Amino acid and peptide concentrations in seedlings. Cucumber seeds (outdoor ridge, variety Best of All, Carters Tested Seeds, Raynes Park, London, S.W. 20) were grown in moistened vermiculite without added nutrients at 26° in the dark. Seedlings were harvested after 1, 2, 3 and 4 days' growth. They were macerated in ethanol-water mixtures to yield final concentrations of 75% (v/v) ethanol and extracted with continuous shaking for 24 hr. at laboratory temperature. Extraction was repeated twice and the amino acid fractions were separated from the combined extracts with small columns of Zeo-Karb 215 (Plaisted, 1958). These fractions were reduced to small volume and portions equivalent to 0.1 g. dry wt. of seedling material were spotted on two-dimensional chromatograms run in solvent A followed by solvent B. After development with ninhydrin the intensities of the amino acid and peptide spots were compared visually (see Table 1).

SUMMARY

1. A peptide, γ -L-glutamyl- β -pyrazol-1-yl-L-alanine, was isolated from seeds of cucumber (*Cucumis sativus*). The peptide was obtained as a monohydrate, m.p. 198–200° (decomp.), $[\alpha]_D^{20} + 7.2^\circ$ in water.

2. The peptide was hydrolysed completely to L-glutamic acid and β -pyrazol-1-yl-L-alanine by N-hydrochloric acid at 100° for 2 hr.

3. During the early stages of growth of cucumber seeds the total amount of peptide decreased rapidly although the amounts of most amino acids increased in the plants.

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Effects of Salicylate and γ -Resorcyate on the Metabolism of Radioactive Succinate and Fumarate by Rat-Liver Mitochondria and on Dehydrogenase Enzymes

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Salicylate has been reported to interfere with the oxidation of several intermediates of the tricarboxylic acid cycle in homogenates of rat liver and kidney (Kaplan, Kennedy & Davis, 1954). It was concluded that the drug inhibited succinate dehydrogenase and might also have affected malate dehydrogenase and isocitrate dehydrogenase in the tissue preparations. This paper is concerned with the effects of salicylate and γ -resorcyate (2,6-dihydroxybenzoate) on the incorporation of ^{14}C from $[1,4-^{14}\text{C}_2]$ succinate and $[1,4-^{14}\text{C}_2]$ fumarate into the

soluble metabolic intermediates of isolated rat-liver mitochondria. The distribution of ^{14}C in the presence of the drugs suggested that they interfered with the conversion of malate into oxaloacetate and with the metabolism of citrate. Salicylate and γ -resorcyate were found to inhibit malate dehydrogenase and isocitrate dehydrogenase *in vitro*, the mechanism of inhibition involving competition with the appropriate coenzyme. γ -Resorcyate, but not salicylate, decreased the incorporation of ^{14}C from the labelled succinate

into the soluble intermediates of the mitochondrial preparations and inhibited succinate dehydrogenase *in vitro*. A preliminary account of part of this work has been published (Bryant & Smith, 1962).

EXPERIMENTAL

Preparation of mitochondria. Male rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet no. 41, were killed by cervical fracture. Liver mitochondria, separated from 12 g. wet wt. of liver by the method of Schneider & Hogeboom (1950), were finally suspended in 2 ml. of 0.25 M-sucrose.

Radioactive experiments. Samples (50 μ l.) of the mitochondrial suspensions were mixed with 25 μ l. of a solution containing the radioactive substrate, either [1,4- 14 C₂]-succinate (0.5 μ C, 0.02 μ mole) or [1,4- 14 C₂]-fumarate (0.5 μ C, 0.04 μ mole) obtained from The Radiochemical Centre, Amersham, Bucks., together with 0.03 mM-cytochrome c, 0.1 mM-ATP, 1.0 mM-ADP (all from Sigma Chemical Co.), 2.0 mM-MgSO₄ and 10.0 mM-KCl dissolved in 0.1 M-potassium phosphate buffer, pH 7.4 (Gomori, 1955). Sodium salicylate and sodium resorcyate, when present, were added in the phosphate buffer to give final concentrations of 5 mM. The mixtures were incubated in stoppered 5 ml. tubes for periods from 2 sec. to 30 min. at 37° under aerobic conditions, and were shaken at 60 cyc./min. At the end of the incubation period the mitochondrial enzymes were inactivated by the addition of 300 μ l. of boiling ethanol. After centrifuging for 5 min. at 500g, the mitochondrial pellet was resuspended in 100 μ l. of cold 20% (v/v) ethanol and the centrifuging repeated. The radioactive substances in the combined supernatants were separated by two-dimensional paper chromatography, visualized by radioautography and the 14 C was measured by the techniques described by Smith & Moses (1960).

