THE FATE OF ACETANILIDE IN MAN[1]

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Therapeutic agents, in general, undergo chemical alteration in the body to form derivatives of the parent compound. Isolation and pharmacological assay of the various transformation products may yield valuable information in the search for better therapeutic agents, in studies on the mechanism of drug action and in the elucidation of normal metabolic processes.

The therapeutic effect of a drug could be limited by a chemical change which results in a less active or an inactive product. Knowledge of the nature of this metabolic product may suggest the introduction of substituents in the parent drug that would prevent the transformation and thereby enhance its activity. On the other hand, it is possible that the parent drug itself is inert but produces a therapeutic effect incidental to the formation of a highly active metabolic product. Knowledge of the structure of this derived product may suggest a synthesis of more effective derivatives. Again, the toxicity of the drug could reside in a derived product the formation of which, if its structure be known, may be blocked by an appropriate modification of the parent drug.

Fundamental studies on the mechanism of drug action attempt to correlate the effect of a drug on enzymatic activity with its pharmacological activity. The compound possessing the pharmacological action may be a product derived in the body from the parent drug. It is obvious in this case that the studies should be made not with the parent drug but with the active derived product.

It is unlikely that special enzymes exist in the body for the chemical transformation of each drug. Rather, it is likely that a drug undergoes chemical change by becoming involved in biochemical reactions which ordinarily handle normally occurring substances. The presence of a particular enzyme system within the animal is indicated by the transformation of the foreign compound. Knox used the transformation of quinine to 2-hydroxy quinine by various tissues as an indicator in the isolation in relatively pure form of the so-called quinine oxidase. He found this enzyme normally to be involved in the metabolism of nicotinamide (1).

The study of drugs from the above points of view has been considerably facilitated by two recent developments: 1. a scheme whereby simple and sensitive analytical methods for nitrogenous bases in biological material may readily be devised; included in this scheme is a technique for identifying the substance measured by means of its solubility characteristics (2, 3); 2. the technique of counter-current distribution which has been applied to the fractionation and isolation of substances from biological material (4).

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The aniline analgesics were selected for study because of their relatively simple chemical structure. The present investigation is concerned with the fate of acetanilide in the body. Studies on other drugs will follow.

A recent monograph on acetanilide presents the conflicting views of various investigators concerning the metabolism of acetanilide (5). It is generally believed, however, that acetanilide owes both its therapeutic and its toxic action to its conversion in the body to p-aminophenol which is then conjugated with sulfuric or glucuronic acid.

Recent work, reported since the publication of the above monograph, indicated that in the human, 70–90 per cent of administered acetanilide is excreted in the urine as conjugated p-aminophenol (6). Neither free p-aminophenol nor aniline was found. N-acetyl p-aminophenol and hydroxyl-conjugated N-acetyl p-aminophenol were found in the blood and plasma, but no free p-aminophenol (7). The compounds found were identified only on the basis of non-specific chemical methods without proof that a single substance was being measured identical with an authentic compound. The same studies also showed that the occurrence of methemoglobin after the administration of acetanilide was not due to the formation of p-aminophenol (8).

METHODS AND MATERIAL. Acetanilide and its metabolites were identified and estimated by the methods described in the previous paper (9).

Methemoglobin was determined by a slight modification of the method of Evelyn and Malloy (10). The subjects used for the experiments described below were normal subjects or patients with various chronic diseases without renal or liver involvement.

EXPERIMENTAL. A single lot of acetanilide was used for the entire study. This was a commercially available sample which was assayed for purity by the counter-current distribution technique using a procedure involving 25 plates (4). The results indicated that the acetanilide was at least 99.5 per cent pure.

Absorption and excretion of acetanilide. Information was obtained relative to the absorption from the gastrointestinal tract and the renal excretion of acetanilide, and the part that each of these processes plays in the physiological disposition of the drug after the oral dosage. Human subjects received 2 grams of the drug daily, in two doses, 6 hours apart, over a period of 6 days. Urine and stool collections were made during the last 72 hours of administration. The drug recovered in the stools in each instance amounted to about 0.1 per cent of the administered dose, indicating almost complete absorption from the gastrointestinal tract. Previous experiments had shown that the drug was not destroyed after incubation in stool suspensions for 24 hours at 37°C. The urinary excretion, in these as well as in single dose experiments, accounted for only 0.1–0.2 per cent of the administered acetanilide. Therefore, almost all the drug underwent metabolic alteration in the body.

