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A RADIOCHEMICAL METHOD FOR CARBAMOYL-PHOSPHATE SYNTHETASE-I: APPLICATION TO RATS FED A HYPERPROTEIC DIET

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Abstract- A method for the measurement of carbamoyl-phosphate synthetase I activity in animal tissues has been developed using the livers of rats under normal and hyperproteic diets. The method is based on the incorporation of ^{14}C-ammonium bicarbonate to carbamoyl-phosphate in the presence of ATP-Mg and N-acetyl-glutamate. The reaction is stopped by chilling, lowering the pH and adding ethanol. Excess bicarbonate is flushed out under a gentle stream of cold CO_2. The only label remaining in the medium was that incorporated into carbamoyl-phosphate, since all ^{14}C-CO_2 from bicarbonate was eliminated. The method is rapid and requires only a low pressure supply of CO_2 to remove the excess substrate. The reaction is linear up to 10 min using homogenate dilutions of 1:20 to 1:200 (w/v). Rat liver activity was in the range of 89±8 nkat/g. Hyperproteic diet resulted in a significant 1.4-fold increase. The design of the method allows for the processing of multiple samples at the same time, and incubation medium manipulation is unnecessary, since the plastic incubation vial and its contents are finally counted together.

Keywords- carbamoyl-phosphate synthetase I, urea cycle, hyperproteic diets

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Introduction

Carbamoyl-P (CP) synthetase is a critical enzyme in the control of the disposal of 2-amino N from amino acids via the urea cycle [1]. CP synthetases use either ammonia as N donor: CP-synthetase-I [2] (EC 6.3.4.16), or glutamine [3] (EC 6.3.5.5). CP-synthetase-I is found in liver mitochondria [4] and catalyzes the incorporation of CO_2 and NH_3 to form CP with the consumption of 2 moles ATP per mol of CP formed [5].

Measurement methods for CP synthetase-I have been developed using different approaches, most of them relying on the coupling of the synthesis of CP with ornithine transcarbamylase to yield citrulline [6], which is, then, estimated by radiochemical [7], or colorimetric [8] methods. The analysis of other byproducts such as ADP, coupled to dehydrogenases after a chain of added enzymes, has been also used in the past [2,9]. Usually, the key step was the separation of CP from labelled precursors [10], or, in coupled reactions, ornithine [10,11]. The problems caused by a long chain of coupled reactions, the availability of the complementary enzymes and labelled substrates and the cumbersome and time-consuming procedures of column separation are an unwanted additional source of variation and built-in error.

The marked decrease of enzyme-based amino acid metabolism studies, from a peak in the early 1980s resulted in the removal from most scientific suppliers' catalogues of labelled substrates, reagents and enzymes needed for coupling or label-transfer reactions, which right now makes more difficult the assay of common enzymes such as CP synthetase. In addition, nowadays there is an enormous distance in knowledge between energy, glucose, and fatty acids metabolism and regulation in health and, especially, in metabolic disease with the sketchy and incomplete actualization of the role amino acids play under common metabolic challenges. In contrast, high-protein diets are widely used to fight obesity [12,13], often with limited knowledge of their potential effects.

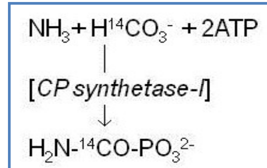
High-protein diets increase urea excretion [14], but high-energy diets lower urea synthesis [15] and increase nitrate production [16] irrespective of protein intake. We are trying to analyze how excess energy may affect so deeply amino acid catabolism, and this implies the analysis of gene expression, but primarily the direct measurement of protein levels and/or enzyme activities. The present study is centred precisely on the actualization of a method for the measurement of CP-synthetase-I in tissues, using a well established incubation medium with bicarbonate, ammonium, ATP, and N-acetyl-glutamate as activator of the reaction [17] and testing the method both in control and hyperproteic diet-fed rats. The differences with the classic procedures stem from the use of inexpensive and easily available ^{14}C-bicarbonate, and the discrimination of the excess substrate from labelled CP at the end of the reaction by

flushing out excess bicarbonate with a stream of cold CO_2.

Materials and Method

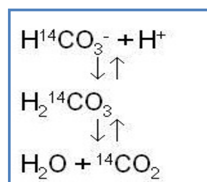
Outline of the Method

Sample homogenates were incubated with ATP, ammonium ions and bicarbonate (containing ^{14}C-bicarbonate), as well as N-acetyl-glutamate as activator. The reaction:



(Reaction-1)

yields labelled carbamoyl-phosphate; but much excess labelled bicarbonate remains in the incubation medium; it is flushed out with a stream of non-labelled CO_2 in a slightly acidified medium:



(Reaction-2)

The specific activity of the remaining carbonate is rapidly diluted by cold CO_2; thus after a time, the only label present in the incubation medium is that of CP. Counting the medium we can estimate the CP formation from the specific activity of the labelled bicarbonate added to the reaction mixture.