Enzyme experiments. Pig-heart malate dehydrogenase, pig-heart isocitrate dehydrogenase, NAD and NADP were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Succinate dehydrogenase was prepared from sheep heart by the method described by Umbreit, Burris & Stauffer (1957). Malate-dehydrogenase activity was measured spectrophotometrically by the method of Burton & Wilson (1953), except that a glycine buffer, pH 9.6, was used. Isocitrate-dehydrogenase activity was measured by the spectrophotometric method of Ochoa (1955) and succinate-dehydrogenase activity was estimated manometrically according to the directions given by Umbreit, Burris & Stauffer (1957).

RESULTS

Effects of salicylate and γ -resorcyate on the distribution of 14 C from [1,4- 14 C₂]-succinate and [1,4- 14 C₂]-fumarate among the soluble intermediates of rat-liver mitochondria. The results in Table 1 show the amounts of radioactivity from [1,4- 14 C₂]-succinate incorporated into the soluble intermediates of rat-liver mitochondria in the presence or the absence of either salicylate or γ -resorcyate. In the absence of the drugs, 14 C was found in fumarate, malate,

citrate, aspartate, glutamate and alanine, showing that 14 C was transferred around the tricarboxylic acid cycle. The radioactive aspartate and glutamate presumably originated by transamination of oxaloacetate and α -oxoglutarate respectively, and the labelled alanine by transamination of pyruvate formed by decarboxylation of the oxaloacetate. Salicylate and resorcyate increased the incorporation of the isotope into fumarate, malate, citrate and aspartate, and in the presence of resorcyate, but not of salicylate, there was a decreased utilization of the labelled succinate.

When the densities of the mitochondrial suspensions, based on the initial weights of liver used for their preparation, were increased, so their capacity to transfer 14 C from the labelled succinate beyond malate was also increased (Table 2). Thus less radioactivity remained in fumarate and malate and a greater proportion of the 14 C was not recovered in the separated soluble intermediates as the mitochondrial suspensions became more concentrated. With the more dense mitochondrial preparations, the effects of salicylate and resorcyate in causing increased accumulation of 14 C in fumarate and malate, and a corresponding decrease in the proportion of radioactivity not recovered in the soluble intermediates, became more apparent.

The results in Table 3 show that when [1,4- 14 C₂]-fumarate was used as the labelled substrate, salicylate and resorcyate increased the incorporation of 14 C into malate but did not alter the amounts of labelled fumarate remaining at the end of the experiment.

Reducing the incubation period caused a decreased utilization of the labelled succinate by the mitochondria (Table 4). Salicylate produced little effect but resorcyate increased the amounts of residual labelled succinate, particularly in the experiments incubated for periods of 20 and 30 min.

Table 1. Incorporation of 14 C from [1,4- 14 C₂]-succinate into the soluble intermediates of rat-liver mitochondria in the presence or absence of either 5 mM-salicylate or 5 mM-resorcyate

The reaction medium and incubation conditions are described in the text. The incubation time was 30 min.

	10 ⁻³ × Radioactivity in sample (counts/min.)		
	Control	Salicylate	Resorcyate
Unchanged succinate	0.8	0.7	3.7
Fumarate	0.2	11.5	15.3
Malate	0.3	27.6	48.7
Citrate	0.1	3.2	3.0
Aspartate	1.2	5.0	5.1
Glutamate	1.0	1.3	1.1
Alanine	0.1	0.6	0.6

Table 2. Incorporation of ^{14}C from $[1,4\text{-}^{14}\text{C}_2]\text{succinate}$ into fumarate and malate by rat-liver mitochondrial suspensions of varying density in the presence or absence of either 5 mM-salicylate or 5 mM- γ -resorcyate

Mitochondria equivalent to 0.5–10 g. of rat liver were finally suspended in 1 ml. of 0.25 M-sucrose solution. Experimental details were as given in Table 1.

Initial wt. of liver from which mitochondria were separated	Radioactivity (% of that initially present in labelled succinate)					
	^{14}C in fumarate + malate			^{14}C not recovered in soluble intermediates		
	Control	Salicylate	Resorcyate	Control	Salicylate	Resorcyate
0.5	60	63	64	2	1	1
2.0	88	94	97	2	2	0
4.0	94	95	90	1	0	0
5.0	43	81	84	25	9	2
6.0	2	54	59	93	31	30
10.0	0	45	54	98	41	39

Table 3. Incorporation of ^{14}C from $[1,4\text{-}^{14}\text{C}_2]\text{-fumarate}$ into malate by rat-liver mitochondria in the presence or absence of either 5 mM-salicylate or 5 mM- γ -resorcyate

Experimental details were as given in Table 1.

