The Effect of Carbonic Anhydrase on the Action of Yeast Carboxylase

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In the decarboxylation of pyruvic acid by yeast, the question of an initial formation of carbonic acid or of carbon dioxide may be investigated by the use of carbonic anhydrase. The use of this enzyme in such investigations was first suggested by Roughton and co-workers (Meldrum & Roughton, 1933; Ferguson & Roughton, 1934; Roughton, 1935). Roughton (1935) published a diagram (Krebs & Roughton, 1934) showing the effect of the enzyme on the carbon dioxide pressure when urease acts on urea.

The relation of ferment to substrate was such that the total urea-splitting occurred in a very short time. The liberation of free carbon dioxide as a primary product was shown by the rapid rise of the pressure in the manometer, followed by its slow decline (over about 10–15 min.) as carbon dioxide was taken up as HCO_3^- by the buffered medium. In the presence of a sufficient concentration of carbonic anhydrase no such initial fling and return of the carbon dioxide pressure occurs.

In repeating such experiments with carboxylase the difficulty lay in obtaining a preparation strong enough to act on the pyruvate substrate with adequate rapidity. Recently, Krebs & Roughton (1948) have so far fulfilled this requirement as to obtain results showing that, for the conditions studied, carbon dioxide is mainly the primary product.

In the investigation of the carboxylase action with carbonic anhydrase we used a somewhat different approach, namely strong concentrations of pyruvate, so as to produce a linear or near-linear rise of pressure over the time studied (of the order of 5 min.). Initial experiments (Conway & Mac-Donnell, 1945) seemed to indicate an increased carbon dioxide tension in the presence of carbonic anhydrase. These preliminary experiments were technically unsatisfactory. Also, the theoretical change of pressure arising from carbonic anhydrase would be very slight at the initial pH of 5.5. Owing, however, to the inadequate buffering this pH very rapidly changed to higher values, which appears to account for the differences found.

Subsequent experiments, better controlled (see O'Malley, 1947), supported the original conclusions and an account was delayed pending further clarification. Simultaneously, Krebs (1948) and Conway & O'Malley (1948) read communications concerning

the effect of carbonic anhydrase on decarboxylation. Krebs advanced definite proof that, under the conditions studied, carbon dioxide was a primary product (though, as judged from the evidence here given, it was not necessarily the sole primary product of the carbon dioxide system). Our results supported the conclusion that whether carbon dioxide or carbonic acid was the main primary product depended on the conditions, and we gave equations, the derivations of which are described in this paper, for the theoretical evaluation of the effect of carbonic anhydrase, assuming a linear or near-linear rate of decarboxylation, as with strong pyruvate substrate. More recently, after a study of the best theoretical conditions, using the strong pyruvate substrate, a type of experiment was designed which would appear to furnish clear-cut results, the pressure changes being compared with the theoretical figure. This type of experiment only will be described in the present account.

THEORETICAL

If yeast carboxylase acts in a small volume of concentrated pyruvate solution, well buffered at pH 7.0, and CO_2 is liberated into a gaseous phase of constant volume, with adequate shaking and at constant temperature, equations may be deduced, described below, relating the CO_2 in the gaseous phase (and hence the change in pressure) with the time of action. It is assumed that the rate of decarboxylation is constant over the short period studied.

Symbols

n_1, n_2 = the number of moles of free CO_2 in the gaseous and liquid phases, respectively; n_3, n_4 = the number of moles of HCO_3^- and of H_2CO_3 in the liquid phase, respectively; V_1, V_2 = the volume, in litres, of the gaseous and liquid phases (V_2 throughout is 3.4×10^{-3} l., and $V_1 = 4.6 \times 10^{-3}$ l. in most of the experiments); k_1, k_2 = the velocity constants of the reactions:

respectively (k_1 and $k_2 = 1.5$ and 720 min.^{-1} at 18° from the data collected by Roughton (1935)); a_{H^+} = the hydrogen-ion activity in the liquid phase,

and corresponds in the experiments to pH 7.0, as determined by the glass electrode;

$$K' = a_{H^+} \times n_3/n_4 = 10^{-3.5}, \text{ approx.};$$

$\lambda = \frac{n_2}{V_2} / \frac{n_1}{V_1}$, or the Ostwald absorption coefficient ($= 0.7$ approx.) as deduced from a consideration of the fluid composition in the decarboxylation studies described and various solubility data for CO_2 in the *International Critical Tables*; $S = Rt$, where S is the sum of the moles of the carbonic acid system, R a constant and t the time in minutes.

In addition, the following symbols have been used for convenience:

$$m = V_2 \lambda / (V_2 \lambda + V_1);$$

$$k_3 = k_2 / (1 + K' / a_{H^+});$$

$$\beta = k_3 + k_1 m.$$

From the above data the value of m is 0.34 and of k_3 and β 0.23 and 0.74 min.^{-1} , respectively.

