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The *in Vivo* Biosynthesis of Embryonic Proteins after Maternal Administration of Phenytoin in the Mouse (42206)

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Abstract. Pregnant A/J mice received 60 mg phenytoin/kg body weight on Day 10 of gestation. Eighteen hours after phenytoin injection, animals were injected (ip) with 20 μCi/g of [^{35}S]methionine. After 6 hr of incorporation animals were sacrificed and the embryos were removed. Protein synthesis in the embryo, as measured by [^{35}S]methionine incorporation into trichloroacetic-precipitable protein, was analyzed by SDS–PAGE and quantitation of autoradiograms. The results of gel electrophoresis indicate that in embryonic primary palates and limb buds from phenytoin-treated mothers there is an increase in synthesis of 66-, 50-, 44-, and 13-kDa proteins and a decrease in synthesis of an 18-kDa protein compared with values for the control counterpart. No role has been assigned to the 66-, 44-, or 13-kDa proteins but the 50-kDa band comigrates with tubulin and the 18-kDa band comigrates with calmodulin. Palatal cells *in vitro* stained positively with specific antibody to both these proteins. An adverse effect of the anticonvulsant drug phenytoin, when administered to pregnant A/J mice is an increase in the incidence of cleft lip with or without cleft palate [CL(P)] in their offspring. These alterations in protein synthesis may be a direct or secondary result of maternal phenytoin treatment and may play a role in CL(P) formation *in vivo*.

Phenytoin (diphenylhydantoin; Dilantin) a drug used extensively for the control of epileptic seizures, has been associated with the production of birth defects (1, 2). In recent years, clinical studies have suggested that there is an increase in the frequency of malformations in offspring of women who have taken phenytoin during pregnancy (3, 4). These birth defects include craniofacial malformations, in particular, cleft lip with or without cleft palate [CL(P)].

Animal studies have demonstrated that phenytoin alters DNA and protein synthesis during critical periods of palatal development. Tassinari *et al.* (5) demonstrated that when phenytoin was administered during the time of secondary palatal closure, DNA and protein levels were significantly altered in various embryonic tissues (i.e., fetal palate and mandible). Maternal phenytoin administration during the time of primary palatal closure resulted in a threefold decrease in DNA synthesis in primary palates of embryos from phenytoin-treated mothers compared with controls. Protein synthesis, however, was increased twofold in primary palates of embryos from phenytoin-treated mothers in comparison to their control counterparts (6). Transmission and scanning electron microscopy indicates that there is more extracellular matrix produced in the area that forms the primary palate in embryos from phenytoin-treated mothers (7).

The identification of these embryonic proteins that are affected after maternal administration of phenytoin, may aid in providing a clue to the possible cause(s) of the birth defects. Therefore, an investigation was conducted to determine which specific proteins were affected after maternal administration of phenytoin.

Materials and Methods. On gestational Day 10, six pregnant A/J mice (Jackson Laboratories, Bar Harbor, Me.) were injected ip with 60 mg/kg body weight of phenytoin (Parke Davis, Morris Plains, N.J.) and another six with vehicle (40% propylene glycol with 10% alcohol in water for injection, adjusted to pH 12 with sodium hydroxide). Injections were staggered every 20 min to allow time for embryo removal. Eighteen hours after phenytoin injection, all animals were injected ip with 20 μCi/g body weight of [^{35}S]methionine (New England Nuclear, Boston, Mass., NEG-009H,

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1064.1 Ci/mmole). After 6 hr of incorporation, females were sacrificed by cervical dislocation. Embryos were dissected free of the uterine wall and embryonic membranes and placed immediately in ice-cold Eagle's minimum essential medium (EMEM) containing 0.02% NaN_3 to stop incorporation.

