706. *Nucleotides. Part XVIII.* The Synthesis and Properties of Adenosine-5′ Uridine-5′ Phosphate.*

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Adenosine-5′ uridine-5′ phosphate (I) has been synthesised by the interaction of silver 2′ : 3′-*iso*propylidene adenosine-5′ benzyl phosphate and 2′ : 3′-*iso*-propylidene 5′-iodo-5′-deoxyuridine in boiling toluene. It is stable towards alkali and ribonuclease. These findings are discussed in relation to current theories of ribonucleic acid structure and ribonuclease action. Rapid degradation to adenosine and uridine is brought about by crude Russell's viper venom.

Investigations of the structure of nucleic acids have been largely concerned in recent years with the establishment of the nature of the internucleotide linkage and the determination of the ratio and sequence of purine and pyrimidine bases. The methods employed have broadly followed two paths. On the one hand, chemical and enzymic hydrolysates have been examined by the techniques of ion-exchange chromatography (Carter and Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 2604; Cohn, *J. Cell. Comp. Physiol.*, 1951, **38**, Suppl. 1, 21; Koerner and Sinsheimer, *J. Amer. Chem. Soc.*, 1952, **74**, 283), paper chromatography (Carter, *ibid.*, 1950, **72**, 1466; Chargaff, *Experientia*, 1950, **6**, 201), and electrophoresis (Gordon and Reichard, *Biochem. J.*, 1951, **48**, 569; Markham and Smith, *Nature*, 1951, **168**, 406; Smith and Markham, *Biochim. Biophys. Acta*, 1952, **8**, 350). The discovery of isomeric *a* and *b* ribonucleotides, the isolation of a number of di- and tri-nucleotides, and the rejection of the " tetranucleotide " theory of nucleic acid structure have resulted. On the other hand, synthetical work, which has been in progress in this laboratory for a number of years, has culminated in the elucidation of most of the structural features of the nucleotides. It seemed desirable to extend the latter investigations to include the synthesis by unequivocal methods of diribonucleoside phosphates and diribonucleotides in order to investigate the stability of the various possible phosphodiester linkages to hydrolysis by alkali and enzymes. In addition, the effect of the purine and pyrimidine moieties of the diribonucleoside phosphates and diribonucleotides on ribonuclease specificity might be determined.

The resistance to alkali of the phosphoryl groups of ribonucleotides (previously designated as 3′-phosphates) and ribose-5′ phosphate led Levene and Tipson (*J. Biol. Chem.*, 1935, **109**, 623) to postulate a $C_{(2')}$–$C_{(3')}$ internucleotide linkage which would undergo alkaline cleavage at $C_{(2')}$. Gulland and Smith (*J.*, 1947, 338; 1948, 1527) claimed to have synthesised uridine-2′ and cytidine-2′ phosphates and diuridine-2′ : 2″ phosphate (*J.*, 1948, 1532) in order to test this hypothesis. Michelson and Todd (*J.*, 1949, 2476) also attempted the synthesis of the 2′-phosphates of guanosine, adenosine, and cytidine. It was subsequently shown, however, that the synthetic route employed in all these cases afforded 5′-substituted nucleosides (Brown, Haynes, and Todd, *J.*, 1950, 408,

* Part XVII, preceding paper.

3299). Independent investigations had resulted meanwhile in the separation of each of the nucleotides from the alkaline hydrolysis of yeast ribonucleic acid into two isomers, neither of which corresponded with the known synthetic nucleoside-5′ phosphates (Carter and Cohn, *Fed. Proc.*, 1949, **8**, 190; Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 1471, 2811; Loring, Luthy, Bortner, and Levy, *ibid.*, p. 2811; Loring and Luthy, *ibid.*, 1951, **73**, 4215; Carter, *loc. cit.*). It is now generally accepted that the *a* and the *b* isomers are 2′- and 3′-phosphates though not necessarily respectively; the recent synthesis of *a* and *b* adenylic acids by direct phosphorylation of 5′-trityl adenosine (Brown and Todd, *J.*, 1952, 44) supports this view. Both *a* and *b* ribonucleotides are stable to alkali under conditions which cause hydrolysis of yeast ribonucleic acid to mononucleotides. Thus, although the $C_{(2')}$–$C_{(3')}$ linkage cannot be completely excluded on this ground, since both *a* and *b* nucleotides are formed by alkaline hydrolysis of ribonucleic acid, the original premises of Levene and Tipson are unsound.

