TOXICOLOGY AND APPLIED PHARMACOLOGY 60, 142–150 (1981)

Potassium Loss as a Causative Factor for Skeletal Malformations in Rats Produced by Indacrinone: A New Investigational Loop Diuretic

R. T. ROBERTSON, D. H., MINSKER, D. L. BOKELMAN, G. DURAND,* AND P. CONQUET*

*Merck Sharp & Dohme Research Laboratories West Point, Pennsylvania 19486; and *Riom, France*

Received January 28, 1981; accepted March 23, 1981

Potassium Loss as a Causative Factor for Skeletal Malformations in Rats Produced by Indacrinone: A New Investigational Loop Diuretic. ROBERTSON, R. T., MINSKER, D. H., BOKELMAN, D. L., DURAND, G., AND CONQUET, P. (1981). *Toxicol. Appl. Pharmacol.* **60**, 142–150. Indacrinone (MK-196), an investigational loop diuretic, was administered to female CRCD rats from Days 6 to 17 of gestation at doses of 40, 80, or 120 mg/kg/day. Dose-related increases in wavy ribs and malformations of the scapula and humerus were apparent in fetuses from all drug-treated groups. Studies on the individual enantiomers of MK-196 showed that the teratogenic potency was associated with the relative saluretic activity of the enantiomers. Furosemide, a widely used diuretic with pharmacologic activity similar to that of MK-196, was administered to rats on Days 6 to 17 at doses of 37.5, 75, 150, or 300 mg/kg b.i.d. Dose-related increases in wavy ribs occurred in all drug treatment groups. At 150 mg/kg b.i.d. there were malformations in the scapula and humerus identical to those described for MK-196. Providing a 1% solution of KCl during the dosing period reduced the incidence of wavy ribs in fetuses from MK-196-treated dams by 90% and eliminated the scapular and humeral malformations. Coadministration of amiloride was also found to antagonize the teratogenicity of MK-196. The results indicate that high doses of diuretics with similar pharmacologic activities can produce identical skeletal malformations in rats, and that this teratogenicity is related to hypokalemia.

Fetal limb malformations in rats involving primarily the ulna and/or digits have been associated with administration of several diuretics including acetazolamide, ethoxazolamide, and dichlorphenamide (Layton and Hallesy, 1965; Wilson *et al.*, 1968; Hallesy and Layton, 1967). The pharmacologic activity of these diuretics is similar in that all are carbonic anhydrase inhibitors and exert their diuretic effects through inhibition of H+ secretion by the renal tubule (Rector, 1973). The forelimb malformations produced by these compounds are most often right-sided or bilateral and may not be directly related to the activity of these diuretics as carbonic anhydrase inhibitors (Storch and Layton, 1973). Hirsch and Scott (1979) showed that the sensitivity of various strains of mice to acetazolamide corresponded to differences in the effectiveness with which acetazolamide inhibited carbonic anhydrase in fetuses of these strains. The first indications that inhibition of carbonic anhydrase might not be the proximate teratogenic action were studies by Ellison and Maren (1972) which demonstrated that the teratogenicity of acetazolamide in rats could be effectively antagonized by providing supplemental potassium in the drinking water, and by coadministration of the potassium-sparing diuretics, amiloride and triamterene.

Indacrinone (MK-196; 6,7-dichloro-2-

0041-008X/81/100142-09$02.00/0

methyl-1-oxo-2-phenyl-5-indanyloxy acetic acid) is an investigational loop diuretic with saluretic-antihypertensive activity that has no activity as an inhibitor of carbonic anhydrase (De Solms *et al.*, 1978). The compound has a single, optically active carbon atom and exists as a racemic mixture. The teratogenicity of MK-196 and its individual enantiomers has been studied in rats. Teratogenic studies also have been conducted in rats using furosemide, a loop diuretic of similar pharmacologic activity but different structure, as a reference compound. Finally, based on data published by Ellison and Maren (1972), the role of potassium in the expression of teratogenicity produced by MK-196 was evaluated through potassium supplementation via the drinking water and by coadministration of amiloride.

