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Acute effects of glucagon on citrulline biosynthesis

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Mitochondria isolated from livers of rats fed on different diets showed altered capacity to synthesize citrulline. Glucagon, 15 min after injection, increased citrulline biosynthesis, except after the high-protein diet. A significant correlation between citrulline biosynthesis and *N*-acetylglutamate content with and without glucagon treatment was shown when rats were fed on a standard or a carbohydrate diet. Different diets modified carbamoyl phosphate synthetase I (EC 6.3.4.16) and *N*-acetylglutamate synthase (acetyl-CoA : L-glutamate *N*-acetyltransferase, EC 2.3.1.1) activities. Glucagon did not modify these activities.

Glucagon increases urea production in the perfused liver (Mallette *et al.*, 1969) or isolated hepatocytes (Bryla *et al.*, 1977; Triebwasser & Freedland, 1977). This effect is partly related to increased citrulline biosynthesis, as shown in mitochondria isolated from liver of treated rats (Yamazaki & Graetz, 1977) or in hepatocytes incubated with glucagon (Bryla *et al.*, 1977; Triebwasser & Freedland, 1977). It has been assumed that the stimulation of citrulline biosynthesis was related to an increased rate of mitochondrial respiration leading to an elevated concentration of ATP (Bryla *et al.*, 1977; Yamazaki, 1975), a substrate of carbamoyl phosphate synthetase I (EC 6.3.4.16). But more recently it has been reported that glucagon can stimulate citrulline biosynthesis after acute treatment of rats (Hensgens *et al.*, 1980; Cathelineau *et al.*, 1980) or after treatment of isolated hepatocytes (Hensgens *et al.*, 1980) by increasing mitochondrial *N*-acetylglutamate, the activator of carbamoyl phosphate synthetase I. However, other workers (Titheradge & Haynes, 1980) claimed that *N*-acetylglutamate concentration did not change after administration of glucagon. Because of these conflicting results, we have tried to obtain new information on the exact effect of acute glucagon treatment.

It has been reported that *N*-acetylglutamate content and citrulline or urea biosynthesis in rat liver mitochondria are correlated and related to the protein content of the diet (Shigesada & Tatibana, 1971; McGivan *et al.*, 1976; Saheki *et al.*, 1977). We decided to study the acute effect of glucagon on citrulline biosynthesis and *N*-acetylglutamate concentration in rats fed on various diets. Our aim was to clarify the effect of the hormone, especially in animals with low basal *N*-acetylglutamate concentrations when fed on a low-protein diet.

Experimental

Albino Wistar male rats (150–250 g) were used. The rats were housed at room temperature and fed *ad libitum* on the following diets: a standard laboratory diet with 19% protein; a diet without protein, which was 10% (w/v) dextrin–maltose and 10% (w/v) glucose in water + minerals (0.1% NaCl and 0.1% KCl) + vitamins (2 ml of Hydrosol Polyvitaminé Roche per litre; Produits Roche S.A., Neuilly-sur-Seine, France); a diet with 40% lipid and 60% carbohydrate without protein; and a diet with 70% protein (all the diets except the carbohydrate one were obtained from U.A.R., Villemoisson, France). A minimum of ten animals were kept on each diet for 3 days. Some animals were also tested after 2 days and 4 days of special feeding. Half of the animals were used as controls and were injected intraperitoneally with saline (0.1 ml of 0.9% NaCl), and the other animals were injected in the same way with glucagon Novo (Novo Industrie Pharmaceutique, Paris, France) (20 μg of glucagon/100 g body wt.). At 15 min after the injection, the animals were decapitated, and the livers quickly removed for the preparation of mitochondria. The effect of the hormone was judged by the measurement of blood glucose concentration on a sample drawn from the inferior vena cava. Glucagon treatment increased plasma glucose concentration (means $\pm$ S.D.) from 8.7 ± 1.3 to 12.7 ± 1.4 mM in rats on standard, carbohydrate or high-protein diets. No change in glucose concentration was found in rats on the carbohydrate + lipid diet (8.7 ± 1.9 mM). Mitochon-

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dria were prepared by the method of Hogeboom (1955) in cold 0.27 M-sucrose solution in 2 mM-Tris/HCl, pH 7.4. Mitochondrial protein was determined by the biuret method (Layne, 1957). Controls of respiration were performed with a Clark oxygen electrode in either State III or State IV (Chance & Williams, 1956), with freshly isolated mitochondria. Respiratory control ratios (State III/State IV) were 5.02 ± 1.18 for control rats and 5.02 ± 0.84 for glucagon-treated rats with succinate as respiratory substrate.

