# Differential Binding of $^{14}$C-Cortisone in Fetal, Placental, and Maternal Liver Tissue in A/Jax and C57BL Mice

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*When $^{14}$C-cortisone was given to pregnant A/Jax and C57BL mice, the concentration of radioactivity firmly bound to fetal tissue was significantly higher in A/Jax mice than in C57BL mice. Differences in the binding of cortisone to tissue proteins may explain the differential incidences of cleft palate in A/Jax and C57BL mice.*

The gross teratologic effects of cortisone on the fetal development of mice have been investigated extensively. Cortisone injected into pregnant mice on days 11, 12, 13, and 14 of pregnancy will cause cleft palate without cleft lip at a frequency that is dependent on the maternal and paternal genotypes.[1,2] In addition to cortisone, cleft palate can be induced by a large number of environmental conditions and drugs including corticoids, salicylates, vitamin A, and antimitotic agents such as X rays, 6-aminonictinamide, and nutritional deficiencies.[3] Although cleft palate is the major structural malformation associated with cortisone administration, other minor embryopathic features including shortening of the head, mandibular alteration, and spina bifida have been reported.[4] Correspondingly, increases in resorption, decreases in fetal weight, and increases in cleft palate frequency after cortisone administration point to the fact that a significant change in fetal or maternal metabolism, or both, has occurred.[5] These facts tend to support our hypothesis that cortisone affects biochemical events in numerous tissues, and that cleft palate specifically results because of the temporal and spacial vulnerability of palatal closure. It has been suggested that cortisone decreases palatal shelf rotational force and consequently causes cleft palate by delaying shelf rotation to the horizontal position.[6]

The specific biochemical alterations induced by cortisone that are responsible for cleft palate are almost completely unknown. In fact, it is not certain whether cortisone directly affects the fetal tissues, or whether its teratologic action is exerted by altered maternal metabolism. The possible modes of developmental interference that cortisone could exert on the mouse palate are many. Cleft palate could result from alterations in placental function since numerous hormones, including cortisone, have been shown to affect placental transfer function and reduce placental blood flow.[7,8] A high rate of mucopolysaccharide synthesis in the palate before palatal closure has been shown to be retarded considerably after cortisone administration.[9,10] Mitosis of cells in palatal shelf mesenchyme in A/Jax mice also has been shown to be decreased by cortisone.[11]

The mechanism of action of cortisone has been subject recently to intense investigation; studies indicate that cortisone acts directly on genetic material by binding with nuclear protein and thus causing induction of specific enzyme systems.[12–19] Cortisone administered in vivo was found in the cytoplasm of hepatic cells in its methanol-soluble form and bound firmly to nuclear proteins and not to deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Nuclear-bound cortisone could not be removed by methanol washings.[17] In the nucleus, the major portion of administered cortisone has been found in the form of cortisone and cortisol, but in the cytoplasm rapid conversion to inactive metabolites had taken place.[15,16] The cytoplasmic cortisone soon disappears, but the nuclear steroid re-

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mains much longer for a period of time that corresponds to the period of enzyme induction. The final result is increased synthesis of numerous gluconeogenic enzymes such as tyrosine-transaminase; thus the amino acid pool is decreased by conversion into glucose.[12,15] When vitamin $B_6$, pyridoxine, the coenzyme of transminases, was administered simultaneously with cortisone, the frequency of cleft palate was decreased substantially.[20]

A "placental barrier" to cortisol has been reported in sheep.[21] It may be speculated that a difference in the transplacental passage of cortisone may exist among different genotypes to "deliver" different drug doses to the fetus and therefore cause differences in cleft palate frequency. Likewise, since binding to nuclear protein seems to moderate the cellular response to cortisone, difference in binding of cortisone may be associated with reported strain differences in cortisone sensitivity.

The purpose of this investigation was to compare two genetically different strains of mice that differ in sensitivity to cortisone-induced cleft palate and in the amounts of unconjugated, conjugated, and bound cortisone and metabolites in the fetuses, placentas, and maternal livers. This was done to determine if differences exist in transplacental passage, relative rate of conjugation, and amounts of free and bound cortisone and metabolites in the two strains of mice.