METHODS

Animals

Female CRCD rats (Charles River, Wilmington, Mass.) that weighed 200 to 300 g were individually housed with males of the same strain. Upon observation of sperm in the daily vaginal lavage (Day 0 of gestation), the females were individually identified by ear notching and dye marking, weighed, and sequentially assigned to treatment groups. Females were housed three or less per box in an air-conditioned room maintained at 23°C with a 12-hr light and 12-hr dark cycle. Body weights of females were recorded on Days 0, 6, 8, 10, 12, 14, 16, 18, and 20 of gestation, and drug doses were based on the most recent body weight. All females were sacrificed on Day 20 of gestation and the reproductive status of each female recorded. The uterine contents of each female were removed, and fetuses were weighed and coded so that external, visceral, and skeletal examinations could be conducted without knowledge of treatment group. All fetuses were examined externally and a visceral examination by dissection was performed on every third fetus in each litter. In addition, a visceral examination was performed on any externally malformed or dead fetus. The heads of fetuses designated for visceral examination were fixed in Bouin's solution and examined by freehand serial sectioning. All fetuses were stained with alizarin red and examined for skeletal alterations (Wilson, 1965).

Drug Administration

MK-196 study. In the initial teratogenic evaluation of MK-196, groups of 20 mated rats each were administered the compound at 40, 80, or 120 mg/kg/day from Days 6 through 17 of gestation. A control group of animals received the vehicle, a 0.5% aqueous suspension of methyl cellulose (400 cp), in the same volume and dosing regimen. Unless otherwise indicated, this same vehicle, volume, dosing regimen, and appropriate vehicle control groups were used in all subsequent studies. Groups of 20 mated female rats were administered the (+) enantiomer of MK-196 at doses of 20, 40, and 80 mg/kg/day, or the (−) enantiomer at doses of 15, 30, and 60 mg/kg/day. The lower doses of the (−) enantiomer were based on an unreported range-finding study in which maternal mortality resulted from doses of the (−) isomer in excess of 60 mg/kg/day.

Furosemide study. Furosemide was used as a reference compound and was administered to groups of 20 rats according to a twice-a-day dosing schedule at dose levels of 37.5, 75, 150, and 300 mg/kg. A twice-a-day dosing regimen was used because comparative studies in humans and rats have indicated that MK-196 has a considerably longer duration of diuretic activity than furosemide (Irvin *et al.*, 1981).

Potassium supplementation study. Since range-finding studies in our laboratory had shown significant ($p \leq 0.05$; analysis of variance) dose-related reductions in maternal plasma potassium during administration of MK-196 at ≥40 mg/kg/day, the effect of potassium supplementation via the drinking water on the teratogenicity of MK-196 was examined in two groups of 25 mated rats administered 80 mg/kg/day from Days 6 through 17 of gestation. Two identically constructed groups of rats served as vehicle controls. One drug-treated and one vehicle control group were provided a 1% solution of potassium chloride as the only source of drinking water during the dosing period. The remaining drug-treated and vehicle control groups were provided tap water throughout the study. Preliminary studies in female rats had shown that providing supplementary potassium in the drinking water at concentrations ≥0.5% was sufficient to maintain potassium balance (total consumption/total excretion) throughout five daily doses of MK-196 at 80 mg/kg/day.

Amiloride coadministration study. An experiment that examined the effect of amiloride coadministration on the teratogenicity of MK-196 was designed so that five groups of 17 mated female rats each were administered an 80 mg/kg/day dose MK-196 on Days 6 through 17 of gestation. Four groups of these rats were administered amiloride at 0.25, 0.5, 1.0, or 2.0 mg/kg b.i.d. during this same time period. The doses of amiloride were administered 10 min prior to and 6 hr following the daily dose of MK-196. The vehicle control group contained an identical number of animals and

was administered the vehicle once daily during this same time period. Fetuses in this study were examined only for skeletal malformations.

