THE METABOLISM OF SPECIES OF STREPTOMYCES

V. The Role and the Pathway of Synthesis of Organic Acids in Streptomyces coelicolor[1]

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Received for publication September 18, 1951

An earlier study (Cochrane and Dimmick, 1949) demonstrated the formation of succinic and other acids by *Streptomyces coelicolor* (Müller) Waksman and Henrici, grown in aerated media. The present investigation was concerned with two related problems: the general significance of organic acids in the carbohydrate metabolism of *S. coelicolor*, and the metabolic pathway or pathways by which they originate.

The importance of succinate, and by inference of the other dibasic 4-carbon acids, was gauged by a study of its production during growth on carbon compounds other than glucose. In addition, the relations between glucose consumption and succinate accumulation were investigated in order to exclude the possibility that acid production, as in many fungi, is the result of luxury consumption of carbohydrate supplied in excess of energy demands (Foster, 1949).

With regard to the metabolic origin of dicarboxylic acids, experimental designs were formulated to test principally three possibilities, all of which have been suggested or demonstrated in other organisms: (1) carboxylation of pyruvate or a 3-carbon derivative of it, followed by reduction of the product (oxaloacetate or malate); (2) direct condensation of acetate or other 2-carbon compound derived from it; and (3) the existence of a metabolic block in a tricarboxylic acid cycle. A fourth suggestion, that 4-carbon acids may arise by direct fixation of formate (Stokes, 1949), was not tested.

METHODS

Unless otherwise stated, the organism was grown as previously described (Cochrane and Dimmick, 1949) for 5 days in a glucose-asparagine-salts-$CaCO_3$ medium supplemented with yeast extract (0.25 g per L). Cells harvested from this medium were of course contaminated with undissolved $CaCO_3$; where this was undesirable, the organism was grown for 3 days in Difco nutrient broth supplemented with glucose (0.04 M), potassium phosphate buffer (0.033 M, pH 7.4), and $MgSO_4$ (0.001 M). All growth and biosynthesis flasks were incubated at 25 C on a reciprocating or a rotary shaker.

Dry weight, sugar, total acids, succinic acid, and volatile acids were determined as described earlier (Cochrane and Dimmick, 1949). Lactic acid was determined by the method of Barker and Summerson (1941), pyruvate and α-

[1] This investigation was supported in part by a research grant (1495) from the National Institutes of Health and in part by the Denison Research Fund of Wesleyan University.

ketoglutarate by the methods of Friedemann and Haugen (1943), and citrate by the method of Perlman *et al.* (1944).

In tracer experiments with carbon-14, succinate was isolated as the silver salt and oxidized with persulfate (Osburn and Werkman, 1932); the CO_2 evolved was passed into standardized $Ba(OH)_2$—$BaCl_2$, and the resultant $BaCO_3$ was collected on a sintered glass disk of known weight and area. Total carbon was estimated by back titration prior to filtering off the $BaCO_3$. Radioactivity of the $BaCO_3$ was determined in a methane flow proportional counter.

TABLE 1

*The production of succinic acid by Streptomyces coelicolor during growth**

EXPERIMENT	CARBON SOURCE†	DRY WEIGHT	FINAL pH	TOTAL ACID	SUCCINIC ACID
		mg		*m.e. per L*	*m.e. per L*
1	None	4.7	8.82	0.34	0.21
	Glucose	137.0	7.97	10.13	8.50
	Cellobiose	142.4	7.40	12.48	9.52
	Maltose	156.2	7.45	9.18	8.55
	Levulose	153.6	6.82	6.02	5.52
	Mannose	165.0	7.80	11.33	9.13
	Galactose	139.8	7.15	3.84	3.15
	Rhamnose	149.2	6.25	7.55	6.04
2	None	<1.0	8.60	0.31	0.23
	Glucose	125.7	6.63	13.53	11.81
	Xylose	153.0	7.20	9.70	8.27
	Arabinose	116.3	7.38	9.44	9.47
	Mannitol	161.0	7.58	15.10	13.16
	Glycerol	128.2	6.05	10.58	8.38
	Na malate	56.6	9.10	—	0.83
	Na fumarate	55.7	9.12	—	0.20

* Basal medium: asparagine 0.0075 M, K_2HPO_4 0.003 M, $MgSO_4$ 0.001 M, yeast extract 0.25 g per L, $CaCO_3$ 3.0 g per L, minor elements. Period of growth 6 days, on reciprocating shaker at 25 C.

