Biochem. J. (1963) **88**, 346

Effects of Salicylate on Glutamate Dehydrogenase and Glutamate Decarboxylase

By B. J. GOULD, A. K. HUGGINS AND M. J. H. SMITH
Empire Rheumatism Council Research Unit, King's College Hospital Medical School, London, S.E. 5

(*Received* 22 *February* 1963)

Salicylate produces an increased incorporation of ^{14}C from [3-^{14}C]pyruvate into glutamate in preparations of rat liver and kidney (Huggins, Smith & Moses, 1961*b*). It also inhibits glutamate–pyruvate transaminase [L-alanine–2-oxoglutarate aminotransferase, EC 2.6.1.3] in rat serum and in extracts of rat tissues (Steggle, Huggins & Smith, 1961; Huggins, Bryant & Smith, 1961*a*). The present paper is concerned with the effects of salicylate on glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase, EC 1.4.1.3] and glutamate decarboxylase [L-glutamate 1-carboxy-lyase, EC 4.1.1.15]. A preliminary account of part of this work has been published (Gould, Huggins & Smith, 1963).

EXPERIMENTAL

Materials. Ox-liver glutamate dehydrogenase (free of ammonium sulphate) and NAD were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; glutamate decarboxylase (acetone-dried powder of *Escherichia coli*) from Sigma Chemical Co., St Louis, Mo., U.S.A.; pyridoxal phosphate from L. Light and Co. Ltd., Colnbrook, Bucks.; L-glutamic acid from British Drug Houses Ltd., Poole, Dorset; and [*carboxy*-^{14}C]salicylate from The Radiochemical Centre, Amersham, Bucks. Other chemicals were of analytical grade.

Enzyme assays. Glutamate-dehydrogenase activity was measured by the method of Strecker (1955) except that the amount of NAD was increased from 0·3 μmole to 13·5 μmoles to obtain maximum activity with the enzyme preparation. The reaction was started by the addition of 0·08 mg. of glutamate dehydrogenase contained in 10 μl. of 0·05M-potassium phosphate buffer, pH 7·6. Assays were performed at room temperature (20$\pm$2°) in a Hilger Uvispek spectrophotometer with silica cells of 1·0 cm. light-path. Measurements of extinction were made at 366 mμ, and not at 340 mμ, to avoid interference caused by the absorption of salicylate at the lower wavelength. Initial rates were determined from readings made at 10 sec. intervals over a period of 2 min.

Glutamate-decarboxylase activity was determined manometrically at 37° as described by Najjar & Fisher (1954), except that 3M-acetate buffer, pH 5·0 (Umbreit, Burris, & Stauffer, 1957), was used.

Dialysis experiments. Glutamate decarboxylase (5 mg.) in 5 ml. of 1M-acetate buffer, pH 5·0, was dialysed against 50 ml. of the same buffer at 0°; the dialysing medium was replaced ten times over a period of 48 hr. Further samples of the enzyme preparation (5 mg./5 ml. of 1M-acetate buffer, pH 5·0) were exposed to either 0·25M-salicylate alone or to 0·25M-salicylate plus 10 μc (10 μmoles) of [*carboxy*-^{14}C]salicylate for 15 hr. at 0° and then dialysed as described above.

RESULTS

Glutamate dehydrogenase. Table 1 shows that salicylate inhibited the activity of ox-liver glutamate dehydrogenase and that the degree of inhibition increased with salicylate concentration.

The glutamate dehydrogenase was exposed for 60 min. at 0° to salicylate concentrations ranging from 0·1 to 1M. Samples (10 μl. containing 0·08 mg. of glutamate dehydrogenase) of these mixtures were added to the other reactants (0·2 ml. of 0·5M-potassium L-glutamate, 0·2 ml. of 67·5 mM-NAD and 2·6 ml. of 0·05M-potassium phosphate buffer, pH 7·6) of the assay system for glutamate dehydrogenase. No inhibitory effect of the salicylate could be detected in the final reaction mixtures. Thus 1 : 300 dilutions of glutamate-dehydrogenase preparations, which had been preincubated with con-

Table 1. *Effect of the concentration of salicylate on the inhibition of glutamate dehydrogenase*

The enzyme preparation had a specific activity of about 3 units/mg. (see Cooper, Srere, Tabachnick & Racker, 1958). It was diluted 2 : 5 with 0·05M-phosphate buffer, pH 7·6. Reaction mixtures contained: 2·6 ml. of 0·05M-phosphate buffer, 0·2 ml. of 0·5M-potassium L-glutamate, 0·2 ml. of 67·5 mM-NAD and 0·01 ml. (0·08 mg.) of the enzyme preparation, which was added to start the reaction. The salicylate was added in 0·2 ml. of phosphate buffer, the appropriate decrease being made in the volume of buffer added. Measurements were made of the change of E_{366} in a Hilger Uvispek spectrophotometer at 10 sec. intervals over a period of 2 min. at room temperature (20$\pm$2°) in a silica cell of 1 cm. light-path, and were used to calculate the initial rates. Each value represents the mean of six determinations.