RESULTS

Teratogenic Evaluation of MK-196

Sixteen rats in the 120 mg/kg/day dosage group and three rats in the 80 mg/kg/day dosage group died between Days 10 and 19 of gestation. No mortality or clinical signs of toxicity were observed in females in the 40 mg/kg/day dosage group. There were dose-related decreases in maternal body weight during the dosing period among surviving females at 120 and 80 mg/kg/day (Fig. 1). At 40 mg/kg/day there was a decreased mean maternal body weight gain during the dosing period compared to the control group. There were no treatment-related effects on the incidence of resorptions and dead fetuses in any group, and the average live fetal weight per litter was not affected by drug administration. External and visceral examination of fetuses did not reveal any evidence of a teratogenic effect.

Skeletal examination revealed dose-related increases in the incidence of "wavy" ribs in all drug-treatment groups (Fig. 2). Malformations of the scapula, specifically distortion of the glenoid angle and splitting along the infraspinus process also increased in a dose-related fashion in all drug-treatment groups (Plates 1 and 2). In the 80 and 120 mg/kg/day groups, malformations of the humerus, including twisting and/or shortening of the bone, accompanied scapular malformations in 15 and 6 fetuses, respectively. Scapular and/or forelimb malformations were always associated with wavy ribs, and the scapula and humerus malformations were either bilateral or occurred only on the right side.

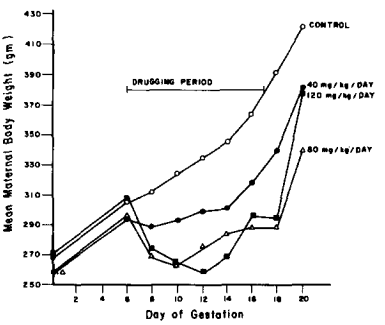

FIG. 1. Mean maternal body weight of rats administered MK-196.

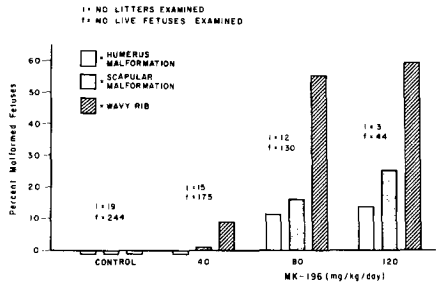

FIG. 2. Effect of MK-196 administered from Days 6 to 17 of gestation on the development of the fetal rat skeleton.

Teratogenic Evaluation of the Individual Enantiomers of MK-196

There was a retardation of weight gain during the dosing period among rats treated with the (+) enantiomer of MK-196. Effects of the (−) enantiomer were more pronounced and included dose-related decreases in body weight during the drugging period at 30 and 60 mg/kg/day and a retardation in body weight gain in the 15 mg/kg/day group. Treatment-related skeletal malformations observed in fetuses from groups treated with the (+) enantiomer were limited to wavy ribs (Fig. 3). In the 20 mg/kg/day group, only two fetuses from two litters had wavy ribs. Although this malformation was not observed in the concurrent control group, this incidence of wavy ribs is comparable to that which has been observed in individual historical control groups in our laboratory. Dose-related increases in wavy ribs were observed in the 40 and 80 mg/kg/