Deduction of the equations relating n_1 to the time t

With H_2CO_3 as the primary product of decarboxylation, the rate of total free CO_2 production may be written

$$\begin{aligned} \frac{dn_1}{dt} + \frac{dn_2}{dt} &= k_2 \frac{n_4}{V_2} V_2 - k_1 \frac{n_2}{V_2} V_2 \\ &= k_2 n_4 - k_1 n_2, \end{aligned} \quad (1)$$

where CO_2 is the primary product,

$$\begin{aligned} \frac{dn_1}{dt} + \frac{dn_2}{dt} &= k_2 n_4 - k_1 n_2 + \frac{dS}{dt} \\ &= k_2 n_4 - k_1 n_2 + R, \end{aligned} \quad (2)$$

Proceeding from equation (1),

$$\frac{dn_1}{dt} \times \frac{V_2 \lambda}{V_1 m} = k_2 n_4 - k_1 n_1 \frac{V_2 \lambda}{V_1}. \quad (3)$$

n_4 can also be expressed in terms of n_1 from

$$S = Rt = n_1 + n_2 + n_3 + n_4, \quad (4)$$

$$= n_1 \frac{V_2 \lambda}{V_1 m} + n_4 \frac{k_2}{k_3}. \quad (5)$$

Inserting the value of n_4 from equation (5) in equation (3) one obtains

$$\frac{dn_1}{dt} + \beta n_1 = \frac{V_1 m R k_3}{V_2 \lambda} \times t, \quad (6)$$

and proceeding from equations (2) and (5) in a similar way there follows

$$\frac{dn_1}{dt} + \beta n_1 = \left(\frac{V_1 m R k_3}{V_2 \lambda} \times t \right) + \frac{R V_1 m}{V_2 \lambda}. \quad (7)$$

With respect to the variables n_1 and t , equations (6) and (7) are linear differential equations of standard

form. Integrating between zero time and time t with $n_1 = 0$ when $t = 0$, and with H_2CO_3 the primary product, there follows

$$\frac{n_1}{R} = \frac{m k_3 V_1}{\beta V_2 \lambda} \left\{ t - \frac{1}{\beta} (1 - e^{-\beta t}) \right\}. \quad (8)$$

With CO_2 the primary product, there follows

$$\frac{n_1}{R} = \frac{m k_3 V_1}{\beta V_2 \lambda} \left\{ t + \frac{k_1 m}{k_3 \beta} (1 - e^{-\beta t}) \right\}. \quad (9)$$

For the manometric conditions used, $e^{-\beta t}$ approaches close to zero within a few minutes, so that equations (8) and (9) may then be written

$$n_1 = \gamma (t - 1/\beta) \quad (10)$$

and

$$n_1 = \gamma (t - k_1 m / \beta k_3), \quad (11)$$

where

$$\gamma = \frac{R m k_3}{\beta} \times \frac{V_1}{V_2 \lambda}.$$

If carbonic anhydrase is present in the fluid in sufficient amount to increase greatly the values of k_1 and k_2 , then the two equations (10) and (11) reduce to

$$n_1 = \gamma t. \quad (12)$$

If a line be drawn representing moles of CO_2 in the gaseous phase (n_1) for any value of t (assuming a linear relation), this line will have the same slope (γ) in equations (10)–(12). But, if sufficient carbonic anhydrase is added and with H_2CO_3 the primary product of decarboxylation, this line increases to a higher level by $\gamma \times 1/\beta$. The ratio of this increase to the slope is $1/\beta$. It is obvious that such a ratio holds independently of whatever units, besides moles, are used to express the CO_2 content of the gaseous phase, whether $\mu\text{l.}$ or pressures observed directly on the scale of a Warburg manometer. It is also independent of the rate of decarboxylation, provided this is constant or nearly constant throughout the observations and the shaking is adequate.

If CO_2 be the primary product, then on adding carbonic anhydrase the corresponding ratio of the change resulting to the slope is $-k_1 m / \beta k_3$ and the line of n_1 against t falls to a lower level.

These expected theoretical curves are shown in Fig. 1. Here it may be assumed that after some minutes' action the manometer tap is closed and after 3 min. the fluid in the side arm containing carbonic anhydrase (sufficiently strong to increase the k_1 and k_2 values 20 times or more) is mixed with that in the central chamber.

It may be noted that the effect of the carbonic anhydrase is relatively much greater but in an opposite direction when the primary product is free CO_2 than when it is H_2CO_3 (or HCO_3^-).

METHODS

Carboxylase preparation. The method of preparation was that of Green, Herbert & Subrahmanyam (1941).

Dried yeast, very kindly supplied by Prof. Krebs, was mixed with 3 times its weight of 0.067M-sodium phosphate buffer (pH 7.2) and incubated at 37° for 1 hr. An equal volume of water was added and the mixture centrifuged.

