*Journal of General Microbiology* (1974), **82**, 319–335
*Printed in Great Britain*



# Biochemical and Genetic Characteristics of Deletion and other Mutant Strains of *Salmonella typhimurium* LT2 Lacking α-Keto Acid Dehydrogenase Complex Activities

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(*Received* 9 *January* 1974)

SUMMARY

Mutants of *Salmonella typhimurium* LT2 requiring acetate or succinate for aerobic growth on glucose were isolated. One acetate-requiring mutant, s8, lacked the activity of the overall pyruvate dehydrogenase complex due to a deficiency in the pyruvate dehydrogenase component (E1p) and was therefore designated an *aceE* mutant. Another mutant, s6, which required succinate (or lysine plus methionine), lacked activities of the overall α-ketoglutarate dehydrogenase complex and the α-ketoglutarate dehydrogenase component (E1kg) and was designated a *sucA* mutant. Genetic studies with these mutants established that like *Escherichia coli* K12, *Salmonella typhimurium* LT2 has an *aceE* gene linked to *aziA* and *aroP* thus: *leu-aziA-aroP-aceE*, and a *sucA* gene linked to *nadA* thus: *sucA-nadA-gal*.

Two deletion strains, SM16 and SM51, which required acetate, or better, acetate plus lysine plus methionine, were found to lack the overall activities of both α-keto acid dehydrogenase complexes. Biochemical and immunological studies showed this to be due to deficiences in pyruvate dehydrogenase and dihydrolipoyltransacetylase (the E1p and E2p components of the pyruvate complex) and failure to synthesize lipoamide dehydrogenase (the E3 components of both complexes). These deletion strains also behaved as if they lacked the general aromatic permease (*aroP*), and SM51, which requires nicotinate, was probably deleted for the *nadC* gene. Genetic studies confirmed that these strains were deleted in the *leu-aziA-nadC-aroP-aceE,aceF,lpd-pan* region. The results also indicated that the gene–protein-relationships of the α-keto acid dehydrogenase complexes are similar in *S. typhimurium* and *E. coli*.

## INTRODUCTION

The pyruvate and α-ketoglutarate dehydrogenase complexes of *Escherichia coli* K12 are multienzyme complexes containing dehydrogenase (E1), transacylase (E2) and lipoamide dehydrogenase (E3) components. They catalyse the oxidative decarboxylation of pyruvate or α-ketoglutarate and mutants lacking these activities require acetate or succinate respectively for aerobic growth on glucose. Genetic studies have established the existence of two pairs of closely-linked structural genes for the E1 and E2 components, *aceE* (E1p) and *aceF* (E2p) at 2 min and *sucA* (E1kg) and *sucB* (E2kg) at 16 min in the *E. coli* linkage map (Taylor & Trotter, 1972). Recently, mutants lacking lipoamide dehydrogenase (E3) activity have been isolated (Guest & Creaghan, 1972, 1973, 1974; Guest, 1974; Alwine, Russell & Murray, 1973). Studies with these have indicated that a single gene (*lpd*), adjacent to the distal gene (*aceF*) of the *ace* region, specifies the E3 components of both multienzyme complexes.

The gene–protein relationships for the α-keto acid dehydrogenase complexes of *Salmon-*

*ella typhimurium* LT2 have not been investigated previously. However, two deletion strains, SMI6 and SM51, have been reported to lack α-ketoglutarate dehydrogenase (E1kg), lipoamide dehydrogenase (E3) and overall α-ketoglutarate dehydrogenase complex activities (Carrillo-Castaneda & Ortega, 1970). These strains required lysine plus methionine for growth on glucose, they lacked phospho*enol*pyruvate carboxykinase activity, they were resistant to low concentrations of streptomycin and one of them (SM51) also required nicotinate. It was suggested, as a result of genetic studies, that they were deleted in the segment of the *S. typhimurium* chromosome corresponding to the *sucA* to *nadA* region of *E. coli*. This conclusion implies that there is an *lpd* gene linked to *suc* in *S. typhimurium* and hence a different organization of the genes for the α-keto acid dehydrogenase complexes compared with *E. coli*. To investigate this possibility, representative *ace* and *suc* mutants of *S. typhimurium* have been isolated. Biochemical and genetic studies with these and with the deletion strains, kindly provided by Dr M. Ortega, now indicate that the gene–protein relationships of the α-keto acid dehydrogenase complexes are similar in both organisms.

## METHODS

*Bacterial strains.* Mutants requiring acetate and succinate were isolated in *Salmonella typhimurium* LT2 which was obtained from Dr D. A. Smith. The deletion strains SMI6 and SM51 were spontaneous auxotrophic mutants found to be resistant to low concentrations of streptomycin (20 μg/ml) (Carrillo-Castaneda & Ortega, 1970). Other strains of *S. typhimurium* LT2 used include: *leuA37* and 0002 (*aziA,gal,H1$^{b}$,H2$^{enx}$,hisA,metA,nml, rha,trpB*) from D. A. Smith; the nicotinate-requiring mutants *nic-502,504,505* and *506* and the pantothenate mutant *pan-501* from M. Alper: HUI11 (*metP,gal*) and *aroP504* from P. Ayling. The following strains of *Escherichia coli* K12 were also used: W3110, prototroph; WGA, (*gal,trpA*); representative *ace* and *suc* mutants *aceE2*, (A2T3); *aceF10*, (A10); *sucA1*, (WI485*sucA1*) and *sucB17* (W3110*sucB17*); and the lipoamide dehydrogenase mutants ABI325*lpd3*, WGA*lpd5*, H*lpd6* and H*lpd10*. Details of these strains have been described previously (Herbert & Guest, 1969; Guest & Creaghan, 1973).

*Abbreviations.* The genetic nomenclature conforms to the recommendations of Demerec, Adelberg, Clark & Hartman (1968) and the symbols for gene loci are those used for *E. coli* by Taylor & Trotter (1972). Consequently, genes determining NAD biosynthesis are designated *nad* rather than *nic* and genes determining a requirement for succinate for growth on glucose medium, e.g. genes for the dehydrogenase and transsuccinylase components of the α-ketoglutarate complex, are designated *suc*. Confusion arising from the use of *sut* and *suc* to specify a gene affecting the utilization of succinate as a carbon and energy source (Sanderson, 1970, 1972) could be avoided if the first symbol, *sut*, was reserved for this purpose. The *sut* locus could correspond to the succinate dehydrogenase gene, *sdh* (Creaghan & Guest, 1972) or a gene governing the transport of dicarboxylic acids, *dctB* or *ct* (Lo, Rayman & Sanwal, 1972) which map near *gal* in *E. coli* K12.

