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The Acute Action of Ammonia on Rat Brain Metabolism *in vivo*

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1. Acute NH_4^+ toxicity was studied by using a new apparatus that removes and freezes the brains of conscious rats within 1s. 2. Brains were removed and frozen 5min after intraperitoneal injection of ammonium acetate (2–3min before the onset of convulsions). Arterial [NH_4^+] rose from less than 0.01 to 1.74mM at 4–5min. The concentrations of all glycolytic intermediates measured, except glucose 6-phosphate, were increased by the indicated percentage above the control value as follows: glucose (by 41%), fructose 1,6-diphosphate (by 133%), dihydroxyacetone phosphate (by 164%), α-glycerophosphate (by 45%), phosphoenolpyruvate (by 67%) and pyruvate (by 26%). 4. Citrate and α-oxoglutarate concentrations were unchanged and that of malate was increased (by 17%). 5. Adenine nucleotides and P_i concentrations were unchanged but the concentration of creatine phosphate decreased slightly (by 6%). 6. Brain [NH_4^+] increased from 0.2 to 1.53mM. Net glutamine synthesis occurred at an average rate of 0.33μmol/min per g. 7. The rate of brain glucose utilization was measured *in vivo* as 0.62μmol/min per g in controls and 0.81μmol/min per g after NH_4^+ injection. 8. The arteriovenous difference of glucose and O_2 increased by 35%. 9. No significant arteriovenous differences of glutamate or glutamine were detected. Thus, although much NH_4^+ was incorporated into glutamine the latter was not rapidly released from the brain to the circulation. 10. Plasma [K^+] increased from 3.3 to 5.4mM. 11. The results indicate that NH_4^+ stimulates oxidative metabolism but does not interfere with brain energy balance. The increased rate of oxidative metabolism could not be accounted for only on the basis of glutamine synthesis. We suggest that increased extracellular [NH_4^+] and [K^+] decreased the resting transmembrane potential and stimulated Na^+,K^+-stimulated adenosine triphosphatase activity thus accounting for the increased metabolic rate.

Increased blood [NH_4^+] may result in coma after extended periods, whereas an acute increase to relatively high concentrations causes convulsions (McIlwain & Bachelard, 1971). Within brain NH_4^+ is removed by reductive amination of α-oxoglutarate and amidation of glutamate to form glutamine (Weil-Malherbe, 1962; Berl, 1971). It was initially suggested that toxicity was due to depletion of citric acid-cycle intermediates (Recknagel & Potter, 1951; Bessman & Bessman, 1955). However, studies of acute NH_4^+ toxicity in anaesthetized animals have shown no change in [α-oxoglutarate] in brain (Hindfelt & Siesjö, 1971) and an increased rate of CO_2 fixation (Berl *et al.*, 1962; Waelsch *et al.*, 1964) probably through pyruvate carboxylase (Felicioli *et al.*, 1966). Suggestions that NH_4^+ interferes with energy stores by various means (Schenker *et al.*, 1967; Clarke, 1971) seem unlikely as recent results have shown no change in cerebral ATP concentration after acute NH_4^+ administration to rats (Hindfelt & Siesjö, 1971).

The development of a new instrument, the brain-freezer, which removes and freezes rat brain within 1s, has permitted study of NH_4^+ toxicity in conscious rats (Veech *et al.*, 1973). The metabolic derangements accompanying acute NH_4^+ toxicity were examined more closely than ever before by measuring key intermediates of the glycolytic pathway and citric acid cycle, indicators of the energy state and representative amino acids in brains of unanaesthetized rats.

Materials and Methods

Materials

Rats. Male albino rats of the Wistar–Furth strain weighing about 225g were from Carworth, New York, N.Y., U.S.A. All rats were starved for 48h before experimentation.

Reagents. Enzymes and co-enzymes were from Boehringer Mannheim Corp., New York, N.Y., U.S.A. Glutaminase, prepared from pig kidney (Kvamme *et al.*, 1970), was kindly given by Dr. H. Weil-Malherbe. All other chemicals were of reagent grade.

