The Effects of Salicylate on the Activity of Rat Liver Tyrosine-2-Oxoglutarate Aminotransferase *in vitro* and *in vivo*

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1. Salicylate, in concentrations of 0.25 mM and above, enhances the basal activity of tyrosine-2-oxoglutarate aminotransferase in homogenates of rat liver incubated in the absence of added pyridoxal 5'-phosphate (endogenous activity). The effect is decreased by increasing the concentration of the cofactor. 2. The intraperitoneal administration of sodium salicylate enhances the activity of rat liver tyrosine aminotransferase; the major effect during the first hour being on the enzyme in the absence of added pyridoxal phosphate. Actinomycin D prevents the induction of the enzyme by cortisol and tryptophan. Induction by pyridoxine or salicylate is 50% inhibited by actinomycin D. The effects of the injections of various combinations of cortisol, pyridoxine and salicylate were also studied in the absence or presence of actinomycin D. 3. It is suggested that salicylate induces rat liver tyrosine aminotransferase by displacing its protein-bound cofactor and that a cofactor-type induction of the hepatic enzyme occurs in pyridoxine-treated rats.

Salicylate displaces albumin-bound pyridoxal 5'-phosphate (Dempsey & Christensen, 1962) and stimulates the activity of rat liver tryptophan-2-oxoglutarate aminotransferase *in vitro* (Gould & Smith, 1965). Tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5), which is more strongly activated by pyridoxal 5'-phosphate than tryptophan aminotransferase (Lin *et al.*, 1958) has been used in the present work to test the possibility that salicylate may activate aminotransferase enzymes by displacing protein-bound cofactor. Experiments are also described that support the view (Greengard & Gordon, 1963) that a cofactor-type induction of liver tyrosine aminotransferase occurs in pyridoxine-treated rats.

Materials and Methods

Chemicals

Cortisol 21-acetate, dimethylformamide, 2-oxoglutaric acid, pyridoxal 5'-phosphate, pyridoxine hydrochloride, sodium diethyldithiocarbamate, L-tryptophan and L-tyrosine were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Actinomycin D was a gift from Merck Sharp and Dohme, Hoddesdon, Herts., U.K. All other chemicals (from BDH, Poole, Dorset, U.K.) were of AnalaR grade except for sodium salicylate which was of British Pharmacopoeial grade.

Animals and injections

Male (200-300 g) Wistar rats, maintained on Medical Research Council cube diet no. 41B, were

killed (at 14.00 h) by stunning and cervical dislocation. All the chemicals were given intraperitoneally 4 h before death. Animals treated with pyridoxine received an extra injection 2 h before death. Additional experiments were performed with salicylate in which groups of rats were killed at between 0.5 and 9 h after the injection. Cortisol acetate was dissolved in dimethylformamide and 0.1 ml (2 mg/100 g body wt.) was injected; the corresponding control rats received an equal quantity of the solvent. Tryptophan (20 mg) was dissolved in the minimum amount of M-NaOH and diluted to 1 ml with 0.9% (w/v) NaCl after the pH had been adjusted to 7.3 with M-HCl; each rat received 1 ml/100 g body wt. Pyridoxine hydrochloride (100 mg) was dissolved in 1 ml of 0.9% NaCl after the pH had been adjusted to 7.3 with 10 M-NaOH; each rat received 1 ml/100 g body wt. Sodium salicylate (400 mg/kg) and actinomycin D (0.7 mg/kg) were dissolved in 0.9% NaCl. The control animals received an equal quantity of 0.9% NaCl.

Preparation of homogenates

The liver was removed within 30 s of the death of the animal and was homogenized for 1 min at 1100 rev./min in 9 vol. of a solution containing 140 mM-KCl and 2.5 mM-KOH at 0°C in a glass homogenizer with a loose-fitting Teflon pestle. The homogenates were used within 5 min of preparation.

Determination of tyrosine aminotransferase activity

The activity of the enzyme was determined in liver homogenates by the method of Diamondstone (1966)

either in the absence (endogenous activity) or presence (total activity) of an excess of added pyridoxal 5'-phosphate. All the components of the assay mixture (except the liver homogenate and the aqueous diethyldithiocarbamate solution) were dissolved in 0.2M-potassium phosphate buffer, pH 7.3 (6.1 ml). The ice-cold incubation mixture (final volume 6.4 ml) contained the following final concentrations: L-tyrosine 6 mM, pyridoxal 5'-phosphate (where necessary) 37.5 μ M, 2-oxoglutarate 9.4 mM, diethyldithiocarbamate 3.75 mM and 10 mg or less of liver (wet wt.). Samples (6.4 ml) of the reaction mixture were incubated for 5 min with shaking at 37°C in glass-stoppered 25-ml conical flasks under air. The reaction was stopped by the addition of 1.2 ml of 1.8M-trichloroacetic acid; the flasks and contents were shaken for a further 2 min and then filtered on Whatman no. 1 filter paper. To a measured portion of the filtrate (3.8 ml) was added 0.2 ml of 10M-NaOH and the mixture was immediately shaken with a mechanical stirrer. After standing at room temperature for 30 min, the E_{331} of the solution was measured with a Unicam SP.800 spectrophotometer. Test solutions were read against blanks that were similarly prepared throughout except that the trichloroacetic acid was added before the 2-oxoglutarate solution before a 7 min incubation at 37°C. Tyrosine aminotransferase activity was expressed in μ mol of 4-hydroxyphenylpyruvate formed/h per g (wet wt.) of liver as the mean value of quadruplicate flasks per rat. In the experiments *in vitro*, sodium salicylate and pyridoxal 5'-phosphate were dissolved in the potassium phosphate buffer.

