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Embryotoxic Effects of Salicylates: Role of Biotransformation[1]

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Embryotoxic Effects of Salicylates: Role of Biotransformation. GREENAWAY, J. C., BARK, D. H., AND JUCHAU, M. R. (1984). *Toxicol. Appl. Pharmacol.* **74**, 141–149. The three major metabolites of salicylate, *o*-hydroxyhippurate (salicylglycine, salicyluric acid), 2,5-dihydroxybenzoate (gentisic acid), and 2,3-dihydroxybenzoate, were examined for their capacities to elicit dysmorphogenesis, embryolethality, and growth retarding effects in an embryo culture system. The effects were compared with those produced by the parent salicylate. At the highest concentrations tested (1.9 mM), none of the three metabolites produced significant increases in the number of malformed embryos or in embryolethality. At the same concentration, all three agents reduced crown-rump lengths and somite numbers slightly but significantly ($p < 0.01$), and the dihydroxy metabolites also reduced the embryonic protein content ($p < 0.01$). In contrast, the parent salicylate produced large increases in embryolethality (embryolethality in controls was 6% or less) and malformed embryos at equivalent or lower concentrations. Preincubation of the parent salicylate with various biotransforming systems did not affect embryotoxicity significantly. The most rapid biotransformation of salicylate *in vitro* was achieved with mitochondrial preparations of monkey kidney as the enzyme source but quantities metabolized were not sufficient to prevent malformations in the culture system. Increased serum protein concentration in the culture medium, however, markedly reduced the capacity of added salicylate to cause malformations. An examination of the kinetics of the dysmorphogenic effects of parent salicylate indicated that 5 hr of exposure elicited nonsignificant increases in numbers of malformations. A significant malformation rate was produced by 9 hr of exposure. In contrast, effects on embryonic growth parameters and embryolethality were greatest after a 24-hr exposure period. The results strongly suggest that the parent salicylate, rather than generated metabolites, was primarily or solely responsible for the malformations observed and that the duration of exposure of embryos to unmetabolized salicylate may be the critical factor for determining teratogenic outcome.

Concern for the embryotoxic effects of salicylates remains strong due to the frequent and often heavy usage of these chemicals as medicinal agents during pregnancy (Corby, 1978). Although not currently regarded as a highly significant teratogenic hazard in humans, several considerations provide cause for caution: (1) salicylates are well-established teratogens in various experimental animals; (2) definitive establishment of the teratogenicity of any chemical in humans is extremely difficult; (3) certain individuals (e.g., rheumatoid arthritics) ingest very large quantities of salicylates and exhibit high plasma levels that reflect the well-known dose-dependent pharmacokinetics of salicylates; (4) some studies (e.g., McNiel, 1973; Richards, 1969; Turner and Collins, 1975) have linked aspirin ingestion with birth defects in humans.

Studies of the mechanisms whereby salic-

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ylates may produce embryotoxic effects have suggested that the action is primarily a direct effect on the embryo rather than a predominantly indirect effect on maternal homeostasis (Kimmel *et al.*, 1971; Koshakji and Schubert, 1973; McGarrity *et al.*, 1978; Greenaway *et al.*, 1982). One study suggests that the kinetics of the plasma levels may be the most important determinants of teratogenesis (Kimmel and Young, 1983). Whether such a direct effect is produced by the parent chemical or by a metabolite(s), however, is not known.

Reputedly, the rat is sensitive to the teratogenic effects of salicylates whereas humans and nonhuman primates are regarded as resistant (Shepard, 1983; Wilson *et al.*, 1977), but the basis for the presumed species difference remains speculative. Differences in embryonic target organ tissue sensitivity or in salicylate disposition would seem to be the most likely reasons for such species differences. If dispositional differences play a major role, it is possible that biotransformational dissimilarities could be extremely important, particularly if metabolites of salicylates display varying degrees of embryotoxicity. The effects of these substances on embryonic parameters have not been reported to our knowledge.

