greatly influenced in vivo enzyme activity, the basic aminoacids being the most effective. The isoenzyme study revealed that in protein-enriched media, the slow moving component, normally present but barely visible in minimal medium, appeared predominantly. If the synthesis of this enzyme is stimulated by aminoacids, then one could expect the same in the aminoacid supplemented media also. However, this enzyme was not predominant in cultures enriched with aminoacids, so it could be small peptides rather than aminoacids that play a role in the synthesis of this enzyme. Hence, the increased activity observed in cultures grown in media supplemented with basic or aromatic aminoacids could be either through the protection of the existing major isoenzyme from degradation, or by activation of the activity of the enzyme species as suggested by in vitro studies.

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Reaction of pyridoxal-5′-phosphate with γ-carboxyglutamic acid

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Summary. Pyridoxal-5′-phosphate (PLP) reacts with γ-carboxyglutamic acid (Gla) to form a stable complex absorbing at 325 nm. It is suggested that a condensation occurs in which the formyl group of PLP reacts with the α-amino group and the γ carbon atom of Gla to give a pyrrolidine derivative.

The recently discovered amino acid γ-carboxyglutamic acid (Gla) has been found in proteins involved in blood clotting[1-4], in plasma proteins[5,6] and in bone proteins[7-9]. It has been found free in the urine of healthy humans[10]. Studies have been undertaken to see if Gla is used by enzymes involved in glutamate breakdown. In this context Federici et al.[11] have recently shown that Gla is a competitive inhibitor of glutamate dehydrogenase with NAD^+ and glutamate, and an activator, additive to ADP, of the reverse reaction. We have found however that Gla is not used by bacterial glutamate decarboxylase (type V from *E. coli,* Sigma Chem. Co., 25 units/mg protein) at pH 4.7, even with enzyme concentrations 5 times higher than those able to decarboxylate glutamate in standard conditions; furthermore Gla does not inhibit the enzyme, also at concentrations 10 times higher than the substrate concentration. The spectrum of holo glutamate decarboxylase, characterized between pH 3 and 5 by an absorption maximum at 415 nm, attributed to a hydrogen-bonded aldimine, is also not affected by the addition of 10^{-2} M Gla (synthesized according to Fernlund et al.[12]) at pH 5.0.

On the other hand, in the course of the experiments with glutamate decarboxylase, we noticed a rapid disappearance of the characteristic yellow colour of the pyridoxal-5′-phosphate (PLP, Fluka AG) added to the assay mixture, upon addition of Gla. This suggests the formation of a colorless adduct between the 2 reagents. The present communication reports results concerning this interaction.

When PLP and Gla are added in aqueous solution over a wide range of pH, disappearance of the maximum at

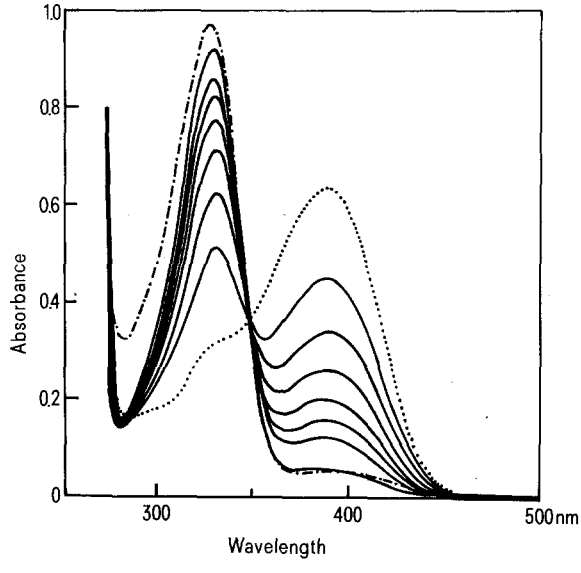
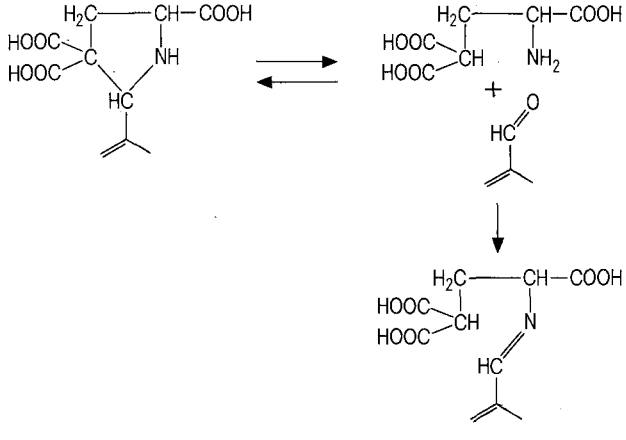


Fig. 1. Absorption spectra of a reaction mixture containing Gla and PLP at pH 4.75 as a function of time. The reaction mixture contains 1.38 mM PLP and 8.3 mM Gla in 0.3 M pyridine-acetate buffer, pH 4.75, 25 °C. Light path 0.1 cm. Spectra recorded with an ACTA 5260 Beckman spectrophotometer. · · · · ·, 1.38 mM PLP; ——, absorption spectra taken 10, 20, 30, 40, 50, 60, and 90 min after the addition of Gla; — · — ·, spectrum of the reacted mixture after addition of 0.3 M ethanolamine at final pH 7.9.