Fig. 1. Diagram showing the full theoretical effect of carbonic anhydrase on the moles of CO_2 liberated into the gaseous phase when carboxylase in a certain strength acts on strong pyruvate solution, and for an initial period produces CO_2 in linear relation with time. pH buffered to 7.0, and temperature 18°. At the beginning of the carboxylase action the manometer is open to the atmosphere, then closed. 3 min. after the closure, carbonic anhydrase is introduced from the side arm, which contains otherwise the same fluid composition as the central fluid. ---, the presumed course of the CO_2 liberation after adding carbonic anhydrase. —, the curve when a carbonic anhydrase inhibitor is included in both fluids from the outset. (a) This shows the effect of adding carbonic anhydrase, when H_2CO_3 is the primary product of decarboxylation. The ratio of the increase in level ($\gamma \times [1/\beta]$) to the slope (γ) is $1/\beta$. (b) This shows the effect of adding carbonic anhydrase when CO_2 is the primary product. The ratio of increase is here $-k_1m/k_3\beta$.

The crude extract obtained was mixed with M-calcium acetate and 0.5M-sodium phosphate buffer (pH 7.2), then centrifuged. The carboxylase was precipitated twice by half-saturation with $(\text{NH}_4)_2\text{SO}_4$ and then fractionated. The precipitate at 0.4 saturation was discarded and the solution brought to half-saturation. For some experiments this suspension was fractionated a second time, the precipitate at 0.47 saturation being discarded.

A different method of extraction was used for fresh Dublin yeast. The pressed yeast was frozen with solid CO_2 or liquid O_2 . After thawing it was centrifuged and the crude extract treated as above. Fractionation with $(\text{NH}_4)_2\text{SO}_4$ led to a considerable loss of activity, whereas the dried Sheffield yeast gave more satisfactory results, and yielded clearer solutions.

The carboxylase preparation was kept at 0° suspended in half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and used in this form. It retained its activity indefinitely.

When crude yeast extract was used it was prepared freshly before the experiment. The activity may be judged from the following typical result. At 18° and pH 7, 0.25 ml. of the $(\text{NH}_4)_2\text{SO}_4$ suspension diluted to 2 ml. with 0.25M-sodium phosphate buffer, on addition of 1 ml. of 2M-sodium pyruvate yielded 34 μl . CO_2 during the second 2 min.

Carbonic anhydrase preparation. Carbonic anhydrase was prepared from ox blood, following the method of Keilin & Mann (1940). The ethanol- CHCl_3 extract is referred to as 'yellow fluid' in the following experiments. This was further purified by dialysing for 3 hr. against running tap water, and precipitated by saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected, dissolved in water and filtered. From 100 ml. 'yellow fluid' 5 ml. of solution containing carbonic anhydrase were thus prepared.

By a microdiffusion method (Conway & McDonnell, 1951; modified procedure of Conway & O'Rourke, 1945) the purified carbonic anhydrase preparation in a dilution of 2000 times (total phosphate buffering of approx. 0.1M at pH 7.0), and acting on an 0.025M concentration of NaHCO_3 , gave an increase of k_1 and k_3 to at least 3.25 times (the maximum observable by the procedure without further dilution).

In the carboxylase experiments it was, however, considered advisable to use the carbonic anhydrase preparation in much more concentrated form owing to possible inhibitory effects; the final dilution was accordingly 34 times. If no additional inhibition occurred this would increase k_1 and k_3 about 200 times.

The protein content of the carbonic anhydrase preparation was determined by heating the acidified solution to 100° for 10 min. The precipitate was collected by centrifuging, washed twice and dried. 2 ml. of solution yielded 0.0162 g. of protein, i.e. 0.81% (w/v).

Pyruvate solution. 2M-Sodium pyruvate was prepared from redistilled pyruvic acid (90%), neutralizing this with 2.4N-NaOH. The solution was adjusted electrometrically to pH 7.0.

Oxaloacetate solution. 2M-Sodium oxaloacetate was made immediately before each experiment from crystalline oxaloacetic acid, prepared by the method of Wohl & Osterlin (1901).

α -Ketoglutarate solution. α -Ketoglutarate was prepared by the method of Wislicenus & Waldmüller (1911). The sodium salt (2M solution) was prepared before each experiment and adjusted to pH 7.0.

Manometric procedure. The following solutions were made up:

(A) Carboxylase-buffer preparation at pH 7.0, containing 1 vol. of the carboxylase preparation, 4 vol. of 0.5M-sodium phosphate buffer (pH 7.0) and 3 vol. of water.

(B) Carboxylase-buffer-anhydrase preparation, at pH 7.0, containing 1 vol. of the carboxylase preparation, 4 vol. of 0.5M-phosphate (pH 7.0) and 3 vol. of the carbonic anhydrase solution.

Such preparations were adjusted, if necessary, to pH 7.0, using the glass electrode.

