and it has unspecific, toxic effects on *Pseudaulacaspis pentagona* (Hom., Diaspididae) and its parasitoid, *Prospaltella berlesei* (Hymen., Aphelinidae)[21]; this suggests that it may have inhibitory effects on other polysubstrate monooxygenases involved in xenobiotic metabolism in insects.

Acknowledgments. The authors are grateful to Prof. Shalom W. Applebaum for allowing us to use his unpublished results.

[a] To whom reprint requests should be addressed.
2 Koolman, J., Insect Biochem. *12* (1982) 225.
3 Wilkinson, C. F., in: Bioregulators for Pest Control, vol. 276, pp. 161–176. Ed. P. A. Hedin. ACS Symp. Series, 1985.
4 Matolcsy, G., Varjas, L., and Bordás, B., Acta phytopath. hung. *10* (1976) 455.
5 Ragsdale, N. N., and Sisler, H. D., Biochem. biophys. Res. Commun. *46* (1972) 2048.
6 Kulcsár, P., Darvas, B., and Varjas, L., Abstr. Int. CNRS Symp., p. 125. Strasbourg 1983.
7 Fónagy, A., Darvas, B., and Kulcsár, P., Abstr. 1st Int. Congr. Dipterology, p. 75. Budapest 1986.
8 Darvas, B., Fónagy, A., and Kulcsár, P., Acta phytopath. ent. hung. (1990) in press.
9 Ohtaki, T., Jap. J. med. Sci. Biol. *19* (1966) 97.
10 Darvas, B., Rees, H. H., Kuwano, E., Bélai, I., Matolcsy, G., Timár, T., Hoggard, N., and Tag El-Din, M. H., Abstr. IX. Ecdysone Workshop, Paris 1989.
11 Darvas, B., Abstr. 2nd Int. Cong. Dipterology, Bratislava (1990) in press.
12 Wilkinson, C. F., and Murray, M., Drug Metab. Rev. *15* (1984) 897.
13 Bidmon, H. J., Käuser, G., Möbus, P., and Koolman, J., in: Proc. 3rd Neem Conf., Nairobi, pp. 253–271. Eds H. Schmutterer and K. R.-S. Ascher. GTZ GmbH 1986.
14 Bollenbacher, W. E., Smith, S. L., Wielgus, J. J., and Gilbert, L. I., Nature *268* (1977) 660.
15 Hoffmann, J. A., and Hetru, C., in: Endocrinology of Insects, pp. 65–68. Eds G. H. Downer and H. Laufer. Alan R. Liss, New York 1983.
16 Rees, H. H., Nova Acta Leopoldina, NF 56, *255* (1984) 267.
17 Clarke, G. S., Baldwin, B. C., and Rees, H. H., Pest. Biochem. Physiol. *24* (1985) 220.
18 Nebert, D. W., and Gonzales, F. J., A. Rev. Biochem. *56* (1987) 945.
19 Kappler, C., Kabbouh, M., Durst, F., and Hoffmann, J. A., Insect Biochem. *16* (1986) 25.
20 Applebaum, S. W., personal communication.
21 Darvas, B., and Zsellér, H. I., Acta phytopath. hung. *20* (1985) 341.

0014-4754/91/030261-03$1.50 + 0.20/0

Tamoxifen 'sex reverses' alligator embryos at male producing temperature, but is an antiestrogen in female hatchlings

V. A. Lance and M. H. Bogart[a]

Center for Reproduction of Endangered Species, Zoological Society of San Diego, P.O. Box 551, San Diego (California 92112, USA) and [a] Division of Medical Genetics M-039, University of California, San Diego/La Jolla (California 92093, USA)

Received 1 August 1990; accepted 24 September 1990

Summary. Tamoxifen is an anticancer drug widely used in the treatment of estrogen-dependent breast cancer. In hatchling alligators it acts as a pure antiestrogen in that it completely blocks the effect of estradiol-induced oviductal hypertrophy and completely blocks the estradiol-induced hepatic vitellogenin secretion. Paradoxically, when injected into alligator eggs incubated at 33 °C, a temperature which would normally result in 100% male hatchlings, tamoxifen 'sex reverses' the embryos into apparently normal female hatchlings.
Key words. Tamoxifen; sex determination; alligators.