In spite of the repeated failure to isolate nucleoside-5′ phosphates from alkaline hydrolysates of ribonucleic acids and their known stability to alkali, early enzymic experiments (Gulland and Jackson, *J.*, 1938, 1492) did, in fact, indicate the participation of $C_{(5')}$ in at least some of the internucleotide linkages. Incontrovertible proof was forthcoming when Cohn and Volkin (*Nature*, 1951, **167**, 483) separated by ion-exchange chromatography the 5′-phosphates of adenosine, guanosine, cytidine, and uridine from digests of ribonucleic acid treated successively with ribonuclease and intestinal phosphatase in presence of arsenate to inhibit phosphomonoesterase. Very recently, Cohn and Volkin (*Arch. Biochem.*, 1952, **35**, 465) have isolated diphosphates of cytidine and uridine which are believed to have phosphoryl groups at $C_{(3')}$ (or $C_{(2')}$) and $C_{(5')}$.

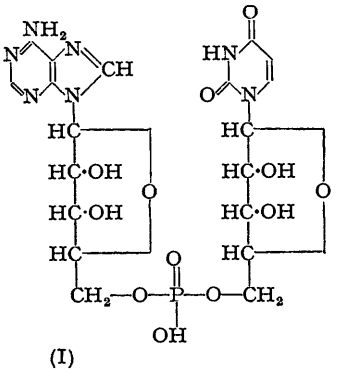
From the evidence cited above and considerable data relating to the interconversion of glycerol α- and β-phosphates and *a* and *b* ribonucleotides by acid, and the alkali-lability of glycerol α-(methyl hydrogen phosphate) and of esters of the *a* and *b* nucleotides, Brown and Todd (*J.*, 1952, 52) have advanced an explanation of the hydrolytic behaviour of ribonucleic acids. A $C_{(3')}$–$C_{(5')}$ (or $C_{(2')}$–$C_{(5')}$) internucleotide linkage is postulated for ribonucleic acids, and cleavage of the macromolecule by alkali is preceded by the formation of a cyclic triester involving the vicinal $C_{2'}$- (or $C_{3'}$-)hydroxyl group. Hydrolysis of the $C_{(5')}$–O–P linkage then occurs and the resulting nucleoside 2′ : 3′-cyclic phosphates (Brown, Magrath, and Todd, *J.*, 1952, 2708) ultimately afford mixtures of 2′- and 3′-phosphates. Taken alone, this mechanism of hydrolysis would permit $C_{(2')}$–$C_{(2')}$, $C_{(2')}$–$C_{(3')}$, $C_{(3')}$–$C_{(3')}$, $C_{(2')}$–$C_{(5')}$, and $C_{(3')}$–$C_{(5')}$, but would exclude $C_{(5')}$–$C_{(5')}$, as possible internucleotide linkages. Moreover, the concomitant formation of *a* and *b* ribonucleotides during hydrolysis is adequately explained without necessarily involving a $C_{(2')}$–$C_{(3')}$ linkage.

The mechanism of ribonuclease action is less clear. Markham and Smith (*loc. cit.*) believe that degradation of ribonucleic acids by ribonuclease simulates that by alkali with the important difference that $C_{(3')}$–$C_{(5')}$ (or $C_{(2')}$–$C_{(5')}$) internucleotide linkages are cleaved only if $C_{(3')}$ (or $C_{(2')}$) refers to a ribose residue bearing a pyrimidine base. Some confirmation of this qualification has been evinced from a study of the action of ribonuclease on cyclic nucleoside-2′ : 3′ phosphates (Brown, Dekker, and Todd, *J.*, 1952, 2715). Cyclic phosphates of uridine and cytidine are cleaved by ribonuclease to yield only the *b* nucleotide whereas cyclic adenosine-2′ : 3′ phosphate is unaffected by the enzyme (cf. Markham and Smith, *loc. cit.*). Recently, Allen (*Fed. Proc.*, 1951, **10**, 155), Becker and Allen (*J. Biol. Chem.*, 1952, **195**, 429), and Cavalieri, Kerr, and Angelos (*J. Amer. Chem. Soc.*, 1951, **73**, 2567) have observed that ribonuclease digests of ribonucleic acids show an increased uptake of periodate. On the other hand, Schmidt and his co-workers (*J. Biol. Chem.*, 1951, **192**, 715) obtained completely negative results in a similar investigation. Brown and Todd (*J.*, 1952, 52) have pointed out that, if the observations of Allen (*locc. cit.*) and Cavalieri, Kerr, and Angelos (*loc. cit.*) are valid, ribonuclease action must be more complex than indicated above, since an increased uptake of periodate by ribonuclease digests can arise only if some $C_{(3')}$–O–P (or $C_{(2')}$–O–P) linkages are severed as well as $C_{(5')}$–O–P linkages. In order to clarify this remarkable disparity, the action of ribonuclease on simple model substrates including diribonucleoside phosphates is under investigation in this laboratory.