One flask was set up as follows (after smearing a little white Vaseline around the opening of the side arm as a precaution against any premature mixing of its contents with the main fluid): 2 ml. of solution *A* in central compartment; 0.266 ml. of solution *B* in side arm; 1 ml. of the sodium pyruvate solution (also adjusted to pH 7.0 as described above) was then added to the centre and 0.133 ml. to the side arm simultaneously, noting the time. The flask was attached to the manometer and set shaking in a water bath at 130 oscillations/min. The water bath was at room temperature (normally about 18°) as were all solutions. After 2 min. the tap connecting the manometer to the air was shut and manometric readings taken every 30 sec. After four readings (five or six in some experiments) the side-arm contents were mixed in as quickly as possible (taking no longer than 7 sec. after a little experience) and readings continued for another few minutes. The change in rate of CO₂ production immediately after the addition of side-arm contents indicated the effect of active carbonic anhydrase.

The experiments using inhibited carbonic anhydrase were the same except for the presence of sulphanilamide or thiophen-2-sulphonamide included in the buffer to give concentrations of 0.033 and 0.0014% (w/v), respectively (both in the central and side-arm fluids).

In this type of experiment it was sought to have the composition of the side arm and central fluid as similar as possible at the outset except for the presence of carbonic anhydrase in the side arm.

The dilution of the carboxylase preparation described above was 12 times, for the central and side-arm fluids, and the dilution of the carbonic anhydrase preparation in the final mixed fluids was 34 times.

The only difference in fluid composition between the experiments with active and inhibited carbonic anhydrase was the inclusion in the latter of a small amount of sulphanilamide or thiophen-2-sulphonamide. Such experiments with the inhibited anhydrase gave fully adequate controls, the inhibitor being present in equal concentrations in the central and side-arm fluids.

The activity of the carbonic anhydrase in the mixture in the Warburg flask at the end of such experiments (uninhibited) was tested by a microdiffusion procedure previously mentioned. The buffered mixtures from the flasks were used directly without further dilution.

Manometer cups. As shown in the theoretical section, the relation of the volume of the gaseous phase to that of the liquid phase is of some importance, at least for the theoretical evaluation. In the experiments described the cup had a volume of approximately 8.0 ml. In some of the later experiments a larger cup of the same type was used with a volume of 11.5 ml.

RESULTS

Diffusion effects

In the Theoretical section it was assumed that shaking was sufficient to prevent significant diminution of the gaseous exchange rate. Such limitation produced by diffusion in manometry has been dealt with, for example, by Roughton (1941).

Concerning this effect, if we suppose that car-

boxylase is acting on strong pyruvate at pH 7.0 under the conditions described in the Methods section and that the slope of n_1 (CO₂ liberated into the gaseous phase) against time is linear but the shaking inadequate, then compared with adequate shaking the effect is similar to an increase in solubility of the gas in the liquid phase or of the λ value as given above. We have carried out a series of experiments to test the adequacy of the shaking on various decarboxylation rates. In these the effect of shaking on various slopes of CO₂ discharge into the gas phase against time was observed. The conditions were the same as for the experiments described above under Methods, except that the contents of the side arm were included in the central chamber from the beginning of the experiment, and different dilutions of the original carboxylase preparations used.

Fig. 2. Effect of shaking rate on CO₂ liberation when carboxylase of different strengths (as in *A*, *B*, *C* and *D*) acts on strong pyruvate at 18°. Conditions described in text.

The results are shown in Fig. 2. It will be seen that with decarboxylation conditions giving a slope of 10 μl./min. with shaking rate of 130 cyc./min., increased shaking will have no appreciable effect. If the evolution of CO₂ were about 13 μl./min. with similar shaking, the curve (between *B* and *C* in Fig. 2) could be extrapolated to give a maximum of about 14 μl./min. Such a slope is of interest here, since in the first group of experiments, described below, showing the formation of H₂CO₃ as the primary product, the average rate of CO₂ discharge into the gaseous phase from 2.5 to 5 min. and without carbonic anhydrase inhibition was likewise 13 μl./min. (The figure from Table 1, summarizing such experiments, is 15 μl./min.; the difference arises from the adjustment of the data by a factor constant for each experiment to give 30 μl. at the 2 min. period.)

With fully adequate shaking the value of $1/\beta$, or the ratio of the increase of CO_2 after carbonic anhydrase to the slope (assuming H_2CO_3 as primary product), is $1/0.74 = 1.35$ (β being taken from data given under Symbols). However, as 15% of the total mixed fluids has been subjected to the action of carbonic anhydrase in the side arm, the expected $1/\beta$ value is, rather, 1.15. If allowance is made in turn for shaking not being fully adequate, in the form that λ is 1.1 times 0.7 (the figure stated under Symbols) then an observed $1/\beta$ figure of 1.1 may be anticipated. (If the apparent λ value were even as high as 2×0.7 , the resulting $1/\beta$ figure would be approximately 0.9.)

With CO_2 the primary product, the expected $k_1 m / \beta k_3$ is 2.55. Allowing for apparent λ values of 1.1×0.7 , or as high as 2.0×0.7 , it becomes 2.6 and 2.9 times respectively. Thus, considerable increase in the apparent λ value, due to inadequate shaking, would have but a small effect on $1/\beta$ or on $k_1 m / \beta k_3$.