In some teleost and amphibian species functionally reproductive adults of either sex can be produced by treating the larvae with androgens or estrogens, though there are a number of paradoxical exceptions in which androgens feminize and estrogens masculinize[1,2]. In amniote vertebrates it has not been possible to 'sex reverse' embryos in the male direction with androgens, but estrogens will sex reverse reptile embryos into apparently normal females[3–5], and will produce feminized but sterile birds[1]. Tamoxifen has been reported to block the feminizing action of estradiol in chick embryos[6] and of DES in quail embryos[7]. Treatment of bird embryos with tamoxifen alone has given conflicting results. In one study no effect was noted[6], but in two other studies a partial 'masculinizing' of the left ovary and partial development of the normally regressed right gonad were reported[8,9]. Despite this apparent masculinizing action, the Mullerian duct did not appear to be affected. Similar 'masculinizing' effects have been noted in turtle embryos treated with tamoxifen[4].

The sex of alligators is determined by the temperature at which the eggs are incubated. Eggs incubated at 33 °C produce 100% male hatchlings, and eggs incubated at 30 °C produce 100% female hatchlings[10–12]. Injection of estrogen into eggs incubated at 33 °C will 'sex reverse' the embryos and produce female hatchlings[3], but the converse is not true. It has not been possible to produce male hatchlings from eggs incubated at 30 °C by injecting androgens[13]. If, as has been suggested, ovarian development is dependent upon synthesis of estrogen in the developing gonad[4,5,14], then it may be possible to 'sex reverse' embryos at female producing temperatures by

blocking the effect of endogenous estrogen production by the developing ovary[4]. Since tamoxifen acts as a potent 'pure' antiestrogen in chick oviduct[15], blocks the feminizing action of estradiol in chick and quail embryos[6,7], and inhibits the estrogen-stimulated calcium-binding protein synthesis in the liver of laying hens[16], a similar antiestrogenic effect should be manifest in crocodilians, given the taxonomic affinities of the two groups. We report here that tamoxifen is indeed a pure antiestrogen in hatchling female alligators, but that when injected into alligator eggs incubated at male producing temperature, tamoxifen 'sex reverses' the embryo in the female direction.

Materials and methods

Alligator eggs were collected from nests within one or two days of oviposition, packed in vermiculite and shipped to San Diego where they were placed in constant temperature incubators at 30 °C (female producing temperature) or 33 °C (male producing temperature). After one week the eggs were candled to check viability and assigned to treatment groups. A small hole was drilled in the shell and hormone or drug injected in a 100 µl volume using a 30-gauge needle. The hole was sealed with paraffin wax and the egg returned to the incubator. Treatments consisted of estradiol-17B (50 µg/egg), tamoxifen (100 µg/egg) or vehicle (corn oil) between nine and twelve days after oviposition. Each treatment group consisted of eggs from at least three separate clutches, and treatments were assigned such that eggs from a single clutch received each treatment at each temperature. At estimated day of hatch (day 61 for 33 °C eggs and day 74 for 30 °C eggs) the embryos were removed from the eggs and the gonads dissected and fixed in Bouin's solution for histology. Histological slides were coded and the sex assigned by two independent observers, and the results tabulated. Since tamoxifen appeared to act as an estrogen in the alligator embryo, we decided to test

