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METABOLISM OF [1-^{14}C]γ-HYDROXYBUTYRIC ACID BY RAT BRAIN AFTER INTRAVENTRICULAR INJECTION

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Abstract—Brain tissue was shown to be capable of metabolizing [1-^{14}C]γ-hydroxybutyric acid (GHB) to succinic acid and amino acids interconvertible with Krebs tricarboxylic acid cycle intermediates after intraventricular injections *in vivo* into rats under chloral hydrate anesthesia. The extent of GHB metabolism was confirmed by three different methods of analysis and indicated that oxidation to succinic acid is the principal if not the only metabolic pathway for GHB metabolism in the brain. Analysis of radioactive amino acid products after administration of both [1-^{14}C]GHB and ^{14}C-labeled succinic acid indicated a similar profile of amino acid formation. Simultaneous injection of malonate with either GHB or succinic acid produced an effective inhibition of succinate metabolism and a buildup of labeled succinate in the GHB experiments. However, simultaneous injection of pyrazole with GHB had no effect on the metabolism of GHB. The production of γ-aminobutyric acid from GHB was shown to result primarily after GHB was metabolized to succinic acid and not by a transamination of succinic semialdehyde.

Attention has been focused on the overall neuropharmacology and biochemistry of γ-hydroxybutyric acid (GHB) because of the gross behavioral effects produced as well as its more specific effects on the life cycle of putative neurotransmitters such as acetylcholine and dopamine, and its close structural resemblance to γ-aminobutyric acid (GABA).

GHB appears to produce a general suppressant action on the entire cerebrospinal axis. Muscular relaxation is produced mainly through spinal cord depression rather than by a direct effect on the neuromuscular junction [1]. Unlike most other central nervous system depressants, GHB is very well tolerated and is toxic to the respiratory system only at very high doses. This has led to the use of the sodium salt of GHB as an anesthetic or anesthetic adjuvant.

GHB causes marked increases in the brain levels of acetylcholine [2] and dopamine [3]. The time course of the alteration in the level of acetylcholine is not well correlated with the behavioral effects produced [4], whereas the onset and duration of the increase in dopamine levels coincides with the anesthetic or sedative properties of the drug [5, 6].

GHB is metabolized quite rapidly by the rat. The half-life of an anesthetic dose (5·8 m-equiv./kg) in the rat is about 1 hr [7]. The drug appears to be almost completely cleared from the body 4 hr after administration. Labeled CO_2 can be shown to evolve from the animal while very little intact drug is found in the urine; thus it appears as if this drug is cleared largely by metabolism. The actual route(s) for the metabolism of GHB is open to speculation. Some evidence has been reported that the initial step in metabolism involved β-oxidation [8]. Alternately, GHB could enter the Krebs cycle via oxidation of the γ-hydroxyl group to yield first succinic semialdehyde and then succinic acid. Proof of the latter pathway has been indirect and based upon the production of small quantities of radiolabeled amino acids interconvertible with Krebs cycle intermediates [9]. However, these same species of amino acids would appear even if the β-oxidation pathway were operable, resulting from the incorporation of labeled acetate into the cycle. Furthermore, attempts to trap labeled succinate following the metabolism of labeled GHB have not been successful [8]. The structurally similar endogenous compound, GABA, can be produced *in vivo* from GHB [9, 10], but not in large enough amounts to suggest it is the active form of the compound.

Interest in the metabolism of GHB also arises because it has been shown to be a naturally occurring metabolite of mammalian brains, present in small but significant amounts. For example, 1·45 nmoles/g is present in rat brain, 3·23 nmoles/g in cat brain and 4·10 nmoles/g in guinea-pig brain [11]. It has also been suggested that GHB may act as a regulator of the sleep states by altering neuroglial metabolism [12]. Finally, in view of the interaction of GHB with dopaminergic neurons [13], the drug has been tested clinically in a host of neurological disorders [14].*

* M. Van Woert and R. H. Roth, unpublished data.