Citrulline synthesis was studied by the method of Charles *et al.* (1967), by incubating 3–5 mg of mitochondrial protein for 2.5 and 5 min at 37°C. Citrulline formed was measured colorimetrically by the method of Ceriotti & Spandrio (1965) with an automatic apparatus. Basal *N*-acetylglutamate was measured in freshly isolated mitochondria by the method of Meijer & Van Woerkom (1978), by using the ability of *N*-acetylglutamate to activate carbamoyl phosphate synthetase I (ammonia) activity.

Frozen and thawed batches of mitochondria were disrupted by addition of 4 vol. of distilled water, and used for the assay of enzymic activities. Carbamoyl phosphate synthetase I (ammonia) (EC 6.3.4.16) and ornithine transcarbamoylase (EC 2.1.3.3) were measured by the methods of Brown & Cohen (1959) and Snodgrass (1968) respectively. *N*-Acetylglutamate synthase (EC 2.3.1.1) was determined by the method of Coudé *et al.* (1979). All the results are expressed as means ± S.D.

$NaH^{14}CO_3$ (56 Ci/mol) for the measurement of *N*-acetylglutamate and L-[1-^{14}C]glutamic acid (0.5 Ci/mol) for the *N*-acetylglutamate synthase reaction were purchased from Amersham.

Results and discussion

Citrulline synthesis by mitochondria from control and glucagon-treated rats in several dietary states was determined in the presence of two oxidizable substrates, succinate and L-glutamate, and in the presence of ATP + uncouplers with or without exogenous *N*-acetylglutamate. Uncoupled conditions were used so that ATP production was no longer the limiting factor for citrulline synthesis. In uncoupled conditions ATP can enter the mitochondria (Graafmans *et al.*, 1968). When citrulline biosynthesis (Table 1) was determined in control rats on standard diet with ATP + oligomycin + 2,4-dinitrophenol, it was not significantly different from that observed with succinate, but slightly higher ($P < 0.05$) than with L-glutamate. However, citrulline synthesis was much lower than in rats on protein-free diets whatever the source of energy used. McGivan *et al.* (1976) have also described the low capacity of mitochondria to synthesize citrulline when rats were fed on 20% glucose without protein. When *N*-acetylglutamate was added, citrulline biosynthesis was always higher whether standard (Hensgens *et al.*, 1980) or protein-free diets were given (Table 1), suggesting that *N*-acetylglutamate was a limiting factor for citrulline synthesis.

When rats were fed on a 70%-protein diet, citrulline biosynthesis was strongly increased as compared with control rats on the standard diet when succinate or L-glutamate was used as source of energy. With the high-protein diet, addition of *N*-acetylglutamate in uncoupled conditions surprisingly inhibited the citrulline biosynthesis observed with succinate ($P < 0.01$) or with ATP + uncouplers ($P < 0.02$).

When the activities of the mitochondrial enzymes of the urea cycle were measured after the standard, carbohydrate or high-protein diet, we found respectively 61 ± 14, 32 ± 9 and 78 ± 20 nmol/min per mg of protein for carbamoyl phosphate synthetase I (ammonia) and 2040 ± 725, 2130 ± 61 and 2500 ± 540 nmol/min per mg for ornithine transcarbamoylase. As reported by McGivan *et al.* (1976) and Schimke (1962), we found that carbamoyl phosphate synthetase was altered by the different diets, whereas ornithine transcarbamoylase was not significantly modified. The variations of the carbamoyl phosphate synthetase I were too small to explain the large discrepancies observed for citrulline biosynthesis on the different diets. As shown in Table 1, *N*-acetylglutamate content was probably the determining factor in the regulation of citrulline production on the different diets. All the values were significantly different from that obtained with standard diet ($P < 0.02$ for carbohydrate diet, $P < 0.001$ for carbohydrate + lipid and for high-protein diet).

When rats on standard or carbohydrate diet were injected with glucagon, citrulline synthesis was greatly increased whatever the source of energy used. This increase was also observed in the presence of ATP, but it was much less when *N*-acetylglutamate was added (Table 1). However, glucagon had a very slight effect on citrulline synthesis in rats fed on a carbohydrate + lipid diet, and no effect on that in rats fed on a high-protein diet. These experiments suggest that ATP was not, as reported by several workers, the only determining factor in the increase in citrulline synthesis after glucagon treatment (Bryla *et al.*, 1977; Yamazaki, 1975). Indeed, for all but the high-protein diet, when ATP was added together with oligomycin + 2,4-dinitrophenol, citrulline synthesis after glucagon treatment was always higher than that observed with control rats. On the other hand, glucagon did not modify significantly the values of activities of carbamoyl phosphate synthetase I and ornithine transcarbamoylase. In rats on standard, carbohydrate, carbohydrate + lipid and high-protein diets, these activities were respectively 51 ± 15,

