His-Glu-Ala-Leu-($His_2$,Asp,Tyr)-(Thr,Glu)-Lys

$T_1C_3$ $T_1C_1$ $T_1C_2$

*Peptide* $T_1C_3$. The first step of the Edman degradation showed PTH-His (45%) and the second step showed PTH-Glu (40%). *C*-Terminal leucine was found by hydrazinolysis and its sequence is:

His-Glu-Ala-Leu

in agreement with the *N*-terminal sequence of peptide C–5.

*Peptide* $T_1C_1$. The first step of the Edman degradation showed PTH-His (72%) and the second step showed PTH-Asp (36%). *C*-Terminal tyrosine was determined by digestion with carboxypeptidase A and hydrazinolysis. Therefore its sequence is:

His-Asp-His-Tyr

*Peptide* $T_1C_2$. Its *N*-terminal residue was found by Edman degradation to be threonine (90%) and its sequence is therefore:

Thr-Glu-Lys

Another Edman step was not attempted since it was found to contain glutamine, which might cyclize to the pyrrolidone.

Since each one of the above three peptides contains only 1 glutamic acid or aspartic acid residue and they do not contain serine, it was possible to determine if the glutamic acid or aspartic acid is amidated by analysing the amino acids released from the isolated peptides after digestion with leucine aminopeptidase. Peptide $T_1C_2$ has glutamine, peptide $T_1C_1$ has asparagine and peptide $T_1C_3$ has glutamic acid.

The summary of these results gives the following sequence for the C–5 peptide, which is probably bound to the rest of the heavy chain through methionine:

(. . . Met)-His-Glu-Ala-Leu-His-Asp($NH_2$)-His-Tyr-Thr-Glu($NH_2$)-Lys-Ser-Ile-Ser-Arg-Ser-Pro-Gly

Considering the high recovery of this peptide from the heavy chain it can be concluded that in rabbit $\gamma$-globulin, which is prepared by a chromatography on DEAE-Sephadex, both heavy chains have predominantly the same unique sequence at their *C*-terminal end. A parallel work on human myeloma heavy chain has shown that it contains a very similar *C*-terminal octadecapeptide with only two replacements (P. J. Piggot, personal communication). Amino acid sequence studies of tryptic peptides by R. L. Hill and collaborators (unpublished work) are in agreement with the sequence reported here.

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## Subcellular Distribution of some Folic Acid-Linked Enzymes in Rat Liver

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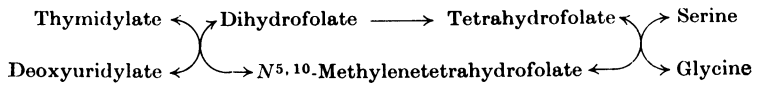
Although much is known about the folic acid-linked enzymes of liver (Friedkin, 1963), work on their intracellular location has not been reported. The distribution of three such enzymes, tetrahydrofolate dehydrogenase (EC 1.5.1.3), methyltransferase and thymidylate synthetase, is examined here. Tetrahydrofolate-dehydrogenase activity results in the production of reduced forms of folic acid; methyltransferase and thymidylate synthetase are concerned with the utilization of reduced

Table 1. *Specific activities of folic acid-linked enzymes in subcellular fractions of rat liver*

Values in parentheses indicate the percentage distribution of each enzyme amongst the three fractions.

| | Specific activity (mμmoles/hr./mg. of protein) | | | | | |
|---|---|---|---|---|---|---|
| | Normal liver | | | Regenerating liver | | |
| Fraction | Methyl-transferase | Tetra-hydrofolate dehydrogenase | Thymidylate synthetase | Methyl-transferase | Tetra-hydrofolate dehydrogenase | Thymidylate synthetase |
| Supernatant | 4·3 (51) | 170 (88) | <0·2 | 4·0 (33) | 170 (90) | 3·0 (40) |
| Mitochondrial | 5·7 (36) | 44 (12) | <0·05 | 11 (48) | 40 (10) | 1·4 (10) |
| Nuclear | 10 (14) | <5 (<1) | <1·0 | 20 (19) | <5 (1<) | 33 (50) |




Scheme 1.

folates as cofactors in processes associated respectively with amino acid and nucleic acid metabolism. Typical determinations of the specific activities of these enzymes in, and percentage distributions among, the supernatant, mitochondrial and nuclear fractions of normal and regenerating (48 hr.) rat liver are shown in Table 1.

Mitochondrial fractions were prepared by the technique of Hogeboom (1955) and lysed with 0·1% deoxycholate, and the soluble protein was fractionated with ammonium sulphate, as appropriate for each enzyme. The supernatant (100000*g*) from the original mitochondrial preparation was fractionated likewise. Nuclei were isolated by the procedure of Widnell & Tata (1964), or in larger-scale experiments by that of Gill (1965), and disrupted by repeated freezing and thawing; this solubilized about 10% of the total nuclear protein, with the release of barely 2% of the DNA. The low recovery of protein (about 1mg./g. wet wt. of liver) precluded purification of these particular extracts.

Tetrahydrofolate dehydrogenase was assayed spectrophotometrically with dihydrofolate as substrate (Brown, Neal & Williams, 1965), and methyltransferase by the spectrophotometric method of Rosenthal, Smith & Buchanan (1965) with homocysteine as acceptor. Thymidylate synthetase was determined by the chromatographic method II of Wahba & Friedkin (1962), with [$^{14}$C]formaldehyde as tracer instead of [$^{32}$P]dUMP; it was not found to be possible to apply their spectrophotometric assay III to the partially purified enzymes of the present study (cf. Blakley & McDougall, 1962).