EXPERIMENTAL

Throughout these experiments, male albino rats weighing from 200–300 g were anesthetized with a dose of chloral hydrate equivalent to 400 mg/kg of body weight administered intraperitoneally. Once anesthetized, they were placed into a sterotaxic assembly and an intraventricular injection of radiolabeled GHB or succinic acid was made in the following manner. An incision, 1 to 1·5 inch long, was made in the scalp and the tissue adhering to the skull was scraped away. A 3-mm burr hole was made 1·4 to 1·5 mm to the right of the bregma. The injection of radiolabeled compound into the lateral ventricle was made after lowering the tip of a Hamilton syringe (blunt tip, 22 gauge needle) containing 10–20 μl of compound dissolved in Ringer's solution from 3·7 to 4·0 mm below the surface of the skull.

[1-^{14}C]GHB (Na^+ salt) and [1-^{14}C]succinic acid were obtained from the Schwarz/Mann Co. (Orangeburg, N.Y.). GHB (sp. act., 8 mCi/m-mole) was dissolved in 1 ml Ringer's solution (Cutter Labs, Berkeley, Calif.) so that a 20-μl injection corresponded to 250 nmoles GHB. Succinic acid (sp. act., 53 mCi/m-mole) was also dissolved in 1 ml Ringer's solution with the resulting concentration being 37·8 nmoles/20 μl injection.

In several experiments a simultaneous injection of sodium malonate and GHB or succinic acid was made by first preparing 624 mg/ml of sodium malonate in Ringer's solution and diluting one to one with succinic acid or one to two parts of labeled GHB as prepared above. Thus 20 μl contained 28·2 μmoles malonate and 167 nmoles GHB and 42·3 μmoles malonate and 18·9 nmoles succinate.

Unless the time course for the metabolism of GHB was being studied, the rat was sacrificed (by decapitation) 20 min after making the intraventricular injection. The skull was cut open and the brain immediately removed and placed into 4 ml of ice-cold 80% ethanol and homogenized with a Teflon–glass homogenizer. After centrifuging at 12,000 g for 20 min, the resulting supernatant was stored in the freezer (−5°) until ready for analysis. The supernatant was concentrated by flash evaporation and additional protein was removed from the remaining aqueous phase by acidifying to pH 1 with 2 N HCl and centrifuging at 37,000 g for 20 min. Alternatively, for some experiments, 0·5 ml of 2 M trichloroacetic acid was added to the supernatant before flash evaporation to facilitate a more effective removal of protein.

The amino acid analysis was performed on a J.E.O.L. JLC-5AH automated amino acid analyzer using 500–700 μl of the deproteinized supernatant. Separation was attained using an 0·8 × 50 cm column containing Jeolex-200 cation-exchange resin of the polystyrene type. The sample was eluted with 0·2 N sodium citrate buffers (pH 3·25 and 4·25) in the manner described by Spackman *et al.* [15], using a temperature of 50° and a flow rate of 40 ml/hr. The effluent was collected in 2-ml fractions by means of an automatic fraction collector.

Anion-exchange chromatography was performed using AG 1-X8 anion-exchange resin (Bio Rad Laboratories, Richmond, Calif.). The resin, equilibrated with 0·1 N HCl in 0·25 N NaCl, was packed into a 50-ml burette to occupy a bed volume of 51 × 1 cm. Just prior to adding the sample, 0·5 ml of 0·25 M Tris-HCl, pH 7·4 buffer was allowed to settle into the column and 0·6 ml of deproteinized sample, adjusted to pH 7·5, was mixed with 0·4 ml of strong Tris-HCl buffer to give a 0·25 M, pH 7·4 mixture. This buffered sample in a 1-ml volume was added to the column and eluted with the HCl–NaCl solution described above. Fractions containing 3 ml each were collected every 3·7 min using an automtic fraction collector. The fractions were counted in 17 ml scintillation solution (1 liter Triton X-100, 2 l. toluene, 5·5 g PPO and 0·3 g dimethyl POPOP*).

Assay for GHB or succinic acid metabolites by thin-layer chromatography (TLC) was conducted using the deproteinized supernatant by spotting 6–10 μl of metabolite solution on precoated Silica gel plates (Eastman Kodak Co., Rochester, N.Y., stock No. 6060) and developing 10 cm in butanol–acetic acid–water (5:1:4). Migration of the ^{14}C activity was monitored by cutting the chromatogram into 1-cm sections and placing into a counting vial. The Silica gel was scraped from the plastic backing and 10 ml of counting solution was added.

