LXXVII. HYDROGENLYASES.

IV. THE SYNTHESIS OF FORMIC ACID BY BACTERIA.

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THE formic hydrogenlyase enzyme of bacteria has been studied in detail by Stephenson and Stickland [1932; 1933] and by Yudkin [1932]. This enzyme catalyses the reaction:

Recent work has shown the importance of H_2 and CO_2 in reactions induced by bacteria [Gaffron, 1935; Roelofsen, 1934; Kubowitz, 1934]. It was therefore of interest to determine whether the formic hydrogenlyase system could be reversed. Stephenson and Stickland [1932] found a quantitative formation of H_2 from formic acid, but since, in their experiments, the other reaction product, carbon dioxide, was absorbed, an equilibrium was scarcely to be expected. An early observation of Harden [1901] indicates the possibility of such an equilibrium. In the fermentation of glucose by *Bact. coli* he found that an increase of pressure reduced the production of H_2 and CO_2 and increased the production of formic acid.

In the experiments to be described it will be shown that the formic hydrogenlyase system can be reversed, and that if washed suspensions of *Bact. coli* and other organisms are shaken in a gas mixture of H_2 and CO_2 , gas is taken up and formic acid is produced.

Preparation of bacterial suspensions.

The cultures were obtained from the National Collection of Type Cultures. *Bact. coli* (Escherich) was used for most of the work. It was grown under the optimum conditions for the formation of the formic hydrogenlyase enzyme [Yudkin, 1932]. Tryptic caseinogen broth containing 0.5 % formic acid as sodium formate was sown from a 12-hour culture of *Bact. coli* on broth. After 14 hours' growth the organism was centrifuged down and washed twice on the centrifuge with distilled water. The growth from 1 l. of medium was usually finally suspended in 10 ml. water. Such a suspension averaged 0.8 ± 0.2 mg. N/ml. (6.6 ± 1.6 mg. dry weight/ml.).

Measurement of gas absorption.

Gas changes were measured in Warburg manometers. The cups contained bacterial suspension and NaHCO_3 of final concentration 0.025 *M*. The manometers were filled with a gas mixture of 5 % CO_2 in H_2 ; the p_{H} was 7.4. Controls were done with 5 % CO_2 in N_2 and with H_2 (using phosphate buffer). Conical cups with side-bulbs and inner cups, total volume 18–20 ml., were used for most of the work.

Gas changes will usually be expressed as Q values ($\mu\text{l.}/\text{hour}/\text{mg.}$ dry weight). The total nitrogen of the suspensions was determined and converted to dry weight by use of the figures for N % of dry weight given by Yudkin [1932].

It will be realised that if H_2 and CO_2 combine with production of formic acid the only gas change to be measured in the manometers would be the H_2 uptake; the production of one equivalent of formic acid from one equivalent of CO_2 would liberate again one equivalent of CO_2 from the bicarbonate buffer.

The manometers were equilibrated in the bath for 20 min. before the initial readings were taken. All experiments were done at 38° unless otherwise stated.

EXPERIMENTAL.

Table I gives the results of an experiment designed to show the gas uptake from a $\text{H}_2\text{--CO}_2$ mixture. It will be seen that there is a considerable gas uptake from $\text{H}_2\text{--CO}_2$ whilst the blanks in $\text{N}_2\text{--CO}_2$ and in H_2 are negligible.

Table I.

Gas used	...	...	5% CO_2 in H_2	5% CO_2 in N_2	H_2
<i>Bact. coli</i> suspension (ml.)			0.5	0.5	0.5
0.125 M NaHCO_3 (ml.)			0.6	0.6	—
0.2 M phosphate p_{H} 7.4 (ml.)			—	—	0.6
Water (ml.)			1.9	1.9	1.9
mm. Brodie's solution/hour			—200	1	—6
$\mu\text{l. H}_2/\text{hour}$			—256	—	—8
Q_{H_2}			—76	—	—2

It is now necessary to establish that the only gas change occurring is an uptake of H_2 , for, as has been pointed out, if formic acid is the product the CO_2 uptake would be balanced by a corresponding evolution of CO_2 from the bicarbonate buffer. Use was made of the principle of Warburg's two vessel method. The gas uptake was measured using small and large volumes of fluid in the cups. (The volume of the cups was 10–12 ml.) If the only gas uptake is H_2 then $\mu\text{l.}$ gas change per ml. bacterial suspension should be the same; if CO_2 is also absorbed, then owing to the relatively high solubility of CO_2 the measured gas uptakes in the two cases will differ. The results of such experiments are given in Table II.