	$10^{-3} \times$ Radioactivity in sample (counts/min.)		
	Control	Salicylate	Resorcyate
Unchanged fumarate	14.6	13.6	14.5
Malate	47.7	56.8	55.5
Not recovered in soluble intermediates	8.0	0	0

Table 4. Disappearance of ^{14}C from $[1,4\text{-}^{14}\text{C}_2]\text{-succinate}$ incubated with rat-liver mitochondria for varying time-intervals

Experimental details were as given in Table 1.

Time (sec.)	$10^{-3} \times$ Radioactivity in sample (counts/min.)		
	Control	Salicylate	Resorcyate
2	45.5	49.0	48.5
30	42.0	44.5	48.0
120	26.5	31.0	42.0
1200	1.4	0.6	16.0
1800	0.6	0.5	12.0
3600	0.2	0.4	0.8

Effects of salicylate and γ -resorcyate on enzyme activities *in vitro*. (a) Malate dehydrogenase. Fig. 1 shows that salicylate and resorcyate inhibited the activity of pig-heart malate dehydrogenase *in vitro* and the inhibition caused by salicylate became greater as the concentration of the drug was increased (Fig. 2). 1.25 mM-Salicylate has no effect but 2.5 mM-salicylate produced an inhibition of approximately 25 %, which increased to values ranging from 40 to 50 % with salicylate concentrations above 5 mM. The percentage inhibition of the malate-dehydrogenase activity produced by 10 mM-salicylate was not appreciably changed

Fig. 1. Effects of 5 mM-salicylate and 5 mM- γ -resorcyate on pig-heart malate-dehydrogenase activity *in vitro*. Experimental details were as given in Table 5. $\circ$, Control; $\bullet$, salicylate; $\blacktriangle$, resorcyate.

Fig. 2. Effect of concentration of salicylate on inhibition of pig-heart malate-dehydrogenase activity *in vitro*.

either by varying the ratio of enzyme to salicylate (Table 5) or by altering the malate concentration in the reaction mixtures (Table 6).

When the NAD concentration was varied the degree of inhibition of the enzyme activity caused by salicylate and resorcyate was changed (Table 7).

Table 5. *Effect of amount of malate dehydrogenase on inhibition produced by salicylate*

Enzyme preparation was Boehringer pig-heart malate dehydrogenase (specific activity approx. 36 units/mg.; Cooper, Srere, Tabachnick & Racker, 1958) dialysed against phosphate buffer, pH 7.4, and diluted with glycine buffer, pH 9.6. All solutions were prepared in glycine buffer. Reaction mixtures contained 1 ml. of 30 mM-sodium malate, 1 ml. of 0.75 mM-NAD, 0.01 ml. of the enzyme preparation and 1 ml. of either glycine buffer or 30 mM-salicylate solution. Enzyme activity was estimated by measuring the change in $E_{340\text{ m}\mu}$ in a Hilger Uvispek spectrophotometer at 15 sec. intervals over a period of 2 min. Each observation represents the mean of six different estimations.

10 ⁻⁵ × Concn. of enzyme in reaction mixture (μg./ml.)	Inhibition by 10 mM-salicylate (%)
80	40
20	40
10	39
5	37

Table 6. *Effect of concentration of malate on inhibition of malate dehydrogenase by salicylate*

Experimental details were as given for Table 5.

Concn. of malate (mM)	Inhibition by 10 mM-salicylate (%)
1.0	37
2.0	33
3.0	40
12.5	37
25.0	35
37.5	33
50.0	36
500.0	35

Table 7. *Effect of concentration of nicotinamide-adenine dinucleotide on inhibition of malate dehydrogenase by salicylate and γ-resorcyate*

Experimental details were as given for Table 5. Figures for NAD represent final concentrations.

Concn. of NAD (mM)	Inhibition (%)	
	10 mM- Salicylate	10 mM- Resorcyate
0.08	57	100
0.15	53	—
0.30	39	—
0.60	35	60
1.20	21	41
2.40	0	17

Increasing the NAD concentration from 0.08 to 2.40 mM completely removed the inhibitory activity of 10 mM-salicylate and reduced that caused by 10 mM-resorcyate from 100 to 17%.