Methods

Analytical methods. Metabolites were determined spectrophotometrically or fluorimetrically by enzymic techniques: ADP and AMP (Adam, 1963); ATP (Lamprecht & Trautschold, 1963); alanine (Pfleiderer, 1963*a*); NH_4^+ (Kirsten *et al.*, 1963); aspartate (Pfleiderer, 1963*b*); citrate (Moellering & Gruber, 1966); creatine phosphate (Lowry *et al.*, 1964); glycogen (Lowry & Passonneau, 1972*a*); fructose 1,6-diphosphate and dihydroxyacetone phosphate (Bücher & Hohorst, 1963); glucose (Slein, 1963); glucose 6-phosphate (Hohorst, 1963*a*); glutamate (Bernt & Bergmeyer, 1963); α-glycerophosphate (Hohorst, 1963*b*); lactate (Hohorst, 1963*c*); malate (Hohorst, 1963*d*); α-oxoglutarate (Bergmeyer & Bernt, 1963) O_2 (Van Slyke & Neil, 1924); phosphate (Guynn *et al.*, 1972); phosphoenolpyruvate (Lowry & Passonneau, 1972*b*); pyruvate (Bücher *et al.*, 1963). Where possible, several metabolites were determined in the same cuvette by sequential addition of the appropriate enzymes (lactate, malate and α-glycerophosphate; dihydroxyacetone phosphate, fructose 1,6-diphosphate, α-oxoglutarate and pyruvate). Labile metabolites were measured immediately after extraction and the extracts were deep-frozen (−90°C). The remaining metabolites were determined within a few days.

Glutamine measurement presented special difficulties (see Lund, 1970). Poor recoveries were obtained because of contamination of commercial glutaminase with glutamate decarboxylase (Sigma grade IV and to a lesser but significant extent Sigma grade V). However, glutaminase prepared from pig kidney (Kvamme *et al.*, 1970) had no glutamate decarboxylase activity. The incubation mixture had final concentrations of 100mM-potassium phosphate buffer, pH9.0, 10mM-dithiothreitol, 2mM-EDTA, 0.25mg of enzyme/ml (3.5 units/mg) and up to 0.5mM-glutamine. The samples were incubated at 25°C for 1h and then centrifuged at 1000*g* to remove debris. A portion was removed and assayed for glutamate. Recovery of added glutamine was quantitative.

Removal and treatment of brain. The supratentorial portion of the brain was removed and frozen within 1s (Veech *et al.*, 1973). The brain samples were ground under liquid N_2 and extracted as described by Nelson *et al.* (1966) for all metabolites except P_i, which was determined after methanol extraction (Hawkins *et al.*, 1973) to avoid P_i contamination by bone.

Brain utilization of glucose in vivo. The rate of glucose utilization was measured by a technique based on that described by Gaitonde (1965). Briefly, the accumulation of ^{14}C in the acid-soluble fraction of brain (excluding glucose) is measured after a single intravenous injection of [2-^{14}C]glucose. The rate of glucose utilization is calculated according to the equation:

$$\text{Rate of glucose utilization } (\mu\text{mol/min per g}) = \frac{^{14}\text{C accumulated in brain (d.p.m./g)}}{\int_0^t \text{glucose specific radioactivity (d.p.m.} \cdot \text{min} \cdot \mu\text{mol}^{-1})}$$

On the basis of the assumption that brain cell and plasma glucose rapidly equilibrate, the integral was evaluated by plotting plasma glucose specific radioactivity against time and measuring the area beneath the curve by planimetry.

Results

Blood NH_4^+ concentrations

$[NH_4^+]$ is normally in the range 0.002–0.007mM in rat blood (Lund *et al.*, 1970; Williams *et al.*, 1972). At 4–5min after intraperitoneal injection of ammonium acetate the concentration rose to 1.74mM (Table 1). Between 7 and 10min the rats convulsed.

Blood gases and pH

Blood pH remained constant after NH_4^+ injection, although arterial CO_2 pressure dropped markedly (Table 2). This may have resulted from the more rapid penetration of NH_4^+ into tissues as compared with acetate resulting in decreased plasma $[HCO_3^-]$ (Webster & Gabusda, 1958; Hindfelt & Siesjö, 1971).