Fig. 3. Curves showing rate at which equilibrium is attained when free CO_2 is liberated from 3.4 ml. of a mixture as described in text. The shaking was 120 cyc./min.

Rate at which a fixed amount of CO_2 is discharged into the gaseous phase from a mixture buffered at pH 7.0

0.25M-Phosphate buffer (pH 7.0) containing a little of the carbonic anhydrase preparation was bubbled with carbon dioxide gas for some time, then diluted with different volumes of the 0.25M-phosphate solution, containing carbonic anhydrase, to give suitable readings in the next stage. 3.4 ml. of the diluted mixture were quickly introduced into the central chamber of the cup and placed in position. Fig. 3 shows the results of shaking at 120 cyc./min., plotting $\mu\text{l. CO}_2$ against time. The experiments were suitably controlled using the phosphate solution with carbonic anhydrase, but without CO_2 . The results with two different levels of CO_2 (15 and 28 $\mu\text{l.}$) are given. The rate at which equilibrium is attained is practically independent of the amount

discharged, up to the level studied. With a shaking rate of 130 cyc./min., these percentages would be reached a little sooner.

The effect of a pH difference between side-arm contents and that of central chamber

With a type of experiment described in the Methods section where the pressure development is observed for some minutes and the contents of the side arm then mixed with the central fluid it was considered advisable to examine first the effect of pH differences deliberately produced, the contents of the chambers being otherwise the same.

Results with a difference of 0.3 pH are here described. The enzyme mixture had the following composition: 4 parts 0.5M-phosphate buffer at pH 7; 3 parts water; 1 part carboxylase preparation (ammonium sulphate suspension).

Half of this mixture was adjusted electro-metrically to pH 6.8 and the other half to 7.1. These

Fig. 4. The curves show the development of pressure during carboxylase action on strong pyruvate, the mixtures buffered to 6.8 or to 7.1 at 18° , when the side-arm contents (0.4 ml.) having the same composition but a somewhat different pH are introduced into the central contents (3.0 ml.). A, Central fluid at pH 6.8, side-arm fluid at pH 7.1; B, Central fluid at pH 7.1, side-arm fluid at pH 6.8.

were evacuated of gas. 2 ml. of the mixture at pH 6.8 were introduced into the centre and 0.266 ml. of the mixture at pH 7.1 into the side arm. 1 ml. of 2M-pyruvate at pH 6.8 was added to the centre and 0.133 ml. of 2M-pyruvate at pH 7.1 simultaneously to the side arm, and the experiment continued as described in the Methods section. After four readings the side-arm fluid was mixed with the centre fluid.

The average of five sets is shown in Fig. 4. The average of five sets using pH 7.1 in the centre and 6.8 in the side arm is also shown.

It will be seen from Fig. 4 that no appreciable change of level or slope occurs on mixing.

This may be explained by the compensatory effect of the pH rise or fall in one fluid as it is mixed with the other.

While, in the other experiments to be described, much care was expended in having the contents of both regions initially at the same pH, it will be seen that even a difference far beyond any that could presumably arise will not explain the results with carbonic anhydrase described below.

Incidentally, such experiments provide additional controls for those which follow, with regard to any effects that might result from tipping in the side-arm contents, as distinct from the action of carbonic anhydrase.

Experimental conditions showing the primary production of H_2CO_3 (or HCO_3^-) in pyruvate decarboxylation

The experiments were carried out as described under Methods. The carboxylase preparation used was prepared by fractional ammonium sulphate precipitation from the dried yeast supplied by Prof. Krebs. The carbonic anhydrase preparation was used undiluted.

The fluid in the side arm was mixed with that in the centre after four readings at 30 sec. intervals. The first of such readings, 30 sec. after closing the manometer tap, was taken as zero level.

Each of these ten experiments showed the characteristic rise to a new level on mixing with the fluid containing active carbonic anhydrase.

The accompanying experiments with inhibitor were carried out in the same way, except that sulphanilamide was incorporated in the buffer solution of both components to give a concentration of 0.033 % (w/v). In nine out of the ten experiments with inhibitor there was no similar increase of pressure to a new level after tipping in the side-arm contents. The exception would appear to be due to some accident as in a large number of experiments carried out in other series using sulphanilamide (or thiophen-2-sulphonamide) it was not repeated.

It is true that after tipping in the side-arm contents and using such inhibitors the first reading is a little above the level of the original curve but the subsequent readings follow this curve. In Table 1 is given the averages and standard deviations for the individual experiments, and the results of subtracting the means for the experiments with active from those with inactive carbonic anhydrase. To express the degree of variability between the different experiments, and to remove that arising merely from some differences between slopes of n_1 against time (attributable largely to small temperature changes on different days and some difference in the carboxylase action), each reading in a given experiment was multiplied by a factor, this factor bringing the 2 min. reading to 30 μl . (this being near to the mean value in the series).