† Concentration to supply 3.6 g carbon per L.

For studies of biosynthesis by resting cells, the small pellets of mycelium were centrifuged down and washed twice by centrifugation with a solution of the inorganic salts of the growth medium, including minor elements. The cells were then suspended in sterile potassium phosphate buffer of the desired pH; substrates and inhibitors, sterilized by filtration, and sterile water were added aseptically to yield finally a suspension in which there was twice as much cell material per unit volume as in the original growth medium.

RESULTS

The production of succinic acid by growing cells. From the data of table 1 it appears that *S. coelicolor* accumulates succinate from a variety of carbon sources and that in general the succinate accounts for almost all the ether extractible

acids. Presumably, all of the carbohydrates tested are metabolized by a common terminal pathway, and succinate is a normal product thereof.

Negative data omitted from table 1 show that the organism is unable to grow with sucrose, lactose, sorbose, dulcitol, citrate, acetate, or lactate as the sole source of carbon. The failure of succinate to accumulate in large amounts with malate or fumarate as substrate is probably attributable to the rapid utilization of these acids; *S. coelicolor* is able to utilize succinate for energy when other carbon sources are lacking. Over shorter time periods and with resting cells it is easy to demonstrate conversion of both fumarate and malate to succinate (see later).

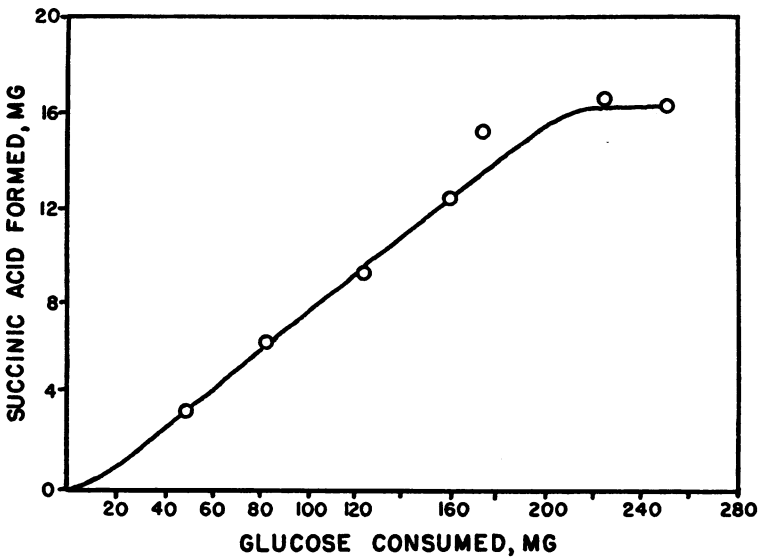

Figure 1. The relation between glucose consumption and the formation of succinic acid by washed cells of *Streptomyces coelicolor*. Conditions as described in table 2.

The dynamics of succinate biosynthesis. Washed cells were suspended in buffer (pH 7.2) with varying concentrations of glucose. At the end of 24 hours residual glucose and accumulated succinate were determined. The results (figure 1) indicate that succinate, and by inference precursor organic acids, is formed at all tested levels of glucose consumption, and that over a wide range of carbohydrate concentration there is a linear relation between sugar consumed and acid formed. The interpretation is that succinate is a constant product of glucose metabolism and is not formed only in a system "overloaded" with carbohydrate.

The flattening of the curve of figure 1 suggests either that at high glucose levels there is a mechanism of glucose consumption which does not form succinate, or that some intermediate reaction has become limiting. Analysis of associated data points to the latter interpretation, since the ratio of total ether-extractible acids to succinic acid changes gradually from approximately 1.0

at lower sugar concentrations to almost 2.0 at the highest concentration tested. It will be shown later in this paper that the rate limiting step is the fixation of carbon dioxide, with the inference that it is pyruvic acid which accumulates at high sugar concentrations.

Precursors of succinate in biosynthesis by resting cells. Failure in growth experiments to demonstrate biosynthesis of succinate from fumarate and malate (table 1) pointed to the necessity of a different experimental design for study of precursor organic acids. Recourse, consequently, was had to washed cell experiments of short duration in media buffered against the drastic pH changes accompanying utilization of acids.