Chemicals

Inorganic chemicals, ethanol and acetic acid were purchased from Panreac (Montcada, Barcelona Spain) and all were of analytical quality. Organic compounds and albumin were obtained from Sigma (St Louis, MO USA). Labelled $NaH^{14}CO_3$ (specific activity 1.48-2.22GBq/mmol) was obtained from Perkin Elmer (Bad Neuham, Germany). Plastic polyethylene tubing was purchased from Becton-Dickinson (Sparks, MD USA).

Sample Preparation

Adult (90 days old) male rats (Zucker lean) were used. Two groups of 6 animals each were randomly selected and fed during one month (i.e. from days 90 to 120) with either the standard rat chow (controls, with a protein energy content of 20%), or a hyperproteic diet (40% of protein-derived energy). On day 120, the rats were killed and liver samples were frozen in liquid nitrogen and kept at -80ºC, for later use in enzyme analyses.

The protocol of animal handling was authorized by the Ethics Committee on Animal Experimentation of the University of Barcelona.

Tissue samples were homogenized in 20 volumes of chilled 50mM triethanolamine/HCl buffer pH 8.0, containing 1mM dithiothreitol and 10mM of Mg-acetate, using a cell disruptor (IKA, Stauffen, Germany). Homogenates were diluted, when needed, with homogenizing buffer. Protein content in homogenates was measured with the Lowry method [18].

There were no significant differences in enzyme activity using fresh and frozen tissue samples under the conditions described. However, the activities measured using similarly frozen (and stored) homogenates resulted in losses of up to 50% of the enzyme activity.

Incubation

The conditions of incubation were adapted from those described in the literature for analysis of activity of the rat liver enzyme [8]. Incubations were carried out at 30ºC in thin-walled 1.5ml capped polyethylene tubes (Eppendorf tubes), using a block heater. The incubation mixture (final volume 0.200mL) contained (final concentrations) 5mM ATP, 10mM Mg-acetate, 1mM dithiothreitol, 1 g/L bovine serum albumin (defatted), and 5mM disodium N-acetyl-glutamate, in 50mM thriethanolamine/HCl buffer pH 8.0.

To 0.100mL of incubation medium, 0.050mL of homogenate was added. The reaction was started immediately by adding 0.050mL of 200mM ammonium bicarbonate, containing 5 kBq of $NaH^{14}CO_3$, i.e. with 50 kBq/mmol specific activity. After gently shaking, the reaction was allowed to run for up to 10 minutes.

The reaction was stopped by the addition of 0.100ml of ethanol: acetic acid (20:1 v/v), shaking and leaving the uncapped tubes on ice. Zero time was established by adding (to medium and homogenate) the ethanol: acetic acid first, and then the ammonium bicarbonate. Control tubes in which the homogenate was substituted by the same volume of homogenizing buffer were also included in each series.

CO_2 Flushing and Labelled Carbamoyl-Phosphate Estimation

A series of 20 cm pieces of capillary tubing were tightly fitted in both ends with the serrated steel tubes from hypodermic needles, obtaining tubes with two steel-needle point ends. One of the extremes of the tube was stuck in a closed plastic (or rubber) tube connected to a low-pressure source of carbon dioxide. The other was introduced in the incubation mixture of the Eppendorf tubes, containing an incubated sample, or a control (no homogenate) with reagents alone. The gas was left to bubble gently through the samples for 5-10 minutes at a rate of 10-15mL/min (i.e. 4.5-6.8mMol). The tubes were maintained in a rack on ice, and the whole flushing operation was carried out in a ventilated hood, since $^{14}CO_2$ was lost.

We used advantageously a Kipp gas generator [19] (Anorsa, Barcelona Spain), producing CO_2 from HCl and marble ($CaCO_3$) chunks. The gas outlet was fitted to a short length of plastic/rubber tube which was capped. The only way the gas could leave the system was through the two-pronged capillary tubes stuck on this outlet tube. This setup acted as a manifold, supporting up to 20 flushing capillaries at any time. The use of a Kipp over a gas cylinder (which we also tested) has the advantage of producing low-pressure gas on demand for sustained periods of time. Pressure was an issue here, to prevent excessive evaporation or dispersion of the incubation medium, but enough to maintain the sample in turmoil and to provide enough gas in small bubbles to allow a good interchange between the sample carbonate and the gas stream.