In order to gain further insights into these important questions, we investigated the possibility that metabolites of salicylate might contribute importantly to observed dysmorphogenic and/or other embryonic effects. To avoid the complications of potentially confounding maternal factors, we chose to study the effects of salicylate and its major metabolites in an embryo culture system. In addition, we investigated the bioconversion of salicylate to various metabolic products under varying conditions including those utilized for the culturing of embryos. The results obtained provide evidence that the parent salicylate is largely, if not entirely, responsible for the dysmorphogenic effects observed in rats and that the kinetics of embryo exposure to the parent salicylate are an important determinant of embryotoxicity and dysmorphogenesis.

METHODS

Animals. Embryos utilized for culturing were taken from primigravid Sprague–Dawley rats purchased from Tyler Laboratories (Bellevue, Wash.). The morning after copulation was designated as the start of Day 0. These animals were received between Days 3 and 6 of pregnancy and were housed in the animal facility of the Central Laboratory for Human Embryology. The animals received feed (Purina Lab Chow) and water *ad libitum*. A 14-hr light, 10-hr dark lighting cycle was maintained. At 9:00 A.M. on Day 10 of pregnancy, the animals were anesthetized with ether, and blood was collected from the abdominal aortas. (This blood was used to provide serum for the medium used to culture the embryos.) Uteri were then removed under aseptic conditions and placed in cold, sterile Hank's balanced salt solution (HBSS).

Embryo culture system. A modification of the rat embryo explant culture system of Fantel *et al.* (1979) was utilized. This system is based upon methods developed by New (1978). After removal of uteri on Day 10 of gestation, decidua, and remnants of trophoblast, parietal yolk sac and Reichert's membrane were carefully removed with the aid of a stereoscopic dissecting microscope. The visceral yolk sac, ectoplacental cone region, and amnion were left intact. The embryos were cultured in a medium consisting of 7 ml of Waymouth's medium (752/1) GIBCO, Grand Island, N.Y.), supplemented with glutamine, 3.5 ml of rat serum, and 3.5 ml of human serum. Blood from healthy, adult volunteers was used to provide the human serum. Both rat and human sera were centrifuged immediately after collecting and were heat-inactivated at 56°C for 20 min. Penicillin (100 units/ml) and streptomycin (50 μg/ml) were added to each 125-ml culture bottle containing 14 ml of complete medium. Prior to addition of embryos (10 ± 2 somites and litters evenly distributed between bottles), the medium was prewarmed and pregassed with 5% CO_2, 5% O_2, and 90% N_2 to obtain a constant pH of 7.3. For study of the kinetic aspects of salicylate teratogenicity, embryos were cultured with 1.9 mM Na salicylate from 0 time. At 5 and 9 hr, embryos were rinsed with Waymouth's medium and transferred to fresh medium without salicylate for the duration of the culture period at 37°C in a roller apparatus. The following morning the media were regassed with 95% O_2 and 5% CO_2, and culturing was continued for an additional 5 hr. After 24 hr of culturing, embryos were examined under a dissecting microscope for viability and abnormalities as well as for indices of growth and development. All embryos were examined without knowledge of treatment. Viable embryos were those exhibiting active yolk sac circulation and heart beat. Only viable embryos were assessed for malformations and growth parameters. Crown–rump length, somite number, limb development, and malformations were recorded. Protein content was determined by the method of Bradford (1976). Repre-

sentative embryos were photographed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3 for histological examination. Light microscopy was performed on representative sections.

Chemicals. Sodium salicylate, *o*-hydroxyhippurate (salicylglycine, salicyluric acid), 2,5-dihydroxybenzoate (gentisic acid), and 2,3-dihydroxybenzoate were purchased from Aldrich Chemical Co., Milwaukee, Wis. Salicylic acid, NADPH, NADH, ATP, glycine, CoA, uridine diphosphoglucuronic acid (UDPGA), glucose-6-phosphate (G6P), and G6P-dehydrogenase (G6PDH) were obtained from Sigma Chemical Co., St. Louis, Mo. The salicylic acid derivatives were purified by recrystallization from distilled water. All melting points were equal to or higher than the specified ranges (Merck, Aldrich). Purity also was checked by reverse phase high performance liquid chromatography (HPLC) with a Waters C-18 Microbondapak column and by monitoring the absorbance of the eluate at 254 nm. Purity was determined to be greater than 99% in each case. [7-^{14}C]Salicylic acid (53.8 Ci/mol; 98.5% purity) was purchased from New England Nuclear Corp., Boston, Mass. and was not further purified, but purity was documented by a ^{14}C fraction profile of an HPLC separated aliquot. All other chemicals used were reagent grade and were of the highest purity available commercially.

