Carbonic Anhydrase as a Tool in Studying the Mechanism of Reactions Involving H_2CO_3 , CO_2 or HCO_3^-

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In the study of enzymic processes where CO_2 or HCO_3^- or H_2CO_3 are reactants, either as end products or as starting material, it is often of interest to know the order in which the three forms of carbonic acid of the reversible system

arise or react. For example, when urea is acted upon by urease in a buffer solution the end products are NH_4^+ , CO_2 , and HCO_3^- and the question presents itself whether CO_2 , or HCO_3^- , or H_2CO_3 is the primary form of carbonic acid. Similar problems arise in studying the mechanism of decarboxylation of α - or β -ketonic acids and of the bacterial fission of formic acid into H_2 and CO_2 .

As Roughton and his colleagues (Meldrum & Roughton, 1933; Ferguson & Roughton, 1934; Roughton, 1935) have already pointed out, carbonic anhydrase can be used as a tool in studying this kind of problem. The present paper is an attempt to discuss the scope of this tool, to elaborate the experimental conditions under which it can be used and to define the conclusions which can be drawn from various types of experiments. Applications of the method to the study of urease and yeast carboxylase are given.

Theoretical considerations

General. In this paper the term carbonic acid is used to mean the sum of the true carbonic acid (H_2CO_3) and the forms arising therefrom by electrolytic and molecular dissociation. Under physiological conditions, i.e. in the region of neutrality, carbonic acid is present in four forms:

with CO_2 and HCO_3^- predominating. The composition of the equilibrium mixture in a closed system, with a gas phase and a liquid phase, is governed by the volumes of the gas and liquid phases, the dissociation constants of carbonic acid, pH, and the absorption coefficient of CO_2 .

The effect of carbonic anhydrase on the course of a reaction in which carbonic acid takes part depends on the nature of the primary product (whether CO_2 or H_2CO_3) and on the factors controlling the secondary reactions leading to the formation of the

equilibrium mixture. Among the latter, the pH and the nature and concentration of the buffers are outstanding in importance.

The use of carbonic anhydrase is limited by the following two requirements:

(1) The rate of the reaction in which carbonic acid is formed or used must be rapid in comparison with the rates of the uncatalyzed reaction

Otherwise no observable effect will be caused by addition of the enzyme.

(2) The reaction must take place under conditions where carbonic anhydrase is active; substances inactivating carbonic anhydrase must be absent.

In the following discussions specific cases showing the effects of carbonic anhydrase are analyzed in detail and illustrated by manometric experiments. The treatment refers to conditions where a relatively small amount of substrate is added to an enzyme sufficiently powerful to decompose the substrate completely in a matter of minutes.

Effects of carbonic anhydrase if CO_2 is the primary form of carbonic acid. If CO_2 is the primary product and the pH is in the region of 5.0 carbonic anhydrase should not have an appreciable effect because at that pH CO_2 does not form bicarbonate to an appreciable extent.

If, in contrast, the pH is higher, say in the region of 7.0 or higher, some CO_2 will enter into secondary reactions yielding mainly HCO_3^- . The change of CO_2 pressure in a closed system will then depend on the relative rates of (1) the primary reaction yielding CO_2 , (2) the secondary reaction converting CO_2 into HCO_3^- , and addition of carbonic anhydrase should diminish the rate of CO_2 evolution in the early stages, but the precise effect of carbonic anhydrase will vary according to the relative rates of (1) and (2). Thus if (1) is much more rapid than (2), a rapid evolution of CO_2 in excess of the equilibrium will be followed by a slow absorption of the excess (Table 1 and Fig. 1); as the rate of (1) approaches that of (2), the amount of excess CO_2 diminishes (Table 3, Figs. 3 and 4). In both cases the addition of carbonic anhydrase will accelerate

the rate of (2) and therefore lower the CO_2 pressure in the early stages of the reaction, so that the system is in equilibrium throughout the reaction, but the effect of this enzyme is of course less marked in the second case. If the rate of (1) is equal to the rate of (2), CO_2 will not be evolved in excess of the equilibrium, and in this case carbonic anhydrase will have no demonstrable effect.