Plasma concentration-time curves. The plasma concentrations of acetanilide and the per cent methemoglobin after the administration of single oral doses of 1 and 2 grams to man, in the post-absorptive state, are shown in table I. The results shown here are typical of 15 such experiments. As a rule, the absorption was rapid and maximal plasma levels were reached within 1 to 2 hours. The

plasma level fell rapidly, the drug being almost completely metabolized in about 7 hours. The methemoglobin followed a similar curve but with some time-lag.

Acetanilide and its transformation products found in urine. Information concerning the metabolic products of acetanilide was obtained in the urine of subjects given 1 gram orally. The urines were secured for the succeeding 24 hours and examined for the drug and its transformation products. The urinary excretion of acetanilide and its transformation products subsequent to this time, was negligible. About 80 per cent of the drug was accounted for as conjugated N-

TABLE I

Plasma acetanilide and per cent methemoglobin following oral administration of one and two gram doses of acetanilide to man

TIME	SUBJECT D				SUBJECT E			
	1 gram		2 grams		1 gram		2 grams	
	Acetanilide	Methemoglobin	Acetanilide	Methemoglobin	Acetanilide	Methemoglobin	Acetanilide	Methemoglobin
hrs.	*mgm./L.*	*per cent*	*mgm./L.*	*per cent*	*mgm./L.*	*per cent*	*mgm./L.*	*per cent*
0	—	1.4	—	0.3	—	1.5	—	2.2
1	5.6	3.9	17.0	7.2	9.0	4.5	11.0	5.0
2	5.4	6.0	12.0	12.0	7.2	7.7	19.0	10.0
4	2.8	6.6	6.8	13.2	5.0	8.0	11.5	17.5
7	0.5	2.8	1.6	6.0	1.1	4.3	4.2	13.5

TABLE II

The metabolic fate of acetanilide in man

Recovery of acetanilide and its metabolic products from the urine of subjects given single oral doses of acetanilide.

The urine was collected over a period of 24 hours. The proportion of the various metabolites is expressed in percentage of the amount of acetanilide administered.

ACETANILIDE ADMINISTERED	ACETANILIDE	N-ACETYL p-AMINOPHENOL	CONJUGATED N-ACETYL p-AMINOPHENOL	ANILINE
grams	*per cent*	*per cent*	*per cent*	*per cent*
1.0	0.11	3.9	82	0.05
1.0	0.19	3.8	70	0.05
2.0	0.10	3.4	78	0.05
2.0	0.10	3.2	86	0.03

acetyl p-aminophenol,[2] about 4 per cent as free N-acetyl p-aminophenol, and traces as aniline. Less than 0.2 per cent of the parent drug was excreted unchanged. The results shown in table II are representative of 10 similar experiments. That the conjugated p-aminophenol also was acetylated was surmised from the absence of free amino groups in plasma or urine other than that due to the small amount of aniline present. The conjugated N-acetyl p-aminophenol

[2] The conjugated N-acetyl p-aminophenol is the total conjugated p-aminophenol less the N-acetyl p-aminophenol.

has been shown to be esterified at the OH group, partly with sulfuric and partly with glucuronic acid (7). Free p-aminophenol was not detected. An attempt was made to demonstrate the presence of a substance in urine or plasma capable of converting hemoglobin to methemoglobin. Urine or plasma from subjects receiving acetanilide was added to hemolyzed red cells. One hour incubation of this solution at 37°C. failed to result in the formation of methemoglobin.

It is to be noted that the above substances were not analyzed merely on the basis of non-specific chemical reactions but by methods which identified the material measured as a single substance identical with the authentic compound (9).

