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The Effects of Salicylate on the Activity of Rat Liver Tryptophan Pyrrolase *in vitro* and *in vivo*

By A. A.-B. BADAWY and M. J. H. SMITH
Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London S.E.5, U.K.

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1. Salicylate, in concentrations of 0.05mM and above, inhibits the basal activity of tryptophan pyrrolase in homogenates of rat liver and the activity induced by cortisol but not that induced by tryptophan. The inhibition is abolished by adding haematin to the reaction mixtures. 2. The intraperitoneal injection of 400mg of sodium salicylate/kg in the rat causes a decrease in the tryptophan pyrrolase activity in the liver at 30min, the activity is restored to normal at 2h, increases to sixfold after 5h and returns to the basal value at 12h. The activation of the enzyme by salicylate is prevented by the administration of cycloheximide but not by pretreatment with actinomycin D. The effects of the combined injection of salicylate and cortisol are additive, whereas those of salicylate plus tryptophan are not. The injection of salicylate causes a progressive increase in the holo-/apo-enzyme ratio and an increased content of tryptophan in the liver over a period of 3h. 3. It is suggested that salicylate inhibits tryptophan pyrrolase activity *in vitro* and *in vivo* by interacting with iron protoporphyrins and causes a later enhancement of the enzyme activity *in vivo* by a mechanism involving the release of tryptophan from its binding sites on circulating albumin and on other proteins.

It has been reported that the injection of salicylate stimulates the activity of tryptophan pyrrolase in rat liver (Chiancone, 1964) but that the drug inhibits the activity of the enzyme *in vitro* (Satoh & Moroi, 1969). The present paper provides evidence suggesting that salicylate activates the enzyme *in vivo* by a mechanism involving the release of protein-bound tryptophan and inhibits the enzyme activity by interacting with iron protoporphyrins. A preliminary account of part of this work has been published (Badawy & Smith, 1971).

MATERIALS AND METHODS

Chemicals. Cycloheximide, dimethylformamide, cortisol 21-acetate, L-tryptophan and L-kynurenine sulphate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Hoddesdon, Herts., U.K. Haemin (haematin hydrochloride), 2,2′-bipyridyl, 3- and 4-hydroxybenzoic acid were from British Drug Houses Ltd., Poole, Dorset, U.K. All other chemicals were of analytical grade except for the sodium salicylate, which was of British Pharmacopoeial grade.

Animals and injections. Male (150–300g) Wistar rats, maintained on Medical Research Council cube diet no. 41B, were killed by stunning and cervical fracture. All the chemicals were given intraperitoneally 3h before death except that actinomycin D was injected 1h before the administration of cortisol, tryptophan or salicylate. Additional experiments were performed with salicylate in which groups of animals were killed at 15, 30 and 45min and 1, 2, 4, 5, 6, 8 and 12h after the injection. Cortisol acetate was dissolved in dimethylformamide and 0.1ml (2mg/100g body wt.) injected; the corresponding control rats received an equal quantity of the solvent. Tryptophan (100mg) was dissolved in the minimum amount of M-NaOH and diluted to 1ml with 0.9% (w/v) NaCl after the pH had been adjusted to 7.3 with M-HCl; each rat received 1ml/100g body wt. The other substances were dissolved in 0.9% (w/v) NaCl and given in the following doses; sodium salicylate 400mg/kg, cycloheximide 50mg/kg, actinomycin D 0.7mg/kg. The control animals received an equal volume of 0.9% (w/v) NaCl.

Preparation of homogenates. The liver was removed within 30s of the death of the animal and was homogenized for 1min at 1100rev./min in 7vol. of a solution containing 140mM-KCl and 2.5mM-NaOH at 0°C in a glass homogenizer with a loose-fitting Teflon pestle.