## Materials and Methods

*Animals.*—Ten C57BL and A/Jax female mice, 2 to 4 months old and weighing 23 to 26 gm each before pregnancy, were used in this study. All mice were kept in the same environment before and after injection of cortisone. Females were mated with males of the corresponding strain to yield fetuses of the same inbred strain. Female mice were inspected for vaginal plugs every eight hours; conception time, designated as time zero, was determined by observation of a vaginal plug.

*Steroid.*—Nonradioactive cortisone was supplied as cortisone acetate in saline suspension (25 mg/ml).* One hundred microcuries of ring A-labeled $^{14}C$-cortisone was supplied in benzene-ethanol solution† (specific activity 58.8 millicuries(mCi)/m*M*). The solvents of the radioactive cortisone were removed by evaporation using a gentle stream of 30° nitrogen blown over the surface. The radioactive residue was then redissolved in a 1 ml solution of 25% ethanol-sterile physiologic saline (volume/volume) and yielded a concentration of 5 microcuries (μCi)/50 μl.

*Injection and Tissue Recovery.*—On day 12.5 of pregnancy, a loading dose of 2.5 mg (0.1 ml) cortisone acetate (that dose is necessary to produce cleft palate at a frequency of 17 and 100% in C57BL and A/Jax mice, respectively[1]) was injected intraperitoneally and was followed immediately by a second injection of 5 μCi $^{14}C$-cortisone per 25 gm mouse. Thirty minutes after injection, the approximate plasma half-life of cortisone in mice, mothers were killed by cervical dislocation. The individual fetuses, placentas, and three excised portions of maternal liver from each mother were removed carefully, weighed and frozen at −17 C until processing. Fetuses and placentas were pooled into groups of two and assayed; each of three maternal liver samples were assayed individually.

*Extraction of Cortisone and Metabolites.*—The extraction procedure is shown in Figure 1. The unbound radioactivity was extracted from the tissues by a modified technique of Mikhail, Wiqvist, and Diczfalusy[22] and Migeon[23] with methanol, and the methanol extract then was separated into unconjugated and conjugated radioactive steroid by partioning between water and dichloromethane. The radioactivity remain-

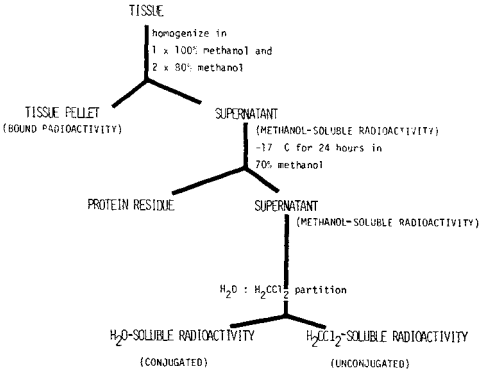


FIG 1.—Extraction technique used for separating the radioactive steroid into unconjugated, conjugated, and bound fractions.

* Merck Sharpe & Dohme Inc., St. Louis, Mo.
† New England Nuclear Corp., Boston, Mass.

ing in the tissue residue after methanol extraction was considered bound to the tissue.[17]

Each tissue sample first was homogenized with a tissue homogenizer* with a motor-driven, Teflon pestle in 10 ml of 100% methanol, and twice in 80% methanol. The particulates were centrifuged at 5,000 rpm and supernatants were removed between homogenizations. Routinely, the particulates were reextracted a fourth time with 100 and 80% methanol and ethanol; less than 1% of the remaining radioactivity in the remaining tissue could be removed further. After methanol extraction, radioactivity in the particulates was considered "bound" to the tissue. The tissue particulates were dissolved in solubilizer,† decolorized with benzoyl peroxide, and added to 15 ml of Bray's solution.[24] The supernants were pooled and evaporated to dryness. The residue from the supernatants was then redissolved in 10 ml 70% methanol and stored for 48 hours at −17 C to precipitate the proteins. The proteinfree supernatant was evaporated to dryness and the resulting residue was partitioned between dichloromethane and water using five separatory funnels with 30 ml in each phase. The dichloromethane-soluble and water-soluble fractions were collected, evaporated, and redissolved in 15 ml Bray's solution.

