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Amber Mutants of the α-Ketoglutarate Dehydrogenase Gene of *Escherichia coli* K12

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SUMMARY

Of 21 *suc* mutants of *Escherichia coli* K12 five were shown to be *amber* mutants by their sensitivity to *amber* suppressors. In addition to lacking activity for the overall α-ketoglutarate dehydrogenase complex and the α-ketoglutarate dehydrogenase component (E1), the *amber* mutants had little or no dihydrolipoyl transsuccinylase (E2) and 20 to 30% less dihydrolipoyl dehydrogenase (E3) than the parental strain. In common with non-suppressible *suc* mutants they also had reduced amounts of succinylCoA synthetase and succinate dehydrogenase. Fine structure mapping by P1-transduction showed that some of the *suc-amber* sites were located in the *sucA* gene. It is concluded that expression of the *suc* region is polarized from *sucA* to *sucB*, i.e. synthesis of the E1 component precedes that of the E2 component. Double *amber* mutants with lesions in the pyruvate and α-ketoglutarate dehydrogenase genes (*aceE,sucA*) were constructed but their E3 activity was never less than 30% of that of the parental strain. The results are consistent with the existence of separate genes for the dihydrolipoyl dehydrogenase component of the pyruvate and α-ketoglutarate dehydrogenase complexes, but other possibilities could not be ruled out. Further studies on the positions of the four closely-linked tricarboxylic acid cycle genes specifying citrate synthase (*gltA*), succinate dehydrogenase (*sdh*) and two components of the α-ketoglutarate dehydrogenase complex (*sucA* and *sucB*) indicate the relative order: *gltA . . sdh . . sucA . sucB . . tolII . . . gal.*

INTRODUCTION

The α-ketoglutarate dehydrogenase complex (α-kgdhc) is a multi-enzyme complex which catalyses the oxidative decarboxylation of α-ketoglutarate via a series of enzyme-bound intermediates. It contains three enzyme components, α-ketoglutarate dehydrogenase (E1), dihydrolipoyl trans-succinylase (E2) and dihydrolipoyl dehydrogenase (E3) and their activities may be summarized as follows:

$$\alpha\text{-Ketoglutarate} + \text{Lip}S_2\text{-E2} \xrightarrow{\text{TPP-E1}} \text{Succinyl-S-LipSH-E2} + CO_2$$

$$\text{Succinyl-S-LipSH-E2} + \text{CoA} \longrightarrow \text{SuccinylCoA} + \text{Lip}(SH)_2\text{-E2}$$

$$\text{Lip}(SH)_2\text{-E2} + NAD^+ \xrightarrow{\text{FAD-E3}} \text{Lip}S_2\text{-E2} + NADH + H^+$$

$$\alpha\text{-Ketoglutarate} + \text{CoA} + NAD^+ \xrightarrow{\alpha\text{-kgdhc}} \text{SuccinylCoA} + CO_2 + NADH + H^+.$$

In *Escherichia coli* two types of mutant lacking α-ketoglutarate dehydrogenase complex activity have been found amongst mutants which require succinate or lysine plus methionine for aerobic growth on glucose (Herbert & Guest, 1969). Those occurring most frequently lacked E1 activity, *sucA* mutants, but a few *sucB* mutants, which lacked only E2 activity

were isolated. The corresponding genes are closely linked and they map in a region near the *gal* operon which also contains genes for at least two other tricarboxylic acid cycle enzymes, citrate synthase, (*gltA*; Ashworth, Kornberg & Nothmann, 1965) and succinate dehydrogenase (*sdh*; U. Henning, personal communication).

The analogous pyruvate dehydrogenase complex (pdhc) catalyses the oxidative decarboxylation of pyruvate and whereas the E1 and E2 components from each complex differ in composition and specificity, the E3 components are similar and functionally interchangeable (Reed & Oliver, 1969; Ginsburg & Stadtman, 1970). Mutants lacking pdhc require acetate for aerobic growth on glucose and extensive studies by Henning and co-workers have established the existence of two closely-linked structural genes, *aceE* for the E1 component and *aceF* for the E2 component, plus a regulatory element in the *leu* region (Henning, Dennert, Hertel & Shipp, 1966; Henning, Dietrich, Murray & Deppe, 1968; Flatgaard, Hoehn & Henning, 1971). Nearly a third of the *ace* mutants were pleiotropically negative (o^-), they produced no detectable E1 or E2 and much reduced amounts of the E3 component and since about half were *aceE*-nonsense mutants it was concluded that translation of the *ace* region proceeds from E1 to E2. No mutants lacking E3 activity have been found amongst *suc* or *ace* mutants and this has raised the question of whether one gene provides the E3 component for both complexes or whether there is an E3 gene associated with each of the *ace* and *suc* regions. If there is only one gene then a single mutation may affect the activity of both complexes and lead to a requirement for both acetate and succinate. With two genes a mutation in one would probably go undetected because of complementation by the other E3 gene product. Polarized synthesis of the flavoprotein in *ace* mutants is compatible with the existence of an E3 gene linked to the distal end of the *ace* region but the possibility of one or two unlinked but co-ordinately regulated genes cannot be ruled out.

The aim of the present work was to isolate and study the properties of *suc*-nonsense mutants in order to determine the polarity of the *suc* region and to obtain material useful for investigating the problem of the number of dihydrolipoyl dehydrogenase genes. The position of the *sucA* and *sucB* genes relative to other linked genes was also investigated further.