Table II.

	Exp. 1		Exp. 2	
	2	5	2	5
<i>Bact. coli</i> suspension (ml.)				
$\mu\text{l. H}_2/35$ min.	—31.1	—75.2	—22.7	—55.7
$\mu\text{l. H}_2/35$ min./ml. suspension	—15.5	—15.1	—11.3	—11.1
Q_{H_2}	—71	—69	—52	—51

A temperature of 25° was used in this case since the uptake of gas is more constant; at 38° the uptake in the cups containing the larger volume of fluid fell off rather rapidly. 1 ml. of M NaHCO_3 solution was diluted with 22 ml. water and the mixture equilibrated with the $\text{H}_2\text{--CO}_2$ gas mixture for 30 min. at 25° . 2 ml. *Bact. coli* suspension were then added. The resulting suspension was measured into the manometer cups and the manometers filled with gas. Readings were commenced after a further 20 min. equilibration in the bath, when the uptake had become steady, and were taken at 5-min. intervals. The results show sufficient agreement to justify the conclusion that H_2 uptake is the only gas change measured.

It may be mentioned at this point that the Q_{H_2} values obtained with various batches of *Bact. coli* varied from 50 to 90. Further, the activity fell off fairly rapidly with time, the Q_{H_2} value being halved on standing 24 hours at 0°.

If formic acid is produced and liberates from the bicarbonate present a quantity of CO_2 equal to that absorbed during its synthesis, then there should be a disappearance of bicarbonate from the system. This was demonstrated in the following experiment. Warburg cups contained 0.5 ml. *Bact. coli* suspension, 0.6 ml. 0.031 *M* $NaHCO_3$, 0.7 ml. water and 0.2 ml. 2*N* H_2SO_4 (in the side-bulb). The manometers were filled with the usual 5 % CO_2 in H_2 mixture. After equilibration the acid in one set of manometers was tipped into the main cup and the evolution of CO_2 measured. This gave the amount of bicarbonate present at the beginning of the experiment. Meanwhile, in the second series of manometers, the uptake of H_2 was measured for 80 min. At the end of this period the acid was tipped into the main cup, the CO_2 output giving in this case a measure of the bicarbonate finally present. The tipping in of the acid is, of course, sufficient to stop any enzyme action. The results of two experiments are given in Table III.

Table III.

	Exp. 1	Exp. 2
Initial bicarbonate (μ l. CO_2)	364	364
Final bicarbonate (μ l. CO_2)	230	228
Bicarbonate used (μ l. CO_2)	134	136
H_2 uptake (μ l. H_2)	117	126
H_2/CO_2	1.13	1.07

It is thus established that bicarbonate, as well as H_2 , disappears from the system and further that these disappear in approximately equimolecular proportions. The experiments summarised in Table II also show that for each molecule of H_2 absorbed one molecule of a monobasic acid is formed (or a half molecule of a dibasic acid and so on).

Detection of formic acid.

One method for the detection and estimation of formic acid depends on the fact that it is a volatile acid and can reduce mercuric chloride to mercurous chloride [Dakin, 1913]. The growth of *Bact. coli* from 1 l. of broth-formate medium was finally suspended in 15 ml. water. To 10 ml. of this suspension were added 1 ml. *M* $NaHCO_3$ and 14 ml. water (A). The other 5 ml. were diluted with 1 ml. 0.5 *M* phosphate buffer p_H 7.4 and 6.5 ml. water (B). 10 ml. of (A) were placed in a 75 ml. pot and the pot filled with the 5 % CO_2 in H_2 gas mixture and shaken for 6 hours at 38°. Controls were done with 10 ml. (A) in 5 % CO_2 in N_2 and with 10 ml. (B) in H_2 alone. The contents of each pot were then treated as follows. 1 ml. of phosphoric acid was added and the acid mixture distilled *in vacuo* almost to dryness, the distillate being collected under 5 ml. of 0.1 *N* $NaOH$. 25 ml. of 50 % alcohol were added and the distillation repeated. The distillate was evaporated to 10 ml. on the water-bath, acidified with 5 ml. of 5 % acetic acid (formate-free) and 40 ml. of a saturated solution of mercuric chloride added. This mixture was heated in a sealed flask on the boiling-water bath for 6 hours. The precipitated calomel was filtered off through a weighed Gooch crucible, washed with 5 % HCl , dried to constant weight at 90°, and weighed. The results of a typical experiment are given in Table IV and indicate clearly that the product is a volatile acid which can reduce mercuric chloride to calomel. Formic acid is the only common volatile acid with this property. The next step is to show that, assuming the acid to be formic acid, the total volatile acid produced is quantitatively equal to the formic acid as estimated by the calomel method. For this