The patterns of the activities and distributions of the dehydrogenase and of the transferase were similar in the normal and the regenerating tissues. It is striking that there was no measurable activity of the former enzyme in either nuclear fraction; this was confirmed by spectrophotofluorimetric assay (Brown & Williams, 1965). By contrast there was a marked general increase in the activity of thymidylate synthetase in regenerating liver. The presence of this particular enzyme in the nuclear protein is not unexpected in view of the extensive range of enzymes associated with nucleotide metabolism that Rees, Ross & Rowland (1962) have found to be present in isolated rat-kidney nuclei. The total activity of thymidylate synthetase in the three liver fractions was comparable with the peak values found by Maley & Maley (1960) (cf. Maley, Lorenson & Maley, 1965) in the supernatants from homogenates of livers excised at various times after partial hepatectomy. If, as these authors suggest, the rise in synthetase in the regenerating tissue is intimately concerned with DNA biosynthesis, it is surprising that there is no concomitant increase in tetrahydrofolate dehydrogenase, since the two enzymes comprise a coupled system for the utilization and regeneration of tetrahydrofolate (Scheme 1). A simple explanation of this apparent anomaly could be that, despite its low molecular weight, the dehydrogenase was present in the protein fraction that was not solubilized on lysis of the nuclei; this would contrast with the ready release of the enzyme on lysis of the mitochondria. An alternative possibility is that the dihydrofolate produced by thymidylate-synthetase activity in the nucleus leaves the nucleus for reduction back to tetrahydrofolate. This would presumably occur at a site rich in both the dehydrogenase and in systems capable of generating reduced nicotinamide nucleotides. We suggest, in view of the following observations, that

the mitochondrion could be the site of this reaction.

Lysates of mitochondria from both normal and regenerating liver were found to be relatively rich in tetrahydrofolate dehydrogenase. Contamination by supernatant enzyme can be ruled out, since repeated washing of the intact mitochondrial fractions did not affect the results. This finding is significant on two counts: (1) Mitochondria are known to accumulate several cations, notably $Ca^{2+}$, which *in vitro* markedly stimulate the activity of tetrahydrofolate dehydrogenase; this indicates that the mitochondrial enzyme may have a more significant role in dihydrofolate metabolism than is suggested by the activity and distribution results presented here. (2) The mitochondrial dehydrogenase appears to be relatively inaccessible to exogenous methotrexate; thus there was only a partial loss of enzyme activity on incubating intact mitochondria, for 15–30min., in iso-osmotic sucrose or potassium chloride at pH 7·4, with 0·1$\mu$M-methotrexate, i.e. a tenfold excess over that needed to inhibit the available enzyme totally. Moreover, when dihydrofolate and $NADPH_2$ were included in the incubation mixtures, the decrease in extinction of the supernatant at 340m$\mu$ was independent of concentrations of methotrexate from 0·1$\mu$M to 10mM. This, and the fact that only small decrements were observed in blanks, suggests that there was an active reduction of the dihydrofolate, not affected by the inhibitor. Similar experiments with [3′,5′-$^3H_2$]methotrexate (The Radiochemical Centre, Amersham, Bucks.) confirmed that there was no uptake by the mitochondria of the drug itself, since, at 0·1$\mu$M, substantially all of it could be recovered in the supernatant, and on simple washing. This would be unlikely to dissociate an enzyme–inhibitor complex if one had, in fact, been formed. It is relevant that Hakala (1965*a*,*b*) has found that there is a considerable barrier to the entry of methotrexate into sarcoma 180 cells cultured *in vitro*, but that $Ca^{2+}$ stimulates the influx.

The present results are consistent with there being a folate-mediated metabolic link between liver nuclei and mitochondria (cf. Bell, 1965). It seems that the enzymic synthesis of thymidylate from deoxyuridylate is effected, in part at least, in the cell nucleus. The complementary reaction, however, by which dihydrofolate is regenerated from tetrahydrofolate, is apparently carried out by the supernatant enzyme or by that in the mitochondria, or both. Since this coupling between the two enzymes is indirect, it is feasible that changes in the concentration of one, as in hepatic regeneration, need not necessarily be reflected by changes in the concentration of the other.

Such a scheme necessitates a net transport of partially and of fully reduced folates between nuclei and mitochondria. In view of the general occurrence in the three liver fractions of methyltransferase, it is likely that this enzyme serves to make these metabolites available to the thymidylate cycle from their methyl derivatives, which are known to be present in the general cytoplasmic pool.

An indication that mitochondrial tetrahydrofolate dehydrogenase is of distinct physiological significance is provided by its relative inaccessibility to methotrexate. This, taken with the fact that thymidylate synthetase is not sensitive to this particular antimetabolite, suggests that liver nucleic acid replication need not be inhibited by the drug. This actually seems to be so, since methotrexate fails to repress liver regeneration even at acutely toxic doses (Brown *et al.* 1965).

It is likely that a knowledge of the intracellular distribution of folate-linked enzymes in tumours would be of value in interpreting the results of studies *in vivo* of their therapy with antifolate drugs.

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