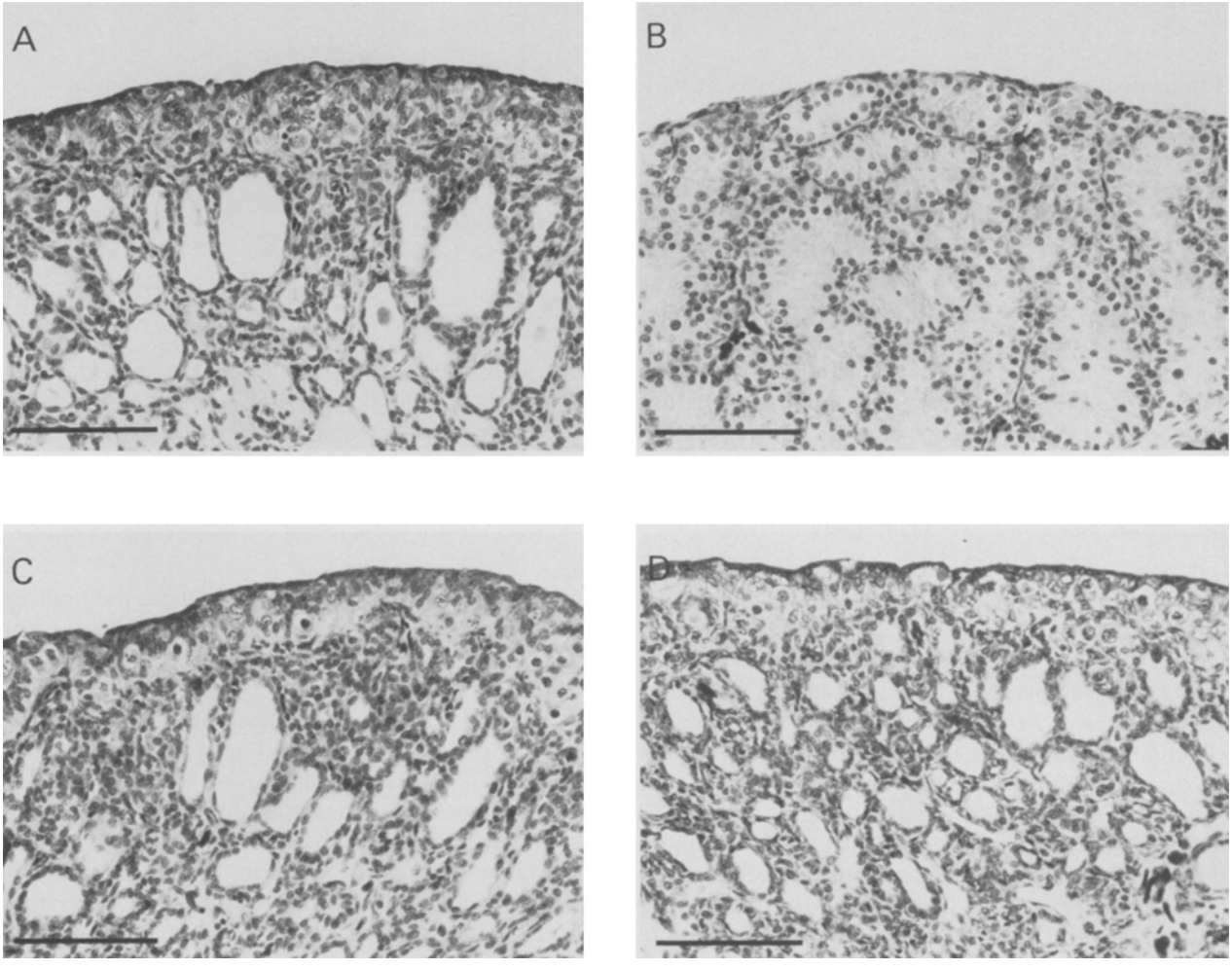

Figure 1. Histological sections of alligator gonads just before hatching. *A* Ovary from control embryo incubated at 30 °C collected at day 74 of incubation. *B* Testis from control embryo incubated at 33 °C collected at day 61 of incubation. *C* Ovary from embryo treated with tamoxifen incubated at 30 °C and collected on day 74 of incubation. *D* Ovary from embryo treated with tamoxifen, incubated at 33 °C (male-inducing temperature) and collected on day 61 of incubation.

its estrogenic activity on liver and oviduct, two well-characterized estrogen-sensitive tissues in hatchling alligators[17,18]. Three-month-old alligators (50–250 g, n = 28) hatched from eggs incubated at 30 °C (all females) were injected intramuscularly as follows: Group 1: estradiol-17B, 1 mg/kg, one injection only (E2 × 1); Group 2: estradiol-17B, 1 mg/kg for three days (E2 × 3); Group 3: Tamoxifen 10 mg/kg for 3 days (Tmx × 3); Group 4: estradiol-17B 1 mg/kg plus tamoxifen 10 mg/kg, one injection only (E2 + Tmx); and Group 5: vehicle (vegetable oil) alone, one injection only (Control), and sacrificed 6 days after the last injection. A blood sample was taken immediately before hormone or drug was injected and again at the end of the experiment using heparinized 1-ml syringes. Blood was centrifuged and the plasma separated for calcium and zinc analysis by atomic adsorption spectrophotometry, and the ovaries and oviducts fixed in Bouin's solution for histology. The calcium and zinc data were subjected to a single factor ANOVA and a multiple means comparison test. Differences were considered significant at the 0.05 level.