Effects of carbonic anhydrase if HCO_3^- or H_2CO_3 is the primary form of carbonic acid. As the reactions $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ are extremely rapid in comparison with the reaction $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$, the effects of carbonic anhydrase are expected to be the same if either H_2CO_3 or HCO_3^- is the primary product of the reaction. In both cases carbonic anhydrase will accelerate the evolution of carbon dioxide if the pH is at or near the neutral point. An example is the standard procedure of Meldrum & Roughton (1933) of mixing a bicarbonate solution with phosphate buffer in the presence and absence of enzyme.

As the pH rises, the formation of CO_2 from bicarbonate decreases in magnitude until it becomes negligible above pH 8.0. Effects of carbonic anhydrase are therefore not expected at that pH. On the acid side of the neutral point, say between pH 5.0 and 6.0, the rate of the uncatalyzed decomposition of H_2CO_3 is very rapid and the manometric measurement of the acceleration by carbonic anhydrase becomes difficult.

EXPERIMENTAL

General technique. The measurements consisted of the manometric determination of the change in the pressure of CO_2 within a closed system. The experiments were carried out with Warburg manometers, which were more convenient for the present work than the boat method. Conical flasks of about 20 ml. capacity were used, having a side arm from which the substrate was added, but no centre well as the latter hinders the rapid movement of the fluids. In each experiment two manometers with flasks differing in volume by less than 1% were used. Identical quantities of the enzyme (urease or carboxylase), buffer, substrate and crude carbonic anhydrase were added to both flasks and an inhibitor of the latter enzyme (thiophen-2-sulphonamide) to one of the flasks. The two flasks therefore differed only by the presence of a small amount (usually 0.02 mg./ml.) of inhibitor. The possible alternative of adding carbonic anhydrase to one flask only may cause complications as impurities in the enzyme may have buffering capacities which affect the equilibrium. It is essential that the two flasks

should be equal in volume as the ratio $\frac{\text{CO}_2}{\text{total carbonic acid}}$ in the equilibrium mixture depends on v_a and v_r . The changes in CO_2 pressure therefore are not directly comparable if vessels of different sizes are used.

The quantity of substrate added was relatively small, equivalent to a maximum yield of 1000–2000 μl . carbonic acid. Under these conditions the end point was reached within a few minutes and the pressure changes did not exceed the scope of the manometer scales.

An important control in this type of experiment is the measurement of the final value of the CO_2 output. The rates in two parallel manometric vessels are comparable only if the pH's and the concentrations of the buffers are identical. The final CO_2 value is determined by the pH and the buffer concentration and its measurement is therefore a check on the identity of conditions in the two flasks.

The manometers were first shaken slowly until equilibrium was established. The contents of the side arms of the two flasks were then mixed simultaneously by two people and the rate of shaking was increased to about 150 periods/min., with an amplitude of 7 cm. at the level of the bottom of the flasks. Two small glass beads (2 mm. diam.) were placed in the solution to increase the effectiveness of shaking.

In experiments in which a substrate was added from the side arm to an enzyme the pH of substrate and enzyme solutions was carefully adjusted to the same value, as checked by colorimetric and electrometric measurements, and the initial amounts of CO_2 and HCO_3^- in both solutions were kept as low as possible. If the solutions contain HCO_3^- differences in pH cause pressure changes on mixing which may vitiate the results of the enzymic process.

The temperature of the bath was low, usually 15° or below. Owing to the relatively high temperature coefficient of the uncatalyzed reaction $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$ (see Roughton, 1943) the effects of carbonic anhydrase are likely to be greater the lower the temperature.

Urease. Jack beans obtained from the Imperial College of Tropical Agriculture, Trinidad, were broken up in a coffee grinder and the husks were separated from the remainder by sieving. The bean meal (6 g.) was shaken for 10 min. with water or buffer solution (50 ml.) and centrifuged. At 15°, 2 ml. of the supernatant decomposed 2.5 mg. urea completely in about 40 sec.

Carboxylase. Freshly pressed brewer's yeast was spread in thin layers and allowed to dry in the air. This required less than 24 hr. The dried material was stored in a cool, dry place. The carboxylase preparation was made on the day on which it was used by incubating a mixture of 20 g. dried yeast and 60 ml. water for 2 hr. at 30° and centrifuging; the supernatant was used as carboxylase. At 15° and pH 5.5, 3 ml. solution, on addition of 782 μl . pyruvate, liberated 230 μl . CO_2 during the 1st min. and 316 μl . during the 2nd min. The rate of pyruvate decomposition was of the same order at pH 7.0.