*Media.* The citrate-free medium of Herbert & Guest (1970) was used with glucose (0·2 and 0·4 %), acetate (50 mM), succinate (50 mM) or galactose (0·4 % plus bromothymol blue at a final concentration of 0·002 %) as substrates. In the genetical studies the minimal medium E of Vogel & Bonner (1956) was used but only with glucose as the substrate. Minimal media were supplemented as required with acetate (2 mM), succinate (2 mM), L-lysine (40 μg/ml), L-methionine (20 μg/ml), other L-amino acids (30 μg/ml) and vitamins (10 μg/ml). L-Broth (Lennox, 1955) and Bacto Nutrient Broth (Difco) were used as complete media. All media were solidified with 1·5 % agar (Difco) except in the preparation of

lysates when the top and bottom layers contained 0·6 and 1·0 % agar respectively. Anaerobic cultures were incubated in an atmosphere of $H_2$ with 5 % $CO_2$.

*Enzymology.* Ultrasonic extracts of organisms grown in citrate-free medium containing glucose (0·2 %) plus other necessary supplements were prepared and assayed for protein content according to the methods of Guest & Creaghan (1973). Samples of each mutant culture were tested to ensure that reversion had not occurred.

Enzyme activities were measured in the region of proportionality between initial reaction velocity and protein concentration at 25 °C and are quoted as μmol of substrate transformed/mg protein/h. The assay for lipoamide dehydrogenase (lpdh or E3), the pyruvate and α-ketoglutarate dehydrogenase complexes (pdhc and kgdhc) and the α-ketoglutarate dehydrogenase component (E1kg) have been described previously (Creaghan & Guest, 1972). Some indication of the pyruvate dehydrogenase (E1p) activity was obtained by using the method for E1kg with pyruvate as substrate, but it should be noted that the flavoprotein pyruvate oxidase is also active in this system. Reconstitution of the activities for overall pyruvate and α-ketoglutarate dehydrogenase complexes was measured by mixing extracts of mutants defective in one or more components in pairs, or with purified E3 components according to the complementation assay described previously (Guest & Creaghan, 1973).

*Immunology.* The preparation of antiserum raised against purified lipoamide dehydrogenase from *E. coli* B and the methods used for immunodiffusion in agarose have been described previously (Guest & Creaghan, 1974). In quantitative experiments one unit of lipoamide dehydrogenase was defined as the amount which oxidized 1 μmol dihydrolipoate in 1 h under the conditions of the assay. Then, 1 unit of antiserum was defined as the amount neutralizing 1 unit of enzyme. Antiserum was assayed by incubating graded amounts with *E. coli* K12 extracts containing 2 to 3 units of lpdh for 20 min at room temperature in a final volume of 0·25 ml saline. After centrifuging (20 min, 10000*g* at 4 °C) to remove precipitated antibody–antigen complex the lpdh activity remaining in the supernatant fluid was assayed. A similar procedure was adopted in experiments in which antiserum was pretreated with bacterial extracts. Antiserum (approximately 3 units in a final volume of 0·3 ml) was incubated for 20 min at room temperature with an amount of bacterial extract (usually 2 to 2·5 mg protein) which would normally remove 90 % of the antibody after centrifuging. The residual antibody activity in 0·2 ml of the supernatant fluid was then assayed by the method described above.

*Isolation of mutants.* Mutants of *S. typhimurium* requiring acetate or succinate for aerobic growth in glucose minimal medium were isolated following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to the methods of Roth (1970) and Guest & Creaghan (1973). Treated cultures were expressed in the presence of acetate plus succinate (2 mM of each), selected with penicillin in the absence of these supplements and mutants detected by replica-plating. Mutants with a variety of different phenotypes were defined by further nutritional tests and from these, representative *ace* (s8) and *suc* (s6) were chosen for further study. The final characterization of these mutants was based on enzymological studies.

*Transduction with phage P22 and linkage analysis.* Phage P22 lysates were prepared by confluent lysis in nutrient agar overlayers on L-agar plates. Soft layers seeded with $5 \times 10^8$ bacteria of donor strain and $10^6$ phage were incubated at 37 °C for 16 h. The top layers were collected, shaken with nutrient broth plus chloroform, and stood for 8 h at 2 °C before centrifuging to remove agar. The supernatant lysates were titrated with *S. typhimurium* LT2 as indicator and generally contained between $5 \times 10^{10}$ and $5 \times 10^{11}$ p.f.u./ml.

Phages P22 and P22*int4* were used in some experiments, but a derivative of P22, HT104/2, which gives higher frequencies of transduction (Schmieger, 1972), was used routinely. Transduction mixtures contained $4 \times 10^8$ stationary phase recipient organisms and $4 \times 10^9$ phage in a final volume of 2 ml nutrient broth. After incubating for 20 min at 37 °C, appropriate dilutions in nutrient broth were plated directly on selective medium. Selective media were enriched with Bacto Nutrient Broth (1 %, v/v) except for glucose-based media, and transductant colonies were scored after 2 to 5 days, depending on the medium.

Transductants were purified on the corresponding selective medium before investigating the segregation of unselected markers by replica-plating on non-permissive and permissive media. Glucose-based media were used for selecting and scoring Leu, Pan and Nad markers. To limit the inoculum size and vitamin transfer in scoring the Nad and Pan markers two rounds of replica-plating were performed, in which the first replica-plate was used immediately as the master plate in the second round. Ace$^+$ and Suc$^+$ transductants were selected on succinate and acetate minimal media respectively, and unselected Ace and Suc markers were scored on the same media and also on glucose-based media. Galactose minimal medium was used for selecting and scoring the fermentation marker. Tests for resistance to azide were performed on nutrient agar containing sodium azide (3 mM). Azi$^R$ transductants were selected on this medium, but only after the transduced cultures had been centrifuged, resuspended in fresh broth and incubated for 2 h at 37 °C to allow expression of this characteristic. AroP and MetP were scored by radial streaking of saline suspensions of test colonies on plates of glucose medium and placing a filter paper disc impregnated with 0·02 μmol azaserine (*o*-diazo-acetyl-L-serine) for AroP or 3 μmol or α-methyl-DL-methionine for MetP, at the centre of the plate. Resistance to low concentrations of streptomycin was tested on L-agar containing streptomycin sulphate (20 μg/ml). Chlorate resistance was tested by plating on nutrient agar containing glucose (0·2 %) and $KClO_3$ (0·1 %) and incubating anaerobically. The AroG phenotype was determined by plating on glucose minimal medium supplemented with L-tyrosine (28 μg/ml) plus L-tryptophan (35 μg/ml); on this medium *aroG* mutants fail to grow because two of the three DAHP synthetases are repressed and the phenylalanine-repressible enzyme (the *aroG* gene product) is defective.

*Materials.* Purified pig heart lipoamide dehydrogenase was from Sigma and a sample of the *E. coli* B enzyme was kindly provided by Dr C. H. Williams Jun. (Department of Biochemistry, University of Michigan, Ann Arbor, Michigan, U.S.A.). DL-Dihydro-α-lipoate was prepared by the method of Gunsalus & Razzell (1957). The sources of some other materials were: 3-acetyl NAD, Boehringer, Männheim, Germany; azaserine, Calbiochem; and α-methyl-DL-methionine, Sigma.