Arteriovenous differences of metabolites across brain

The mean arteriovenous difference of glucose and O_2 were increased 35% and 33% respectively above the control values (Table 3). The quantity of O_2 removed from blood was in excess of that required to combust all glucose by about 20%. The additional fuel required was probably supplied by ketone bodies, not measured in these experiments, which can account for this quantity in 48h-starved rats (Hawkins *et al.*, 1971). Little, if any, glucose removed from the blood was converted into blood lactate by normal rats (4%), whereas 13% was converted after NH_4^+ injection. The arteriovenous difference of $[NH_4^+]$ was 0.57mM at 4–5min whereas arteriovenous differences of glutamate and glutamine were undetectable.

Table 1. *Concentrations of metabolites in arterial and venous blood across the brain*

The values are means (±S.E.M.) with the number of observations indicated in parentheses. Arteriovenous differences were measured across brain of conscious rats as described by Hawkins & Veech (1973). For other details see the Materials and Methods section.

Treatment and time	Blood sample	[Glucose] (mM)	[Lactate] (mM)	[NH_4^+] (mM)	[Glutamate] (mM)	[Glutamine] (mM)
None	Artery	5.10±0.22	2.64±0.38	<0.01	0.141±0.008	0.982±0.028
0min (8)	Vein	4.53±0.19	2.68±0.37	<0.01	0.141±0.008	0.994±0.034
Ammonium acetate in-	Artery	5.74±0.21	3.20±0.45	1.74±0.19	0.124±0.008	1.06±0.04
jected 4–5min (8)	Vein	4.97±0.16	3.43±0.52	1.08±0.10	0.128±0.008	1.07±0.03

Table 2. *Blood gases and pH after ammonium acetate injection*

The values are means (±S.E.M.) of four individual measurements. For other details see the Materials and Methods section.

Treatment and time	Blood sample	pH	CO_2 (kPa)	CO_2 (Torr)	O_2 (mM)
None	Artery	7.44±0.009	5.38±0.27	(40.4±2.0)	8.46±0.24
0min	Vein	7.37±0.003	6.67±0.11	(50.1±0.8)	4.22±0.28
Ammonium acetate in-	Artery	7.49±0.026	3.46±0.15	(26.0±1.1)	8.36±0.22
jected 4–5min	Vein	7.37±0.004	5.81±0.21	(43.6±1.6)	2.70±0.56

Table 3. *Arteriovenous differences of metabolites across the brain*

The values are means (±S.E.M.) of the arteriovenous differences and are derived from the experiments contained in Table 1. The symbols + and − indicate appearance or removal of a metabolite and the symbols * and ** indicate the statistical significance of the arteriovenous differences at the 5% and 1% levels respectively as compared with controls. For other details see Table 1.

Treatment and time	[Glucose] (mM)	[Lactate] (mM)	[NH_4^+] (mM)	[Glutamate] (mM)	[Glutamine] (mM)	[O_2] (mM)
None 0min	−0.57±0.05	+0.04±0.04	—	0.000±0.005	+0.011±0.04	4.23±0.16
Ammonium acetate injected 4–5min	−0.77±0.07*	+0.23±0.10**	−0.57±0.12**	+0.004±0.003	+0.02±0.04	5.64±0.50*

Brain glucose utilization

Brain glucose utilization was 0.624±0.026 (S.E.M.) μmol/min per g in control rats and increased by 29% after NH_4^+ injection to 0.806±0.035. Values are the mean of nine individual determinations and the difference between the two groups of animals is statistically significant at the 1% level. From the rate of glucose utilization and the arteriovenous difference whole-brain blood flow may be calculated:

$$\text{Blood flow (ml/min per g)} = \frac{\text{glucose utilization (}\mu\text{mol/min per g)}}{\text{arteriovenous difference (}\mu\text{mol/ml)}}$$

Thus by using the values given above and in Table 3, blood flow was about 1.1ml/min per g in untreated rats and 1.05ml/min per g after NH_4^+ injection.

Intermediary metabolites of brain

The brain content of glycogen was decreased and the concentrations of all the glycolytic intermediates, with the exception of glucose 6-phosphate, were raised

substantially (Table 4). The concentrations of citrate and α-oxoglutarate remained constant and the concentration of malate was increased. The calculated concentration of oxaloacetate decreased as a consequence of the decreased cytoplasmic [NAD^+]/[NADH] ratio (see below).