TABLE 2

*The biosynthesis of succinate by resting cells of Streptomyces coelicolor**

SUBSTRATE†	AEROBIC		ANAEROBIC‡	
	Substrate utilization	Succinic acid	Substrate utilization	Succinic acid
	per cent	*m.e. per L*	*per cent*	*m.e. per L*
Glucose 0.02 M	91.6	5.55	0.7	<0.05
Gluconate 0.02 M	—	2.71	—	—
Pyruvate 0.02 M	60.2	0.85	0.0	<0.05
Malate 0.02 M	>90.0	6.01	54.0	2.12
Fumarate 0.02 M	97.0	5.35	51.2	2.87
Citrate 0.02 M	61.3	1.07	34.0	<0.05
α-Ketoglutarate 0.02 M	27.8	<0.20		
Ethanol 0.05 M	—	<0.05		
Acetate 0.02 M	31.0	<0.05		
Propionate 0.02 M	10.2	<0.05		
Glycerol 0.05 M	—	2.28		
Glutamate 0.02 M	—	<0.05		
Aspartate 0.02 M	—	<0.05		

* Cells grown 5 days in glucose-asparagine-yeast extract-salts-$CaCO_3$ medium.

† KH_2PO_4-K_2HPO_4 buffer (M/60, pH 6.0). Incubation 16 hours on reciprocating shaker at 25 C.

‡ Nitrogen atmosphere.

The results of some typical experiments with single substrates, under aerobic (air) or anaerobic (nitrogen atmosphere) conditions, are collected in table 2. Aerobically, apart from glucose, glycerol, and gluconate, which are readily available energy sources, succinate was formed in good yield only from closely related dicarboxylic acids. It is particularly significant that neither citrate nor α-ketoglutarate, both of which were utilized, yielded succinate in amounts comparable to the yield from fumarate and malate. Ethyl alcohol, acetate, and propionate were completely inactive.

If malate and fumarate are involved, the role of pyruvate requires further study, since in aerobic experiments the yield from pyruvate was low, and under anaerobic conditions pyruvate was not appreciably utilized. The carboxylation of pyruvate or other 3-carbon compound requires energy, and the possibility

arises that the organism is unable to couple pyruvate oxidation with carboxylation. The same restriction might of course account for other negative results in single substrate experiments.

To test this possibility, glucose was supplied to resting cells at a level known from the data of figure 1 to yield by itself a small amount of succinate and to be largely consumed in the time period chosen. The effect of relatively large concentrations of suspected precursors supplied along with glucose as an energy source was then determined. The data (table 3) indicate first that pyruvate can contribute substantially to succinate accumulation if a source of energy is provided. High yields from malate and fumarate accord with single substrate data (table 2), as do low yields from citrate and α-ketoglutarate. The mechanism of the depressant effect of glutamate was not investigated; its inclusion in a medium converts it into a growth medium, since the nitrogen of glutamate is

TABLE 3

*The biosynthesis of succinate by resting cells of Streptomyces coelicolor**

SUBSTRATE(S)†	SUCCINIC ACID	
	Yield	Increase over control
	m.e. per L	*per cent*
Glucose 0.0125 M (control)	5.32	—
Glucose 0.0125 M plus pyruvate 0.05 M	12.79	130
Glucose 0.0125 M plus malate 0.05 M	11.70	120
Glucose 0.0125 M plus fumarate 0.05 M	14.86	179
Glucose 0.0125 M plus citrate 0.05 M	5.88	10
Glucose 0.0125 M plus α-ketoglutarate 0.05 M	5.40	1.5
Glucose 0.0125 M plus acetate 0.05 M	0.06	—
Glucose 0.0125 M plus acetate 0.005 M	5.93	12
Glucose 0.0125 M plus glutamate 0.05 M	1.71	—

* Conditions as in table 2.

† Acid substrates adjusted to pH 6 with NaOH, sterile filtered.

available to the organism. Glutamate was tested because of the possibility that it might enter into atricarboxylic acid cycle. The data of course do not dispose of that possibility; they do show that whatever pathway exists for glutamate metabolism does not contribute to succinate accumulation.

Acetate is a special problem in that, as shown in table 3, it is toxic at a concentration as high as 0.05 M. At lower concentrations (0.005–0.01 M) toxicity disappears and there is slow utilization of the acetate. All attempts to demonstrate succinate biosynthesis directly from acetate were unsuccessful, including the use of malonate to enhance accumulation of succinate (Barron *et al.*, 1950). Manometric data (Cochrane and Peck, unpublished) indicate that the rate of acetate utilization by intact cells is so low that acetate as such is unlikely to be an obligatory intermediate in the formation of succinate from glucose.