After gas flushing, the incubation tubes were capped, held over empty 6ml scintillation counting pony vials, and then they were cut with heavy scissors. The lower part of the tube and its contents fell inside the vial; then scintillation cocktail was added and the vial

closed; after energetic shaking, the tubes were left to stand for about 10hrs. (to prevent any possible effect of chemiluminescence and to allow residual CO_2 to leave the liquid phase) and counted.

In order to determine whether part of the labelled CO_2 flushed out could came from carbamoyl-phosphate, we added unlabelled carbamoyl-phosphate (to a final concentration of 1μM) to samples after 5 min incubation. The presence of cold carbamoyl-phosphate did not alter the label countings, i.e. under the conditions of work and elimination of excess carbonate, carbamoyl-P is not broken up.

Calculations, Liver Enzyme Activity

Typically, blanks and zero-time tubes were in the range of 100-150 dpm. The zero values were discounted as background from all incubated sample counts. The amount of CP formed was derived from the net counts and the specific activity of the bicarbonate reagent. For all samples, in addition to a zero value, at least three incubation times were used, typically 2.5, 5 and 10 min; in all cases, initial velocities were calculated. This was usually made easier because there was a good linearity for up to 10 minutes in samples adequately diluted. Insufficiently diluted homogenates resulted in slightly lower Vi values and a loss of linearity. [Fig-1] shows the linear relationship between CP label found and incubation time. Repeated analyses of the same sample of liver (different homogenates) yielded practically the same enzyme activity (s.e.m. of 4.4% of the mean value, N=6).

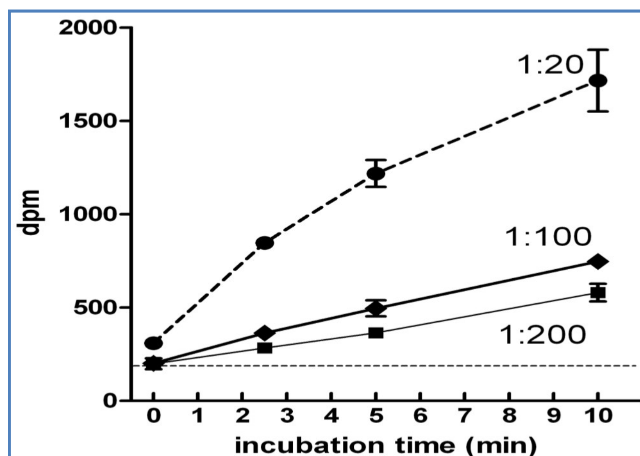


Fig. 1- Relationship between CP synthesis and time in rat liver homogenates

The data show the CP dpm obtained using the method described in the text for analysis of 0.050mL of rat liver homogenate (equivalent to about 2.5 mg of fresh tissue or 0.5 mg of protein) over time (dilution 1:20 w/v), further dilution to 1:100 and 1:200 vs. the initial tissue weight yielded more straight lines. The values are the mean ± sem of triplicate measurements. Linearity of the experimental data: 1:20 (r=0.982, p=0.018); 1:100 (r=0.998; p=0.002); 1:200 (r=0.998; p=0.002).

Results

Liver Carbamoyl-Phosphate Synthetase I Activity

Measurements in six different male rat liver samples yielded a CP synthetase-I activity of 89±8 nkat/g of tissue, i.e. 444±38 nkat/g protein (standard diet), and 122±8 nkat/g of tissue, i.e. 704±94 nkat/g protein (hyperproteic diet). The differences between groups were significant ($P<0.01$ tissue; $P<0.001$ protein; Student's *t* test).

Effectiveness of the Gas-flushing System

[Fig-2] shows the relationships between times of flushing on the bicarbonate label remaining on tubes not containing homogenate. The loss of label is rapid (depends on the flow of CO_2) and becomes practically asymptotic in a few minutes when using flow speeds of CO_2 in the range of 8-20μmol/s. The flow of gas was estimated by timing the accumulation of gas in an inverted graduated tube full of water and using one of the two-pronged capillaries to fill it up with gas.