Determination of tryptophan pyrrolase activity. The activity of the enzyme in the liver homogenates was determined by measuring the conversion of L-tryptophan into kynurenine (Feigelson & Greengard, 1961). Samples (15ml) of the homogenate were added to a solution containing 15ml of 0.2M-sodium phosphate buffer, pH7.0, 5ml of 0.03M-L-tryptophan and 25ml of water at 0°C. Samples (3ml) of the mixture were incubated with shaking at 37°C in glass-stoppered 25ml conical flasks in an atmosphere of O_2 for appropriate time-intervals up to 105min. The reaction was stopped by the addition of 2ml

of 15% (w/v) metaphosphoric acid; the flasks and contents were shaken for a further 3 min and then filtered with Whatman no. 1 filter paper. To a measured portion (2.5 ml) of the filtrate was added 1.5 ml of 1 M-NaOH and the kynurenine present determined by measuring the E_{365} with a Unicam SP. 800 spectrophotometer. Tryptophan pyrrolase activity was obtained from the slope of the linear phase of a plot of μmol of kynurenine produced per g wet wt. of liver against time of incubation. The holo-/apo-enzyme ratio was calculated by measuring tryptophan pyrrolase activity in the presence of 2 μM-haematin (total activity) and in its absence (holoenzyme activity). In the experiments *in vitro* sodium salicylate, other hydroxybenzoates and bipyridyl were dissolved in the sodium phosphate buffer and the haematin was dissolved in 0.1 ml of 0.1 M-NaOH and added to the mixtures before incubation.

Determination of tryptophan and salicylate in liver. Tryptophan was determined by the method of Hess & Udenfriend (1959) in the supernatant fractions from 1 g samples of liver which had been heated with 3.2 ml of water in a boiling-water bath for 2 min, cooled, homogenized as described above, and centrifuged at 10000*g* for 10 min after the addition of 2 ml of 20% (w/v) trichloroacetic acid, further homogenization and thorough cooling. Salicylate was measured by the method of Trinder (1954) in samples (5 g) of liver extracted by the method of Yesair & Coutinho (1970).

RESULTS

Effects of salicylate on rat liver tryptophan pyrrolase in vivo. The results (Fig. 1) show that the intraperitoneal injection of 400 mg/kg body wt. of salicylate caused an initial decrease in the activity of the enzyme at 30 and 60 min; the activity returned to that found in corresponding control animals at 2 h, then increased to sixfold at 5 h and fell to normal after 12 h. The results, from groups each of four rats, were analysed by the *t* test and a significant difference ($P<0.02$) between the control and salicylate values occurred at 30 and 60 min and 3, 4, 5, 6 and 8 h. The salicylate concentrations in the liver reached a maximum of 1.81 mM at 30 min and decreased to 1.02 mM after 2 h, and the tryptophan concentrations increased to between 23.0 $\pm$ 0.5 and 26.2 $\pm$ 0.8 μg/g wet wt. of liver over the time-period 15 min to 3 h, compared with the zero-time control value of 13.7 $\pm$ 1.5 μg/g wet wt. of liver. The control animals showed a holo-/apo-enzyme ratio for the liver enzyme of 0.76 and the injection of salicylate caused this to increase to 1.26 at 3 h, 1.35 at 4 h and 1.55 at 5 h.

Tryptophan pyrrolase activity in rat liver is induced by the injection of either cortisol or tryptophan and the results in Table 1 show that the effects of the combined injection of either cortisol plus tryptophan or cortisol plus salicylate were additive whereas that of salicylate plus tryptophan was not. The injection of dimethylformamide alone gave results identical with the saline-treated controls. The results in Table 2 show that cycloheximide

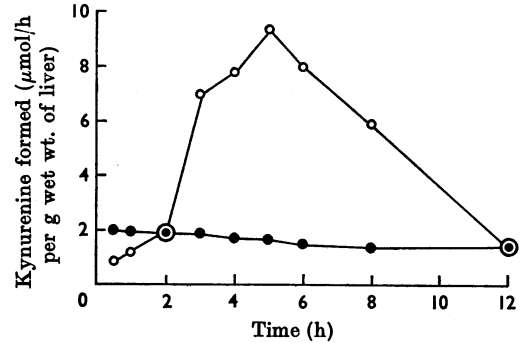

Fig. 1. Effects of salicylate on rat liver tryptophan pyrrolase activity *in vivo*. The enzyme activity was determined as described in the Materials and Methods section. Each point represents the mean value for four rats. ○, Rats injected with 400 mg of sodium salicylate/kg body wt. at zero time; ●, rats injected with 0.9% (w/v) NaCl at zero time.