METHODS

Organisms. Escherichia coli K12 strains W3110(W), W3110 *gal,trpA*(WGA) and several of their *suc* mutant derivatives have been described previously (Herbert & Guest, 1969). Strains carrying the following suppressor mutations in W3110 R^-, $trpA\text{-}E_{del}$ were obtained from Dr C. Yanofsky: $sup1^+$ (a *his*-linked *amber*-suppressor which inserts serine); $sup3^+$ (a *trp*-linked *amber*-suppressor which inserts tyrosine) and $sup4^+$ and $supC^+$ (*trp*-linked *ochre*-suppressors which insert tyrosine). The *trpA* mutation of WGA and its derivatives is $trpA9761_{amber}$ which can be suppressed by all of these suppressors (Yanofsky & Ito, 1966). Strains carrying suppressor mutations were constructed by transduction with PI*kc*. The presence of *amber* or *ochre* suppressors was detected by a cross-streak test using lysates of two T4 mutants, N58 (*amber*) and 427 (*ochre*). Two pleiotropically negative *aceE* mutants, K1-1*aceE*64 and 131 (*HfrH, pps, thy*, ace_{amber}, *PI*) which lack E1 and E2 components of the pyruvate dehydrogenase complex and are suppressed by $sup1^+$ and $sup3^+$ were kindly provided by Dr U. Henning (Henning *et al.* 1966; Dietrich & Henning, 1970). Leucine-requiring derivatives of WGA and the WGA*suc-amber* mutants were conjugated with the *ace* mutants by cross-streaking on a selective medium of glucose supplemented with acetate, succinate and tryptophan but no leucine or thymine. The desired strains (*ace,gal,trp,*suc^+ or *suc*) were obtained by testing colonies of leu^+ recombinants which grew in the tail of the donor streak.

Strains lacking citrate synthase, W620*glt* (*gltA, gal, ura*) and W1485*glt* (*gltA*) have been described previously (Herbert & Guest, 1968) and succinate dehydrogenase mutants were kindly provided by Dr U. Henning, W945*sdh* (*sdh, gal, thr, thi*) and Dr C. A. Hirsch, P678*sdh* (*sdh, gal, thr, leu, thi*; Hirsch, Rasminsky, Davis & Lin, 1963). PI-sensitive *gal*$^+$ derivatives were prepared by PI-transduction with W3110 as donor at a multiplicity of infection of 0·05.

Media. Minimal medium E of Vogel & Bonner (1956) was used in all experiments except in the mutant isolations where the citrate-free medium described by Herbert & Guest (1970) was used. The substrates were glucose (0·2 or 0·4%), acetate or succinate (50 mM) and galactose (0·5%, plus bromothymol blue at 0·002%). Other appropriate supplements were added to satisfy the growth requirements of mutant organisms and media were solidified with Difco Agar (1·5%, w/v). L-Broth and L-agar were used for growth and maintenance of organisms (Lennox, 1955).

Enzyme assays. Ultrasonic extracts were prepared from organisms grown in glucose minimal medium and harvested in late log phase (Herbert & Guest, 1968). Samples of each culture were tested to ensure that reversion had not occurred. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951) and specific activities expressed as μmol substrate transformed/mg protein/h at 25 °C unless stated otherwise.

α-Ketoglutarate dehydrogenase complex (*α-kgdhc*) *and pyruvate dehydrogenase complex* (*pdhc*) were measured at pH 8·5 by the method of Amarasingham & Davis (1965) with 3-acetylNAD as the electron acceptor.

α-Ketoglutarate dehydrogenase (*E*1) was assayed spectrophotometrically at 420 nm and pH 6·0 with ferricyanide as the electron acceptor according to Hager & Korberg (1961).

Dihydrolipoyl trans-succinylase (*E*2) was assayed at pH 7·3 and 30 °C by determining the extra thioester formed on adding dihydrolipoate to a succinylCoA generating system according to Knight & Gunsalus (1962). Dihydrolipoate (10 μmol), protein at 10 mg/ml of reaction mixture and no extra succinylCoA synthetase were used.

Dihydrolipoyl dehydrogenase (*E*3) was assayed spectrophotometrically at 366 nm by recording the dihydrolipoate-dependent reduction of 3-acetylNAD. The reaction mixture contained (μmol/ml final volume): potassium phosphate pH 7·8, 100; 3-acetylNAD, 0·8; potassium dihydrolipoate, 5; and extract containing 0·5 mg protein. Some determinations were made at pH 7·0 but it was later found that a threefold increase in rate could be obtained at the optimum pH, 7·8.

SuccinylCoA synthetase (*scs*) was assayed by two methods. The specific activities quoted were determined spectrophotometrically at 230 nm by the method of Bridger, Ramaley & Boyer (1969). The method of Kaufman (1955) was also used.

Succinate dehydrogenase (*sdh*) was assayed spectrophotometrically with 2,6-dichlorophenol indophenol as the final electron acceptor (Herbert & Guest, 1970). The oxidation of 1 mol of succinate corresponded to the reduction of 1 mol of dye.

Genetic techniques. Transduction with phage P1*kc* was according to Guest (1969); mixtures containing 10^9 recipient organisms, 5 μmol $CaCl_2$ and 2×10^9 P1*kc*/ml of L-broth were incubated 20 min and, after washing, appropriate dilutions were plated on selective media. All selective media except glucose-based media were enriched with Bacto Nutrient Broth (0·2%, v/v). Recombinant colonies were counted after 3 to 5 days at 37 °C depending on the selective medium, and purified on the same medium before scoring the inheritance of non-selective markers.

RESULTS

Isolation of α-ketoglutarate dehydrogenase amber mutants. Twenty-one independently isolated *suc* mutants were obtained from cultures of *Escherichia coli* strain WGA after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0·1 mg/ml) or u.v. radiation according to the methods described by Roth (1970). Screening was initially for mutants requiring succinate for aerobic growth on glucose minimal medium. The *suc* mutants were then distinguished from other succinate-requiring strains by their ability to respond to lysine plus methionine (but not lipoate), their ability to grow anaerobically without supplements and their inability to use acetate as carbon and energy source. These mutants, designated WGA*suc35* to *55* all lacked α-ketoglutarate dehydrogenase complex activity and activity for the dehydrogenase component (E1). Transduction with W3110 (*suc*$^+$,*gal*$^+$) as donor indicated that all the *suc* mutations were co-transducible with *gal*; between 34 and 61% of *suc*$^+$ transductants were *gal*$^+$ (average frequency of cotransduction, 46%).

