51. THE FORMATION OF SUCCINIC ACID IN YEAST

By ARNOŠT KLEINZELLER

From the Department of Biochemistry, University of Sheffield

(Received 26 March 1941)

PASTEUR [1860] was the first to observe succinic acid among the substances formed during alcoholic fermentation. In his experiments the yield of succinic acid was 0.5–0.7 % of the fermented sugar. Ehrlich [1909] found that fermenting yeast is capable of converting glutamic acid into succinic acid and advanced the view that all the succinic acid formed during fermentation is derived from glutamic acid; the latter was assumed to arise from the autolysis of dead yeast cells. This view has been widely accepted.

It will be shown in this paper that in addition to Ehrlich's mechanism there is a second mechanism of succinic acid formation in which sugar is the parent substance. Under normal conditions most, if not all, of the succinic acid is derived from this source.

EXPERIMENTAL

Methods. Suspensions of yeast in 1 % KH_2PO_4 or 0.1 *M* phosphate buffer pH 7.4, or 0.5 % NaHCO_3 were used, and 3 ml. of the suspension containing 2–40 mg. yeast (dry weight) were measured into the main compartment of conical manometer cups. The substrate was measured into the side-arm and the volume brought up to 4 ml. with water unless otherwise stated. The gas space contained air, or expired air (i.e. 5 % CO_2 in air), or, in anaerobic experiments, CO_2 . In the anaerobic experiments yellow phosphorus was placed in the centre chamber to remove traces of O_2 .

The manometers were shaken at 25°. When equilibrium was established the substrates were added from the side-arm and the cups were shaken until the CO_2 output had almost ended. The yeast suspension was then deproteinized with tungstic acid and the succinate was estimated manometrically [see Krebs, 1937]. The results are expressed in $\mu\text{l.}$ as usual.

In most of the experiments baker's yeast was used, but a few experiments were carried out with brewer's yeast, washed suspensions of pure cultures of *Torulopsis utilis*, or a strain of *Saccharomyces cerevisiae* adapted to the use of galactose (received from Dr Marjory Stephenson, see Stephenson & Yudkin [1936]).

(1) *Effect of media.* Two factors were found to affect the formation of succinate by fermenting yeast: the concentrations of H^+ and of CO_2 and bicarbonate.

Generally no succinate was formed when 1 % KH_2PO_4 was used as a medium, whilst 85–245 $\mu\text{l.}$ succinate were formed from 50 mg. of glucose when 0.5 % NaHCO_3 saturated with 5 % CO_2 was used. The importance of the CO_2 pressure is demonstrated by Exps. 1 and 2, Table 1, where two media of approximately equal pH but of different CO_2 concentration were compared. No succinate was formed in the absence of glucose.

Table 1. *Succinate formation by baker's yeast in different media*

(1 ml. 5 % glucose; 3 ml. medium.)			
No.	mg. yeast (dry wt.)	Medium	μ l. succinate found
1 and 2	18.6	1 % KH_2PO_4 ; air; CO_2 absorbed by NaOH	0; 0
		0.1 M phosphate buffer pH 7.4; air; CO_2 absorbed by NaOH	0; 0
3	19.5	0.5 % NaHCO_3 ; 5 % CO_2 in air	85; 92
		1 % KH_2PO_4 ; air; CO_2 absorbed by NaOH	37
		0.5 % NaHCO_3 ; 5 % CO_2 in air	152
		0.5 % NaHCO_3 ; deproteinized immediately	15
4	10.0	1 % KH_2PO_4 ; CO_2	0
	20.0	1 % KH_2PO_4 ; CO_2	0
	40.0	1 % KH_2PO_4 ; CO_2	0
	10.0	0.5 % NaHCO_3 ; CO_2	108
	20.0	0.5 % NaHCO_3 ; CO_2	109
	40.0	0.5 % NaHCO_3 ; CO_2	119

(2) *Effect of glucose.* The amount of succinate formed during the fermentation in the presence of NaHCO_3 and CO_2 is proportional to the quantity of sugar fermented; it increases linearly with the amount of sugar added and with time. Per 100 mol. of sugar fermented about 1.35–3.96 mol. of succinate are formed. Slightly larger quantities seem to be formed when low concentrations of yeast are used (see Table 2).

