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DISTRIBUTION AND METABOLISM OF DIPHENYLHYDANTOIN-^{14}C IN FETAL AND MATERNAL TISSUES OF THE PREGNANT MOUSE†*

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Abstract—The distribution of diphenylhydantoin-^{14}C in A/JAX mice at 17·5 days of gestation was studied by whole-body autoradiography. The drug was given intravenously, and the mice were frozen 6·5 min, 20 min, 1 hr, 3 hr and 9 hr later. Whole-body sagittal sections, processed for autoradiography, revealed rapid localization of radioactivity in the adrenal cortex, liver, kidney, aorta, myocardium, Harder's gland, corpora lutea of ovary, and wall of intestine of mother. Equilibrium between maternal and fetal concentrations of radioactivity was achieved by 1 hr after injection. Fetal myocardium, liver, intestine, adrenal and yolk sac were the first tissues to have detectable amounts of radioactivity and also the highest concentrations at equilibrium. Placenta, fetus, and maternal brain, kidney, liver, heart, ovary, and adrenal were removed from the frozen sections, extracted and analyzed by a specific radiochromatographic technique. Unchanged diphenylhydantoin-^{14}C in these tissues accounted for most (88–99 per cent) of the radioactivity in the chromatograms, while 0·2–5 per cent of the radioactivity was identified as its major metabolite, *p*-hydroxyphenylphenylhydantoin-^{14}C (HPPH-^{14}C). These studies demonstrate that diphenylhydantoin crosses the placenta rapidly and is distributed ubiquitously into fetal tissues. The specific localizations suggest that the drug may be interfering with steroid hormone metabolism or action.

THE TERATOGENIC action of diphenylhydantoin has been demonstrated in Swiss-Webster and A/JAX mice.[1,2] The suggestion also has been made that this drug may be responsible for the cleft lip and palate observed in some children born to epileptic mothers receiving diphenylhydantoin.[3,4] These effects have been attributed to impairment of folic acid metabolism but, as yet, no clear mechanism for its teratogenic action has been elucidated.[5]

This investigation was performed in an effort to obtain information on the maternal and fetal distribution as well as possible sites of action of diphenylhydantoin in pregnant animals. The disposition of diphenylhydantoin-^{14}C in pregnant mice at 17 days of gestation was studied by whole-body autoradiography. Although the teratogenic action of the drug is effected prior to this stage of gestation, no specific fetal distributions of teratogenic compounds are seen in early or mid-gestation.[6] Late

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gestation was chosen for this study so that larger fetuses would make fetal tissue distributions more discernible. If a compound is metabolically altered in the body, this technique gives no information on the chemical identity of the radioisotope. Consequently, specific sections of maternal or fetal tissue were analyzed by a thin-layer radiochromatographic assay which achieved quantitative identification of diphenylhydantoin-^{14}C and its major metabolite. These metabolic data were correlated with the tissue distribution of radioactivity observed by autoradiography.

METHODS

Autoradiography

A/JAX mice were mated overnight once a week and examined the following morning for the presence of vaginal plugs. Those with plugs were considered to be in day zero of pregnancy. At noon on day 17 of gestation, five mice were each injected intravenously with 0·2 ml of a solution containing approximately 9 μc of 5,5-diphenylhydantoin-4-^{14}C (DPH-^{14}C) obtained from New England Nuclear Corp. (sp. act. 4·73 mc/m-mole). The DPH-^{14}C was dissolved by adding 2·0 equivalents of NaOH and then brought to volume with 0·9% NaCl. The mice were briefly anesthetized with ether before freezing by immersion in a solution of hexane cooled with dry ice at 6·5 min, 20 min, 1 hr, 3 hr and 9 hr after injection. They were then sectioned and processed for autoradiography by taking whole-body sagittal sections onto No. 800 Scotch tape by a modification[7] of the technique originally described by Ullberg.[8] Areas of interest were sectioned by taking a 100 μ thick section, a 20 μ thick section, and another 100 μ thick section, in that order. The 20 μ sections were processed for autoradiography by placing against Kodak industrial type AA X-ray film. The autoradiograms were used as negatives to produce prints; consequently, white areas in the figures correspond to radioactivity.