Nonsense mutants were then sought by testing for joint suppression of the *trp-amber* and *suc* mutations of all the WGA*suc* strains by transduction with PI-lysates of strains containing the *amber* or *ochre* suppressors, *sup1*$^+$, *3*$^+$, *C*$^+$ and *4*$^+$. Since the suppressor strains are deleted for the *trp* operon, Trp$^+$ transductants could only arise by suppression and because none of these suppressors is linked to *suc*, simultaneous expression of the Suc$^+$ phenotype could only occur in strains with suppressible *suc* mutations. As expected, Trp$^+$ (*trp*$^-$,*sup*$^+$) transductants were obtained in all the crosses but comparable numbers of Trp$^+$,Suc$^+$ transductants were only recovered with five recipient strains (Table 1). Some five to ten Trp$^+$ transductants from each cross were purified, tested for the presence of the suppressors by streaking over *amber* and *ochre* mutants of phage T4 and further checked for their ability to grow in the absence of succinate and for growth on enriched acetate medium. Only the same five *suc* mutations were readily suppressed and the effects of the different suppressors can be seen in terms of ability of the WGA*suc*,*sup*$^+$ derivatives to grow on acetate (Table 1). Similar results were obtained with glucose medium but the existence of weak suppression was often obscured by reversion and subsequent cross-feeding. This problem of differentiating between weak suppression and the effects of reversion also meant that some weakly suppressible mutants may have been overlooked. The five suppressible mutants gave different responses to the same group of suppressors, but they were all suppressed by at least one *amber* suppressor indicating that their *suc* mutations are of the *amber* type. Only *suc36* responded well to both serine- and tyrosine-inserting suppressors. Two mutants, *suc35* and *suc53*, showed a high degree of specificity for suppression by serine-insertion or tyrosine-insertion respectively and *suc44* and *47* also showed marked preference for one suppressor. No significant differences between the effects of *supC* and *sup4* were observed throughout this work and only one set of results is recorded. To investigate the effects of the suppressors on the *suc* mutations alone, the WGA*suc*,*sup*$^+$ strains were inoculated into tryptophan-supplemented medium. The resulting growth curves (Fig. 1) show essentially similar patterns of suppression to those found for joint suppression of *trp* and *suc* lesions on unsupplemented solid media and this confirms that the suppressor-specificities are determined by the *suc* mutation. One exception is WGA*suc44sup3*$^+$ which grew better than expected. Good suppression led to growth equivalent to that of W3110. Growth of the unsuppressible mutant WGA*suc23* was not stimulated by introducing the suppressors.

No *suc-ochre* mutants were detected but this is probably not surprising in view of the relatively low efficiency of *ochre* suppressors, the use of a selection which requires joint suppression of two markers (*trp* and *suc*), the high degree of suppressor-specificity exhibited

Table 1. *Transfer of amber and ochre suppressors to WGAsuc mutants by PI transduction*

Suc trp_{amber} recipients were transduced with PI lysates of trp_{del} sup^+ donors. Transductants with a Trp^- phenotype were selected on succinate-supplemented glucose medium (G + suc, scored after 2 days). Joint suppression of the *suc* and *trp* mutations was scored after 3 days on unsupplemented glucose medium (G) and after 5 days on enriched acetate medium (enA). Controls to test for reversion of the recipient markers were also included. Trp^+ transductants (trp^-, suc^-, sup^+) selected on G + suc were purified on the same medium and tested for simultaneous suppression of *suc* by streaking suspensions on enA.

		Selected transductants per 10^7 PI			
Recipient	Donor	Trp^+ (G + suc)	Trp^+Suc^+ (G)	Trp^+Suc^+ (enA)	Growth of $trp,^-suc^-,sup^-$ recombinants on enA
WGA*suc35*	$sup1^+$	160	125	54	++
	$sup3^-$	143	12	0	−
	$sup4^-$	49	6	0	−
WGA*suc36*	$sup1^-$	194	178	115	++
	$sup3^-$	160	191	101	++
	$sup4^+$	62	54	17	+
WGA*suc44*	$sup1^-$	67	48	38	+
	$sup3^+$	138	0	2	±
	$sup4^+$	8	0	0	−
WGA*suc47*	$sup1^+$	23	21	3	+
	$sup3^+$	73	58	21	++
	$sup4^-$	13	13	0	−
WGA*suc53*	$sup1^+$	83	59	0	±
	$sup3^+$	68	68	20	++
	$sup4^+$	23	22	6	−

++, denotes good growth, visible after 2 days; +, poorer growth visible after 3 days; ±, faint growth after 4 days; −, no growth after 5 days.

by the *suc* nonsense mutants and the difficulties associated with defining weak *suc*-suppression. For example, even with WGA*suc47* and *53*, which responded to the insertion of tyrosine by the *amber* suppressor, suppression was not detectable with $supC^+$ and $sup4^+$.

Genetic mapping of the suc-amber sites

In order to define the polarity of the α-ketoglutarate dehydrogenase system it was necessary to assign the *suc-amber* sites to one of the *suc* genes. The methods used for fine structure mapping of *suc* mutants have been described in detail previously (Herbert & Guest, 1969).

The donor strains used were suc^-gal^+, the recipients were suc^-gal^- and transduction mixtures were plated on enriched acetate plus tryptophan and enriched galactose plus succinate media to select suc^+ and gal^+ transductants respectively. The distances between *suc* mutant sites were determined by expressing the number of suc^+ transductants as a percentage of the number of gal^+ transductants, a procedure which gives distances comparable to those based on his^+ selection. The order of the *suc* sites relative to *gal* was also determined by scoring 50 to 100 suc^+ transductants for inheritance of the non-selective gal^+ marker of the donor. Normal distributions (30 to 65% gal^+) indicate that the recipient *suc* site is closer to *gal* whereas low values (less than 10% gal^+), indicate that the *suc* site of the donor is closer. In a few crosses intermediate distributions have been found and for the purpose of map construction values $\geqslant 22\%$ or $\leqslant 18\%$ have been regarded as normal or low respectively but the