Additional evidence that the apparent p-aminophenol resulting from the hydrolysis of its conjugated derivatives in urine was a single substance rather than a mixture, and the actual isolation and precise identification of this substance was gained with the aid of Craig's counter-current distribution technique. The urine was acidified by the addition of $\frac{1}{5}$ volume of concentrated HCl and heated in an autoclave at 15 pounds for 1.5 hours. The urine was now adjusted to pH 7 by the addition of K_2HPO_4 and the apparent p-aminophenol extracted with ether. The apparent p-aminophenol was then returned to 0.1 N HCl. The material was then subjected to a counter-current distribution involving 8 transfers, according to the method of Craig (4). The distribution was effected in a series of separatory funnels, between the immiscible solvent pair: isoamyl alcohol and pH 5.8 phosphate buffer. The partition coefficients of the apparent p-aminophenol in each separatory funnel was then determined by measuring the concentration in each phase. The partition coefficients were found to be the same, within analytical error, for the apparent p-aminophenol in each separatory funnel. It was concluded, therefore, that the material which reacted chemically as p-aminophenol was not a mixture but a single substance.

The aqueous phases of the middle three separatory funnels were acidified with HCl. The total apparent p-aminophenol in each separatory funnel was transferred to the aqueous phases by shaking. The three aqueous phases were combined and the pH adjusted to 7. The apparent p-aminophenol was extracted into ether and then returned to dilute HCl. This latter phase was evaporated to dryness on a water bath. The residue was taken up in 30 per cent acetic acid, and benzaldehyde added to form a benzylidene derivative. After two recrystallizations from 30 per cent ethyl alcohol, crystals melting at 179–183°C. were obtained. The mixed melting point with the benzylidene derivative of pure p-aminophenol was unchanged. This showed that the apparent p-aminophenol isolated from urine was identical with the known substance.

Acetanilide and its transformation products found in plasma. The plasma levels of acetanilide and its transformation products after the oral administration of 1 gram doses of the drug are shown in table III. These results are representative of 13 similar experiments. It is seen that the acetanilide levels declined rapidly while those of N-acetyl p-aminophenol increased, suggesting that acetanilide was quickly oxidized to the latter compound. The plasma levels of acetanilide and N-acetyl p-aminophenol permitted an estimate of the amounts of these substances in the body. The calculation was based on the experimental finding for

dogs that both compounds are distributed fairly uniformly throughout total body water. Thus in the case of subject D, if the acetanilide were distributed in 70 per cent of the body weight, the amount of administered acetanilide which remained in the subject would correspond to 28 per cent in 2 hours and 2.5 per cent in 7 hours. The corresponding amounts for N-acetyl p-aminophenol in terms of the administered acetanilide would be 41 per cent in 2 hours and 11 per cent in 7 hours. A rise in conjugated N-acetyl p-aminophenol was coincident

TABLE III

Plasma acetanilide, N-acetyl-p-aminophenol, conjugated N-acetyl-p-aminophenol and aniline after the oral administration of 1 gram of acetanilide to man

TIME	SUBJECT F				SUBJECT G			
	Acetanilide	N-Acetyl p-Amino-phenol	Conj. N-Acetyl p-Amino-phenol	Aniline	Acetanilide	N-Acetyl p-Amino-phenol	Conj. N-Acetyl p-Amino-phenol	Aniline
hrs.	*mgm./L.*	*mgm./L.*	*mgm./L.*	*mgm./L.*	*mgm./L.*	*mgm./L.*	*mgm./L.*	*mgm./L.*
1	9.3	7.9	2.1	0.05	14.0	7.6	6.9	0.09
2	5.8	9.4	5.6	0.04	9.3	7.9	9.7	0.08
4	2.7	6.2	10.4	0.02	5.5	6.6	12.8	0.05
7	0.5	2.6	7.0	0.01	1.2	3.0	11.5	0.02

TABLE IV

Distribution of acetanilide and aniline in the dog

The distribution of acetanilide and aniline was examined in dog tissues. The studies were made 4 hours after the oral administration of 2.9 grams of acetanilide. The dog weighed 23 kilograms.

TISSUE	CONCENTRATION OF ACETANILIDE	CONCENTRATION OF ANILINE
	mgm./kgm.	*mgm./kgm.*
Plasma	70	11.4
Whole blood	63	15.0
Kidney	59	11.2
Heart	60	10.3
Muscle	57	11.2
Lung	58	12.1
Liver	73	15.2
Cerebrospinal fluid	58	7.3
Brain	60	5.8

with a fall in free N-acetyl p-aminophenol as the latter compound is conjugated. Aniline levels were low in comparison with the other substances, suggesting that only a minor fraction of the acetanilide was deacetylated. However, as will be shown later, this small amount of aniline plays a significant role in the toxicity of acetanilide. No free p-aminophenol was demonstrated.