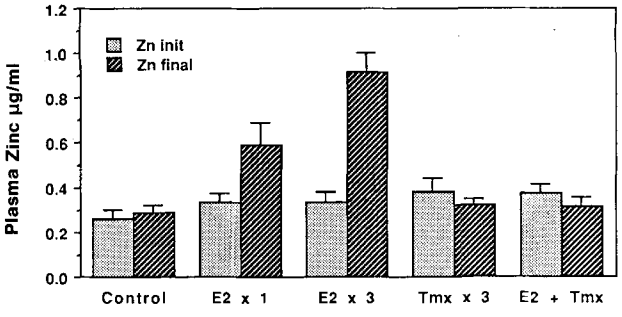

Figure 2. Plasma zinc in hatchling alligators treated with estradiol and tamoxifen. Bars represent mean values plus SEM of treatment groups before and after hormone and drug injections.

Results

All untreated eggs produced males at 33 °C and females at 30 °C and injection of vehicle alone had no effect on predicted sex ratio (table 1). Eggs injected with estradiol produced female hatchlings at male inducing incubation

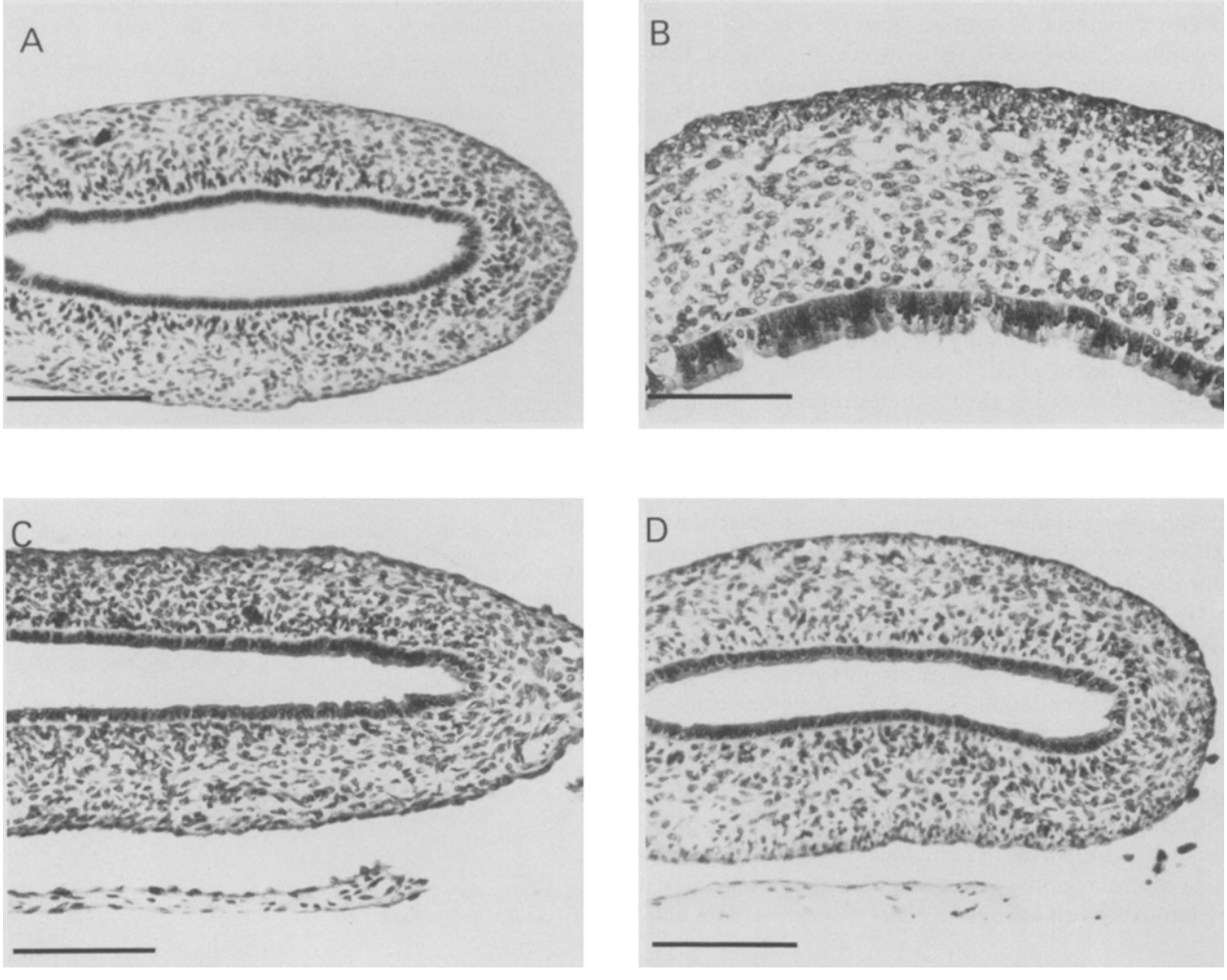

Figure 3. Histological sections of hatchling alligator oviducts. *A* Control, *B* E2 × 1 at the same magnification showing hypertrophy of both the epithelial lining and the muscular wall, *C* Tamoxifen alone, and *D* E2 + tamoxifen; bar = 100 µm.

Effect of estradiol and tamoxifen on gonadal differentiation in alligator embryos

Temperature	Treatment	Number	Gonad
30 °C	None	14	Female
	Vehicle	11	Female
	Estradiol-17B	5	Female
	Tamoxifen	5	Female
33 °C	None	15	Male
	Vehicle	12	Male
	Estradiol-17B	9	Female
	Tamoxifen	5	Female

temperature, and estradiol did not appear to affect normal ovarian development in eggs at female inducing temperature. Tamoxifen in the 30 °C eggs had no detectable effect on the ovary or Mullerian ducts, but tamoxifen in the 33 °C eggs produced embryos with ovaries histologically similar to normal female embryos from untreated 30 °C eggs (fig. 1). Hatchlings injected with estradiol alone had significant ($p < 0.05$) increases in plasma calcium (data not shown), and plasma zinc (fig. 2). Tamoxifen alone did not stimulate any detectable increase in plasma calcium or zinc in the alligator hatchlings, and when given simultaneously with estrogen prevented the estrogen-induced increases in these two cations (fig. 2). Likewise, tamoxifen alone had no detectable estrogenic effect on the oviducts or ovaries of hatchling alligators, and when given simultaneously with estradiol was able to prevent the estrogen-induced oviductal hypertrophy (fig. 3). Thus in hatchling alligators tamoxifen acted as a pure antiestrogen.

