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Effects of salicylate on RNA polymerase activity and on the incorporation of orotic acid and thymidine into the nucleic acids of rat foetuses *in vitro*

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Salicylate (10 mM) significantly inhibited the activity of RNA polymerase and the incorporation of radioactivity from orotic acid-5-^{3}H and thymidine-6-^{3}H into whole 13 day and 16 day rat foetuses. The polymerase activity and the incorporation of the labelled orotate were significantly inhibited by 2 mM salicylate in the 16 day foetus only. The inhibitory action of salicylate on RNA biosynthesis may be related to its effects in causing an increased incidence of foetal death and congenital malformation in pregnant rodents.

The chronic administration of acetylsalicylic acid to pregnant rats and mice causes a high incidence of foetal death (Obbink & Dalderup, 1964). A single injection of either methyl or sodium salicylate during gestation in these species not only produces foetal death and premature birth (Eriksson & Larsson, 1968) but also several congenital malformations of the litters carried to full term (Warkany & Takacs, 1959; Larsson, Ericson & Bostrom, 1963). The major foetal abnormalities comprise skeletal anomalies and disturbances of vascular and neural development. The day or days of pregnancy on which the salicylates are given appear to determine which type of abnormality predominates. Thus, the highest incidence of skeletal anomalies in mice were observed after injection on the 9th day of gestation whereas vessel anomalies were the most prominent after injections on the 15th day (Larsson & Eriksson, 1966). These findings suggest that each organ or system may have a critical period when it is most susceptible to the teratogenic action of salicylate.

Salicylate could be teratogenic either by interfering with placental function or by directly affecting metabolic reactions in the foetal tissues. The drug uncouples oxidative phosphorylation reactions (Brody, 1956), interferes with the biosynthesis of mucopolysaccharides (Larsson & Bostrom, 1965), inhibits the activities of dehydrogenase, decarboxylase and aminotransferase enzymes (Smith, 1968) and impairs protein synthesis (Dawkins, Gould & Smith, 1966) in animal tissues. A further, and perhaps more relevant, action is that salicylate inhibits the activity of nucleic acid polymerases prepared from rat liver (Janakidevi & Smith, 1969) and interferes with the biosynthesis of nucleic acids in adult mice (Janakidevi & Smith, 1970). We have, therefore, studied the effects of sodium salicylate on the activity of RNA polymerase and on the incorporation of radioactivity from labelled orotic acid and thymidine into the nucleic acids of rat foetuses at varying stages of development.

EXPERIMENTAL

Animals

Female rats of the Wistar strain, 300 to 400 g, were mated and the day in which sperm was found in vaginal smears was considered to be the first day of pregnancy.

Groups, each of six animals, were killed by stunning and cervical fracture on the 13th, 15th, 16th and 19th day of pregnancy and the foetuses, freed from the foetal membranes, were placed in an ice-cold solution containing 0·1M tris-HCl, pH 7·5, 0·01M $MgCl_2$ and 0·25M sucrose (TMS medium).

Materials

Orotic acid-5-^{3}H (specific activity 1Ci/mmol), thymidine-6-^{3}H (specific activity 5Ci/m mol) and UTP-5-^{3}H (specific activity 1·5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Calf thymus DNA, UTP, CTP, GTP, ATP and orcinol were obtained from the Sigma Chemical Co., St. Louis, RNA from the Boehringer Corporation (London) Ltd. and diphenylamine from Hopkins and Williams Ltd., Chadwell Heath, Essex. Sodium salicylate was B.P. grade, all other chemicals were of analytical grade and glass distilled water was used throughout.

Measurement of RNA polymerase activity

Between 30 and 40 foetuses were homogenized in 10 volumes of ice-cold 0·32M sucrose, containing 3 mM $MgCl_2$, using an all-glass homogenizer. The nuclei were isolated and purified as described previously for adult rat liver. RNA polymerase activity was estimated at 17°, to minimize interference from ribonuclease, by measuring the incorporation of radioactivity from UTP-5-^{3}H into RNA in a Beckman LS 200B liquid scintillation system, using GF/A (2·1 cm) glass fibre discs (Janakidevi & Smith, 1969). The RNA product from the incubation mixtures had a DNA-like base composition.

