Inhibitory Effect of Salicylate on the Incorporation of L-[U-^{14}C]Leucine into the Protein of Rat Tissue Preparations *in vitro*

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1. The inhibitory effect of salicylate, in concentrations ranging from 0·1 to 20mM, on the incorporation of radioactivity from L-[U-^{14}C]leucine into the protein of isolated rat diaphragm muscle and of cell-free systems from rat liver was studied.
2. The lowest salicylate concentrations producing significant inhibitions of amino acid incorporation were as follows: isolated rat diaphragm, 0·1mM; rat-liver mitochondrial–microsomal system, 0·1mM; rat-liver microsomal system, 0·3mM.
3. Salicylate concentrations of 2·5mM and above were found to inhibit creatine-kinase activity *in vitro*.

Conflicting reports have appeared on the effects of salicylate *in vitro* on the incorporation of ^{14}C-labelled amino acids into protein. Manchester, Randle & Smith (1958) found that 5mM-salicylate diminished the incorporation of radioactive glycine, lysine and glutamate into the protein of isolated rat diaphragm muscle. Similar results with 10mM-salicylate, radioactive glutamate and proline, and slices of rat costal cartilage were obtained by Bellamy, Huggins & Smith (1963). In contrast, it was claimed by Weiss, Campbell, Deibler & Sokoloff (1962) that 6mM-salicylate stimulated the incorporation of labelled leucine into the protein of cell-free preparations from rat liver. A similar effect was stated to occur with lower salicylate concentrations, ranging from 0·6 to 3mM.

It was implied by Weiss *et al.* (1962) that this discrepancy may be more apparent than real because of the different preparations employed. They suggested that the use of salicylate concentrations below 5mM, with the isolated diaphragm, may reveal a stimulation of amino acid incorporation, and that conversely it was likely that salicylate concentrations exceeding 6mM would diminish, and even reverse, the stimulatory effect of salicylate on amino acid incorporation in cell-free preparations.

The present paper shows that the addition of salicylate, in concentrations ranging from 0·1 to 20mM, produces an inhibition but not a stimulation of the incorporation of [^{14}C]leucine into the protein either of isolated rat diaphragm muscle or of a number of cell-free systems prepared from rat liver.

EXPERIMENTAL

Materials

Chemicals. Creatine kinase, the sodium salt of ADP and the disodium salt of creatine phosphate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. AMP, tris, α-oxoglutaric acid and all other non-radioactive chemicals used were obtained from British Drug Houses Ltd., Poole, Dorset. L-[U-^{14}C]Leucine (8·0, 10·7 and 155·0mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Deionized water was used throughout.

Animals. Male rats (body wt. 90–110g.) of the Wistar strain, maintained on M.R.C. cube diet no. 41, were killed by stunning and cervical fracture.

Diaphragm experiments

Preparation. The diaphragm was removed by cutting round the sternal and rib boundaries, care being taken to cause the minimum of damage to the muscle fibres. The excised diaphragm was washed in Krebs–Ringer phosphate (Cohen, 1957), pH 7·4, which had been gassed with O_2 and to which glucose had been added to give a concentration of 250mg./100ml. This solution was subsequently used as the incubation medium. The diaphragm was divided into two hemidiaphragms and a central portion, which was discarded.

Incubation. The hemidiaphragms were blotted carefully with Whatman no. 50 filter paper and incubated with shaking for 2hr. at 37° in 10cm. × 1cm. glass tubes sealed with Parafilm (Gallenkamp Ltd., London, E.C.2). Each hemidiaphragm was incubated in 1ml. of medium to which the L-[U-^{14}C]leucine (0·2μc, 0·02μmole) had been added. Salicylate, when present, was added to the incubation

medium to give final concentrations ranging from 0·1 to 5 mM. The experiments were arranged so that one of each pair of hemidiaphragms was incubated in the absence and the other in the presence of salicylate. At the end of the incubation period the diaphragms were removed and homogenized in 10% (w/v) trichloroacetic acid, and the resulting homogenate was mixed with the original medium. The precipitated protein was separated by centrifugation at 600*g* and heated with 5 ml. of 5% (w/v) trichloroacetic acid at 90° for 20 min. After centrifugation the insoluble material was treated with 4 ml. of 0·4 N-NaOH and recentrifuged, and the protein in the separated supernatant was precipitated with 1 ml. of 50% (w/v) trichloroacetic acid. The precipitated protein was washed successively with 5 ml. portions of ethanol, ethanol–ether–chloroform (2:2:1, by vol.), acetone and ether, and finally gently dried.