Results

Effects of salicylate on rat liver tyrosine aminotransferase in vivo

The results (Fig. 1) show that the intraperitoneal administration of sodium salicylate (400 mg/kg body wt.) caused an initial increase in the endogenous activity of the enzyme at 30 and 60 min (33 and 90% respectively); the endogenous activity continued to rise, reaching sevenfold at 5 h, then fell to twofold after 9 h. The total enzyme activity (that measured in the presence of an excess of pyridoxal 5'-phosphate) did not rise until after 1 h, was fivefold at 5 h and fell to normal after 9 h.

Endogenous and total activity of tyrosine aminotransferase in rat liver was induced by injection of cortisol, tryptophan, pyridoxine and salicylate as shown in Table 1. Actinomycin D abolished the induction of endogenous enzyme activity by cortisol and decreased the induction of endogenous enzyme activity by tryptophan, pyridoxine and salicylate by 65, 65 and 50% respectively.

The effects of the injections of various combina-

Fig. 1. *Effects of salicylate on rat liver tyrosine aminotransferase activity in vivo*

The enzyme activity was determined as described in the Materials and Methods section. Each point represents the mean value for four rats except that the zero-time points are means of twenty animals. ○, Total enzyme activity (that measured in the presence of 37.5 μ M-pyridoxal 5'-phosphate); ●, endogenous enzyme activity (that in the absence of added cofactor).

tions of cortisol, pyridoxine or salicylate, with or without actinomycin D (Table 2), show that actinomycin D decreased the combined effects of cortisol plus pyridoxine or cortisol plus salicylate to values observed after actinomycin D had been given with pyridoxine or with salicylate respectively (Table 1). In the presence or absence of actinomycin D, the effect of the combined injection of pyridoxine plus salicylate (Table 2) was not additive when compared with the sum of the corresponding separate effects (Table 1).

Effects of salicylate on rat liver tyrosine aminotransferase in vitro

The results (Table 3) show that salicylate, in concentrations of 0.25 mM and above, significantly increased the endogenous activity of the enzyme in

Table 1. *Effect of actinomycin D on the induction of rat liver tyrosine aminotransferase by cortisol, tryptophan, pyridoxine and salicylate*

Each rat received an intraperitoneal injection of either 0.9% (w/v) NaCl, cortisol acetate (20 mg/kg), L-tryptophan (20 mg/100 g), or sodium salicylate (400 mg/kg) with or without actinomycin D (0.7 mg/kg) 4 h before being killed. Pyridoxine hydrochloride (100 mg/100 g) was given 4 and 2 h before death and where necessary, actinomycin D (0.7 mg/kg) was given with the first injection of pyridoxine. The injection of dimethylformamide gave results identical with the saline controls. The results are given as the means $\pm$ S.E.M. of each group of four rats.

Injection	4-Hydroxyphenylpyruvate formed (μ mol/h per g wet wt. of liver)			
	No addition		With actinomycin D	
	Endogenous activity	Total activity	Endogenous activity	Total activity
0.9% NaCl	30.8 $\pm$ 1.6	132.5 $\pm$ 14.6	28.6 $\pm$ 1.5	94.3 $\pm$ 4.6
Cortisol	70.6 $\pm$ 6.5	470.9 $\pm$ 40.0	32.6 $\pm$ 1.0	141.2 $\pm$ 6.5
Tryptophan	118.6 $\pm$ 6.6	544.3 $\pm$ 50.0	59.0 $\pm$ 8.7	143.7 $\pm$ 22.5
Pyridoxine	163.2 $\pm$ 15.9	469.8 $\pm$ 33.3	75.2 $\pm$ 3.8	251.7 $\pm$ 3.0
Salicylate	166.7 $\pm$ 27.1	716.0 $\pm$ 26.6	95.9 $\pm$ 7.2	403.5 $\pm$ 48.3

Table 2. *Effects of the combined injections of cortisol, pyridoxine and salicylate, with or without actinomycin D, on the total activity of rat liver tyrosine aminotransferase*

Experimental details are as in Table 1 except that the three inducers were given in various combinations. The results are given as the means $\pm$ S.E.M. of each group of four rats.