Table IV.

Gas mixture	...	...	5% CO ₂ in H ₂	5% CO ₂ in N ₂	H ₂
Bacterial suspension (ml.)			10 (A)	10 (A)	10 (B)
Calomel formed (mg.)			54.6	1.2	0.5
Formic acid (mg.)			5.33	0.11	0.05

purpose it is necessary to obtain a larger amount of product. The growth of *Bact. coli* from 2 l. of broth-formate medium was suspended in 60 ml. 0.04 *M* NaHCO₃ and a stream of the 5% CO₂ in H₂ mixture passed through for 10 hours at 38°. The suspension was then acidified and the volatile acid estimated by a method described for acetic acid by Stephenson and Whetham [1922]. The neutralised distillates were evaporated and the formic acid estimated in duplicate by the calomel method. The results, given in Table V, show that the total volatile acid is in good agreement with the formic acid as estimated by its reducing property.

Table V.

Gas mixture	Volatile acid method		Calomel method	
	Volatile acid, ml. 0.1 <i>M</i>	H. COOH equivalent mg.	H. COOH mg.	Volatile acid equivalent, ml. 0.1 <i>M</i>
5% CO ₂ in H ₂	8.77	—	37.8	—
5% CO ₂ in N ₂	0.23	—	0.4	—
Difference	8.54	39.2	37.4	8.13

H. COOH (by calomel)/H. COOH (by titration) = 95.2%.

Identification of formic acid by biological methods.

Further evidence that the product of the reduction of CO₂ by H₂ in the presence of *Bact. coli* is formic acid is provided by the decomposition of the product by *Bact. coli* itself. Anaerobically in N₂ the formic hydrogenlyase enzyme decomposes formic acid to H₂ and CO₂, and, if the CO₂ is absorbed, H₂ is produced quantitatively [Stephenson and Stickland, 1932]. Aerobically formic acid is decomposed by *Bact. coli*:

The oxygen uptake is theoretical [Cook and Stephenson, 1928] and may be measured if the CO₂ is absorbed.

The acid was prepared on a large scale as already described, distilled into dilute NaOH, evaporated, carefully neutralised and made up to 10 ml. Formic acid was estimated in duplicate on 2 ml. portions by the calomel method. For the anaerobic decomposition the Warburg cups contained 0.5 ml. *Bact. coli* suspension, 1 ml. phosphate buffer *p*_H 7.4, 0.2 ml. of the distillate (in the side-bulb) and filter-paper rolls moistened with 0.2 ml. of 10% KOH in the centre-tubes for CO₂ absorption. Controls were done with 0.2 ml. formate of known strength (approximately equal to that of the distillate) and with 0.2 ml. water. The manometers were filled with N₂. After equilibration the substrate was tipped in from the side-bulb and the gas evolution measured to completion. The aerobic decomposition experiment was performed in the same way except that the manometers contained air and the oxygen uptake was measured. The results of an experiment are given in Table VI and provide excellent quantitative evidence that the distillate contains formic acid. The slightly over-theoretical oxygen uptake has always been observed and no explanation can be offered for this.

Table VI.