Measurement of radioactivity. The protein was placed on 1·0 cm.^{2} Polythene disks and counted in triplicate at infinite thickness with a Nuclear–Chicago D47 thin-window gas-flow counter. Each sample was counted until at least 2000 counts were recorded.

Experiments with cell-free liver preparations

Preparation of homogenates. The livers were quickly removed and placed in ice-cold homogenizing medium. When once chilled, the tissue fractions were maintained between 0° and 2° during all subsequent operations. The livers were blotted on Whatman no. 50 filter paper, weighed, minced with scissors and homogenized in 5–6 g. portions in either 0·25 M-sucrose or in a solution (tris–sucrose) containing sucrose (0·25 M), KCl (75 mM), $MgCl_2$ (10 mM) and tris–HCl buffer, pH 7·8 (35 mM) (1 g. of liver/2·5 ml. of medium). Homogenization was performed with an ice-chilled motor-driven loose-fitting all-glass Potter–Elvehjem homogenizer. The motor speed was the minimum required to prevent binding of the pestle and homogenization was continued for only about 30 sec. irrespective of the degree of completeness.

Fractionation and reconstitution. Two different preparations, referred to below as the mitochondrial–microsomal and the microsomal systems, were used. All centrifugations were performed in a Spinco model L preparative ultracentrifuge.

Mitochondrial–microsomal system. Intact cells, nuclei and cell debris were removed from the crude homogenate by centrifugation for 10 min. at 700*g*. The supernatant was decanted and centrifuged for 60 min. at 54000*g*. The combined mitochondrial and microsomal fractions contained in the sediment were resuspended in the appropriate homogenizing medium to a final volume of 1·4 ml./g. of original liver wt. To this suspension was added half its volume of the 54000*g* supernatant.

Microsomal system. This was similar to the above except that the crude homogenate was centrifuged for 15 min. at 15000*g* to remove the mitochondrial fraction with the intact cells, nuclei and cell debris. The supernatant was centrifuged for 60 min. at 54000*g* and the microsomal sediment resuspended in the appropriate medium to a final volume of 0·7 ml./g. of original liver wt. The microsomal suspension was then mixed with an equal volume of the 54000*g* supernatant.

Incubation. The various suspensions were incubated at 37°, with gentle shaking, under the conditions given in the Tables.

Extraction of protein for radioactive assay. After incubation the protein was precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The precipitate, after being heated at 90° in 5% (w/v) trichloroacetic acid for 20 min., was dissolved in 2 ml. of 3 N-thioglycollic acid by warming to 80° and the protein reprecipitated by the addition of 1 ml. of 1·5 N-NaOH. The precipitate was washed successively with 5 ml. quantities of cold 5% (w/v) trichloroacetic acid, ethanol, ethanol–ether–chloroform (2:2:1, by vol.), acetone and ether, and finally gently dried.

Measurement of radioactivity. The protein was dissolved in formic acid (AnalaR), transferred to 8 cm.^{2} aluminium disks, dried under an infrared lamp and counted in triplicate at infinite thinness, with a Nuclear–Chicago D47 thin-window gas-flow counter. Each sample was counted until at least 2000 counts were recorded. The weight of protein was determined by weighing and the results were expressed as counts/min./mg. of protein.

Creatine-kinase assay. The effect of salicylate on creatine-kinase (ATP–creatine phosphotransferase, EC 2.7.3.2) activity was investigated by using the following reaction mixture: tris–HCl buffer, pH 7·0, 100 μmoles; magnesium acetate, 12·5 μmoles; creatine phosphate, 5 μmoles; creatine kinase, 3 μg.; potassium salicylate, 0–25 μmoles; incubated at 20°. The final volume of the reaction mixture was 2·5 ml. and the reaction was started by the addition of 1·25 μmoles of ADP. Samples (1 ml.) were taken after 30 and 90 sec., over which period the reaction rate was linear. The creatine was estimated by the method of Eggleton, Elsden & Gough (1943) except that the final extinction was measured at 525 mμ with a Unicam SP. 800 spectrophotometer.

