Vol. LXXII. Fascicule 2. MARS 1964

ARCHIVES INTERNATIONALES DE PHYSIOLOGIE ET DE BIOCHIMIE

(Continuation des
ARCHIVES INTERNATIONALES DE PHYSIOLOGIE,
fondées en 1904 par Léon FREDERICQ et Paul HEGER)

PUBLIÉES PAR

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METABOLISM OF *dl*-CARNITINE AND CROTON-BETAINE IN THE CHICK EMBRYO

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(2 figures)

ABONNEMENTS:

VAILLANT-CARMANNE, S. A., IMPRIMEUR-ÉDITEUR

4, PLACE SAINT-MICHEL, LIÈGE (BELGIQUE)

Titre abrégé pour les citations : *Arch. Internat. Physiol. Bioch.*, 1964, 72

Publication périodique paraissant cinq fois par an.

8-16-12 IMPRIMÉ EN BELGIQUE

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METABOLISM OF *dl*-CARNITINE AND CROTON-BETAINE IN THE CHICK EMBRYO

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(2 figures)

The presence of carnitine in animal tissues has been known for a long time (GULEWITSCH and KRIMBERG, 1905). The studies of FRAENKEL *et al.* (1952) and also of LECLERCQ (1954) have shown that it is a vitamin for the larvae of *Tenebrio molitor*. Croton-betaine is obtained chemically by dehydration of carnitine ; so far, its occurence has not been demonstrated *in vivo*. But γ-butyrobetaine is a precursor of carnitine (BREMER, 1962) and the latter, at least in bacteria, can be partially degraded to glycine-betaine (LINDSTEDT and LINDSTEDT, 1962 *a*) ; the overall transformation is the splitting of 2 C from a substituted fatty acid and it is possible that croton-betaine might appear as an intermediate. Present experimental evidence on the hydroxylation of γ-butyrobetaine to carnitine does not point however in this direction (LINDSTEDT and LINDSTEDT, 1962 *b*).

LIÉBECQ-HUTTER (1960) has observed that croton-betaine stimulates *in vitro* the growth and the periostic ossification of tibias from chick embryos taken after a 6 day incubation. Carnitine has not this effect. However MEHLMAN and WOLF (1962) have observed an important increase of the amount of carnitine in chick embryos between the 12th and the 16th day of incubation.

(1) *Associé du Fonds National Belge de la Recherche Scientifique.*

The action of croton-betaine on the lay-down of collagen in chick bones might be explained if it were a precursor of a required amino acid the quantity of which is the limiting factor of the biosynthesis of the protein in the experimental conditions. Proline or hydroxyproline, very abundant in collagen, might be this amino acid. It is possible to think of a metabolic pathway whereby the γ-amino-crotonic acid, product of demethylation of the croton-betaine, might be reductively carboxylated (with energy from some ATP-like substance) to an α-keto-acid; this keto-acid, analogous to α-keto-δ-amino-valeric acid which is a known intermediate in proline biosynthesis in birds (BOULANGER and OSTEUX, 1955) would close into a $\Delta_{1,3}$-pyrrolidine-2-carboxylic acid and then be reduced to the Δ_3-pyrroline derivative. This latter compound might be either reduced to proline or hydroxylated into hydroxyproline.

To explain why carnitine is without action on the growth of the chick bone, one might argue that the ring closure is easier with a *cis* double bond (1) than with the β-hydroxy compound. On the other hand, the conversion of carnitine to croton-betaine seems to be very slight *in vivo*.

Experimental

1) *Preparation of dl-carnitine and croton-betaine labelled with tritium outside the methyl groups.* (VERLY *et al.*, 1963).

300 mg of β-hydroxy-γ-amino-butyric acid have been exposed at room temperature to 21 curies of tritium hydrogen under 250 mm Hg pressure for 15 days. The labelled compound was freed from labile tritium atoms by repeated solution in water followed by evaporation.

The tritiated β-hydroxy-γ-amino-butyric acid, without any purification to remove the products of radiolysis, was dissolved in KOH solution and treated with an excess of methyl iodide in methanol. The carnitine formed in the reaction was extracted

(1) The exact steric configuration of the chemically prepared crotonbetaine is unknown, but it is supposed to be predominantly *cis*.

with phenol and, after addition of diethyl ether, back-extracted in water; it was then purified by passing through a columm of Amberlite CG-45 (OH) (CARTER and BHATTACHARYYA, 1953).

