The de novo biosynthesis of purine and pyrimidine precursors of nucleic acid requires folic acid cofactors in the form of tetrahydrofolate derivatives (8). The reduction of folate and dihydrofolate to tetrahydrofolate is catalyzed by folate reductase (dihydrofolate reductase) (15, 29). This enzyme is inhibited by very low levels of aminopterin or methotrexate (9, 18, 28). With folate as substrate, folate reductase can be measured in equivalents of drug by the titration of enzymatic activity with these folate analogs (26, 27).

In the methylation of deoxyuridime-5'-monophosphate by thymidylate synthetase to form thymidine-5'-monophosphate, N5, N10-methylenetetrahydrofolate is oxidized to dihydrofolate (5, 24). In order to reactivate and recycle the dihydrofolate, it must again be reduced to tetrahydrofolate (7). This coupled relationship of dihydrofolate reductase and thymidylate synthetase for deoxyribonucleic acid biosynthesis suggests that dihydrofolate reductase activity should be elevated in tissues with a high proportion of dividing cells. Elevated levels of thymidylate synthetase activity have been found in the chick embryo (14), regenerating rat liver (13), and hepatomas of the rat (14, 10) in association with increased mitotic activity. Silber et al. (23) have reported elevated levels of dihydrofolate reductase activity in the early stages of chick embryonic development. However, the distribution of folate reductase activity in tissues of adult members of a number of species (1) and the enzyme pattern of regenerating rat liver (17) are not associated with the mitotic index of a given tissue. The observation of elevated levels of folate reductase in white blood cells of patients with leukemia (2, 16, 20, 27) and in a few other neoplastic human tissues prompted further investigation of the level of activity in embryonic tissues of the rat and the human. The chick embryo was also re-examined to determine if the elevated level of activity reported by Silber et al. (23) could be correlated with an increased number of drug-binding sites.

MATERIALS AND METHODS

Folate reductase was assayed with folate as the substrate (26). Enzyme activity was expressed either as moles of product formed or, after titration of enzyme activity, in terms of mole equivalents of methotrexate required to titrate the enzyme (26). A high coefficient of correlation was observed in this laboratory (20) between the measurement of folate reductase activity by the conversion of folate to tetrahydrofolate, titration with methotrexate, and the method for converting tetrahydrofolate to N10-formyltetrahydrofolate (3). A quantitative enzymatic conversion of folate to an aromatic amine was observed with folate previously standardized by reduction with zinc (26). Zakrzewski has observed that 2-mercaptoethanol and ascorbate each form complexes with tetrahydrofolate that interfere with the diazotizable amine assay; this probably resulted in the difficulties reported by Huenne.

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Studies on Folic Reductase
II. Enzyme Activity of Embryonic Organs of the Chicken, Rat, and Human

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WITH THE TECHNICAL ASSISTANCE OF JOHN FINESILVER, KATICA TOMIC, AND DANNA REEDER

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SUMMARY

The level of folate reductase activity in the liver of the chick embryo was highest during the earlier stages of development. The enzyme level of human fetal liver was higher than that previously reported for the adult. Lower levels of enzyme activity were observed in fetal human lung, kidney, viscera, heart, and adrenal. The level of activity in the rat embryo liver was similar to the levels observed in the liver of the adult. Folate reductase activity in embryo liver does not uniformly parallel mitotic rate in the species examined and is suggested to have a permissive role in cell divisions.
CHART 1.—Titration of folate reductase activity in the presence of rate-limiting concentrations of nicotinamide adenine dinucleotide phosphate, reduced. The enzyme used in this study had been purified from rat liver. A 20% homogenate (gm/ml) in 0.01 M tris(hydroxymethyl)aminomethane, pH 7.0, was centrifuged at 20,000 x g for 25 min. The supernatant fluid was adjusted to pH 7.0 with 0.1 M NaOH, and the protein precipitating between 60 and 90% saturation with (NH₄)₂SO₄ was redissolved in a minimum of 0.01 M Tris(hydroxymethyl)aminomethane, pH 7.0, and dialyzed overnight against 6 liters of water.