A more rigorous test for the incorporation of acetate carbon into succinate was possible through the use of acetate-2-C^{14} as substrate (table 4). Acetate of high

specific activity was supplied alone and with glucose. While acetate carbon is shown to be fixed in succinate, the incorporation is not large; it can be calculated from the data of table 4 that the trapping in succinate of activity disappearing from acetate was slightly under 2 per cent.

These results, and the yield data shown earlier, indicate that bulk formation of succinate cannot be accounted for by direct biosynthesis from acetate. There is, consequently, no point in determining the location of C^{14} in succinate formed from acetate-2-C^{14}, since the position of the label alone fails to discriminate between the two most likely mechanisms for the origin of succinate from acetate: oxidation of acetate *via* a tricarboxylic acid cycle, and direct condensation of acetate. The carbon-14 results might suggest that, in addition to the bulk formation of succinate, there is a contribution from acetate by one or the other of these routes.

TABLE 4

*The incorporation of C^{14} from acetate-2-C^{14} into succinate by resting cells of Streptomyces coelicolor**

	SUBSTRATE(S)†	
	Acetate-2-C^{14}, 100 μM	Acetate-2-C^{14}, 100 μM plus glucose, 800 μM
Initial specific activity of acetate, mμc/mg C.	727	727
Final specific activity of acetate, mμc/mg C..	678	492
Residual acetate, μM.....	63	108
Succinate formed, μM.....	—	12.5
Specific activity of succinate, mμc/mg C....	—	15.2

* Cells grown 64 hours on rotary shaker in Difco nutrient broth + glucose 0.04 M + KH_2PO_4-K_2HPO_4 buffer (M/60, pH 6.0) + $MgSO_4$ 0.001 M.

† Buffer KH_2PO_4-K_2HPO_4 (M/60, pH 6.0), volume 40 ml, incubation 6 hours on rotary shaker.

Parenthetically, the data of table 4 provide other information. Acetate appears, contrary to conclusions reached earlier with less sensitive methods (Cochrane and Dimmick, 1949), to be involved in glucose metabolism; the dilution of the specific activity of acetate during the oxidation of glucose and the slight increase in acetate concentration both lead to this conclusion. This again is consonant with the operation of a metabolic cycle for acetate oxidation which proceeds over succinate but does not contribute substantially to accumulation of succinate. With acetate-2-C^{14} a sole substrate, little dilution of activity occurred.

The effect of enzyme poisons on succinate biosynthesis. Data shown in table 5 indicate that succinate biosynthesis by resting cells from glucose is inhibited completely by pyrophosphate and arsenite, and partially by fluoride. Pyrophosphate inhibits by a nonspecific complexing of essential ions (Lardy, 1949), and hence cannot be used here as evidence for any specific reaction. Fluoride, while commonly acting through its effect on enolase, may poison other systems also (Bonner and Thimann, 1950; Hunter, 1947; Stephenson, 1949); some such other

effects may be involved in the present case since, as shown in table 5, the inhibition by fluoride is not relieved by pyruvate.

Arsenite at 0.01 M, while it may have other effects, usually prevents the metabolic breakdown of keto acids (Green *et al.*, 1948; Walker, 1949). In experiment 1 of table 5, the data on total acids indicated that some acid other than succinic was present at approximately 8.7 m.e. per L. The extract was treated with 2,4-dinitrophenylhydrazine, the precipitated hydrazone purified by several recrystallizations from ethyl acetate, and the melting point determined to be 213 to 215 C (theoretical for pyruvic acid 2,4-dinitrophenylhydrazone 218 C). These results are interpreted to mean that pyruvic acid is a normal

TABLE 5

*The effect of inhibitors on biosynthesis of succinate by resting cells of Streptomyces coelicolor**

