Release of Folate by Rat Liver and Spleen Slices

By Erik M. Magnus, Hugh Dempsey, and C. E. Butterworth, Jr.

There is relatively little information concerning the transfer of folate between intracellular and extracellular compartments of various tissues. This is particularly true as far as red blood cells and plasma are concerned. Plasma folate appears to be somewhat more labile and subject to depletion under conditions of deprivation or increased needs, for example, in pregnancy and cancer1-3 while red cell folate seems to require a longer period for depletion. Although the red cell folate concentration is 10 to 20 times that of plasma, there appears to be little exchange between the two. Herbert and Zalusky have presented a limited amount of evidence4 which suggests that mature human red cells are incapable of incorporating synthetic PGA, while reticulocytes apparently retain this function. A decline in plasma folate has been observed within 24 to 48 hours after vitamin B-12 therapy of pernicious anemia, suggesting enhanced utilization of folate.5-7 However, pernicious anemia patients as well as a subject who was nutritionally deficient in both folate and vitamin B-12, did not display an upsurge in red cell folate until 5 to 7 days after vitamin B-12 therapy indicating that most folate is probably incorporated during the early stages of erythropoiesis in the marrow.8 The bulk of evidence indicates that red cell folate exists as a polyglutamate form of 5-methyl tetrahydrofolate, while the same compound circulates in plasma as the monogluttamate.8,10 Whitehead and Cooper11 have recently shown that unaltered synthetic pteroylglutamate is capable of crossing the intestinal mucosa and entering the portal venous plasma. It has been suggested that this compound enters the liver cells where it is converted to the reduced 5-methyl derivative and either stored or released for peripheral utilization. Chanarin and McLean12 have shown that parenteral injections of pteroylglutamate cause increased quantities of N5-methyl-THFA to appear in the serum and urine by displacement from tissue. In view of general interest in folic acid as a nutrient, and the use of folic acid antagonists as drug therapy in a wide range of disorders, the following studies were undertaken in order to examine the exchange of folate compounds between plasma and cells of liver, spleen and blood. The

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basic technic has consisted of measuring the folate activity of *L. casei* assays of supernatant fluid after incubation of tissue slices or red blood cells in vitro, under varying conditions. Appropriate allowances have been made for the folate content of additives in order to determine the size and rate of net changes in folate concentrations with the passage of time. The findings are believed to indicate that: (a) Folate is released from liver slices into the bathing solution by the action of endogenous and exogenous factors: (b) intact red cells are relatively inert as either donors or recipients of N^5^-methyl-THFA and (c) spleen slices release folate when incubated with an extract of chicken pancreas, but remove folate from plasma suggesting that folate is bound or utilized by splenic cells.

**METHODS**

Incubation experiments were carried out in three different incubation systems, as shown in the Tables below. The experiments were performed with homologous rat organ and blood from 17 male Sprague-Dawley rats, weighing 250 to 300 Gm. The rats, which had been maintained on a diet adequate in folic acid, were lightly anesthetized with ether and sacrificed by exanguination. Blood was withdrawn from the heart into a sterile heparinized syringe. Plasma was removed and the red cells washed twice by centrifugation in the cold with 0.9 per cent saline. Liver and spleen tissue was sliced into sections 1.0 to 2.0 mm. thick, placed on a waxed paper and weighed on an analytical balance. Portions were trimmed from the edges to provide the desired weight.

**Part I:**

Experiments were carried out on 6 separate occasions with homologous tissue and blood from 6 animals. The incubations were performed utilizing 50 ml. Erlenmeyer flasks, containing saline in different combinations with plasma, liver and red blood cells (see Table 1). The flasks, covered with parafilm and maintained aseptically, were incubated with frequent agitation by tilting, in a waterbath at 37 C. Folate content was estimated in the supernatants after 15, 60 and 255 minutes incubation, respectively. Folate estimations were performed on the sediment (red cells) from the flasks containing red cells (see Table 2).