Assay of diphenylhydantoin-^{14}C and p-*hydroxyphenylphenylhydantoin-^{14}C (HPPH-^{14}C) in tissue sections*

Extraction. A modification of the method described by Mirkin and Koski was employed.* Tissue sections, 100 μ thick, corresponding to a contiguous 20 μ thick autoradiographed section were used for extraction. Areas of interest were dissected with a scalpel under low magnification. In most experiments, the contents of ten sections were pooled for a single extraction. The pooled sections were added to an extraction vessel containing conc. HCl (0·1 ml) and ethyl ether (12 ml) which was shaken for 15 min and centrifuged (low speed) for 5 min. A 10-ml aliquot of the ethyl ether layer was transferred into an extraction tube containing 1 N NaOH (4 ml), shaken for 10 min and centrifuged for 5 min. An aliquot of the NaOH phase (2–3 ml) was removed, and two similar NaOH extractions carried out on the remaining volume of ethyl ether. The pooled NaOH extracts were placed in a vessel containing conc. HCl (1 ml) and ethyl acetate (8 ml), shaken for 10 min and centrifuged for 5 min. An aliquot (7 ml) of the ethyl acetate phase was removed and placed in a tapered evaporating tube, after which an additional 5 ml of ethyl acetate was added to the acidified mixture and a second extraction performed. The pooled ethyl acetate extracts were

* B. L. Mirkin and D. Koski, unpublished observations.

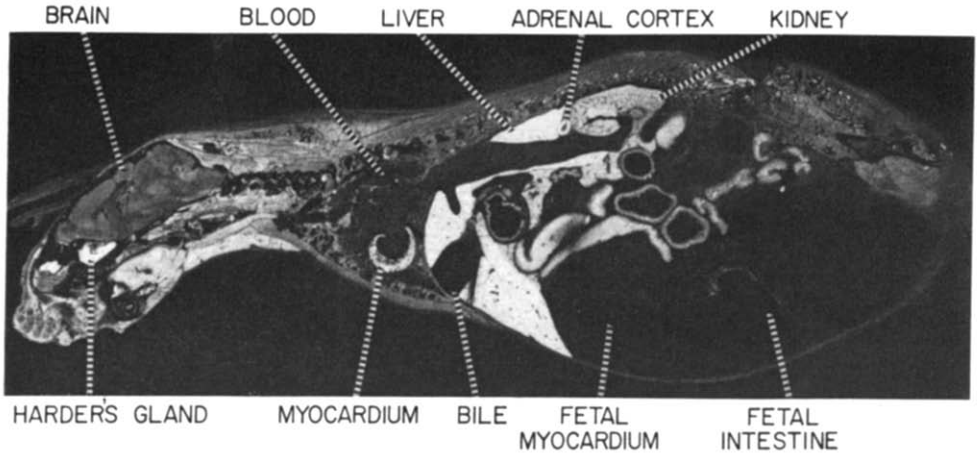

FIG. 1. Print of a whole-body autoradiogram of a pregnant mouse frozen 6·5 min after i.v. injection. White areas correspond to radioactivity. Note the high activity in the myocardium and adrenal cortex of the mother. Some radioactivity is visible in the fetal myocardium and intestine.