Discussion

In mammals tamoxifen may act as a weak estrogen or as an antiestrogen depending on the species and target tissue being investigated[19], but in birds[15], lizards, and frogs[20] tamoxifen is clearly antiestrogenic. Our results show that tamoxifen is a potent pure antiestrogen in hatchling alligators in that it completely blocked the estradiol-induced oviductal hypertrophy and completely blocked the estradiol-induced vitellogenin secretion by the liver, as indicated by calcium and zinc in the plasma. Our results showing complete sex reversal of 'male' embryos treated with estradiol are in agreement with previous studies on the feminizing action of estradiol on alligator embryos[3]. However, our results showing complete ovarian differentiation in 33 °C alligator embryos ('males') treated with tamoxifen alone suggest that the drug may have acted as an estrogen in this system. On the other hand tamoxifen acts as a potent antiestrogen in hatchling alligators. A similar paradoxical effect of tamoxifen was reported in zebra finches. Estrogen-sensitive song control regions of the brain were hypermasculinized by tamoxifen, an estrogenic effect, whereas a pure antiestrogenic effect was seen in the oviducts[21]. Tamoxifen is believed to exert its antiestrogenic or weakly estrogenic effects via interaction with an estrogen receptor[19,22]; however, a second non-estrogen tamoxifen receptor has been identified[23]. Curiously, tamoxifen has also been shown to inhibit the action of a non-aromatizable androgen, dihydrotestosterone, in chick embryos[24,25]. Based on similar paradoxical results with antiandrogens and tamoxifen in frog larvae, Rastogi and Chieffi[20] proposed that embryonic sex inductors have no structural affinity with the sex steroids produced by the adult animal. Our results suggest that the receptor in embryonic alligator gonad that interacts with tamoxifen differs from the estrogen receptor in the liver and oviducts of hatchlings of this species.