**Part II:**

The incubation series for each of 6 rats was performed and handled as described above. The Erlenmeyer flasks contained liver or spleen slices in different combinations with saline, plasma and chicken pancreas conjugase (see Table 3). Folate estimations were performed in the supernatants after 15, 60 and 255 minutes, respectively.

Chicken pancreas conjugase was prepared according to the method of Mims and Laskowski, stored in small bottles in the frozen state, and thawed and diluted 1:50 immediately before use.

**Part III:**

The incubation series for this system consisted of liver or spleen slices in combination with different concentrations of plasma or chicken pancreas (see Fig. 1). The experiment was carried out on 5 separate occasions, with homologous tissue and plasma from 5 animals. Folate estimations were performed after 2 and 4 hours incubation. The total folate released after each incubation period, in all incubation systems, was calculated, including a correction for folate contained in samples removed for assay. Whenever plasma or chicken pancreas was added to, or was an integral part of the incubation fluid, their assayed folate values were subtracted. The red cells were washed twice in saline before plasma was added as a source of conjugase prior to assay. Supernatants as
Table 1.—Changes in Folate Activity of Supernatant Fluid After Incubation of Liver Slices with Plasma and/or Saline Solution, in the Presence and Absence of Red Blood Cells

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>Net Change in L. casei Activity of Supernate in ng. (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. In the absence of Red Blood Cells:</td>
<td></td>
</tr>
<tr>
<td>15 Min</td>
<td>60 Min</td>
</tr>
<tr>
<td>255 Min</td>
<td></td>
</tr>
<tr>
<td>Plasma-saline, 1:10 (blank)</td>
<td>5.5±0.6</td>
</tr>
<tr>
<td>Liver-saline</td>
<td>13.3±5.0</td>
</tr>
<tr>
<td>Liver-saline (+ plasma added after incubation)</td>
<td>18.1±3.7</td>
</tr>
<tr>
<td>Liver-saline (+ plasma added before incubation)</td>
<td>33.5±4.5</td>
</tr>
<tr>
<td>B. In the presence of Red Blood Cells:</td>
<td></td>
</tr>
<tr>
<td>18.0±2.9</td>
<td></td>
</tr>
<tr>
<td>37.4±2.5</td>
<td></td>
</tr>
<tr>
<td>110.7±17.6</td>
<td></td>
</tr>
<tr>
<td>Liver-saline</td>
<td></td>
</tr>
<tr>
<td>Liver-saline (+ plasma added after incubation)</td>
<td>29.1±3.7</td>
</tr>
<tr>
<td>Liver-saline (+ plasma added before incubation)</td>
<td>39.3±4.3</td>
</tr>
</tbody>
</table>

Values represent the folate activity released (mean ± S.E.M.) in nanograms per 0.1 Gm. of liver from parallel experiments on 6 rats using autologous rat liver, plasma, or red cells as indicated.

well as red cell samples were, after dilution with buffer, kept at 37 C. for 90 minutes before assay. All samples from one experiment were assayed in the same batch, and compared with standard curves prepared simultaneously.

The folate content of liver and spleen was determined in 20 rats of similar weight and from the same stock. This value was used to compare the total folate content of the liver and spleen, respectively, with the folate released during the incubation experiments. Liver and spleen tissue was homogenized and digested with chicken pancreas conjugase prior to assay with Lactobacillus casei.

The microbiological assay of the supernatants was carried out as reported for serum by Baker et al.15 with some modifications,16 using Lactobacillus casei (ATCC No. 7469). The estimation of red cell folate was carried out with activated hemolysates, as described by Grossowicz et al.17 The folate content of the supernatants is given as ng of total released folate per 0.1 Gm. of tissue, or as ng per 0.1 ml. when plasma or chicken pancreas was incubated alone.

**RESULTS**

The results from the experiments in Part I are given in Tables 1 and 2. Values represent the folate activity in nanograms (mean ± S.E.M.) released into the supernatant fluid per 0.1 Gm. of tissue, or per 0.1 ml. of liquid in the case of controls, or per ml. of red cells.

