SUMMARY

Adult pregnant mice were given i.v. injections of \([3H]3\)-methylcholanthrene (20 sCi in 1.1 μg/mouse) or \([14C]3\)-methylcholanthrene (1.0 μCi in 48 μg/mouse). Ethanol extracts of their tissues were chromatographed on Sephadex LH-20. Three groups of 3-methylcholanthrene metabolites were obtained: one group as yet unidentified, one containing the hydrocarbon and hydroxylated derivatives, and a third consisting of conjugated metabolites from the treated adult mice and their fetuses. The conjugated metabolites in tissues and in bile were separated into two fractions; one was acted on by \(\beta\)-glucuronidase and to a lesser extent by arylsulfatase, and the other was resistant to these enzymes but completely susceptible to acid hydrolysis. The hydrolysis resulted in altered chromatographic behavior characteristic of the hydroxy compounds, which also appear in tissue. The enzyme-resistant conjugates were predominant in brain, muscle, and lung, and the enzyme-labile conjugates were predominant in the kidney, liver, and bile of adult mice. These conjugated metabolites were also demonstrated in fetal mice; some appeared in the fetus as early as the thirteenth day of gestation, the most immature fetus so far examined. The resistant group was predominant in the early developmental stages of the fetus and the susceptible group was increased in the excretory organs such as the kidney, liver, and contents of the intestinal tract as the fetuses approached term.

INTRODUCTION

It is well known that fetuses are easily affected by chemical carcinogens given to their mothers. Tumors were induced in fetal mice and rats by giving the mothers injections of 3-MC\(^2\) (22, 28, 30) or 7,12-dimethylbenzanthracene (29), and lethal, deleterious, or teratogenic effects were reported in fetuses of mice given injections of 3-MC (12, 21), in those of rats given 3,4-benzo(a)pyrene (20) or 7,12-dimethylbenzanthracene, (3) and in chicks given injections of 3-MC (19). The presence in the fetus of these hydrocarbons given to the pregnant mothers was detected with the aid of radioactive chemicals (1, 28, 30). In our previous paper (28), it was shown that 3-MC given to pregnant mice near term appears to produce lung tumors in their fetuses, the hydrocarbon being finally excreted in the urine and feces \textit{in utero}. This indicates that polycyclic hydrocarbons may be metabolized at least in the fetal kidneys and liver. Generally speaking, aromatic hydrocarbons have been found to be metabolized by arylhydrocarbon hydroxylases in liver microsomes, and this may play a role in carcinogenesis as an inhibitor (32) or accelerator (8, 9) for the induction of proximate carcinogens. In the study of transplacental carcinogenesis or teratogenesis by these hydrocarbons it is, therefore, necessary to determine when and where the enzymes involved in detoxification and elimination of the carcinogens occur in the fetus.

MATERIALS AND METHODS

Polycyclic Hydrocarbons. 3-MC was obtained from Fluka AG Chemische Fabrik, Buchs, Switzerland, (CH-9470), and 1-hydroxy-3-MC, 2-hydroxy-3-MC, cis- and trans-1,2-dihydroxy-3-MC, 1-keto-3-MC, 2-keto-3-MC, and 11,12-dihydro-11,12-dihydroxy-3-MC were prepared in this laboratory, as described previously (27). \([6-14C]3\)-MC (5.47 mCi/mmol) and \([3H]3\)-MC (15 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. The latter was purified by column chromatography on Sephadex LH-20 in ethanol in this laboratory, and its final specific activity was 4.8 Ci/mmol.

Animals. DDD strain young adult male mice, weighing about 25 g at about 2 months of age, were used. All were raised in the Animal Center of Kyoto University. To obtain pregnant mice, 3 females were housed with a male overnight and the fetuses were considered to be at Day 0 of the pregnancy.
development when the vaginal plug was found the next morning.

