THE EFFECT OF ASCORBIC ACID ON THE CITROVORUM FACTOR-LIBERATING ENZYME OF CHICK LIVER*

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In a recent report from this laboratory (1) results were presented showing that dried brewers' yeast contains a citrovorum factor (CF) largely in bound form, and that this bound CF can be released by an enzyme in hog kidney which is strikingly similar, if not identical, to hog kidney folic acid conjugase. These results showed further that the CF-liberating enzyme of hog kidney, under the conditions used, was activated by cysteine and to a lesser extent by hydrogen sulfide, but not by ascorbic acid or other reducing agents.

However, when the experiments were extended to a study of the CF-liberating enzymes of chick liver, it was found that these livers contained a relatively high level of CF in bound form and that ascorbic acid acted as an activator of the CF-liberating enzyme in chick liver.

Nichol and Welch (2) reported an increase in CF upon incubation of folic acid and ascorbic acid in the presence of rat liver slices. These results have been interpreted to indicate that ascorbic acid aids in the conversion of folic acid to CF. Dietrich, Monson, and Elvehjem (3) also obtained results which have been taken to indicate that vitamin B12 and ascorbic acid are concerned in the conversion of folic acid to CF in chick liver.

It is the purpose of this report to show that the major effect of ascorbic acid when incubated with chick livers is to aid in the release of CF from a bound form in the liver rather than to aid in conversion of folic acid to CF.

EXPERIMENTAL

Single comb white Leghorn chicks of mixed sex, the progeny of hens fed an all-plant ration, were used in these studies. The chicks were housed in heated batteries with raised wire floors. Feed and water were supplied ad libitum. The chicks were wing-banded and placed on the experimental diet at 1 day of age. The diet consisted of crude casein 20, corn-starch

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ASCORBIC ACID AND CF-LIBERATING ENZYME

64, gelatin 5, cellophane 3, hydrogenated vegetable fat (Primex) 2.5, KCl 0.6, dicalcium phosphate 0.5, NaCl 0.5, MgSO₄ 0.25, methionine 0.3. All the known vitamins and required trace minerals were added in adequate amounts, including 2 γ of vitamin B₁₂ and 100 γ of folic acid per 100 gm. of diet.

The chicks were sacrificed at about 4 weeks of age and the livers were removed for use in the incubation studies. In order to insure adequate amounts of vitamin B₁₂ in the livers, the chicks were given 20 γ of vitamin B₁₂ by pipette 15 hours before they were killed.

Citrovorum factor activity was measured with Leuconostoc citrovorum, ATCC 8081, with the basal medium and techniques described earlier (1). Leucovorin¹ was used as the standard.

RESULTS AND DISCUSSION

Bound Citrovorum Factor in Chick Liver—In order to ascertain whether CF occurred in the conjugated form in chick liver, a sample of chick liver was incubated with a preparation of hog kidney enzyme solution which was known to release CF from its conjugated form in yeast.

The chick liver sample was prepared by steaming a portion of the liver for 30 minutes immediately upon removal of the liver from the chick. The liver was then homogenized in distilled water in a Waring blender. The solid material was removed by centrifugation at 3000 r.p.m. and the supernatant liquid used as the substrate in the test for conjugated CF. The hog kidney enzyme solution was prepared by the method of Hill and Scott (1) from kidney obtained from a freshly slaughtered hog.

The equivalent of 10 mg. of liver was incubated with the equivalent of 25 mg. of hog kidney. The incubation was carried out in 50 ml. acetate buffer, pH 4.5.

The results of this study, presented in Table I, show that a manyfold increase in the free CF content of chick liver occurred, indicating that the CF of chick liver, as the CF of dried brewers' yeast, exists largely in conjugated form.

Effect of Ascorbic Acid upon Citrovorum Factor Content of Chick Liver Incubation Mixtures—In view of the findings by Nichol and Welch (2) and Dietrich et al. (3) that more CF appears when liver is incubated with ascorbic acid than is measured when the liver is incubated alone, an experiment was conducted to determine whether or not added ascorbic acid would cause an increased release of CF from the chick livers in use here. The results of several incubation studies showed that, whereas the CF content of the liver when incubated alone was very low, a marked increase in CF

¹ Kindly supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.
occurred when 10 mg. of the liver were incubated in the presence of 50 mg. of ascorbic acid at pH 7.0. When ascorbic acid was incubated with the liver, the levels of CF were of the same magnitude as those produced previously by incubation of chick liver with hog kidney.

On the other hand, when the liver was steamed for 30 minutes before the incubation was carried out, much less CF appeared, whether or not ascorbic acid was added. These results indicate that an enzyme, largely inactivated by steaming, is involved in increasing the CF content of chick liver when ascorbic acid is added.