Final concn. of salicylate (mM)	Inhibition (%)
5	5
10	9
20	20
50	40
100	72
200	88

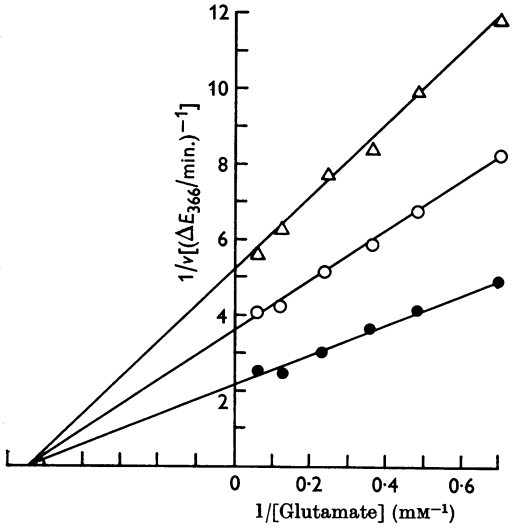

Fig. 1. Non-competitive inhibition of glutamate dehydrogenase by salicylate: effect of various concentrations of glutamate and salicylate. Each reaction mixture, prepared directly in the silica cuvettes (1 cm. light-path) of a Hilger Uvispek spectrophotometer, contained in a final vol. of 3 ml.: 2·6 ml. of 0·05M-phosphate buffer, pH 7·6, which contained the salicylate when present, 0·2 ml. of 67·5 mM-NAD, and glutamate as indicated. The reaction was started by mixing into the cuvette 0·01 ml. (0·08 mg.) of enzyme preparation and measurements were made of the change of E_{366} at 10 sec. intervals over a period of 2 min. at room temperature (20±2°). ●, Control; ○, 20 mM-salicylate; △, 40 mM-salicylate. Calculated values were: K_m, 1·82 mM; K_i, 29·7 mM.

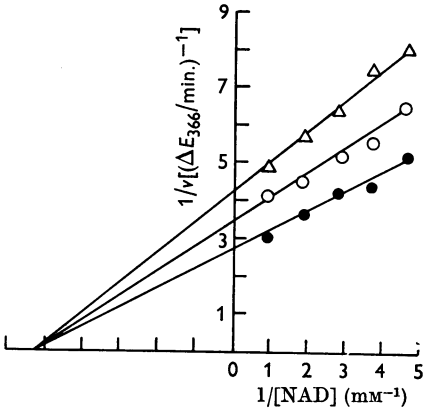

Fig. 2. Non-competitive inhibition of glutamate dehydrogenase by salicylate: effect of various concentrations of NAD and salicylate. The experimental conditions were the same as in Fig. 1 except that each reaction mixture contained 0·2 ml. of 0·5M-potassium L-glutamate and NAD as indicated. ●, Control; ○, 20 mM-salicylate; △, 40 mM-salicylate. Calculated values were: K_m, 0·192 mM; K_i, 70·2 mM.

centrations of salicylate sufficient to produce inhibitions of enzyme activity greater than 70%, caused complete reversal of the inhibitory effect of the salicylate.

The possibility of competition between salicylate and either glutamate or NAD was investigated by reciprocal-plot analysis (Lineweaver & Burk, 1934). The results (Figs. 1 and 2) show that both the substrate and the coenzyme gave characteristic non-competitive plots with salicylate.