Analysis of salicylate biotransformation. For studies of salicylate biotransformation *in vitro,* a number of different homogenate subfractions prepared from livers and kidneys of adult female rats (Sprague–Dawley) and monkeys (*Macaca nemestrina,* obtained from the U.W. Primate Center) were utilized. Whole homogenates were prepared by first finely mincing the tissues with scissors and then homogenizing the minces in a Potter homogenizing vessel with a Teflon pestle (3 passes). The homogenate consisted of 1 g of tissue (wet weight) per 2 ml of a solution containing 0.01 M potassium phosphate (pH 7.5) and 0.2 M sucrose. The following subfractions were prepared from the homogenates; a supernatant fraction obtained by centrifuging the homogenate at 600*g* for 15 min (600 g), a supernatant fraction obtained by centrifuging the homogenate at 3000*g* for 10 min (S-3), a supernatant fraction obtained by centrifuging the homogenate at 9000*g* for 10 min (S-9), and a mitochondrial-enriched particulate fraction obtained by centrifuging the 600 g fraction at 9000*g* for 10 min (P-9). The P-9 fraction was resuspended in 2 volumes of 0.1 M potassium phosphate buffer. The 600 g, S-3, S-9, and P-9 preparations were utilized as enzyme sources in studies of the biotransformation of salicylate.

For biotransformation studies of salicylate, incubation vessels contained 5–10 mg protein from the enzyme source and cofactors in the following final concentrations: 0.9 mM NADPH, 4.2 mM G6P, 3 units of G6PDH, 4.2 mM ATP, 0.4 mM CoA, 83 mM glycine, 0.8 mM UDPGA, 1.5–1.9 mM salicylate, and 0.4 μCi of [7-^{14}C]salicylic acid. Incubations were carried out in darkness at 37–38°C at pHs from 7.4 to 8.0 (Tris buffer, 0.1 M) in an atmosphere of air. Total volume of the reaction mixtures was 1.2 ml. Incubations were run from 30 min to 3 hr, and reactions were stopped by the addition of 0.2 ml of 6 N HCl. The incubation mixtures were extracted twice with 6.5 volumes of ethyl ether (peroxide-free). The ether phase was taken to dryness with a stream of nitrogen gas and was then resuspended in 200 μl of a methanol solution containing the metabolite standards (each at 0.5 mg/ml) (Fig. 2). Samples (20 μl) of the methanol solution were injected into a Beckman HPLC system with a Waters Microbondapak reverse phase C-18 column (3.9 × 300 mm) at room temperature. Ten microliters yielded a full peak deflection at a sensitivity setting of 0.1 and a wavelength of 254 nm. The solvent system was methanol–0.1 M acetic acid utilized as a three-step mobile phase: (1) 15:85 isocratic for 10 min, (2) 15:85 to 35:65 linear gradient for 10 min, (3) 35:65 to 100:0 linear gradient for 2 min. Fractions were collected for an additional 5 min such that 90 fractions were collected (ISCO, Model 1850 fraction collector) in 27 min. Salicylate biotransformation was determined by counting the fractions collected in a Model 8000 Beckman liquid scintillation counter and determining the percentage of total counts (dpm) from each sample that corresponded with peaks eluting with the metabolite standards.

