DETERMINATION OF FOLIC ACID AND CITROVORUM FACTOR IN ANIMAL TISSUE*  

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Much work has been done on the liberation of bound folic acid from tissues of animal origin. In general, there exist in animal tissues two distinct conjugase systems. One has an optimum pH around 7 and is present in such tissues as rat liver (1–3) and chicken pancreas (2, 4, 5). The other has a pH optimum around 4.5 and is present in a wider range of tissue. Hog kidney is a notable example (4, 6, 7).  

Upon the demonstration that the Leuconostoc citrovorum factor stimulated the growth of both Streptococcus faecalis R and Lactobacillus casei, the two organisms generally used in the microbiological assay of folic acid, and in view of the structural and metabolic similarities of these two substances, it became apparent that the whole field must be reinvestigated in respect to the components of the total liberated microbiological activity before further studies could be undertaken on the metabolic functions of these components. This paper deals with a comparison of the total L. citrovorum and S. faecalis activity released by autolysis from various animal tissues.  

EXPERIMENTAL  

S. faecalis activity (FA) was determined with S. faecalis R and the medium of Luckey et al. (8). L. citrovorum activity (CF) was determined with the medium of Sauberlich and Baumann (9) with L. citrovorum 8081 as the test organism. An 18 hour turbidimetric assay was employed in all cases.  

Disodium phosphate-citric acid buffers (MacIlvaine) were employed throughout. In general the following procedure was used: The animals...
were decapitated and bled, and the tissues were removed immediately, rinsed in ice water, blotted, cut into small segments, and dropped into a dry ice-acetone mixture. The frozen tissues were then weighed and homogenized in 4 times their weight of cold distilled water in a Potter-Elvehjem homogenizer (10). 1 ml. of homogenate was then added to 10 ml. of buffer, covered liberally with toluene, and incubated at 37° for 22 hours. After incubation, the pH was adjusted to around 7 with 5 N KOH. The samples were then boiled for 5 minutes in a boiling water bath, cooled, diluted to volume, filtered, and assayed. In the determination of the free CF and FA, the frozen homogenate was added to the buffer at pH 7.0 and boiled immediately. In the incubation studies the procedure was the same except that the freezing step was eliminated. In all cases, duplicate determinations were made on each animal. Stock animals maintained on a typical stock ration were employed.

Results

Small amounts of free FA were found in the livers of three different species (Table I). On the other hand, at the levels assayed, no free CF was found. Thus it appears that in liver tissue all the measurable CF is bound. Values for FA and CF obtained upon autolysis of liver tissue from three different species are presented in Table II. The optimum pH for maximum release of FA was found to be 7.0, 4.5, and 4.5 for the rat, the chick, and the guinea pig, respectively. The optimum pH for release of CF was found to be 4.5 and 5.5 for the chick and the guinea pig, respectively. However, in the case of the rat two peaks were obtained, the one at pH 4.5 and the other at pH 6.0. These results clearly show that under normal conditions both FA and CF are present in the livers of all three species. Since good recoveries of both FA and CF can be obtained and since, under the conditions of macerative blending employed throughout, little conversion of FA to CF occurs,¹ it may be assumed that the observations reported here are not due to conversion of FA to CF.

Studies on the time of incubation necessary for maximum release of CF are shown in Table III. In the case of both the rat and the chick there was a sharp increase in activity during the first 2 hours. This was in turn followed by a slower increase of activity, which leveled off around 22 hours. After 30 hours there was a sharp decrease in activity.

The tissue concentrations of FA and CF were determined for various tissues of the guinea pig, rat, and chick, with use of the optimum pH for release. These results are presented in Table IV. Maximum release of FA occurred at either pH 4.5 or 7.0, depending on the tissue and the spe-

¹ Unpublished data from this laboratory.
cies concerned. On the other hand, the optimum pH for maximum release of CF was found to occur at 6.0 or 4.5, depending, as in the case of FA, on the species and the tissue involved.

**Table I**

*Free Folic Acid and Citrovorum Factor in Livers of Various Species*

All values are averages of three different animals run in duplicate.