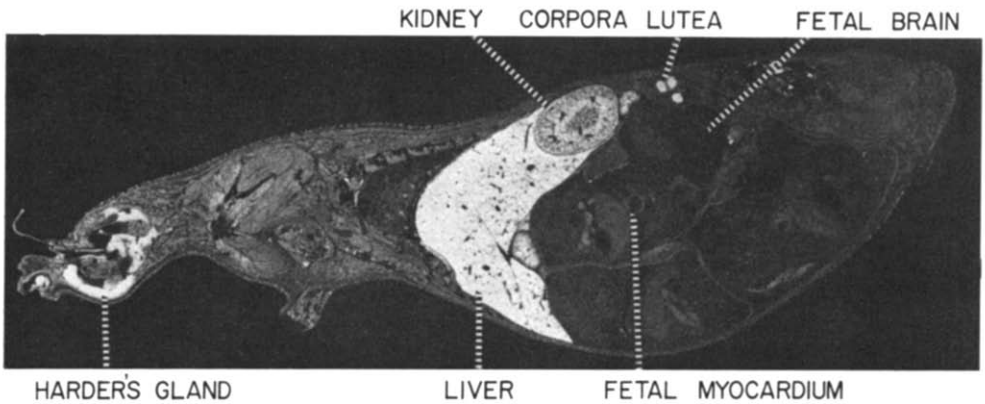

FIG. 2. Print of a whole-body autoradiogram of a pregnant mouse frozen 20 min after i.v. injection. Note the high concentration in the corpora lutea of the ovary.

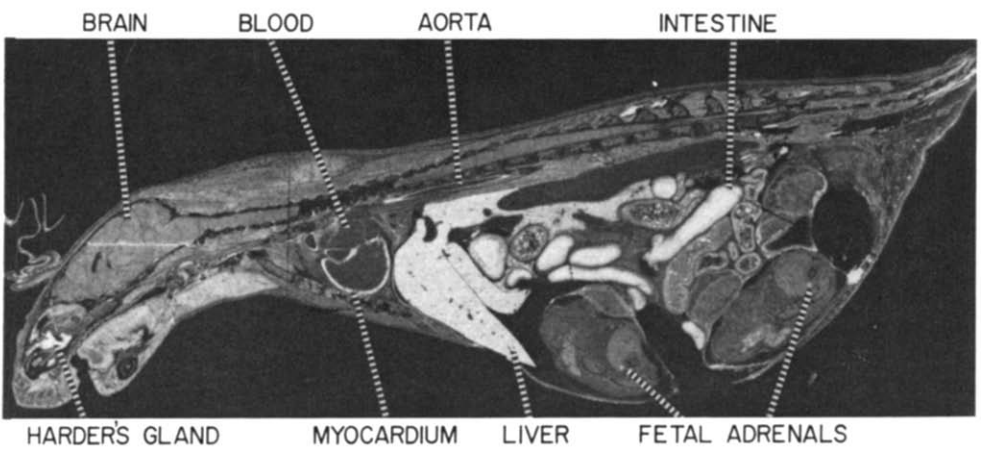

FIG. 3. Print of a whole-body autoradiogram of a pregnant mouse frozen 1 hr after i.v. injection. Note the high concentration in the fetal adrenals.

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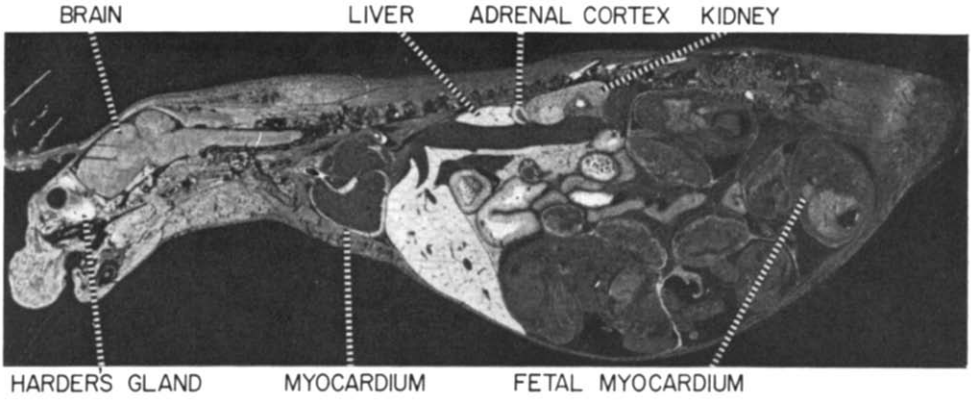

FIG. 4. Print of a whole-body autoradiogram of a pregnant mouse frozen 3 hr after i.v. injection. Fetal and maternal tissues of initially high activity remain higher than that of other tissues.