**Treatment with Chemicals.** Male or pregnant female mice were given i.v. injections of 20 μCi (1.1 μg) of [3H]-MC or 1.0 μCi (48 μg) of [14C]-MC in 0.15 ml of a vehicle, bovine serum. The male mice were sacrificed at various intervals after treatment. Immediately after sacrifice, organs to be examined were removed. The intestine was subjected to extraction of 3-MC metabolites after irrigation with water to remove its contents, if any. The pregnant mice were sacrificed 5 hr later and their placentas and fetuses were immediately removed. Fetal organs were taken from fetuses on Day 18 of development and subjected to the same treatment. The fetal intestinal tracts were so delicate that irrigation was almost impossible. The tracts were found to be colored with bile, faintly on Day 16 and distinctly on Day 17.

In order to examine the possible transplacental transfer of conjugated metabolites, the 3H-labeled fractions were separated chromatographically from intestinal extracts of mice treated with 20 μCi of [3H]-MC 3 hr prior to sacrifice and then injected i.v. into pregnant mice, and the maternal and fetal tissues were taken 30 min later for examination.

**Radiometry.** Each organ was weighed immediately after removal, a part of the fresh tissue was digested in a tissue solubilizer (Packard Instrument Co., Inc., Downers Grove, Ill.), and its radioactivity was measured with a Nuclear Chicago liquid scintillation counter in a toluene-PPO-POPOP system. Specific radioactivity was calculated as dpm/100 mg of tissue.

**Column Chromatography.** Sephadex LH-20 was allowed to swell in ethanol and was packed in a column (gel bed volume, 8 ml). The tissue, 100 mg of each organ, was extracted with ethanol, and the extracts were evaporated to remove its contents, if any. The extract of 3-MC metabolites after irrigation with water to remove its contents, if any. The pregnant mice were sacrificed 5 hr later and their placentas and fetuses were immediately removed. Fetal organs were taken from fetuses on Day 18 of development and subjected to the same treatment. The fetal intestinal tracts were so delicate that irrigation was almost impossible. The tracts were found to be colored with bile, faintly on Day 16 and distinctly on Day 17.

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**TLC.** Silica gel (Merck HF-254, Merck, Darmstadt, West Germany) film (20 x 20 sq cm in size and 0.25 mm in depth) on a glass plate was activated at 110° for 30 min just before use. A test substance dissolved in ether was applied to the plate and developed 1-dimensionally with benzene or 2-dimensionally, 1st with benzene and subsequently with a benzene:ethanol (19:1) mixture. When standard metabolites from the feces or standard synthesized derivatives were added to the test substance in order to identify its constituents, the developed plate was illuminated under UV light and its fluorescent spots were removed for measurement of their radioactivity.

**Hydrolysis.** A portion of the various fractions obtained by Sephadex chromatography from ethanol extracts of the intestine was incubated with 0.02 ml of Helicase (L'Industrie Biologique Française, Gennevilliers, France) in 2.0 ml of acetate buffer (0.1 M, pH 4.5) at 37° for 3 hr. Helicase consists of β-glucuronidase and arylsulfatase. Otherwise, 0.3 ml of a substrate solution was incubated with 0.1 mg of β-glucuronidase Type I (Sigma Chemical Co., St. Louis, Mo.) in 0.5 ml of phosphate buffer (0.025 M, pH 6.8) at 37° for 30 min. For hydrolysis with arylsulfatase, 0.3 ml of the substrate was incubated with 0.1 ml of NaCl solution of bacterial arylsulfatase (0.1 mg) from limpets (Sigma Type 3) in 0.5 ml of sodium acetate buffer (0.2 M, pH 4.5) at 37° for 30 min. Acid hydrolysis was carried out in 1 N hydrochloric acid at 100° for 5 min or 65° for 4 hr.