0.2 ml. distillate (0.538 mg. H.CO ₂ H by calomel method)						0.2 ml. 0.05 M H.COONa (0.46 mg. H.CO ₂ H)					
H ₂ evolved (μl.)			O ₂ uptake (μl.)			H ₂ evolved (μl.)			O ₂ uptake (μl.)		
Theo- retical	Found	%	Theo- retical	Found	%	Theo- retical	Found	%	Theo- retical	Found	%
262	249	95	131	144	110	224	216	96	112	123	110

Further evidence that the distillate contains formic acid is provided by its behaviour with a formic dehydrogenase preparation from *Bact. coli*. This preparation [Stickland, 1929] is highly specific for formic acid; traces only of lactic and succinic dehydrogenases may be present. Thunberg tubes were set up containing 1 ml. phosphate buffer p_H 7.4, 1 ml. 1 : 5000 methylene blue, 1 ml. of a suitable dilution of the enzyme preparation and 1 ml. of the substrate. Distillate containing approximately $M/200$ formic acid (by the calomel method) was used. The tubes were evacuated and the time taken for the reduction of the methylene blue noted. The results are given in Table VII and show that the

Table VII.

Substrate	Reduction time
Water blank	> 6 hours
Formate $M/200$	2 min. 30 sec.
Lactate $M/200$	> 6 hours
Succinate $M/200$	> 6 hours
Distillate	2 min. 30 sec.

distillate contains formic acid. Owing to the very high affinity of the formic dehydrogenase enzyme for its substrate a quantitative estimation of formic acid by this method is not possible. A rough idea may be obtained by running parallel dilutions of the distillate and of formate of known concentration. Some results are given in Table VIII and show the concentration of formic acid as estimated by this method and by the calomel method to be of the same order.

Table VIII.

Dilution	Reduction times	
	Distillate ($M/15$ formate by calomel method) min.	Formate $M/15$ min.
1 : 20	5	4.5
1 : 80	7	6.75
1 : 160	9	9
1 : 320	50% reduced only	50% reduced only

The evidence that the product of the reduction of CO₂ by H₂ in the presence of washed suspensions of *Bact. coli* is formic acid may be summarised as follows:

- (1) The manometric data agree with this assumption.
- (2) It is a volatile acid.
- (3) It reduces mercuric chloride.
- (4) The H₂ formed when it is decomposed by *Bact. coli* anaerobically, and the oxygen uptake when oxidised aerobically agree exactly with the values obtained for formic acid by other methods.
- (5) It reduces methylene blue in the presence of the specific formic dehydrogenase preparation.

Quantitative relation between H₂ uptake and formic acid produced.

The H₂ uptake of *Bact. coli* suspension from 5 % CO₂ in H₂ gas mixture was measured in a series of eight manometers, the bacterial suspension being tipped in from the side-bulb after equilibration and readings started at once. At the end of the experiment the contents of the cups were acidified with phosphoric acid and the total formic acid content estimated by the calomel method in the usual way. The results of two experiments are shown in Table IX.

Table IX.

H ₂ uptake μl.	H.CO ₂ H calc. mg.	H.CO ₂ H found mg.	% found of calc.
2931	6.02	5.79	96
2803	5.76	5.58	97

Since it has already been shown that the ratio H₂/HCO₃' is 1, the reaction is proved to be, under the actual experimental conditions:

Nothing definite can be said, however, as to the possibility of the primary reaction being:

Effect of the partial pressure of carbon dioxide.

For these experiments the manometers were filled with H₂ and varying pressures of CO₂ generated in the cup by mixing NaHCO₃ with acid. The final concentration of NaHCO₃ was also varied with the pressure of CO₂ in order to maintain a constant p_{H} . The effect may also be due, therefore, to the increase in NaHCO₃ concentration. The partial pressure of H₂ decreases proportionally. The results are plotted in Fig. 1 and show that the H₂ uptake increases with increasing partial pressure of CO₂ up to about 0.25 atmosphere.

Fig. 1. Effect of partial pressure of CO₂ (1 atmosphere = 10,000 mm. Brodie's solution).

Effect of bicarbonate concentration.

The results of an experiment in which various concentrations of bicarbonate were used with the usual 5 % CO₂ in H₂ gas mixture are shown in Fig. 2. The p_{H} also varies with the bicarbonate concentration, so that the net effect is probably due in part to the influence of p_{H} and in part to the concentration of bicarbonate.

In the case of the lower concentrations of bicarbonate the falling off of the rate of gas uptake with time is due to the exhaustion of bicarbonate (which disappears during the reaction). At the highest bicarbonate concentration, 0.1 *M*, the slow rate is no doubt due to the high p_H of 8.1.