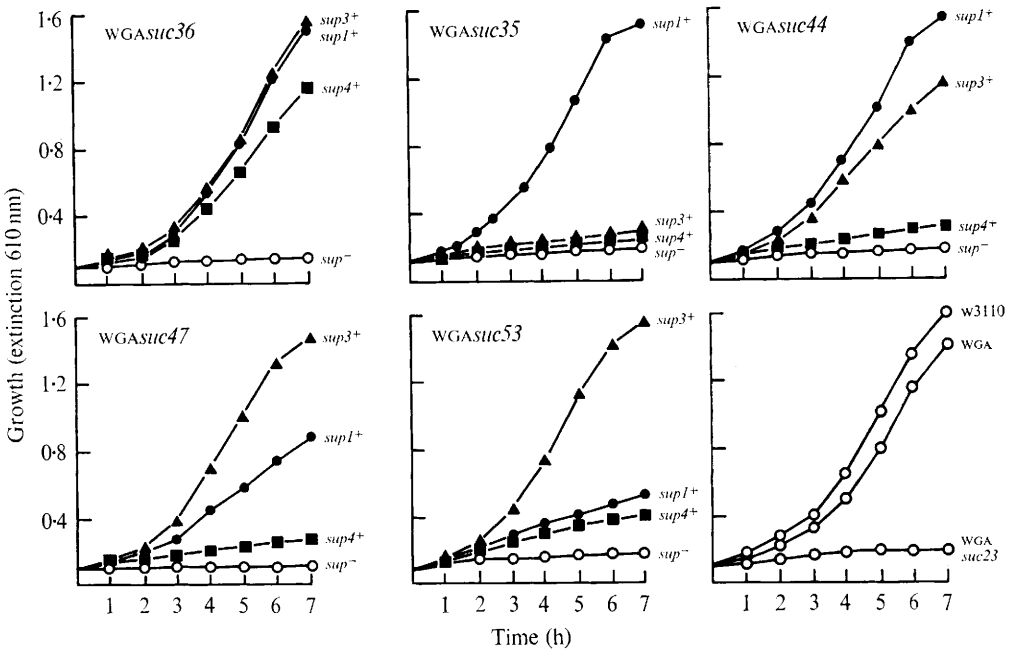

Fig. 1. The effects of *amber* and *ochre* suppressor mutations on the growth of WGA*suc-amber* mutants in tryptophan-supplemented glucose medium. Cultures were shaken at 37 °C in 250 ml Erlenmeyer flasks fitted with side arms. Each flask contained 10 ml of medium E plus glucose (0·4 %, w/v) and L-tryptophan (30 μg/ml) inoculated to give an initial extinction at 610 nm of 0·1 (equivalent to 10^8 bacteria/ml) with a washed suspension (0·2 ml) of organisms grown for 16 h in the same medium plus succinate (4 mM). Growth was recorded for the unsuppressed mutants (○) and for derivatives carrying the suppressor mutations, *sup1*$^+$ (●), *sup3*$^+$ (▲) and *supC*$^+$ and *4*$^+$ (■).

corresponding orders should not be considered reliable. The results obtained with a set of mutants which were not suppressed by *sup1*$^+$ or *sup3*$^+$ are summarized in Fig. 2 (upper part); these included 7 *sucA* mutants (*23*, *26*, *37*, *38*, *39*, *40* and *55*) and 2 *sucB* mutants (*11* and *17*). The relative positions of four of the *amber* mutants were then determined by crossing them with this set of mutants (Fig. 2, lower part). The results showed conclusively that all four *amber* sites are to the left of the two *sucB* sites and that the *suc35* and *36* sites fall within the *sucA* gene. The exact position of *suc44* and *53* are less certain because intermediate *gal*$^+$ distributions were observed in the two important crosses. Satisfactory results could not be obtained with *suc47* as it appeared to be unstable and it also gave poor lysates with PI*kc*. High frequencies of recombination have often been observed with *amber* mutants as parents (Drapeau, Brammar & Yanofsky, 1968; Kemper, 1970) and particularly between pairs of *amber* mutants (Henning *et al.* 1968). Several of the distances reported here seem to be high and it would not have been possible to locate the *amber* sites from the recombination frequencies alone.

Relative order of four tricarboxylic acid cycle genes

The structural genes for citrate synthase (*gltA*) and succinate dehydrogenase (*sdh*) and the *suc* genes are all located in a small segment of the *Escherichia coli* chromosome close to the *gal* operon. The gene order *gltA*..*sucA*.*sucB*...*gal* has been established (Herbert & Guest, 1968; 1969) but the position of the *sdh* gene is less certain. A mutant (*S*$^-$), tentatively identified as having an *sdh* mutation because it caused an 80 % loss of succinate dehydrogenase