Glutamate decarboxylase. Table 2 shows that salicylate inhibited the activity of glutamate decarboxylase and that the degree of inhibition became greater as the salicylate concentration was increased. Preincubation of the enzyme preparation with pyridoxal phosphate for 45 min. stimulated glutamate-decarboxylase activity; the degree of stimulation appeared to be maximal at a pyridoxal phosphate concentration of 0·67 mM. The degree of inhibition caused by 20 mM-salicylate was decreased by about 50% by preincubation of the enzyme preparation with 0·67 mM-pyridoxal phosphate but was not further diminished even when the coenzyme concentration during preincubation was raised tenfold (Table 3). Thus the degree of reversibility is independent of the coenzyme concentration. The results given in Table 4 show that the inhibitory action of salicylate could be decreased by preincubation of the enzyme preparation with pyridoxal phosphate but that the subsequent addition of the coenzyme to an inhibited enzyme had no effect. The percentage inhibition of glutamate-decarboxylase activity by 20 mM-salicylate was not appreciably changed by varying the glutamate concentration between 25 and 400 mM.

Glutamate decarboxylase showed no decrease in activity after dialysis against 1M-acetate buffer,

Table 2. *Effect of the concentration of salicylate on the inhibition of glutamate decarboxylase*

All solutions were prepared in 3M-acetate buffer, pH 5·0. Reaction mixtures consisted of: 1 ml. of the enzyme preparation (1 mg.) in the side arm and 2 ml. of 0·2M-L-glutamic acid. The salicylate was added in 0·2M-L-glutamic acid. The reaction was started by tipping the enzyme from the side arm and the evolution of CO_2 was followed manometrically at 5 min. intervals for 45 min. Inhibitions were calculated from Q_{CO_2} over the initial period (that for the control was 550). Each value represents the mean of six determinations.

Final concn. of salicylate (mM)	Inhibition (%)
5	0
10	12
20	23
40	42
75	76
100	84
150	97

pH 5·0, for 48 hr. at 0°. However, exposure of the enzyme preparation to 0·25 M-salicylate for 15 hr. at 0° caused a complete loss of activity that was not restored by dialysis alone or by dialysis followed by the addition of 6·7 M-pyridoxal phosphate. These results indicated that inhibition by salicylate is irreversible.

The glutamate-decarboxylase preparation was exposed to 0·25 M-salicylate plus 10 μmoles of [*carboxy*-^{14}C]salicylate for 15 hr. at 0° and then dialysed as described in the Experimental section. Triplicate samples (10 μl.) of the mixtures, before and after dialysis, were spotted on Whatman no. 1 filter paper, and the radioactivity in each spot was counted directly for 1 min. with a Scott-type Geiger–Müller tube (Fuller, 1956), flushed continuously with helium that had previously been passed through ice-cold ethanol. Both sides of the paper were counted and the values were averaged. The accuracy of each count was approximately proportional to the square root of the number of disintegrations recorded. The average counts/min. were 1800 ± 50 before dialysis and 300 ± 20 after dialysis. Thus about 20% of the added radioactivity remained bound to the protein after a dialysis procedure involving ten changes of the dialysing medium.

Table 3. *Effects of the concentration of pyridoxal phosphate on the glutamate-decarboxylase activity in the presence or the absence of salicylate*

The experimental details were as given for Table 2 except that the enzyme preparation (1 ml.) was preincubated with the pyridoxal phosphate for 45 min. before the start of the reaction. The values for pyridoxal phosphate represent final concentrations in the reaction mixture (3 ml.). Enzyme activity is expressed as a percentage of the commercial enzyme–coenzyme mixture showing maximum activity.

Final concn. of added pyridoxal phosphate (mM)	Final concn. of salicylate (mM)	Enzyme activity (%)
0	0	80
0·01	0	85
0·02	0	87
0·04	0	89
0·09	0	100
0·67	0	100
6·67	0	100
0	20	76
0·67	20	89
6·67	20	88

DISCUSSION

The inhibition of the dehydrogenase is non-competitive with both glutamate and NAD. It therefore differs from that reported for malate dehydrogenase and isocitrate dehydrogenase, where salicylate competes with NAD (Bryant, Smith & Hines, 1963).

The initial experiments with glutamate decarboxylase suggested that the inhibitory action of salicylate may be due to competition with the coenzyme, pyridoxal phosphate, but the degree of reversibility was found to be independent of the coenzyme concentration. When the glutamate concentration was varied between wide limits there was no change in the percentage inhibition of the enzyme activity by salicylate. Salicylate does not therefore interfere with the reaction between substrate and enzyme. The dialysis experiments show that salicylate causes an apparently irreversible inhibition of glutamate decarboxylase and the radioactivity experiment demonstrates the presence of bound salicylate in an inactivated, and subsequently dialysed, enzyme preparation. Pyridoxal phosphate decreased the inhibitory effect of salicylate only when the coenzyme was preincubated with the enzyme, and its subsequent addition to an inhibited enzyme preparation had no effect. A possible explanation of these results is that, during preincubation with the enzyme, pyridoxal phosphate combines with certain sites and hinders the subsequent binding of salicylate to the same sites. This suggestion is supported by the work of Davison & Smith (1961) and Dempsey & Christensen (1962), who found that both pyridoxal phosphate and salicylate combine with ϵ-amino groups in bovine albumin.