The tritiated carnitine so obtained still contained numerous radioactive impurities. These were removed by chromatography on Dowex-50 (H) using N HCl as the eluent (FRIEDMAN *et al.*, 1955), followed by continuous flow electrophoresis in the Spinco Model CP apparatus (600 volts, 80 mA; solution of 8.75 ml of acetic acid and 2.5 ml of pyridine per liter of water : pH 4). 82 mg of pure tritiated carnitine were obtained with a specific radioactivity of 0.88 mC per millimole. The radio-chemical purity was checked by paper electrophoresis followed by a continuous recording of the radioactivity along the paper.

The dehydration of carnitine to croton-betaine was performed by treatment with concentrated sulphuric acid at 130° for 4 hrs. The isolated croton-betaine was purified by chromatography on Dowex-50 (H). The radio-chemical purity was established as before by paper electrophoresis followed by recording of the radioactivity. The overall yield was about 50 % and the specific radioactivity 0.91 mC per millimole, i.e. essentially the same as that of carnitine.

This identity of the specific radioactivities of carnitine and the derived croton-betaine is interesting in the context of the discussion on the localization of the stable tritium atoms within the molecules of β-hydroxy-γ-amino-butyric acid after an exchange with tritium hydrogen according to WILZBACH's method.

2) The action of the labelled compounds on the elongation of tibias from chick embryo was tested *in vitro* according to LIÉBECQ-HUTTER (1960). The tritiated croton-betaine induced a stimulation of bone growth similar to that observed previously for the unlabelled compound.

3) *Experiment with tritiated dl-carnitine.*

A small window was opened (HARKMARK and GRAHAM, 1951) just above the embryo, in the shell of hen eggs incubated for 5 days. 400 µg of the tritiated dl-carnitine, dissolved in 0.1 ml

of Tyrode solution, were instilled onto the embryo area. Then the hole was masked with a siliconed coverslip sealed with paraffin and the incubation continued until the 9th day.

On this day, the eggs were opened and 17 living embryos collected forming a total of 23 g. The activity injected into the 17 eggs can easily be calculated to be 8.26×10^7 disintegrations per minute (d/min.).

The embryos were thoroughly ground with 50 ml of water in a Virtis 45 homogenizer. In order to precipitate the proteins, the homogenate was treated with an equal volume of 1 % picric acid ; the mixture was ground again in the Virtis before centrifugation. To remove from the sediment a possible contamination with free unmetabolized tritiated carnitine, it was resuspended in 40 ml of 0.5 % picric acid solution containing 100 mg of unlabelled carnitine and then centrifuged down again ; this operation was performed 3 times. The pellet was finally washed 4 times with ethanol and twice with ether.

The proteins of the centrifugate were hydrolyzed in 10 ml of 6 N HCl boiling under reflux for 20 hrs. After evaporation to dryness under vacuum, the last traces of HCl were eliminated by 3 washings with water followed by evaporation. To remove the picric acid still contained in this hydrolysate, the residue, dissolved in water, was percolated through a column of 6 ml of Dowex-2 in the chloride form and the amino acids were eluted with 15 ml of 0.02 N HCl ; the eluate was evaporated to dryness under vacuum. This last residue was dissolved in 10 ml of water.

The radioactivity of the solution was assayed by liquid scintillation counting in a Packard Tri-Carb spectrometer. To 0.1 ml were added 1 ml of 0.2 N hyamine hydroxide in methanol and 11 ml of a scintillating solution (10 g PPO, 250 mg POPOP and 100 g of naphthalene per liter of dioxane). To obtain the yield of counting, a standard of tritiated naphthylacetamide of known absolute radioactivity was dissolved in a scintillating mixture prepared in an identical way. The total radioactivity of the hydrolysate was 55.000 d/min.

The remaining 9.8 ml of the amino-acid solution were poured on a columm of Dowex-50 (H) (Stein and Moore, 1950) of

61 cm in height and 2 cm diameter. The elution was carried out successively with 1.5 N, 2.5 N and 4.0 N HCl and 5 ml fractions were collected.

The chemical assay of the amino acids was performed with ninhydrin (MOORE and STEIN, 1954). But, as proline and hydroxyproline were of special interest, the former was localized with the Chinard reagent (CHINARD, 1952) and the latter with the reagent of NEUMAN and LOGAN (1950).

For the radioactivity determinations, 2.5 ml of each fraction was evaporated to dryness. To the residue were added 1 ml of 0.2 N hyamine hydroxide in methanol and 11 ml of a scintillating solution (4 g PPO, 100 mg POPOP per liter of toluene). Standardization and assay in the Tri-Carb were made as usual. The results are indicated in Figure 1.