**RESULTS**

**Column Chromatographic Studies of 3-MC Metabolites in Tissue.** Chart 1 shows column chromatographic profiles of 3-MC metabolites in tissues 1 and 3 hr after an i.v. injection of 20 μCi of [3H]-MC. Every tissue extract was found to contain 3 peaks, P1, PII, and PIII, which were presumed from their elution profiles and component analyses to correspond, respectively, with the peaks (F1, FII, and FIII)
observed previously in fecal extracts (27). PI was highest at early periods after injection. PII was low in kidney, liver, and lung and nearly negligible in brain and muscle. PIII was remarkable in liver and kidney. This peak in liver separated into 2 peaks, PIII-a and PIII-b; this was also true in kidney, where PIII-b was predominant. PIII, although it was small in quantity, was predominantly PIII-a in the lung, muscle, and brain. A group of peaks, appearing right after the void elution volume, was observed in the lung, liver, and kidney but not in the brain or muscle. This fraction with very fast mobility was not seen previously in either feces or bile, so these peaks are designated as fP complex in the present paper.

Chart 2 shows 3-MC metabolite peaks in the fetal body and placenta at the various developmental stages. Three fractions, fP complex, PI and PII complex, and PIII complex were also observed in all the extracts examined, and they are thought to be the same as those seen in adult tissues. The most remarkable change in the profiles with development was the increasing amount of PIII. The ratio of specific radioactivity of the liver to the residual corpus was constant at about 1.6 from Day 14 to Day 18 of gestation. In contrast, the ratio of the intestine to the residual corpus activity increased with development, ranging from about 1.2 on Days 14 and 15, 1.7 on Day 16, 2.2 on Day 17, to 4.7 on Day 18. This indicates the increasing excretion of 3-MC metabolites in the bile when the fetus approaches term, because the specific activity of the residual corpus was rather constant. As shown in the chart, PIII began to split into 2 peaks, PIII-a and PIII-b, on Day 17 and the separation became definite on the following day. The chromatographic profiles of the placentas appeared to be almost the same in all the developmental stages so far examined, with PI quite predominant.

Chart 3 illustrates 3-MC metabolite peaks in organs on the 18th day of development of fetuses from mice given i.v. injections of 20 μCi of [3H]3-MC 5 hr prior to sacrifice. PIII complex was observed, with predominant PIII-a in the lung and predominant PIII-b in the kidney. In the intestine, although contaminated with the bile, the peak split in 2 and the height of these peaks was quite remarkable.

**TLC Analyses of PI and PII Complex.** Two-dimensional TLC analysis of PI of the adult liver exhibited all the...
components described previously in fecal FI (27) as well as some others. cis- and trans-1,2-Dihydroxy-3-MC, 2-
dihydroxy-3-MC, 2-keto-3-MC, and intact 3-MC, which are all described as components of fecal FI, were detected. In addition, 1-hydroxy-3-MC and possibly 11,12-dihydro-11,12-dihydroxy-3-MC were new components. Other fluorescent and radioactive spots on TLC are still under investigation. Components of liver PII appeared to be the same in their mobility on TLC as did those of fecal FI, except that PII was sometimes contaminated with a small amount of the parent hydrocarbon.

In fetal tissue extracts, it was not easy to separate PII from PII, as may be seen in Charts 2 and 3. Therefore, PII and PIII complex in admixture was run on 1-dimensional TLC. Table 1 shows the data obtained. Coincidentally with the data in fecal FI and FI (27), dihydroxy compounds were detected in Fraction 1, monohydroxy compounds were detected in Fractions 2 and 3, ketones were detected in Fraction 5, and parent 3-MC was detected in Fraction 10. Fractions 9 and 10 might have been contaminated with some ketones. Parent 3-MC was the major component, and dihydroxy compounds, followed by monohydroxy compounds, came next in the body after removal of all visceral organs of fetuses on Day 18 of gestation. The other metabolites, if any, were small in amount. Chromatographic profiles of extracts of placenta from either 14-day-old or 18-day-old fetuses were similar to those of the residual body. In contrast, dihydroxy compounds were the major metabolites in the fetal liver and kidney and even in the fetal lung. A slight increase in the amount of monohydroxy compounds was also seen in these organs. The increment of these hydroxy compounds resulted in a significant decrease of the parent chemicals, as a matter of course. The whole-body extracts of fetuses on Day 18 reflected the metabolic pattern of fetal visceral organs, but those on Day 14 did not, an increase in the amount of monohydroxy compounds being characteristic in the fetus at the early stage of development.