Incorporation experiments

Single foetuses were each placed in 1 ml of the TMS medium containing 5 μCi of either the labelled orotic acid or thymidine plus either enough sodium salicylate to give a final salicylate concentration of either 2 or 10 mM or sufficient sodium chloride to produce the same final concentration of sodium. The mixture was incubated, with shaking, for 2 h at 37°. At the end of the incubation period the foetus was removed, washed with ice-cold TMS medium and homogenized in 3 ml of 6% (v/v) perchloric acid. The homogenate was centrifuged at 3000 *g* for 15 min and the residue re-extracted with 2 ml of the perchloric acid followed by 1 ml of water. The final residue was washed with two quantities of ethanol–ether mixture (3:1) to remove lipid material and the RNA and DNA extracted according to the directions of Widnell & Tata (1964; 1966). The specific activities of the extracted nucleic acids were measured by estimating the RNA content by the orcinol method (Hurlbert, Schmitz & others, 1954), the DNA content by the diphenylamine technique (Burton, 1952) and the radioactivity, in aliquots of 0·1 ml, using the Beckman liquid scintillation system and glass fibre discs.

RESULTS

The incorporation of radioactivity from the labelled orotic acid and thymidine into the whole foetuses obtained between the 13th and 19th day are given in Fig. 1. It was not possible to obtain adequate material to perform similar incorporation experiments on whole foetuses from earlier stages of pregnancy. In these and subsequent experiments an incubation period of 2 h was chosen because preliminary work showed that the incorporation of radioactivity from the orotic acid and thymidine into the nucleic acids of whole rat foetuses was linear over a period of 4 h.

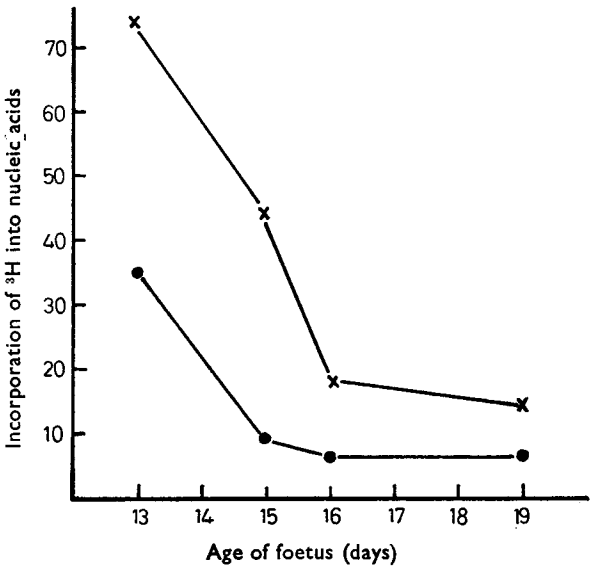

FIG. 1. Incorporation of orotic acid-5-^{3}H and thymidine-6-^{3}H into the nucleic acids of whole rat foetuses varying from 13 to 19 days old. Individual results represent the mean values from three or four experiments: ●, from orotic acid, expressed as counts/min × 10^{-2}/mg RNA isolated; ×, from thymidine, expressed as counts/min × 10^{-3}/mg DNA isolated.

The results in Fig. 1 show that the incorporation of the labelled precursors was highest in the 13 day foetus, had markedly decreased in the 16 day foetus and did not apparently change with increasing age of the foetus. The 13th and 16th day foetuses were therefore chosen to investigate the effects of salicylate.

The results, given in Table 1, show that the 16 day foetus was more sensitive than the 13 day foetus to the effects of salicylate. The high concentration of the drug (10 mM) significantly inhibited RNA polymerase activity and the incorporation of tritium from both orotic acid and thymidine in both foetuses where 2 mM salicylate caused significant inhibition of the polymerase and the orotate incorporation in the 16 day foetus only.

Table 1. *Effects of sodium salicylate on RNA polymerase activity and on the incorporation of orotic acid and thymidine into rat foetuses* in vitro. Each value is given as the mean ± standard deviation, the number of whole foetuses used in each experiment being given in parentheses. The results have been analysed by the *t*-test and * indicates a statistically significant decrease ($P < 0.05$) between the control and salicylate values.