Table 1. *Time course of decarboxylation experiments*

(This gives the averages for ten sets of decarboxylation experiments, carried out on different days, using the same dried-yeast sample as the source of the carboxylase preparation. The side-arm contents, of the same composition as the fluid in main chamber but containing carbonic anhydrase, were tipped in 2 min. after closing the manometer tap. The reading $\frac{1}{2}$ min. after closing the tap was taken as zero, and, to reduce variability from one experiment to another, the readings for each experiment were multiplied by a factor to bring the 2 min. period to 30 μl . (The actual average rate up to 2 min. of the observation period was approximately 17 $\mu\text{l}/\text{min.}$, and towards the end of the observation period about 13–14 $\mu\text{l}/\text{min.}$))

Min.	$\mu\text{l. CO}_2$		
	Carbonic anhydrase active, tipped in after 2 min.	Carbonic anhydrase inhibited with sulphanilamide, tipped in after 2 min.	Differences between (2) and (3)
(1)	(2)	(3)	(4)
0.5	0 $\pm$	0 $\pm$	0 $\pm$
1	10 $\pm$ 0.3	11 $\pm$ 0.4	-1 $\pm$ 0.5
1.5	21 $\pm$ 0.8	22 $\pm$ 0.3	-1 $\pm$ 0.9
2	30	30	0
2.5	48 $\pm$ 0.6	43 $\pm$ 1.1	+ 5 $\pm$ 1.3
3	60 $\pm$ 1.1	53 $\pm$ 0.9	+ 7 $\pm$ 1.4
3.5	67 $\pm$ 1.2	60 $\pm$ 1.1	+ 7 $\pm$ 1.6
4	76 $\pm$ 1.7	68 $\pm$ 1.6	+ 8 $\pm$ 2.3
4.5	84 $\pm$ 1.6	75 $\pm$ 1.9	+ 9 $\pm$ 2.4
5	93 $\pm$ 2.0	83 $\pm$ 2.2	+10 $\pm$ 2.9
5.5	100 $\pm$ 2.3	89 $\pm$ 2.3	+11 $\pm$ 3.0

The means in cols. 2 and 3 are followed by their s.d.

Col. 4 gives the differences between the means and the s.d. of the differences.

A further series of four sets of experiments was carried out with a similar carboxylase preparation, obtained in turn from another consignment of dried yeast received from Prof. Krebs in March 1949. The results were the same as before. The four sets are summarized in Fig. 5*a*.

and after the increase to a higher level the further development of pressure follows practically parallel with the original curve.

The total average value of $1/\beta$ for the sixteen sets of experiments (sixteen with inhibitor and sixteen without) was 0.8.

Fig. 5. Effect on CO_2 liberation into gaseous phase when carbonic anhydrase from side arm (0.4 ml.) is introduced into 3 ml. of carboxylase-pyruvate mixture in centre (pH 7.0; 18°). The side-arm fluid has the same composition as the central fluid except for carbonic anhydrase. Conditions described in text; O—O, curve before and after addition of inhibited carbonic anhydrase; ●—●, the effect when uninhibited carbonic anhydrase is added; —, theoretical line for CO_2 as full primary product. (a), average of four experiments using uninhibited and four companion experiments using the enzyme inhibited with sulphanilamide; (b), conditions same as (a), but using thiophen-2-sulphonamide as carbonic anhydrase inhibitor (two sets of experiments); (c), general conditions same as for previous diagrams, but 'yellow fluid' was used as the carbonic anhydrase preparation and a direct water extract of dried yeast as the carboxylase preparation; (d), conditions as for (a), but with a more impure carboxylase preparation from a different yeast: for details see text.

Two sets were in turn carried out using thiophen-2-sulphonamide as the inhibitor, as described under Methods, and are shown in Fig. 5*b*. (For the data in Fig. 5 no factors were used to adjust to a common level at the 2 min. period.)

It will be seen from a comparison of Fig. 5*a* and *b*, with Fig. 1*a* that the results agree closely with the theoretical form, when the primary product is not free carbon dioxide. While the trend of pressure production is not exactly linear, it is nearly linear

As considered above, the expected value with H_2CO_3 as the primary product is about 1.0. The difference may not be significant, as the theoretical calculation of β depends on data which are not known with exactitude. It shows that at least much the greater fraction of the primary product was H_2CO_3 .

Each of the sixteen experiments showed a similar rise of pressure to a new level when the content of the side arm containing active carbonic anhydrase was mixed into the main chamber.

It was apparent to us that with the conditions described such results could be indefinitely repeated with carboxylase preparations obtained from the yeast samples received in November and March and prepared as described according to the procedure of Green *et al.* (1941).

Experimental conditions in which the primary product of decarboxylation was chiefly CO₂

In May 1949 we sought, as described in the Methods section, to prepare from a Dublin pressed yeast a carboxylase of purity similar to that from the dried Sheffield yeast. The preparation in this case was not very successful and gave a turbid instead of a clear solution, although on previous occasions we had obtained purer preparations from a similar source. Using such a preparation and the carbonic anhydrase preparation described under Methods, we found that after tipping in the side-tube contents the pressure production was at a somewhat lower level than with the inhibited mixture. The average results of two sets of experiments are given in Fig. 5c, in which the theoretical line for CO₂ as the full primary product is also given.