EXPERIMENT	SUBSTRATE AND INHIBITOR	INITIAL pH	INCUBATION	SUCCINIC ACID	INHIBITION
			hours	*m.e. per L*	*per cent*
1	Glucose 0.05 M (control)	7.3	24	9.25	
	plus arsenite 0.01 M	7.3	24	0.05†	100
	plus pyrophosphate 0.02 M	7.3	24	0.05	100
	plus fluoride 0.025 M	7.3	24	5.58	39
2	Glucose 0.05 M (control)	7.3	24	3.06	
	plus fluoride 0.025 M	7.3	24	1.76	42
	plus fluoride 0.025 M and pyruvate 0.05 M	7.3	24	1.88	39
3	Malate 0.02 M (control)	5.1	16	5.94	
	plus cyclohexanol 0.04 M	5.1	16	1.01	83
	plus malonate 0.01 M	5.1	16	3.45	42
4	Citrate 0.02 M (control)	5.4	16	1.59	
	plus malonate 0.01 M	5.4	16	0.89	44

* Conditions as in table 2.

† Pyruvic acid isolated as 2,4-dinitrophenylhydrazone.

intermediate in the formation of succinate, arsenite preventing the final conversion.

In the malonate experiments of table 5 the reaction was run at pH 5.3 in order to maximize penetration of the inhibitor into the cells. Since there was, in these and other experiments, an observable effect of malonate, at least some of the acid must have penetrated. The important feature, however, is the direction of the effect: with either malate or citrate as substrate the effect of malonate is to decrease succinate accumulation. While not crucial, the malonate data afford evidence against the origin of succinate *via* oxidative steps of a conventional tricarboxylic acid cycle, and focus attention on the reductive pathway from pyruvate as a possible route.

Cyclohexanol is known (Ajl, 1950) to inhibit fumarase; inhibition of succinate

accumulation with malate as substrate is to be expected if a reductive pathway is involved.

The role of carbon dioxide in succinate biosynthesis. Definitive evidence for the fixation of carbon dioxide was obtained with carbon-14, the data of table 6 showing incorporation of $C^{14}O_2$ into the succinate molecule. No mechanism other than the reductive carboxylation of pyruvate is presently known to accomplish this reaction (Wood, 1946).

TABLE 6

*Fixation of $C^{14}O_2$ in succinic acid**

ADDITION TO BIOSYNTHESIS MEDIUM†	FINAL pH	SUCCINIC ACID	
		Yield	Specific activity
		mg	*mμc per mg C*
None	6.2	2.7	0.00
$Na_2C^{14}O_3$, 0.1 mμc per ml	6.2	2.4	2.65
$Na_2C^{14}O_3$, 0.1 mμc per ml	6.3	3.5	1.62

* Cells grown 4 days in glucose-asparagine-yeast extract-salts-$CaCO_3$ medium on rotary shaker at 25 C.

† Biosynthesis medium: glucose 0.025 M, KH_2PO_4-K_2HPO_4 buffer (0.03 M, pH 7.4), $MgSO_4$ 0.001 M, $CaCO_3$ 0.1 per cent. Volume 25 ml; incubated 24 hours on rotary shaker at 25 C.

TABLE 7

*The effect of CO_2 on succinate biosynthesis by resting cells**

ADDITIONS TO BIOSYNTHESIS MEDIUM†	ATMOSPHERE	TOTAL ACID (TA)	SUCCINIC ACID (SA)	RATIO TA/SA
		m.e. per L	*m.e. per L*	
$CaCl_2$ 0.001 M‡	CO_2-free air	4.88	1.24	3.9
$CaCl_2$ 0.001 M‡	10% CO_2 in air	7.04	7.19	1.0
$CaCO_3$ 0.6%	Air	15.22	12.15	1.3

* Cells grown 3 days in nutrient broth plus glucose 0.04 M, KH_2PO_4-K_2HPO_4 buffer (M/60, pH 7.4), and $MgSO_4$ 0.001 M, on reciprocating shaker at 25 C.

† Biosynthesis medium: glucose 0.025 M, KH_2PO_4-K_2HPO_4 buffer (M/60) $MgSO_4$ 0.001 M, minor elements, adjusted to pH 6.0. Incubation 10 hours on rotary shaker at 25 C.

‡ Ca approximately equivalent to soluble Ca in presence 0.6 per cent $CaCO_3$.

The extremely low recovery (about 0.25 per cent) of added activity in the experiment of table 6 is probably attributed to loss of CO_2 at the low pH resulting from acid accumulation. Whatever the cause, the low specific activity of the succinate could, in principle, result merely from equilibration among the dicarboxylic acids and pyruvate in the terminal phases of a dicarboxylic or tricarboxylic acid cycle (Foster *et al.*, 1949), without necessarily involving net fixation of CO_2.