The metabolic system was modified when rat embryos were to be added to the incubation medium. For glycine conjugation, incubation was conducted in 3 ml Waymouth's medium and ATP was reduced to 2.8 mM. Both S-3 and 600 g supernatant fractions from kidney or liver of rats and monkeys were utilized as enzyme sources. The embryos were started in separate culture at the time the preincubation of the metabolic system was begun. Following preincubation, samples were taken for ether extraction, HPLC separation, and quantitation of metabolites. Embryos were transferred to the preincubated medium after 3 to 6 hr, and an additional 4 ml of Waymouth's medium and 7 ml of sera were included. After a total of 24 hr of incubation, comparisons were made between:

(1) embryos cultured with salicylate only from 0 time (beginning of preincubation);

(2) embryos cultured with "metabolized" salicylate from the end of the incubation period;

(3) embryos cultured with "unmetabolized" salicylate from the end of the incubation period—to control for any time-specificity of the salicylate effect;

(4) embryos cultured with the enzyme fractions and cofactors but no salicylate;

(5) embryos cultured in medium only.

A minimum of two separate experiments and 10 embryos were assessed for each type of enzyme preparation. Additionally, embryos were cultured with rat S-9 or 600 g fraction, 1 mM UDPGA, and 1.6 mM salicylate to assess the contribution of glucuronidation to salicylate teratogenicity *in vitro.*

RESULTS

Salicylate (tested both as the acid form and as the sodium salt) and three of its major metabolites each were added directly to separate bottles of embryos at the beginning of the culture period. Results of these experiments are presented in Table 1. At a final concentration of 1.9 mM, sodium salicylate produced embryolethality in 26 of 34 embryos and 38% of the remaining viable embryos exhibited malformations. At the same concentration, the tested metabolites caused no embryolethal effects or detectable malformations. However, each of these metabolites produced significant reductions in crown–rump lengths and somite numbers.

The typical malformations produced by exposure of the embryos to the parent salicylate are illustrated in Fig. 1. Abnormal closure of the neural tube and/or dilation or edema of the cephalic portion of the neural tube were the most frequent malformations observed in this study as well as in a previous study (Greenaway *et al.*, 1982) in which the morphologic changes and concentration effect relationships were presented in detail. Light microscopic examination of sections prepared for histology also provided similar results.

Lack of malformations in embryos exposed to the salicylate metabolites suggested that biotransformation could prevent or ameliorate the effect of the parent chemical. We therefore incubated salicylate with various active metabolic systems before adding embryos to test whether metabolic inactivation could be detected *in vitro* by embryotoxic parameters. At the same time, we monitored the activity of the metabolic systems utilized. The metabolites generated were extracted and separated with high pressure liquid chromatography (Fig. 2). We compared metabolism by enzyme sources from a resistant species (monkey) and a sensitive species (rat). A metabolite profile obtained with monkey kidney P-9 fraction is illustrated in Fig. 3. Under the conditions utilized, the glycine conjugate (salicylic acid) was the major metabolite formed in all tissue homogenate subfractions except S-9 fractions from the kidneys and livers of rats (Table 2). The only other major metabolites identifiable were 2,5-dihydroxybenzoic acid (gentisic acid, the major metabolite in studies with rat liver S-9) and 2,3-dihydroxybenzoic acid (the major

TABLE 1

EFFECTS OF SODIUM SALICYLATE AND THREE OF ITS MAJOR METABOLITES[a] ON CULTURED RAT EMBRYOS

Chemical	N^b	Crown–rump length[c] (mm)	Somites[c]	Protein[c] (μg)	Malformed[d] (%)	Viable (%)
Medium only	27	2.9 ± 0.2	21.3 ± 0.6	203 ± 23	0	96
Sodium salicylate[e]	34	2.6 ± 0.3[f]	17.9 ± 1.6[f]	165 ± 61[f]	38[f]	24[f]
Salicyluric acid	18	2.7 ± 0.1[f]	20.3 ± 0.4[f]	214 ± 19	0	83
Gentisic acid	15	2.7 ± 0.1[f]	19.9 ± 0.5[f]	182 ± 5[f]	0	87
2,3-Dihydroxybenzoate	19	2.7 ± 0.1[f]	19.9 ± 0.7[f]	155 ± 15[f]	0	95

[a] Each chemical was added at the beginning of the 24-hr culture period at a final concentration of 1.9 mM.