Citrovorum Factor-Liberating Enzyme in Chick Liver—In order to obtain proof that the liver contained a CF-liberating enzyme, the liver enzyme preparation was incubated with a yeast extract known to contain conjugated CF. The yeast extract was prepared by steaming 5 gm. of dried brewers' yeast for 30 minutes in 100 ml. of distilled water. The solid material was then centrifuged at 3000 r.p.m. The clear supernatant liquid

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>CF per gm. liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick liver substrate (steamed), 10 mg.</td>
<td>200</td>
</tr>
<tr>
<td>Hog kidney enzyme, 25 mg.</td>
<td>0</td>
</tr>
<tr>
<td>&quot; &quot; + steamed liver substrate</td>
<td>6600</td>
</tr>
</tbody>
</table>

TABLE I

Release of CF from Liver

Failure of Folic Acid to Increase Citrovorum Factor in Chick Liver—It appears from the above results that chick liver contains a CF-liberating enzyme which is activated by ascorbic acid. However, the possibility exists that the increase in free CF obtained upon the addition of ascorbic acid to the liver incubation mixture was due to the conversion to CF of folic acid present in the liver or in the yeast. An experiment was conducted, therefore, to determine whether or not folic acid is converted to CF by chick liver under the conditions of these experiments. In this study fresh chick liver was cut in two immediately upon removal from the chick. Half of the liver was dropped into boiling water and heated for 5 minutes.
This portion was then made up as a source of substrate as previously described. The other half of the liver was prepared as a source of the enzyme.

The equivalent of 10 mg. of the liver enzyme was incubated alone and with the equivalent of 100 mg. of steamed liver as a source of the substrate. These were also incubated with ascorbic acid with and without the addition of folic acid. The results are presented in Table III.

In this experiment the addition of folic acid did not cause an increase in

\[
\text{TABLE II} \\
\text{Liberation of CF from Yeast Extract by Chick Liver in Presence and Absence of Ascorbic Acid}
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<table>
<thead>
<tr>
<th>Incubation mixtures</th>
<th>CF content (μg/ml)</th>
<th>CF released (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick liver enzyme, 10 mg.</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Dried brewers' yeast extract, 25 mg.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chick liver enzyme + dried brewers' yeast</td>
<td>315</td>
<td>115 (from yeast)</td>
</tr>
<tr>
<td>&quot; &quot; &quot; + ascorbic acid</td>
<td>840</td>
<td>640 (&quot; liver)</td>
</tr>
<tr>
<td>&quot; &quot; &quot; + &quot; &quot; + dried brewer's yeast</td>
<td>2190</td>
<td>1350 (&quot; yeast)</td>
</tr>
</tbody>
</table>

\[
\text{TABLE III} \\
\text{Failure of Folic Acid to Increase CF Content of Chick Liver}
\]

<table>
<thead>
<tr>
<th>Incubation mixtures</th>
<th>CF content (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick liver enzyme, 10 mg.</td>
<td>45</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 10 &quot; + chick liver substrate, 100 mg.</td>
<td>38</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 10 &quot; + ascorbic acid, 50 mg.</td>
<td>300</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 10 &quot; + &quot; &quot; 50 &quot; + folic acid, 5 γ.</td>
<td>195</td>
</tr>
<tr>
<td>Chick liver enzyme, 10 mg. + liver substrate + ascorbic acid</td>
<td>465</td>
</tr>
<tr>
<td>&quot; &quot; &quot; 10 &quot; + &quot; &quot; + &quot; &quot; + &quot; &quot; + folic acid</td>
<td>435</td>
</tr>
</tbody>
</table>

CF content of the incubation mixtures. On the other hand, the addition of ascorbic acid increased the CF content of the liver enzyme solution alone, and further increased the CF content when liver enzyme was incubated together with liver substrate.

These results indicate, therefore, that the primary rôle of ascorbic acid in increasing the CF content of chick liver homogenates is due to activation of the enzyme which liberates CF from a bound form present in the liver. The results of Nichol and Welch (2), Dietrich et al. (3), and more recently of Broquist, Stokstad, and Jukes (4) indicate that ascorbic acid also acts in some way in the conversion of folic acid to CF \textit{in vivo} and in rat liver.
but further studies on this possibility should take into account the effect of ascorbic acid in activating the CF-liberating enzyme.

The mechanism through which ascorbic acid acts in the activation of the CF-liberating enzyme is unknown. The activity of many enzymes is increased by the addition of reducing agents. Mims et al. (5) showed that hog kidney folic acid conjugase could be reactivated by ascorbic acid following inactivation by various conjugase inhibitors. Hill and Scott have shown that activation of hog kidney folic acid conjugase (6) and the CF-liberating enzyme of hog kidney (1) by cysteine is probably due to the transfer of a sulfhydryl group to an inactive form of the enzyme, thereby rendering it active. It appears possible that chicken liver might contain some free cysteine or cystine which is not present in hog kidney. However, under conditions favoring oxidation of the sulfhydryl group the enzyme may be inactive. Ascorbic acid, due to its reducing properties, could activate the enzyme either by reducing any cystine present to cysteine or by restoring the enzyme to the sulfhydryl form.

**SUMMARY**

1. Chick liver contains a conjugated form of the *citrovorum* factor.
2. Chick liver also contains a *citrovorum* factor-liberating enzyme.
3. The *citrovorum* factor-liberating enzyme is activated by ascorbic acid.
4. The increase of CF in chick liver homogenates incubated with ascorbic acid is apparently due to the release of CF from a conjugate.
5. No increase was obtained in the CF content of chick liver homogenates when folic acid was added to the incubation mixtures in the presence of ascorbic acid as compared with incubation mixtures to which ascorbic acid was added alone.

**BIBLIOGRAPHY**

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