COUNTING.—Each sample was assayed on a liquid scintillation counter‡ three times for ten minutes each. Quench was determined by adding internal standard and was found to be nearly constant between similar tissue samples. Counts given in the data are corrected for quenching.

STATISTICAL ANALYSIS.—Statistical analysis of recovered radioactivity was performed by computer with the $t$ statistic for the mean of two samples to determine $\bar{x}$, $s$, and $t$.[25]

* Arthur H. Thomas Co., Philadelphia, Pa.
† NCS Solubilizer, Amersham/Searle, Arlington Heights, Ill.
‡ Packard Tri-Carb, Packard Instrument Co., Inc., Downers Grove, Ill.

## Results

The concentrations of $^{14}$C-cortisone and its metabolites recovered in the dichloromethane-soluble (unconjugated), water-soluble (conjugated), and bound fractions in the various tissues analyzed from C57BL and A/Jax mice are summarized in the table. The concentrations of bound radioactivity in the fetuses of both strains are illustrated in Figure 2.

The mean concentrations of radioactive steroid in corresponding fetuses, placentas, and maternal livers were higher in A/Jax mice than in C57BL mice in all extracted fractions (unconjugated, conjugated, bound, and total). However, our data indicate that the higher concentrations found in the tissues of the A/Jax strain were not significantly different than those in the tissues of the C57BL strain, except in the bound fraction of the fetuses, since the differences were small with overlapping ranges. In the fetuses, both the mean concentration of radioactive steroid bound to the tissue after extraction of unconjugated and conjugated steroid with

TABLE
RADIOACTIVITY IN UNCONJUGATED, CONJUGATED, AND BOUND FRACTIONS IN FETAL, PLACENTAL, AND MATERNAL LIVER TISSUE OF A/JAX AND C57BL MICE
(in cpm/gm tissue; one half hour after injection of $^{14}$C-cortisone)

| | Methanol Soluble | | | | Methanol Insoluble | | | |
|---|---|---|---|---|---|---|---|---|
| | Unconjugated | | Conjugated | | Bound | | Total | |
| | $\bar{x}$* | $s$† | $\bar{x}$ | $s$ | $\bar{x}$ | $s$ | $\bar{x}$ | $s$ |
| Fetus | | | | | | | | |
| A/Jax | 49,706 | 5,222 | 16,530 | 3,264 | 1,828 | 351 | 68,064 | 8,386 |
| C57BL | 39,673 | 3,718 | 14,496 | 2,912 | 899 | 151 | 55,068 | 4,620 |
| Placenta | | | | | | | | |
| A/Jax | 908,504 | 42,279 | 870,242 | 381,610 | 5,769 | 854 | 1,784,515 | 535,271 |
| C57BL | 543,656 | 25,106 | 387,544 | 197,337 | 4,288 | 753 | 935,488 | 350,487 |
| Maternal Liver | | | | | | | | |
| A/Jax | 1,015,106 | 470,998 | 4,128,663 | 3,189,529 | 20,341 | 6,026 | 5,163,115 | 2,515,320 |
| C57BL | 613,553 | 295,756 | 3,495,678 | 1,991,448 | 15,646 | 3,364 | 5,164,110 | 2,275,653 |

* $x$, Mean concentration.
† $s$, ± Standard deviation of the mean.