[b] Total number of embryos cultured.

[c] Numbers are means (±SD) determined from viable embryos.

[d] Percent of viable embryos exhibiting malformations.

[e] Salicylic acid exhibited roughly equal embryotoxicity (data not shown).

[f] Significantly different from control ($p < 0.01$). Student's t test was utilized for analyses of differences in crown–rump length, somite numbers, and protein concentration. For analyses of differences in viability and malformations, the χ^2 criterion was utilized with Yates' correction for continuity. All statistical methods are described by Edwards *et al.* (1954).

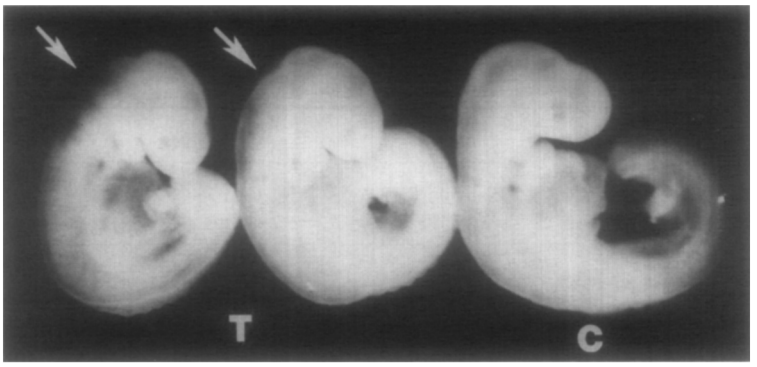

FIG. 1. Morphology of Day 11 rat embryos (T) cultured with 1.9 mM sodium salicylate from Day 10, compared to control embryo (C). Arrows indicate dilated rhombencephalon.

metabolite produced with rat kidney S-9 as enzyme source). Monkey tissues exhibited a three- to sixfold greater capacity than rat tissues to convert salicylate to metabolites extractable into ether following acidification of the aqueous phase. Experiments also were performed with human placental tissues. Under the same conditions in which tissues from rats and monkeys exhibited easily demonstrable glycine conjugation and hydroxylation reactions, the placental preparations did not generate detectable quantities of these metabolites.

Analyses indicated that less than 1% of the originally added radioactivity remained in the aqueous phase subsequent to ether extractions unless an active glucuronidating system was present in the reaction mixtures. Inclusion of UDPGA and an active enzyme source (e.g., rat hepatic S-9) resulted in significant increases in measured activity in the aqueous phase, presumably as a result of the more preferential partitioning of salicylate glucoronides into the aqueous phase. No significant difference was seen in growth of embryos exposed to salicylate, UDPGA, and rat S-9 when compared to embryos cultured with salicylate only.

Preculturing of several combinations of ac-

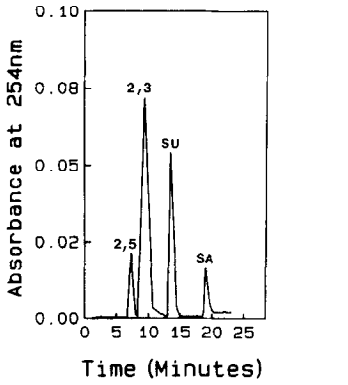

FIG. 2. Chromatogram of HPLC separation of salicylate and metabolite standards (5 μg each): 2,5-dihydroxybenzoic acid (2,5) retention time (RT) 7.2 min, 2,3-dihydroxybenzoic acid (2,3) RT 9.7 min, salicyluric acid (SU) RT 14.21 min, salicylic acid (SA) RT 20.14 min.