Table 1. *Effects of the separate and combined injections of cortisol, tryptophan and salicylate on the activity of rat liver tryptophan pyrrolase*

Each rat received an intraperitoneal injection of either 0.9% (w/v) NaCl, cortisol (20 mg/kg body wt.), L-tryptophan (100 mg/100 g body wt.) or sodium salicylate (400 mg/kg body wt.) either separately or in various combinations, 3 h before being killed. The results are given as the mean of each group of four animals minus the value (1.89) for the corresponding group that had received saline only.

Injection	Liver tryptophan pyrrolase activity (μmol of kynurenine formed/h per g wet wt.)	Sum of separate injections
Cortisol	3.56	—
Tryptophan	9.97	—
Salicylate	5.04	—
Cortisol + tryptophan	11.97	13.53
Cortisol + salicylate	7.33	8.60
Tryptophan + salicylate	4.01	15.01

Table 2. *Effects of cycloheximide and actinomycin D on the induction of rat liver tryptophan pyrrolase by cortisol, tryptophan and salicylate*

Experimental details are as in Table 1 except that some groups of animals received either cycloheximide (50mg/kg body wt.) at the same time or actinomycin D (0.7mg/kg body wt.) 1h before the inducing agents. The results are given as the means ±S.E.M. of each group of four rats.

	Liver tryptophan pyrrolase activity (μmol of kynurenine/h per g wet wt.)		
Injection	No addition	With cycloheximide	After actinomycin D
0.9% (w/v) NaCl	1.89±0.03	1.53±0.09	1.64±0.06
Cortisol	5.45±0.41	1.41±0.05	1.39±0.25
Tryptophan	11.86±0.92	3.37±0.80	10.85±0.41
Salicylate	6.93±0.65	1.52±0.05	5.88±0.40

Table 3. *Effect of salicylate on the activity of tryptophan pyrrolase in homogenates of rat liver.*

Results are given as the means ±S.E.M. of homogenates from four separate animals incubated in either the absence or the presence of salicylate. They have been analysed by the *t* test and values for *P* are included.

Salicylate concn. (mM)	Enzyme activity (μmol of kynurenine/h per g wt wt. of liver)		
	Control	Salicylate	*P*
0.01	1.53±0.11	1.56±0.12	—
0.05	1.32±0.10	0.82±0.11	0.02
0.10	1.03±0.10	0.43±0.05	0.005
1.00	1.74±0.37	0.43±0.14	0.02
2.00	1.43±0.07	0.00	0.001

abolished the increased pyrrolase activity caused by cortisol, tryptophan and salicylate, whereas pretreatment with actinomycin D inhibited the induction of the enzyme by cortisol only. The cortisol-induced enzyme resembles the basal pyrrolase and differs from the enzyme activity induced by either tryptophan or salicylate in being significantly inhibited ($P = 0.001$) by salicylate (1mM) *in vitro*, showing a lag phase on incubation and possessing a holo-/apo-enzyme ratio of 0.50.

Effects of salicylate on rat liver tryptophan pyrrolase in vitro. The results (Table 3) show that salicylate, in concentrations of 0.05mM and above, significantly inhibited the basal activity of tryptophan pyrrolase in homogenates of rat liver. The initial lag phase, which lasts 30–45min with rat liver homogenates, was not affected by the drug and the inhibition became evident when the enzyme activity became linear with time. No stabilization of the basal enzyme activity was produced by incubating the homogenates with a non-inhibitory concentration (0.01mM) of salicylate. Additional experiments were performed in which the homogenate was incubated at 37°C in either the absence or presence of 0.435mM-L-kynurenine and the rate of disappearance of the kynurenine was not altered by salicylate (1mM).

The addition of haematin to the reaction mixtures in concentrations up to 2μM increased the pyrrolase activity, and at higher concentrations caused some inhibition (Fig. 2), and abolished the lag phase. The inhibitory effect of salicylate (1mM) was considerably decreased by the presence of 2μM-haematin and the inhibition observed in the presence of 8μM-haematin disappeared in the presence of the salicylate. 2,2′-Bipyridyl (0.1mM) caused a 54% inhibition ($P<0.005$) of the basal pyrrolase activity which was abolished by the addition of 2μM-haematin, but 3- and 4-hydroxybenzoate (0.1mM) did not affect the enzyme activity *in vitro*.