(b) Isocitrate dehydrogenase. The results in Fig. 3 show that salicylate and resorcyate inhibited isocitrate-dehydrogenase activity *in vitro*. When the concentrations of the drugs were increased, the degree of inhibition of the enzyme activity was also increased (Table 8). The degree of inhibition caused by 5 mM-salicylate was reduced by increasing the NADP concentration, being completely removed when the coenzyme concentration was 0.176 mM (Table 9).

Fig. 3. Effects of 5 mM-salicylate and 5 mM-γ-resorcyate on pig-heart isocitrate-dehydrogenase activity *in vitro*. Experimental details were as given in Table 8. ○, Control; ●, salicylate; ▲, resorcyate.

Table 8. *Effect of concentrations of salicylate and γ-resorcyate on inhibition of isocitrate dehydrogenase*

All solutions were prepared in veronal buffer, pH 7.4. Enzyme preparation was 0.5 mg. of pig-heart isocitrate dehydrogenase (Boehringer) in 1 ml. of buffer. Reaction mixtures contained 0.05 ml. of the enzyme preparation, 0.1 ml. of 18 mM-MnCl₂, 0.1 ml. of 6 mM-DL-isocitrate and 0.1 ml. of 0.528 mM-NADP, being made up to a final volume of 3 ml. with either veronal buffer or salicylate solution. Enzyme activity was estimated by measuring the change in $E_{340\text{ m}\mu}$ in a Hilger Uvispek spectrophotometer at 15 sec. intervals over a period of 2 min. Each observation represents the mean of six different estimations.

Concn. of salicylate or resorcyate (mM)	Inhibition (%)	
	Salicylate	Resorcyate
2.5	8	21
5.0	17	45
10.0	39	100
15.0	62	—
20.0	69	—

Table 9. *Effect of concentration of nicotinamide-adenine dinucleotide phosphate on inhibition of isocitrate dehydrogenase by salicylate*

Experimental details were as given for Table 8. Figures for NADP represent final concentrations.

$10^{-3} \times$ Concn. of NADP (mM)	Inhibition by 5 mM- salicylate (%)
4.4	30
8.8	22
17.6	17
22.0	14
44.0	10
88.0	4
176.0	0

Fig. 4. Effects of 10 mM-salicylate and 10 mM- γ -resorcyate on sheep-heart succinate-dehydrogenase activity *in vitro*. Enzyme preparation (Umbreit *et al.* 1957) was suspended in phosphate buffer, pH 7.4 (10 g./40 ml.). Each Warburg flask contained 2 ml. of this suspension and 1 ml. of either phosphate buffer or a solution of the drug in the buffer; 0.2 ml. of 10% (w/v) KOH soln. was placed in the centre well and 0.2 ml. of 0.3M-sodium succinate in the side arm. The flasks were incubated at 37° and equilibration for 30 min. was allowed for oxidation of any endogenous substrates before the succinate was added from the side arm by tipping. ○, Control; ●, salicylate; ▲, resorcyate.

(c) Succinate dehydrogenase. Fig. 4 shows that 10 mM-salicylate had no effect on succinate-dehydrogenase activity *in vitro* but that 10 mM-resorcyate caused an inhibition of approximately 15%.

DISCUSSION

Rat-liver mitochondria incorporated radioactivity from [1,4-¹⁴C₂]succinate into fumarate, malate, citrate, aspartate, glutamate and alanine. Salicylate and resorcyate appeared to interfere with the transfer of ¹⁴C between the labelled soluble

intermediates at several points. The most prominent action of both drugs was an increased accumulation of ¹⁴C into fumarate and malate (Table 1). This effect became more pronounced as the capacity of the mitochondrial preparations to transfer ¹⁴C beyond malate was enhanced by increasing the density of the mitochondrial suspensions (Table 2). Thus salicylate and resorcyate appeared to interfere directly with the metabolism of malate, because the increased incorporations of ¹⁴C found in aspartate, alanine and citrate, in the presence of the drugs, were relatively much less (Table 1). The results with [1,4-¹⁴C₂]fumarate (Table 3) showed that neither salicylate nor resorcyate influenced the conversion of the labelled fumarate into labelled malate, suggesting that the increased incorporation of ¹⁴C in fumarate in the succinate experiments (Table 1) was secondary to an interference with the transfer of ¹⁴C beyond malate. One possible explanation of these results was that salicylate and resorcyate decreased the conversion of oxaloacetate into aspartate because the drugs have been shown to inhibit transaminase enzymes *in vitro* (Huggins, Smith & Moses, 1961; Huggins, Bryant & Smith, 1961). The subsequent accumulation of oxaloacetate might in turn have inhibited malate dehydrogenase (Gutfreund, Ebner & Mendiola, 1961). However, the formation of radioactive aspartate from the labelled succinate was increased in the presence of salicylate and resorcyate (Table 1), and a more probable alternative mechanism was that the drugs affected the conversion of malate into oxaloacetate.