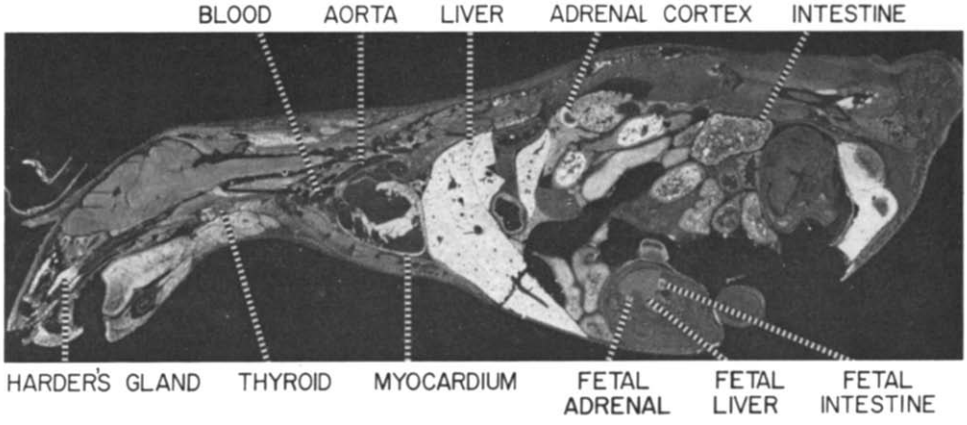

FIG. 5. Print of a whole-body autoradiogram of a pregnant mouse frozen 9 hr after i.v. injection. Myocardium, liver, adrenal and intestine of both mother and fetus continue to show high concentrations of radioactivity.

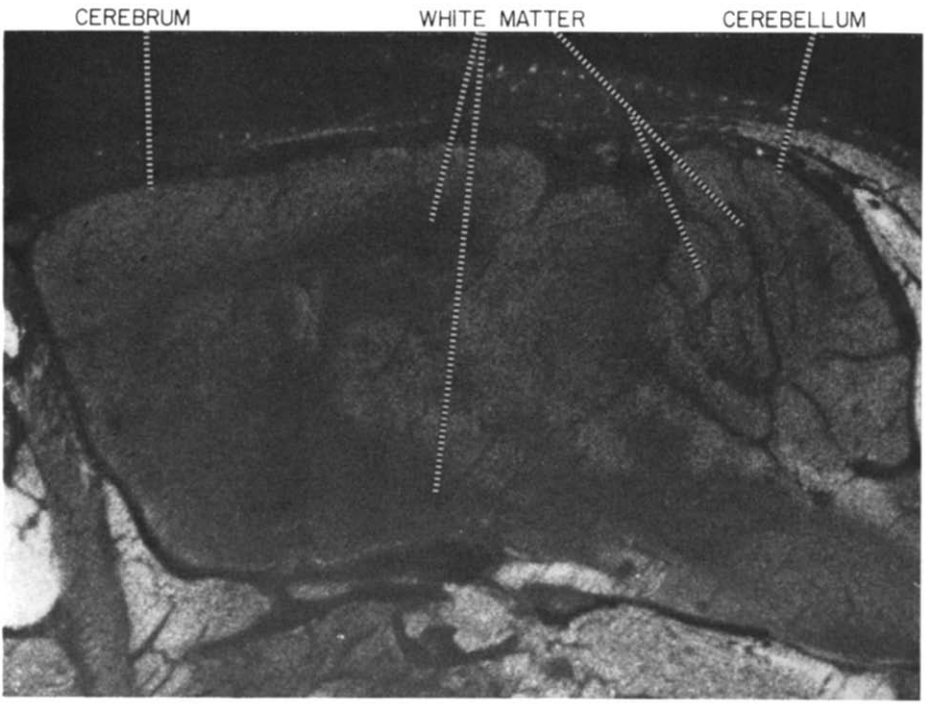

FIG. 6. Enlargement of an autoradiogram of the brain from the mouse frozen at 6·5 min after injection. Note that some of the white matter in the cerebellum is higher and some lower in activity than the gray matter.