Experiments with bicarbonate present, but not CO_2 (in H_2 alone), and with CO_2 but no bicarbonate (at acid p_H values), gave no H_2 uptake. It is impossible to dissociate these results from possible p_H effects. It can be said that in the actual conditions when H_2 is absorbed both CO_2 and bicarbonate are present, and that the rate of absorption seems to depend on the concentration of both.

Fig. 2.

Fig. 2. Effect of bicarbonate concentration.

A. —○— $M/10 \text{ NaHCO}_3$; p_H 8.1.	E. —○— $M/160 \text{ NaHCO}_3$; p_H 6.9.
B. —○— $M/20 \text{ NaHCO}_3$; p_H 7.8.	F. —▲— $M/320 \text{ NaHCO}_3$; p_H 6.6.
C. —■— $M/40 \text{ NaHCO}_3$; p_H 7.5.	G. —●— $M/640 \text{ NaHCO}_3$; p_H 6.9.
D. —×— $M/80 \text{ NaHCO}_3$; p_H 7.2.	

Fig. 3.

Fig. 3. Effect of KCN.

Effect of poisons.

The system was found to be sensitive to the same poisons as the reverse reaction studied by Stephenson and Stickland [1932]. It was completely inhibited by toluene, chloroform and 1 % sodium fluoride. The effect of KCN is shown in Fig. 3.

Effect of temperature.

The Q_{H_2} for the reaction was measured at 38° and at 20° with the same bacterial suspension. The Q_{H_2} at 38° was -71 and at 20° -51. The decrease of the rate of reaction with temperature is less than usually found in reactions brought about by *Bact. coli*; the effect of temperature is offset to some extent in this case by the mass effect due to the increased solubility of CO_2 at the lower temperature.

Effect of growth conditions.

Stephenson and Stickland [1932] and Yudkin [1932] found that washed suspensions of *Bact. coli* contained the enzyme formic hydrogenlyase only when the organism was grown in a medium containing formate or substances giving rise to formate by the action of the bacterium. The results summarised in Table X show that, as far as the matter was studied, this is the case also for the

Table X.

Growth medium	Q_{H_2} values	
	Synthesis of formate	Decomposition of formate
Broth-formate	-70	91
Broth	-26	35
Broth agar (Roux)	-1	1
Extracted broth-formate	-67	93
Extracted broth	-6	10

reverse system dealt with in this paper. As the stock tryptic broth was found to contain small amounts of formic acid it was extracted at p_H 2 with ether for 72 hours. After this time the ether extract contained no formate. For comparison Q_{H_2} values for the decomposition of formate (measured in N_2 - CO_2 mixture, formate 0.03 *M* in bicarbonate buffer) are also given. The experiments with extracted broth clearly show the adaptive nature of the enzyme. The Q_{H_2} values for the synthesis and for the splitting of formate run parallel; this is to be expected if the same enzyme is responsible for both reactions.

Distribution of the enzyme.

Yudkin [1932] studied the distribution of the formic hydrogenlyase enzyme in some detail. The distribution of the power to induce the reverse reaction, in so far as it was studied, was found to be similar in every way to that of the formic hydrogenlyase enzyme (Table XI).

Table XI.

Species	Growth medium	Q_{H_2} values	
		Synthesis of formate	Decomposition of formate
<i>Bact. coli</i> (Esch.)	Broth-formate	-70	91
<i>Bact. coli</i> (3813)	Broth-formate	-63	89
<i>Bact. lactis aerogenes</i> (124)	Broth-formate	-51	64
	Broth agar (Roux)	0	0
<i>Bact. dispar</i>	Broth-formate	0	0
<i>Bact. freundii</i>	Broth-formate	-75	83
<i>Bact. cloacae</i>	Broth	0	0
	Broth-formate	0	0
	Broth-glucose	-26	56

Comparison of Tables X and XI with Table II of Stephenson and Stickland [1932] also shows that the presence of the formic hydrogenlyase enzyme is essential if the synthesis is to occur.

Determination of the equilibrium point.