The only diribonucleoside phosphate so far synthesised is diuridine phosphate (Gulland

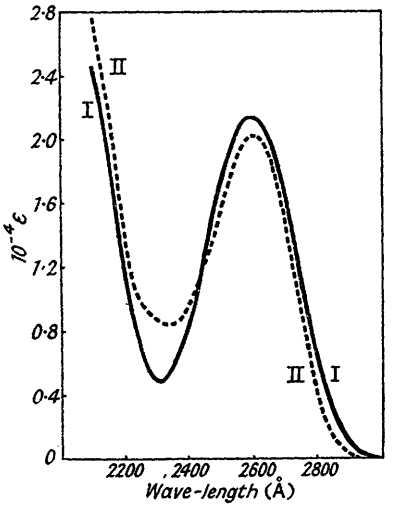
and Smith, *J.*, 1948, 1532), which, as mentioned above, is now known to possess a $C_{(5')}$–$C_{(5')}$ linkage. The synthetical route employed, which involved the reaction of 2 mols. of benzylidene uridine with 1 mol. of phenyl dichlorophosphinate, has several deficiencies. First, removal of the phenyl residue requires conditions which would almost certainly degrade a diribonucleoside phosphate containing any phosphodiester linkage except the type $C_{(5')}$–$C_{(5')}$. Secondly, reaction of phenyl dichlorophosphinate with dissimilar nucleosides would inevitably afford mixtures of diribonucleoside phosphates, thereby increasing the difficulty of isolating pure products. Finally, in the absence of a reagent which will block $C_{(3')}$ and $C_{(5')}$ (or $C_{(2')}$ and $C_{(5')}$) of the ribose moiety, unequivocal synthesis of diribonucleoside phosphates with ester linkages involving $C_{(2')}$ and/or $C_{(3')}$ is impossible.

The method which we have employed in the present work involves the heterogeneous reaction in anhydrous inert solvents of a silver salt of a suitably protected nucleotide with 2′ : 3′-*iso*propylidene 5′-iodo-5′-deoxyuridine. This method may be limited to the synthesis of diribonucleoside phosphates in which the $C_{(5')}$-position of uridine is esterified, since it has recently been demonstrated that attempts to prepare 5′-iodo-5′-deoxy-deriv-



(I)

Adenosine-5′ uridine-5′ phosphate in (I) N/100-HCl *and* (II) N/100-NaOH.



atives of adenosine and cytidine by the well-known Oldham–Rutherford method (*J. Amer. Chem. Soc.*, 1932, **54**, 366) lead to *cyclo*nucleoside salts by a novel intramolecular alkylation (Clark, Todd, and Zussman, *J.*, 1951, 2952). It is not known whether 5′-tosyl guanosine exhibits similar behaviour. Moreover, 2′-iodo-2′-deoxy- and 3′-iodo-3′-deoxy-ribonucleosides are as yet unknown and it is probable that the halogen substituent would, in any case, be too inert for these substances to be practicable intermediates for the synthesis of diribonucleoside phosphates by this route. Nevertheless, it seemed desirable to explore the potentialities of the reaction outlined, since by its use a limited number of unsymmetrical diribonucleoside phosphates might be made accessible.