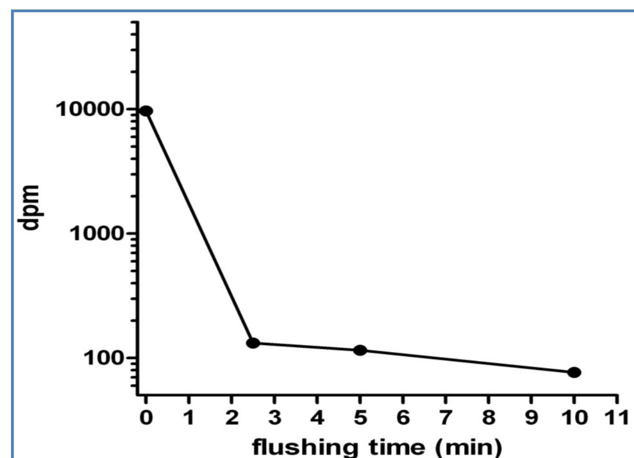


Fig. 2- Effect of CO_2 flushing on the loss of $H^{14}CO_3^-$ label in the medium used for incubations in the absence of homogenate.

The conditions for reaction stoppage and flushing are described in the text. The flow of CO_2 was 250μL/s at about 100 kPa, i.e. 12μmol/s.

We decided to use capillaries with two serrated needle extremes, since one was needed to connect to the Kipp outlet tube, and the other was had to be kept vertical within the tube; the metal needle weight and rigidity allowed it to remain in vertical position indefinitely. This way, the distribution of bubbles was uniform, and they emerged from the bottom in all incubation tubes. The use of polyethylene tubing with no attached needle resulted in a lower repetitiveness of results.

The addition of ethanol stopped effectively the reaction by decreasing the hydration envelopes of proteins, and compounded the effect of cold. Another reason why ethanol was used was its lowering of superficial tension, resulting in the practical elimination of foam from the incubation medium proteins. The absence of foam decreases the spill over of medium; CP is not soluble in ethanol [20], the amount of alcohol added (final concentration 33% by volume) may not be enough to precipitate CP, but enough to reduce its availability and limit the eventual action of phosphatases present in the medium (also inhibited by the change in pH).

The addition of acetic acid decreased the pH to 4,5, which further impeded treaction 1, and yielded enough protons to drive reaction 2 to the right, favouring the decomposition of carbonic acid and the emission of $^{14}CO_2$, helped by the diluting mass effect of the cold CO_2 stream. However, the decrease in pH was not sufficient to break up the CP in its components, especially in a medium which

contained a considerable amount of ammonium ions buffering the acetate.

When trying to establish the precise specific activity of the ammonium bicarbonate reagent, we observed that the direct estimation of radioactivity through liquid scintillation counting resulted in significant and variable losses of radioactivity. This was attributed to spontaneous decomposition of ammonium bicarbonate in equilibrium with the atmosphere. This was solved by adding 0.210mL (i.e. equivalent to the sequestering power of 1mMol of CO_2) of Carbo-Sorb E (Perkin Elmer) before the incorporation of the scintillation cocktail. This resulted in repetitive counting results and the prevention of losses. However, in the samples, part of the label was lost because of limited exchange with atmospheric (and dissolved air) CO_2; i.e., label counting of non-flushed samples (in which no acetic acid was added) was in the range of 89-95% of that expected (i.e. the amount of label added). Since the concentration of bicarbonate in the incubation medium was well in excess of that needed to sustain the reaction, and these losses did not modify the specific activity of bicarbonate, we decided to ignore this limited loss of label.

Discussion

The method presented has the advantage of simplicity; several samples can be analyzed in parallel, requires a short time of incubation, yielding a good linearity and repetitiveness, and requires minimal manipulation of the incubation medium. The main problem its utilization may pose is, perhaps, the long-term stability of homogenates (which lose activity when frozen) coupled with the necessity of previously finding the most adequate dilution of the homogenates in order to obtain the highest Vi (V_{max}) values. On the other side, the method shows considerable sensitivity, since it needs only a few mg of tissue for analysis; if needed, sensitivity can be increased simply by using less-diluted homogenates or, primarily, by increasing the specific activity of bicarbonate in the reaction mixture.

We used albumin to decrease the effect of proteases, and dithiothreitol as –SH group protector, but its presence should be removed when adapting the present method to the analysis of CP synthetase II, since its activity is inhibited by dithiothreitol [21].

The approach, and conditions of the incubation medium (and homogenization) used here are shared by other methods previously published. The main difference we introduced is, perhaps the direct measurement of CP formation without coupling this reaction to that of ornithine transcarbamylase [6] to yield citrulline, which is later measured by radioenzymatic [7] or colorimetric [8] procedures.