<table>
<thead>
<tr>
<th>Species</th>
<th>FA, per gm. fresh weight</th>
<th>CF, per gm. fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>0.24</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.18</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Chick</td>
<td>0.18</td>
<td>&lt;0.006</td>
</tr>
</tbody>
</table>

**Table II**

*Effect of pH on Concentration of S. faecalis and L. citrovorum Activity in Liver Tissue*

The values are in micrograms per gm. of fresh weight and are averages of four different animals run in duplicate.

<table>
<thead>
<tr>
<th>pH</th>
<th>Rat</th>
<th>Chick</th>
<th>Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA</td>
<td>CF</td>
<td>FA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>0.60</td>
<td>0.28</td>
<td>10.12</td>
</tr>
<tr>
<td>5.0</td>
<td>0.58</td>
<td>0.20</td>
<td>3.95</td>
</tr>
<tr>
<td>5.5</td>
<td>0.72</td>
<td>0.20</td>
<td>3.80</td>
</tr>
<tr>
<td>6.0</td>
<td>0.80</td>
<td>0.32</td>
<td>1.80</td>
</tr>
<tr>
<td>7.0</td>
<td>1.72</td>
<td>0.20</td>
<td>1.35</td>
</tr>
<tr>
<td>8.0</td>
<td>1.30</td>
<td>&lt;0.01</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**Table III**

*Effect of Time of Incubation on Release of Citrovorum Factor from Liver Tissue*

The CF values are averages of two animals run in duplicate and are expressed in micrograms per gm.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr.</td>
</tr>
<tr>
<td>Chick, pH 4.5</td>
<td>0.20</td>
</tr>
<tr>
<td>Rat, pH 6.0</td>
<td>0.15</td>
</tr>
</tbody>
</table>

The concentration of CF followed that of the FA activity. The largest amounts were found in the liver. Smaller concentrations were found in other tissues in the following decreasing order of activity: kidney, spleen, pancreas, and muscle.

*Enzymatic Release of CF*—Taka-diastase has long been used to release
FA from animal tissues (11). However, only a slight increase of CF was observed when boiled liver tissue was incubated with taka-diastase. Pep-​sin and trypsin were also found to be ineffective in increasing the CF activity of animal tissues.

**Bioautographic Studies**—By means of the procedure of Winsten and Eigen (12), modified by increasing the time of development to 50 hours, bioautographic studies were carried out on extracts of liver tissues incubated at the optimum pH for maximum release of CF. In all three species, rat, chick, and guinea pig, the CF released by autolysis was identical; *i.e.*, the same *R*<sub>F</sub> values were obtained regardless of whether the optimum pH was 4.5 or 6.0. These values in turn correspond very closely to those obtained with the CF standard (Leucovorin, Lederle). Furthermore, since only one spot appeared when synthetic CF was added to the tissue extract, it can be assumed that the active substances in the extract and the standard are identical or so closely related that separation could not be obtained by the methods employed.

**DISCUSSION**

The knowledge that FA occurs naturally, for the most part in conjugated forms, *i.e.* tri- and heptaglutamates, has led to speculation that CF may occur similarly. The observation that maximum release of CF, in many animal tissues, occurs at a pH different from that at which maximum release of FA takes place, strongly suggests that in these cases the CF moiety is bound in a manner different from that of FA. Thus, data re-
ported here indicate the possible existence of two general forms of bound CF, the one being released at a pH different from that of the tissue FA and the other being released at the same pH as the FA present.

Preliminary data are presented which show that the measurable liver CF released at various H+ ion concentrations is identical or similar to synthetic CF (Leucovorin, Lederle). This demonstrates that, under the conditions employed here, the measurable CF activity released from liver tissue by autolysis exists as one component and is very closely related to, if not identical with, synthetic CF.

SUMMARY

1. Maximum release of CF was obtained by autolysis at pH 4.5 or 6.0, depending on the tissue analyzed. The optimum incubation time was found to be 22 to 30 hours.

2. No measurable quantity of CF was obtained when the tissues were frozen immediately upon removal. However, small quantities of FA were observed.

3. The CF released from various liver tissues at various H+ ion concentrations was found, by means of chromatographic analysis, to be similar if not identical to synthetic CF. Under the conditions employed only the one active component was observed.

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