## RESULTS

### *Nutritional and other properties of mutants*

Representative *ace* and *suc* mutants of *Salmonella typhimurium* LT2 were selected from a spectrum of different mutants requiring either acetate or succinate for aerobic growth on glucose (see Methods). The *ace* mutant (s8) was characterized by a requirement for acetate for aerobic growth on both glucose and succinate minimal media and an ability to grow on unsupplemented acetate minimal medium (Table 1). The *suc* mutant (s6) required succinate or lysine plus methionine for aerobic growth on glucose and would not grow on succinate or acetate minimal media (Table 1). Neither mutant responded to supplements of

Table 1. *Growth of Salmonella typhimurium strains on different media*

Washed suspensions of organisms were streaked on plates of minimal salts media with substrates and supplements as indicated (10 μg nicotinic acid/ml was added to all media for SM51). Growth recorded after 48 h was scored as follows: ++, very good; +, good; ±, poor; −, none. No growth was obtained with any of the strains with glucose medium supplemented with lactate, citrate, fumarate, glutamate or aspartate, or when these compounds were used as sole carbon and energy sources.

| | Growth of strain: | | | | |
|---|---|---|---|---|---|
| Substrate and supplement | LT2 (parent) | s8 (*ace*) | s6 (*suc*) | SM16 | SM51 |
| Glucose | ++ | − | − | − | − |
| Glucose (anaerobic) | + | + | + | + | + |
| Glucose + acetate | ++ | + | − | + | + |
| Glucose + succinate | ++ | − | + | − | − |
| Glucose + acetate + succinate | ++ | + | + | + | + |
| Glucose + lysine + methionine | ++ | − | + | ± | ± |
| Glucose + lysine + methionine + acetate | ++ | + | + | + | + |
| Glucose + lipoate | ++ | − | − | − | − |
| Succinate | ++ | − | − | − | − |
| Succinate + acetate | ++ | + | − | − | − |
| Acetate | ± | ± | − | − | − |

lipoate, lactate, fumarate, citrate, glutamate or aspartate on glucose aerobically, but they grew anaerobically on unsupplemented glucose medium. The mutants failed to use lactate, citrate, fumarate, glutamate and aspartate as substrates; fumarate and the two amino acids were also very poor substrates for the parental strain. These responses were confirmed by growth tests in liquid media and the results clearly resembled those obtained with the corresponding mutants of *E. coli*.

Tests with the deletion strains, SM16 and SM51, confirmed previous observations that they required lysine plus methionine for aerobic growth on glucose but grew anaerobically without supplements (Table 1), that they would not use succinate as a carbon source, were resistant to streptomycin (20 μg/ml) and that SM51 required nicotinate (Carrillo-Castaneda & Ortega, 1970). However, the response to lysine plus methionine was very poor and there was no response to succinate, which replaces these amino acids with *suc* mutants of *E. coli* (Herbert & Guest, 1968). Further tests showed that good growth could be obtained with a single supplement of acetate, but unlike typical *ace* mutants they could not grow on acetate-supplemented succinate medium or acetate minimal medium (Table 1). Growth tests in liquid media showed that the response to acetate could be improved by further supplements of succinate or, better, lysine plus methionine (Fig. 1). The deletion strains were similar in all respects except for the nicotinate requirement of SM51.

The phenotypes of SM16 and SM51 were further investigated in an attempt to establish the position and extent of their deletions. In *S. typhimurium* and *E. coli* the *nadA* gene is often deleted with the *aroG*, *gal* and *chlD* genes but deletions involving *nadA* and the *suc* region have not been found (Stouthamer, 1969; Sanderson, 1972; Shapiro & Adhya, 1969). Neither deletion strain was AroG$^-$, Gal$^-$ or Chl$^R$. Further tests, based on the possibility that the strains were deleted for *ace* and adjacent genes showed them to be Azi$^s$ and MetP$^+$ but AroP$^-$, i.e. lacking the general aromatic amino acid permease (Ames & Roth, 1968; Brown, 1970). It was thus concluded that SM16 and SM51 are deleted for *aroP* and at least one *ace* gene and that the nicotinate requirement of SM51 stems from the simultaneous deletion of a *nad* gene, probably *nadC* which is close to *aroP* in *E. coli* (Guest, 1974).

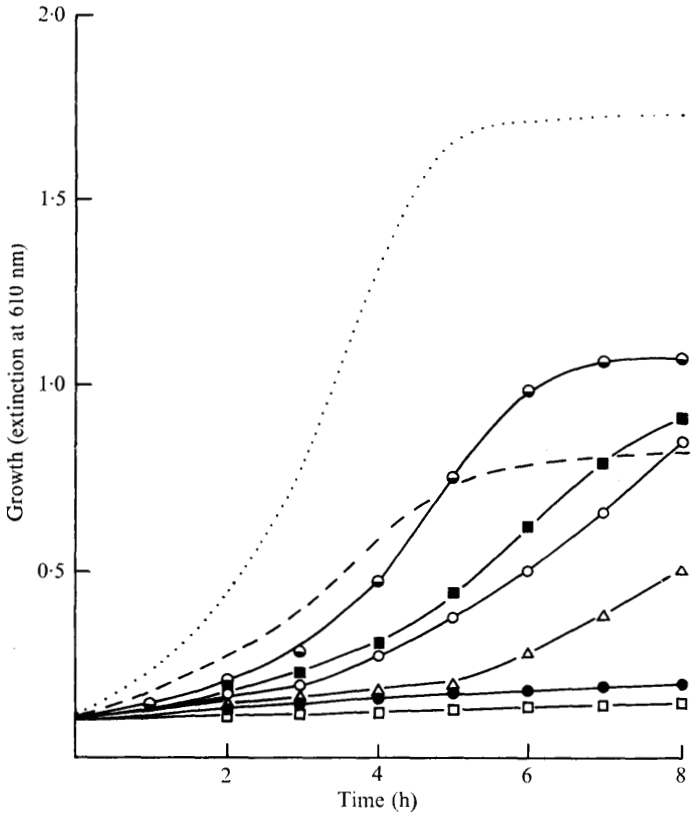


Fig. 1. Growth of *Salmonella typhimurium* LT2 and the deletion strain SM16. Cultures were shaken at 37 °C in 250 ml Erlenmeyer flasks fitted with optically matched side-arms. Each flask contained 10 ml citrate-free medium plus glucose (0·4 %, w/v) inoculated with 0·2 ml of a washed suspension of organisms (grown for 16 h in the same medium plus acetate and succinate for SM16) to give an initial extinction at 610 nm of 0·1 ($10^8$ bacteria/ml). Anaerobic growth was measured in sealed flasks containing an atmosphere of $H_2$ with 5 % $CO_2$. ····, Aerobic growth of LT2 with or without acetate+succinate; – – – –, anaerobic growth of LT2 without supplements. ——, Growth of the deletion strain SM16: aerobically with no supplements or succinate (□), lysine+methionine (●), acetate (○), acetate+succinate (■), or acetate+lysine+methionine (◒); and anaerobically with no supplements (△).