This type of reaction has been utilised previously in the carbohydrate and nucleotide field. The Cori ester and its L-enantiomorph have been synthesised by the interaction of trisilver phosphate and the appropriate 2 : 3 : 4 : 6-tetra-acetyl α-bromoglucose in benzene (Cori, Colowick, and Cori, *J. Biol. Chem.*, 1937, **121**, 465; Wolfrom and Pletcher, *J. Amer. Chem. Soc.*, 1941, **63**, 1050; Potter, Sowden, Hassid, and Doudoroff, *ibid.*, 1948, **70**, 1751). Trisilver phosphate was also employed by Meagher and Hassid (*ibid.*, 1946, **68**, 2135) for the synthesis of maltose-1 and xylose-1 phosphates. The β-isomer of the Cori ester was surprisingly formed when silver dibenzyl phosphate was substituted for trisilver phosphate (Zervas, *Naturwiss.*, 1939, **27**, 317; Wolfrom, Smith, Pletcher, and Brown, *J. Amer.*

Chem. Soc., 1942, **64**, 23). Reithel (*ibid.*, 1945, **67**, 1056) obtained β-D-galactose-1 phosphate in an analogous fashion. In this department uridine-5′ phosphate and uridine-5′ pyrophosphate (UDP) have been synthesised conveniently and in good yield by the interaction of 2′ : 3′-*iso*propylidene 5′-iodo-5′-deoxyuridine in benzene with silver dibenzyl phosphate and silver tribenzyl pyrophosphate respectively (Anand, Clark, Hall, and Todd, *J.*, 1952, 3665).

In applying this method to the synthesis of adenosine-5′ uridine-5′ phosphate (I) several variations were investigated. Although some reaction occurred between the disilver salt of adenosine-5′ phosphate or silver adenosine-5′ benzyl phosphate and 2′ : 3′-*iso*propylidene 5′-iodo-5′-deoxyuridine in boiling toluene, best results were obtained when silver 2′ : 3′-*iso*propylidene adenosine-5′ benzyl phosphate was employed. Boiling benzene and dioxane were also tried as reaction media but were inferior to toluene. Isolation of the fully protected diribonucleoside phosphate has proved impossible. In consequence, *iso*propylidene and benzyl groups were removed by hydrolysis with dilute sulphuric acid, a method which proved useful during the synthesis of adenosine-5′ benzyl phosphate (Baddiley and Todd, *J.*, 1947, 648). In all cases reaction mixtures were examined by paper chromatography, but complete purification by this technique alone was not possible owing to the presence of a number of by-products which have not been further investigated. Ion-exchange chromatography on Dowex 2 resin in the formate cycle, with 0·08N-formic acid as the eluting agent, proved entirely satisfactory, since most of the impurities appeared to be neutral and were readily eluted from the column by water or 0·02N-formic acid. Fractions from the column containing adenosine-5′ uridine-5′ phosphate were combined, concentrated under reduced pressure, and freeze-dried. The free nucleotide and its barium and brucine salts have all resisted crystallisation. That our product was pure, however, seemed certain, since it had the correct analytical composition and travelled as a single spot on paper chromatograms irrigated with four different solvent systems; furthermore, it migrated homogeneously when subjected to paper electrophoresis.

Definite proof of structure was obtained from physical and degradative evidence. On periodate oxidation, 2 mols. of oxidant were consumed. Potentiometric titration with alkali revealed no secondary phosphoryl dissociation. Of the two groups titrating in the range pH 3—10, one ($pK_a = 3{\cdot}8$) can be attributed to the dissociation of the NH_3^+ group of adenine and the other ($pK_a = 9{\cdot}5$) is undoubtedly due to the enolic hydroxyl group of uracil. The ultra-violet absorption spectra (see Figure) in acid and alkaline solution are in accordance with the published data for adenosine and uridine and their derivatives. In particular, it is interesting that the shift of the minimum in alkaline solution is similar to that observed for uridine but smaller in magnitude. Russell's viper venom, which is known to possess phosphodiesterase and phosphomonoesterase activities (Hurst and Butler, *J. Biol Chem.*, 1951, **193**, 91), brought about complete hydrolysis within 2 hours to adenosine and uridine. The nucleosides were identified by paper chromatography and determined by cutting out the spots, elution, and measuring the optical density at the ultra-violet absorption maximum. A value of 0·98 was computed for the ratio, mols. of adenosine : mols. of uridine, in good agreement with the theoretical figure. Purified phosphodiesterase from Russell's viper venom (Hurst and Butler, *loc. cit.*) caused random fission of the phosphodiester to mononucleotides and nucleosides.