The proportion of yeast present to succinate formed, which would be expected to be fairly constant if Ehrlich's hypothesis were correct, shows very wide variations (Table 1, Exp. 4). If a small quantity of yeast is used, the yield of succinate exceeds the total dry weight of yeast (Table 2, Exp. 3). This is conclusive proof of the formation of succinate from added glucose.

Table 2. *Succinate formation from glucose*

Baker's yeast in 3.0 ml. 0.5 % NaHCO_3 , 5 % CO_2 in air in Exps. 1 and 2; CO_2 in Exp. 3.

No.	mg. yeast (dry wt.)	Glucose added	Duration of exp. (hr.)	μ l. succinate formed
1	23.4	0	23	0
		1.0 ml. 5 %	6	134
		1.0 ml. 10 %	8	274
		2.0 ml. 10 %	23	434
2	19.5	0	0	15
		1.0 ml. 5 %	1	19
		1.0 ml. 5 %	3	100
		1.0 ml. 5 %	5	152
3	2.0	1.0 ml. 10 %	90	407

(=2.14 mg.)

(3) *Effect of O_2 .* The yield of succinate was found to be the same in aerobic and anaerobic experiments, although fermentation is more rapid in the absence of O_2 (Table 3).

Table 3. *Formation of succinate under aerobic and anaerobic conditions*

20.4 mg. baker's yeast (dry weight) in 3 ml. 0.5 % NaHCO_3 ; 1 ml. 5 % glucose.

No.	Gas mixture	μ l. succinate found
1	5 % CO_2 in air	137
	CO_2 ; phosphorus	148
2	5 % CO_2 in air	175
	CO_2 ; phosphorus	174

(4) *Succinate formation from various sugars.* The amounts of succinate formed from glucose fructose and sucrose were found to be of the same order. This confirms Pasteur's [1860] results. A strain of *Saccharomyces cerevisiae* adapted to the use of galactose formed the same quantities of succinate from glucose and from galactose.

Table 4. *Formation of succinate from various sugars*

Exp. 1. 23.4 mg. baker's yeast in 3 ml. 0.5 % NaHCO_3 ; 5 % CO_2 in air; 1 ml. 5 % sugar added.
Exp. 2. 19.3 mg. *S. cerevisiae* adapted to use of galactose, in 3 ml. 0.5 % NaHCO_3 ; 1 ml. 10 % sugar added; 5 % CO_2 in air.

Exp.	Sugar added	$\mu\text{l. succinate}$ found
1	Glucose	164
	Fructose	190
	Saccharose	184
2	0	0
	Glucose	183
	Galactose	151

Table 5. *Effect of various substrates on the formation of succinate in baker's yeast*

(3 ml. yeast suspension, 1 ml. substrate. Medium, 0.5 % NaHCO_3 ; 5 % CO_2 in air, unless otherwise stated. Brackets indicate experiments carried out on the same suspension and therefore directly comparable.)

mg. yeast	Additions	$\mu\text{l. succinate}$ found
23.4	1.0 ml. 0.2 M Na acetate	0
	1.0 ml. 0.2 M Ba acetate	0
	1.0 ml. 0.2 M Na acetate*	0
	1.0 ml. 0.2 M Ba acetate*	0
19.5	0	20
	50 mg. glucose	161.5
	1.0 ml. 0.25 M glycerol	6.5
	1.0 ml. 0.25 M α -glycerophosphate	19
	50 mg. glucose; 0.5 ml. 0.2 M pyruvate	157
23.4	50 mg. glucose	149
	50 mg. glucose; 0.5 ml. 0.2 M fumarate	138
	50 mg. glucose; 0.5 ml. 0.2 M aspartate	184
	1.0 ml. 0.25 M fumarate	29
	1.0 ml. 0.25 M aspartate	10
	50 mg. glucose	157
19.5	50 mg. glucose; 0.5 ml. 0.2 M oxaloacetate	187
	1.0 ml. 0.25 M oxaloacetate	24.5
	50 mg. glucose	132
15.5	50 mg. glucose; 0.5 ml. 0.2 M L-tartrate	138
	1.0 ml. 0.25 M L-tartrate	0
	50 mg. glucose	134
19.5	50 mg. glucose; 0.5 ml. 0.2 M L-malate	194
	50 mg. glucose; 0.5 ml. 0.2 M α -hydroxyglutarate	183
	1 ml. 0.25 M L-malate	45
	1 ml. 0.25 M α -hydroxyglutarate	21
	50 mg. glucose	160
19.5	1.0 ml. 0.25 M α -ketoglutarate	156
	50 mg. glucose	134
19.5	50 mg. glucose; 0.5 ml. 0.2 M glutamate	450
	50 mg. glucose; 0.5 ml. 0.2 M α -ketoglutarate	174
	50 mg. glucose	141
19.5	50 mg. glucose; 0.5 ml. 0.2 M glutamate	493
	50 mg. glucose; 0.5 ml. 0.2 M α -hydroxyglutarate	195
	50 mg. glucose	132
	50 mg. glucose; 0.5 ml. 0.2 M arginine	131
15.5	50 mg. glucose; 0.5 ml. 0.2 M ornithine	138