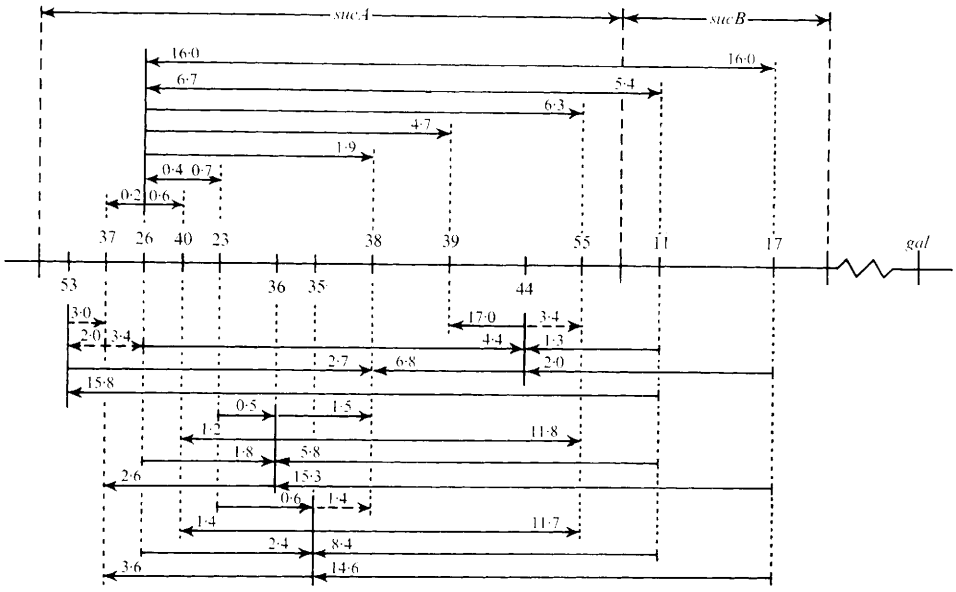

Fig. 2. Fine structure map of the *suc* region based on recombination frequencies (*suc*$^+$ relative to *gal*$^+$ transduction) and distribution of the non-selective *gal*$^+$ marker in *suc*$^+$ transductants. The direction of the transductions is indicated by the arrow which points to the recipient and the corresponding recombination frequency is placed at the arrow head. A solid arrow indicates that the order of *suc* mutant sites relative to *gal* is based on a conclusive *gal*$^+$ distribution, a broken arrow indicates that an intermediate distribution was obtained and the order is unreliable (see Text).

activity has been located to the left of *sucA* and possibly between *gltA* and *sucA* (Herbert & Guest, 1970). More precise mapping was prevented by poor selectivity of the media used with the partially-blocked *S*$^-$ strain. By contrast, strains P678*sdh* and W945*sdh* were almost completely deficient in succinate dehydrogenase activity and enriched acetate medium proved satisfactory for selecting *sdh*$^+$ recombinants, so the position of the *sdh* gene with respect to *gltA*,*sucA*,*sucB* was investigated. All four TCA cycle genes were 35 to 55% cotransducible with *gal* and any two of the TCA cycle markers were over 90% linked by transduction. The results of three-factor recipient crosses are shown in Table 2. Clearly, *sucA* and *sucB* lie between *sdh* and *gal* (crosses I and II) and between *gltA* and *gal* (crosses III and IV) and the *sdh* gene is closer to *gal* than *gltA* (cross V). The results are summarized in a genetic map showing the relative orders of, and distances between, these TCA cycle genes (Fig. 3). Most of the crosses involving the *sdh* marker were repeated with P678*sdh* and its *gal*$^+$ derivative and essentially similar results were obtained.

The positions of the TCA cycle genes were also investigated with several spontaneous *chlD* and *tol* deletion strains lacking segments of the *tolII* to *attλ* region. Although some of these were unable to use acetate or succinate as substrates they all possessed normal amounts of succinate dehydrogenase, succinylCoA synthase, α-ketoglutarate dehydrogenase complex and its E1 and E3 components. This indicates that these deletions do not extend into the TCA cycle genes which must therefore be to the left of *tolII*.

Table 2. *Three-factor reciprocal crosses between tricarboxylic acid cycle mutants*

The map distances represent the number of transductants expressed as a percentage of the total number of *gal*$^+$ transductants selected from a corresponding sample of the same transduction mixture and they are comparable to distances based on the frequency of *his*$^+$ transduction.

				Transductants		Distribution of donor *gal*$^+$		
Cross	Donor (PI)	Recipient	Selection on enA medium*	No./10^8 P1	Map distance	No. scored	*gal*$^+$ (%)	Order indicated
Ia	W*sucA26*	W945*sdh*	*sdh*$^+$, *sucA*$^+$	860	9·2	100	6·0	*sdh.sucA...gal*
Ib	W945*sdhgal*$^+$	WG*sucA26*	*sdh*$^+$, *sucA*$^+$	124	6·4	110	65	
IIa	W*sucB11*	W945*sdh*	*sdh*$^+$, *sucB*$^+$	850	18·4	124	4·8	*sdh.sucB....gal*
IIb	W945*sdhgal*$^+$	WG*sucB11*	*sdh*$^+$, *sucB*$^+$	35	10·1	58	45	
IIIa	W*sucA26*	W620*glt*	*gltA*$^+$, *sucA*$^+$	214	9·7	88	6·8	*gltA.sucA...gal*
IIIb	W1485*glt*	WG*sucA26*	*gltA*$^+$, *sucA*$^+$	88	8·7	56	59	
IVa	W*sucB11*	W620*glt*	*gltA*$^+$, *sucB*$^+$	17	12·4	44	4·6	*gltA.sucB...gal*
IVb	W1485*glt*	WG*sucB11*	*gltA*$^+$, *sucB*$^+$	29	15·1	94	48	
Va	W945*sdh*$^-$*gal*$^+$	W620*glt*	*gltA*$^+$, *sdh*$^+$	39	5·0	173	8·7	*gltA.sdh....gal*
Vb	W1485*glt*	W945*sdh*	*gltA*$^+$, *sdh*$^+$	58	2·7	122	44	

* Enriched acetate medium.

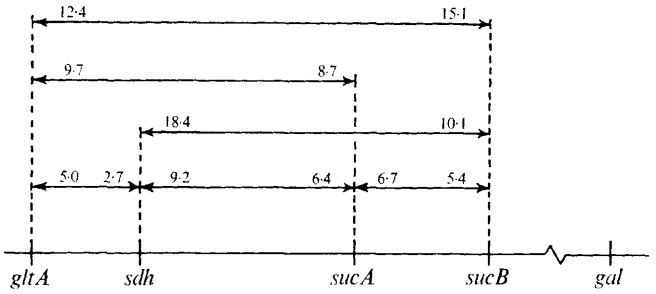

Fig. 3. Genetic map showing position of TCA cycle loci relative to *gal*. The map distances are from the crosses outlined in Table 2 except for the *sucA* to *sucB* values which are from Herbert & Guest (1969).