In fact, the true novelty of the method described here is the elimination of surplus bicarbonate/CO_2 by flushing it out under a stream of CO_2 at a mild acidic pH. This simplifies considerably the process, making it shorter, and remarkably cheaper. It also allows for the parallel analysis of multiple samples because of the simple gas supply manifold described and the small amount of sample needed. Under the conditions tested, the label in CP can be easily measured and there are no losses due to CP breakup by alkaline phosphatases [22].

The levels of activity in rat liver found using the method described here are higher than those found using the approach in which citrulline formation is analyzed under conditions of excess ornithine transcarbamylase [6,23], in spite of wide variability in the actual liver enzyme activities obtained using different approaches. The rats receiving a hyperproteic diet showed a more marked CP synthetase I activity than controls, in this case, both in relation to liver weight and protein content, the activity measured was in the range of 1.4-fold increase. This is in agreement with enhanced activity of the enzyme under high protein/ammonium loads [24,25] and the increased activity of the urea cycle observed in rats receiving high protein diets [14,26]. The effect of diet on the enzyme activity measured reinforces the effectiveness of the method described for the measurement of this key urea cycle enzyme.

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References

[1] Chabas A., Grisolia S. and Silverstein R. (1972) *Eur. J. Biochem*. 29, 333-342.

[2] Rubio V., Ramponi G. and Grisolía S. (1981) *Biochim. Biophys. Acta.,* 659, 150-160.

[3] Mori M. and Tatibana M. (1977) *Biochim. Biophys. Acta.,* 483, 90-99.

[4] Clarke S. (1976) *J. Biol. Chem*., 251, 950-961.

[5] Cohen N.S. and Raijman L. (1980) *J. Biol. Chem*., 255, 3352-3357.

[6] Brown G.W. and Cohen P.P. (1959) *J. Biol. Chem*., 234, 1769-1774.

[7] Lof C., Wanders R.J.A. and Meijer A.J. (1982) *Eur. J. Biochem*., 124, 89-94.

[8] Pierson D.L. (1980) *J. Biochem. Biophys. Meth*., 3, 31-37.

[9] Guthörlein G. and Knappe J. (1968) *Eur. J. Biochem*., 7, 119-127.

[10] Christopherson R.I. and Finch L.R. (1976) *Anal. Biochem.,* 73, 342-349.

[11] Rowe W.C., Huggins A.K. and Baldwin E. (1970) *Anal. Biochem*., 35, 167-176.

[12] Clifton P.M., Bastiaans K. and Keogh J.B. (2009) *Nutr. Metab. Cardiovasc. Dis*., 19, 548-554.

[13] Leidy H.J., Carnell N.S., Mattes R.D. and Campbell W.W. (2007) *Obesity,* 15, 421-429.

[14] Ashida K. and Harper A.E. (1961) *Proc. Soc. Exp. Biol. Med.* 107, 151-156.

[15] Barber T., Viña J.R., Viña J. and Cabo J. (1985) *Biochem. J.* 230, 675-681.

[16] Green L.C., Ruiz de Luzuriaga K., Wagner D.A., Rand W., Istfan N., Young V.R. and Tannenbaum S.R. (1981) *Proc. Nat. Acad. Sci., USA,* 78, 7764-7768.

[17] Cheung C.W. and Raijman L. (1980) *J. Biol. Chem.,* 255, 5051-

5057.

[18]Lowry O.H., Rosebrough R.W., Farr A.L. and Randall R.J. (1951) *J. Biol. Chem*., 193, 265-275.

[19]Griffin J.J. (1860) *A Popular Manual of Experimental Chemistry. Second Division: Non-metallic Elements.,* Griffin J.J. and Griffin R., *London,* 616-618.

[20]Jones M.E., Spector L. and Lipmann F. (1955) *J. Am. Chem. Soc.,* 77, 819-820.

[21]Trotta P.P., Pinkus L.M. and Meister A. (1974) *J. Biol. Chem.,* 249, 1915-1921.

[22]Herzfeld A. and Knox W.E. (1972) *Cancer Res*., 32, 1837-1841.

[23]Brown T., Hug G., Lansky L., Bove K., Scheve A., Ryan M., Brown H., Schubert W.K., Partin J.C. and Lloyd-Still J. (1976) *N. Engl. J. Med.,* 294, 861-867.

[24]Zaragozá R., Renau-Piqueras J., Portolés M., Hernández-Yago J., Jordá A. and Grisolía S. (1987) *Arch. Biochem. Biophys.* 258, 426-435.

[25]McGivan J.D., Bradford N.M. and Mendez-Mourão J. (1976) *Biochem. J*., 154, 415-421.

[26]Bingham S.A. (2003) *J. Nutr*., 133, 921S-924S.