Column Chromatographic Analyses of PII. Column chromatographic identification of PII subfractions, PII-a and PII-b, was carried out by examining a mixture of a test PII fraction with a standard PII. The standard was obtained from adult intestinal extracts. The PII fraction to be tested was prepared by labeling with 3H, and the standard PII was labeled with 14C. The application on chromatography of mixtures was of use not only in identifying but also in separating the PII subfractions. For example, as shown in Chart 4, the bile extract, which appears in chromatography as a well-defined single peak, revealed its subpeaks, PII-a and PII-b, in the mixture. The elution positions of PII or its subfractions fluctuated in every chromatogram. The reason for this is not yet known, but water content, if any, may be a factor in the changeable mobility of the hydrophilic fractions. Chart 4 indicates the occurrence of 2 subfractions in all PIII fractions examined. PIII-b was predominant in the kidney and bile and nearly equal in amount to PII-a in the liver. In contrast, PIII-b was minor in the lung and muscle and negligible in the brain.

PII from whole fetal bodies on Days 13 and 18 were examined in the same manner as above. As illustrated in Chart 5, PII of the whole fetal body on Day 13 was disassociated into 2 subpeaks, a large PII-a and a small PIII-b. The latter increased in amount towards term (Chart 5, Whole Body 18th Day). Chromatograms of each organ extract from fetuses on Day 18 of gestation are also illustrated in Chart 5. The ratio of PIII-b to PIII-a was low in the lung and liver and high in the kidney.

Transplacental Transfer of 3-MC and Its Metabolites. PIII-a and PIII-b in the adult intestine were collected from 4 mice treated i.v. with 3H-labeled 3-MC. The pooled PIII-a was given i.v. to a pregnant mouse on Day 18 of gestation and PIII-b was given on Day 17. The radioactivity in the maternal liver, placenta, and fetuses 30 min after injection is shown in Table 2. An immediate accumulation in the maternal liver of the PIII subfractions and the distribution

Table 1
Relative amounts of 3-MC metabolites in PI and PIII complex in the placenta, fetus, and fetal organs

The full TLC specimen from the starting point to the solvent front development was cut into 10 strips of the same width, and each strip was eluted in benzene to count its radioactivity. Therefore, Fraction 1 contains substances with Rf 0.0 to 0.1, Fraction 2 contains substances with Rf 0.1 to 0.2, and so on. Dihydroxy compounds migrate with Fraction I, monohydroxy compounds migrate with Fractions 2 and 3, and intact 3-MC migrates with Fraction 10.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fetus</th>
<th>Placenta</th>
<th>Whole Body</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Residual corpus</th>
<th>Placenta</th>
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<td>1</td>
<td>24.4</td>
<td>17.3</td>
<td>46.0</td>
<td>43.8</td>
<td>48.3</td>
<td>42.3</td>
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<td>19.5</td>
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<td>2</td>
<td>13.0</td>
<td>8.5</td>
<td>9.3</td>
<td>10.5</td>
<td>8.2</td>
<td>10.7</td>
<td>6.2</td>
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<td>2.5</td>
<td>3.8</td>
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<td>5</td>
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<td>2.4</td>
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<tr>
<td>6</td>
<td>0.8</td>
<td>0.3</td>
<td>0.8</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>1.3</td>
</tr>
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<td>0.5</td>
<td>0.6</td>
<td>0.0</td>
<td>0.2</td>
<td>0.6</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td>1.7</td>
<td>1.8</td>
<td>1.5</td>
<td>1.6</td>
<td>0.1</td>
<td>0.4</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>5.7</td>
<td>3.5</td>
<td>2.1</td>
<td>3.4</td>
<td>3.1</td>
<td>6.6</td>
<td>4.1</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>38.8</td>
<td>56.7</td>
<td>30.2</td>
<td>25.5</td>
<td>25.1</td>
<td>18.5</td>
<td>61.7</td>
<td>57.0</td>
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</tbody>
</table>
of radioactivity to the placenta and fetus were observed. The radioactivity of placentas was only one-tenth or less of the activity of maternal liver, and the radioactivity of fetuses was only one-fiftieth or one-hundredth of the activity of maternal liver. Chromatographic analyses showed that fP, P1, and PI, in addition to the PIII-a or PIII-b administered, were detected in the maternal liver, and fP, P1, and PI were detected in the fetus. Neither PI11-a nor PIII-b was demonstrated in the fetus in this experiment.