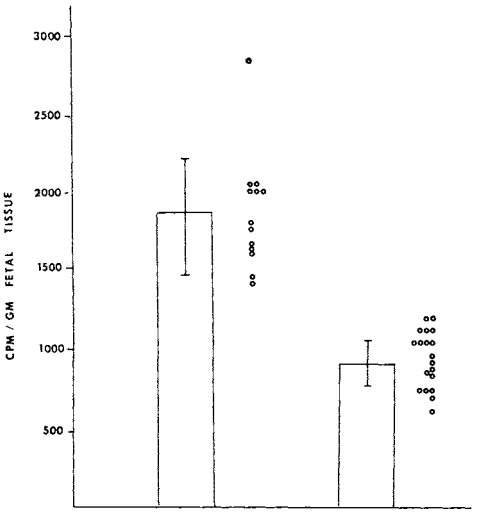


FIG 2.—Concentration of bound radioactivity found in the fetuses of A/Jax and C57BL mice a half hour after injection of $^{14}C$-cortisone. *Bars,* mean concentration of activity in fetuses and standard deviation from the mean; *circles,* concentration of activity in two pooled fetuses.

methanol, and the ratio between bound and unconjugated (free) steroid, was two times higher in A/Jax fetuses than in C57BL fetuses and was significantly different, $P < 0.001$.

## Discussion

Our data have shown that a half hour after an injection of $^{14}C$-cortisone into 12.5 day pregnant C57BL and A/Jax mice, the concentrations of radiosteroid found in each of the tissues analyzed (fetus, placenta, and maternal liver) were slightly higher in A/Jax mice than in C57BL mice. However, these differences in radioactive concentrations are small with overlapping ranges, and this indicates that the reason for the large differential susceptibility to cleft palate reported in these two genotypes is not due to differences in total uptake of cortisone in the fetus, placenta, or maternal liver. It also supports our conclusion that there are no differences in placental permeability to cortisone when it is given as a single dose.

After extraction of the free radioactivity with methanol and fractionation of the extract into an unconjugated and conjugated steroid fraction, no significant difference was found in the concentrations of radioactivity between the two strains in either of the two fractions in similar tissues. Beato[15,16] has shown that unconjugated cytoplasmic cortisone soon disappears after cortisone administration by rapid conversion to inactive metabolites and by binding in unaltered form to nuclear protein. Since the levels of unconjugated steroid were the same in both strains, it appears that the amount available for binding to the tissue, presumably nuclear protein,[19] is the same in A/Jax and C57BL mice. Also, since the concentration of conjugated steroid was about equal, the rates of detoxification of steroid appear the same in both strains.

Significantly, the amount of radioactivity found bound to fetal tissue after rigorous extraction with methanol was considerably higher in A/Jax mice; the genotype exhibited a much higher incidence of cleft palate after cortisone administration. Whether this methanol-insoluble radioactivity is bound to nuclear protein or some other cellular components cannot be ascertained by this experiment. Levin, Daughaday, and Bremer[26] reported a physical tissue binding of cortisone and hydrocortisone in numerous rat tissues. The binding was found to be accomplished by tissue proteins. Sekeris[17] has reported methanol-insoluble cortisone bound to nuclear proteins, probably accomplished by covalent bonds.[27]

Since the amount of radioactivity bound to fetal tissue was higher in cortisone-sensitive A/Jax mice, it may be speculated that quantitative differences in the ability of the tissue to bind cortisone in A/Jax and C57BL fetuses, presumably at its active site, could be responsible for the reported differential incidences of steroid-induced cleft palate. Possible reasons for differences in binding of cortisone could include differences in compartment permeabilities at the subcellular level, binding proteins, or merely the number of available binding sites between the two strains.

## Conclusions

When $^{14}C$-cortisone was injected into A/Jax and C57BL mice on day 12.5 of pregnancy, the radiosteroid extracted with methanol a half hour after injection was found in about equal concentrations in corresponding fetal, placental, and maternal liver tissue samples from both strains. The amount of recovered activity that was bound firmly to the fetal tissues was significantly higher in

A/Jax mice than in C57BL mice in corresponding tissues. It appears that differences in placental permeability or rates of detoxification of cortisone cannot explain the differential incidence of cortisone-induced cleft palate between A/Jax and C57BL mice; however, differences in the binding of cortisone to tissue proteins may explain the reported differential teratologic sensitivity to cortisone in A/Jax and C57BL mice.

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