Acetanilide and its transformation products found in tissues. The distribution of acetanilide and aniline 4 hours after the oral administration of acetanilide to a dog, is shown in table IV. The concentration of acetanilide in most organ

tissues was about 80 per cent of that in plasma. The extent to which acetanilide was bound to the non-diffusible constituents of plasma, presumably plasma albumen, was determined by dialysis against isotonic phosphate buffer of pH 7.4 and at 37°C. for 18 hours. Visking membranes were utilized for the dialysis bags. None of the acetanilide was found to be bound in plasma. These results indicated that acetanilide was distributed uniformly throughout most body fluids with negligible localization in cellular tissues. Even in the cerebrospinal fluid and the brain the levels were such as to indicate little if any hindrance by the blood-brain barrier to the passage of the drug. In the above respects the acetanilide behaved like urea.

Aniline levels in various tissues were fairly uniform, although the cerebrospinal fluid and brain levels were distinctly lower than those in the other tissues. Here again, there was little if any localization of the compound in body tissues. Free p-aminophenol was not demonstrated in any tissue.

Site and rate of the transformation of acetanilide in the body. The role of the kidney and the liver in the transformation of acetanilide in the body was studied. The rate of the disappearance of acetanilide from the blood was compared with that of bromsulfalein, a compound presumed to be removed chiefly by the liver (11). Acetanilide and bromsulfalein were administered simultaneously to normal individuals in 3 experiments by means of a constant intravenous infusion at such a rate that the concentration of the substances in the peripheral blood remained unchanged. Under these conditions, the rate of the transformation of acetanilide by the body may be assumed to equal the infusion rate since urinary excretion of acetanilide is negligible. The concentration of acetanilide in the peripheral blood was compared with that in the blood leaving the kidney and the liver. The blood draining the organs was obtained by the venous catheterization technique (11).

The concentration of acetanilide in the renal vein blood did not differ significantly from that in the peripheral venous blood indicating that little, if any, role was played by the kidney in the transformation of acetanilide *in vivo*. On the other hand, the concentration of acetanilide in the hepatic vein blood was found to be considerably lower than that in the peripheral venous blood, indicating that considerable amounts of acetanilide had been transformed by the liver. If it is assumed that the drug was removed only by the liver, then the hepatic blood flow per minute would be $\frac{L}{X_1 - X_2}$ where L is the removal rate per minute of the compound by the liver (equal to the infusion rate), X_1 is the peripheral blood concentration and X_2 is the concentration in the blood leaving the organ. The hepatic blood flow estimated from the data for bromsulfalein and acetanilide were in good agreement. This is in accord with the belief that the removal of acetanilide is also limited for the most part to the liver. The details of this work will be published subsequently.[3]

Fate of aniline and N-acetyl p-aminophenol in the body. The important role of aniline and N-acetyl p-aminophenol in the overall pharmacological action of acetanilide prompted a study of their fate in the organism. One hundred mgm.

[3] Berliner, R. W., and Kennedy, T. W.: To be published.

of aniline hydrochloride were administered orally to 6 human subjects. Urine was collected for a period of 24 hours. About 80 per cent of the aniline was found in the urine as conjugated N-acetyl p-aminophenol. The absence of free amino groups other than that which could be accounted for as aniline was taken as an indication that the conjugated p-aminophenol was also acetylated. No free p-aminophenol was demonstrated. Only about 0.6 per cent of the administered aniline was excreted unchanged. It is of interest that in the dog, the conjugated p-aminophenol resulting from the administration of aniline contained a free amino group. This fits the well-known observation that the dog does not acetylate amino groups.

One gram doses of N-acetyl p-aminophenol were administered to 6 human subjects. About 85 per cent of the administered compound was found in the urine as total conjugated p-aminophenol. No free aniline was demonstrated in plasma or urine. About 3 per cent of the compound was recovered unchanged. The remainder could not be accounted for.

The role of aniline in the formation of methemoglobin. Both methemoglobin and aniline were found in the blood of man after the administration of acetanilide.

