then during homogenization and differential centrifuging the drug would be washed from the mitochondria. It follows from this that contact with salicylate in itself has no irreversible effect on mitochondrial oxidative phosphorylation.

When the permeability of mitochondria to salicylate was examined *in vitro*, by a suspension method analogous to that of Amoore (1958), and the effect of this procedure on oxidative phosphorylation was studied, the results clearly demonstrated that at 0° the binding of salicylate to mitochondria is both rapid and labile; one wash in hypertonic sucrose medium removed at least 78% of the salicylate from the mitochondria. All the mitochondria examined after this procedure carried out oxidative phosphorylation nearly as efficiently as the controls isolated under the same conditions.

The demonstration of oxidative phosphorylation by liver mitochondria (*a*) from salicylate-poisoned rats, and (*b*) from untreated rats but treated with 5 mM-sodium salicylate after isolation, indicates that the salicylate radical does not produce irreversible effects on the mitochondrial enzymes associated with oxidative phosphorylation. The lability of the binding of salicylate to mitochondria is compatible with the transient nature of the effects of the drug on metabolic rate and electrolyte distribution in man and experimental animals (Hetzel *et al.* 1959; Charnock *et al.* 1961).

SUMMARY

1. The addition of 5 mM-sodium salicylate to rat-liver mitochondria *in vitro* completely inhibited the phosphorylation associated with the oxidation of succinate and β-hydroxybutyrate; the P:O ratio with α-oxoglutarate was reduced to below unity.

2. Mitochondria isolated from the livers of rats poisoned with salicylate carried out the oxidative phosphorylation of succinate, α-oxoglutarate and β-hydroxybutyrate as efficiently as those from untreated rats.

3. Liver mitochondria isolated after suspension in 5 mM-sodium salicylate in 0·44 M-sucrose carried out oxidative phosphorylation nearly as efficiently as the controls.

4. Salicylate was taken up by mitochondria at 0°, but the binding was extremely labile. From 78 to 95 % of the salicylate taken up was removed by one rapid wash in sucrose medium.

5. The intracellular fluid of the livers of salicylate-poisoned rats contained concentrations of salicylate from 0·8 to 4·0 mM. These concentrations approximate to the concentrations of salicylate in the plasma of these animals.

6. The presence of salicylate in the intracellular fluid of the livers of rats poisoned with salicylate *in vivo* is compatible with a physiological uncoupling effect of salicylate on mitochondria *in vivo*. Uncoupling cannot be demonstrated in mitochondria isolated from intoxicated rats because the salicylate is washed out during the isolation procedure.

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Ubiquinone and Vitamin K in Bacteria

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The term 'ubiquinone' covers a family of 5,6-dimethoxytoluquinones with polyisoprenoid side chains; the member most commonly found in animals has a C_{50} side chain, i.e. with 10 isoprene units (Morton, 1961). The ubiquinones recall the naphthaquinones of the vitamin K series; vitamin K_1, found in plants, has a phytyl (C_{20}) side chain with only one double bond. The vitamins of the K_2 group are often found in micro-organisms and have side chains with different numbers of isoprene units but always have one double bond per C_5 unit. The length of the side chain may be indicated by a figure after the name, e.g. 'ubiquinone-50' and 'vitamin K_2-40' indicating C_{50} and C_{40} side chains, composed of 10 and 8 isoprene units respectively. The ubiquinones are also known as the 'coenzymes Q', the side chain being designated by the number of isoprene units incorporated; thus 'Q-10' is synonymous with 'ubiquinone-50'.

Ubiquinone is widely distributed in animal tissues, being found mainly, if not exclusively, in the mitochondria, where it occurs at about 1 μmole/g. dry wt. It is believed to play a part in electron transport, but its precise role is still unknown. Vitamin K occurs in animal tissues at a much lower concentration. It has not so far been isolated from animal tissues, though isotope experiments have recently shown that vitamin K_2-20 can be formed in the liver from other K vitamins present in the diet (Martius & Esser, 1958; Martius, 1961). The amount present is too small for chemical or spectroscopic determination but the sensitive bioassay (blood coagulation in chicks) indicates that the richest tissue, liver, contains about 0·01 μmole/g. (dry wt.); 0·04 μmole/g. is present in the mitochondria (Green, Sondergaard & Dam, 1956).

Like ubiquinone, vitamin K is also believed to be a catalyst in the energy metabolism of the cell. Its