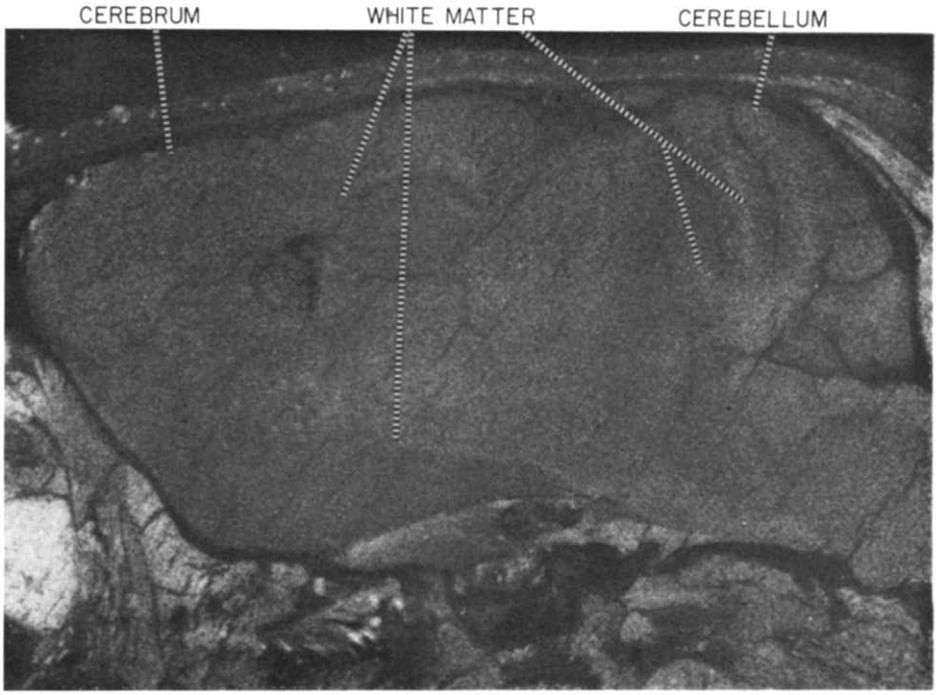

FIG. 7. Enlargement of an autoradiogram of the brain of the mouse frozen 1 hr after injection. Note that all the white matter has a higher concentration than gray matter.

evaporated to dryness under a stream of air or nitrogen, redissolved in absolute methyl alcohol (50 μl) and applied to the thin-layer chromatogram.

Thin-layer radiochromatography. The thin-layer chromatographic apparatus consisted of an Eastman Chromogram Developing System utilizing cellulose acetate backed chromatogram sheets (20 × 20 cm) coated with Silica gel containing fluorescent indicator. Each chromatogram sheet was divided into strips 3 cm wide with the origin 2·5 cm above the lower edge of the sheet. Non-isotopic reference standards consisting of DPH and HPPH (10 mg/ml) in 0·01 N NaOH-methanol were used as markers to locate isotopic correlates of each of these compounds. A 6-μl aliquot of the resuspended extract containing isotopic DPH-^{14}C and HPPH-^{14}C as well as a 3-μl aliquot of the non-isotopic DPH and HPPH reference standard were applied to each strip. The application spot was dried by exposure to a hot-air blower to minimize spreading. The chromatogram was developed in an ascending manner and removed from the chamber after the solvent front had migrated a minimum of 17·0 cm from the origin. The solvent system consisted of chloroform (81 ml), acetone (9·0 ml), anhydrous methyl alcohol (9·8 ml), and glacial acetic acid (0·2 ml).