Biochemical studies with suc-amber mutants

The *amber* mutants were assayed for α-ketoglutarate dehydrogenase complex and its component activities in order to detect pleiotropic effects associated with the nonsense mutations (Table 3). In addition to lacking the overall complex and E1 activities, no E2 activity could be detected in four of the mutants (*suc35*, *36*, *47* and *55*) and a much reduced level was found in *suc44*. The assay for E2 depends on the presence of succinylCoA synthetase (scs) and although the *amber* mutants contained this enzyme, the specific activities were lower than that of the parental strain (Table 3). However, the activities were not much lower than are sometimes found in some non-suppressible *sucA* mutants where the E2 activity is apparently unaffected (Table 3). Furthermore, earlier assays for scs using the method of Kaufman (1955) which is similar to the E2 assay gave activities two to three times greater than those quoted in Table 3 for the *suc* mutants and since the lowest rates of succinylCoA synthesis are several times greater than the highest rates of succinyl lipoate synthesis, it seems unlikely that lack of scs could account for the failure to detect E2 activity in the *amber* mutants. All the *sucA* mutants contained E3 but whereas the specific activities of the *amber* mutants were reproducibly 20 to 30% lower than the parental activity, those of selected non-suppressible mutants were more comparable to the parental level (Table 3). Since the amount of the pdhc was the same for most of the mutants it appears that the pleiotropy observed in the E2 of *amber* mutants also extends to E3. The amount of succinate dehydrogenase (sdh) was also lower in the *suc* mutants (Table 3).

The effects of *amber* and *ochre* suppressors on enzyme synthesis in the *amber* mutants were also examined (Table 3). Up to 75% of the parental α-ketoglutarate dehydrogenase component (E1) and complex activities could be recovered with appropriate suppressors and the suppressor specificities of the *amber* sites, seen in terms of the restoration of enzyme activity, closely paralleled the pattern of growth responses observed in minimal media. Only WGA*suc44 sup3* which grew without succinate in glucose medium failed to show detectable E1 or complex activity. This may indicate that tyrosine-insertion at the *suc44* site produces a labile E1 protein which can supply sufficient succinate to maintain a suboptimal rate of growth but is inactivated before it can be assayed. The suppressors also corrected the polar effects on E2 and E3 synthesis (Table 3). This suppression appeared to be related directly to the restoration of an active E1 rather than being independent of its catalytic properties. However, there is only one clear example, WGA*suc35sup3*$^+$, where the polarity might have been corrected regardless of the failure to produce an active E1; this anomaly could be due to the inability to detect small amounts of E2 activity arising from a partial correction of the polar effects. The suppressor mutations also increased the amounts of scs and sdh in proportion to their effects on the synthesis of active E1.

Table 3. *Specific activities of the α-ketoglutarate dehydrogenase complex and related enzymes in suc-amber mutants in the absence and presence of amber suppressors*

Organisms were grown in medium E with glucose (0·2 %, w/v), L-tryptophan (30 μg/ml) and succinate (4 mM). The enzymes were assayed in ultrasonic extracts as described in the Methods section and the average activities for determination with several different extracts are quoted. Component E3 was assayed at pH 7·0. Mutants WGA*suc26*, *37*, *40* and *55* were not suppressible by *sup1*$^+$ *or sup3*$^+$ (α-kgdhc = activity of the overall complex; scs = succinylCoA synthetase; sdh = succinate dehydrogenase; and − = specific activity less than 0·01).

		Enzyme specific activities					
Strain	Suppressor	α-kgdhc	E1	E2	E3	scs	sdh
W3110	None	0·48	2·80	0·14	0·76	11·3	11·0
WGA	None	0·43	2·60	0·14	0·78	7·4	7·0
WGA*suc35*	None	—	—	—	0·56	1·4	3·2
	sup1	0·22	0·94	0·06	0·71	6·6	5·0
	sup3	—	—	—	0·59	3·8	4·2
	sup4	—	—	—	0·52	—	—
WGA*suc36*	None	—	—	—	0·62	3·1	3·8
	sup1	0·17	1·20	0·06	0·76	7·5	5·0
	sup3	0·21	1·40	0·05	0·83	5·8	4·7
	sup4	0·11	0·22	0·03	0·71	4·4	4·2
WGA*suc44*	None	—	—	0·04	0·66	2·1	5·0
	sup1	0·09	0·90	0·10	0·69	4·0	—
	sup3	—	—	0·05	0·73	—	—
	sup4	—	—	0·04	0·71	2·0	—
WGA*suc47*	None	—	—	—	0·53	1·2	2·4
	sup1	0·05	0·30	0·02	0·65	—	—
	sup3	0·21	1·46	0·04	0·66	2·4	—
	sup4	—	—	0·02	0·68	—	—
WGA*suc53*	None	—	—	—	0·59	2·4	3·0
	sup1	0·05	0·82	0·06	0·61	4·1	3·4
	sup3	0·27	2·00	0·09	0·68	6·3	4·1
	sup4	—	—	—	0·70	—	—
WGA*suc26*	None	—	—	0·14	0·72	1·1	2·8
WGA*suc38*	None	—	—	0·16	0·76	2·3	2·8
WGA*suc40*	None	—	—	0·17	0·76	8·2	2·7
WGA*suc55*	None	—	—	0·18	0·70	7.0	2·1

Two *aceE-amber* mutations of the *o*° type were introduced into WGA and its *suc-amber* derivatives to investigate the effect of *ace*, *suc* double-*amber* combinations on E3 activity (Table 4). The *aceE64* and *aceE131* mutations lowered the E3 activity of the parental strain by approximately 50 % and 35 % respectively (without altering overall α-kgdhc activity) and further reductions were found with the double mutants. However, at its lowest, 30 % of the parental E3 activity still remained and this could not be reduced further by growth in rich media. Some properties of the dihydrolipoyl dehydrogenase activities of WGA and the double-*amber* strain WGA*aceE64sucA35* were examined. The pH versus activity profiles were identical and the activities observed with reduced glutathione, 2,3-dimercaptopropanol and cysteine as substrates were less than 2 % of the values obtained with dihydrolipoate. Attempts were also made to distinguish between complexed and uncomplexed E3 activity by sedimentation. Approximately, 10 % of the activity in extracts of WGA remained in the supernatant fraction after centrifuging under conditions which would sediment particles with molecular weights greater than 1 million. Slightly more non-sedimenting activity was found in the double-*amber* mutants but they still contained the equivalent of 15 % of parental

Table 4. *The effects of combining sucA and aceE amber mutations on the specific activity of dihydrolipoyl dehydrogenase (E3)*