Carbonic anhydrase. Most experiments were made with the chloroform-ethanol preparation of Roughton & Booth (1946), ox blood serving as the starting material. In a few cases laked ox blood was used. In the following protocols 'carbonic anhydrase' refers to the undiluted chloroform-ethanol extract, unless stated otherwise. To obtain maximum effects it was necessary to add about 0.1 ml. of this to 2 or 3 ml. of the reaction mixture.

Inhibitors. Thiophen-2-sulphonamide, kindly supplied by the American Cyanamide Company, was used as a standard inhibitor; 0.1 ml. of a 0.05% aqueous solution was added to the solution. According to Davenport (1945), the discoverer of the inhibitory action of this compound, thiophen-2-sulphonamide, is about 6 times as inhibitory as is sulphanilamide at 0°. After most of the work reported in this paper was completed it was found that *p*-toluene-sulphonamide is still more effective as an inhibitor than thiophen-2-sulphonamide, and this substance was used in

some experiments. Controls indicated that these sulphonamides had no significant effect on the activity of urease or yeast carboxylase when carbonic anhydrase was absent.

Addition of buffers. As the magnitude of the secondary reactions of carbonic acid depends on the concentration of buffers, phosphate was added to the enzyme solutions in most experiments; the amounts added are stated later.

Experiments with urease

Our first experiments with urease were carried out in 1934 and have already been referred to briefly (Roughton, 1935). The experimental conditions in

Table 1. *Effect of carbonic anhydrase on the course of CO₂ evolution from urea in the presence of urease*

(Each flask contained 2 ml. jack-bean extract (6 g. ground jack beans shaken with 50 ml. 0.05 M-phosphate buffer pH 7.4; centrifuged and supernatant used, pH 6.9) and the additions stated below in the main compartment, 0.25 ml. 10% urea ($\equiv 930 \mu\text{l. CO}_2$) in the side arm; 15°; v_0 18.0; K_{CO_2} 1.952.)

Time after mixing (min.)	CO ₂ evolved ($\mu\text{l.}$)	
	Flask 1	Flask 2
	(0.2 ml. carbonic anhydrase, 0.1 ml. 0.05% thiophen-2-sulphonamide)	(0.2 ml. carbonic anhydrase 0.1 ml. water)
0.5	552	129
1.0	566	137
1.5	510	138
2.0	470	138
2.5	427	138
3.0	386	138
4.0	326	138
5.0	275	138
6.0	230	138
8.0	185	138
10.0	164	138
13.0	148	138
18.0	138	138
26.0	138	138

Fig. 1. Effect of carbonic anhydrase on the evolution of CO₂ in the system urease-urea (15°).

which effects of carbonic anhydrase can be demonstrated are given in Table 1 and the results are shown graphically in Fig. 1. It will be seen that in the absence of carbonic anhydrase urea equivalent to 930 $\mu\text{l. CO}_2$ yielded 566 $\mu\text{l. CO}_2$ within 60 sec. (61 %

of the theoretical yield). Of this amount 428 $\mu\text{l.}$ were reabsorbed in the course of 18 min. In the presence of carbonic anhydrase there was no excess

Table 2. *CO₂ evolution from a mixture of methylamine carbamate and phosphate buffer with and without carbonic anhydrase*

(Both flasks contained in the main compartment 3 ml. 0.1 M-phosphate buffer, pH 6.5, and the additions stated below, and in the side arm 0.3 ml. 1.2 N-methylamine carbamate-carbonate solution prepared by passing CO₂ through a solution of methylamine until it turned alizarine yellow to pink orange. The total CO₂ content of the solution (carbamate + carbonate) was 0.470 M and the pH was 10.4 (see Faurholt, 1924); 15°.)