The concentration of formate in equilibrium with a given $\text{H}_2\text{-CO}_2$ gas mixture in the presence of *Bact. coli* was determined in the following experiments. A preliminary experiment showed the equilibrium concentration of formate to lie between $0.1\text{ }M$ and $0.01\text{ }M$. Manometers were set up containing NaHCO_3 in final concentration of $0.025\text{ }M$ and formate in various final concentrations between $0.1\text{ }M$ and $0.0125\text{ }M$. *Bact. coli* suspension was placed in the side-bulbs and the manometers were filled with the 5% CO_2 in H_2 mixture. After equilibration the bacterial suspension was tipped in and the initial rates of H_2 uptake or evolution measured (in order that the concentration of bicarbonate should not decrease appreciably). The experiments were done both at 25° and at 37° . The initial rate of evolution or absorption of H_2 , less the controls in H_2 and in $\text{N}_2\text{-CO}_2$, were plotted against the logarithm of the formate concentration. The mean values from a number of experiments are plotted in Fig. 4. From these curves

Fig. 4. Determination of equilibrium point.

the concentration of formate at which the gas exchange is zero may be obtained. This point, at which H_2 is being absorbed at the same rate as it is evolved, gives the equilibrium concentration of formate in the given experimental conditions. These values, together with other experimental data to be used in the next section, are given in Table XII.

Table XII.

	Series 1	Series 2
Temperature ($^\circ$ absolute, $^\circ$ K.)	298.1	310.7
Formate concentration (M)	0.0306	0.0204
Bicarbonate concentration (M)	0.025	0.025
Partial pressure of H_2 (atmos.) (corrected for atmospheric pressure and pressure of water vapour)	0.916	0.884

Calculation of the free energy of the reaction.

From the equilibrium concentrations of the reactants it is possible to calculate the free energy change of the reaction. It is also possible, from the known free energy values of the reactants, to compute the theoretical free energy change. If the equilibrium measured is a real one these two values should agree within the limits of the errors involved.

Calculation has been made by the conventional procedures of Lewis and Randall [1923]. The free energy change at the standard state¹ ($\Delta F_{\text{obs.}}$) for the reaction,

is related to the activities of the reactants ($[\text{HCOO}']$, etc.) as follows:

$$\Delta F_{\text{obs.}} = -RT \ln K_{\text{obs.}} = -RT \ln \left(\frac{[\text{H.COO}'][\text{H}_2\text{O}]}{[\text{HCO}_3'][\text{H}_2]} \right),$$

where $K_{\text{obs.}}$ is the equilibrium constant.

Following the usual conventions, the activity of hydrogen is taken as its partial pressure and the activity of water as its mol. fraction (calculated as 0.998 at the experimental conditions); the activities of the anions are expressed as molal activities ($\gamma \times \text{molal concentration}$). The activity coefficient (γ) of HCO_3' was calculated from the ionic strength as described by Hastings and Sendroy [1925], and equalled 0.7615 at the equilibrium point. As no value could be found in the literature for the activity of HCOO' , its activity coefficient was taken as equal to that of HCO_3' at the ionic strength in question. Then taking the experimental data of the last section referring to 298.1° K.,

$$\Delta F_{\text{obs.}} = -RT \ln \left(\frac{0.0306 \times 0.7615 \times 0.998}{0.025 \times 0.7615 \times 0.916} \right) = -171 \text{ calories.}$$

The theoretical free energy change of the reaction at the standard state will now be calculated from known free energy values. The data used were: free energy of H.COOH (aq. 1 mol.) = -87,590 cal. [Kay and Parks, 1934]; free energy of water = -56,720 cal. [Giauque and Ashley, 1933]; free energy of CO_2 (g. 1 atm.) = -94,443 cal. [Gordon, 1933]; ionisation constant of H.COOH = 1.772×10^{-4} [Harned, 1934]; saturated solution of CO_2 = 0.03353 *M* [MacInnes and Belcher, 1933]; first ionisation constant of H_2CO_3 , p_K = 6.3655 [Shedlovsky and MacInnes, 1935]. All these values are for 298° K. No values for the ionisation constants of the two acids or for the solubility of CO_2 appear to be available for the ionic strength existing at the standard state; this may be a source of error in the following calculations.

If the free energy of H_2 be represented as F_{H_2} and so on, then for the reaction:

$$\Delta F_0 = (F_{\text{HCOO}'} + F_{\text{H}_2\text{O}}) - (F_{\text{H}_2} + F_{\text{HCO}_3'}) \quad \dots\dots(1),$$

if ΔF_0 is the free energy change of the reaction.