Two legs from different embryos were fixed in Carnoy solution immediately after removal from the shells. Histoautoradiographies were done on slices (4 μ thick) using Gevaert nuclear emulsion Scientia 7.15 or stripping film Kodak AR-10. They were stained by Ehrlich hematoxylin after development by D-19. Exposure time varied from 7 to 14 days. No significant radioactivity could be detected by comparison with controls which received an instillation of Tyrode solution only. Administration of ^{14}C-alanine in the same experimental conditions has previously shown a high labelling of the periosteal ground substance.

4) *Experiment with tritiated croton-betaine.*

The scheme of this experiment is very similar to the preceeding one.

400 μg of tritiated croton-betaine were instilled into each egg on the embryo area after 5 days of incubation. The embryos were removed on the 9th day. 11 surviving embryos (15.2 g) (total activity injected into the 11 eggs = 6.19×10^7 d/min) were homogenized.

0.5 ml of the homogenate was digested in 4 ml of N hyamine hydroxide in methanol at 60° in darkness for 48 hrs ; afterwards, the volume was brought to 12 ml with dioxane. The radioactivity was determined after addition of a scintillating solution

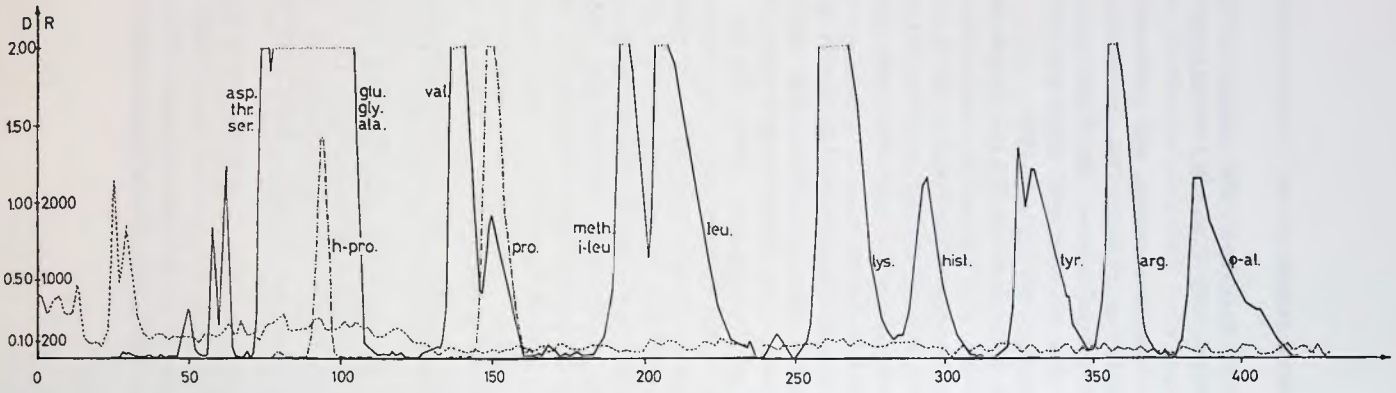

FIG. 1. — Chromatography of the protein amino acids from chick embryos treated with ^{3}H-dl-carnitine.

——————— ninhydrine
—·—·—·—·—·— Chinard reaction or Neuman and Logan reagent
— — — — — — — radioactivity

Abscissae are fraction numbers. Ordonnea : the left scale gives the absorbancy at the chosen wavelength ; the other one indicates the radioactivity in disintegrations per minute per 5 ml fraction.
The eluent was 1.5 N HCl from fraction 1 to fraction 136, 2.5 N HCl from 137 to 299 and 4.0 N HCl from 300 to the end.

and internal calibration with a standard. The total activity of the homogenate was 5.85×10^6 d/min which means that about 10 % of the injected croton-betaine gained access to the embryos.

The precipitation with picric acid was as indicated above, except that the washings for the decontamination of the precipitate were performed with a solution of non-radioactive croton-betaine instead of carnitine. The hydrolysis of the proteins of the sediment has been described and also the removal of the picric acid adsorbed on it. As previously, the amino acids were finally dissolved in 10 ml of water; the solution, which had a total radioactivity of 73 000 d/min, was chromatographed on Dowex-50 (H). The results are drawn in Fig. 2.

Histoautoradiographies were made on legs fixed in Carnoy solution directly after the 9th day of incubation. No labelling could be seen after exposure time varying from 7 to 14 days.