CHART 2.—A, The supernatant fluid, centrifuged at 20,000 x g for 20 min, of an homogenate of rat liver was diluted with 0.01 M tris(hydroxymethyl)aminomethane, pH 7.0, to give the desired concentrations (gm/ml). Sixty μl of homogenate were incubated at 37°C as described under Chart 1 for 40, 80, 120, and 160 min, respectively. B, The enzyme dilutions in Chart 2A are plotted against the amount of methotrexate that was required to titrate the enzyme activity.
TABLE 1

<table>
<thead>
<tr>
<th>Days post-insemination</th>
<th>Placenta</th>
<th>Embryo</th>
<th>Embryonic liver</th>
<th>Maternal liver</th>
<th>Embryo minus liver</th>
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<td>7</td>
<td></td>
<td></td>
<td></td>
<td>12.8</td>
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<td>10</td>
<td>2.80b</td>
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<td></td>
<td>6.15</td>
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<tr>
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<td>2.52b</td>
<td>7.20</td>
<td></td>
<td>11.5</td>
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<td>3.71</td>
<td>7.11</td>
<td></td>
<td>8.68</td>
<td></td>
</tr>
<tr>
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<td>6.24</td>
<td></td>
<td>6.55</td>
<td></td>
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<td>15</td>
<td>2.63</td>
<td>5.75</td>
<td></td>
<td>7.23</td>
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</tr>
<tr>
<td>16</td>
<td>3.06</td>
<td>1.21</td>
<td></td>
<td>8.06</td>
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</tr>
<tr>
<td>17</td>
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<td>5.53</td>
<td></td>
<td>7.86</td>
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</tr>
<tr>
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<td>7.10</td>
<td>9.14</td>
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<tr>
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<td>8.25</td>
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<td>23</td>
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<tr>
<td>24</td>
<td>10.0</td>
<td></td>
<td>7.52</td>
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</tbody>
</table>

a The mmole equivalents of methotrexate/gm protein.

b Combined placenta and embryo.

c Seven days postpartum.

d Lactating rat.

homogenates were prepared. The placenta and embryos were pooled for assay on Day 10, 11, and 12 of gestation. On Day 13, a pooled litter of embryos was large enough for assay, although the pool of placentas was still inadequate. A pool of embryonic livers from most of the pups of a litter was inadequate for assay before the 17th day. The values reported for adult liver were for the animal from which the embryos were obtained (Table 1).

The value for folate reductase activity of the combination of rat placenta and embryo for Day 10, 11, and 12 of gestation was lower than the value obtained for either placenta or embryo on Day 13 or 14 when they were first assayed separately. The value for embryonic liver at Day 17 and thereafter was in the range found for the liver of pregnant animals. The values for the 1-week-old rat and for the liver of the rat after 1 week of lactation were in the range of values for normal animals.

Chick embryo liver.—The level of folate reductase in the liver of the chick embryo was higher at the earlier stages of the embryonic development than at hatching (Chart 3). Each point on the chart represents a pool of embryo livers. The 8- and 9-day values were obtained with a pool of livers from 36 embryos. The apparent turnover number/mole equivalent of methotrexate was very low for the chick embryo. Such a variation in turnover number was first observed by Werkheiser (25) in studies of the enzyme from different organs of the same animal.

Human embryo.—Tissues were obtained from 3 fetuses at the time of surgical intervention in the 13th, 15th, and 16th week of normal pregnancies. The homogenate of fetal liver was used to characterize the conditions for the assay of human tissues with folate as substrate (Chart 4). The conditions for the rat liver are optimal for the assay of human liver. The small size of other fetal organs prevented a similar check of the optimal conditions for these tissues. Lack of tissue did not permit drug titrations and restricted the assay of embryonic organs of the human to a measurement of the amount of product formed/mg/hr (Table 2). These results were obtained by incubating the homogenate with substrate for 2 hr. The reaction was linear for this length of time (Chart 5).