Unchanged energy balance of brain

There were no changes in the concentrations of adenine nucleotides or P_i and hence no change in the phosphorylation state of the adenine nucleotides in the brain (Table 5). A slight decrease in the concentration of creatine phosphate was found.

NH_4^+ and amino acid content of brain

The concentration of NH_4^+ in brain rose from 0.2 to 1.53mM. Much NH_4^+ was incorporated into glutamine (1.65μmol/g) and a lesser amount into alanine (Table 6). The concentration of aspartate was decreased, consistent with the lowered concentration

Table 4. *Effect of ammonium acetate on the concentrations of glycolytic and citric acid-cycle intermediates in rat brain*

The values are means (±S.E.M.) of six to eight individual determinations. The symbols * and ** indicate statistical significance at the 5% and 1% levels respectively. Rats were injected intraperitoneally with 2M-ammonium acetate, pH7 (10mmol/kg body wt.), or an equivalent volume of 0.154M-NaCl and killed at 5min. For other details see the Materials and Methods section. The oxaloacetate concentration was calculated from the formula:

$$[\text{Oxaloacetate}] = \frac{[\text{pyruvate}] \times [\text{malate}]}{[\text{lactate}]} \times \frac{K_{MDH}}{K_{LDH}}$$

where K_{MDH} and K_{LDH} are the equilibrium constants of lactate dehydrogenase and malate dehydrogenase respectively (Williamson *et al.*, 1967).

Metabolite	Concn. in control (mM)	Concn. after injection of ammonium acetate (mM)
Glycogen	2.80 ±0.05	2.00 ±0.13**
Glucose	0.745 ±0.044	1.05 ±0.07**
Glucose 6-phosphate	0.154 ±0.004	0.150 ±0.004
Fructose 1,6-diphosphate	0.009 ±0.001	0.021 ±0.002**
Dihydroxyacetone phosphate	0.011 ±0.001	0.029 ±0.003**
α-Glycerophosphate	0.081 ±0.002	0.129 ±0.004**
Phosphoenolpyruvate	0.003 ±0.0005	0.005 ±0.0005*
Pyruvate	0.099 ±0.003	0.125 ±0.005**
Lactate	1.25 ±0.05	2.39 ±0.11**
Citrate	0.319 ±0.004	0.302 ±0.010
α-Oxoglutarate	0.208 ±0.007	0.204 ±0.007
Malate	0.249 ±0.006	0.291 ±0.006**
Oxaloacetate	0.0049±0.0001	0.0038±0.0001**

Table 5. *Effect of ammonium acetate injection on adenine nucleotide and creatine phosphate concentrations of brain*

The values are means (±S.E.M.) of six to eight determinations. The symbol * indicates statistical significance at the 5% level. For other details see Table 4.

Metabolite	Concn. in control (mM)	Concn. after injection of ammonium acetate (mM)
ATP	2.49 ±0.03	2.48 ±0.03
ADP	0.556±0.013	0.544±0.010
AMP	0.051±0.002	0.056±0.001
Creatine phosphate	4.12 ±0.07	3.86 ±0.04*
P_i	2.03 ±0.05	2.13 ±0.07
$\frac{[ATP]}{[ADP][HPO_4^{2-}]}$ (mM^{-1})	3.90 ±0.11	3.62 ±0.08

of oxaloacetate available for transamination and maintenance of equilibrium with α-oxoglutarate and glutamate (Duffy *et al.*, 1972; Miller *et al.*, 1973).

Redox state changes of brain

The cytoplasmic [NAD^+]/[NADH] ratio, calculated from the reactants of lactate dehydrogenase, was markedly decreased from 717 to 471 (Table 7). This decrease was not reflected by the reactants of α-glycerophosphate dehydrogenase, probably because of the low activity of this enzyme in brain (Balázs, 1970).

The removal of NH_4^+ by brain is catalysed by glutamate dehydrogenase. Although there was a large increase in [NH_4^+], there was no change in α-oxoglutarate or glutamate concentrations. Assuming glutamate dehydrogenase to be near equilibrium in brain (Miller *et al.*, 1973), this indicated an increase in the mitochondrial [NAD^+]/[NADH] ratio. Whereas in general the redox states of the NAD couples change in parallel in the cytoplasm and mitochondria,

Table 6. *Brain concentrations of some amino acids and NH_4^+ after ammonium acetate injection*

The values are means (±S.E.M.) of eight determinations. The symbol ** indicates statistical significance at the 1% level. For other details see Table 4.