Enzymatic and Nonenzymatic Cleavage of PIII. In a preliminary experiment, the bile was shown to be highly susceptible to Helicase. Therefore, the susceptibility of PIII subfractions obtained from intestinal extracts to Helicase, Sigma Type I β-glucuronidase, and limpet arylsulfatase was examined. As shown in Table 3, PIII-b was susceptible to all these enzymes but PIII-a was not. A slight reduction of PIII-a after hydrolysis with Helicase may be due to contamination with susceptible PIII-b. Throughout these experiments, nonenzymatic breakdown was noted.

Acid hydrolysis of PIII-a in 1 N HCl at 100° for 5 min

Table 2
Distribution of radioactivity in pregnant mice given injections of [3H]-labeled PIII subfractions

<table>
<thead>
<tr>
<th>Fraction administered</th>
<th>Pregnant mouse total (cpm/mg)</th>
<th>Liver (cpm/mg)</th>
<th>% Placenta (cpm/mg)</th>
<th>Fetus (cpm/mg)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIII-a</td>
<td>14.2</td>
<td>34.2</td>
<td>3.9</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>P1</td>
<td>30.6</td>
<td>fP</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>PI1</td>
<td>PII</td>
<td>23.5</td>
<td>PI</td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>PI11a</td>
<td>PII11</td>
<td>46.0</td>
<td>PII</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PI11a</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>PIII-b</td>
<td>13.7</td>
<td>66.3</td>
<td>4.0</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>P1</td>
<td>4.1</td>
<td>fP</td>
<td>27.4</td>
<td></td>
</tr>
<tr>
<td>PI1</td>
<td>PII</td>
<td>18.6</td>
<td>PI</td>
<td>54.9</td>
<td></td>
</tr>
<tr>
<td>PI11b</td>
<td>PII11</td>
<td>74.1</td>
<td>PII</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PI11b</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>
resulted in a remarkable reduction of the fraction, as shown in Table 3. The hydrocarbon moiety of the breakdown products appeared in fP, P, and PII, the occurrence of P being the most conspicuous. The same treatment of PIII-b caused complete disappearance of this fraction and appearance of fP and PII. Prolonged treatment in acid of these PIII subfractions resulted in an increment of fP complex.

DISCUSSION

In a study by Sims (23), 3-MC was incubated with liver homogenates of the rat or mouse, and its metabolites were extensively investigated. Chromatographic and fluorometric analyses of these metabolites on TLC revealed that the ether-soluble fraction contained the parent 3-MC, its mono- and dihydroxy and ketone derivatives, K-region dihydrodiol, and some other metabolites. Conjugated metabolites were found in the aqueous fraction. As beautifully summarized by Falk et al. (6) in the case of benzo(a)pyrene, polycyclic hydrocarbons are generally considered to be metabolized mainly to hydroxy compounds in the animal body and are excreted in the bile and urine as conjugates with certain acids such as glucuronic, mercapturic, and sulfuric acids.