Embryonic primary palates (see Fig. 1) and limb buds were dissected free, placed in 0.5 ml of EMEM and homogenized on ice, after which ice-cold trichloroacetic acid–tannic acid (TCA–TA, 5–0.125%, respectively, final concentration) was added. Samples were allowed to precipitate on ice for 15 min prior to sedimentation. The homogenates were centrifuged at 1500*g* for 5 min, after which supernatant fluids were aspirated and the pellets resuspended and washed four times in TCA–TA. The final pellets were resuspended in 1 ml of ice-cold deionized water. Samples (100 μl) were lyophilized and hydrolyzed in 200 μl of 3 *M* toluene sulfonic acid for 24 hr at 106°C. The ninhydrin reaction for protein determination (8) was performed on each sample. Leucine was used as the standard. Results reported are based on an *n* value of six mothers with three embryos per mother and means of duplicate samples from each group.

Gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulphate (SDS) on the remainder of the TCA–TA precipitate according to Laemmli (9), using a sample buffer containing 2% SDS, 2 *M* urea, and 1% (v/v) 2-mercaptoethanol. Samples were heated for 2 min at 90°C in sample buffer before electrophoresis. Bands were visualized by Coomassie blue staining and by autoradiography with the use of fluorography (10, 11). Gels were exposed to Kodak X-Omat AR X-ray film at −70°C. Molecular weight markers were from Bio-Rad Laboratories, (Bio-Rad Corp., Richmond, Calif.). Autoradiograms were scanned with a Hoefer densitometer (Model 65300, Hoefer Scientific Institute, San Francisco, Calif.). The analog output from the densitometer was directed to an Apple 2+ Computer, DYSC integrator equipped with an A/D convertor, timer and memory cards, and a software package for resolution of peaks and valleys (Dynamic Solutions Corp., Pasadena, Calif.).

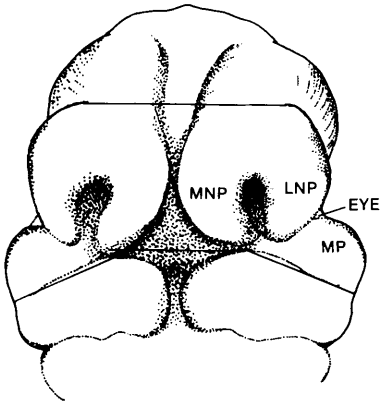

FIG. 1. Drawing of a Day 11 A/J embryo indicating the medial nasal process (MNP), lateral nasal process (LNP), and the maxillary process (MP) which comprise the primary palate. Tissue taken for the study came from the outlined area.

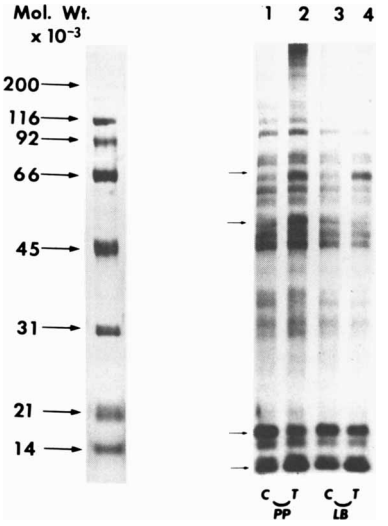

FIG. 2. Autoradiogram of a 10% SDS–PAGE gel stained with Coomassie brilliant blue. Lanes 1 and 3 contain protein samples of primary palatal tissue (PP) and limb buds (LB), respectively, from embryos of control mothers (C). Lanes 2 and 4 contain protein samples of primary palatal tissue and limb buds, respectively, from embryos of phenytoin-treated mothers (T). Marker proteins for calibration are shown on the right.

