Table 1. Distribution of erythrocyte PGM1 and milk PGM4 phenotypes in 140 Mexican women. The phenotype PGM2 1 was found in all samples.

Phenotype PGM1	Phenotype PGM4								Number of samples
	1	2	3	1–2	1–3	2–3	1–4	2–4	
1	14	18	1	26	5	8	10	7	89
1–2	3	11	1	11	4	11	1		41
2		1		1		2			4
1–4				2					2
1–7		1		2					3
2–5				1					1
Number of samples	17	31	1	43	9	21	11	7	Total 140

tive results. Thermostability tests with whole hemolyzate and milk were performed, with the samples being incubated for 15 minutes at 40°, 45°, 50°, 55°, 60°, 65°, and 70°C before electrophoresis. The PGM4 activity disappeared at 60°C, PGM1 at 65°C, and PGM2 at 70°C. When glucose 1-phosphate, glucose 1,6-diphosphate, nicotinamide adenine dinucleotide phosphate, methyl thiazolyl blue, or phenazine methosulfate were omitted in the staining mixture no PGM1, PGM2, or PGM4 isozymes were observed. The omission of G6PD gave faint bands in the position of the endogenous enzyme.

These results show the presence of a different set of PGM isozymes in human milk, coded by a specific gene with at least four alleles probably located in an autosome. Each product of the alleles PGM4*1, PGM4*2, and PGM4*3 gives three anodal bands, the most intense (probably the primary isozyme) being intermediate. This pattern is quite different from the patterns given by PGM1, PGM2, and PGM3, whose secondary isozymes appear toward the anode; PGM1 and PGM3 show one primary and one secondary isozyme, and PGM2 shows one primary and two secondary isozymes (*6*). In the human nonlactating mammary gland, PGM activity is due to PGM1 and PGM2 (*7*). However, these isozymes could not be found in any of the milk samples studied, indicating a switch-off of the PGM1 and PGM2 genes and a simultaneous switch-on of the PGM4. This switching is reversible and probably dependent on hormonal activation of lactation. Since PGM is indispensable for the production of glucose 1-phosphate, which is the first intermediate on the pathway to synthesis of the galactose moiety of lactose (*8*), and since lactose is the main carbohydrate of milk, the need of an efficient PGM has probably been an evolutionary selective force for an independent locus (*9*). Although there are several instances of polymorphism among milk-specific proteins (*10*), to our knowledge this is the first report of a distinct gene for a widely distributed protein being functionally restricted to the lactating mammary gland, since no evidence of its activity has been found in tissues or fibroblasts previously studied (*3*).

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References and Notes

1. Genotype and phenotype designations are based on the International System for Human Gene Nomenclature, *Cytogenet. Cell Genet.* **25**, 96 (1979).
2. J. M. Parrington, G. Cruickshank, D. A. Hopkinson, E. B. Robson, H. Harris, *Ann. Hum. Genet.* **32**, 27 (1968).
3. P. J. McAlpine, D. A. Hopkinson, H. Harris, *ibid.* **34**, 169 (1970).
4. N. Spencer, D. A. Hopkinson, H. Harris, *Nature (London)* **204**, 742 (1964).
5. C. B. Quick, R. A. Fisher, H. Harris, *Ann. Hum. Genet.* **35**, 445 (1972).
6. R. A. Fisher and H. Harris, *ibid.* **36**, 69 (1972).
7. We have electrophoretically assayed mammary gland homogenates from six different women and found phenotypes identical to those in the corresponding erythrocytes. PGM1 activity predominated over PGM2, as happens in other tissues.
8. C. L. Davis and D. E. Bauman, in *Lactation: A Comprehensive Treatise*, B. L. Larson and V. R. Smith, Eds. (Academic Press, New York, 1974), vol. 2, p. 3.
9. In studies of PGM in milk from other mammals we have found evidence of a similar phenomenon in the swine and rat (J. M. Cantú and B. Ibarra, unpublished data).
10. G. F. Voglino and A. Ponzone, *Nature (London)* **238**, 149 (1972); M. P. Thompson and H. M. Farell, in *Lactation: A Comprehensive Treatise*, B. L. Larson and V. R. Smith, Eds. (Academic Press, New York, 1974), vol. 3, p. 109.
11. We thank E. Franco-Gamboa, A. Alcaraz, and R. Troyo for skillful technical assistance and D. A. Hopkinson for his valuable comments when this work was presented at the 6th International Congress on Human Genetics, 13 to 18 September 1981, Jerusalem, Israel (Book of Abstracts, p. 35).