Computation of $F_{\text{H.COO}'}$. If K_1 is the ionisation constant of H.COOH then, free energy of ionisation of H.COOH = $-RT \ln K_1$ = +5119 cal.

from which since

$$F_{\text{H.COO}'} = F_{\text{H.COOH}} + F_{\text{of ionisation}},$$

$$F_{\text{H.COO}'} = -82,471 \text{ cal.}$$

Computation of $F_{\text{HCO}_3'}$. If α is the molal solubility coefficient of CO_2 then, free energy of solution of CO_2 = $-RT \ln \alpha$ = +2013 cal.,

also

$$F_{\text{CO}_2 (\text{aq. sat.})} = F_{\text{CO}_2 (\text{g.})} + F_{\text{of solution}} = -92,430 \text{ cal.}$$

¹ The standard state is defined as 1 molal activity in the case of the solutes, 1 atmos. pressure in the case of H_2 and a mol. fraction of unity in the case of water.

Assuming the activity coefficient of CO_2 (aq.) to be 1, the free energy change of converting CO_2 (aq. sat.) to CO_2 (1 molal activity) = $-RT \ln \frac{0.03353}{1} = +2013$ cals., from which, since

$$F_{\text{CO}_2} (\text{aq. 1 molal}) = F_{\text{CO}_2} (\text{aq. sat.}) + F_{\text{of concentration}},$$

$$F_{\text{CO}_2} (\text{aq. 1 molal}) = -90,417 \text{ cal.}$$

Therefore $F_{\text{H}_2\text{CO}_3} = F_{\text{CO}_2} + F_{\text{H}_2\text{O}} = -147,137$ cal.

If K_1' is the first ionisation constant of H_2CO_3 , then, free energy of ionisation of $\text{H}_2\text{CO}_3 = -RT \ln K_1' = +8688$ cals., so that

$$F_{\text{HCO}_3'} = F_{\text{H}_2\text{CO}_3} + F_{\text{of ionisation}} = -138,449 \text{ cal.}$$

Now since $F_{\text{H}_2} = 0$ and $F_{\text{H}_2\text{O}} = -56,720$ cals., substituting these values and those calculated above for $F_{\text{HCOO}'}$ and $F_{\text{HCO}_3'}$ in equation (1) we find

$$\Delta F_0 = -742 \text{ cal.}$$

This value differs by 571 cal. from the value of 171 cal. obtained for ΔF_{obs} . The agreement is very satisfactory and is well within the order of accuracy usually found in such calculations. It may be noted that according to Parks and Huffman [1932] the free energy of formic acid may be in error ± 300 cals., whilst the latest value for the free energy of CO_2 taken above varies by 300 cal. from earlier values. The ionisation constants of the two acids have been revised considerably in recent years and may be subject to further revision. Other possible sources of error have already been pointed out.

Calculation of the heat of the reaction.

From the equilibrium constants at two temperatures it is possible, by means of the Van't Hoff isochor, to calculate the heat of the reaction. This should agree with the heat of the reaction computed from known thermal data if the equilibria measured are real. Using the results of the equilibrium experiments at 298.1°K . and 310.7°K ., the equilibrium constants were calculated as in the last section. Then if K_T and K_{T_1} be these constants for 310.7°K . and 298.1°K . respectively,

$$\frac{K_T}{K_{T_1}} = \frac{0.0204 \times 0.916}{0.0306 \times 0.884}.$$

From the Van't Hoff isochor

$$\Delta H_{\text{obs.}} = \frac{R(TT_1)}{T - T_1} \ln \frac{K_T}{K_{T_1}},$$

where $\Delta H_{\text{obs.}}$ is the heat of the reaction and T and T_1 are the absolute temperatures corresponding to K_T and K_{T_1} , from which, $\Delta H_{\text{obs.}} = -5407$ cal.

Calculation of ΔH from known thermal data. The following ΔH values were used; all were obtained from Parks and Huffman [1932]:

$\text{H. COOH} (l)$, $\Delta H = -99,750$ cals.;

$\text{CO}_2 (g)$, $\Delta H = -94,240$ cal.;

$\text{H}_2\text{O} (l)$, $\Delta H = -68,310$ cal.

All are for 298°K .