Table 1. *Citrulline synthesis by rats fed on various diets and effect of acute glucagon treatment*
Mitochondria were isolated from liver after 3 days of the diet and were incubated in a standard medium for determination of citrulline synthesis. The standard medium consisted of: 50mM-Tris/HCl, pH 7.4, 35mM-KCl, 5mM-KH_2PO_4, 1mM-EDTA, 1mM-$MgCl_2$, 10mM-ornithine, 16.6mM-$KHCO_3$, 10mM-NH_4Cl; 10mM-succinate, or 10mM-glutamate, or 2mM-ATP (+10μg of oligomycin/ml + 0.04mM-2,4-dinitrophenol, with or without 5mM-*N*-acetylglutamate), were added. The *N*-acetylglutamate values represent the endogenous amount found in freshly isolated mitochondria. Results are expressed as means ± S.D. for the numbers of experiments in parentheses. Statistical significance was calculated by using Student's *t* test (ns, not significant; N.D., not determined).

	Citrulline synthesis (nmol/min per mg of protein)				*N*-Acetylglutamate (nmol/mg of protein)
Diets	Succinate	L-Glutamate	ATP + oligomycin + 2,4-dinitrophenol	ATP + *N*-acetylglutamate + oligomycin + 2,4-dinitrophenol	
Standard					
control	28.2 ± 8.2 (12)	26.7 ± 5.9 (12)	32.8 ± 6.0 (9)	N.D.	0.80 ± 0.16 (9)
+ glucagon	55.2 ± 13.4 (12)	42.6 ± 11.4 (12)	44.6 ± 7.7 (9)	N.D.	1.16 ± 0.15 (9)
P	<0.001	<0.001	<0.01		<0.001
Carbohydrate					
control	1.8 ± 1.8 (13)	4.4 ± 2.4 (13)	5.7 ± 5.2 (13)	16.7 ± 3.6 (8)	0.65 ± 0.12 (13)
+ glucagon	12.3 ± 4.7 (13)	16.1 ± 4.4 (13)	20.8 ± 7.5 (13)	26.0 ± 5.7 (6)	1.02 ± 0.37 (11)
P	<0.001	<0.001	<0.001	<0.02	<0.001
Carbohydrate + lipid					
control	0.4 ± 0.4 (9)	2.0 ± 1.0 (7)	1.1 ± 0.9 (9)	16.5 ± 3.6 (4)	0.54 ± 0.09 (11)
+ glucagon	0.9 ± 0.6 (9)	4.4 ± 2.0 (7)	3.0 ± 1.0 (9)	24.8 ± 6.9 (4)	0.71 ± 0.14 (11)
P	ns	<0.02	<0.01	ns	<0.01
High-protein					
control	71.8 ± 16.3 (4)	43.0 ± 13.0 (4)	40.2 ± 6.0 (4)	28.9 ± 2.5 (4)	1.63 ± 0.30 (7)
+ glucagon	65.5 ± 7.0 (6)	38.5 ± 6.6 (6)	44.8 ± 12.4 (6)	26.4 ± 7.8 (4)	1.78 ± 0.48 (7)
P	ns	ns	ns	ns	ns

38 ± 13, 42 ± 7 and 69 ± 15 nmol/min per mg of protein for carbamoyl phosphate synthetase I and 2450 ± 1069, 2107 ± 426, 2147 ± 388 and 2600 ± 712 nmol/min per mg of protein for ornithine transcarbamoylase after glucagon treatment. But glucagon significantly increased the *N*-acetylglutamate concentration in mitochondria in all dietary conditions except the high-protein diet, where *N*-acetylglutamate was already high (Table 1). Hensgens *et al.* (1980) and Cathelineau *et al.* (1980) have previously reported such an increase in *N*-acetylglutamate after glucagon treatment of rats fed on standard diet. Hensgens *et al.* (1980) have also described the lack of glucagon effect on citrulline and urea synthesis by hepatocytes with high mitochondrial content of *N*-acetylglutamate, as we observed with the high-protein diet. In these two cases the mitochondrial content of *N*-acetylglutamate was not modified by glucagon.