* 0.1 M phosphate buffer; air.

(5) *Effect of other substrates.* The effect of the following substances on the formation of succinate was tested in the presence and in the absence of glucose: acetate, pyruvate, lactate, glycerol, α -glycerophosphate, oxaloacetate, *l*-malate, fumarate, *l*-tartrate, γ -aminobutyrate, *l*-glutamate, α -hydroxyglutarate, α -ketoglutarate, *dl*-ornithine, *l*-arginine. The final concentration of these substances when added alone was 0.0625 *M*, when added with glucose 0.025 *M* (unless otherwise stated).

In absence of glucose α -ketoglutarate was the only substance to yield succinate [see Neuberg & Ringer, 1915]. In presence of glucose 2- and 3-carbon compounds had no effect on the formation of succinate. Oxaloacetate, *l*-malate and aspartate increased the yield of succinate by about 20 % and α -ketoglutarate and α -hydroxyglutarate about 30 %, whilst fumarate, *l*-tartrate, γ -aminobutyrate, arginine and ornithine had no effect (Table 5).

The succinate formation from *l*(+)-glutamic acid, first shown by Ehrlich [1909], is confirmed. The quantity of succinate formed depends on the composition of the medium; only a small amount is formed when the medium is weakly acid and CO_2 is absorbed by NaOH; considerable quantities appear in 0.5 % NaHCO_3 saturated with 5 % CO_2 .

No difference was found in the yield of succinate from glutamic acid under aerobic or anaerobic conditions.

Table 6. *Formation of succinate from l(+)-glutamate in baker's yeast*

Glutamate concentration *M*/40 unless otherwise stated.

No.	mg. yeast	Substrates		Medium	$\mu\text{l.}$ succinate found
		Glucose (50 mg.)	Glutamate		
1	23.4	+	—	0.5 % NaHCO_3 ; 5 % CO_2 in air	157
		+	+	" "	500
		—	+	" "	6
2	18.6	+	—	0.5 % NaHCO_3 ; 5 % CO_2 in air	82
		+	+	" "	402
		+	+	0.1 <i>M</i> phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4 ; air; CO_2 absorbed by NaOH	0
3	18.6	+	—	0.5 % NaHCO_3 ; 5 % CO_2 in air	92
		+	+	" "	396
		+	+	0.1 <i>M</i> phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4 ; air; CO_2 absorbed by NaOH	0
4	20.4	+	—	0.5 % NaHCO_3 ; 5 % CO_2 in air	137
		+	+	" "	305
		+	+	0.5 % NaHCO_3 ; CO_2 ; phosphorus	330

* 0.0625 *M* glutamate.

Table 7. *Effect of NH_4Cl on the formation of succinate in baker's yeast*

16.1 mg. yeast in 3 ml. 0.5 % NaHCO_3 ; 5 % CO_2 in air; 50 mg. glucose.

