High-Molecular-Weight Folates of Rat Liver

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It has been frequently claimed that polyglutamate forms of folates are found in animals (Noronha & Silverman, 1962; Shin et al., 1972; Brown et al., 1974) on the basis of column chromatography of these folates and chromatography of 'authentic' standards and/or microbiological assay before and after conjugase treatment. The present communication describes attempts to identify folate polyglutamates in normal rat livers.

Two types of columns are normally used to separate and identify folate polyglutamates: a column of Sephadex G-15, on which folate polyglutamates leave the column close to the void volume and in a smaller volume than authentic folate monoglutamate markers, or anion-exchange columns, on which folate polyglutamates are reported to be eluted from the column at higher ionic strength than folate monoglutamates.

In our experiments, normal Wistar rats (150g body wt.) received oral doses of [2-14C]folic acid (2μCi; 75μg/kg). Animals were killed 48h after administration of the dose, and the livers removed immediately and treated to prevent breakdown of folate polyglutamates by conjugase by placing finely divided liver pieces into boiling aqueous buffers (pH7.0, 9.0 and 11.0) containing suitable antioxidants (Shin et al., 1972; Brown et al., 1974; J. M. Scott, personal communication). Liver extracts were subjected to gel-filtration chromatography on columns of Sephadex G-15. On all chromatograms a large peak of radioactivity which left the column in an effluent volume slightly greater than the void volume was observed. This peak travelled in front of 4a-hydroxy-5-methyltetrahydrofolic acid (Blair et al., 1975), which is the first folate monoglutamate to leave a Sephadex G-15 column.

The same liver extracts were then subjected to chromatography on a DEAE-cellulose column (Whatman DE52). Liver extracts were diluted to give the same conductivity as the starting buffer before being loaded on to the column. Radioactive material was eluted from the column by using a 0-1.5M gradient of NaCl in phosphate buffer, pH7.0. Three peaks of radioactivity eluted from the DEAE-cellulose columns. The two major peaks co-chromatographed with authentic 5-methyltetrahydrofolinic acid and folic acid, a third minor peak remained unidentified. Elution of DEAE-cellulose columns with NaCl gradients up to 3M failed to produce any peaks at high ionic strength, as did chromatography at pH6.0. Rechromatography of the peak from the Sephadex G-15 column on DEAE-cellulose gave similar results. As it was possible that the high-molecular-weight folate was breaking down on the DEAE-cellulose column, the peak from a DEAE-cellulose column was rechromatographed on a column of Sephadex G-15. Chromatograms showed that the radioactivity was resolved into three peaks, one of which was eluted in the same position as 4a-hydroxy-5-methyltetrahydrofolinic acid and another just after the void volume.

Several groups of workers have used DEAE-Sephadex chromatography to demonstrate polyglutamates. We therefore chromatographed liver extracts on DEAE-Sephadex at pH7.0 by using a 0.3-1.5M gradient of NaCl. The liver extract separated into two fractions, neither of which corresponded to authentic monoglutamates, but both of which were eluted from the column in the same region as folate monoglutamates. The lysosomal enzyme conjugase hydrolyses the peptide links in polyglutamates to produce monoglutamates. This hydrolysis is said to be very rapid. Placing livers in a boiling pH7.0 buffer should effectively prevent breakdown of folate polyglutamates. However, Scott (J. M. Scott, personal communication) suggested that conjugase may not be deactivated under these conditions at pH7.0, that its action is sufficiently fast for breakdown of folate polyglutamates to occur and therefore that pH9.0 or 11.0 might be more appropriate for the extraction procedure. Extraction of liver folates with boiling buffer at pH9.0 or 11.0 failed to reveal the presence of polyglutamates. In both cases chromatograms showed peaks of radioactivity that were eluted on DEAE-cellulose at the same ionic strength as folate monoglutamates.
Previous work demonstrated that the $^3$H label in $[3',5',9-^3$H]folic acid exchanges in the rat (Barford & Blair, 1975) and that DEAE-cellulose chromatograms of liver extracts from rats receiving $[^3$H]folic acid showed the anticipated peaks at high tube number, which did not appear when $[^1$C]folic acid was administered (Beavon, 1973).

We have been able to demonstrate the presence of high-molecular-weight folates in extracts of livers and tumours by using Sephadex G-15 chromatography. DEAE-cellulose chromatography did not show peaks at high ionic strength as reported for folate polyglutamates. A folate polyglutamate would be more highly charged than a monoglutamate and should be eluted from the DEAE-cellulose column after the monoglutamates. This does not happen with the high-molecular-weight folates obtained from livers of rats receiving oral doses of $[2-^{14}$C]folic acid. The experiments reporting the presence of folate polyglutamates in the literature have nearly all used $[3',5',9-^3$H]folic acid as a marker for polyglutamate formation. Osborne-White & Smith (1973) reported the presence of peaks at high ionic strength on DEAE-cellulose in extracts of livers from sheep after giving large doses of $[2-^{14}$C]folic acid over several days. Lavoie et al. (1974) reported that $[5-^{14}$C]methyltetrahydrofolate did not form polyglutamates in lymphocytes, and used the absence of peaks at high ionic strength on DEAE-cellulose chromatography to support this claim, but reported the presence of polyglutamates in lymphocytes after $[^3$H]folic acid incubation.

Our work has failed to produce conclusive evidence for the presence of folate polyglutamates in rats. We have demonstrated the presence of a high-molecular-weight form of folate in rats, but it does not seem to have the necessary charge for a polyglutamate. It is possible that the high-molecular-weight fraction contains other amino acids, resulting in a net charge that is little different from the charge on 5-methyltetrahydrofolate, and that the 'peaks at high tube number' reported by other workers are artifacts caused by using $[^3$H]folic acid of very high specific radioactivity.


Hydrolytic Rupture of Ascorbate by Adenosine 3':5'-Cyclic Monophosphate Phosphodiesterase*

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Ascorbic acid is a γ-lactone which on hydrolytic rupture should give the corresponding acid, thus:

\[
\begin{array}{c}
\text{CH}_2\text{OH} \\
\text{HOCH} \\
\text{H} \\
\text{OH} \\
\text{O} \\
\text{O} \\
\end{array}
\]

Ascorbate

\[
\begin{array}{c}
\text{CH}_2\text{OH} \\
\text{HOCH} \\
\text{H} \\
\text{OH} \\
\text{O} \\
\end{array}
\]

Delactonized ascorbate

* Not presented at the Meeting, owing to the death of Dr. Lewin.

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