RESULTS

Disappearance of [1-^{14}C]*GHB from brain.* Figure 1 demonstrates that the total radioactivity remaining in the brain after an intraventricular injection of [1-^{14}C]GHB declines rapidly with time, with half of the isotope being removed in less than 5 min. The zero time data were obtained by adding the radioactive GHB to the excised brain just prior to homogenization.

From this experiment, it was decided that allowing 20 min for metabolism *in vivo* would be sufficient time for GHB metabolites to be produced. Consequently, the analysis for GHB metabolites as described in the following sections is based upon the fraction of the total GHB that remained at 20 min after the intraventricular injection.

This disappearance curve is based upon data obtained from one animal at each time point. However, in 26 separate experiments in which the rats were sacrificed 20 min after the intraventricular injection of labeled GHB, 21·2 ± 4·5 per cent of the injected label was retained in the brain. This compares very well with the data presented in Fig. 1 where at 20 min approximately 20 per cent of the injected radioactivity is still present in the brain.

Amino acid analysis of [1-^{14}C]*GHB and* [1-^{14}C]*succinic acid and their metabolites.* Figure 2 demonstrates

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

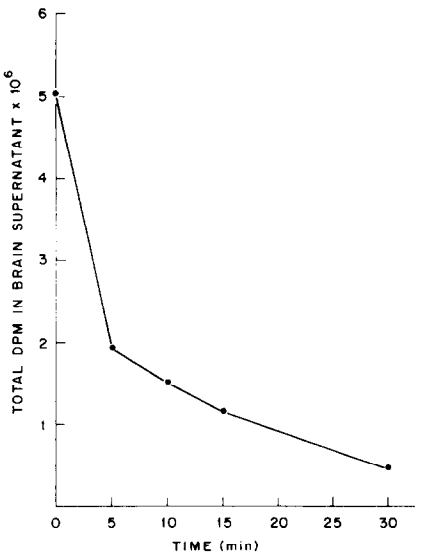

Fig. 1. Total ^{14}C remaining in brain after intraventricular injection of [1-^{14}C]GHB. Ordinate: total dis/min in supernatant after homogenization of the dissected brain (see Experimental); abscissa: time (min) allowed for GHB metabolism.

that for each major peak obtainable from [1-^{14}C]succinic acid metabolism there is also a corresponding peak obtainable when the metabolites from [1-^{14}C]GHB are assayed by amino acid analysis. The dual nature of the first peak for the GHB metabolism (samples 13 24) was also obtained when samples were prepared at zero time and assayed by this method. This dual peak is probably a result of the fact that in acid solution GHB forms an equilibrium with the lactone form, γ-butyrolactone (GBL) [16]. There was no evidence for radioactive peaks in tubes 27, 33 or 41 when the zero time sample was run. The peaks at 27, 33, 41 and 54 correspond to the elution profile for aspartic acid, glutamine, glutamic acid and alanine, respectively, as judged by standard samples previously run through this amino acid analyzer column. The identity of the peak at tube 27 was confirmed by mixing [^{14}C]aspartic acid with the GHB metabolites. In this instance a single peak greater by the amount of added [^{14}C]aspartic acid was obtained. In order to identify further the glutamine and glutamate peaks, a Li^+ instead of Na^+ buffer was used. In this experiment there were two peaks (other than a dual peak representing unbound material), one corresponding to aspartic acid and the other to glutamine/glutamate and containing the expected combined total of the peaks at 33 and 41 observed when the column was eluted with the Na^+ buffer.

In addition to the peaks shown, a small quantity of radioactivity was eluted in tube 121 where authentic GABA was shown to be eluted. This peak was always smaller than the peaks at 27, 33 and 41 illustrated in Fig. 2.

The total radioactivity under peaks 27, 33, 41 and 54 comprises 65·8 per cent of the total radioactivity found in the brain after administration of succinic acid and indicates a very rapid breakdown of this compound. For GHB, the total under these same peaks represents 23 per cent of the total radioactivity eluted. Thus it appears that the initial step of GHB metabolism is slow compared to the rapid metabolism of succinic acid.