Adenosine-5′ uridine-5′ phosphate is stable to treatment with 0·5N-sodium hydroxide at 37° for 16 hours, conditions which effect complete hydrolysis of ribonucleic acids to mononucleotides. The absence of the $C_{(5')}$–$C_{(5')}$ internucleotide linkage in ribonucleic acids is thus proved, since such linkages if present would remain unbroken and products other than simple nucleotides would result. Moreover, ribonuclease has no action on adenosine-5′ uridine-5′ phosphate, a result which accords with current theories of ribonuclease specificity (Brown, Dekker, and Todd, *loc. cit.*, Markham and Smith, *loc. cit.*). Synthesis of further examples of diribonucleoside phosphates are under investigation. In particular, it is hoped to obtain compounds possessing a $C_{(3')}$–$C_{(5')}$ (or $C_{(2')}$–$C_{(5')}$) internucleotide linkage, since such compounds should undergo ready alkaline hydrolysis to a mixture of *a* and *b* adenylic acids and uridine, thus constituting a rigid test of Brown and Todd's theory (*loc. cit.*) regarding hydrolytic behaviour and structure of the ribonucleic acids.

Experimental

Silver 2′ : 3′-iso Propylidene Adenosine-5′ Benzyl Phosphate (first prepared in this Department by Dr. H. S. Mason).—2′ : 3′-*iso*Propylidene adenosine-5′ dibenzyl phosphate (3·0 g.), previously dried over phosphoric oxide in a high vacuum, was heated in freshly distilled 4-methylmorpholine (25 g.) for 1 hour in a stoppered flask at 100°. 4-Methylmorpholine was then removed under reduced pressure, the residue dissolved in water (100 c.c.), and the solution extracted with chloroform (3 × 100 c.c.). 2% Aqueous silver nitrate was added until precipitation of *silver 2′ : 3′-iso propylidene adenosine-5′ benzyl phosphate* was complete. The solid was centrifuged and washed by resuspension with distilled water until the supernatant liquid was colourless and the silver salt tended to become colloidal. It was then freeze-dried, with exclusion of light, to a fine white powder (3·0 g.) (Found : Ag, 21·3, 21·4; N, 11·4. $C_{20}H_{23}O_7N_5PAg$ requires Ag, 21·6; N, 11·9%).

Adenosine-5′ Uridine-5′ Phosphate.—Silver 2′ : 3′-*iso*propylidene adenosine-5′ benzyl phosphate (748 mg.) and 2′ : 3′-*iso*propylidene 5′-iodo-5′-deoxyuridine (505 mg., 1 mol.; Levene and Tipson, *J. Biol. Chem.*, 1934, **106**, 113) were suspended in dry toluene (100 c.c.) and heated under reflux for 8 hours with exclusion of light and moisture. Intimate contact of the reactants was maintained by placing several glass beads in the reaction flask and agitating by means of a stainless-steel paddle stirrer. After reaction the solution was filtered and the silver salts were washed thoroughly with warm ethanol. The combined filtrate and washings were evaporated under reduced pressure to small bulk, and the residue was evaporated twice with ethanol to remove toluene. The ethanolic solution (25 c.c.) was refluxed with N/50-sulphuric acid (80 c.c.) for 1½ hours. Sulphate was removed by the calculated amount of barium hydroxide solution. Barium sulphate was removed by filtration and washed thoroughly with hot water. The combined filtrate and washings were adjusted to pH 8·0 with aqueous ammonia and run on to a column (13·7 × 3·0 cm.) of Dowex 2 resin (250—500 mesh; formate cycle). Elution was carried out successively with 0·02N-, 0·05N-, and 0·08N-formic acid. The bulk of impurities were removed by the first two solvents, as revealed by paper chromatography of various fractions. *Adenosine-5′ uridine-5′ phosphate* was eluted by 0·08N-formic acid (optical density ratio, $D_{280}/D_{260} = 0{\cdot}30 \pm 0{\cdot}015$). Fractions having an optical density greater than 0·2 × maximum optical density were combined and concentrated under reduced pressure to small bulk and freeze-dried to a white hygroscopic fluffy solid (227 mg). The product was redissolved in a minimum of water and precipitated by addition of ethanol and ether, being then obtained as a white, amorphous, hygroscopic powder which decomposed at 180—200° without melting (Found, in material dried at 100°/0·1 mm. for 16 hours : C, 39·6; H, 3·8; P, 5·4, 5·9; N, 17·3. $C_{19}H_{24}O_{12}N_7P$ requires C, 39·8; H, 4·0; P, 5·4; N, 17·1%).