TABLE V

Correlation of aniline and methemoglobin levels in the blood after the administration of aniline and acetanilide to man

SUBSTANCE ADMINISTERED	AMOUNT ADMINISTERED	PLASMA ANILINE MAXIMUM LEVEL	MAXIMUM METHEMOGLOBIN
	grams	*mgm./L.*	*per cent*
Acetanilide	2.0	0.07	13.5
Acetanilide	2.0	0.05	9.0
Aniline hydrochloride	0.10	0.07	11.5
Aniline hydrochloride	0.10	0.05	10.0

Methemoglobin was also found after the oral administration of aniline. This suggested that the methemoglobin in the blood after the ingestion of acetanilide might have been formed as a result of the aniline present. The evidence for this was that the amount of methemoglobin is correlated with the plasma aniline concentration subsequent to the administration of either acetanilide or aniline (table V). A similar relationship between the aniline and methemoglobin levels held also in the case of the dog. About 20 times as much acetanilide as aniline was required to produce the same amounts of methemoglobin in man. This indicated that only a small fraction of the acetanilide was deacetylated to form aniline. Whole blood, incubated for 1 hour at 37°C. with either drug at a concentration of 100 micrograms per ml. of blood showed no accumulation of methemoglobin. It is concluded from this that the hemoglobin in the body was not oxidized to methemoglobin directly by aniline, but by some product derived from it in the organism.

The nature of the actual methemoglobin-forming agent is not known. It has been considered to be p-aminophenol. This hypothesis is made unlikely since free p-aminophenol was not demonstrated in the blood after the administration

of either acetanilide or aniline. In addition, both *in vivo* and *in vitro* experiments have shown that considerable concentrations of free p-aminophenol in blood were required to promote the formation of a measurable amount of methemoglobin (8). It is possible that phenylhydroxylamine is the actual methemoglobin forming agent. One mgm. per kilo of this compound administered intravenously to a dog resulted in the conversion of 45 per cent of its hemoglobin to methemoglobin. This is a considerably greater quantity of methemoglobin than would be expected on the basis of a stoichiometric reaction between phenylhydroxylamine and hemoglobin. This suggests that phenylhydroxylamine is involved in a cyclic reaction in which the oxidizing compound is being reformed. Concerning the fate of phenylhydroxylamine in the body, a considerable fraction of the administered compound rearranges in the organism to p-aminophenol which is then excreted in the conjugated form.

TABLE VI

A comparison of plasma N-acetyl p-aminophenol levels after oral administration of equimolecular doses of N-acetyl p-aminophenol and acetanilide

The dose of N-acetyl p-aminophenol was 1 gram, and that of acetanilide was 0.9 gram.

TIME	PLASMA N-ACETYL P-AMINOPHENOL, MGM./L.			
	Subject X		Subject Y	
	After N-Acetyl p-aminophenol	After Acetanilide	After N-Acetyl p-aminophenol	After Acetanilide
hours				
1	2.9	1.1	5.8	3.5
2	8.2	3.1	10.9	4.1
3	4.6	6.5	7.5	4.8
5	2.6	3.0	2.9	3.9
8	0	1.0	0	2.7

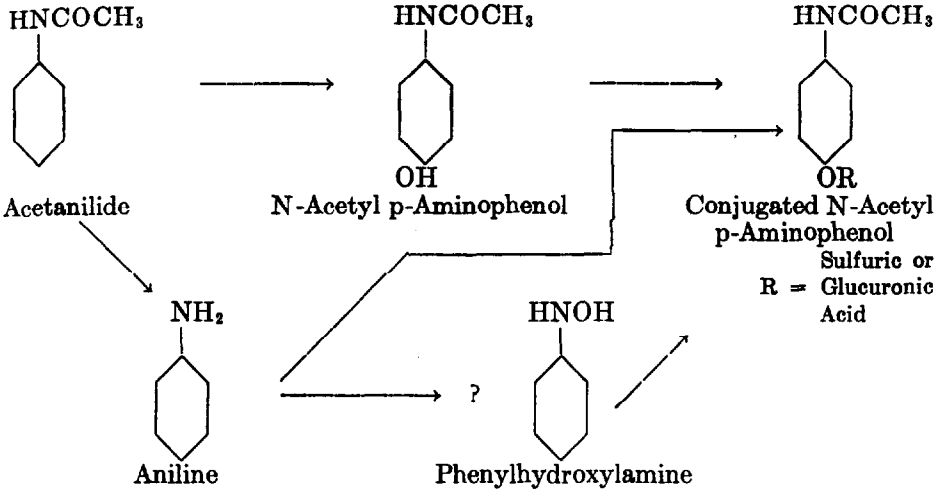
The characteristic toxic symptoms of acetanilide overdosage may be largely explained in terms of the anemic anoxia resulting from the formation of methemoglobin, and anemia due to the destruction of red cells (12). Aniline poisoning also results in methemoglobin formation and the destruction of red cells (12). It is probable that the overall toxicity of acetanilide is derived mainly from the small amount of aniline produced in the body.

