

## EFFECT OF CARBAMYL PHOSPHATE ON STABILITY OF SOME ENZYMES OF THE UREA CYCLE; HIGH SENSITIVITY OF GLUTAMATE DEHYDROGENASE

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Received 30 December 1971

### 1. Introduction

Carbamyl phosphate (CP), at physiological concentrations, can inactivate glutamate dehydrogenase rapidly [1]; carbamylation by CP occurs often with many biological acceptors, including hormones [2]. Protein carbamylation takes place in some cases readily as with histones, while other proteins are resistant [3].

It appeared of interest to determine the relative susceptibility to CP of the main enzymes concerned with its production and utilization in ureotelic animals. Moreover, studies were carried out to test whether these effects could also be demonstrated with mitochondria and under conditions where there is de novo biosynthesis of CP. It was reasoned that in the presence of ornithine, the physiological acceptor for urea synthesis, citrulline would be formed rapidly and there would be protection, while in the absence of ornithine CP would accumulate and inactivate enzymes.

As shown in this paper, of the main enzymes connected with CP metabolism glutamate dehydrogenase is most susceptible.

### 2. Materials and methods

Crystalline pyruvate kinase was from Boehringer (Mannheim). CP and N-carbamyl-DL-aspartic acid were purchased from Sigma.

Calf brain glutamine synthetase was prepared according to Elliot [4]. Rat liver aspartate transcarbamylase was obtained as previously described [5] but homogenization was carried out with 9 ml 0.25 M sucrose per g liver and the centrifugation at high speed

was for 3 hr. Frog liver cetyltrimethylammonium bromide (CTAB) extracts [6], used in the purification of CP synthetase, contain the bulk of the glutamate dehydrogenase present in the mitochondria [7] and were used as such when assaying for this activity; the acetone fraction [6] was used to test susceptibility to CP both of the synthetase and of ornithine transcarbamylase. Beef brain acyl phosphatase was prepared as described elsewhere [8]. Rat liver mitochondria were obtained according to Hogeboom [9]. The final pellet was suspended either in isotonic KCl or sucrose so that 1 ml suspension contained the mitochondria from 1 to 1.5 g liver (wet weight).

Protein was estimated by standard procedures using bovine serum albumin as standard [10, 11]. Stability to CP was tested by incubating enzyme preparations with CP under the conditions outlined in the tables.

Glutamate dehydrogenase was tested as previously described [12] with NAD and glutamate. Ornithine transcarbamylase and acyl phosphatase were assayed as described before [13, 8]. Glutamine synthetase was determined according to Elliot [4] except that incubation was for 30 min at 37°. Aspartate transcarbamylase was estimated by incubating in 1 ml 20  $\mu$ moles CP, 40  $\mu$ moles aspartate, 100  $\mu$ moles phosphate buffer, pH 8.0, and the enzyme; 20 min incubation at 37°. Carbamyl aspartate was measured colorimetrically [14]. CP synthetase was measured as described elsewhere [13] except that incubations were for 40 min, 8  $\mu$ moles ATP were used, and neither an ATP-regenerating system nor ornithine transcarbamylase was added. Citrulline formation was linear for 40 min as determined colorimetrically [13, 14].

### 3. Results and discussion

The stability to CP of several enzymatic systems involved in the utilization of ammonia and/or related to urea cycle are summarized in table 1. As shown, glutamate dehydrogenase and to a much less extent CP synthetase are unstable. Under the conditions used no loss in enzymatic activity due to CP was observed with the other enzymes tested.

As illustrated, glutamate dehydrogenase is very sensitive to CP. The importance of this enzyme in regulating cellular activity has often been pointed out, therefore experiments on stability of this dehydrogenase were conducted under more physiological conditions. Table 2 shows that incubation of rat liver mitochondria under isotonic and aerobic conditions with CP results in a marked decrease in glutamate dehydrogenase activity. Other experiments were carried out under different conditions ( $\alpha$ -ketoglutarate or glutamate, as respiratory substrates; ATP-regenerating system); they confirm and extend the results illustrated in table 2. Interestingly, it was not possible to demonstrate with respiring mitochondria extensive inactivation at low concentrations of CP (10 mM CP inactivated ca. 15% after 2 hr).

Due to the instability of rat liver mitochondria and particularly of the CP synthetase system thereof, it was not possible to demonstrate clearly whether accumulation of CP in these organelles would result in glutamate dehydrogenase inactivation. Moreover, it should be noted that many effectors, including ATP, are powerful modifiers of the activity and the stability of glutamate dehydrogenase. Indeed, the intimate details which regulate this complex enzyme remain unclear in spite of excellent and extensive studies.

The glutamate dehydrogenase present in the preparations was always kept in the presence of a high concentration of ATP. As illustrated in table 3 CP is an effective inactivator even at high concentrations of ATP (the calculated % of activity left with CP is the same with and without ATP when compared with their proper controls).

An approximation to more physiological conditions was attempted by the use of preparations containing all the components necessary for CP synthesis. Table 4 shows the effect of CP synthesized *de novo* by frog liver preparations on glutamate dehydrogenase stability. When ornithine is present in the incubation

Table 1  
Effect of CP on stability of various enzymes.