Attempts were made to classify the *nad* lesion of SM51 by nutritional tests in liquid media using depleted inocula of SM51 and well-characterized *nadA* and *nadC* mutants of *E. coli*. According to Gholson, Tritz, Matney & Andreoli (1969), *nadC* mutants, which lack quinolinate phosphoribosyltransferase, should respond to nicotinate but not quinolinate, whereas *nadA* and *nadB* mutants with early blocks in NAD biosynthesis should respond to both supplements. This could only be demonstrated with poor reproducibility for a very narrow range of quinolinate concentrations using the *E. coli* strains and the results with SM51 were inconclusive. The main problem was that the *nadA* mutants required much higher concentrations of quinolinate (20 μg/ml) compared with nicotinate (0·1 μg/ml) to obtain a good response and it was difficult to assess whether responses to quinolinate were simply due to its decarboxylation to nicotinate.

Table 2. *Specific activities of the pyruvate and α-ketoglutarate dehydrogenase complexes and related enzymes*

Organisms were grown in citrate-free medium with glucose (0·2 %, w/v), and acetate (2 mM), succinate (2 mM) and nicotinic acid (10 μg/ml) as required. The enzymes were assayed in ultrasonic extracts as described in Methods and the average activities for determinations with at least two different extracts are quoted. Specific activities are recorded as μmol of substrate transformed/mg protein/h. Abbreviations: kgdhc = α-ketoglutarate dehydrogenase complex; E1kg = α-ketoglutarate dehydrogenase; pdhc = activity of the overall pyruvate dehydrogenase complex; E1p = pyruvate dehydrogenase plus oxidase; E3 = lipoamide dehydrogenase.

| | Enzyme specific activity | | | | |
|---|---|---|---|---|---|
| Strain | kgdhc | E1kg | pdhc | E1p | E3 |
| LT2 (prototroph) | 1·40 | 4·30 | 2·60 | 1·22 | 1·10 |
| SM16 | < 0·01 | 1·32 | < 0·01 | 0·46 | < 0·01 |
| SM16*T1* (prototroph) | 0·54 | 2·22 | 2·17 | 1·33 | 1·01 |
| SM51 | < 0·01 | 1·43 | < 0·01 | 0·63 | < 0·01 |
| SM51*T1* (prototroph) | 1·04 | 2·61 | 2·90 | 0·83 | 1·09 |
| S8 (*ace*) | 1·26 | 2·08 | 0·18 | 0·75 | 0·65 |
| S6 (*suc*) | < 0·01 | < 0·10 | 1·68 | 0·98 | 1·11 |

Table 3. *Complementation between extracts of Salmonella typhimurium mutants and Escherichia coli ace and suc mutants*

The pdhc activities of mixtures of crude extracts containing 0·4 mg protein of the *S. typhimurium* strains plus an equal amount of the *E. coli ace* mutant in 1 ml of reaction mixture were measured by the complementation assay (see Methods). The kgdhc activities were measured with equal 0·3 mg protein mixtures of test strains with *E. coli suc* mutants. Activities are expressed as total activity in the sample in μmol/h. The combined activities of participating extracts tested separately, were between 0·04 and 0·10 μmol/h for pdhc and less than 0·01 μmol/h for kgdhc, and these have been subtracted.

| | Activity of complex (μmol/h) | | | |
|---|---|---|---|---|
| Test strain | Complementing strain | pdh complex | Complementing strain | kgdh complex |
| *aceE2* | *aceF10* | 0·26 | — | — |
| S8 (*ace*) | *aceF10* | 0·51 | — | — |
| SM16 | *aceF10* | < 0·01 | *sucA1* | 0·04 |
| SM51 | *aceF10* | < 0·01 | *sucA1* | 0·04 |
| S6 (*suc*) | — | — | *sucA1* | < 0·005 |
| S8 (*ace*) | *aceE2* | < 0·01 | — | — |
| SM16 | *aceE2* | < 0·01 | *sucB17* | 0·07 |
| SM51 | *aceE2* | < 0·01 | *sucB17* | 0·12 |
| S6 (*suc*) | — | — | *sucB17* | 0·04 |
| *sucA1* | — | — | *sucB17* | 0·04 |

*Enzymological studies*

Ultrasonic extracts were assayed directly for the overall pyruvate dehydrogenase (pdhc) and α-ketoglutarate dehydrogenase complex (kgdhc) activities and their components, α-ketoglutarate dehydrogenase (E1kg) pyruvate dehydrogenase (E1p) and lipoamide dehydrogenase (E3) (Table 2). Mutant S8 had less than 10% of parental pdhc activity and a lowered E1p activity, consistent with it being an *ace* mutant; the kgdhc activity was little affected. Mutant S6 resembled a typical *sucA* mutant in lacking overall kgdhc and E1kg activities only. The deletion strains lacked the overall activities of both complexes, pdhc and kgdhc, they had low E1p activities, and consistent with the findings of Carrillo-Castaneda & Ortega (1970) they had no detectable lipoamide dehydrogenase (E3) activity

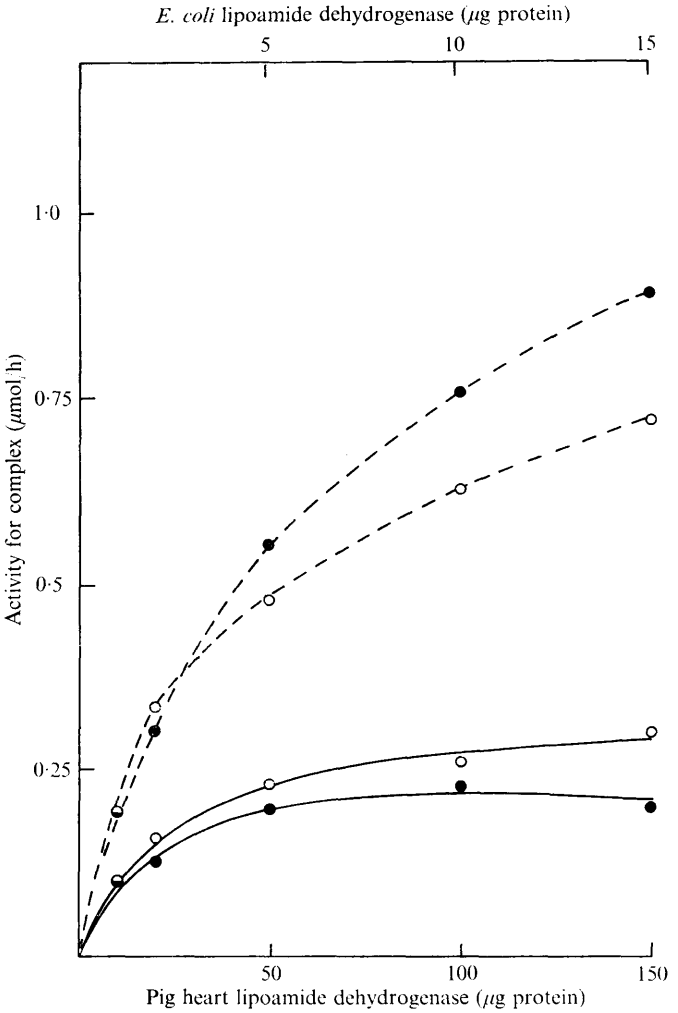


Fig. 2. Complementation of extracts of SM16 and SM51 for overall α-ketoglutarate dehydrogenase complex activity by lipoamide dehydrogenase purified from pig heart (——) or *E. coli* B (– – –). Duplicate samples containing extract of SM16 (●) or SM51 (○) equivalent to 0·6 mg protein were mixed with the purified enzyme and assayed for kgdhc by the complementation method.