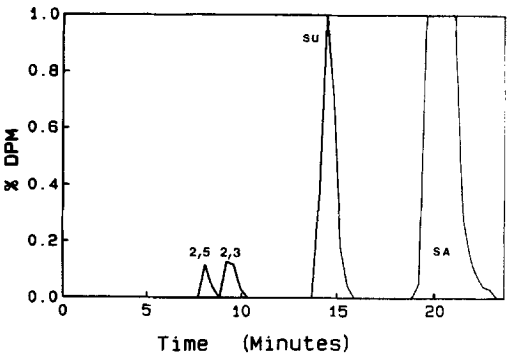

FIG. 3. ^{14}C profile of extract from incubation medium separated by HPLC demonstrating biotransformation of salicylate *in vitro* with monkey kidney mitochondrial fraction as the enzyme source.

TABLE 2

GENERATION OF ETHER-EXTRACTABLE SALICYLATE METABOLITES[a] AFTER INCUBATION WITH VARIOUS ENZYME SOURCES

Enzyme source	Metabolites		
	Salicyluric acid	Gentisic acid	2,3-Dihydroxy benzoic acid
Rat kidney S-9[b] (5)	1.4 ± 0.8	2.8 ± 2.0	6.8 ± 4.7
Monkey kidney S-9[b] (4)	32.1 ± 11.8	ND[d]	3.7 ± 2.4
Rat kidney P-9[c] (6)	10.7 ± 2.5	2.3 ± 0.8	2.5 ± 1.2
Monkey kidney P-9[c] (4)	121.8 ± 3.7	ND[d]	3.1 ± 1.6
Rat liver S-9[b] (4)	0.2 ± 0.2	12.4 ± 7.8	4.8 ± 3.0
Monkey liver S-9[b] (3)	8.8 ± 4.3	3.7 ± 1.5	2.8 ± 1.7
Rat liver P-9[c] (4)	1.6 ± 0.5	0.1 ± 0.1	ND[d]

[a] Numbers in the table are mean specific activities (±SE) expressed as pmol/mg protein/min after a 1-hr incubation at 37°C in air. Numbers in parentheses are the number of experiments performed for each enzyme source. Protein concentrations in the incubation vessels were all between 5 and 10 mg/ml. Cofactors included glycine, NADPH, G6PDH, ATP, CoA, and G6P in concentrations given in the Methods section.

[b] The 9000*g* (10 min) supernatant fraction (see methods).

[c] The 9000*g* (10 min) particulate fraction obtained from the 600*g* (15 min) supernatant fraction.

[d] Not detectable.

tive metabolic systems with salicylate prior to adding embryos to the culture system did not reduce the malformation incidence or other embryotoxic effects. Preincubation of salicylate with monkey kidney mitochondrial fractions (the most metabolically active studied) for as long as 6 hr before inclusion of embryos did not significantly alter the embryotoxic effects of salicylate. However, analyses of the data indicated that a maximum of 4% of parent salicylate was converted to metabolites under the most optimal incubation conditions utilized in the biotransformation studies. (More than 96% of the parent salicylate could be recovered as the unchanged compound.) This conversion would reduce the concentration of salicylate in the culture medium from 1.9 mM to approximately 1.8 mM and would not be sufficient to diminish the embryotoxic effects significantly under the conditions utilized. Rates of salicylate metabolism measured in this study are similar to those previously reported in the literature (Irjala, 1972).

Because salicylate is known to bind extensively to plasma proteins and because we observed greater salicylate embryotoxicity in these experiments than in previous experiments (Greenaway *et al.*, 1982) in which the protein concentration in the culture medium was higher, we examined the possibility that the embryotoxic effects could be ameliorated by increasing the concentration of serum protein in the culture medium. Results of these experiments are presented in Table 3. A direct comparison revealed that a twofold increase in the protein concentration (effected by utilizing a medium with 100% serum rather than 50% serum) resulted in significant decreases in salicylate-elicited malformations and embryolethality.