**Comment**

The total folate released from liver slices is increased approximately two-fold when plasma is present in the incubation system in comparison to incubation with saline only. Folate released from liver-saline incubation is only slightly influenced if plasma is added to the supernatant after incubation. The presence of red blood cells in the incubation system does not significantly alter the total released folate. (Red blood cells incubated with saline alone, or with saline plus plasma give only minimal release of folate to the supernatant).
RELEASE OF FOLATE

Table 2.—Failure of Red Cell Folate to Change After Incubation in the Presence of Folate Released from Liver Slices in vitro

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>15 Min</th>
<th>60 Min</th>
<th>255 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC - liver - saline</td>
<td>479±82</td>
<td>450±86</td>
<td>468±84</td>
</tr>
<tr>
<td>RBC - liver - plasma - saline</td>
<td>477±90</td>
<td>472±81</td>
<td>481±92</td>
</tr>
<tr>
<td>RBC - saline</td>
<td>475±98</td>
<td>458±89</td>
<td>449±72</td>
</tr>
<tr>
<td>RBC - plasma - saline</td>
<td>471±88</td>
<td>447±74</td>
<td>477±73</td>
</tr>
</tbody>
</table>

Red cells from the previous experiment (Table 1) were isolated, washed three times in saline, hemolyzed and assayed. Autologous plasma was added after hemolysis to accomplish deconjugation of bound forms. Note that although the red cells were exposed to high concentrations of folate (presumably N5-methyl tetrahydrofolate) no significant variation occurred in folate content within the cells.

Table 3.—Comparison of the Effects of Chicken Pancreas and Rat Plasma on the Release of Folate from Bound Forms in Rat Liver and Spleen

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>Net Change in ( L_\text{casei} ) Activity of Supernate in ng. (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver-saline</td>
<td>9.1± 3.1</td>
</tr>
<tr>
<td>Liver-saline *</td>
<td>12.7± 2.9</td>
</tr>
<tr>
<td>Liver-chicken pancreas-saline</td>
<td>8.0± 3.2</td>
</tr>
<tr>
<td>Liver-plasma-saline</td>
<td>17.6± 8.4</td>
</tr>
<tr>
<td>Liver-plasma-saline *</td>
<td>6.5± 6.1</td>
</tr>
<tr>
<td>Spleen-saline</td>
<td>4.8± 2.4</td>
</tr>
<tr>
<td>Spleen-saline *</td>
<td>3.9± 2.7</td>
</tr>
<tr>
<td>Spleen-plasma-saline</td>
<td>-3.4± 6.1</td>
</tr>
<tr>
<td>Spleen-plasma-saline *</td>
<td>-8.8± 7.8</td>
</tr>
<tr>
<td>Spleen-chicken pancreas-saline</td>
<td>9.2±11.1</td>
</tr>
</tbody>
</table>

Values represent the folate activity released (mean ± S.E.M.) in nanograms per 0.1 gm. of liver or spleen, corrected by subtraction of any added folate, from parallel experiments on 6 rats using autologous rat liver, spleen and plasma as indicated.

* Chicken pancreas extract was added to supernate only, in order to deconjugate any polyglutamate which might have been released during incubation.

findings suggest that rat plasma contains one or more factors which favor the release of folate from liver slices. The effect could be due in part to binding or transport proteins, since it seems to be different from endogenous releasing factors (presumably conjugases) which are normally present in liver. Plasma also seems to differ from chicken pancreas conjugase in its action on spleen (see below).

The red cell folate was unchanged whether the red blood cells had been incubated with liver-saline, liver-plasma, saline alone or plasma-saline.

The results from incubation experiments in Part II are given in Table 3.