(Table 2). However, in contrast to previous findings both strains had significant α-ketoglutarate dehydrogenase (E1kg) activities, so it appeared that the lack of kgdhc activity stemmed from the absence of E3 rather than a combined lack of E1kg and E3. Attempts to measure dihydrolipoyltranssuccinylase (E2kg) activity by the method used for *E. coli* (Creaghan & Guest, 1972) were unsuccessful with *S. typhimurium* LT2. It should also be noted that the E1p assay is ambiguous as it measures pyruvate dehydrogenase and also the flavoprotein pyruvate oxidase; the latter probably accounts for approximately half of the activity recorded for the wild-type. Prototrophic transductants, SM16*T1* and SM51*T1*, selected on glucose minimal medium with P22*int4*.LT2 lystate regained in a single step the activities lacking in the deletion strains (Table 2).

In the complementation studies summarized in Table 3, the restoration of overall pdhc

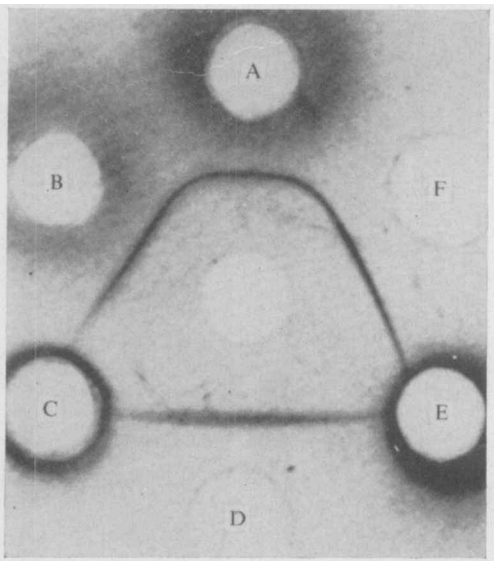


Fig. 3. Immunodiffusion in agarose with antiserum in the centre well (10 $\mu$l of a tenfold dilution and lateral wells containing ultrasonic extract equivalent to 100 $\mu$g protein of *S. typhimurium* LT2 (A), *E. coli* K12 (B), SM51 (C) and SM16 (E), or 1 $\mu$g of lipoamide dehydrogenase purified from *E. coli* B (D and F). The gel was developed for 40 h at room temperature, washed in saline then water and dried before staining with Ponceau S.

and kgdhc activities in extracts of the *S. typhimurium* mutants by addition of standard *E. coli ace* and *suc* mutants was investigated. Strain S8 was complemented by *aceF10* but not by *aceE2* for pdhc activity. This strain may therefore be designated as an *aceE* mutant. The overall kgdhc activity generated by complementation was generally low but strain S6 was complemented by extracts of *sucB* but not *sucA* mutants of *E. coli* (Table 3). This confirmed the earlier conclusion that S6 is a *sucA* mutant of *S. typhimurium*. Similar studies with SM16 and SM51 showed that they were complemented by *sucA* and *sucB* mutants but not by *aceE* and *aceF* mutants (Table 3). Further complementation studies using purified lipoamide dehydrogenases from *E. coli* B and pig heart (Fig. 2) also indicated that the deletion strains contain both the E1kg and the E2kg components because overall kgdhc activity was restored. However, no overall pdhc activity was reconstituted, confirming that the deletion strains lack one and probably both of the E1p and E2p components of the pdh complex as well as the lipoamide dehydrogenase (E3) activity. Similar results were obtained when an extract of an *ace,suc* double-amber mutant, WG*aceE64sucA35* (Guest & Creaghan 1973), was used as the source of lipoamide dehydrogenase activity for complementation.

### *Immunological studies*

The deletion strains were further characterized in immunological studies using a rabbit antiserum raised against purified *E. coli* lipoamide dehydrogenase. In double diffusion experiments with this antiserum, crude extracts of *S. typhimurium* LT2 (Fig. 3A) gave a single precipitin line which fused completely with the lines developed by crude extracts of *E. coli* K12 (Fig. 3B) and purified lipoamide dehydrogenase (Fig. 3F). This indication of complete immunological identity between the lipoamide dehydrogenases of the two species was confirmed in the quantitative tests illustrated in Fig. 4. These titrations showed that the

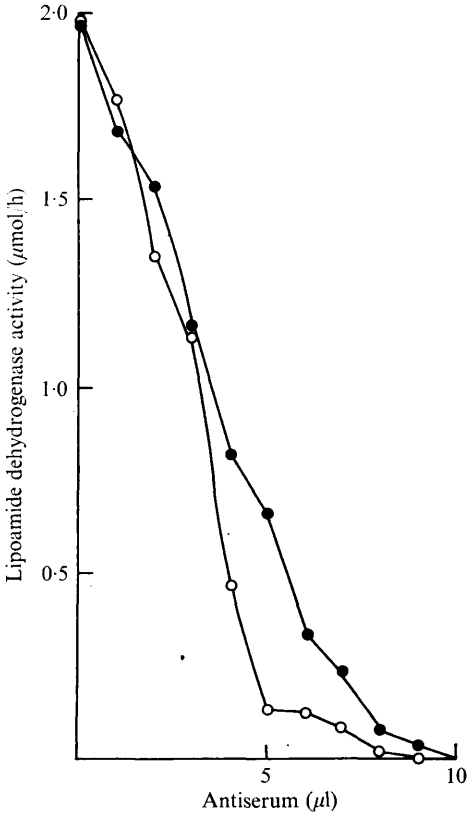


Fig. 4. The effect of antiserum on the lipoamide dehydrogenase activities of ultrasonic extracts of ●, *S. typhimurium* LT2 (1·6 mg protein) and ○, *E. coli* W3110 (1·4 mg protein). Samples containing 2 units of enzyme activity were incubated with antiserum in buffered saline and after centrifuging the lipoamide dehydrogenase activity remaining in the supernatant fluid was assayed (see Methods).