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Prenatal Exposure to Phenobarbital Permanently Decreases Testosterone and Causes Reproductive Dysfunction

Abstract. *Exposure of rats to phenobarbital during late prenatal development decreased the concentration of testosterone in plasma and the brain during the late fetal, early postnatal, pubertal, and adult periods. By decreasing the production of testosterone in the brain during the period of sexual differentiation, phenobarbital may lead to sexual dysfunction in later life.*

Phenobarbital (PB), when administered during pregnancy to a variety of species including humans, produces both anatomic and neurologic malformations in the offspring (*1, 2*). Recently, we demonstrated that prenatally administered PB is also capable of modifying the development of reproductive function in male and female offspring (*3, 4*). Phenobarbital acts during neuroendocrine development in the late fetal and neonatal periods to produce reproductive disorders in both male and female offspring (*5*). In male offspring there is a delay in testicular descent, with subsequent infertility and decreased production of testosterone and gonadotropin throughout adulthood (*3*).

The mechanism underlying these effects of PB in early life has not been determined. During the fetal and early neonatal periods, testicular hormones masculinize anatomic sexual differentiation and support the maturation of re-

Table 1. Testosterone synthesis and concentration in brain and plasma of male rats exposed to phenobarbital in utero. Values are means ± standard deviations for six animals per group.

Treatment	Age	Testosterone in plasma (ng/ml)	Testosterone in brain (pg/g)	Testosterone synthesis (nanograms per testis)
Saline	21 days	1.10 ± 0.38	70.5 ± 25.2	3.14 ± 0.71
Phenobarbital	21 days	0.28 ± 0.04*	15.0 ± 5.3*	0.45 ± 0.10*
Saline	120 days	5.06 ± 1.28	554.0 ± 120.0	56.8 ± 18.9
Phenobarbital	120 days	2.66 ± 0.05*	375.2 ± 65.7*	32.1 ± 14.2*

*Significantly different from corresponding control value at $P < .01$ (Student's t-test).

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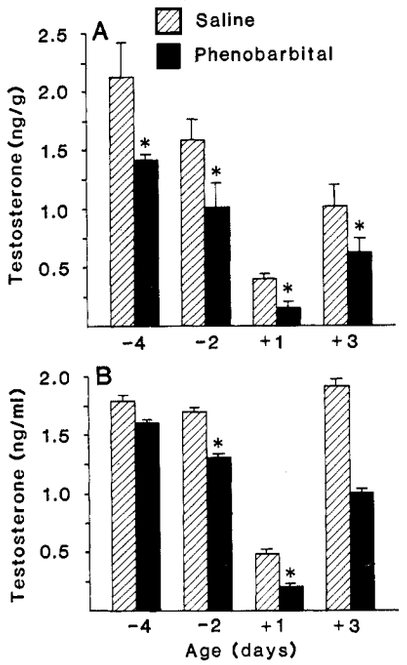

Fig. 1. Effect of prenatal PB treatment on the concentration of testosterone in the brain (A) and plasma (B) in offspring before and after birth. Each value is the mean ± standard deviation for six animals. Asterisks indicate significant differences at $P < .01$ (Student's t-test).

productive function (*6–9*). The effects produced in male offspring following prenatal exposure to PB, such as small anogenital distance, decreased concentrations of androgen and gonadotropin in adulthood, and other reproductive dysfunctions (*3*), resemble the effects of neonatal or perinatal androgen deficiency (*9, 10*). Therefore, it is reasonable to speculate that PB administration in utero can decrease fetal or neonatal testosterone and thereby produce defects in masculine sexual differentiation.