Comment

Liver slices incubated with chicken pancreas or with plasma, gave significantly higher folate values in supernatants than when incubated with saline.
Fig. 1.—Net changes in folate activity (F.A.A.) for L. casei, in supernatant fluid after incubation of rat tissue slices with increasing concentrations of either rat plasma or chicken pancreas. Each bar represents the mean value of five replicate experiments in which 0.1 Gm. of rat liver or spleen was incubated. The bar to the left in each pair represents the mean value after two hours; the bar to the right represents the mean value after four hours. Final results have been corrected by subtracting the quantity of exogenous folate added in the form of either plasma or pancreas extract, thus accounting for the negative values.

only. While chicken pancreas and plasma have a similar action on liver, they differ markedly when exposed to splenic tissue. When spleen slices were incubated with plasma, a slight decline in folate values of supernates was observed, while slices incubated with chicken pancreas regularly showed an increase in folate value of supernates with the passage of time.

The results from incubation experiment Part III are given in Figure 1.

Comment

Incubation of liver with increasing concentrations of plasma in the incubation fluid produced a corresponding, almost linear, increased release of liver folate. In contrast, only minor increases in folate release occurred with higher concentrations of chicken pancreas in the incubation fluid. Thus 0.2 ml. of plasma was comparable in activity to 0.4 ml. of chicken pancreas extract diluted 1:50. Expressed in another way, the enzyme activity of rat plasma was roughly equivalent to a 1:25 dilution of chicken pancreas extract.

Splenic tissue incubated with plasma gave a net negative folate value after the first two hours, and a somewhat less negative value after the next two hours. No significant change occurred with increasing concentrations of plasma in the incubation system. Incubation of spleen with chicken pancreas, on the other hand, yielded more folate both with the passage of time and with increasing concentration of chicken pancreas in the incubation fluid.

Folate Content of Liver and Spleen

Folate content of liver tissue was found to be 6850 ng per Gm. with a range
of 3270 and 11250 ng per Gm. Folate content of spleen tissue was found to be 1970 ng per Gm.; with a range of 960 to 3460 ng per Gm. Using these values and the data in Table 3, it may be seen that liver slices released a maximum of 25 to 30 per cent of the folate present (198/685). Approximately 20 per cent of the total available folate was released from spleen slices in the presence of chicken pancreas (40/197).

Discussion

The data indicate that nearly twice as much folate is released from liver slices in vitro if plasma is present in the incubation mixture, than if plasma is added to the supernatant fluid after incubation. Chicken pancreas extract, a known source of polyglutamyl conjugase, exhibited a somewhat similar effect. Under the experimental conditions described, rat plasma was roughly comparable to a 1:25 (v/v) dilution of conventionally prepared pancreatic extract. These effects are thought to be directly related to release of bound folate forms from tissue, since neither agent caused appreciable enhancement of folic acid activity if added to supernates after incubation. Thus this relationship appears to be somewhat different from the mechanism of interaction between plasma and red cells as described by Toennies et al. These authors reported the release of inactive folate precursors by lysis of washed red cells; treatment of hemolysates with plasma brought about biologic activity in the assay system, presumably by converting polyglutamates to a shorter chain length which could be utilized by the assay organisms. In the experiments described above there was no evidence that biologically inactive, water-soluble folate is released from liver slices. It does not seem likely that an exogenous material such as chicken pancreas extract would induce the release of folate from intact liver cells. However it would be reasonable to expect that enzymes such as the conjugases present in either plasma or pancreas could react with bound forms of folyl polyglutamate on the surface of subcellular particles and membranes of injured cells. In this way folate could be released without necessarily going through an inactive polyglutamate phase. The findings suggest that approximately half of the folate available from liver exists in an insoluble form bound to cellular structures. It is possible that plasma also contains folate-binding proteins, similar to those described in milk by Ghitis in addition to deconjugating enzymes as described by Toennies et al.