Metabolite	Concn. in control (mM)	Concn. after injection of ammonium acetate (mM)
Glutamate	11.6 ±0.1	11.3 ±0.1
Glutamine	6.71 ±0.2	8.36 ±0.11**
Aspartate	2.75 ±0.04	2.32 ±0.06 **
Alanine	0.425±0.022	0.574±0.027**
NH_4^+	0.18 ±0.03	1.53 ±0.15**

Table 7. *Redox state of brain nicotinamide nucleotide couples*

The values are means (±S.E.M.) calculated from individual metabolite concentrations listed in Table 4. The symbol ** indicates statistical significance at the 1% level. [$NADP^+$]/[NADPH] ratios were calculated from the reactants of 'malic' enzyme ([$NADP^+$]/[NADPH] = [pyruvate][CO_2]/[malate]K), and isocitrate dehydrogenase ([$NADP^+$]/[NADPH] = [α-oxoglutarate][CO_2]/[isocitrate]K), where the equilibrium constants were 34.4mM and 1.17M respectively (Krebs & Veech, 1969; Londesborough & Dalziel, 1968). Cytoplasmic and mitochondrial free [NAD^+]/[NADH] ratios were calculated from the reactants of lactate dehydrogenase ([NAD^+]/[NADH] = [pyruvate]/[lactate]K), and glutamate dehydrogenase ([NAD^+]/[NADH] = [α-oxoglutarate][NH_3]/[glutamate]K), where the equilibrium constants were 0.111mM and 3.87μM respectively (Williamson *et al.*, 1967). The concentration of isocitrate was assumed to be 5% that of citrate (Goldberg *et al.*, 1966) and brain tissue CO_2 was taken to be 1.17mM (Krebs & Veech, 1969).

Ratio	Control value	Value after injection of ammonium acetate
[NAD^+]/[NADH] (lactate dehydrogenase)	717 ±16	471 ±11**
$10^3 \times$[$NADP^+$]/[NADPH] (isocitrate dehydrogenase)	7.27±0.21	7.57±0.42
$10^3 \times$[$NADP^+$]/[NADPH] ('malic' enzyme)	8.48±0.21	7.93±0.22
[NAD^+]/[NADH] (glutamate dehydrogenase)	0.80±0.13	7.06±0.70**

Table 8. *K^+ and Na^+ concentration of brain and plasma*

All values are means (±S.E.M.) of four to six individual determinations. For other details see the Materials and Methods section. Brain concentration was calculated assuming an 80% water content and no correction was applied for extracellular contamination.

Treatment	Plasma [K^+] (mM)	Plasma [Na^+] (mM)	Brain [K^+] (mM)
Control	3.3±0.1	140±1	116.0±1.25
Ammonium acetate injected	5.4±0.2	140±1	115.0±1.0

changes in opposite directions have been reported (Williamson *et al.*, 1967).

Increased plasma [K^+] after NH_4^+ administration

In brain, and presumably other tissues, NH_4^+ may exchange with K^+ causing K^+ efflux (Vrba *et al.*, 1958; Tower *et al.*, 1961; Rybova, 1959). After ammonium acetate injection, plasma [K^+] rose from 3.3 to 5.4mol/l but no change of [Na^+] occurred (Table 8).

Discussion

Incorporation of NH_4^+ into glutamine

NH_4^+ taken up by brain is largely incorporated into glutamine (Weil-Malherbe, 1962; Berl, 1971), and we confirmed this. A quantitatively significant net increase in the incorporation of NH_4^+ into glutamate, aspartate or alanine did not occur. During the time-period studied there was no appreciable efflux from the brain of glutamine or glutamate. Thus if glutamine is the vehicle for removal of NH_4^+ from brain (Lund, 1971), this process is slow and restoration of normal concentrations of glutamine must require relatively long periods. This is emphasized by observations that rats with plasma [NH_4^+] raised from 7 to 32 μM by porta-caval anastomosis had brain glutamine concentrations 250% those of controls after 6 weeks (Williams *et al.*, 1972).