Salicylate and resorcyate inhibited pig-heart malate-dehydrogenase activity *in vitro* (Fig. 1). The inhibitory action of salicylate was found to be reversible since varying the ratio of enzyme to salicylate did not appreciably change the degree of inhibition (Table 5). When the malate concentration was varied between wide limits there was no change in the percentage inhibition of the enzyme activity by the drug (Table 6). Salicylate did not therefore interfere with the reaction between substrate and enzyme. In contrast, when sufficient NAD was added to the reaction mixtures, the inhibition produced by salicylate was completely removed and the more pronounced inhibition caused by similar concentrations of resorcyate largely disappeared (Table 7). Salicylate and resorcyate therefore inhibited malate dehydrogenase *in vitro* by a mechanism involving competition with the coenzyme. Euler & Ahlstrom (1943) reported that salicylate inhibited lactate-dehydrogenase activity *in vitro* and that the inhibition could be reversed by the addition of NAD. The increased incorporations of ¹⁴C into malate observed when the mitochondrial suspensions were incubated with the labelled succinate or fumarate

in the presence of either salicylate or resorcyate are therefore explicable in terms of a direct inhibitory action of the drugs on the mitochondrial malate dehydrogenase.

A further implication of these results was that salicylate and resorcyate may inhibit any dehydrogenase requiring NAD as a cofactor. Such an action on mitochondrial isocitrate dehydrogenases, which require either NAD or NADP as coenzymes (Ernster, 1959), could explain the accumulation of labelled citrate in the succinate experiments (Table 1). Some support for this suggestion is provided by the present results (Fig. 3; Table 9), which show that both drugs inhibit pig-heart isocitrate-dehydrogenase activity *in vitro* and that the inhibitory action of salicylate is reversed by the addition of NADP. The potential inhibitory range of the drugs may therefore be extended to include those dehydrogenases that required NADP as a coenzyme. In addition, the radioactivity experiments (Tables 1 and 4) indicated that resorcyate, but not salicylate, decreased the utilization of the labelled succinate by the mitochondrial preparations and resorcyate inhibited sheep-heart succinate-dehydrogenase activity *in vitro* (Fig. 4). Salicylate was inactive in the present experiments although other workers (Nitzescu & Cosma, 1923; Kaplan *et al.* 1954) have reported that the drug appears to inhibit the enzyme activity. However, the earlier work was based on measurements of oxygen uptake in relatively complex systems, such as homogenates, and an apparent effect of salicylate on succinate oxidation could have resulted from the drug primarily inhibiting malate dehydrogenase.

The increased incorporations of ^{14}C into fumarate, malate and citrate in the radioactive experiments (Table 1) are explicable in terms of salicylate and resorcyate inhibiting malate dehydrogenase and isocitrate dehydrogenase. Inhibition of the latter enzyme would also be expected to cause an increased accumulation of oxaloacetate, which would in turn increase the formation of radioactive aspartate and alanine. It is possible that such an increased accumulation of oxaloacetate may reinforce the inhibitory action of the drugs on malate dehydrogenase and thus contribute to the increased incorporation of ^{14}C into malate and fumarate.

SUMMARY

1. The effects of salicylate and γ -resorcyate on the incorporation of ^{14}C from $[1,4-^{14}\text{C}_2]$ succinate and

$[1,4-^{14}\text{C}_2]$ fumarate into the soluble intermediates of rat-liver mitochondria have been studied.

2. In the absence of the drugs the mitochondria incorporated the isotope from the labelled succinate into fumarate, malate, citrate, aspartate, glutamate and alanine.

3. Salicylate and resorcyate increased the incorporation of ^{14}C from the $[1,4-^{14}\text{C}_2]$ succinate into fumarate, malate and citrate but did not alter the utilization of the $[1,4-^{14}\text{C}_2]$ fumarate. Resorcyate, but not salicylate, decreased the utilization of the radioactive succinate.

4. Salicylate and resorcyate inhibited malate-dehydrogenase and isocitrate-dehydrogenase activities *in vitro* by competing with either NAD or NADP. Resorcyate, but not salicylate, inhibited succinate-dehydrogenase activity *in vitro*.

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