Fig. 1 shows the relationship between citrulline synthesis and *N*-acetylglutamate content in mitochondria from rats fed on a standard or a carbohydrate diet. When citrulline synthesis was measured with succinate or ATP + oligomycin + 2,4-dinitrophenol, the correlation was significant with both diets. Data obtained from control and glucagon-treated rats were plotted together to draw each correlation curve. In uncoupled mitochondria isolated from rats loaded with a mixture of amino acids, Stewart & Walser (1980) also reported a relation between *N*-acetylglutamate content and carbamoyl phosphate synthetase I activity in intact mitochondria. In the present experiments, when succinate was used as oxidizable substrate, the slopes of the curves were very different, and for a given concentration of *N*-acetylglutamate citrulline synthesis was always lower with rats fed on a carbohydrate diet. These curves had the same slope with ATP + uncouplers (Fig. 1), but the citrulline synthesis was always different. With succinate an impaired uptake of ornithine or an increased efflux of *N*-acetylglutamate might be responsible, together with a decreased carbamoyl phosphate synthetase activity, for a lower citrulline synthesis by the mitochondria isolated from rats fed on a carbohydrate diet. When ATP + uncouplers were added, the movements of metabolites across the mitochondrial membrane were no longer vectorial. In these conditions the only difference that could be seen was the difference in the citrulline synthesis owing to a decreased carbamoyl phosphate synthetase activity.

On the other hand, the intercept of the slope with the abscissa shows that citrulline synthesis was nil when *N*-acetylglutamate concentration was 0.40 nmol/mg of protein in rats fed on a standard

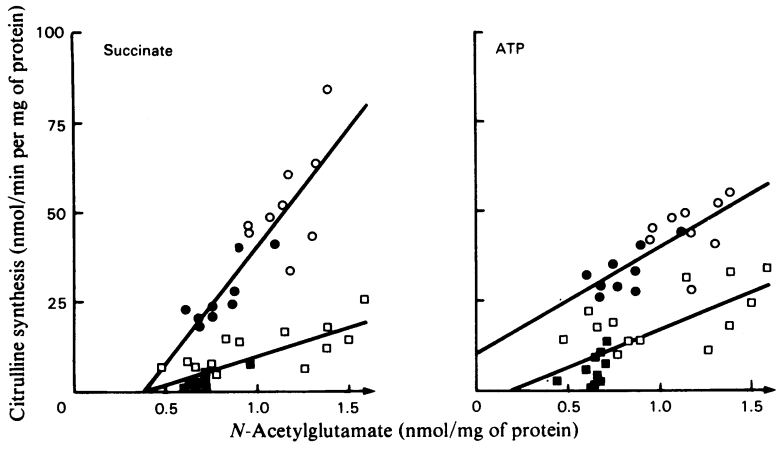

Fig. 1. *Relationship between citrulline synthesis and N-acetylglutamate concentration in mitochondria from rats fed on a standard or a carbohydrate diet in control and glucagon-treated rats*

Each point represents results of a single experiment. ●, Control rats fed on standard diet; ■, control rats fed on carbohydrate diet; ○, glucagon-treated rats fed on standard diet; □, glucagon-treated rats fed on carbohydrate diet. When citrullinogenesis was measured in succinate medium, $r = 0.86$, $y = 65x - 25$, $P < 0.001$, for rats fed on standard diet, and $r = 0.81$, $y = 16.3x - 6.5$, $P < 0.001$, for rats fed on carbohydrate diet. When citrullinogenesis was measured in ATP medium, $r = 0.73$, $y = 28.4x + 10.7$, $P < 0.001$, for rats fed on standard diet, and $r = 0.73$, $y = 21.2x - 4.1$, $P < 0.001$, for rats fed on carbohydrate. The *N*-acetylglutamate values represent the endogenous amount found in freshly isolated mitochondria.

diet or a carbohydrate diet. These last results imply that part of the *N*-acetylglutamate was unable to activate carbamoyl phosphate synthetase I (McGivan *et al.*, 1976). However, with ATP + uncouplers the line for values with standard diet extrapolates back to the origin and that drawn for values with carbohydrate diet cuts the abscissa at a concentration of *N*-acetylglutamate (0.19 nmol/mg of protein) much lower than with succinate. In these uncoupled conditions much more *N*-acetylglutamate appears to be available for the activation of carbamoyl phosphate synthetase I.