Anion-exchange chromatography of metabolites of [1-^{14}C]*GHB and the effects of sodium malonate on their production.* The elution pattern of [1-^{14}C]GHB and its metabolites from the AG 1-X8 anion-exchange column is shown in Fig. 3. In a separate experiment, GHB and succinic acid were applied to the column and eluted; the tube showing the peak activity for each is marked on the abscissa of Fig. 3. This figure shows that for the malonate-treated animals there is a reduced produc-

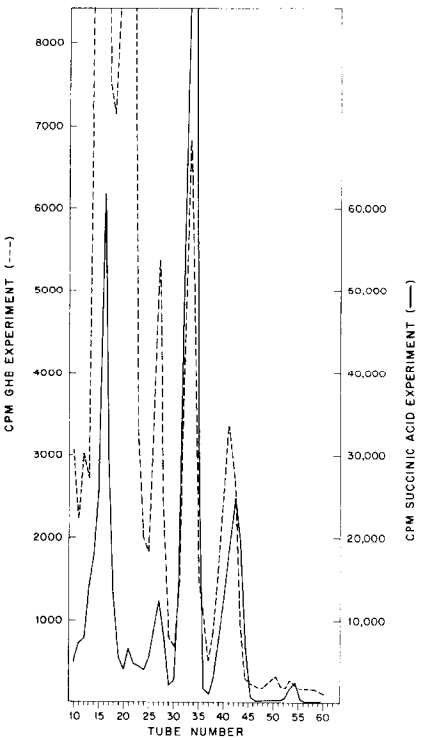

Fig. 2. Amino acid analysis of the metabolites of [1-^{14}C]succinic acid and GHB. Ordinate: radioactivity contained in each 2-ml fraction. The solid line represents succinic acid metabolites and the data units are represented on the right. The dashed line represents [1-^{14}C]GHB metabolites and the units are represented on the left. Abscissa: tube number; additional fractions were collected out to tube 130. A small peak at tube 121 was obtained containing all basic radioactive compounds. A small quantity of [^{14}C]tyrosine was also added to the sample as a radioactive marker and it consistently eluted at tube 92.

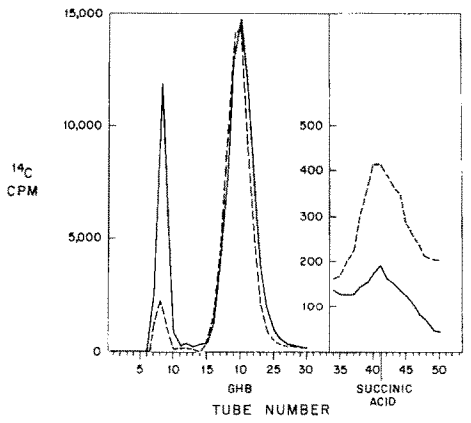

Fig. 3. Anion-exchange chromatography of GHB and its metabolites. Ordinate: cpm in each fraction; abscissa: fraction tube number. The tubes that showed the peak for GHB and succinic acid radiolabeled standards in separate experiments are also designated on the abscissa. After tube 33, the ordinate units are changed to illustrate more clearly the small amount of succinic acid produced relative to the total amount of radioactivity. Dashed line, GHB and sodium malonate simultaneously injected intraventricularly; solid line, GHB and metabolites in untreated animals.

tion of compounds not binding the column (tubes 6–11) and an increase in succinic acid relative to the control animal. Table 1 shows that the amount of isotope not binding the anion-exchange resin very nearly equals the amount of isotope binding the cation-exchange resin for the six experiments shown. In both cases this fraction is representative of the total amino acids produced. Table 1 shows both quantitatively and as a percentage that the brain of malonate-treated animals always contains an increase in labeled succinate. The amount of isotope remaining as GHB is a higher percentage for the malonate-treated animal than for the controls. This could be due to inhibition of the dehydrogenase that first oxidizes GHB or to inhibition of succinic semialdehyde dehydrogenase either directly or as a result of a buildup of endogenous succinate.