Additions (final conc. 0.025 <i>M</i>)	$\mu\text{l.}$ succinate found
0	82
<i>l</i> -Glutamate	364
α -Ketoglutarate	250
α -Hydroxyglutarate	141.5
α -Ketoglutarate; NH_4Cl	78
α -Hydroxyglutarate; NH_4Cl	83
NH_4Cl	92

α -Ketoglutarate and α -hydroxyglutarate have been assumed to be intermediates in the succinate formation from glutamate [Ehrlich, 1909; Neuberg & Ringer, 1915] and it is therefore remarkable that under identical conditions the yields of succinate from these acids were smaller than those from glutamate. This result is not affected by the addition of equimolecular quantities of NH_4Cl (Table 7). Since the permeability of the yeast cell may influence the rate of reaction we do not regard our results as contradictory to the hypothesis of Neuberg & Ringer.

(6) *Effect of growth.* Little or no succinate was found, even in presence of NaHCO_3 , when the fermentation proceeded in a medium in which growth occurs. Two media were tested, a modified Czapek-Dox medium (2 g. NaNO_3 , 1.32 g. Na_2HPO_4 , $2\text{H}_2\text{O}$, 0.5 g. MgSO_4 , $7\text{H}_2\text{O}$, 0.5 g. KCl , 0.01 g. FeSO_4 , $7\text{H}_2\text{O}$ in 1 l. tap water) and the medium of Fink & Krebs [1938] (2.6 g. $(\text{NH}_4)_2\text{HPO}_4$, 3.3 g. $(\text{NH}_4)_2\text{SO}_4$, 2 g. K_2SO_4 , 1.5 g. MgSO_4 , $7\text{H}_2\text{O}$, in 1 l. tap water).

The recovery of succinate added to yeast fermenting in 0.5 % NaHCO_3 , or 1 % KH_2PO_4 , was quantitative, whilst a considerable amount of succinate was taken up by the yeast fermenting in the complex medium (Table 8).

Table 8. *Effect of growth on the formation of succinate in baker's yeast*

20.0 mg. yeast in 3 ml. medium; 5 % CO_2 in air.

No.	Medium	Additions		Succinate found, μl .
		Glucose mg.	Succinate μl .	
1	Czapek-Dox	—	—	0
		50	—	39
	0.5 % NaHCO_3	50	—	138
2	Fink-Krebs	—	—	0
		50	—	0
	0.5 % NaHCO_3	50	—	115
3	0.5 % NaHCO_3	50	—	247
		50	672	943
	Fink-Krebs	50	—	45
	"	50	672	638

(7) *Experiments on brewer's yeast and on Torulopsis utilis.* Experiments with varying quantities of sugar were carried out on fresh brewer's yeast and on a pure culture of *Torulopsis utilis*. As in the case of baker's yeast the yield of succinate was found to be proportional to the quantity of sugar fermented (Table 9).

Table 9. *Succinate formation from glucose in different yeasts*

Exp. 1. 15 mg. brewer's yeast in 3 ml. 0.5 % NaHCO_3 ; 5 % CO_2 in air; glucose.

Exp. 2. 20.2 mg. *Torulopsis utilis* in 3 ml. 0.5 % NaHCO_3 ; 5 % CO_2 in air; glucose.

No.	mg. glucose added	Duration of exp. (hr.)	μl . succinate found
1	0	0	0
	0	30	10
	50	6½	87
	100	24	167
	200	30	268
2	0	22	0
	25	2½	58
	50	4	107
	100	6½	195
	200	22	236

(8) *Presence of fumarase in yeast.* In view of the fact that fumarase takes part in the synthesis of succinate in some organisms [Krebs & Eggleston, 1940; Smyth, 1940] it was thought of interest to examine the fumarase activity of yeast. Fumarate was estimated according to Krebs *et al.* [1940]. No evidence was found of the presence of fumarase in baker's yeast. Added malate was not converted into fumarate within pH 5-8. This explains why malate and fumarate behave differently (Table 5). The conversion of malate into succinate must be regarded as a direct reduction which does not involve the formation of fumarate.

DISCUSSION

(1) *Source of succinate.* The experimental results show that in normal conditions of fermentation sugar is the main parent substance of succinate (Exp. 5, Table 1). The fact that 1 mg. of yeast can form 1.07 mg. succinic acid proves that the succinate cannot be derived from the yeast protoplasm, as assumed by Ehrlich. It must be formed from the added substrate, i.e. glucose.