## Table 4. *Test for neutralization of lipoamide dehydrogenase antiserum by extracts of S. typhimurium and E. coli strains*

Samples of antiserum (equivalent to 2·3 units before preincubation with 1·5 mg protein in extracts of the test strain and centrifuging) were assayed for residual activity by measuring their effect on extract of wild-type *E. coli* containing 2·5 units of lipoamide dehydrogenase. Representative *CRM*+ and *CRM*− *lpd* mutants of *E. coli* were used as controls.

| Test strain | Antiserum (units remaining) |
|---|---|
| None | 2·3 |
| SMI6 | 2·5 |
| SM5I | 2·3 |
| H*lpd6* (*CRM*−) | 2·0 |
| H*lpd10* (*CRM*−) | 2·2 |
| AB1325*lpd3* (*CRM*+) | 0·2 |
| WGA*lpd5* (*CRM*+) | 1·0 |

Table 5. *Linkage relationships in the regions of the aceE and sucA genes of Salmonella typhimurium*

All transductions were mediated by a high-frequency transducing derivative of phage P22, HT104/2, except crosses 1A and 1B where P22*int4* was used.

| Cross | Donor | Recipient | Selection | Transductants/ $10^6$ phage | Number scored | Unselected donor marker | Cotransduction frequency (%) |
|---|---|---|---|---|---|---|---|
| 1A | *leuA37* | s8 | Ace$^+$ | 0·5 | 98 | Leu$^-$ | < 1 |
| 1B | s8 | *leuA37* | Leu$^+$ | 0·1 | 70 | Ace$^-$ | < 2 |
| 2 | *pan-501* | s8 | Ace$^+$ | 10 | 150 | Pan$^-$ | < 1 |
| 3 | 0002 | s8 | Ace$^+$ | 4 | 100 | Azi$^R$ | 14 |
| 4 | *aroP504* | s8 | Ace$^+$ | 20 | 200 | AroP$^-$ | 72 |
| 5 | HUI11 | s8 | Ace$^+$ | 5 | 50 | MetP$^-$ | < 2 |
| 6 | *nic-502* | s8 | Ace$^+$ | 12 | 100 | Nad$^-$ | < 1 |
| 7 | *nic-505* | s8 | Ace$^+$ | 15 | 100 | Nad$^-$ | < 1 |
| 8A | HUI11 | s6 | Suc$^+$ | 28 | 100 | Gal$^-$ | < 1 |
| 8B | s6 | HUI11 | Gal$^+$ | 2 | 100 | Suc$^-$ | < 1 |
| 9A | *nic-502* | s6 | Suc$^+$ | 2 | 200 | Nad$^-$ | 12 |
| 9B | s6 | *nic-502* | Nad$^+$ | 1 | 100 | Suc$^-$ | < 1 |
| 10 | *nic-505* | s6 | Suc$^+$ | 4 | 100 | Nad$^-$ | < 1 |
| 11 | 0002 | *aroP504* | Azi$^R$ | 11 | 100 | AroP$^-$ | 39 |
| 12 | 0002 | *leuA37* | Leu$^+$ | 8 | 120 | Azi$^R$ | 3 |
| 13 | *aroP504* | *leuA37* | Leu$^+$ | 16 | 210 | AroP$^-$ | 1 |
| 14A | 0002 | *nic-502* | Nad$^+$ | 11 | 100 | Gal$^-$ | 27 |
| 14B | *nic-502* | 0002 | Gal$^+$ | 5 | 100 | Nad$^-$ | 20 |
| 15 | 0002 | *nic-505* | Nad$^+$ | 20 | 100 | Gal$^-$ | < 1 |

antiserum was almost equally effective in neutralizing the *E. coli* and *S. typhimurium* enzymes. Tests with the deletion strains, SM51 and SM16, gave no detectable precipitin lines (Fig. 3C, E), indicating the absence of cross-reacting material (*CRM*−). In similar tests the prototrophic transductants SM16*T1* and SM51*T1* and mutants s6 and s8 were indistinguishable from wild type. Further quantitative tests with the deletion strains and representative *CRM*+ and *CRM*− *lpd* mutants of *E. coli* showed that the activity of the antiserum was virtually unaffected by preincubation with extracts of the deletion strains and the *CRM*− mutant of *E. coli* (Table 4). The lack of lipoamide dehydrogenase activity and cross-reacting material would therefore suggest that the deletions extend into the *lpd* gene or into some element controlling the expression of the *lpd* gene.

### *Genetic studies*

The results of P22-mediated transductions involving *aceE*, *sucA* and several other mutants of *S. typhimurium* are shown in Table 5. Cotransduction was observed for *aceE* with *aziA* (cross 3) and, at a higher frequency, with *aroP* (cross 4) but no linkage was detected with *leuA* (cross 1), *pan* (cross 2), *metP* (cross 5) or with either of two classes of *nad* mutant (crosses 6 and 7). Considered with the cotransduction frequencies observed for *leuA* with *aziA* (cross 12), *leuA* with *aroP* (cross 13) and *aziA* with *aroP* (cross 11), these linkage data are consistent only with the gene order *leuA-aziA-aroP-aceE*, as illustrated in Fig. 5. The *sucA* marker was linked to *nadA* (cross 9A) but not to *gal* (cross 14), and since *nadA* is linked to *gal* (cross 14) and the relative orientation of *nadA* and *gal* has been established by studies with deletions (Stouthamer, 1969) the order *sucA-nadA-gal* can be deduced (Fig. 5). The failure to detect linkage between *sucA* and *nadA* with s6 as donor (cross 9B) was confirmed but not investigated further. A total of four *nad* mutants, kindly provided by Dr M. Alper,

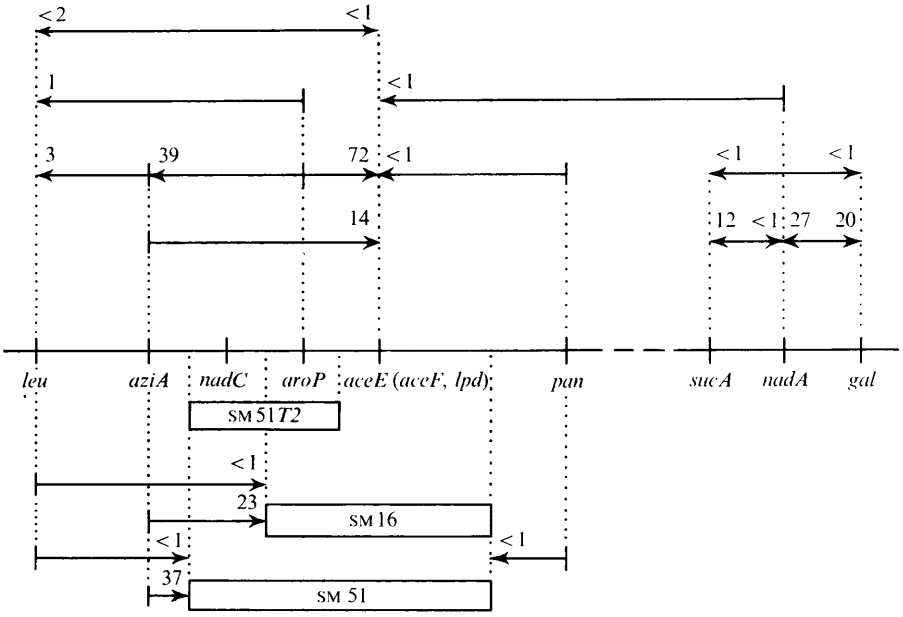


Fig. 5. Linkage map showing the position of *ace* and *suc* loci with respect to other loci. Also shown are the proposed extremities of the SM16, SM51 and SM51*T2* deletions. Cotransduction frequencies are given as the number of recombinants inheriting the unselected marker expressed as a percentage of the number of selected transductants examined. For each cross the corresponding frequency is placed near the head of the arrow pointing to the selected marker.

was examined. Two of them, *nic-502* and *nic-506*, were designated *nadA* by virtue of their linkage to *sucA* and *gal* and by comparison with the distribution of *nad* genes in *E. coli* (Taylor & Trotter, 1972). The others, *nic-504* and *nic-505*, exhibited no linkage with *sucA* (cross 10), *gal* (cross 15) or *ace* (cross 7) and, again by analogy with *E. coli*, are unlikely to be *nadA* or *nadC* mutants.