Then since $\text{CO}_2 (g) \rightleftharpoons \text{CO}_2 (\text{sat. aq.}) + 4844$ cal. [Adolph and Henderson, 1922],

$$\Delta H \text{ of } \text{CO}_2 (\text{sat. aq.}) = -94,240 - 4844 = -99,084 \text{ cal.}$$

So that

$$\Delta H \text{ of } \text{H}_2\text{CO}_3 = \Delta H \text{ of } \text{CO}_2 (\text{aq.}) + \Delta H \text{ of } \text{H}_2\text{O} = -167,394 \text{ cal.}$$

Also $\text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3' + \text{H}^+ - 2075$ cal. [Shedlovsky and MacInnes, 1935], from which,

$$\Delta H \text{ of } \text{HCO}_3' = -167,394 + 2075 = -165,319 \text{ cal.}$$

According to Parks and Huffman [1932],

therefore, ΔH of $\text{HCOOH (aq.)} = -99,750 - 100 = -99,850$ cal.,

and since $\text{H.COOH (aq.)} \rightleftharpoons \text{H.COO'} + \text{H'} + 13$ cal. [Harned, 1934],

$$\Delta H \text{ of } \text{H.COO'} = -99,850 - 13 = -99,863 \text{ cal.}$$

If ΔH_0 is the heat of the reaction:

$$\begin{aligned} \Delta H_0 &= (\Delta H_{\text{H.COO'}} + \Delta H_{\text{H}_2\text{O}}) - (\Delta H_{\text{H}_2} + \Delta H_{\text{HCO}_3'}) \\ &= -2854 \text{ cal.} \end{aligned}$$

This is in reasonable accord with the value of -5407 cal. calculated from the observed equilibria; an agreement of ± 3000 cal. is usually considered satisfactory in such calculations. The main source of error probably lies in the fact that the temperature difference of 12.6° is rather large; the calculation from thermal data is for 298°K. , no values for higher temperatures being available.

DISCUSSION.

It has been shown that the distribution of the enzyme system studied in this paper is identical with that of the formic hydrogenlyase system of Stephenson and Stickland. Further it has been demonstrated that the presence in the washed cells of the synthesising enzyme is conditioned, as with formic hydrogenlyase, by the presence of formate in the culture medium. Both systems are sensitive to the same poisons. It seems justifiable to conclude, therefore, that the same enzyme catalyses both the synthesis and the breakdown of formic acid, that is, that the formic hydrogenlyase enzyme system is reversible. This view is confirmed by the free energy and heat calculations for the reaction. The values calculated from the observed equilibrium agree (within the limits of the accuracy of the data available and the experimental methods) with the values computed from known thermodynamical data, indicating that the observed equilibrium is a real one.

The reversal of the formic hydrogenlyase system may provide a satisfactory explanation for several cases recorded in the literature of inhibition by hydrogen of bacterial reactions producing hydrogen and carbon dioxide. Stephenson and Stickland [1932] found that the breakdown of formate by the formic hydrogenlyase enzyme was inhibited by cylinder hydrogen to the extent of 40%. Since hydrogen from a Kipp's apparatus completely inhibited the reaction they thought that the effect might be due in both cases to impurities in the gas. It now seems possible that the reverse reaction was in fact occurring, for some carbon dioxide would remain in solution as bicarbonate (at $p_{\text{H}} 7$) in spite of the NaOH papers used in their experiments to absorb carbon dioxide.

Kubowitz [1934] found that hydrogen, as compared with argon, partially inhibited the gas production of certain reactions brought about by *Cl. butyricum*:

In argon the concentration of hydrogen produced would be too small for a reverse reaction between hydrogen and carbon dioxide to be of quantitative importance; in an atmosphere of hydrogen, however, a synthesis of formic acid may well explain the low values obtained by Kubowitz.

It is also conceivable that a synthesis of formic acid similar to that described in this paper may help to explain the differences between the work of Gaffron [1935] and that of Roelofsen [1934] concerning the reduction of carbon dioxide by hydrogen in the dark in the presence of the red sulphur bacteria. Gaffron has already suggested that Roelofsen's results were due to an inhibition of carbon dioxide production by hydrogen, quoting the cases mentioned above as examples of such an inhibitory effect of hydrogen on bacterial fermentations.

SUMMARY.

The formic hydrogenlyase enzyme system of bacteria has been shown to be reversible.

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