Phenobarbital was administered to three groups of pregnant Sprague-Dawley CD rats. (Day 1 of pregnancy was the day on which a vaginal smear positive for sperm was first obtained.) At least five pregnant animals were assigned to each group. The first group received PB (40 mg/kg) on day 17 of pregnancy and was killed the following day. The second group received PB on days 17, 18, and 19 and was killed on day 20. The third group received PB on days 17, 18, 19, and 20 and was allowed to deliver. Fetuses from group 1 and group 2 mothers were removed by cesarean section, and the offspring of group 3 were killed 1, 3, 21, or 120 days after birth. Control dams were treated with saline for equivalent periods.

The male fetuses and offspring were killed by decapitation and blood from their trunks was collected in tubes containing heparin. In the case of younger offspring, blood from two to five fetuses or pups was pooled for analysis. The plasma was separated and frozen for testosterone determination by radioimmunoassay (*11*). Brain tissue was also collected to determine testosterone content (*12*), and the testes were used to determine testosterone synthesis by the method of Feldman and Bloch (*13*).

The dose of PB used in this study did not produce any observable effects in the mothers. Both control and PB-treated mothers delivered on day 21 or 22 of pregnancy. At birth, offspring from the PB-treated mothers showed no gross anatomic malformations, and litter size and birth weights were similar to those of control offspring. Phenobarbital injected into the newborn rats produced sleep for a short time. The animals were alert within 2 to 3 hours and appeared to nurse similarly to controls. The onset of puberty and the fertility of animals receiving PB during the prenatal and postnatal periods were determined in separate groups of animals. Both prenatal and postnatal PB treatment altered these reproductive functions significantly (*5*).

Prenatal PB administration significantly reduced the concentration of testosterone in brain tissue and plasma of male offspring during the perinatal period (Fig. 1). Testosterone synthesis by fetal testes was not significantly altered by PB on days 18 and 20 of gestation but was significantly lower when measured 1 and 3 days after birth (Fig. 2). The concentration of testosterone in brain tissue and plasma changed considerably with age in the PB-exposed and control groups; however, the pattern of change was the same in both groups (Fig. 1).

We also examined the long-term effects of prenatal exposure to PB. The concentration of testosterone in brain tissue and plasma was depressed and the rate of gonadal testosterone synthesis was subnormal in the male offspring at 21 and 120 days of age (Table 1).

This study demonstrates that exposure to PB during prenatal life can permanently alter the testosterone titer of plasma and brain and the synthesis of testosterone in the male rat beginning in the perinatal period. The secretion of testosterone during perinatal life is believed to be essential in organizing the male rat brain to direct the male pattern of gonadotropin secretion and adult sexual behavior (*14, 15*). In addition, testosterone in the brain controls male differentiation of the brain (*12*). In the PB-exposed male rats a decrease in testosterone was noted in both plasma and the brain. Therefore, it may be concluded that prenatal PB exposure causes androgen deprivation during the critical period of masculine development which results in abnormal reproductive function in the adult.

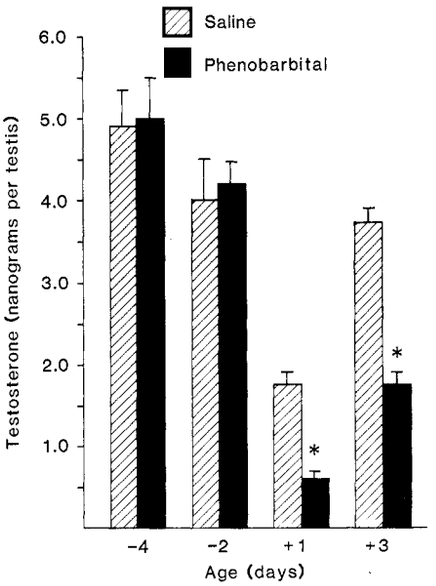

Fig. 2. Effect of prenatal PB treatment on the testicular synthesis of testosterone in offspring before and after birth. Each value is the mean ± standard deviation for six animals.

Phenobarbital has a half-life of 4 to 5 days (*16*); therefore, it is unlikely that the long-term effect of the drug is direct. Rather, prenatally administered PB may alter the programming of the brain, resulting in a permanent change in testicular function. We do not know the stage of testosterone synthesis in which PB introduced a defect. In the adult rat PB is capable of temporarily altering steroidogenesis at both the enzyme and gonadotropin levels (*17–19*). Whether prenatal exposure to PB can induce a permanent alteration in these parameters remains to be determined.