The enzymological studies with the deletion strains indicated that they possess Ace$^-$Lpd$^-$ phenotypes; however, no independent selection procedures for Ace$^+$ and Lpd$^+$ transductants could be devised. Using SM16 as recipient, Ace$^+$Lpd$^+$ transductants were readily selected on glucose minimal medium. With SM51, on the other hand, separate selections for Ace$^+$Lpd$^+$ and Nad$^+$ transductants were possible using glucose + nicotinate and glucose + lysine + methionine + acetate, in addition to a combined selection for Nad$^+$Ace$^+$Lpd$^+$ transductants. In crosses with wild-type donors similar transduction frequencies were observed in all three selections (range, 1 to 10 per $10^6$ phage in different experiments). Tests with 900 Ace$^+$Lpd$^+$ and 450 Nad$^+$ transductants of SM51 yielded only one transductant in which segregation of the unselected characteristic had occurred. This strain, SM51*T2*, was an Ace$^+$Lpd$^+$ transductant which proved to be Nad$^-$. It would therefore appear that all three characteristics are generally inherited in a single step. It is assumed that SM51*T2* arose following some kind of aberrant recombination event. The prototrophic transductants of SM16 and SM51 were also found to be AroP$^+$ and sensitive to low concentrations of streptomycin. By contrast, SM51*T2* became Ace$^+$Lpd$^+$ but remained AroP$^-$ and resistant to low concentrations of streptomycin as well as Nad$^-$. This suggests that the *nad* and *aroP* genes and a gene conferring resistance to low concentrations of streptomycin are clustered to one side of

Table 6. *Linkage relationships between the deletion strains* SM16 *and* SM51 *and other markers*

| Cross | Donor* | Recipient* | Selection | Transductants/ $10^8$ phage | Number scored | Unselected donor marker | Cotransduction frequency (%) |
|---|---|---|---|---|---|---|---|
| 16, 17 | *leuA37* | SM16, SM51 | Ace$^+$Lpd$^+$(Nad$^+$) | 1 | 330 | Leu$^-$ | < 0·3 |
| 18, 19 | *pan-501* | SM16, SM51 | Ace$^+$Lpd$^+$(Nad$^+$) | 1 | 200 | Pan$^-$ | < 0·5 |
| 20 | 0002 | SM16 | Ace$^+$Lpd$^+$ | 2 | 100 | Azi$^R$ | 23 |
| 21 | 0002 | SM51 | Ace$^+$Lpd$^+$Nad$^+$ | 8 | 100 | Azi$^R$ | 37 |
| 22, 23 | *aroP504* | SM16, SM51 | Ace$^+$Lpd$^+$(Nad$^+$) | 1 | 100 | AroP$^-$ | 100 |
| 24 | S8 | SM16 | Ace$^+$Lpd$^+$ | < 0·002 | — | — | — |
| 25 | S8 | SM51 | Ace$^+$Lpd$^+$ | < 0·002 | — | — | — |
| | | | Nad$^+$ | 0·2 | 50 | Ace$^-$ | 100 |
| 26, 27 | SM16, SM51 | S8 | Ace$^+$ | < 0·002 | — | — | — |
| 28 | S6 | SM16 | Ace$^+$Lpd$^+$ | 0·6 | — | — | — |
| 29 | S6 | SM51 | Ace$^+$Lpd$^+$ | 0·8 | — | — | — |
| | | | Nad$^+$ | 0·8 | — | — | — |
| 30, 31 | SM16, SM51 | S6 | Suc$^+$ | 9 | — | — | — |

* Where two donor or recipient strains are indicated, two crosses were performed with similar results.

the *ace* region. Furthermore, since the gene order *aziA-aroP-aceE* has already been indicated, a consideration of the phenotypes of SM16, SM51 and SM51*T2* places the *nad* gene between *aziA* and *aroP* thus: *aziA-nad-aroP-aceE.*

Transductions between the deletion strains and a variety of donor strains are summarized in Table 6. Tests with Ace$^+$Lpd$^+$ (SM16) and Nad$^+$Ace$^+$Lpd$^+$ (SM51) transductants showed no linkage with *leu* (crosses 16 and 17) or *pan* (crosses 18 and 19) but *aziA* was linked to both deletions (crosses 20 and 21). The gradient of cotransduction frequencies for SM51 (37 %), SM16 (23 %) and S8 (14 %) suggests that the SM51 deletion ends closer to *aziA* than the SM16 deletion, which in turn terminates nearer *aziA* than the *aceE* mutation of S8. This further supports the conclusion that the SM51 deletion includes a *nad* gene, presumably analogous to the *nadC* gene of *E. coli*, which lies between *aziA* and *aroP*, and that the SM16 deletion extends through *aroP* but not as far as *nad.* Further confirmation that the deletions include *aroP* came from the use of an *aroP* donor, because all prototrophic transductants were AroP$^-$ (Table 6, crosses 22 and 23). No Ace$^+$Lpd$^+$ transductants could be obtained from either deletion strain using S8 as donor (Table 6, crosses 24 and 25) and although Nad$^+$ transductants of SM51 were recovered they were all Ace$^-$. In reciprocal crosses (26 and 27) with SM16 and SM51 as donors no acetate-independent transductants of S8 could be recovered on enriched succinate medium. These findings confirm that both strains are deleted for *aceE.* By contrast, high yields of transductants (Ace$^+$Lpd$^+$ and Nad$^+$) were obtained from the deletions using S6 as donor and from reciprocal crosses in which Suc$^+$ transductants were selected on enriched acetate medium (Table 6, crosses 28 to 31). This genetic evidence lends further support to the conclusion that the deletions are not in the *suc* region. Similar reciprocal crosses also established that the nicotinic acid requirement of SM51 could not be due to the deletion of either class of *nad* gene represented by *nic-502* and *nic-505.*

In connection with the reported deletion of the phospho*enol*pyruvate carboxykinase gene (*pck*) in SM16 and SM51 it should be noted that, contrary to the observations of Carrillo-Castanedo & Ortega (1970), single-step transductions of SM51 with LT2 as donor failed to yield products capable of using succinate or acetate as sole carbon and energy sources. None of the Ace$^+$Lpd$^+$ transductants of SM51 grew with either of these substrates and even

after a second transduction recombinants could only be recovered on succinate. With SMI6 a small proportion of the Ace+Lpd+ transductants could utilize succinate, but this could have been due to reversion. These results suggest that there are at least two or three unlinked defects which are affecting growth on succinate and acetate respectively. The addition of citrate promoted growth of some of the derivatives on these substrates but this complication could be connected with the general observation that all strains of *S. typhimurium* LT2 grew better in the presence of citrate, especially with acetate or dibarboxylic acids as the main substrate.