Results. Results of gel electrophoresis experiments indicate that maternal administration of phenytoin alters embryonic primary palatal protein synthesis. There was an increase in synthesis of 66-, 50-, 44-, and 13-kDa proteins and a decrease in synthesis of an

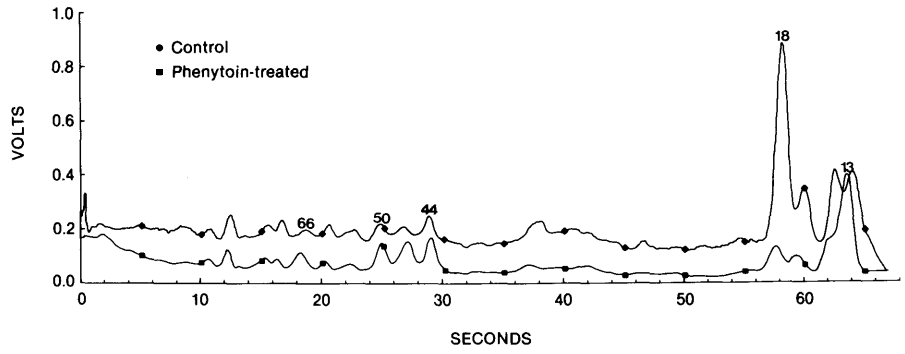

FIG. 3. A densitometric scan of the autoradiogram (lanes 1 and 2) indicates an increase in incorporation of [^{35}S]methionine in some high-molecular-weight proteins (i.e., 66-, 50-, and 44-kDa) as well as a decrease in incorporation of a low-molecular-weight protein (18-kDa) primary palatal tissue from embryos of phenytoin-treated mothers in comparison to controls.

18-kDa protein in comparison to primary palatal proteins from embryos of control mothers (Figs. 2, 3 and Table I). Similar changes in protein synthesis were also observed in limb bud tissue from embryos of phenytoin-treated mothers compared to control counterparts (Fig. 2).

Analysis of the autoradiogram indicates that the 66-kDa protein in palatal tissue from phenytoin-treated mothers was increased 2.85-fold and represented 4.7% of total protein present versus 1.6% for the control (Table I). Greater protein values were also noted for the 50- and 44-kDa proteins in primary palatal tissue from phenytoin-treated animals, in comparison to controls (1.74- and 1.70-fold increase, respectively). Synthesis of the 13-kDa protein was 3.69-fold greater than that of the control value, and represented 41 and 11% of the total protein for the phenytoin-treated and control, respectively. However, synthesis of the 18-kDa protein decreased in primary palatal tissue of phenytoin-treated mothers to 24.1% of the control value, and was 8% of the total protein compared with 33% for the control (Table I).

Discussion. The present investigation has demonstrated that synthesis of specific embryonic proteins is altered after maternal administration of phenytoin. In embryonic tissue from phenytoin-treated mothers, synthesis of 66-, 50-, 44-, and 13-kDa proteins was increased whereas synthesis of an 18-kDa protein was decreased (Table I). The current study is the first to report changes in specific proteins in embryos after maternal administration of phenytoin. However, there have been other more general biochemical studies, to address this problem (5, 12–15).

Sonawane and Goldman (13) demonstrated that when phenytoin was administered to pregnant mice during the critical embryonic period of palatal differentiation, fetal–palatal RNA and protein syntheses were significantly decreased. Garizon *et al.* (14) injected mongolian gerbils intraperitoneally with different dosages of phenytoin, then various body parts were examined for DNA, RNA, and protein contents. They found a slight increase in protein concentration in liver and kidneys. RNA values were decreased in the cerebellum; DNA concentration was decreased in all four tissues examined. Parker and Netzloff (15) discovered that maternal administration of phenytoin inhibited the synthesis of the enzyme (protein) ornithine decarboxylase (ODC) in embryos. They concluded any alterations in ODC activity indicated abnormal development because of its importance in embryogenesis; ornithine decarboxylase is the rate-limiting enzyme in putrescine biosynthesis (15).