The results in Table 1 are considered to indicate that plasma favors release of folate from liver slices and that this release is not influenced by the presence or absence of intact red blood cells. Under the conditions of this experiment the folate content of red cells did not increase, although the concentration of folate in the surrounding fluid rose as much as fifty-fold. Thus the red cell membrane seems to be impermeable to "natural" folate material during in vitro incubation, as well as to synthetic pteroylglutamic acid as reported by Herbert et al.

The net decline in folate activity of supernatant fluid after incubation of spleen slices with plasma contrasts sharply with results obtained with liver (Tab. 3; Fig. 1). Both tissues displayed increased release of folate to the
bathing fluid in the presence of chicken pancreas. These findings suggest a fundamental difference between chicken pancreas conjugase and plasma factors concerned with the release and transport of folic acid. Although there is no readily available explanation for these unexpected results, the data are compatible with the view that plasma contains a transport protein capable of receiving folate from the liver and relinquishing it at other sites. It must be borne in mind that the experiments were carried out under aerobic conditions which are quite different from the usual liver cell environment. Also changes in pH undoubtedly occur during the incubation period and could have significant effects on enzyme activity. Nevertheless it is interesting to speculate, that under aerobic conditions the liver appears to release folate while the spleen tends to bind, inactivate, or utilize folate. It is conceivable that this is part of a physiologic mechanism to salvage folate from red blood cells for subsequent return to the liver. On the other hand the spleen is an active lymphoid organ and the utilization of folate for metabolic activities and growth of surviving cells seems to be a somewhat more acceptable explanation for the observed results. The fact that folate flux into tissue appeared to diminish with time seems to favor the latter view. This observation is believed to merit further investigation in view of the effectiveness of folate antagonists in producing temporary remissions in certain forms of childhood lymphatic leukemia.

**Summary**

1. The effects of rat plasma and chicken pancreas have been appraised concerning their role in releasing bound folate from rat liver and spleen slices in vitro. Net changes in folate concentration of the bathing solution have been determined by bacteriologic assay employing *L. casei* as the test organism.

2. Both rat plasma and chicken pancreas extract enhance the release of folate from liver slices in comparison with saline controls. Rat plasma was as effective in liberating bound folate from liver as a 1:25 dilution of conventionally-prepared chicken pancreas extract. The evidence suggests that a considerable portion of liver folate exists in an insoluble form which is bound to cellular structures.

3. Mature red cells appeared to be impermeable to high concentrations of natural folate released from liver.

4. Spleen slices released folate to the supernatant fluid when incubated with chicken pancreas extract, although at levels approximately one-tenth as great as liver slices. When spleen slices were incubated for short periods with plasma, evidence was obtained favoring a flux of folate into surviving cells.

**SUMMARIO IN INTERLINGUA**

1. Le effectos de plasma de ratto e de pancreas de gallina esseva evaluate con respecto a lor rolo in le liberation de folato ligate ab segmentos de hepate e splen de ratto in vitro. Alterationes in le concentration de folato in le solution del banio esseva determinate per essayage bacteriologic con le uso de *L. casei* como organismo de experimentation.

2. Le plasma de ratti e extracto de pancreas de gallina promove le liberation de folato ab segmentos de hepate in comparation con solution salin. Plasma de ratto esseva tanto efficace in le liberation de folato ligate ab segmentos de hepate como un dilution de 1:25 de convenionalmente preparet extracto de pancreas de gallina. Le evidentia suggestiona
RELEASE OF FOLATE

que un considerabile portion de folato hepatic existe in un forma insolubile que es ligate a
structuras cellular.

3. Erythrocytos matur pareva esser impermeabile a alte concentrationes de folato natural
liberate ab hepate.

4. Segmentos de splen liberava folato ad in le fluido supernatante quando illos esseva
incubate con extracto de pancreas de gallina, ben que a nivellos approximativemente un
decimo de illo trovate con segmentos de hepate. Quando le segmentos de splen esseva in-
cubate durante breve periodos de tempore con plasma, evidentia esseva trovate que sup-
porta le these de un fluxo de folato ad in le cellulas supervivente.

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