RESULTS

Diaphragm experiments. The results (Table 1) show that salicylate concentrations ranging from 0·1 to 5 mM caused a significant inhibition of the incorporation of radioactivity from labelled leucine into the protein of isolated rat diaphragm.

Mitochondrial–microsomal systems. Table 2 shows the results obtained with a mitochondrial–microsomal system prepared according to the directions of Weiss *et al.* (1962). Salicylate significantly inhibited the incorporation of the radioactive leucine into protein at all the concentrations studied. Similar experiments were performed with a second mitochondrial–microsomal system, prepared by using a modified homogenizing medium composed of tris–sucrose containing additional K^+ and Mg^{2+} ions (cf. Tata *et al.* 1963). The results (Table 3) show that the modified mitochondrial–microsomal system exhibited about four times as much activity, assessed by the counts/min./mg. in the protein, as the preparation prepared by the method of Weiss *et al.* (1962). However, salicylate significantly inhibited the incorporation of the labelled leucine into protein in concentrations ranging from 0·6 to 20 mM.

Microsomal systems. The reaction mixtures used with the microsomal systems included an external energy source involving the participation of

Table 1. *Effect of salicylate on the incorporation of* L-[*U*-^{14}C]*leucine into the protein of isolated rat hemidiaphragms*

Experimental details are given in the text. The percentage inhibition was calculated for each pair of hemidiaphragms. The results, for each concentration of salicylate, are expressed as the means ± S.D. of the individual observations.

Concn. of salicylate (mM)	No. of paired hemidiaphragms	Radioactivity in diaphragm protein (counts/min.) Control	Salicylate	Percentage inhibition	Significance of difference (*P*)
0·1	4	811 ± 194	704 ± 162	13·0 ± 2·8	0·005
0·5	6	452 ± 135	366 ± 77	18·5 ± 10·8	0·02
1·0	8	1048 ± 486	684 ± 179	28·1 ± 20·0	0·01
2·0	4	1822 ± 139	1036 ± 54	39·5 ± 17·9	0·05
5·0	4	872 ± 213	179 ± 25	78·3 ± 4·5	0·001

Table 2. *Effect of salicylate on the incorporation of* L-[*U*-^{14}C]*leucine into the protein of a mitochondrial–microsomal system, prepared with* 0·25 M-*sucrose, from rat liver*

Each incubation tube contained, in a final volume of 1·7 ml., the following components: sucrose, 150 μmoles; AMP, 5 μmoles; potassium phosphate buffer, pH 7·4, 20 μmoles; $MgCl_2$, 5 μmoles; potassium α-oxoglutarate, 50 μmoles; L-[U-^{14}C]leucine, 2 μc, 0·013 μmole; potassium salicylate, 0–34 μmoles; sufficient KCl to achieve a final concentration of K^+ ions of 0·1 M. The reaction was started by the addition of 0·45 ml. of mitochondrial–microsomal suspension (see the text) and the mixtures were incubated for 25 min. at 37°. Other experimental details are given in the text. The results are expressed as means ± S.D.

Concn. of salicylate (mM)	No. of expts.	Counts/min./mg. of protein	Significance of difference (*P*)
0	6	36·1 ± 5·7	—
0·1	6	24·1 ± 4·6	0·005
0·6	6	24·5 ± 3·9	0·005
1·0	6	23·2 ± 1·7	0·001
2·0	6	19·9 ± 2·9	0·001
3·0	6	19·3 ± 5·0	0·001
10·0	6	15·1 ± 1·8	0·001
20·0	6	5·8 ± 2·3	0·001

Table 3. *Effect of salicylate on the incorporation of* L-[*U*-^{14}C]*leucine into the protein of a mitochondrial–microsomal system, prepared with tris–sucrose, from rat liver*