After completing development, the chromatogram was air-dried and scanned under ultraviolet light (180–300 nm). The DPH and HPPH spots were clearly visible as areas devoid of fluorescence. These were outlined in pencil, the R_f value was obtained, and the segment 1 cm above and below the center of the spot was removed for scintillation counting. The segment was placed in a counting vial containing 3·0 ml of 0·01 N NaOH–methanol and shaken for one-half hour. A toluene-based liquid scintillation phosphor was added and the radioactive content of each vial assayed in a Packard Tri-Carb spectrometer. Quenching and counting efficiency was determined for each sample to enable conversion of all results into disintegrations per minute.

RESULTS

Distribution of radioactivity in maternal and fetal tissues. Representative whole-body autoradiograms were prepared from each of the five mice and are shown in Figs. 1–5. A very rapid localization of radioactivity was noted in the following maternal tissues: adrenal cortex, liver, kidney, aorta, myocardium, Harder's gland, corpora lutea of the ovary and wall of the intestine. At 6·5 min after injection, the amount of radioactivity was lower in the fetuses than the mother; at 20 min it was only slightly lower in the fetuses; whereas, by 1 hr and thereafter the concentration of isotope in the fetuses was approximately equivalent to that of the mother.

The fetal tissues in which radioactivity was initially detected were generally the same as those which had the highest concentrations after equilibrium. These tissues were the fetal myocardium, liver, intestine and adrenal. These tissues also were the same as those of the mother that had a high affinity. The fetal yolk sac demonstrated a rapid uptake and a consistently high concentration of the isotope; it is seen as a fine white line surrounding each fetus in each of the autoradiograms.

There was rapid penetration of radioactivity into the gray matter of both the cerebrum and cerebellum so that the concentrations were about the same as those present in muscle tissue (Fig. 6). The gray matter maintained this approximate equivalence with muscle at all the time intervals studied. The majority of white matter in both cerebrum and cerebellum had a lower concentration than gray matter prior to 1 hr, but thereafter, the concentration in white matter was slightly higher than that in gray

TABLE 1. DIPHENYLHYDANTOIN-^{14}C AND *p*-HYDROXYPHENYLPHENYLHYDANTOIN-^{14}C IN MATERNAL AND FETAL TISSUE SECTIONS AFTER MATERNAL ADMINISTRATION OF DIPHENYLHYDANTOIN-^{14}C

	Maternal tissue												Fetal tissue			
	Brain*		Kidney*		Liver*		Heart*		Ovary†		Adrenal†		Placenta*		Fetus†	
Time after DPH injection (min)	DPH‡ (%)	HPPH§ (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)	DPH (%)	HPPH (%)
6·5	97	0·3	95	1·6	99	0·5	98	1·0	92	2	91	5	93	0·6	90	3·4
20·0	95	1·1	97	0·9	98	0·7	98	0·5	91	0·8	85	3	95	0·9	93	1·1
60·0	96	1·2	94	1·6	98	0·9	99	0·4	N.S.	N.S.	97	1	92	1·9	89	1·5
180·0	95	1·8	96	1·2	99	0·8	98	0·8	N.S.	N.S.	92	4	88	2·8	94	1·4
540·0	94	0·2	96	1·5	98	0·8	99	0·3	95	1·0	96	2	96	1·2	93	2·1
Mean	95·4	0·9	95·6	1·4	98·4	0·7	98·4	0·6	92·7	1·3	92·2	3·0	92·8	1·5	91·8	1·9
S. E. M.	0·5	0·3	0·5	0·1	0·3	0·1	0·3	0·1	1·2	0·4	2·1	0·7	1·4	0·4	1·0	0·4

* Represents extract of 10 tissue sections; each assay performed in triplicate.
† Represents extract of 12 tissue sections; each assay performed in triplicate. N.S. = no sample.
‡ Diphenylhydantoin-^{14}C.
§ *p*-Hydroxyphenylphenylhydantoin-^{14}C.

(Fig. 7). However, some portions of cerebellar white matter appeared to achieve higher concentrations of radioactivity more rapidly than cerebellar gray matter or cerebral white matter (Fig. 6). No other specific localization of isotope was noted in the maternal brain.