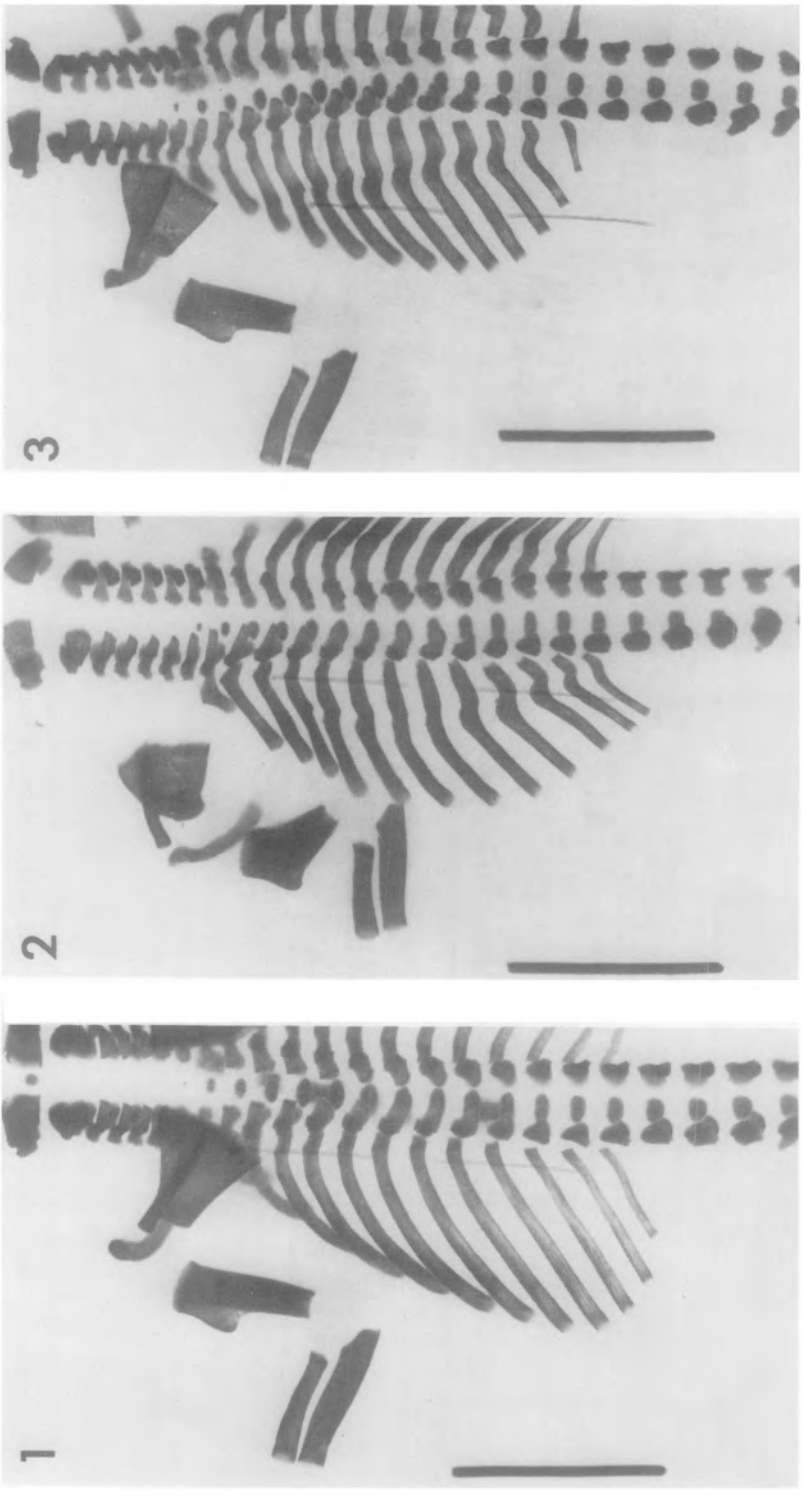

PLATE 1. (1) Dorsal skeleton of fetus from a control rat. (2) Dorsal skeleton of fetus from a rat treated with 80 mg/kg/day of MK-196. Note the malformation of the ribs, scapula, and humerus. (3) Dorsal skeleton of fetus from a rat treated with furosemide at 150 mg/kg b.i.d. Malformation of the ribs and scapula are comparable to those seen in fetuses from MK-196-treated rats.