Table 4. *Effect of pyridoxal phosphate and salicylate on glutamate-decarboxylase activity*

The experimental details were as given in Table 3 except that the enzyme preparation was preincubated alone and in the presence of either pyridoxal phosphate or salicylate, or both. Final concentrations in 3 ml. after addition to substrate were: pyridoxal phosphate, 6·7 mM; salicylate, 40 mM.

Preincubated with enzyme in side arm (1 ml.)	Addition to substrate in flask (2 ml.)	Enzyme activity (%)
—	—	100
—	Salicylate	58
—	Salicylate + pyridoxal phosphate	62
Pyridoxal phosphate	Salicylate	76
Pyridoxal phosphate + salicylate	—	46
Salicylate	Pyridoxal phosphate	15
Salicylate	—	12

A prominent effect of salicylate on the incorporation of ^{14}C from [3-^{14}C]pyruvate into the soluble intermediates of rat tissues is the increased accumulation of ^{14}C in glutamate (Huggins *et al.* 1961*b*). This suggests that salicylate interferes with the further metabolism of glutamate. The more important pathways involved in glutamate metabolism include protein synthesis, conversion into α-oxoglutarate by glutamate dehydrogenase and various transamination reactions, decarboxylation to γ-aminobutyrate and the formation of glutamine. Salicylate inhibits the incorporation of ^{14}C-labelled amino acids into protein (Manchester, Randle & Smith, 1958). It also inhibits both glutamate–pyruvate transaminase (Steggle *et al.* 1961) and glutamine synthetase (Messer, 1958). The present work suggests that the conversion of glutamate into α-oxoglutarate by glutamate dehydrogenase and into γ-aminobutyrate by glutamate decarboxylase may also be inhibited by salicylate. In the radioactivity experiments of Huggins *et al.* (1961*b*) labelled α-oxoglutarate was detected in the isolated brain preparation and its formation was decreased in the presence of salicylate. However, this finding cannot be interpreted as direct evidence for an inhibitory effect of salicylate on glutamate dehydrogenase because a similar action on the glutamate–pyruvate transaminase would also cause a decreased incorporation of ^{14}C from labelled glutamate into α-oxoglutarate. More specific evidence for an interference by salicylate with glutamate-decarboxylase activity was a decreased formation of labelled γ-aminobutyrate from [3-^{14}C]pyruvate.

SUMMARY

1. Salicylate inhibits glutamate-dehydrogenase and glutamate-decarboxylase activities *in vitro*.

2. The mechanism of the inhibition of glutamate dehydrogenase is reversible but does not involve competition with either glutamate or NAD; the inhibition of glutamate decarboxylase is irreversible. Preincubation of glutamate decarboxylase with pyridoxal phosphate reduces the degree of inhibition by salicylate.

We are grateful to the Empire Rheumatism Council and to Monsanto Chemicals Ltd. for grants towards the cost of the work.

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Biosynthesis of Griseofulvin

OBSERVATIONS ON THE INCORPORATION OF [^{14}C]GRISEOPHENONE C AND [^{36}Cl]GRISEOPHENONES B AND A

BY A. RHODES, G. A. SOMERFIELD AND MOIRA P. McGONAGLE
Glaxo Research Ltd., Sefton Park, Stoke Poges, Bucks.

(*Received* 28 *November* 1962)

Inhibition of griseofulvin (I) biosynthesis enabled Rhodes, Boothroyd, McGonagle & Somerfield (1961) to isolate three methylated benzophenone intermediates designated griseophenone C (2,4′,6-trihydroxy-2′,4-dimethoxy-6′-methylbenzophenone) (II; R = R′ = H), griseophenone B (5-chloro-2,4′,6-trihydroxy-2′,4-dimethoxy-6′-methylbenzophenone) (II; R = H; R′ = Cl) and griseophenone A (5-chloro-4′,6-dihydroxy-2,2′,4-trimethoxy-6′-methylbenzophenone) (II; R = CH_3;