The concentration of radioactivity in fetal brain appeared to have the same general relationship to other fetal tissues as the maternal brain did to non-neural maternal tissues. There were no areas of specific accumulation in fetal brain. The white matter in fetal brain was not discernibly higher than the gray matter.

Identification of ^{14}C-isotope in tissue sections. The quantitative analysis of ^{14}C in maternal and fetal tissues was carried out by thin-layer radiochromatographic procedures (see Methods). The ^{14}C content of tissue sections obtained from maternal brain, kidney, liver, heart, ovary and adrenal and the placenta and fetus was found to consist mainly of DPH-^{14}C along with small quantities of HPPH-^{14}C. The assay of more than 400 tissue sections obtained at periods ranging from 6·5 min to 9 hr after DPH-^{14}C administration demonstrated that 94·8 $\pm$ 3·3 per cent of the ^{14}C isotope present was DPH-^{14}C (Table 1). There appeared to be no tendency for HPPH-^{14}C to accumulate in any specific tissue over the time period studied. It is unlikely that glucuronide conjugates were present to a significant extent in most tissues since they are rapidly excreted by the kidney.

DISCUSSION

This investigation has demonstrated the rapid transplacental passage and ubiquitous distribution of DPH-^{14}C in the pregnant mouse. Equilibrium was achieved between mother and fetuses by 1 hr after injection; no further change in the fetal concentration of drug relative to that in maternal tissues was seen after this time interval. The fetal localization of DPH-^{14}C in the mouse was similar to that observed for non-isotopic diphenylhydantoin in both the human[4] and rat fetus.[9,10] A rather high concentration of DPH-^{14}C was present in the following maternal and fetal tissues: liver, brain, kidney, heart, adrenal cortex and corpora lutea.

The intense autoradiographic localization and persistence of DPH-^{14}C in the heart, at all time intervals after injection, indicate a high tissue affinity. Other studies, using non-isotopic diphenylhydantoin, have demonstrated that the concentration of diphenylhydantoin in myocardial tissue (μg/g) is about equal to that of the liver over a 24-hr period.* The marked anti-arrhythmic action of diphenylhydantoin in digitalis intoxication may possibly relate to this high affinity for myocardial tissue.

In view of the well-documented effect of diphenylhydantoin upon steroid metabolism, the high concentration of DPH-^{14}C noted in the adrenal cortex and corpora lutea of the ovary may be of pharmacologic significance. Diphenylhydantoin administration increases the urinary excretion of 6β-hydroxycortisol and decreases the excretion of 17-ketosteroids in man.[11] Although human liver, adrenal, kidney, placenta, and skeletal muscle are all capable of hydroxylating cortisol in the 6 position, liver is the most active.[12] Furthermore, pretreatment of guinea pigs for several days with DPH stimulates the formation of an enzyme system in liver microsomes that hydroxylates cortisol in the 6-β position.[13] The same enzyme may be responsible for

* B. L. Mirkin, unpublished observations.

metabolism of both compounds, since 6-β hydroxylation of cortisol, by rat liver slices, is inhibited by DPH in both male and female rats. This apparently diverts cortisol metabolism to other enzymes since significant enhancement of reduction of cortisol in the 3 position accompanied addition of DPH to incubations of female rat liver slices.[14] The high concentration in the corpora lutea of the ovary has been found for only two other compounds, to our knowledge. One of these, bis-(*p*-hydroxyphenyl)-cyclohexylidenemethane[15] has a structure very similar to that of diphenylhydantoin; the other is progesterone.[16]

Differences in the vascularity of various parts of the brain could account for the slow accumulation of radioactivity in the white matter and also for the differences between white matter in various parts of the cerebellum. A very similar pattern of distribution was seen after the administration of progesterone.[16]

The only clues which these experiments offer regarding the mechanism of the teratogenic action of DPH are that it may be altering the normal pattern of steroid hormone metabolism or action in the mother, fetus, or the yolk sac.

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