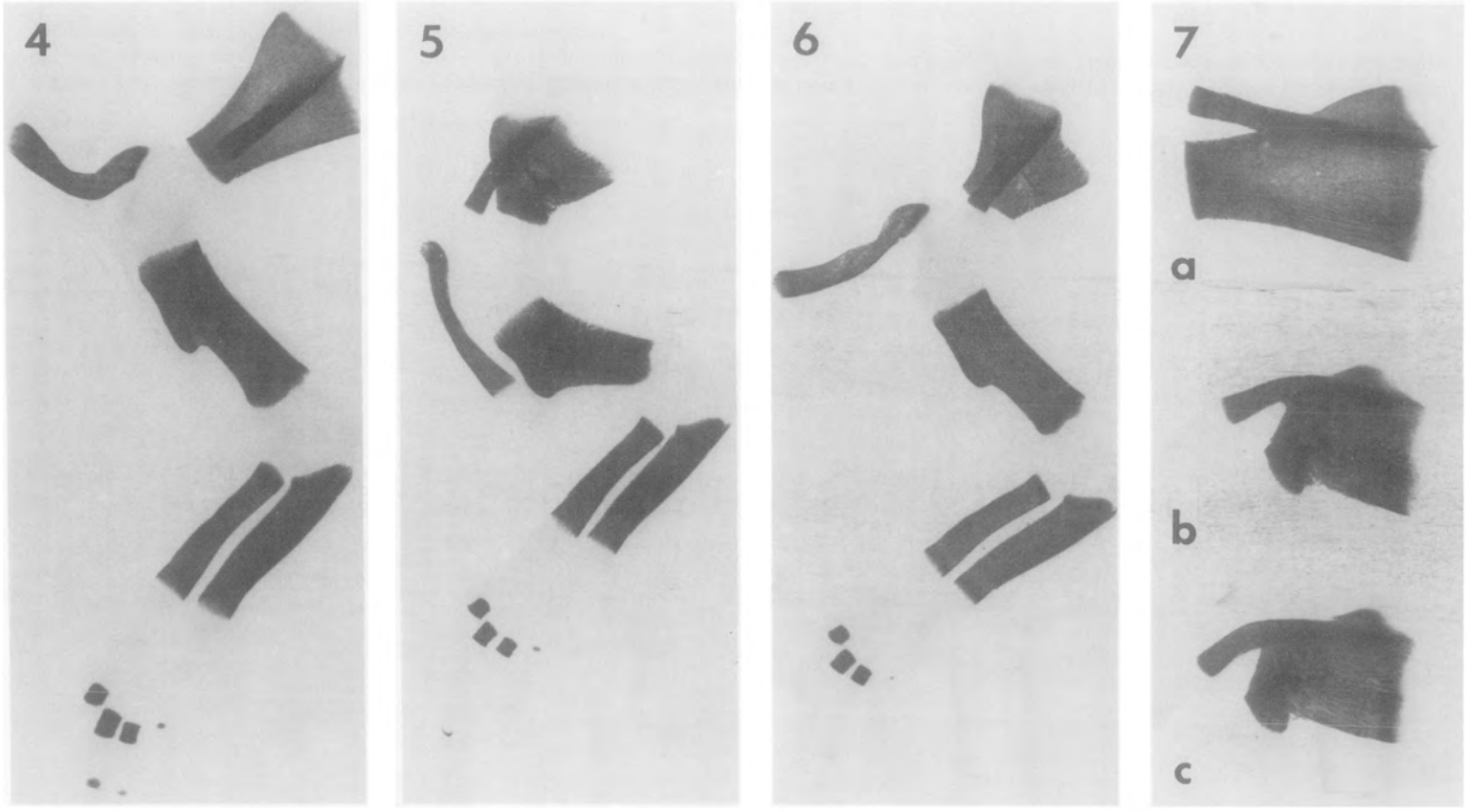

PLATE 2. (4–6) Example of forelimb from control, MK-196-treated, and furosemide-treated dams, respectively. The scapulae and clavicles of both drug-treated fetuses are similarly malformed and the humerus of the MK-196-treated fetus is shortened and thickened compared to the control. (7) a, Scapula from control fetus. b,c, Scapulae from MK-196 and furosemide-treated fetuses. The scapulae show comparable distortions of the glenoid angle and a splitting along the infraspinous process.