In vitro studies also indicate that phenytoin alters DNA and/or protein synthesis (16–18). Previous studies, as well as the present investigation clearly demonstrate the adverse effect of phenytoin on DNA and protein synthesis. The observed changes in protein synthesis of primary palates from embryos of phenytoin-treated mothers may play a negative role in primary palatal development. The 50-, 44-,

TABLE I. QUANTITATION OF EMBRYONIC PALATAL PROTEINS FROM CONTROL AND PHENYTOIN-TREATED A/J MICE[a]

	Peak number	Molecular weight (kDa)	Ret time (sec)	Peak area[b] (volt-sec)	Area %[c]	E/C[d]
Control	1		8.505252	0.026021	1.719615	
	2		10.815321	0.017339	1.145872	
	3		12.495371	0.040328	2.665096	
	4		13.720407	0.004458	.294616	
	5		15.540461	0.026436	1.747043	
	6		16.730497	0.27589	1.823198	
	7	66	18.585552	0.24727	1.634102	2.85
	8		20.580611	0.29127	1.924846	
	9		22.715674	0.29698	1.962600	
	10	50	24.920740	0.43898	2.900983	1.74
	11		27.160806	0.58581	3.871311	
	12	44	29.050862	0.69455	4.589942	1.70
	13		33.110983	0.008433	.557286	
	14		34.511024	0.001914	.126494	
	15		38.046129	0.74040	4.892948	
	16		40.846212	0.32833	2.169767	
	17		41.931244	0.009165	.605689	
	18		46.516380	0.018184	1.201697	
	19	18	58.101724	0.500933	33.104116	0.241
	20		59.991780	0.124769	8.245373	
	21	13	62.581857	0.168067	11.106665	3.69
	22		64.051901	0.177207	11.710471	
Phenytoin-treated	1		.385011	0.000843	0.055692	
	2		1.785053	0.017831	1.178333	
	3		10.570314	0.024696	1.632079	
	4		12.215363	0.047105	3.112909	
	5		13.195392	0.002031	.134248	
	6		15.295454	0.040486	2.744167	
	7		16.275483	0.025592	1.691289	
	8	66	18.200540	0.070541	4.661742	
	9		20.230600	0.025717	1.699496	
	10		22.295662	0.031936	2.110447	
	11	50	24.710733	0.076467	5.053347	
	12		26.670792	0.075181	4.968343	
	13	44	28.770854	0.118062	7.802204	
	14		37.066100	0.067340	4.450110	
	15		39.726179	0.007548	.498886	
	16		41.861242	0.068085	4.499355	
	17	18	57.611710	0.120529	7.965178	
	18		59.326761	0.073282	4.842889	
	19	13	63.526885	0.620570	41.010669	

[a] Proteins from [^{35}S]methionine-labeled palates were subjected to electrophoresis in a 10% SDS–polyacrylamide gel and an autoradiogram was made. Developed bands were quantitated using a DYSC computer integration system (see Methods).

[b] Peak area is the measurement for each band on the autoradiogram in volt–seconds.

[c] Percentage total is the relative amount of each protein band expressed as a percentage of the total.

[d] E/C is the ratio of areas in volt–seconds for designated proteins for the experimental (phenytoin-treated) divided by the control value.

and 18-kDa bands comigrated with purified tubulin, actin, and calmodulin, respectively, in two-dimensional gel electrophoresis; however, immunoblotting experiments are required to substantiate the thought that the altered proteins at these positions are indeed those suggested. Our experiments indicated that immunofluorescence staining for actin and calmodulin demonstrated their presence in A/J mouse embryos during the same ges-

tational period during which this study was conducted (unpublished observation). MacKinney *et al.* (19) demonstrated that 5,5-diphenylhydantoin (phenytoin) inhibited polymerization of purified pig tubulin as much as 50%, as measured by the viscosometric assay and estimation of tubulin length by electron microscopy. Since phenytoin inhibits tubulin polymerization, increased synthesis of tubulin may interfere with cell movement. Decreased calmodulin synthesis could interfere with the indirect influences that calmodulin has on a variety of cellular processes, i.e., regulation of cyclic nucleotide levels (20). No roles have been assigned to the 66- or 13-kDa proteins.

In conclusion, further analyses leading to the identification of these proteins that are altered in their synthesis after maternal administration of phenytoin (particularly in the area of the primary palate) may aid in understanding the association between phenytoin and the induction of birth defects, such as cleft lip and or cleft palate.

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