Consequently, the effect of CO_2 or carbonate on succinate accumulation by resting and growing cells was tested directly. Data of table 7 show that succinate formation is sharply reduced by exclusion of atmospheric CO_2, and that some other acid accumulates under these conditions.

The nature of the precursor acid was investigated further in growing cultures with and without carbonate, preliminary experiments having shown no effect of Ca^{++} supplied as $CaCl_2$ at 0.01 M. Cultures lacking carbonate accumulate pyruvate as the major metabolic acid, while in those supplied carbonate the principal acid is succinic (table 8), in accordance with expectations. The accumulation of pyruvate in aerated cultures of *Aerobacter* has recently been described in detail by Dagley *et al.* (1951) and may be comparable.

The implication of CO_2 in succinate formation parallels earlier studies with many different organisms, e.g., *Escherichia coli* (Elsden, 1938), *Propionibacterium* (Wood and Werkman, 1938), yeast (Kleinzeller, 1941), and fungi (Foster and Davis, 1948 and Cantino, 1949); although in *S. coelicolor* the fixation reaction proceeds only aerobically, never anaerobically.

For purposes of verification, pyruvic acid was isolated as the 2,4-dinitrophenylhydrazone from medium A of table 8; the derivative melted at 215 to 216 C (theoretical 218 C), and the melting point was not changed by admixture with an authentic sample prepared from analytically pure sodium pyruvate.

TABLE 8

The effect of carbonate on acid synthesis by growing cells

NO.	ADDITIONS TO BASAL MEDIUM*	SUCCINIC ACID (SA)	PYRUVIC ACID (PA)	LACTIC ACID	RATIO PA/SA
		m.e. per L	*m.e. per L*	*m.e. per L*	
A	$CaCl_2$ 0.0005 M†	1.42	5.83	0.43	4.11
B	$CaCO_3$ 0.3%	1.98	0.90	0.32	0.45

* Basal medium: nutrient broth plus glucose 0.04 M, KH_2PO_4-K_2HPO_4 buffer (M/60), $MgSO_4$ 0.001 M, adjusted to pH 6.0. Incubation 64 hours on reciprocating shaker at 25 C.

† Ca approximately equivalent to soluble Ca in presence 0.3 per cent $CaCO_3$.

DISCUSSION

Of the three mechanisms for the origin of dicarboxylic acids which were considered, the evidence points to the carboxylation of pyruvate or a 3-carbon compound derived from pyruvate and reduction of the product to succinic acid as the system responsible for the accumulation of succinate in *S. coelicolor*. This sequence of reactions is of course of widespread occurrence among organisms (Wood, 1946).

The precursor experiments, inadequate in themselves to prove the mechanism, are consistent with the previous mechanism; in particular the high yields from fumarate and malate both aerobically and anaerobically can best be explained in this way. The low yield from pyruvate alone (table 2) is believed to reflect the fact that the energy required for the conversion of pyruvate to a 4-carbon acid (Kalnitsky and Werkman, 1944) cannot be obtained by this organism from mechanisms of pyruvate breakdown. When a small amount of glucose is present, the contribution of pyruvate to succinate accumulation is easily measurable aerobically. A possibly comparable situation is reported by Crane and Ball (1951) in the metabolism of ox retina, in which tissue the energy

for anaerobic CO_2 fixation appears to be supplied by the initial steps in glycolysis. In the present case, anaerobic fixation is presumably not possible even with glucose present because the organism is unable to ferment appreciable amounts of carbohydrate. It is possible, on the other hand, that regeneration of cofactors is limiting under anaerobic conditions.

Unequivocal evidence for the fixation reaction is provided by the experiments with carbon-14. The only known mechanism for the incorporation of CO_2 into succinate is the reductive pathway (Wood, 1946), involving probably in bacterial systems the sequence pyruvate—oxalacetate—malate—fumarate—succinate (Utter, 1951). Since, however, the low specific activity of the isolated succinate left open the possibility of simple equilibration without net fixation (Foster *et al.*, 1949), proof for the essential role of fixation rests on the more indirect evidence presented that with both resting and growing cells CO_2 (or carbonate) increases the yield of succinate. In the absence of exogenous CO_2 pyruvate accumulates in isolatable amounts; the small amount of succinate formed presumably involves utilization of metabolic CO_2.