Experiments designed to determine the duration of exposure of embryos to salicylate required for maximal embryotoxic effects are presented in Table 4. For all embryotoxic effects examined (except malformations), the severity of the effect increased with the duration of exposure through the 24-hr culture period. The percentage of malformations observed after a 24-hr exposure period was no greater than that measured after only a 9-hr

TABLE 3

EFFECT OF SERUM IN CULTURE MEDIUM ON SALICYLATE EMBRYOTOXICITY[a]

Measured parameter[b]	2.5 mM salicylate 100% serum	1.9 mM salicylate 50% serum
Crown–rump length	100	90
Number of somites	95	84
Protein (μg/embryo)	101	80
Percent malformed	16	38
Percent viable	89 (25/28)	24 (8/34)

[a] With 100% serum, 1.9 mM salicylate produced no detectable changes in the measured parameters. With 50% serum, 2.5 mM salicylate resulted in 100% embryolethality.

[b] Growth parameters are expressed as a mean percentage of concurrent control values for embryos cultured with the appropriate serum concentration without salicylate. Numbers in parentheses are viable embryos/total embryos in the culture medium. Only viable embryos were assayed for growth and malformations.

exposure. A 9-hr exposure period was sufficient to produce a significant increase in the number of malformed embryos ($p < 0.01$) compared with embryos not exposed to salicylate.

DISCUSSION

Although the concentration of salicylate required to elicit malformations *in vitro* could be regarded as high (1.6–1.9 mM), it should be borne in mind that measured salicylate levels in human maternal blood and tissues may approach this concentration after single doses of salicylate (Elis *et al.*, 1978; Bochner *et al.*, 1981). Additionally, the plasma concentrations associated with anti-inflammatory activity (200–300 μg/ml) are about 6 times that needed to produce analgesia (Swingle and Kvam, 1982) and correspond to molar concentrations of 1.4–2.2 mM. At the higher plasma concentrations, the rate of metabolism is reduced and the plasma half-life is extended. Experiments with rats also have indicated that concentrations of salicylate in the embryo mimic maternal blood concentrations of the unbound form after the first 45 min (Kimmel and Young, 1983). Thus, the concentrations of salicylate used in these experiments could be relevant to human exposure. However, in such considerations, the relative concentrations of bound and unbound forms of the salicylate must be considered since only the unbound form has access to intracellular fetal components. The experiments illustrated in Table 3 are clearly indicative of the importance of protein binding as a determinant of salicylate embryotoxicity. Kimmel *et al.* (1971) and Wilson *et al.* (1977) also have emphasized the importance of protein binding in salicylate teratogenicity. Future experiments should focus more explicitly on the effect of protein binding on the embryotoxic effects of salicylates *in vitro*.

Barring differential tissue sequestration, plasma and tissue levels of salicylate metabolites would not be expected to exceed those of the parent chemical (Bochner *et al.*, 1981). Thus the absence of dysmorphogenic effects from the three major metabolites tested at concentrations equivalent to those of the par-

TABLE 4

EFFECT OF DURATION OF EXPOSURE OF EMBRYOS TO SALICYLATE[a] ON MEASURED PARAMETERS

Measured parameter	Time of exposure (hr) 0	5	9	24
Crown–rump length[b,c]	100 (16)	96 (12)	94 (12)	85 (7)
Number of somites[b,c]	100 (16)	98 (12)	93 (12)	79 (7)
Protein (μg/embryo)[b,c]	100 (16)	99 (11)	83 (12)	70 (7)
Percent malformed[c]	0	25 (3)	58 (7)	57 (4)
Percent viable[c]	94 (16)	86 (12)	100 (12)	42 (7)

[a] Sodium salicylate (1.9 mM final concentration) was present in the culture medium at the beginning of the 24-hr culture period. At the times indicated, embryos were removed from the original culture medium (containing salicylate) and transferred to fresh medium for the remainder of the culture period.

[b] Expressed as percentage of concurrent control values (mean of three experiments).