Time after mixing (min.)	CO ₂ evolved ($\mu\text{l.}$)	
	Flask 1 Carbonic anhydrase (0.2 ml.)	Flask 2 Water (0.2 ml.)
0.33	159	721
0.67	232	960
1.0	270	1033
1.33	288	1040
1.67	301	1025
2.0	310	1000
3.0	321	908
4.0	324	825
6.0	324	682
10.0	324	508
14.0	324	409
20.0	324	355
30.0	324	331

Fig. 2. Effect of carbonic anhydrase on the evolution of CO₂ in a mixture of methylamine carbamate and phosphate buffer (15°). Carbonic anhydrase added, ---x---; water added —•—•—.

production of CO₂, and the final yields of CO₂ were the same with and without the enzyme.

In further experiments the concentrations of the reactants were varied. When less carbonic anhydrase was used there was a small excess production of

CO₂ even in the presence of the enzyme. When the urease solution was diluted 10 times with phosphate buffer pH 7.1 the course of the reaction was protracted and the excess CO₂ was smaller in amount but still marked. The effects of carbonic anhydrase were also demonstrable at 30°. On the other hand, as expected, no effects of carbonic anhydrase were observed at 15° at pH 6.0 (phosphate buffer) or 5.0 (acetate buffer).

The results make it certain that bicarbonate or H₂CO₃ are not primary products in the action of urease. They are in accordance with the view that primary reaction products are either ammonium carbamate or CO₂ + NH₃. When a solution containing a carbamate is added to an approximately neutral buffer, curves of the same general shape as those given by the urea-urease system are obtained, as Ferguson & Roughton (1934) have shown, and as is further illustrated by Table 2 and Fig. 2. The view that ammonium carbamate is an intermediate was first expressed by Fenton (1885) and by Armstrong & Horton (1912), and substantiated by Yamasaki (1920), Mack & Villars (1923), and especially by Sumner, Hand & Holloway (1931). The last-named workers showed conclusively that ammonium carbamate is formed when urease is allowed to act in alkaline medium. It could not be detected in the presence of buffers. The authors rightly point out that the demonstration of the presence of ammonium carbamate does not necessarily prove that this substance is the first intermediate. The reversible reaction

is extremely rapid (Faurholt, 1924), and in the experiments where carbamate was detected it could have arisen either directly from urea or secondarily from CO₂ and NH₃. It is not possible at present to distinguish between these alternatives.

The action of urease and the subsequent series of reactions in the presence of buffers may therefore be written as follows:

Experiments with yeast carboxylase

To 20 ml. yeast extract were added, immediately before use, Na₂HPO₄·2H₂O (178 mg.) and, drop by drop, CO₂-free N-NaOH (1.7 ml.). The resulting turbid mixture turned phenol red pink and showed electrometrically a pH of 7.0. A 0.2M-pyruvate solution of the same pH and phosphate concentration was prepared from a N-stock solution of pyruvic acid, Na₂HPO₄·2H₂O and CO₂-free N-NaOH.

The details of two experiments showing the effects of carbonic anhydrase are given in Table 3 and Figs. 3 and 4. In both experiments carbonic anhydrase retarded the evolution of CO₂ in the manner expected for a reaction yielding CO₂ as the primary product. At 7.5° the excess of CO₂ evolved was greater than at 15° and the effect of carbonic anhydrase correspondingly more marked. A greater effect is expected at a lower temperature if the temperature coefficient of carboxylase (which is about 2.1) is smaller than that of the uncatalyzed hydration of CO₂.

As regards the rate of CO₂ evolution from its substrate the carboxylase was less potent than the urease, and in addition, the rate of reaction dropped more rapidly with decreasing substrate concentration in the case of carboxylase. These two factors account for the differences in the shapes of the curves obtained with the two enzymes, although in both cases CO₂ is the primary product.

In similar experiments at pH 6.0 and 5.0 no effects of carbonic anhydrase were seen.

It will be noted that there is a short lag period before the full rate of CO₂ evolution is reached. It is very unlikely that any significant part of this lag is due to diffusion. Attempts to abolish the lag were unsuccessful. Addition to the main compartment of small amounts of glucose or pyruvate which were decomposed during the equilibration period had no effect.