Age of foetus (days)	Salicylate concentration (mM)	RNA polymerase activity (pmol/mg DNA)	Radioactivity incorporated: From orotate (counts/min mg^{-1} RNA isolated)	Radioactivity incorporated: From thymidine (counts/min mg^{-1} DNA isolated)
13	0	867 ± 213 (3)	3511 ± 267 (3)	74377 ± 19947 (3)
	2	908 ± 167 (3)	4675 ± 313 (3)	90684 ± 24696 (3)
	10	*347 ± 69 (3)	*116 ± 29 (3)	*5760 ± 2242 (3)
16	0	1191 ± 244 (6)	578 ± 45 (4)	16712 ± 2993 (4)
	2	*846 ± 207 (6)	*379 ± 44 (4)	14818 ± 3719 (4)
	10	*596 ± 205 (6)	*191 ± 25 (4)	*8789 ± 1756 (4)

DISCUSSION

The results of the present work show that 2 mM salicylate significantly inhibits the activity of RNA polymerase and the incorporation of radioactivity from tritiurated orotic acid, but not from labelled thymidine, into 16 day but not into 13 day whole rat foetuses. Thus the 2 mM salicylate appears to inhibit preferentially the biosynthesis of RNA rather than that of DNA in the 16 day foetus. The formation of both types of nucleic acid in the 13 and 16 day foetuses is inhibited by 10 mM salicylate.

These observations may bear some relevance to the increased incidence of foetal death and congenital malformations found to occur in pregnant rodents treated with large doses of the drug. Warkany & Takacs (1959) used a single subcutaneous injection of sodium salicylate in doses between 300 and 900 mg/kg in pregnant rats and these would be expected to produce tissue salicylate levels about 1 to 3 mM (Sturman, Dawkins & others, 1968). The increased sensitivity of the 16 day foetus to salicylate may be related to the increased incidence of foetal death and resorption in pregnant rodents which occurs as the drug is administered at an increasingly later stage in the pregnancy (Larsson & Eriksson, 1966). In addition, the predominance of vessel anomalies when salicylate was given on or about the 16th day of pregnancy in the mouse may reflect the relative susceptibility of this system to salicylate at this particular stage of gestation. It must be emphasized that the present results were obtained with whole foetuses and it will be necessary to extend the experiments to include foetuses of different ages and also to investigate separately individual organs and systems.

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Note added in proof. Since this paper was submitted for publication Richards (1969) has reported the results of a retrospective epidemiological study of congenital malformations in human pregnancy. The results showed that the taking of salicylate preparations in the first trimester of pregnancy is associated with significant increases in abnormalities of the central nervous system and the alimentary tract of the foetus. It was concluded that either salicylates have a teratogenic effect in man or that the conditions for which they are given have such an action.

REFERENCES

BRODY, T. M. (1956). *J. Pharmac. exp. Ther.*, **117**, 39–51.
BURTON, K. (1952). *Biochem. J.*, **62**, 315–323.
DAWKINS, P. D., GOULD, B. J. & SMITH, M. J. H. (1966). *Ibid.*, **99**, 703–707.
ERIKSSON, M. & LARSSON, K. S. (1968). *Nature, Lond.*, **220**, 385.
HURLBERT, R. B., SCHMITZ, H., BRUMM, A. F. & POTTER, V. R. (1954). *J. biol. Chem.*, **209**, 23–41.
JANAKIDEVI, K. & SMITH, M. J. H. (1969). *J. Pharm. Pharmac.*, **21**, 401.
JANAKIDEVI, K. & SMITH, M. J. H. (1970). *Ibid.*, **22**, 51–55.
LARSSON, K. S. & BOSTROM, H. (1965). *Acta paediat. scand.*, **54**, 43–48.
LARSSON, K. S., ERICSON, B. & BOSTROM, H. (1963). *Ibid.*, **52**, 36–40.
LARSSON, K. S. & ERIKSSON, M. (1966). *Ibid.*, **55**, 569–576.
OBBINK, H. J. K. & DALDERUP, L. M. (1964). *Lancet*, **1**, 565.
RICHARDS, I. D. G. (1969). *Br. J. prev. soc. Med.*, **23**, 218–225.
STURMAN, J. A., DAWKINS, P. D., MCARTHUR, N. & SMITH, M. J. H. (1968). *J. Pharm. Pharmac.*, **20**, 58–63.
SMITH, M. J. H. (1968). *Clinical Toxicology*, **1**, 387–407.
WARKANY, J. & TAKACS, E. (1959). *Am. J. Path.*, **35**, 315–331.
WIDNELL, C. C. & TATA, J. R. (1964). *Biochem. J.*, **92**, 313–317.
WIDNELL, C. C. & TATA, J. R. (1966). *Biochim. Biophys. Acta*, **123**, 478–492.