The increase in *N*-acetylglutamate concentration after glucagon treatment is observed in a range of concentration much higher than the K_a of the carbamoyl phosphate synthetase I for *N*-acetylglutamate. This K_a was 0.1 mM for the purified enzyme (Elliott & Tipton, 1974) and 0.2 mM in mitochondrial extracts (McGivan *et al.*, 1976). The *N*-acetylglutamate concentrations found in our work are around 1 mM, assuming that 1 mg of mitochondrial protein corresponds to 1 μl (Harris & Van Dam, 1968). Actually McGivan *et al.* (1976) have postulated that part of the *N*-acetylglutamate measured was not available for the activation of the carbamoyl phosphate synthetase I. Their assumption is in agreement with our own observations, where citrulline was nil for 0.40 mM-*N*-acetylglutamate in rats on standard or carbohydrate diet. Therefore the mean values of the available *N*-acetylglutamate become 0.40 mM in untreated rats fed on standard diet and 0.25 mM in untreated rats fed on carbohydrate diet, values closely similar to those calculated by McGivan *et al.* (1976). Available *N*-acetylglutamate after glucagon treatment increases from 0.40 to 0.77 mM for rats on standard diet and from 0.25 to 0.62 mM for those on the carbohydrate diet (Table 1). A variation of *N*-acetylglutamate between these concentrations must be effective to increase significantly the carbamoyl phosphate synthetase activity.

Several factors may directly control the intramitochondrial concentration of *N*-acetylglutamate in different conditions. *N*-Acetylglutamate synthase activity varied widely under the different dietary conditions. This enzyme activity increased from 23.8 ± 7.4 nmol/h per mg of protein with standard diet to 45.9 ± 23.1 nmol/h per mg on the 70%-protein diet and decreased to 11.2 ± 4.3 and 7.6 ± 3.4 nmol/h per mg on carbohydrate and carbohydrate + lipid diets respectively. Fig. 2 shows a significant correlation between intramitochondrial *N*-acetylglutamate concentration and *N*-acetylglutamate synthase activity whatever the dietary state. Glucagon did not modify this activity significantly with the different diets. But after injection of glucagon, glutamate, acetyl-CoA and arginine, substrates and cofactor of *N*-acetylglutamate synthetase, are probably the key factors in the short-term regulation of the intramitochondrial concentration of *N*-acetylglutamate, as previously observed with other stimuli (Shigesada *et al.*, 1978;

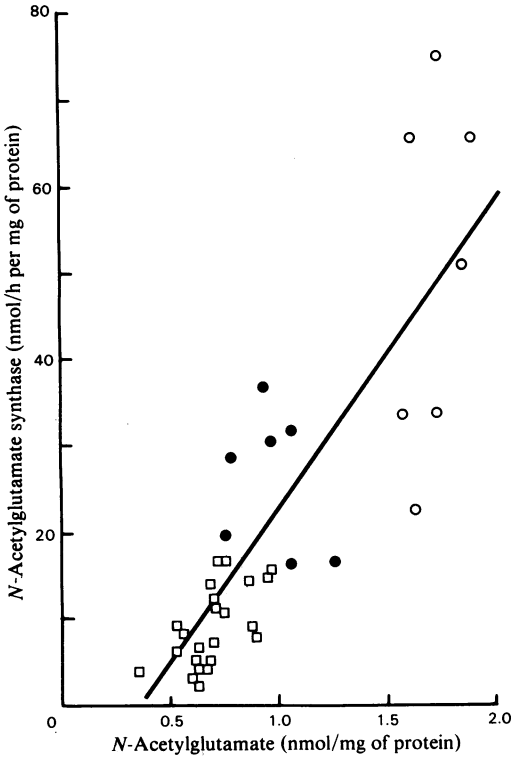

Fig. 2. *Relationship between N-acetylglutamate synthase activity and N-acetylglutamate concentration in mitochondria freshly isolated from rats fed on various diets*

N-Acetylglutamate synthase activity is plotted against endogenous amount of *N*-acetylglutamate in freshly isolated mitochondria ($r = 0.83$, $P < 0.001$). □, Carbohydrate or carbohydrate + lipid diets; ●, standard diet; ○, high-protein diet.

Shigesada & Tatibana, 1971; Stewart & Walser, 1980). Unfortunately the concentrations of these substrates and cofactor were not available in our work on whole animals. However, in isolated hepatocytes, Siess *et al.* (1977), using a rapid method to separate cytosolic and particulate fractions, have shown a 25% increase in acetyl-CoA together with a 60% decrease in glutamate in mitochondria after incubation with glucagon. But, as no information was available on the variation of *N*-acetylglutamate under these conditions, it is difficult to draw any conclusion on the relative role of these metabolites.

Although several authors (Bryla *et al.*, 1977; Yamazaki, 1975) claimed that ATP was responsible for the increased citrulline synthesis after glucagon treatment or that there was no change in intramitochondrial concentration of *N*-acetylglutamate (Titheradge & Haynes, 1980), Hensgens *et al.* (1980) and Cathelineau *et al.* (1980) were able to show an increase in this cofactor by glucagon. This increased *N*-acetylglutamate is probably the main factor controlling the increased citrulline synthesis.

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