Acknowledgments. This work was supported by the Louisiana Department of Wildlife and Fisheries. We would like to thank Ted Joanen and Larry McNease for help with the egg collection, Alex Wempren for preparing the histological specimens, Zdenka Schultze for help with the calcium and zinc assays, and Ken Kelly for help with the photography.

1 Burns, R. K., in: Sex and Internal Secretions, p. 76. Eds W. C. Young and G. W. Corner. Williams and Wilkins, Baltimore 1961.
2 Davis, K. B., Simco, B. A., Goudie, C. A., Parker, N. C., Cauldwell, W., and Snellgrove, R., Gen. comp. Endocr. *78* (1990) 218.
3 Bull, J. J., Gutzke, W. H. N., and Crews, D., Gen. comp. Endocr. *38* (1988) 425.
4 Dorizzi, M., Desvages, G., and Pieau, C., Arch. Anat. micr. *75*(3) (1987) 204.
5 Crews, D., Wibbels, T., and Gutzke, W. H. N., Gen. comp. Endocr. *76* (1989) 159.
6 Weniger, J.-P., Chouraqui, J., Zeiss, A., and Samsel, J., C. r. Acad. Sci. Paris Ser. III *292* (1981) 927.
7 Samsel, J., Zeis, A., and Weniger, J.-P., Biochimie *64* (1982) 369.
8 Salzgeber, B., Reyss-Brion, M., and Baulieu, E., C. r. Acad. Sci. Paris Ser. III *293* (1981) 133.
9 Scheib, D., and Baulieu, E.-E., C. r. Acad. Sci. Paris Ser. III *293* (1981) 513.
10 Ferguson, M. W. J., and Joanen, T., Nature *296* (1982) 850.
11 Ferguson, M. W. J., and Joanen, T., J. Zool., Lond. *200* (1983) 143.
12 Joss, J. M. P., J. Zool., Lond. *218* (1989) 679.
13 Lance, V. A., and Bogart, M. H., (in preparation).
14 Bogart, M. H., J. theor. Biol. *128* (1987) 349.
15 Sutherland, R., Mester, J., and Baulieu, E., Nature *267* (1977) 434.
16 Sommerville, B. A., Luck, M., Scanes, C. G., Harvery, S., and Chadwick, A., IRCS Med. Sci. Libr. Compend. *8* (1980) 864.
17 Elsey, R. M., and Wink, C. S., Comp. Biochem. Physiol. *84*A (1986) 107.
18 Ho, S. M., Kleis, S., McPherson, R., Heisermann, G. J., and Callard, I. P., Herpetologica *28* (1982) 40.
19 Furr, B. J. A., and Jordan, V. C., Pharmac. Ther. *25* (1984) 127.
20 Rastogi, R. K., and Chieffi, G., Gen. comp. Endocr. *26* (1975) 79.
21 Mathews, G. A., Brenowitz, E. A., and Arnold, A. P., Horm. Behav. *22* (1988) 540.
22 Pasqualini, J. R., Sumida, C., Giambiagi, N. A., and Nguyen, B. L., J. Steroid Biochem. *27* (1987) 883.
23 Pavlik, E. J., Nelson, K., van Nagell, J. R. Jr, Donaldson, E. S., Gallion, H. H., and Kenady, D. E., Endocrine Soc., 72nd Annual Meeting (1990) Abstr. No. 149.
24 Weniger, J. P., Samsel, J., and Zeis, A., C. r. Acad. Sci. Paris Ser. III *293* (1981) 451.
25 Stoll, R., Faucounau, N., and Maraud, R., Gen. comp. Endocr. *66* (1987) 218.

0014-4754/91/030263-04$1.50 + 0.20/0