These experiments were also carried out using a water extract of the dried Sheffield yeast as the carboxylase solution, and the 'yellow fluid' as the carbonic anhydrase preparation.

Such solutions were used by Krebs & Roughton (1948) and the result at 18° was similar to theirs at 15°.

We used the solutions prepared as they described but with our technique of adding the side-arm contents during the pressure development. The mixture in side arm and central region was made in the same proportions as described under Methods, but the water extract of the dried Sheffield yeast was used as the carboxylase and the 'yellow fluid' as the carbonic anhydrase preparation.

The results of two sets of experiments are given in Fig. 5d. After mixing with the active carbonic anhydrase the curve of pressure falls to a lower level. The conclusion may be drawn that the primary product under such conditions is mainly free CO₂.

The results differ in some important respects from the theoretical expectation if the primary product is altogether CO₂. Thus, the curve of pressure after the active carbonic anhydrase does not tend to run parallel with that using the inhibited enzyme. Also, the ratio of the difference between the two curves to the slope, even taking the smaller slope, with active carbonic anhydrase and after 4 min., is only 1.5, the expected value with CO₂ as the primary product being about twice this figure. The divergence of the curve from that theoretically expected if CO₂ were the full primary product is shown in Fig. 5d.

From this it may be concluded that CO₂ was produced directly only in some preponderance over H₂CO₃ or its ion.

Experiments with oxaloacetate and α-ketoglutarate

A series of experiments was conducted with sodium oxaloacetate as the substrate in the same concentration as pyruvate and with carboxylase and carbonic anhydrase prepared as described under Methods. The first series (four experiments) is summarized in Fig. 6a. It will be seen that on mixing in the side-arm contents the result was an unexpected change of slope even in the presence of sulphanilamide.

Fig. 6. Results of experiments with oxaloacetate. ○—○, curve before and after addition of inhibited carbonic anhydrase; ●—●, effect when uninhibited carbonic anhydrase is added; —, theoretical line for CO₂ as full primary product. (a) Similar conditions to Fig. 5a, except oxaloacetate used as the carboxylase substrate. Average of four sets inhibited and four sets uninhibited. (b) Similar to (a), but diluted blood used as the carbonic anhydrase preparation. (0.1 ml. of twice-diluted rabbit whole blood in side-arm contents; final blood dilution 1:68 in the mixed fluids.) Average of two sets uninhibited and two sets inhibited. (c) Similar to (b), except that α-ketoglutarate was used as the carboxylase substrate. Average of two sets inhibited and two sets uninhibited.

We carried out a further series using laked blood as the source of the carbonic anhydrase. The blood had a final dilution of 68 times in the mixed fluids. The results are summarized in Fig. 6b (from two experiments) and show a typical picture of the course of pressure development with the primary product chiefly CO₂ and not H₂CO₃ or its ion. The

comparison may here be drawn between Fig. 6*b* and the theoretical Fig. 1*b*. Thus, the difference in tension produced on mixing with the active carbonic anhydrase is rapidly developed and the further rise of pressure is practically parallel with the pressure developed in the presence of the inhibited carbonic anhydrase. Further, this latter continues the curve of pressure up to the mixing point without any deviation on mixing. The $k_1 m/k_3 \beta$ value approached 2.1 and the expected value for full CO_2 production as primary product is about 2.5 (not allowing for inadequacy of shaking). In all such experiments this is the nearest approach we have obtained to a decarboxylation with CO_2 only as the primary product, and with typical theoretical form.

With sodium α -ketoglutarate the results, using diluted blood as the source of the carbonic anhydrase, also showed that CO_2 was the main primary product (Fig. 6*c*), but the depression of the pressure level is far less than the expected figure if CO_2 were the only primary product.

DISCUSSION

From the results described it will appear that preparations of carboxylase from a dried yeast obtained from Sheffield (following the procedure of Green *et al.* 1941) decarboxylated strong pyruvate solution at pH 7.0 with a primary production of carbonic acid (or HCO_3^-). The increase in pressure on mixing in the carbonic anhydrase approached the theoretical amount, but the results are compatible with the view that a small fraction of the primary product of decarboxylation was free carbon dioxide.

In any assessment of such experiments and results as those described above, the following two facts should be noted. First, when carboxylase is acting on strong pyruvate (buffered at pH 7.0 with phosphate) in the main compartment of a Warburg manometer, the curve of pressure is not appreciably changed on tipping in side-arm contents of the same composition to the main fluid and under the experimental conditions described. Secondly, the curve of pressure is not appreciably changed when the side-arm contents, otherwise of the same composition as that in the main chamber, contain carbonic anhydrase inhibited with sulphanilamide or thiophen-2-sulphonamide (the inhibitor being present in both compartments in the same concentration).