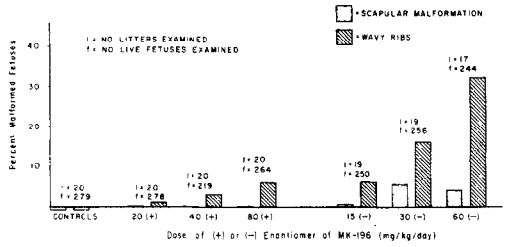

FIG. 3. The effects of the individual enantiomers of MK-196 administered from Days 6 to 17 of gestation on the development of the fetal rat skeleton.

day dosage level groups. There were no malformations of the scapula or humerus observed in any of the fetuses from dams administered the (+) enantiomer of MK-196. In contrast, fetuses from dams treated with the (−) enantiomer showed dose-related increases in wavy ribs and scapular malformations at all dosage levels (Fig. 3).

Teratogenic Evaluation of Furosemide

Thirteen females in the group administered furosemide at 300 mg/kg b.i.d. and one female in 150 mg/kg b.i.d. group died during the period of drug administration. No clinical signs of toxicity were observed in surviving females in any of the drug-treatment groups (Fig. 4). Embryo and fetal toxicities were apparent in the 300 and 150 mg/kg b.i.d. groups as an increase in the resorption rate and decrease in average live fetal weight per litter at both dosage levels.

There was no evidence of a teratogenic effect during external or visceral examinations of fetuses from any furosemide-treated group. Skeletal examination revealed dose-related increases in wavy ribs in all drug-treatment groups (Fig. 5). In addition, five fetuses in the 150 mg/kg b.i.d. group had malformations of the scapula which were morphologically identical to those which had been observed in the MK-196-treated fetuses (Plates 1 and 2). Scapular changes were not observed in fetuses at 300 mg/kg b.i.d., but this was probably the result of a smaller sample population resulting from the maternal and embryonic mortality previously mentioned.

Effect of Potassium Supplementation on the Teratogenicity of MK-196 in Rats

Three females in the MK-196-treated group and one female in the potassium-supplemented, MK-196-treated group died during the period of drug administration. There were decreases in average maternal body weight gains among MK-196-treated females in both groups during the period of drug administration comparable to those previously observed at this dose of MK-196. The average maternal body weight gain of females in the potassium-supplemented group was greater than that of females treated with MK-196 alone during the period of drug administration.

There was no evidence of a teratogenic effect at external or visceral examination of fetuses from either MK-196-treated group. Skeletal examination of fetuses from the group treated with MK-196 showed the expected costal, scapular, and humeral malformations (Fig. 6). The incidence of the specific malformations was comparable to that reported in the initial study at this dosage level. As in the previous study, the malformations of the scapula involved the right side or were bilateral.

In contrast to the results from fetuses exposed to MK-196 alone, there were no

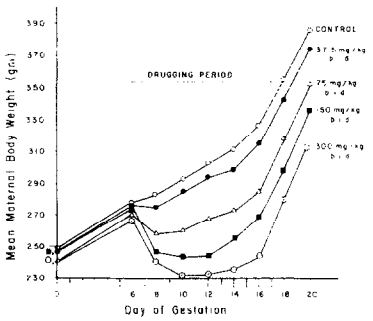

FIG. 4. Mean maternal weight of rats administered furosemide.

scapular or humeral malformations in fetuses from MK-196-treated dams supplemented with potassium during the dosing period (Fig. 6). In addition, the frequency of wavy ribs in this group was reduced by 90% compared to that in fetuses from dams that received MK-196 alone.

The Effect of Amiloride Coadministration on the Teratogenicity of MK-196

There were two or three deaths among dams in each of the MK-196-treated groups including those groups coadministered amiloride. Administration of an 80 mg/kg/day dose of MK-196 with and without amiloride produced decreases in average maternal body weight gain during the period of drug administration. MK-196-treated females that were coadministered amiloride at the three higher dose levels gained more weight during the period of drug administration than animals treated with MK-196 alone or MK-196 plus 0.25 mg/kg b.i.d. amiloride.