Enzyme tested	CP added ( $\mu$ moles)	Incubation time (hr)	Remaining activity (%)
Glutamate dehydrogenase	10	1	54
	25	1	26
	10	2	28
	25	2	0
CP synthetase	33	1	89
	66	1	67
Ornithine transcarbamylase	10–70	1–4	100
Aspartate transcarbamylase	10–40	1	100
Glutamine synthetase	33–50	0.5	100
Acyl phosphatase	200	24	100

Each tube contained in 1 ml (unless otherwise specified) the following: 3.3 mg and 0.43 mg acetone fraction, when testing for stability of CP synthetase and ornithine transcarbamylase, respectively; 7.6 mg CTAB extract, when testing for stability of glutamate dehydrogenase; 0.85 mg glutamine synthetase; 25 mg aspartate transcarbamylase; 0.1 mg acyl phosphatase. Incubations were at 37°. At zero time and at the indicated times suitable aliquots were removed and assayed. Controls remained stable during the incubation.

mixture CP is converted to citrulline and no inactivation of glutamate dehydrogenase is detected. However, in the absence of ornithine, the approx. 20  $\mu$ moles of CP formed, as judged by citrulline synthesis, results in 40% inactivation of glutamate dehydrogenase.

The data presented in this paper confirm the high sensitivity of frog and rat liver glutamate dehydrogenase to CP. Inactivation of the synthetase by CP occurs but to a much limited extent. The high susceptibility of glutamate dehydrogenase under all the conditions tested indicate that control of CP levels by acyl phosphatase may be of much importance. Indeed, the fact that many proteins are susceptible to carbamylation by CP and that this may have important biological implications have acquired additional significance by the demonstration that carbamylation of red cells from sickle cell anemia patients prevents or decreases sickling [15–17].

Unfortunately the experimental conditions

Table 2

Effect of CP on glutamate dehydrogenase from rat liver mitochondria.

Experiments	CP added ( $\mu$ moles)	Incubation time (min)	Activity (units)	Remaining activity (%)
1	0	60	22.0	100
	50	60	12.8	59
	100	60	10.0	46
2	0	90	17.0	100
	50	90	3.6	22
	100	90	2.4	15

Mixtures in 25 ml erlenmeyer flasks contained the indicated CP and 1 ml of rat liver mitochondria suspension prepared as described in the text plus 100  $\mu$ moles Tris-Cl<sup>-</sup> buffer pH 7.4, 30  $\mu$ moles potassium phosphate buffer, pH 7.4, 48  $\mu$ moles succinate, 4  $\mu$ moles ATP and 20  $\mu$ moles MgSO<sub>4</sub>. Total vol: 2 ml. 37°. Air. Incubations were carried out in a shaking bath at 100 oscillations per min and 4 cm displacement. At the end of the incubation the reaction mixtures were centrifuged, the mitochondrial pellet washed twice with 0.15 M KCl, resuspended in KCl, frozen and thawed twice and then centrifuged. Glutamate dehydrogenase activity was determined in the supernatant fluid. The activity is expressed as total units [12] present in the incubations. Glutamate dehydrogenase in the controls remained fully active during the incubation.

Table 3

Effect of CP and ATP on activity of glutamate dehydrogenase.

Conditions		Activity	
( $\mu$ moles)	( $\mu$ moles)	1 hr	2 hr
—	—	14.0	12.0
10	—	9.4	6.0
20	—	8.0	~0
50	—	3.4	~0
—	4.8	21.0	22.0
—	9.6	23.0	24.0
10	4.8	16.0	12.0
20	4.8	11.0	6.0
50	4.8	5.0	0
10	9.6	16.0	12.0
20	9.6	13.0	6.0
50	9.6	5.4	0

Each tube contained in 2 ml: 2 mg CTAB extract, 80  $\mu$ moles phosphate buffer, pH 7.6, and the additions indicated. 1 hr at 38°. 100  $\mu$ l aliquots taken for determination of glutamate dehydrogenase. Activity is given as units present in the incubation mixtures.

Table 4

Effect of biosynthetic CP on frog liver glutamate dehydrogenase.

Addition ( $\mu$ moles)			Citrulline formed ( $\mu$ moles)	Incubation time (hr)	Units glutamate dehydrogenase
KHCO <sub>3</sub>	Acetylglutamate	Ornithine			
—	—	—	0	1	16.0
—	—	—	0	2	15.2
—	—	—	0	3	15.2
+	+	—	0	1	14.0
+	+	—	0	2	12.0
+	+	—	0	3	9.6
+	+	+	14	1	15.2
+	+	+	17	2	15.2
+	+	+	20	3	15.2

Each tube contained: 2.3 mg CTAB extract; 100  $\mu$ moles Tris-Cl<sup>-</sup> buffer, pH 7.4; 20  $\mu$ moles MgSO<sub>4</sub>; 80  $\mu$ moles phosphoenolpyruvate; 10  $\mu$ moles ATP; 50  $\mu$ moles NH<sub>4</sub>Cl and 80  $\mu$ g pyruvate kinase. When indicated 50  $\mu$ moles KHCO<sub>3</sub>, 10  $\mu$ moles acetylglutamate and 52  $\mu$ moles ornithine were added. Final vol: 2 ml. 37°. At the end of the incubation 0.5 ml aliquots were chilled, deproteinized with 5 ml 1 N HClO<sub>4</sub> and citrulline measured. Another portion was chilled in ice and glutamate dehydrogenase activity determined immediately. Activity is expressed as total units [12] present in the incubation. Activity remained unchanged in samples kept at 0°.

necessary to test the *in vivo* significance of enzyme inactivation arising from carbamylation are difficult to attain. Nevertheless, as illustrated here with the main enzymes of CP synthesis and utilization in ureotelic animals, a step has been taken into that direction. By refining or modifying such conditions it may be possible to acquire additional insight into the phenomenon.

### Acknowledgements

This work was supported by a Grant-in-Aid from the U.S. Public Health Service AM 13119 and AM 01855.

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