ON THE HYDROXYLATION OF γ-BUTYROBETAINE TO CARNITINE IN VITRO[x)]

G. Lindstedt and S. Lindstedt.

Departments of Chemistry, Karolinska Institutet
and of Clinical Chemistry, Karolinska Sjukhuset,
Stockholm 60, Sweden.

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It has recently been unequivocally shown that γ-butyrobetaine is hydroxylated to carnitine in vivo (Lindstedt and Lindstedt 1961, Bremer 1962). It appeared possible that the reaction proceeded as a conventional β-oxidation involving an intermediate α-β-dehydrogenation as originally suggested by Linneweh (1929). In support of this hypothesis one could cite the reported biological occurrence of a CoA-ester of γ-butyrobetaine (Hosein 1960) and the fact that carnitine is further degraded to glycine betaine in microorganisms (Lindstedt and Lindstedt 1961).

We have now studied the hydroxylation in vitro and obtained results which make this mechanism unlikely since the reaction is catalyzed by a soluble enzyme system requiring molecular oxygen in the presence of $NADPH_2$ or NADP and ascorbate. These co-factors cannot be replaced by a combination of CoA, ATP, Mg^{++} and FAD.

Preliminary experiments with fractionated rat liver homogenates (33 % in 0.25 M sucrose) showed that enzymic activity was located in the 100,000 x g supernatant and was stable to dialysis against 0.1 M potassium phosphate buffer at pH 7.8. Pooled rat livers were then used to prepare an acetone powder (Colowick and Kaplan 1955) from which the activity could be extracted into the same buffer. One g of acetone powder equivalent to 5 g of rat liver was left over night in 7 ml of buffer and shaken

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at intervals. The suspension was then centrifuged at 20,000 x g for 10 minutes, which resulted in a clear solution. This was dialyzed overnight at 4°C against the same buffer and incubated with γ-butyrobetaine-$C^{14}OOH$ (1.5 mC/mmole) for 6 hours at 37°C in air. Anaerobic experiments were carried out after alternate evacuation and flushing with purified nitrogen. Co-factors were added in amounts given in Tables I and II. After deproteinization with TCA the incubations were analyzed for carnitine and remaining γ-butyrobetaine by ion-exchange chromatography on columns of Dowex-50 x 8 (Lindstedt and Lindstedt 1961). With acetone powder extracts these were the only labelled compounds observed.

Table I shows that CoA, ATP and FAD were inactive as co-factors when added to a dialyzed enzyme preparation while the addition of $NADPH_2$ plus ascorbate resulted in 15 % conversion of γ-butyrobetaine to carnitine. Fe^{++} had a stimulatory effect while incubation under nitrogen resulted in complete loss of activity.

Table I

Conditions for hydroxylation of γ-butyrobetaine to carnitine

Conditions	% conversion
No co-factors added	0.6
ATP, CoA	0.1
ATP, CoA, FAD	0.1
$NADPH_2$, ascorbate	15
$NADPH_2$, ascorbate, Fe^{++}	21
$NADPH_2$, ascorbate, Fe^{++} in N_2	0.1
$NADPH_2$, ascorbate, Fe^{++} + boiled extract	0.1

Composition of incubation medium: dialyzed enzyme preparation from 1.5 g of rat liver (~150 mg of protein), γ-butyrobetaine-$C^{14}OOH$ (0.055 mM), potassium phosphate buffer pH 7.8 (80 mM), $MgCl_2$ (2.7 mM), KCl (27 mM), potassium fumarate (2.7 mM). Co-factors added in final concentration of: ATP (1.3 mM), CoA (0.35 mM), FAD (0.4 mM), $NADPH_2$ (0.45 mM), potassium ascorbate (3 mM), $FeCl_2$ (0.4 mM). Total volume 3.7 ml.

Omittance of either $NADPH_2$ or ascorbate resulted in a tenfold decrease in activity. $NADPH_2$ could be replaced by NADP in the presence of ascorbate while NAD and $NADH_2$ were considerably less effective when substituted for $NADPH_2$ (Table II). The incubations were usually carried out in the presence of fumarate (Levin, Levenberg and Kaufmann 1960) since this addition consistently gave slightly better conversions. Hydroxylation of 20 - 40 % of the substrate was regularly obtained with different acetone powder preparations and the complete system as defined in Table II.

Table II

Co-factor requirements for hydroxylation of γ-butyrobetaine

Co-factor omitted from complete system	Co-factor added	% conversion
None	None	41
$NADPH_2$	None	3.9
Ascorbate	None	3.5
$NADPH_2$, ascorbate	None	0.2
$NADPH_2$	NADP	43
$NADPH_2$	$NADH_2$	6.7
$NADPH_2$	NAD	9.6
$NADPH_2$, ascorbate	$NADH_2$	0.1

Complete system: γ-butyrobetaine-$C^{14}OOH$, $MgCl_2$, KCl, potassium fumarate, $NADPH_2$, ascorbate and $FeCl_2$ (concentrations as in Table I). Final concentration of NADP, $NADH_2$ and NAD 0.45 mM.

It thus appears that the hydroxylation of γ-butyrobetaine to carnitine in rat liver is an example of an enzymic reaction which requires oxygen and a reducing agent. Several hydroxylations in which this type of mechanism operates have been studied in recent years i.e. aromatic and steroid hydroxylations (for review see Mason 1957), the hydroxylation of dopamine to norepinephrine (Levin, Levenberg and Kaufmann 1960) and the formation of hydroxypalmitic acid as an intermediate in the biogenesis of palmitoleic acid (Bloomfield and Bloch 1960).

The exact characterization of the co-factor dependencies and further studies on the mechanism of the present reaction must await purification of the enzyme(s).

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