DISCUSSION

In the rat, the injection of sodium salicylate causes a biphasic response in the activity of hepatic tryptophan pyrrolase (Fig. 1). The initial decrease in activity could arise from a direct action of the drug on the enzyme, since salicylate is present in the liver at concentrations (>1mM) that significantly inhibit the basal pyrrolase activity *in vitro* (Table 3). It has been reported (Satoh & Moroi, 1969) that salicylate inhibits tryptophan pyrrolase activity *in vitro* but these workers used livers from animals that had been pre-treated with tryptophan. In the

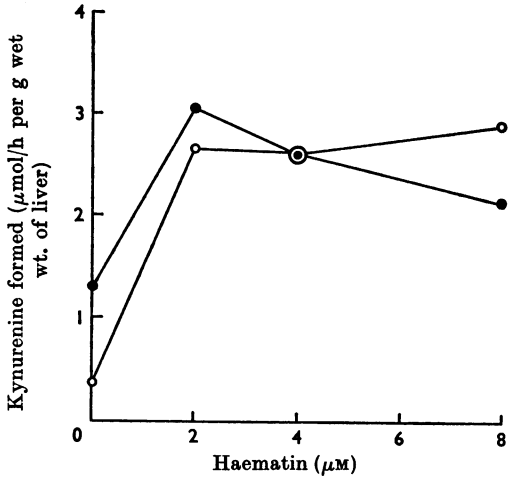

Fig. 2. Effect of haematin on rat liver tryptophan pyrrolase activity *in vitro*. Each point represents the mean value for four rats. ○, Plus salicylate (1mM); ●, no salicylate.

present work it was found that the activity of the tryptophan-induced enzyme was not affected by a concentration (1mM) of salicylate that inhibited the basal activity by 75%. Globin behaves in a similar fashion (Knox, 1966) and a further resemblance between it and salicylate is that haematin reverses their inhibitory effects on the basal pyrrolase activity *in vitro* (Feigelson & Greengard, 1961). The mechanism of the inhibitory action of salicylate may therefore involve an interaction with the iron protoporphyrin activator of tryptophan pyrrolase. The inactivity of the two congeners, 3- and 4-hydroxybenzoate, suggests that salicylate may act as a chelating agent and this suggestion is supported by the observation that the inhibition produced by 2,2′-bipyridyl is abolished by haematin. It has also been reported that salicylate interacts with other haem proteins such as the liver microsomal cytochrome *P*-450 (Bullock, Delaney, Sawyer & Slater, 1970).

The increased activity of tryptophan pyrrolase observed in rats injected with salicylate (Fig. 1) resembles the induction of the enzyme by tryptophan and not that by cortisol. Thus pre-treatment of the animals with actinomycin D prevented the induction of the enzyme by the steroid but not by either tryptophan or salicylate (Table 2). The effects of the combined injections of either cortisol plus tryptophan or cortisol plus salicylate were additive whereas that of salicylate plus tryptophan was not (Table 1). The increased enzyme activity produced by the administration of either tryptophan or salicylate was associated with a holo-/apo-enzyme ratio of about 1.5 and showed no lag phase on incubation, whereas the cortisol-induced enzyme resembled the basal pyrrolase in possessing a ratio of 0.5–0.8 and exhibited a lag phase of 30–45min. Salicylate (1mM) inhibited the basal enzyme and the enzyme induced by cortisol to approximately the same extent but had no effect on the increased enzyme activity observed after the injection of either tryptophan or salicylate.

One possible explanation of the present findings is that salicylate induces rat liver tryptophan pyrrolase activity *in vivo* by displacing tryptophan from its binding sites to circulating and liver proteins. The administration of small doses of the amino acid in the rat produces parallel increases in the concentrations of tryptophan in the liver and in the activity of the hepatic pyrrolase (Civen & Knox, 1959; Yatvin & Pitot, 1970). In the present work it was observed that the concentration of tryptophan in the liver increased twofold at 15min after the injection of a dose of salicylate, that subsequently induced the liver tryptophan pyrrolase (Fig. 1). It has also been shown that salicylate displaces tryptophan from its binding sites to bovine serum albumin *in vitro* (McArthur & Dawkins, 1969) and from circulating human proteins *in vivo* (Smith & Lakatos, 1971).

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