In the present study, the 3-MC metabolites detected in ethanol extracts of liver from mice treated with 3-MC were 1-hydroxy-3-MC and the 11,12-dihydrodiol, in addition to metabolites described in fecal extracts (27). In addition to these constituents of P, another chromatographic Peak PIII was ethanol soluble as well as water soluble and appears to consist of conjugated metabolites of 3-MC. PIII-b, 1 of 2 PIII subfractions, was susceptible in part to β-glucuronidase and in part to sulfatase, yielding derivatives appearing in PII, which we assume to be oxidized at 4 to 10 position(s) of the 3-MC nucleus, as postulated previously (27). Similar products appeared after nonenzymatic hydrolysis of this fraction. In contrast, PIII-a was resistant to enzymatic hydrolysis but susceptible to acid hydrolysis, resulting in mainly P and a small amount of PII. Prolonged treatment of PIII-a with acid resulted in further disruption of PII into fP complex. Information on the chemical nature of PIII-a is insufficient as yet.

The ratio of PIII-b to PIII-a was characteristic for each organ of adult mice. Up to the present, PIII-b has been predominant in the kidney and liver and minor in the brain, muscle, and lung, suggesting that the induction of conjugation is obligatory in excretory organs. In contrast, PIII-a-type conjugation occurs in organs not specialized for excretion. PIII-a-type conjugation was distinct in mice given injections of 3-MC but not in mice treated with 3,4-benzo(a)pyrene, as described in our previous paper (25).

Transplacental transfer to the fetus of chemical carcinogens given to pregnant animals has been suggested by the development of tumors in their offspring (15, 17, 24). Direct detection in the fetal tissue of chemicals injected in the mother has been possible radiochemically (1, 28, 30) and autoradiographically (25, 26, 28), although the metabolites were not distinguishable from the parent chemicals in these reports. The present studies not only confirmed the transplacental transfer but also established the presence in the fetus of metabolites as well as the parent chemicals.

On Sephadex chromatograms, all peaks from adult tissues were seen in fetuses on and after the 13th day of gestation. These findings led to the question as to whether these metabolites were generated in the fetus or transferred from the mother. After the administration of PIII to a pregnant mouse, breakdown products of this fraction were
detected in the maternal liver, placenta, and fetuses, the amounts measured by radioactivity decreasing markedly in this order. The conjugates could be recovered from the maternal liver but were never detected in the fetuses. Thus, parent 3-MC and its nonconjugated metabolites were transferable to the fetus but its conjugated ones were not, indicating that the conjugated metabolites in the fetus were generated in the fetus itself. Chromatographic analyses revealed that the PIII-a-type conjugation occurs generally in immature fetuses, and PIIIB-type conjugation, whose products appear to be glucuronides, sulfates, and some others, develops near term in the excretory organs such as liver and kidneys.

In some earlier studies (1, 7, 10), drug-metabolizing enzymes were reported to be absent or deficient in the fetus or newborn. However, recent studies (4, 16, 18, 31, 33) have agreed that these enzyme activities were detectable in the fetal liver and stimulated by pretreatment of animals with certain compounds. Glucuronide formation is mediated by a microsomal enzyme, glucuronyl transferase (11), which is reported to be deficient in newborn guinea pigs (2, 13) and mice (13, 14). However, Dutton (5), who also observed negligible enzyme activity in early fetal guinea pig liver, found increased activity towards full term. The present studies clearly demonstrated the indisputable occurrence of conjugated metabolites of 3-MC in mouse fetuses, indicating the presence of drug-metabolizing systems, including hydroxylases and conjugating enzymes, in fetal tissues.

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Chromatographic Analyses of 3-Methylcholanthrene Metabolism in Adult and Fetal Mice and the Occurrence of Conjugating Enzymes in the Fetus

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