Inhibition of succinic acid metabolism by malonate and other attempts to inhibit GHB metabolism. As part of an effort to show that the amino acid profile and the buildup of succinate caused by malonate for [1-^{14}C]GHB metabolism was not due to acetate resulting from β-oxidation products being incorporated into the Krebs cycle, intraventricular injections of a mixture of malonate and labeled succinate were made. For these experiments a TLC system was used to assay the extent of metabolism. Table 2 shows that malonate inhibits the succinate metabolism into amino acids by a factor 78/24 = 3·2. Under similar conditions malonate lowers the extent of GHB metabolism into amino acids by a factor of 30/8·5 = 3·5, indicating that the metabolism of both succinate and GHB into amino acids is affected to the same degree by malonate administration.

In experiments using a decarboxylase inhibitor, RO44602 (seryl-trihydroxybenzylhydrazine) [17], and pyrazole, an inhibitor of brain alcohol dehydrogenase [18], no significant inhibition of GHB metabolism was obtained. For example, animals treated with RO44602 (1 g/kg, i.p.) gave 25 per cent GHB metabolism as compared with 22 per cent metabolism for its control as assayed by amino acid analyses. Two animals treated with pyrazole (56 μmoles injected simultaneously with GHB in the same manner used for sodium malonate injection) gave 24 and 25 per cent GHB metabolism as compared with the 23·9 $\pm$ 3·9 per cent average of 15 controls, indicating that this compound also has no significant inhibitory effect on GHB metabolism.

In still another series of experiments, sodium pyruvate was administered either intraperitoneally (1·2 g/kg, 20 min prior to intraventricular injection) or intraventricularly together with labeled GHB (2·35 and 24·3 μmoles sodium pyruvate) in attempts to both alter the redox state (increase the availability of NAD) and flood the TCA cycle with an available precursor to acetate. However, as indicated in Table 2, pyruvate had no

Table 1. Analysis of malonate inhibition of [1-^{14}C]GHB metabolism*

	% Amino acids determined by cation exchange	Total activity eluted (counts/min)	Non-binding amino acids		GHB		Succinic	
			(counts/min)	(%)	(counts/min)	(%)	(counts/min)	(%)
Control†	23	94,810	21,530	23	71,430	75·3	1850	2·0
Control	31	95,690	32,670	34·2	60,790	63·5	2230	2·3
Control	29	79,920	26,380	33	51,860	65	1670	2·4
Malonate*	7	73,530	5080	7	64,260	87·4	4200	5·7
Malonate	10	87,720	8600	9·8	74,710	85	4410	5·2
Malonate	10	69,990	6480	9·3	59,740	85·3	3760	5·4

* The first column represents the per cent of ^{14}C after 20 min of [1-^{14}C]GHB metabolism that binds to cation-exchange resin (i.e. the amino acid fraction). The other columns represent data under the peaks shown in Fig. 3. The non-binding component for anion-exchange resin compares with the fraction binding the cation-exchange resin and is a measure of the total amino acids present. Malonate inhibition was by co-intraventricular injection with [1-^{14}C]GHB (see Experimental).

† Represented in Fig. 3.

Table 2. Inhibition of labeled GHB and succinate metabolism*

Substrate	Time (min)	Inhibitor	Total activity (counts/min)	Activity remaining as substrate (counts/min)	Total activity for metabolites (counts/min)	% Metabolism
Succinate	20		4030	890	3140	78
Succinate	10	malonate	1270	1070	200	16
Succinate	20	malonate	1320	1000	320	24
GHB	20		1320	920	390	30
GHB	20	malonate	1140	1020	120	10
GHB	20	malonate	1290	1200	100	7
GHB	20	pyrazole	990	760	240	24
GHB	20	pyrazole	1370	1030	380	25
GHB	20	pyruvate	1040	730	310	30

* For these experiments a TLC system was used (see Experimental). On these chromatograms, succinic acid and GHB had an R_f value of 0·7, while the potential metabolites (aspartic acid, glutamic acid, glutamine and γ-aminobutyric acid) had R_f values of 0·05 to 0·4. All inhibitors were co-injected into the lateral ventricles as a mixture of either [1-^{14}C]GHB or labeled succinate.

significant effect on GHB metabolism. Animals that were given intraperitoneal injections (not shown) gave 28, 30 and 24 per cent metabolism for [1-^{14}C]GHB.

DISCUSSION

When the products of metabolism of [1-^{14}C]succinic acid and [1-^{14}C]GHB were analyzed by amino acid analysis, similar patterns of elution of ^{14}C compounds were obtained. Of these peaks, all could be identified with the expected elution of amino acids interconvertible with intermediates on the tricarboxylic acid cycle or glycolysis (i.e. aspartic acid, glutamine, glutamic acid, alanine and GABA).