Organisms were grown in medium E with glucose (0·2%, w/v), L-tryptophan (30 μg/ml) and acetate and succinate (each 4 mM). Dihydrolipoate dehydrogenase activities were assayed at pH 7·8 and expressed as μmol/3-acetyl NAD reduced/mg protein/h

Strain	Specific activity of dihydrolipoyl dehydrogenase (E3) in the absence or presence of *aceE-amber* mutations		
	aceE$^+$	*aceE64*	*aceE131*
WGA	2·46	1·25	1·57
WGA*suc35*	1·86	0·83	0·92
WGA*suc36*	2·02	1·08	1·05
WGA*suc44*	2·12	1·12	1·21
WGA*suc47*	2·02	1·07	1·22
WGA*suc53*	1·97	0·90	1·02

activity as sedimentable material. After anaerobic growth on glucose medium plus tryptophan both strains had similar activities corresponding to 25% of the E3 activity in the aerobically grown parent.

DISCUSSION

Of the 21 *suc* mutants of *Escherichia coli* examined here five were clearly *amber* mutants. They lacked the E1 component and had little or no detectable activity for the E2 component. Since two of the *amber* sites were clearly shown to map within the *sucA* gene it could be concluded that expression of the *suc* region is polarized from *sucA* to *sucB* (i.e. E1 synthesis precedes E2 synthesis during translation). This polarity is analogous to that established for the *ace* region. The *suc44* mutation appeared to be the least polar because some E2 activity could still be detected and this is consistent with its being situated close to the *sucB* gene. Unfortunately, the poor sensitivity of the trans-succinylase assay made it difficult to detect less than 15% of wild-type activity reliably so it was not possible to assess the degree of polarity of the other *suc* mutations or to decide whether any of them are absolutely polar (i.e. pleiotropically negative). It is not known whether the E2 enzyme, as assayed, requires E1 protein for full activity. If it does then this could provide an alternative explanation for the observed polarity of the *suc-amber* mutants.

Polarity in the *suc* region appears to extend to synthesis of the E3 component but not to scs or sdh synthesis because the effect on E3 synthesis is most apparent in the *amber* mutants whereas scs and sdh synthesis is similarly affected in both suppressible and non-suppressible *sucA* mutants. The simplest interpretation is that an E3 gene specifying the E3 component of the α-kgdhc is linked to the *suc* region, and that another E3 gene is linked to the *ace* region as suggested by Henning *et al.* (1968). Nevertheless, other possibilities such as the existence of one or two unlinked E3 genes, which are expressed in the presence of E1–E2 partial complex or are repressed by an excess of uncomplexed E3, cannot be ruled out. The reduction in scs and sdh is probably a secondary consequence of the *suc* mutations. The position of the *sdh* gene indicates that it is not part of the *suc* regulatory unit. It also seems unlikely that the *scs* gene forms part of the *suc* region, because although it is repressed during anaerobic growth it is not as severely repressed as α-kgdhc synthesis. However, this still remains a formal possibility and selection for mutants which respond to succinate or lysine plus methionine and can grow anaerobically may preclude the selection of absolute polar mutants, which may lack scs in addition to α-kgdhc.

Ochre mutants were not detected by the genetic tests nor were any other pleiotropic mutants observed amongst the relatively small number of *suc* mutants whose enzymology has been examined in detail. No *sucB-amber* mutants were isolated but this is not unexpected in view of the low frequency with which *sucB* mutants have been recovered (three out of 55 *suc* mutants). One reason for their scarcity may be the ease with which succinyl thioesters hydrolyse spontaneously. Succinate so released could then be converted to succinylCoA without involving the catalytic activity of the trans-succinylase. This could mean that selection would have to be restricted to special types of *sucB* mutant.

The *suc-amber* sites resembled the *ace-amber* sites by exhibiting a variety of different responses to the same group of suppressors. Suppression of the Suc$^-$ phenotype was always associated with the synthesis of active E1, detectable E2 and increased amounts of E3, scs and sdh. Reasons for the failure to observe suppression of the polar effects independent of E1 activity may include the relatively small sample of nonsense mutants examined and the poor sensitivity of the trans-succinylase assay.

The α-kgdhc and pdhc of W3110 and WGA are generally present in the ratio of 1:3 (approx) after growth on glucose. This suggests that 25% of the E3 activity could be associated with α-kgdhc and the remainder with pdhc. The amount of pdhc in *suc-amber* mutants is relatively constant so the *amber* mutations appeared to reduce the total E3 activity by an amount corresponding to the proportion of α-kgdhc present. The *aceE-amber* mutants of the *o*° have shown reductions in E3 activity approaching 70% (Henning *et al.* 1966), i.e. amounts equivalent to the proportion of pdhc present, so it appeared that the E3 activity might be reduced to a very low level in double *amber* mutants. If so, individual polar mutants could have been used to investigate whether or not there are two E3 genes (one linked to each α-keto acid dehydrogenase region); e.g. E3 gene mutants may be found amongst *ace* mutants isolated from a polar *sucA* parental strain, and mutants with defects in the same or a different E3 gene may be found amongst *suc* mutants isolated in a polar *aceE* strain. The experiment designed to test the feasibility of this approach showed that combinations of *aceE-* and *sucA-amber* mutations in WGA did not abolish E3 synthesis. If there are two linked E3 genes, then it could be concluded that one or both types of *amber* mutation fail to exert a sufficiently high degree of polarity on the E3 genes to prevent its synthesis and this is supported by the sedimentation studies which suggest that some E2–E3 partial complex may be synthesized. If polarity is due to degradation of mRNA and can be relieved by amino acid starvation of stringent strains (Morse & Guertin, 1971) it may not be possible to obtain absolute polarity in mutants with defects in the central pathways of metabolism. Attempts to bypass this problem by using rich media were unsuccessful. The sedimentation studies also indicate the presence of some uncomplexed or readily dissociable E3 component which could support the existence of either one or two unlinked E3 genes with a regulatory mechanism which maintains an excess of uncomplexed E3 component. It is also possible that the dihydrolipoyl dehydrogenase activity is not entirely due to the E3 components of the α-keto acid dehydrogenase complexes but may include similar flavoproteins which can couple the oxidation of dithiols or thiols to the reduction of pyridine nucleotides. The results indicated that glutathione reductase was not making a contribution but some of the apparent E3 activity could still be due to thioredoxin reductase which can catalyse dihydrolipoyl-dependent NAD reduction (Moore, Reichard & Thelander, 1964) or the flavoprotein component of the glycine decarboxylation system (Blakley, 1969). If this is so then success in isolating E3 mutants would depend on whether these enzymes can replace E3 under physiological conditions.