(2) *Mechanism of succinate formation from glucose.* The formation of succinate depends on the presence of NaHCO_3 and of CO_2 . A similar relationship between the formation of C_4 -dicarboxylic acids and the presence of NaHCO_3 was observed by Elsdon [1938] and by Krebs & Eggleston [1940], who concluded that oxaloacetic acid is synthesized in animal tissues by condensation of pyruvic acid with CO_2 . It is therefore suggestive to assume that succinic acid in yeast is formed in a similar way. The 'carboxylation' of pyruvic acid would represent a side reaction in the metabolism of pyruvate, the main reaction being the decarboxylation of pyruvic acid. In accordance with this view are the observations that oxaloacetate and malate increase the yield of succinate from glucose, and that yeast is able to bind CO_2 [Ruben & Kamen, 1940].

(3) *Formation of succinate from acetate.* According to Table 5 no succinate was formed from acetate (Na or Ba salt), contrary to the results of Wieland & Sonderhoff [1932], Sonderhoff & Deffner [1936], Sonderhoff & Thomas [1937]. It should be noted that our experimental conditions differ in some ways from those of Wieland and his collaborators. In our experiments fresh yeast, as opposed to 'impoverished' yeast, was used and the relatively short experimental times (24 hr.) excluded bacterial infection, which has previously been accepted by Wieland as an explanation of some of his results [Wieland *et al.* 1936].

SUMMARY

1. The amount of succinate formed in resting suspensions of baker's yeast, brewer's yeast and *Torulopsis utilis* is proportional to the amount of sugar fermented. The yield of succinate varies between 1.35 and 3.96 g. mol. per 100 g. mol. of sugar. The quantity of succinate can exceed the dry weight of the fermenting yeast. This shows conclusively that most of the succinate formed during fermentation is derived from the fermented sugar. Ehrlich's view, according to which succinic acid is derived from the glutamic acid of autolysed yeast cells, is therefore to be discarded.

2. The formation of succinate depends on the presence of bicarbonate in the medium. No succinate is formed in 1 % KH_2PO_4 ; between 0.45 and 1.29 mg. succinate are formed when 50 mg. glucose are fermented by yeast suspended in 0.5 % NaHCO_3 .

3. The amount of succinate formed from various fermentable sugars is of the same order.

4. Oxaloacetate, *l*-malate and *l*-aspartate increase the yield of succinate in the presence of glucose by about 20 %, α -ketoglutarate and α -hydroxyglutarate about 30 %, *l*-glutamate about 200 %, whilst various other substances had no effect.
5. No succinate is found when glucose is fermented by growing yeast cells.
6. Fumarase is absent from baker's yeast.
7. The mechanism of succinate formation from glucose is discussed.

The author wishes to express his gratitude to Dr H. A. Krebs for having suggested this investigation and for his constant help and advice, and also to the Sheffield Co-ordinating Committee for Refugees and to the International Student Service for a maintenance grant.

REFERENCES

- Ehrlich (1909). *Biochem. Z.* **19**, 391.
Elsden (1938). *Biochem. J.* **32**, 187.
Fink & Krebs (1938). *Biochem. Z.* **299**, 1.
Krebs (1937). *Biochem. J.* **31**, 2095.
— & Eggleston (1940). *Biochem. J.* **34**, 1383.
— & Smyth & Evans (1940). *Biochem. J.* **34**, 1041.
Neuberg & Ringer (1915). *Biochem. Z.* **71**, 226.
Pasteur (1860). *Ann. chim. (Phys.)*, 3^e série, **58**, 323.
Ruben & Kamen (1940). *Proc. Nat. Acad. Sci., Wash.*, **26**, 418.
Smyth (1940). *Biochem. J.* **34**, 1598.
Sonderhoff & Deffner (1936). *Liebigs Ann.* **525**, 132.
— & Thomas (1937). *Liebigs Ann.* **530**, 195.
Stephenson & Yudkin (1936). *Biochem. J.* **30**, 506.
Wieland, Crawford & Walch (1936). *Liebigs Ann.* **525**, 119.
— & Sonderhoff (1932). *Liebigs Ann.* **499**, 213.