Adenosine-5′ uridine-5′ phosphate travelled as a single spot on acid-washed Whatman No. 1 paper (Hanes and Isherwood, *Nature*, 1949, **164**, 1107) in the following solvent systems : (1) *n*-butanol–acetic acid–water (40 : 10 : 50; the upper layer was used to irrigate the chromatogram; $R_F = 0{\cdot}08$) (Partridge, *Biochem. J.*, 1948, **42**, 238), (2) *n*-butanol–acetic acid–water (50 : 20 : 30); $R_F = 0{\cdot}26$, (3) *iso*propyl alcohol–ammonia (*d* 0·88)–water (70 : 10 : 20; $R_F = 0{\cdot}29$) and (4) 5% disodium hydrogen phosphate–*iso*amyl alcohol ($R_F = 0{\cdot}61$) (Carter, *loc. cit.*). The product migrated homogeneously when applied to Whatman No. 54 paper buffered at pH 7 with 0·1% ammonium acetate–ammonia solution and subjected to electrophoresis. Treatment with sodium metaperiodate (0·025M) overnight resulted in a consumption of 2·08 mol./mol. (mean of three determinations). Ultra-violet absorption : (1) In N/100-sulphuric acid, max. at 2590—2600 Å (ε 21,300), min. at 2310—2315 Å (ε 4860); (2) in N/100-sodium hydroxide, max. at 2600—2610 Å (ε, 20,300), min. at 2335—2340 Å (ε 8470). Potentiometric titration of adenosine-5′ uridine-5′ phosphate (14·9 mg.) in water (1 c.c.) with 0·105N-sodium hydroxide, with a glass electrode, demonstrated the absence of secondary phosphoryl dissociation and revealed the presence of two groups pK_a 3·8, 9·5 which are attributed to the dissociation of NH_3^+ of adenosine and enolic hydroxyl of uracil respectively.

Action of Alkali on Adenosine-5′ Uridine-5′ Phosphate.—Adenosine-5′ uridine-5′ phosphate (1·8 mg.) was dissolved in 0·5N-sodium hydroxide (0·2 c.c.) and kept at 37° for 16 hours. Paper chromatography, with *iso*propyl alcohol–ammonia (*d* 0·88)–water (70 : 10 : 20), showed that no hydrolysis had occurred.

Action of Ribonuclease on Adenosine-5′ Uridine-5′ Phosphate.—Adenosine-5′ uridine-5′ phosphate (1·9 mg.) was dissolved in water (0·1 c.c.) and a solution (0·6 mg./c.c.) of ribonuclease (0·1 c.c.) added. The pH was adjusted to 8·0 by dilute aqueous ammonia, and the digest kept

at 37° for 16 hours. Paper chromatography, with *iso*propyl alcohol–ammonia–water, revealed that no degradation had occurred.

Action of Russell's Viper Venom on Adenosine-5′ Uridine-5′ Phosphate.—Adenosine-5′ uridine-5′ phosphate (1 mg.) was dissolved in 0·25M-glycine–ammonia buffer (0·1 c.c.; pH 9·0) and treated with a solution of crude Russell's viper venom at 37° for 2 hours. Paper chromatography, with *n*-butanol–acetic acid–water (40 : 10 : 50), showed that hydrolysis to adenosine and uridine was complete. The spots were cut out and eluted with water, and the amounts of nucleoside determined by ultra-violet absorption measurements, giving a ratio, adenosine : uridine = 0·98. This experiment was repeated with the Russell's viper venom phosphodiesterase purified by paper chromatography (Hurst and Butler, *loc. cit.*). Hydrolysis of the diester linkage appeared to occur in a random fashion. Adenosine and uridine were identified by paper chromatography as before.

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