Results of skeletal examination of fetuses in the MK-196-treated group were comparable to those described in previous studies at this dosage level (Fig. 7). Coadministration of amiloride resulted in dose-related decreases in the incidence of wavy ribs as well as decreases in malformations of the scapula and humerus, at all dosage levels (Fig. 7). There were dose-related decreases both in the percentage of fetuses affected with one or more of these malformations and in the number of litters containing one or more malformed fetuses.

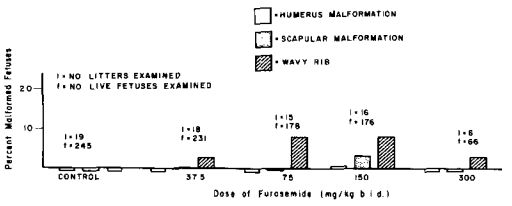

FIG. 5. Effects of furosemide administered from Days 6 to 17 of gestation on the development of fetal rat skeleton.

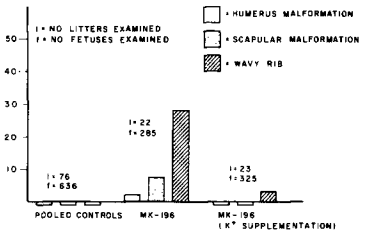

FIG. 6. The effect of potassium-supplemented drinking water on the teratogenicity of an 80 mg/kg/day dose of MK-196 administered to rats on Days 6 to 17 of gestation.

DISCUSSION

The teratology studies described in this report show that MK-196, an investigational loop diuretic, produces dose-related increases in skeletal malformations in rats when administered orally from Days 6 to 17 of gestation at doses of 40, 80, or 120 mg/kg/day. All three doses of MK-196 were maternotoxic, and the two highest dose levels resulted in some maternal mortality. Malformations produced by MK-196 in rats are highly specific and are limited to wavy ribs and malformations of the scapula and humerus, which are predominantly right-sided.

Studies of the teratogenic potential of the individual enantiomers of MK-196 have shown that at comparable doses the (−) enantiomer was more effective in producing fetal skeletal malformations than the (+) enantiomer. For example, the frequency of wavy ribs in the group treated with 15 mg/kg/day of the (−) enantiomer was comparable to that among fetuses treated with 80 mg/kg/day of the (+) enantiomer. The (−) enantiomer is a more potent diuretic than the (+) enantiomer,[1] and at comparable doses, results in a greater saluretic effect. These data suggest that the teratogenic effect of MK-196 and its individual components may be linked to saluretic activity and possible electrolyte imbalances at high doses.

[1] E. Blaine personal communication.

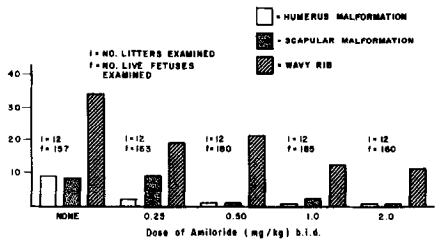

FIG. 7. Effect of amiloride coadministration on the teratogenicity of an 80 mg/kg/day dose of MK-196 administered from Days 6 to 17 of gestation.

Furosemide, a widely used diuretic with pharmacologic activity similar to that of MK-196, also produced malformations of the ribs, scapula, and humerus when administered to rats at maternotoxic doses. The malformations produced by furosemide were morphologically identical to those described following the administration of MK-196 and its individual enantiomers to rats. These results differ from those of Wilson *et al.* (1968), who found that a single dose of 1000 mg/kg furosemide on Day 10 of gestation was not teratogenic in rats.