The experiments leave no doubt that CO₂, and not H₂CO₃ or HCO₃⁻, is the primary product of decarboxylation. This result is in contrast to that of Conway & MacDonnell (1945), who claimed in a preliminary publication to have observed an acceleration of the CO₂ output by carbonic anhydrase, when yeast carboxylase acts upon pyruvate. Prof. Conway has kindly supplied the full experimental details in the procedure employed by Conway & MacDonnell, and it has therefore been possible to repeat their experiments. Conway & MacDonnell worked at pH 5.5 and 37° and added a large excess of pyruvate. Under such conditions we did not find any difference between the rates of CO₂ evolution in the presence and absence of carbonic anhydrase and the results of Conway & MacDonnell thus could not be confirmed. At pH 5.5 and 37° the rate of the uncatalyzed dehydration of H₂CO₃ is very fast, the time for the half-reaction being about 0.8 sec.; no additional manometric effect is therefore to be expected on addition of carbonic anhydrase, and the effects recorded by Conway & MacDonnell cannot have been due to carbonic anhydrase; their origin remains unexplained.

It should be noted that the graphs published by Conway & MacDonnell are not directly comparable with those given in this paper, as Conway & MacDonnell plotted the rate of CO₂ evolution (not the total CO₂ output) against time.

Table 3. *Effect of carbonic anhydrase on the course of CO₂ evolution from pyruvate in the presence of yeast carboxylase*

(For details of carboxylase and pyruvate solution, see text. Each flask contained 3 ml. carboxylase and 0.1 ml. carbonic anhydrase in the main compartment and 0.4 ml. 0.2M-pyruvate in the side arm. Flasks (1) further contained 0.1 ml. 0.05% thiophen-2-sulphonamide solution. K_{CO_2} in Exp. 1, 2.13; in Exp. 2, 2.24.)

Time after mixing (min.)	CO ₂ evolved (μl.)			
	Exp. 1 (15°)		Exp. 2 (7.5°)	
	Flask 1	Flask 2	Flask 1	Flask 2
0.5	11	8	11	10
1.0	45	23	23	18
1.5	92	45	45	26
2.0	140	77	72	42
2.5	200	113	103	56
3.0	251	155	137	80
4.0	356	234	200	122
5.0	412	302	256	164
6.0	469	356	306	205
7.0	511	405	347	241
8.0	522	443	383	274
10.0	542	490	441	335
12.0	554	516	490	389
14.0	—	—	528	421
16.0	—	—	540	458
17.0	—	—	545	466
19.0	—	—	545	485
21.0	—	—	535	490
25.0	585	582	515	499
30.0	—	—	500	502
40.0	591	588	500	502

Fig. 3. *Effect of carbonic anhydrase on the evolution of CO₂ in the system yeast carboxylase-pyruvate (15°).*

The use of Warburg manometers for the estimation of carbonic anhydrase activity

The reaction between bicarbonate and hydrogen ions and the effects of carbonic anhydrase thereon have already been thoroughly investigated by pre-

vious workers (see Roughton, 1943), but the use of the Warburg manometer for the study of this reaction has not been described in detail. The following experimental conditions, which are similar to those used in various forms of the 'boat' method of Meldrum & Roughton (1933), were found to be suitable for the given dimensions of the Warburg flasks.

Solutions. Phosphate buffer (0.1M) prepared by mixing 300 ml. 0.1M-Na₂HPO₄ with 200 ml. 0.1M-KH₂PO₄; NaHCO₃, 0.1M and 0.05M.

Manometric arrangement. For experiments at 0°, 2 ml. phosphate buffer, and additions such as carbonic anhydrase and inhibitors in a total volume of 0.2 ml., were measured into the main compartment and 1 ml. 0.1M-NaHCO₃ into the side arm. For experiments at temperatures between 15 and 40°, 1 ml. buffer was used instead of 2 ml., and 1 ml. 0.05M-NaHCO₃ was placed in the side arm. An experiment at 15° is shown in Table 4. It will be seen that the initial rate was accelerated more than sixfold by the enzyme. At 38° the initial acceleration was about twofold.