Discussion

To test the hypothesis that croton-betaine might be a precursor of proline or hydroxyproline in birds, it was necessary to label the molecule outside the methyl groups because these groups had to be removed before cyclisation could take place. This condition was fulfilled by labelling β-hydroxy-γ-aminobutyric acid by exchange with tritium hydrogen before methylation with ordinary methyl iodide and dehydration of the labelled carnitine to croton betaine.

As LIÉBECQ-HUTTER (1960) has observed the action of croton-betaine on the deposition of collagen in bones from chick embryo *in vitro*, similar, but *in vivo*, conditions were used in the present work : the labelled compound was laid on the embryo areas of hen eggs after 5 days of incubation. There is a good resorption of the product administered in this way as proved in the second experiment where about 10 % of the given radioactivity was found in the embryos 4 days later; the fact had also been established previously in a pilot experiment with ^{14}C-alanine.

The chemical synthesis having given tritiated dl-carnitine as well as tritiated croton-betaine, both were tried experimentally.

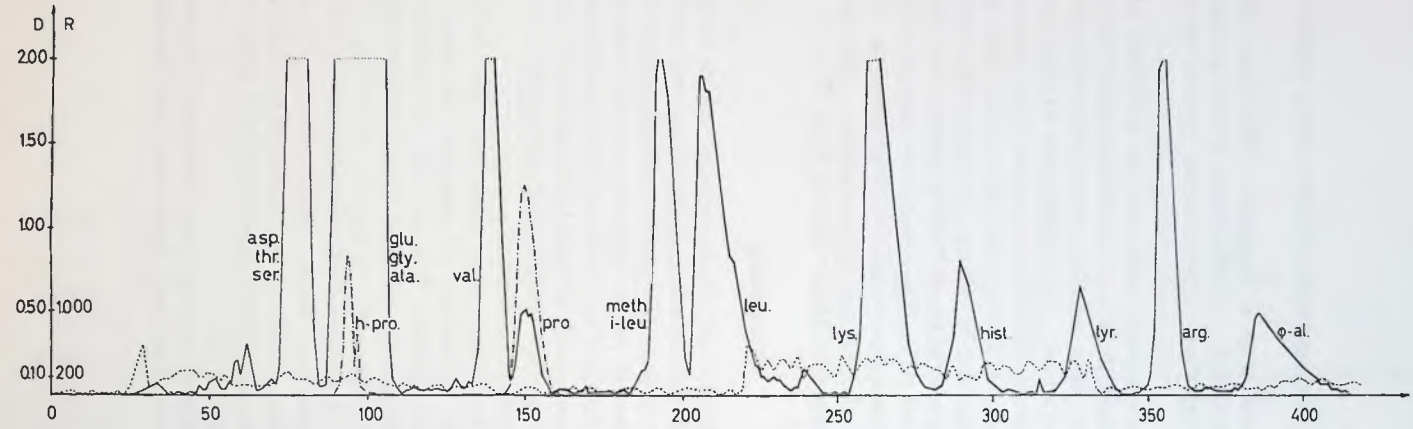

FIG. 2. — Chromatography of the protein amino acids from chick embryos treated with ^{3}H-croton-betaine.

——————— ninhydrine
—·—·—·—·—·— Chinard reagent or Neuman and Logan reagent
— — — — — — — radioactivity

Abscissae are fraction numbers. Ordonnea : the left scale gives the absorbancy at the chosen wavelength ; the other one indicates the radioactivity in disintegrations per minute per 5 ml fraction.

The eluent was 1.5 N HCl from fraction 1 to fraction 136, 2.5 N HCl from 137 to 299, and 4.0 N HCl from 300 to the end.

It is obvious that the sediment obtained by centrifugation of the embryo homogenates after addition of picric acid contains many things besides proteins even after the washings with ethanol and ethyl ether. The amino acids of the hydrolysate of this sediment are partially purified by the treatment of Dowex-2 which leaves an « amino acid fraction » containing about 1/1000 of the administered radioactivity in both experiments. But the chromatography on Dowex-50 shows that this radioactivity is not bound to any amino acid and, in particular, not to the proline or the hydroxyproline.

The conclusion is clear that neither croton betaine nor carnitine is an important precursor of amino acid in the chick embryo, at least through a relatively direct pathway that does not labilize the tritium used as tracer. It does seem that the *in vitro* observation of LIÉBECQ-HUTTER (1960) of a stimulatory effect of croton-betaine on bone growth, which has been confirmed with the synthetic tritiated compound, must be justified by another explanation.

Summary

dl-carnitine and croton-betaine were not found to be amino acid precursors in the chick embryo.

* * *

The authors wish to thank G. FILLET for his careful help in part of this work.

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