The role of N-acetyl p-aminophenol in the analgesic action of acetanilide. N-acetyl p-aminophenol, which is found in both plasma and urine after the administration of acetanilide, appears to be the first step in the major route of the metabolism of the drug. The high concentration of this metabolite in the plasma prompted an appraisal of its analgesic effect. Studies conducted on human subjects, utilizing the Wolff-Hardy technique, showed N-acetyl p-aminophenol to be, dose for dose, about equal in analgesic activity to acetanilide (13). Plasma levels of N-acetyl p-aminophenol achieved after the administration of equimolecular doses of acetanilide and N-acetyl p-aminophenol were of the same order of magnitude, but those obtained after acetanilide persisted longer (table VI).

However, the peak levels of N-acetyl p-aminophenol, when this drug itself was given, were higher and were reached more rapidly, as is to be expected. The results are compatible with the assumption that acetanilide exerted its action mainly through N-acetyl p-aminophenol. Further studies are planned to ascertain whether acetanilide has any analgesic activity *per se* or whether its activity is predicated solely upon its conversion to N-acetyl p-aminophenol. The latter compound, administered orally, was not attended by the formation of methemoglobin nor, at least *in vitro*, did it destroy red cells. It is possible, therefore, that it may have distinct advantages over acetanilide as an analgesic, and it may well serve as a starting point for the synthesis of more effective agents.

Preliminary work in this laboratory has shown that acetophenetidin (p-ethoxyacetanilide), a well-known analgesic, is also transformed in the body to N-acetyl p-aminophenol.

Discussion. The following scheme for the route of metabolism of acetanilide in the human is suggested by the observations described previously.



The main route of metabolism appears to involve two serial steps. The first of these is oxidation with the replacement of the hydrogen atom in the para position of the benzene nucleus by a hydroxy group to form N-acetyl p-aminophenol, an active analgesic. The second step is conjugation of this compound at the hydroxyl group with sulfuric or glucuronic acid. A minor channel of metabolism also involves several serial steps. Part of the acetanilide deacetylates to yield aniline. This compound then adds oxygen to the benzene nucleus to form p-aminophenol which is rapidly conjugated at both the amino and the hydroxy groups. Aniline is also the precursor of the substance, probably phenylhydroxylamine which is responsible for the formation of methemoglobin.

The route of metabolism of acetanilide in the organism differs from that expected on the basis of studies with animal tissues *in vitro*. It has been demonstrated that acetanilide is rapidly hydrolyzed to aniline when it is incubated with rat liver and kidney tissue (14). Yet the intact organism hydrolyzes only a

minor fraction of the drug to aniline, while the major fraction is oxidized to N-acetyl p-aminophenol. Results (unpublished) with other compounds make it apparent that studies *in vitro* concerning the fate of a drug may describe a pathway of metabolism which is of minor importance only, in the intact organism. Many factors come into play in the intact animal which may be eliminated in the simpler experimental conditions *in vitro*. This is not meant to imply that work with isolated tissues is not important. Such work can do much to clarify reactions which first have been shown to occur significantly in the whole animal.

SUMMARY

The route of metabolism of acetanilide in man was shown to be as follows: a minor fraction of the drug deacetylates to form aniline; this compound was shown to be the precursor of the substance which oxidizes hemoglobin to methemoglobin; the major fraction of the drug is oxidized to N-acetyl p-aminophenol; this compound is excreted in a conjugated form. The analgesic action of acetanilide is exerted mainly through N-acetyl p-aminophenol which is an active analgesic. The oxidation of acetanilide occurs mainly in the liver.

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