At low enzyme concentration the acceleration caused by carbonic anhydrase is proportional to the enzyme concentration as shown in Table 5 which gives details of an experiment carried out at 0°. The uncatalyzed reaction, as well as the increase caused by relatively small amounts of the enzyme, proceeded approximately linearly until about one third, i.e. about 80 mm., of the final pressure change had been reached. Later the rate of reaction fell off. In

Fig. 4. *Effect of carbonic anhydrase on the evolution of CO₂ in the system yeast carboxylase-pyruvate (7.5°).*

quantitative experiments the initial third section of the curve was used in calculating the activity of carbonic anhydrase. A reading in the region between 60 and 80 mm. was chosen and from it the rate of reaction for a standard period, usually 30 or 60 sec., was calculated. The application of this

procedure to the data recorded in Table 5 gives the following figures:

	CO ₂ (μl.) evolved in 30 sec.	Increase due to carbonic anhydrase (μl. CO ₂)
No enzyme added	20	—
Enzyme diluted 1/320	49	29
Enzyme diluted 1/160	80	60
Enzyme diluted 1/80	138	118

The increased CO₂ output is thus proportional to the amounts of carbonic anhydrase added. The procedure is, therefore, suitable for quantitative measurements on the concentration of the enzyme in tissues and on the action of inhibitors. It must be emphasized that the rigid precautions in cleanliness—acid washing, rinsing with doubly distilled water, prevention of contamination by gas and metallic impurities—which Roughton & Booth (1946) found necessary, are essential for obtaining reproducible results. Even when these precautions are taken single results cannot be relied upon; duplicate or triplicate experiments are essential.

Table 4. *Effect of carbonic anhydrase on the reaction between NaHCO₃ and phosphate buffer, measured in Warburg manometers*

(The carbonic anhydrase preparation was the chloroform-ethanol extract from ox red cells, diluted 20-fold; the main compartment contained 1 ml. buffer (see text) and the side arm 1 ml. 0.05M-NaHCO₃; 15°; K_{CO₂}, 2.32.)

Time after mixing (min.)	CO ₂ evolved (μl.)	
	Flask 1 Carbonic anhydrase (0.2 ml.)	Flask 2 Water (0.2 ml.)
0.33	196	30
0.67	280	63
1.0	332	98
1.33	354	128
1.67	372	154
2.0	382	179
3.0	399	244
4.0	402	296
9.0	405	392
15.0	405	406

In previous work the reciprocal of the time taken for a given evolution of CO₂ has been used as a measure of the enzyme activity, but if the rate of CO₂ evolution is linear, as is here the case in the

Table 5. *Effect of varying quantities of carbonic anhydrase on the rate of CO₂ evolution from a mixture of phosphate buffer and bicarbonate*

(0°; volume of manometer flasks, 24 ml.; the carbonic anhydrase preparation was obtained from ox blood cells by treatment with chloroform and ethanol (Roughton & Booth, 1946); for further details, see text; the data are averages of duplicate determinations.)

Time after mixing (min.)	Pressure (mm.) observed manometrically				
	With 0.1 ml. enzyme				
	Without enzyme	Diluted 1/320	Diluted 1/160	Diluted 1/80	Undiluted
0.5	10	25	42	69	204
1.0	20	50	80	130	248
1.5	31	73	110	170	259
2.0	42	94	133	195	264
2.5	52	113	157	212	267
3.0	62	128	169	224	267
4.0	79	158	194	241	—
5.0	96	180	210	251	—
7.0	124	212	232	259	—
12.0	168	255	247	262	—
17.0	208	263	263	264	267

early stages, it is equivalent, and simpler, to use the amount of CO₂ evolved in 30 or 60 sec. as a measure of activity.

SUMMARY

1. When a powerful urease preparation is allowed to act upon a limited quantity of urea in a neutral buffer at 15°, CO₂ is rapidly evolved and later gradually reabsorbed. If carbonic anhydrase is added no excess of CO₂ is evolved. These results support the conception that CO₂, and not HCO₃⁻ or CO₃⁼, is a primary intermediate in the action of urease on urea.

2. Carbonic anhydrase has a similar effect in the system yeast carboxylase-pyruvate, under certain conditions, which are described. The results indicate that CO₂, and not HCO₃⁻ or H₂CO₃, is the primary product in the enzymic decarboxylation of pyruvate.

3. The effects which are to be expected when carbonic anhydrase is added to systems in which CO₂ or bicarbonate are reactants are discussed from a general point of view.

4. The use of Warburg manometers for the quantitative determination of carbonic anhydrase activity is described.

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