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REFERENCES

AMARASINGHAM, C. R. & DAVIS, B. D. (1965). Regulation of α-ketoglutarate dehydrogenase formation in *Escherichia coli*. *Journal of Biological Chemistry* **240**, 3664–3668.

ASHWORTH, J. M., KORNBERG, H. L. & NOTHMANN, D. L. (1965). Location of the structural gene for citrate synthetase on the chromosome of *Escherichia coli* K12. *Journal of Molecular Biology* **11**, 654–657.

BLAKLEY, R. L. (1969). *The Biochemistry of Folic Acid and Related Pteridines*. Amsterdam and London: North Holland Publishing Co.

BRIDGER, W. A., RAMALAY, R. F. & BOYER, P. D. (1969). Succinylcoenzyme A synthetase from *Escherichia coli*. In *Methods in Enzymology*, vol. XIII, pp. 70–75. Edited by J. M. Lowenstein. New York and London: Academic Press.

DIETRICH, J. & HENNING, U. (1970). Regulation of pyruvate dehydrogenase complex synthesis in *Escherichia coli* K12: Identification of the inducing metabolite. *European Journal of Biochemistry* **14**, 258–269.

DRAPEAU, G. R., BRAMMAR, W. J. & YANOFSKY, C. (1968). Amino acid replacements of the glutamic acid residue at position 48 in the tryptophan synthetase A protein of *Escherichia coli*. *Journal of Molecular Biology* **35**, 357–367.

FLATGAARD, J. E., HOEHN, B. & HENNING, U. (1971). Mutants of *Escherichia coli* K12 which synthesize the pyruvate dehydrogenase complex constitutively. *Archives of Biochemistry and Biophysics* **143**, 461–470.

GINSBURG, A. & STADTMAN, E. R. (1970). Multienzyme systems. *Annual Review of Biochemistry* **39**, 429–472.

GUEST, J. R. (1969). Biochemical and genetic studies with nitrate reductase *C*-gene mutants of *Escherichia coli*. *Molecular and General Genetics* **105**, 285–297.

HAGER, L. P. & KORNBERG, H. L. (1961). On the mechanism of α-oxoglutarate oxidation in *Escherichia coli*. *Biochemical Journal* **78**, 194–198.

HENNING, U., DENNERT, G., HERTEL, R. & SHIPP, W. S. (1966). Translation of the structural genes of the *E. coli* pyruvate dehydrogenase complex. *Cold Spring Harbor Symposia for Quantitative Biology* **31**, 227–234.

HENNING, U., DIETRICH, J., MURRAY, K. N. & DEPPE, G. (1968). Regulation of pyruvate dehydrogenase synthesis. Substrate induction. In *Molecular Genetics*, pp. 223–236. Edited by H. G. Wittman & H. Schuster. Berlin: Springer-Verlag.

HERBERT, A. A. & GUEST, J. R. (1968). Biochemical and genetic studies with lysine + methionine mutants of *Escherichia coli*: Lipoic and α-ketoglutarate dehydrogenase-less mutants. *Journal of General Microbiology* **53**, 363–381.

HERBERT, A. A. & GUEST, J. R. (1969). Studies with α-ketoglutarate dehydrogenase mutants of *Escherichia coli*. *Molecular and General Genetics* **105**, 182–190.

HERBERT, A. A. & GUEST, J. R. (1970). Two mutations affecting utilization of C_4-dicarboxylic acids. *Journal of General Microbiology* **63**, 151–162.

HIRSCH, C. A., RASMINSKY, M., DAVIS, B. D. & LIN, E. C. C. (1963). A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. *Journal of Biological Chemistry* **238**, 3770–3774.

KAUFMAN, S. (1955). α-Ketoglutaric dehydrogenase system and phosphorylating enzyme from heart muscle. In *Methods in Enzymology*, vol. I, pp. 714–722. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic Press.

KEMPER, B. (1970). Increased recombination frequencies with an *amber* mutation in the *gal*-operon of *E. coli*. *Molecular and General Genetics* **107**, 107–113.

KNIGHT, E. & GUNSALUS, I. C. (1962). Formation and breakdown of acyl lipoic acids. In *Methods in Enzymology* vol. V, pp. 651–656. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic Press.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MOORE, E. C., REICHARD, P. & THELANDER, L. (1964). Enzymatic synthesis of deoxyribonucleotides. V. Purifications and properties of thioredoxin reductase from *Escherichia coli* B. *Journal of Biological Chemistry* **239**, 3445–3456.

MORSE, D. E. & GUERTIN, M. (1971). Regulation of mRNA utilization and degradation by amino acid starvation. *Nature New Biology* **232**, 165–169.

REED, L. J. & OLIVER, R. M. (1969). The multienzyme α-keto acid dehydrogenase complexes. *Brookhaven Symposia in Biology* **21**, 397–412.

ROTH, J. R. ('970). Genetic techniques in studies of bacterial metabolism. In *Methods in Enzymology*, vol. XVIIA, pp. 3–35. Edited by H. Tabor and C. W. Tabor. New York and London: Academic Press.

VOGEL, H. & BONNER, D. M. (1956). A convenient growth medium for *E. coli* and some other microorganisms. *Microbial Genetics Bulletin* **13**, 43–44.

YANOFSKY, C. & ITO, J. (1966). Nonsense codons and polarity in the tryptophan operon. *Journal of Molecular Biology* **21**, 313–334.