Forelimb malformations, specifically ectromelia and/or ectrodactyly, have been produced in rats with acetazolamide, ethoxzolamide, and dichlorphenamide (Wilson *et al.*, 1968; Hallesy and Layton, 1967). The forelimb malformations produced are, as in the case of MK-196, predominantly right-sided or bilateral. Although available data indicate that inhibition of fetal and maternal carbonic anhydrase is a necessary condition for manifestation of the teratogenic effects of these compounds, it may not be a sufficient condition. Ellison and Maren (1972), as previously mentioned, successfully antagonized the teratogenic effects of high doses of acetazolamide in rats by potassium supplementation and by coadministration of amiloride and triamterene. Potassium supplementation in the drinking water can antagonize the teratogenic effects of high doses of MK-196, a diuretic with a structure and pharmacologic activity very different from acetazolamide, but with no effect on carbonic anhydrase. In addition, we have shown that coadministration of amiloride can also effectively ameliorate the teratogenicity of MK-196 in rats. Amiloride is a potassium-sparing diuretic that reduces the potassium loss associated with MK-196 administration.[1] The results of this study add further support to the conclusion that potassium loss at high doses of MK-196 is responsible for the teratogenic effects of this compound in rats.

These studies and the available literature suggest that diuretics of widely differing structures and pharmacologic activities can produce specific skeletal malformations in rats when administered at high doses. It is possible that the teratogenicity produced by these diuretics (including MK-196) is not a direct effect of the drugs, but is secondary to the electrolyte imbalance, primarily potassium depletion, caused by administration of high doses of these compounds.

The high dose levels necessary to produce the teratogenic effects observed with both MK-196 and furosemide in our studies suggest that the use of these drugs at therapeutic doses should pose little risk to the fetus so long as maternal potassium balance is maintained within reasonable limits by dietary and/or therapeutic adjustments.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to the following for their technical support: W. Cusick, A. Jonar, M. Lenhart, N. Moskalski, and J. Sheets, and to K. Bond and R. A. Chonka for assistance in preparing this manuscript.

REFERENCES

BLAINE, E. (1980). *Amiloride: Effect on Indocrinone Induced Potassium Excretion in Rats*. Merck Sharp & Dohme Research Laboratories, West Point, Pa., personal communication.

DE SOLMS, J. S., WOLTENSDORF, O. W., CRAGOE, E. J., WATSON, L. S., AND FANELLI, G. M. (1978). (Acylaryloxy) acetic acid diuretics-2-(2-alkyl-2-aryl-

1-oxo-5-indanyloxy) acetic acids. *J. Med. Chem.* **21,** 437.

ELLISON, E. C., AND MAREN, T. H. (1972). The effect of potassium metabolism on acetazolamide-induced teratogens. *Johns Hopkins Med. J.* **130,** 105–115.

HALLESY, D. W., AND LAYTON, W. M. (1967). Forelimb deformity of offspring of rats given dichlorphenamide during pregnancy. *Proc. Soc. Exp. Biol. Med.* **126,** 6–8.

HIRSCH, K. S., AND SCOTT, W. J. (1979). Acetazolamide teratology: A biochemical basis for strain differences in the mouse. *Teratology* **19,** 30A.

IRVIN, J. W., VLASSES, P. H., HUBER, P. B., FERGUSON, R. K., SCHROGIE, J. J., AND DAVIES, R. O. (1981). Clinical pharmacology of indacrinone. I. Oral dose-response comparison with furosemide. *Clin. Pharm. Ther.*, in press.

LAYTON, W. M., AND HALLESY, D. W. (1965). Deformity by forelimb on rats: Association with high doses of acetazolamide. *Science* **149,** 306–308.

RECTOR, F. C. (1973). Acidification of the urine. In *Renal Physiology, Handbook of Physiology* (J. Orloff and R. W. Berliner, eds.), pp. 431–454. Amer. Physiol. Soc., Washington, D.C.

STORCH, T. G., AND LAYTON, W. M. (1973). Teratogenic effects of intrauterine injection of acetazolamide and amiloride in hamsters. *Teratology* **7,** 209–214.

WILSON, J. C. (1965). In *Teratology; Principles and Techniques* (J. G. Wilson and J. Warkany, eds.), Univ. of Chicago Press, Chicago.

WILSON, J. G., MAREN, T. N., TAKANO, K., AND ELLISON, A. (1968). Teratogenic action of carbonic anhydrase inhibitors in the rat. *Teratology* **1,** 51–60.