Each incubation mixture contained, in a final volume of 1·0 ml., the following components: sucrose, 125 μmoles; AMP, 3 μmoles; tris–HCl buffer, pH 7·8, 17·5 μmoles; $MgCl_2$, 5 μmoles; potassium α-oxoglutarate, 30 μmoles; L-[U-^{14}C]leucine, 1 μc, 0·09 μmole; potassium salicylate, 0–20 μmoles; sufficient KCl to achieve a final concentration of K^+ ions of 0·1 M. The reaction was started by the addition of 0·5 ml. of mitochondrial–microsomal suspension (see the text) and the mixtures were incubated for 25 min. at 37°. Other experimental details are given in the text. The results are expressed as means ± S.D.

Concn. of salicylate (mM)	No. of expts.	Counts/min./mg. of protein	Significance of difference (*P*)
0	5	129·2 ± 11·4	—
0·1	6	131·2 ± 11·0	0·7
0·6	6	44·2 ± 13·1	0·001
1·0	5	39·5 ± 6·6	0·001
2·0	6	22·3 ± 3·0	0·001
3·0	6	35·3 ± 3·9	0·001
10·0	6	35·1 ± 6·4	0·001
20·0	6	21·2 ± 2·7	0·001

creatine kinase. Preliminary experiments were therefore performed to investigate the possible effects of salicylate on this enzyme activity. The results given in Table 4 show that salicylate inhibits creatine-kinase activity *in vitro* at concentrations of 2·5 mM and above. The reaction mixtures used in the subsequent experiments with the microsomal systems were therefore designed to ensure that an adequate excess of creatine kinase was present at all the salicylate concentrations studied. Table 5 shows the results obtained with a microsomal system prepared according to the directions of Weiss *et al.* (1962) and Table 6 gives the results of

Table 4. *Effect of salicylate on creatine-kinase activity*

Experimental details are given in the text. The results are expressed as means ± S.D.

Concn. of salicylate (mM)	No. of expts.	10^2 × Creatine formed (μmoles/min.)	Significance of difference (*P*)
0	10	4·88 ± 0·60	—
2·5	6	2·70 ± 0·68	0·005
5·0	6	2·02 ± 0·30	0·001
7·5	6	1·20 ± 0·53	0·001
10·0	6	0·75 ± 0·38	0·001

Table 5. *Effect of salicylate on the incorporation of* L-[*U*-^{14}C]*leucine into the protein of a microsomal system, prepared with* 0·25M*-sucrose, from rat liver*

Each incubation tube contained, in a final volume of 1·7ml., the components given in Table 2, except that the potassium α-oxoglutarate was replaced by creatine phosphate (40μmoles) and creatine kinase (0·25mg.), and the quantity of the labelled leucine added was 2μc, 0·25μmole. The reaction was started by the addition of 0·3ml. of microsomal suspension (see the text) and the mixtures were incubated for 25min. at 37°. The results are expressed as means±S.D.

Concn. of salicylate (mM)	No. of expts.	Counts/min./mg. of protein	Significance of difference (*P*)
0	5	109·9±4·9	—
0·1	4	101·3±5·2	0·1
0·6	6	105·1±7·4	0·4
1·0	6	102·8±6·2	0·1
2·0	6	102·2±6·8	0·1
3·0	6	100·4±9·7	0·2
6·0	5	77·7±5·9	0·001
10·0	5	75·3±5·7	0·001
15·0	6	65·8±8·0	0·001
20·0	4	46·8±3·9	0·001

Table 6. *Effect of salicylate on the incorporation of* L-[*U*-^{14}C]*leucine into the protein of a microsomal system, prepared with tris–sucrose, from rat liver*

Each incubation tube contained, in a final volume of 1·0ml., the components given in Table 3 except that the potassium α-oxoglutarate was replaced by creatine phosphate (25μmoles) and creatine kinase (0·25mg.) and the quantity of the labelled leucine added was 1μc, 0·065μmole. The reaction was started by the addition of 0·5ml. of microsomal suspension (see the text) and the mixtures were incubated for 25min. at 37°. The results are given as means±S.D.