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References

1. D. E. Manning, A. G. Stoud, J. W. Jemp, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 495 (1971).
2. L. D. Middaugh, C. A. Santos III, J. W. Zemp, *Dev. Psychobiol.* **8**, 305 (1975).
3. C. Gupta, B. H. Shapiro, S. J. Yaffe, *Pediatr. Pharmacol.* **1**, 55 (1980).
4. C. Gupta, B. R. Sonawane, S. J. Yaffe, B. H. Shapiro, *Science* **208**, 508 (1980).
5. C. Gupta and S. J. Yaffe, *Pediatr. Res.* **15**, 1488 (1981).
6. A. Jost, *Prog. Horm. Res.* **8**, 379 (1953).

7. M. G. Stinmakre, *Arch. Anat. Microsc. Morphol. Exp.* **64**, 45 (1975).
8. F. Neuman, R. VonBerswordt-Wallrobe, W. Elger, H. Steinbeck, J. D. Hahn, M. Kramer, *Recent Prog. Horm. Res.* **26**, 337 (1970).
9. J. A. Moguilevsky, P. Scacchi, Y. Epstine, B. Lunenfeld, *Neuroendocrinology* **26**, 65 (1978).
10. A. S. Goldman, M. K. Baker, J. C. Chen, R. G. Wieland, *Endocrinology* **90**, 149 (1972).
11. A. Weinstein, H. R. Lindner, A. Friedlander, S. Ballminger, *Steroids* **20**, 789 (1972).
12. J. C. Butte, J. A. Moore, R. Kakihana, *Life Sci.* **24**, 2343 (1979).
13. S. C. Feldman and E. Bloch, *Endocrinology* **102**, 999 (1978).
14. R. A. Gorski, *Prog. Brain Res.* **39**, 149 (1973).
15. W. C. Young, R. W. Goy, C. H. Phoenix, *Science* **143**, 212 (1964).
16. W. J. Waddell and T. C. Butler, *J. Clin. Invest.* **36**, 1217 (1957).
17. C. A. Barraclough, R. Collu, R. Massa, L. Martini, *Endocrinology* **88**, 1437 (1971).
18. T. Kaneyuki, K. Matsutoshi, T. Shahmori, *Endocrinol. Jpn.* **26**, 345 (1979).
19. C. Gupta and H. J. Karavolas, *Endocrinology* **92**, 117 (1973).
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Calmodulin Binds to Chick Lens Gap Junction Protein in a Calcium-Independent Manner

Abstract. *A biochemically active conjugate of calmodulin and tetramethylrhodamine isothiocyanate (CaM-RITC) was synthesized. When incubated with sections of chick lens, this conjugate bound to the surface membranes of lens fiber cells in the presence or absence of calcium. Incubation of lens sections with antibodies to gap junction protein of lens completely blocked the binding of the conjugate to cell membranes, whereas serum from nonimmunized animals or antibodies to other lens proteins reduced the binding only slightly. By means of a gel overlay procedure, ^{125}I-labeled calmodulin was found to bind to the gap junction protein of lens, also in a calcium-independent manner. These results support the concept that calmodulin may interact with and regulate gap junctions in living cells.*

Gap junctions are cell membrane structures that allow adjacent cells to communicate by permitting passage, directly from cell to cell, of molecules of molecular weight of approximately 1000 or less (*1, 2*). Gap junctions appear to be important in a number of significant functions of normal and abnormal cells and tissues (*3–11*). An increased understanding of the regulation of gap junctions would thus be relevant to a wide range of biological events.

Calmodulin is a ubiquitous calcium-binding protein that has been described as a mediator of calcium action in numerous events in eukaryotic cells (*12*). Gap junction permeability is believed to be regulated by calcium (*1, 2*), although recently *p*H has also been suggested as a potential regulatory factor (*4, 13*). Because the junctions appear to respond to calcium ion concentrations generally within the concentration range of calmodulin affinity for calcium (*1–4, 14, 15*), and because calmodulin affinity for calcium is also affected by *p*H (*15*), it seemed possible that calmodulin might regulate gap junctions by mediating the effects of calcium.