The effect of inhibitors is consistent similarly with a reductive pathway over pyruvate. Malonate partially blocks succinate synthesis in *S. coelicolor;* both in a tricarboxylic acid cycle (Wood, 1946) and a cycle based on acetate condensation (Barron *et al.*, 1950) the effect, if any, of malonate is to increase succinate accumulation. Inhibition by arsenite with concomitant accumulation of pyruvate accords with the hypothesis although the observation is by itself not critical.

While it is conceivable that a block in an oxidative tricarboxylic acid cycle could account for the accumulation of dicarboxylic acids, three observations militate against this interpretation in the present case: (1) the direction of the malonate effect; (2) the low yields aerobically from citrate and α-ketoglutarate; and (3) failure of resting cells to form succinate from citrate anaerobically, in contrast to the rapid anaerobic conversion of malate and fumarate into succinate.

Attempts to detect the operation of a C_2—C_2 condensation mechanism for the origin of succinate have yielded consistently negative results, and there is no reason to postulate any such reaction for this organism. Carbon-14 from acetate-2-C^{14} was found to be trapped in succinate, but it should be emphasized that isotope data alone cannot prove unequivocally the existence of a C_2—C_2 condensation. Fixation of carbon-1 of acetate in the central carbons, or of carbon-2 of acetate in the carboxyl carbons, of a dicarboxylic acid may be achieved in principle by the operation of a tricarboxylic acid cycle as well as by direct condensation. The convincing evidence so far at hand for the direct condensation of acetate is the production of fumarate from acetate by *Rhizopus oryzae* in yields higher than can be accounted for by the tricarboxylic acid cycle (Foster *et al.*, 1949). In *S. coelicolor*, yields of succinate from acetate were so low as to make unnecessary the postulate of direct condensation.

While acetate does not appear to contribute in large measure to succinate accumulation, the experiments with acetate-2-C^{14} are consistent with a pathway

of oxidation of acetate which proceeds over succinate; the only well established pathway of this type is a tricarboxylic acid cycle.

It is concluded that succinate accumulates in the course of normal carbohydrate metabolism and that the mode of origin is by carboxylation of pyruvate or a derivative of it and reduction of the 4-carbon product. Since succinate itself is metabolized, albeit at a slower rate than glucose, it is possible that a considerable fraction of the pyruvate metabolism passes over this pathway, although at present other metabolic reactions must be invoked as a source of energy for the primary fixation. While neither acetate condensation nor a blocked tricarboxylic acid cycle seems to be responsible for the accumulation of succinate, it should be emphasized that the data in no way exclude the operation of one or both of these systems in oxidations which are of minor importance in the accumulation of succinate.

The suggested phylogenetic relationship between the actinomycetes and the propionic acid bacteria (Stanier and van Niel, 1941) was strengthened by the findings of Hungate (1946) that an anaerobic *Micromonospora* produces propionic acid. The origin of succinate in *S. coelicolor* forms the same kind of biochemical link between the two groups, *Propionibacterium* being the "classical" organism for the fixation of CO_2 in succinate by the reductive pathway (Wood and Werkman, 1938; Krebs and Eggleston, 1941). So far, it has not been possible definitely to implicate propionic acid in the metabolism of aerobic actinomycetes. The discovery by Delwiche (1948) of a succinic acid decarboxylase sufficiently active to account for the production of propionate from pyruvate by *Propionibacterium pentosaceum* may offer clues as to the physiological significance to the organism of the reductive formation of succinate.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the hospitality of the Brookhaven National Laboratory, and in particular of Dr. Martin Gibbs, in whose laboratory the carbon-14 experiments reported herein were performed.

SUMMARY

Succinic acid is formed by *Streptomyces coelicolor* from utilizable sugars and polyhydric alcohols and appears to be a constant and normal product of aerobic carbohydrate metabolism.

Evidence is presented that the pathway of succinate formation proceeds from hexose *via* pyruvate, followed by fixation of carbon dioxide and reduction of the product. While carbon-14 from acetate-2-C^{14} is trapped in succinate, the incorporation is so low as to make it impossible that acetate condensation is responsible for the accumulation of succinate.

The conversion of pyruvate into succinate proceeds only aerobically and only in the presence of an oxidizable substrate other than pyruvate.

The dilution of the specific activity of acetate-2-C^{14} during oxidation with glucose is so large as to suggest that glucose is metabolized, at least in large part, *via* acetate.

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