[c] Numbers in parentheses are the numbers of cultured viable embryos used for calculations of values in the table.

ent salicylate strongly suggests that biotransformation of salicylate would normally result in inactivation. This concept is also supported by Kimmel *et al.* (1971) who observed that inhibition of glycine conjugation of salicylate with benzoic acid *in vivo* resulted in increased embryotoxicity. Salicylate, rather than a metabolite, is directly embryotoxic, and teratogenicity need not be mediated through the alterations of maternal homeostasis. Other minor metabolites (such as glucuronides) were not tested directly due to lack of availability but are considered unlikely to be responsible for salicylate's embryotoxic effects. Addition of UDPGA and an active glucuronidating system (data not shown) did not measurably alter the embryotoxic effects of salicylate. Sulfate esters and other metabolites would be generated only in trace amounts and likewise are probably not embryotoxic. Thus, plasma levels of the parent salicylate should represent a principal predictor of ultimate embryotoxicity. Support for this concept has recently been given by Kimmel and Young (1983) who reported that plasma salicylate levels were well-correlated with teratogenic outcome in rats.

The data also are supportive of the idea that species differences in the biotransformation of salicylates may be partially responsible for differences in susceptibility to embryotoxic effects. In all experiments, the data indicate that the primate species is capable of a more rapid biotransformation of salicylate than the rat due to greater formation of the glycine conjugate which is a particularly important metabolite of salicylate in humans. Approximately 80% of administered salicylate is excreted as the glycine conjugate in humans (Swingle and Kvan, 1982). Although the metabolic systems utilized in this study for the biotransformation of salicylate *in vitro* did not reduce salicylate concentrations in the embryo culture media below levels that produce dysmorphogenic effects, metabolic clearance *in vivo* could be expected to reduce plasma/tissue concentrations to nontoxic levels effectively (Bochner *et al.*, 1981). At higher doses of salicylate, however, plasma half-lives in humans have been estimated at 15 to 30 hr. Since only a short period of exposure (5 hr) was required to elicit dysmorphogenic effects in the culture system, the implication is that the more rapid rates of clearance in primates may not always suffice to avert embryotoxic effects. Experimental animals or subjects exhibiting a long salicylate half-life would be at greater risk, particularly if exposure occurred at the more critical periods during gestation.

The significance of the rhombocephalic edema elicited by salicylate in the embryo culture system is difficult to ascertain at present. It was of interest, however, that administration of salicylate to the maternal organism on the morning of day 10 (600 mg/kg) resulted in the appearance of embryos with a very similar appearance as that observed following exposure *in vitro* when examined 24 hr after the drug was administered (data not shown). Upon examination of the paper of Kimmel *et al.* (1971), one might speculate that meningocele might be associated with such a change. However, the high doses utilized in this study probably would not be generally compatible with viability at term (Greenaway *et al.*, 1982). Thus, the relative transience or permanence of the observed morphologic change cannot be ascertained at present.

Our data suggest that plasma protein binding may be more effective than metabolic clearance in preventing the effects of salicylate *in vitro*. Thus, protein binding also may play a highly important role as a determinant of the species differences in salicylate embryotoxicity exhibited by rats and primates. It is known that primates exhibit a higher bound fraction of salicylates than do rodents. It has been noted (Varma and Yue, 1983) that maternal protein deficiency resulted in consistently and significantly greater placental transfer of salicylate. However, the increased placental transfer was attributed to differences in factors influencing placental permeability rather than to changes in serum protein-salicylate binding.

The weight of evidence now indicates that unchanged salicylate, acting directly on the embryo, is responsible for the dysmorphogenic effects of salicylates administered to the maternal organism. This allows a closer focus on the mechanism of the dysmorphogenic and embryotoxic effects of salicylate. Presumably, the mechanism does not involve inhibition of cyclooxygenase because salicylate appears to be as effective as acetylsalicylic acid (Juchau and Fantel, 1981) as an embryotoxin *in vivo* and yet is much less effective as a cyclooxygenase inhibitor (Flower, 1974). In these investigations (data not shown), we found that salicylate was slightly more effective than acetylsalicylic acid as a dysmorphogenic agent in the embryo culture system and that indomethacin, an extremely effective cyclooxygenase inhibitor, did not elicit dysmorphogenic effects at concentrations that profoundly inhibit the cyclooxygenase. Therefore, it would appear that other mechanisms should now be investigated.

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