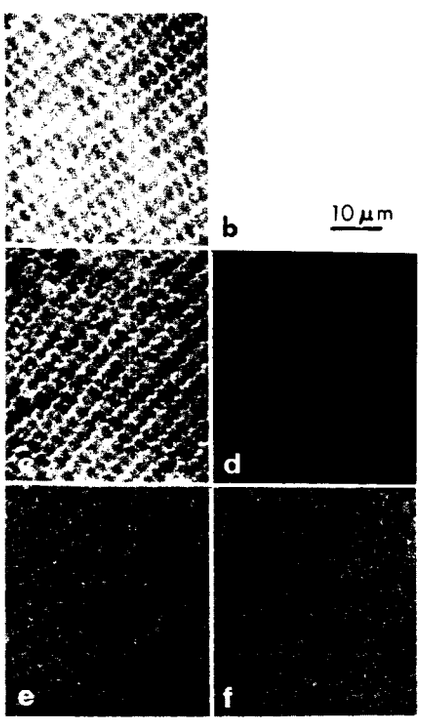

Fig. 1. The distribution of gap junction protein and CaM-RITC binding in chick lens. (a) The protein was located (*21*) on the fiber cell membranes. (b) Lens sections were incubated with CaM-RITC (*20*) in the presence of calcium, and the CaM-RITC bound to fiber cell cytoplasm and membranes. (c) In the presence of EGTA to chelate calcium, the CaM-RITC bound only to the fiber cell surface membranes. (d) Binding of CaM-RITC to the fiber cell membranes in the absence of calcium was blocked completely by incubation of lens sections with rabbit antibodies to lens gap junction protein. CaM-RITC binding was not blocked by (e) incubation with rabbit antibodies to water-soluble proteins of chick lens or (f) incubation with serum from nonimmunized rabbits. For (a and b), exposures were determined by the automatic camera on the microscope and were shorter than exposures for (c) to (f). Exposures for (c) to (f) were manually terminated after the same length of time. All prints were exposed and developed in the same manner.

To examine this possibility, we first determined the location of the calmodulin binding sites in a cell type rich in gap junctions, the lens fiber cell (*10*). Following the rationale of Pardue *et al.* (*16*) for identifying calmodulin binding sites in cells in vitro, we synthesized a conjugate of calmodulin and tetramethylrhodamine isothiocyanate (CaM-RITC) (*17*). The CaM-RITC was judged to be biochemically active because its migration pattern in sodium dodecyl sulfate (SDS)–polyacrylamide gels depended on the presence or absence of calcium; its mobility was identical to the native protein under the same conditions; and because it activated calmodulin-deficient bovine brain phosphodiesterase in a dose-dependent manner identical to that of the native protein (data not shown). The functional integrity of the CaM-RITC was further verified by microinjecting the conjugate into dividing cells and observing its rapid incorporation into the mitotic apparatus (*18*) in a pattern almost identical to that observed in cultured cells by indirect immunofluorescence (*19*). Calmodulin binding sites in chick lens were located as described (*20*). The distribution of the gap junction protein was then observed by indirect immunofluorescence (*21*).

Gap junction protein was located on the surface membranes of the lens fiber cells (Fig. 1a). When lens sections were incubated with CaM-RITC in the presence of 1 m*M* Ca^{2+}, the conjugate was found both in the fiber cell cytoplasm and at the cell surface membrane (Fig. 1b). However, when sections were incubated with CaM-RITC in the presence of 1 m*M* EGTA to chelate the Ca^{2+}, conjugate binding occurred only at the cell surfaces (Fig. 1c) in a pattern similar to the distribution of the gap junction protein as seen by indirect immunofluorescence. Incubation of the lens sections with rabbit antibodies to chick lens gap junction protein in the absence of Ca^{2+} (*22*) blocked CaM-RITC binding to the cell membranes (Fig. 1d). Control sections, in the absence of Ca^{2+}, were incubated with antibodies to water-soluble cytosol proteins of lens (Fig. 1e), with serum from nonimmunized rabbits (Fig. 1f), or with actin antibodies (not shown). These controls reduced but never eliminated CaM-RITC binding to the cell membranes. We believe that this reduction in CaM-RITC binding brought about by incubating the lens sections with antibodies to proteins other than gap junction protein indicates the presence of

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