Enzyme activity was detectable in all tissues studied, in contrast to the observations with tissues from the adult. The level in human embryonic liver was more than twice the value observed for any other human organ and half the level of adult rat liver. The levels of enzyme activity in the 13-week fetus were approximately twice the values observed for the older fetus.
TABLE 2
FOLATE REDUCTASE ACTIVITY OF FETAL HUMAN ORGANS

<table>
<thead>
<tr>
<th>Organ (human)</th>
<th>Folate reductase activity*</th>
<th>13 weeksb</th>
<th>15 weeks</th>
<th>16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td>0.70</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td></td>
<td>e</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>e</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Viscera</td>
<td></td>
<td>1.55c</td>
<td>0.42</td>
<td>0.18</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>2.33</td>
<td>1.30</td>
<td>1.03</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>0.57</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Embryo</td>
<td></td>
<td>0.46d</td>
<td>0.53*</td>
<td></td>
</tr>
<tr>
<td>Adult rat liver</td>
<td></td>
<td></td>
<td>4.03</td>
<td></td>
</tr>
</tbody>
</table>

* The micromoles of tetrahydrofolate/mg protein/hr.
*b Age of fetus at surgical intervention.
*c The small size of the kidney and the adrenal precluded their assay as separate organs. They were, therefore, included with the other organs termed viscera.
*d Embryo minus the above organs and the left leg.
* Skin and muscle.

CHART 5.—Time curve of folic reductase activity with an homogenate of human fetal liver.

DISCUSSION

The measurement of enzyme activity in crude homogenates may be subject to many interfering reactions, e.g., variations of the pH, destruction of the substrates, and modification of the product. Removal of the interfering reactions by purification of an enzyme almost invariably leads to a loss of activity. With crude homogenates, maintenance of high levels of NADPH during the course of the reaction may become a serious problem, even when a NADPH-regenerating system is incorporated. Werkheiser observed this problem in the measurement of folate reductase with preparations of intestinal mucosa where levels of nicotinamide that resulted in a 40% inhibition of folate reductase from rat liver were required to obtain a linear reaction (25). The titration of enzyme activity is relatively independent of the concentration of NADPH when compared with other assays for folate reductase (3, 18).

The number of moles of drug/mole of enzyme will vary with the number of drug-binding sites on the enzyme (25). In the comparison of the level of enzyme activity in various organs, there is a tacit assumption that enzymes from all sources have the same number of drug-binding sites.

The levels of folate reductase activity reported here for adult rat liver are similar to those observed earlier by Werkheiser (25) and by Ngu et al. (17). A greater variation in the level of activity is observed with livers from pregnant rats than in earlier studies with male animals. This variation may arise from a factor related to pregnancy per se. In a previous study (17), we reported that regeneration did not increase the level of folate reductase activity in rat liver after partial hepatectomy. The present data indicate that the level of folate reductase in the embryonic liver of the rat is in the range observed for the normal adult organ. The level of folate reductase activity observed in the rat liver appears to be independent of the rate of cell division in these 2 situations. The enzyme level of the placenta and the embryo does not show a pronounced change with progression of the pregnancy.

The pattern of folate reductase activity in the liver of the chick embryo observed by Silber et al. (23) with a different method suggested a peak of enzyme activity around the 10th–12th day. The present study includes embryos at the 8th and 9th day of embryonic development and indicates that a high level of activity is also present at these earlier stages of development when growth of the liver is more rapid (22). The high levels of enzyme activity measured by drug titration suggest that a larger number of molecules of enzyme are present in earlier embryonic life and confirm the observations reported by these earlier workers using a different assay (23).

Folate reductase was detected in all of the fetal human tissues examined, although it was present only in trace amounts in several tissues. In normal human adult tissues, enzyme is rarely detectable by this assay, and when it is detected it is at a level of less than 0.1% of that in rat liver (19). Elevated levels of folate reductase were detected in a few types of malignant human cells, e.g., Wilms’s tumor and carcinoma of the cervix. White blood cells from patients with leukemia have also been reported to have elevated levels of enzyme activity (2, 16, 20, 27).

Although folate reductase, when it occurs in human tissues, is found in those tissues which show high mitotic indices, there appears to be no necessary relation between rate of cell division and enzyme level. In human and chick embryo liver, enzyme levels are higher than in the adult, but this does not seem true of rat liver at the earliest time measured, 17 days, and the low levels in the whole rat embryo before that stage do not suggest that enzyme levels in the liver are greatly elevated in the younger embryo.

The wide range of folate reductase values observed in tissues with high mitotic rates suggests that this enzyme may be “permissive” rather than rate limiting on cell division.

a Unpublished observations of D. Roberts and T. C. Hall.
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