Concn. of salicylate (mM)	No. of expts.	Counts/min./mg. of protein	Significance of difference (*P*)
0	6	1076·6±50·1	—
0·3	6	955·9±45·3	0·005
0·6	6	942·5±53·7	0·005
1·0	6	913·1±39·8	0·001
2·0	6	779·3±36·6	0·001
3·0	6	685·4±13·5	0·001
6·0	6	598·5±43·3	0·001
10·0	6	530·3±26·2	0·001
15·0	6	495·9±31·0	0·001
20·0	6	430·7±43·8	0·001

similar experiments with a microsomal system prepared with tris–sucrose as the homogenizing medium (cf. Tata *et al.* 1963).

In both instances salicylate inhibited the incorporation of radioactivity from labelled leucine into the microsomal protein. However, the microsomal system prepared with tris–sucrose was more sensitive to the inhibitory action of the salicylate, a significant degree of inhibition being observed with salicylate concentrations of 0·3mM and above.

DISCUSSION

The present results show that salicylate inhibits the incorporation of L-[U-^{14}C]leucine into the protein of isolated rat diaphragm of mitochondrial–microsomal systems and of microsomal systems prepared from rat liver. The findings with the isolated rat diaphragm muscle (Table 1) confirm the conclusion of Manchester *et al.* (1958) that 5mM-salicylate diminishes the incorporation of radioactive amino acids into the protein of this tissue preparation. However, they do not support the suggestion of Weiss *et al.* (1962) that salicylate concentrations below 5mM may cause a stimulation of amino acid incorporation into diaphragm protein. The present work shows that salicylate concentrations ranging from 0·1 to 5mM cause significant inhibitions of the incorporation of radioactivity from labelled leucine into the protein of isolated rat diaphragm muscle.

The results with the mitochondrial–microsomal system, prepared with 0·25M-sucrose as the homogenizing medium (Table 2), showed that salicylate concentrations ranging from 0·1 to 20mM caused a significant inhibition of the incorporation of radioactive leucine into protein. This finding is in direct contrast with the report of Weiss *et al.* (1962), who observed a stimulation of amino acid incorporation in the presence of 0·6–6mM-salicylate under apparently identical experimental conditions. We are unable to account for this discrepancy. However, Tata *et al.* (1963) have criticized the procedure used by Weiss *et al.* (1962) on the grounds that a suboptimum pH and suboptimum concentrations of K^+ and Mg^{2+} ions in the homogenizing medium produced preparations of low activity. In the present work the use of tris–sucrose containing additional K^+ and Mg^{2+} ions as the homogenizing medium was found to produce a more active mitochondrial–microsomal system but had little, if any, effect on the inhibitory action of salicylate (Table 3).

Salicylate is known to uncouple oxidative phosphorylation reactions in respiring mitochondrial preparations at concentrations of 2mM and above (Brody, 1956). It was assumed by Manchester *et al.* (1958) that the inhibitory effect of 5mM-salicylate on protein synthesis in the isolated rat diaphragm resulted from its uncoupling action. However, in the present work, salicylate at concentrations of 0·3mM and above was found to inhibit significantly the incorporation of labelled leucine into the protein

of microsomal preparations supplied with an external energy source. The inhibitory effect of salicylate on protein synthesis must therefore be mediated, at least in part, by a mechanism other than an uncoupling action on oxidative phosphorylation.

It seems possible that the action of salicylate *in vitro* on protein synthesis may be related to certain actions of the drug *in vivo*. Thus 0·4–10mM-salicylate has been shown to inhibit the growth of wheat coleoptiles (Reid, 1957) and there are a number of reports that salicylates cause a negative nitrogen balance in men and in experimental animals (Reid, Watson & Sproull, 1950; Winters & Morrill, 1955). A negative nitrogen balance may result from multiple actions of salicylate, including an interference with the renal tubular absorption of amino acids (cf. Segal & Blair, 1963) and damage to the liver parenchyma (Andrews, Bruton & De Baare, 1962). However, an interference with protein synthesis *in vivo* may also be a contributory factor.

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