Increased oxidative metabolic rate caused by NH_4^+

Brain glucose utilization increased by 29% after NH_4^+ injection and arteriovenous differences of glucose and O_2 increased proportionately. The excess of energy produced from the increased rate of oxidative metabolism must have been consumed by a process that occurred as a consequence of increased [NH_4^+]. Two possibilities are considered: incorporation of NH_4^+ into glutamine; and ionic effects of NH_4^+.

Synthesis of glutamine

Although the synthesis of extra glutamine occurred at an average rate of 0.33 μmol/min per g, this does not necessarily explain the increased rate of oxidative metabolism. Glutamine may be synthesized from glucose and NH_4^+ by pathways present in rat brain (Felicioli *et al.*, 1966) by the following equation:

$$2NH_4^+ + 3NAD^+ + \text{glucose} \rightarrow \text{glutamine} + CO_2 + 3NADH + 5H^+$$

Thus NH_4^+ detoxification requires additional glucose but no increased oxidative metabolism with concomitant production of ATP. Glucose uptake from blood was insufficient to supply substrate for glutamine since O_2 uptake was stoicheiometric. The additional substrate required was approximately accounted for by glycogenolysis (0.16 μmol/min per g) and aspartate disappearance (0.09 μmol/min per g).

Stimulation of Na^+, K^+-stimulated adenosine triphosphatase and oxidative metabolism by NH_4^+

The rate of cell energy metabolism is correlated with ion transport (Whittam & Wheeler, 1970). Stimulation of this process by increased [NH_4^+] and the resulting rise in [K^+] may account, at least in part, for the increased rate of oxidative metabolism observed. This effect could be brought about in two ways. First, in the presence of high [Na^+], nerve Na^+, K^+-stimulated adenosine triphosphatase is stimulated by both NH_4^+ and K^+ with K_m values of 5 and 1.8mM respectively (Skou, 1960). The site of this effect is believed to be extracellular in nerve (Sjodin, 1971), as shown for erythrocytes (Whittam, 1964). By using the above constants and blood [NH_4^+] and [K^+] (Table 7) as an approximation of those existing in brain extracellular fluid an increase of 19% in Na^+,K^+-stimulated adenosine triphosphatase activity is predicted (Dixon & Webb, 1964). Secondly, Na^+,K^+-stimulated adenosine triphosphatase activity can also be influenced by the transmembrane potential. In frog muscle, decreases of about 20mV result in a sharp increase in Na^+ efflux (Horowicz & Gerber, 1965). The resting transmembrane potential (V) may be calculated from:

$$V = -\frac{RT}{F} \ln \left(\frac{[K^+]_i + \alpha[NH_4^+]_i}{[K^+]_e + \alpha[NH_4^+]_e} \right)$$

Thus at rest:

$$V = -62 \log \left[\frac{(116) + (0.2 \times 0.2)}{3.3 + (0.2 \times 0.007)} \right] = -96\,\text{mV}$$

After NH_4^+ injection:

$$V = -62 \log \left[\frac{(115) + (0.2 \times 1.5)}{(5.4) + (0.2 \times 1.74)} \right] = -81\,\text{mV}$$

The subscripts i and e refer to intracellular and extracellular, respectively and α ($\alpha = 0.2$) is the permeability of NH_4^+ relative to K^+ (Binstock & Lecar, 1969). Thus, a decrease in the resting transmembrane potential of 15mV is calculated. This agrees with direct measurements made on cat motor neurons and squid giant axons (Lux *et al.*, 1970; Binstock & Lecar, 1969; Lorente de Nó *et al.*, 1957).

The convulsions produced by high [NH_4^+] prob-

ably do not result from its incorporation into glutamine or the stimulation of oxidative metabolic rate as such. The increased flux through the various pathways resulting from these processes is well within the capacity of brain tissue. Generalized seizures that increase brain oxidative metabolism by 300–400% do not alter brain energy balance (Plum, 1971). A more likely mechanism of the pharmacological action of NH_4^+ is the effect on the electrical properties of nerve cells. When presented extracellularly NH_4^+, like K^+, decreases the resting transmembrane potential (Lorente de Nó *et al.*, 1957; Binstock & Lecar, 1969; Lux *et al.*, 1970), therefore bringing the potential closer to the threshold for firing. This could cause a general increase in nerve-cell excitability and activity, resulting in convulsions.

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