Injections	4-Hydroxyphenylpyruvate formed (μ mol/h per g wet wt. of liver)	
	No addition	With actinomycin D
Cortisol+pyridoxine	602.4 $\pm$ 9.7	251.2 $\pm$ 5.5
Cortisol+salicylate	720.6 $\pm$ 58.3	413.4 $\pm$ 26.2
Pyridoxine+salicylate	512.7 $\pm$ 52.6	340.8 $\pm$ 19.1

Table 3. *Effect of salicylate on the endogenous activity of tyrosine aminotransferase in homogenates of rat liver in vitro*

Results are given as the means $\pm$ S.E.M. of homogenates from four separate animals incubated in the absence of added pyridoxal 5'-phosphate, with or without salicylate. They have been analysed by the *t* test and values for *P* are included.

Concn. of salicylate	Endogenous enzyme activity (μ mol of 4-hydroxyphenylpyruvate formed/h per g wet wt. of liver)		<i>P</i>
(mM)	Control	Salicylate	
0.125	25.9 $\pm$ 0.8	25.2 $\pm$ 1.2	>0.100
0.25	22.1 $\pm$ 0.7	30.7 $\pm$ 1.0	<0.001
0.50	28.6 $\pm$ 0.9	48.5 $\pm$ 2.1	<0.001
1.00	29.0 $\pm$ 1.4	62.9 $\pm$ 5.2	<0.001
2.00	27.8 $\pm$ 2.2	70.9 $\pm$ 5.1	<0.001

homogenates of rat liver incubated in the absence of added pyridoxal 5'-phosphate. The total enzyme activity (that measured in the presence of an excess of the cofactor) was not affected by any of the con-

centrations of salicylate tested. The addition of pyridoxal 5'-phosphate in concentrations of up to 10 μ M increased the activity of tyrosine aminotransferase (Fig. 2) and the stimulatory effect of

salicylate (1 mM) on the endogenous activity was decreased by increasing the concentration of the cofactor and disappeared at maximum enzyme activity.

Discussion

In the adrenalectomized rat liver, the mechanism of induction of tyrosine aminotransferase by pyridoxine is in dispute with respect to the question of sensitivity to actinomycin D (Greengard & Gordon, 1963; Holten *et al.*, 1967). In the present work, it was found that whereas the cortisol induction of the total enzyme activity in the intact rat was almost completely abolished by actinomycin D, the induction by pyridoxine was only decreased by 50% (Table 1). Both inducers increased the total enzyme activity to the same extent whereas pyridoxine was a better activator of endogenous tyrosine aminotransferase (Table 1). The effect of the combined injection of cortisol plus pyridoxine (Table 2) equalled the sum of the cortisol effect plus the actinomycin-insensitive portion of the pyridoxine-induced rise in activity (Table 1) and actinomycin D administered with the combined injection of both inducers gave results (Table 2) similar to those obtained after actinomycin D had been given with pyridoxine alone (Table 1). The failure of Holten *et al.* (1967) to show the above type of 'additive' effect of the combined injection of cortisol plus pyridoxine may be due to the higher dose of free cortisol used by these authors, which resulted in maximum induction of the enzyme activity. The present findings therefore suggest that pyridoxine induces liver tyrosine aminotransferase in the intact rat by two mechanisms; an actinomycin-insensitive, cortisol-like mechanism and a cofactor one involving the saturation of the apoenzyme with pyridoxal phosphate (Greengard & Gordon, 1963).

In the rat, the injection of sodium salicylate enhances the activity of hepatic tyrosine-2-oxoglutarate aminotransferase (Fig. 1). The initial increase in the endogenous activity (during the first hour) suggests that salicylate acts primarily by increasing the availability to the apoenzyme of the pyridoxal phosphate cofactor. This effect is demonstrable *in vitro* (Table 3) and becomes less apparent as the concentration of pyridoxal 5'-phosphate is increased (Fig. 2). The increased activity of tyrosine aminotransferase observed in rats injected with salicylate (Fig. 1) resembles the induction of the enzyme by pyridoxine. Thus both inducers increased the endogenous activity of the enzyme to the same extent and their induction of the total enzyme activity was 50% decreased by actinomycin D (Table 1). The 'additive' effect of cortisol plus pyridoxine and the influence of actinomycin D on this were also

Fig. 2. Effect of pyridoxal 5'-phosphate on rat liver tyrosine aminotransferase activity *in vitro*

Each point represents the mean value for four rats. ○, Plus salicylate (1 mM); ●, no salicylate.

observed after the combined injection of cortisol plus salicylate (Tables 1 and 2). In the presence or absence of actinomycin D, the effect of the combined injection of pyridoxine plus salicylate (Table 2) was not additive with respect to the sum of the corresponding separate effects (Table 1) thus suggesting that the two inducers do not act differently. One possible explanation of the present findings is that salicylate induces rat liver tyrosine aminotransferase by increasing the availability of the cofactor by displacing it from its binding sites on serum and liver proteins; this is known to occur *in vitro* (Dempsey & Christensen, 1962). A similar action on protein-bound tryptophan (Badawy & Smith, 1971a,b) causes salicylate to induce rat liver tryptophan pyrrolase and to enhance the substrate induction of the enzyme activity.

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