Such controls appear to be entirely adequate and those with sulphanilamide or, alternatively, thiophen-2-sulphonamide (two of the inhibitors studied by Krebs (1948)) were carried out with each experiment in which the effect of carbonic anhydrase on the carboxylase system was examined.

On the other hand, preparations of carboxylase can be obtained which decarboxylate strong pyruvate with free carbon dioxide as the major product, agreeing, at least qualitatively, with the observations of Krebs & Roughton (1948). Thus, we used their carboxylase preparation of a water extract of dried Sheffield yeast and the 'yellow fluid' as the carbonic anhydrase preparation, but with the procedure described in this paper.

The addition of the active carbonic anhydrase caused a fall in rate of carbon dioxide production, compared with the control curve with inhibited carbonic anhydrase. The effect, however, was much less than the theoretical requirement if carbon dioxide were the full primary product in decarboxylation (Fig. 5*d*). In this connexion the theoretical curve may be compared with Fig. 6*b*, showing the results with oxaloacetate, and diluted blood as the source of the carbonic anhydrase.

Such results as a whole may be best interpreted by the view that decarboxylation takes place through two paths, the primary product being a mixture of free carbon dioxide and of carbonic acid, the preponderance of one or the other depending, apparently, on the total conditions. This conclusion would not, of course, be invalidated if some special condition (including even the use of the purest enzymes) was found which showed a considerable preponderance of the primary pathway through carbon dioxide.

In the experiments with 'yellow fluid' as the direct source of carbonic anhydrase and the water extract of dried yeast, the question may be raised whether the change in the decarboxylation of pyruvate was due to relative impurities in the carboxylase extract or the 'yellow fluid'. Since a similar result (Fig. 5*c*) was obtained with a relatively impure carboxylase preparation from a Dublin yeast and the carbonic anhydrase preparation described under Methods, it would appear that the carboxylase preparation could furnish the operative impurity. At the same time we leave it at present an open question as to whether a similar result might not arise from the use of a relatively crude source of carbonic anhydrase.

Our interest in such decarboxylation lay chiefly in its being a possible direct source of H^+ ions, as, for example, in the remarkable H^+ and K^+ exchange in fermenting yeast (Conway & O'Malley, 1943, 1946). Here, under suitable conditions, HCO_3^- accumulated in the yeast cells in nearly quantitative relation to the H^+ ions exchanged (Conway & Brady, 1947). Such facts, however, have been better interpreted by an oxidation-reduction theory of the acidic production (Conway & Brady, 1948; Conway, 1949; Conway, Brady & Carton, 1950). The change of interpretation is not related to the question of the path of decarboxylation.

SUMMARY

1. The derivation is given of equations (previously stated by Conway & O'Malley, 1948) from which the full theoretical effect of carbonic anhydrase on the increase of manometric pressure during decarboxylation may be calculated. It is assumed that the decarboxylation, as of strong pyruvate, is a linear function of the time over the observation period. Such equations may be further developed for special conditions where the decarboxylation is a definite, but non-linear function of the time.

2. A carbonic anhydrase preparation (Keilin & Mann, 1940) was introduced from the manometer side arm, being present there in fluid having otherwise the same composition as the central fluid. This contained carboxylase prepared according to the procedure of Green *et al.* (1941) acting on 0.67M-pyruvate at pH 7.0 and 18°. The production of pressure was nearly linear over the time of observation. With the introduction of active carbonic anhydrase there was a rapid rise of pressure which after a few minutes proceeded practically parallel with the previous curve, or with that using inhibited carbonic anhydrase.

3. The mean increase of pressure level, after carbonic anhydrase action, approaches but does not quite reach the expected figure when carbonic acid

(or HCO_3^-) is the primary product of decarboxylation.

4. Using a crude water extract of dried yeast as the carboxylase preparation and the 'yellow fluid' as that of carbonic anhydrase, there was a fall of pressure on introducing the latter. This is in qualitative agreement with the experiments of Krebs & Roughton (1948) and shows a primary production of carbon dioxide. The degree of fall was only a fraction of that to be expected if carbon dioxide was the sole primary product of the carbon dioxide system.

5. Experiments were also carried out with strong concentrations of α -ketoglutarate and oxaloacetate, used as carboxylase substrates. With the introduction of the carbonic anhydrase preparation there occurred a small fall of pressure indicating carbon dioxide as mainly the primary product, but the curves of pressure were not regular. Using diluted blood as the source of the carbonic anhydrase, the result with oxaloacetate was similar to the theoretical picture when carbon dioxide is the primary product of decarboxylation. Similar results with α -ketoglutarate indicated a primary production mainly of carbon dioxide, but to a lesser degree than with oxaloacetate.

6. The conclusion may be drawn that in the decarboxylation of keto-acids by yeast carboxylase, carbon dioxide and carbonic acid (or HCO_3^-) are both primary products, but one or the other may predominate depending on the total conditions.

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