Owing to the comparably small amount of GABA produced and because the malonate-treated animals did not produce an increase in this compound, it is unlikely that a transaminase for converting succinic semialdehyde to GABA is active in brain. Thus, the previously reported observation suggesting that GABA is produced from GHB primarily through the Krebs cycle [9] is consistent with the results presented here.

The possibility that a β-oxidation pathway, as suggested by Walkenstein *et al.* [8], is largely responsible for GHB metabolism is rendered somewhat unlikely by these experiments, although no direct proof is presented. This implication is supported by the consistent appearance of a similar extent of GHB metabolism determined by three alternate methods of analysis and the fact that GHB metabolism is blocked by malonate. Any product(s) of β-oxidation would then have to have the same chromatographic behavior as GHB in the methods used and the enzymes for this pathway would have to be susceptible to malonate inhibition. Specifically, if a β-oxidation pathway were active, then in the presence of malonate, production of the labeled amino acids such as glutamine and glutamate would have occurred at their normal (control) rate. However, this was not observed. Instead there was a comparable reduction (i.e. a factor of 3·2 to 3·5) in overall metabolism for both GHB and succinate caused by malonate. Alternately, the presence of pyruvate would have been expected to inhibit at least partially the incorporation of acetate produced by a β-oxidation pathway into the amino acids, but again no such effect was noted.

The possibility that GHB is metabolized via a direct decarboxylation to propanol is also unlikely, although this could not be studied more thoroughly using [1-^{14}C]GHB. However, the gross symptomatology of propanol intoxication is much different from that of GHB. Also a potent decarboxylation inhibitor (RO44602) did not effectively inhibit GHB metabolism.

The 22–30 per cent figure for the extent of GHB metabolism when 250 nmoles was injected is based upon the amount of total isotope that was left in the brain at the time the animal was sacrified. It represents an even smaller amount of the total GHB injected into the brain, owing to the fact that a large percentage of the injected compound was rapidly cleared from the brain (ventricle), probably as a result of being pumped out via the choroid plexus.

The enzyme involved in the initial step of GHB metabolism to yield succinic acid cannot be easily classified in any of the groups of alcohol dehydrogenases found in brain based upon data presented above. Alcohol dehydrogenase (EC 1.1.1.1), which oxidizes ethanol and other alcohols, is known to be present in rat brain [18]. This enzyme can be distinguished from other dehydrogenases since it is susceptible to inhibition by pyrazole. This enzyme is indeed involved in some aspects of GHB metabolism, but apparently not in the oxidation of GHB to succinate. For example, the compound 1,4-butanediol can be metabolized to GHB after intraventricular [19] or intraperitoneal [20] injection. This conversion is inhibited by pyrazole as attested by the lack of appearance of GHB in the blood after administration of 1,4-butanediol to rats pretreated with pyrazole [20]. Brain high speed

supernatant can reduce succinic semialdehyde *in vitro* to GHB by an enzyme inhibited by pyrazole, but the reverse reaction cannot be demonstrated [21]. Purified preparations of equine liver alcohol dehydrogenase, which readily oxidize 1,4-butanediol and ethanol with nearly the same velocity, do not oxidize GHB [20]. Thus this alcohol dehydrogenase does not appear to be involved in GHB metabolism. This suggestion is further supported by the failure of pyrazole co-injected with [1-^{14}C]GHB to affect the conversion of GHB to succinate. Other experiments (to be published) using a more potent pyrazole analog, 4-methylpyrazole, which induces sleep and loss of the righting reflex, also had no effect on [1-^{14}C]GHB metabolism.

Alcohol dehydrogenases (EC 1.1.1.2) that have been postulated to be related to metabolism of biogenic amines and are distinguishable from those that metabolize ethanol by their apparent insensitivity to pyrazole are also present in brain tissue [22]. However, this enzyme will not reduce succinic semialdehyde to GHB but prefers long chain aliphatic and aromatic aldehydes. Efforts to determine more clearly the class or classes of dehydrogenases that convert GHB to succinate are currently underway in this laboratory.

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