390 nm and increase of the absorption at 325 nm is observed. Figure 1 reports the spectral changes of 1.38 mM PLP and 8.3 mM Gla in 0.3 M pyridine-acetate buffer, pH 4.75, as the reaction progresses. The presence of the isosbestic point suggests that a single equilibrium is being observed between the 2 chromophores directly concerned in the reaction. The decrease at 390 nm is proportional to the absorbance increase at 325 nm. The complex responsible for this absorption maximum is rather stable. At the end of the reaction, as judged by the disappearance of the 390 nm absorption, the addition of a large excess of valine or ethanolamine (final pH 7.9) left the spectrum unchanged. This fact should mean that the equilibrium of the reaction between Gla and PLP is shifted far toward the reaction product, which does not present the characteristics of a Schiff base.

The dependence of the reaction rate on pH is shown in figure 2. Solutions of 1.38 mM PLP and 8.3 mM Gla were incubated at 37 °C in 0.3 M pyridine-acetate buffer at the desired pH. The reaction rate was measured by following the absorption decrease at 390 nm as a function of time. Molar extinction of PLP is a function of pH, and therefore rates are expressed as percentage of PLP reacted in unit of time, instead of absolute concentration changes. Being the reaction rate at pH 7.0 low, another set of experimental values was obtained as follows. The reaction mixture was incubated at the desired pH, samples were taken at various times, diluted 10 times with 1 M phosphate buffer, pH 7.0, in order to stop the reaction, and absorption was read at 390 nm; rates are derived from the progress curve. Both sets of data agree and the maximum of the reaction rate is observed around pH 3.5.

The 330–325-nm peak is known to appear when the aldehyde carbon of PLP changes its hybridization from sp^2 (as in the aldimine form) to sp^3, as found in several cyclic derivatives. For instance during the reaction between PLP and cysteine a similar spectrum is observed, attributed to the thiazolidine ring[13]. The presence in Gla of a highly reactive hydrogen atom on the γ carbon, between the 2 carboxyl groups, would suggest, in the presence of an aldehydic group (as in PLP) and of the α-amino group in Gla, the occurrence of a condensation of the Mannich type[14], as follows:



where only the reactive formyl group of PLP is shown. The suggested reaction pattern leads, through an intermediate Schiff base, to a pyrrolidine derivative. The formation of the Schiff base should be the rate limiting step, as indicated by the fact that its characteristic absorbance at 410 nm is not apparent in the spectrum. However Gla and PLP show a Schiff base peak at pH 9.1, which is promptly converted to the adduct absorbing at 325 nm, as the pH is lowered to

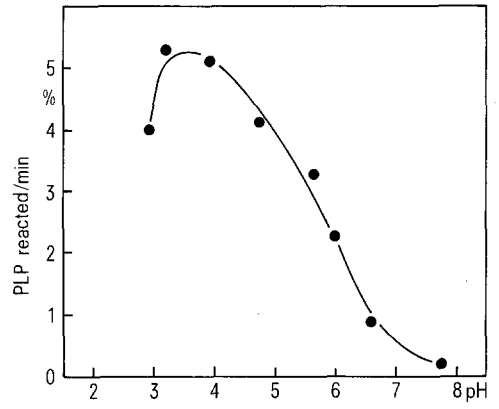
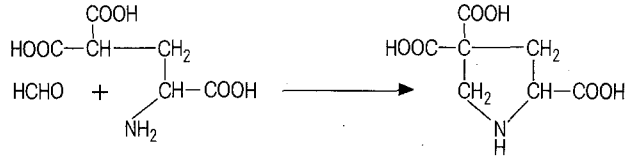


Fig. 2. pH-Dependence of the rate of complex formation between Gla and PLP at 38 °C. See text for experimental data.

4.75. This feature indicates that cyclization is favoured by acidic pHs.

In order to confirm the possibility of the formation of a cyclic derivative, the reaction of Gla with formaldehyde has been studied. 10^{-2} M Gla was incubated at room temperature with 5 M HCHO in 0.3 M pyridine-acetate buffer, pH 3.8. The solution was tested for products from time to time by chromatography on an aluminium cellulose plate (Merck, 0.1 mm, n-butanol:formic acid:water 75:15:10 as solvent). As Gla (R_f=0.19) disappears, a new product (R_f=0.23), giving a yellow spot to ninhydrin, increases. The reaction is almost complete after 16 h, as judged by the disappearance of Gla. Yellow spots are given, as is well known, by imino acids like proline, which has a R_f value of 0.47 in the same system of solvents. In this case the proposed reaction is as follows:



and leads to the formation of 4,4′-dicarboxyl-proline.

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