## DISCUSSION

Studies with the *ace* and *suc* mutants and the deletion strains suggest that the genes determining the α-keto acid dehydrogenase complexes are organized in a similar manner in *Salmonella typhimurium* LT2 and *Escherichia coli* K12. Mutant S8, identified as a pyruvate dehydrogenase (*aceE*) mutant, resembled comparable mutants of *E. coli* and was similarly situated in the *leu-pan* region. The *sucA* mutant, S6, which lacked α-ketoglutarate dehydrogenase, was similar in all respects to the class of *E. coli sucA* mutant which requires succinate for aerobic growth on glucose but fails to grow with succinate as substrate (Herbert & Guest, 1968); whether this latter property is a feature of some *suc* mutants or whether it is due to a second mutation has still to be determined. As in *E. coli* the *sucA* gene was situated near *nadA* and *gal*. The components of both *S. typhimurium* complexes also appeared to be sufficiently similar to those of *E. coli* to permit complementation and presumably the formation of hybrid complexes when extracts of mutants of both species were mixed. Purified *E. coli* lipoamide dehydrogenase complemented the *S. typhimurium* E1kg and E2kg components of SMI6 and SMB1 very efficiently to generate overall activity of the α-ketoglutarate dehydrogenase complex. Furthermore, the lipoamide dehydrogenase components of the two species were immunologically indistinguishable.

Compared with P1-mediated transduction in *E. coli*, P22 presented certain problems initially. These were due mainly to the low frequencies of transduction observed for markers in the regions under investigation, and the relatively small size of the P22 transducing fragment. The first problem was overcome by using one of the high-frequency transducing phages isolated by Schmieger (1972), the second became less troublesome when additional markers were brought into the analysis. If it is assumed that the molecular weights of P22 and P1 DNA are $2{\cdot}8 \times 10^7$ and $7{\cdot}8 \times 10^7$ daltons (Clowes, 1972) and that the transducing phages carry 90 % (Schmieger, 1970) and 100 % (Ikeda & Tomizawa, 1965) as bacterial DNA respectively, then using the mapping function of Wu (1966) which relates joint transduction of markers to the distance between markers thus:

$$\text{Frequency of cotransduction} = \left(1 - \frac{\text{distance between markers}}{\text{length of transducing fragment}}\right)^3$$

it can be calculated that a cotransduction frequency of 1 % with P22 would correspond to a frequency of 41·6 % in P1-mediated cotransduction for markers separated by the same distance.

Assuming that the corresponding markers are separated by approximately the same distances (in terms of DNA length) in *E. coli* and *S. typhimurium*, it is not surprising that with P22 no cotransduction was observed for *aceE* with *leu* (25 % for P1), *aceE* with *pan* (20 % for P1) or *gal* with *sucA* (36 % for P1). One exception is the observed cotransduction of *aroP* with *leu* (1 % for P22) which may not have been expected since these markers are only 30 % cotransducible with P1 in *E. coli* (Brown, 1970). This could mean that the

markers are relatively closer in *S. typhimurium* but it should be noted that other workers have failed to detect any linkage between *aroP* and *leu* (Ames & Roth, 1968). In other cases where linkage has been observed in both systems the cotransduction frequencies, though different, were comparable in terms of the marker separations (in base pairs) when calculated using the Wu relationship and a figure of $1{\cdot}51 \times 10^3$ base pairs per mega-dalton of DNA. For example, cotransduction frequencies for *aceE* with *aroP* of 72 % (P22) and 92 % (P1; Guest, 1974) correspond to separations of 4·1 and 2·8 ($\times 10^3$ base pairs or 'average genes'). Likewise, the cotransduction frequencies for (*a*) *leu* with *aziA* at 3 % (P22) and 56 % (P1; Yura & Wada, 1968) correspond to 19·4 and 13·4 ($\times 10^3$ base pairs), (*b*) *leu* with *aroP* at 1 % (P22) and 30 % (P1; Brown, 1970) correspond to 31 and 39 ($\times 10^3$ base pairs), (*c*) *nadA* with *sucA* at 12 % (P22) and 63 % (P1; Guest, 1974) correspond to 21 and 17 ($\times 10^3$ base pairs) and (*d*) *nadA* with *gal* at 23 % (P22) and 80 % (P1; Guest, 1974) correspond to 15 and 9 ($\times 10^3$ base pairs) respectively. Thus it would appear that not only are the genes in the same relative order, *leu-aziA-aroP-aceE* and *sucA-nadA-gal* in *S. typhimurium* (Fig. 5) and in *E. coli* (Guest, 1974), but the intergenic separations could be comparable.

All the properties of the deletion strains support the conclusion that they are deleted in the *aziA* to *pan* region, rather than in the *gal* region as previously supposed. The nicotinate requirement of SM51 is almost certainly due to the deletion of a *nad* gene, presumably *nadC*, between *aziA* and *aroP*. The biochemical properties of SM16 and SM51 are most readily interpreted if the *aceE*, *aceF* and *lpd* genes are deleted. That the *aceE* gene is deleted can be inferred from the fact that no recombinants were obtained from crosses between s8 (*aceE*) and either deletion strain. No such confirmation is available for *aceF* and *lpd* since the corresponding point mutants have not yet been characterized in *S. typhimurium*. It therefore remains a formal possibility that the *aceF*, *lpd* or both genes are present, but their expession is in some way prevented by the deletions. This again would be most simply explained if the genes are linked and polarized as they are in *E. coli*: *aceE-aceF-lpd*. It is therefore proposed that the strains are deleted in the *leu-aziA-nadC-aroP-aceE,aceF,lpd-pan* region; SM16 between *aroP* and *lpd* and SM51 between *nadC* and *lpd*. Similar deletions have now been isolated in *E. coli* K12 and here the end-points can be analysed with greater precision.

One noteworthy property of the deletion strains is their ability to respond to acetate as sole supplement on glucose medium. *E. coli lpd* mutants which would be expected to have a comparable phenotype, do not respond to this single supplement. This could indicate that in *S. typhimurium* other routes of succinate biosynthesis, e.g. the reductive routes, are less severely repressed or inhibited, or alternatively that an unstable lipoamide dehydrogenase (possibly an incomplete protein) is produced by the deletion strains and this can support growth when acetate is provided.

It has been suggested previously that SM16 and SM51 exhibit a limited resistance to streptomycin because the deletions impose a respiratory deficiency which shifts their metabolism towards anaerobiosis (Carrillo-Castaneda & Ortega, 1970). This resistance was not observed with *ace* or *suc* mutants of *S. typhimurium* or *E. coli* or with *lpd* mutants of *E. coli*, which are more comparable to the deletion strains because they lack activities of both dehydrogenase complexes. Further tests seemed to rule out any connexion with the *aroP* lesion, because *aroP* mutants of *S. typhimurium* and *E. coli* were sensitive, as were *aroP*, *aceE* and *aroP,lpd* double mutants of *E. coli* (Guest, unpublished observations). It would therefore appear that resistance to low concentrations of streptomycin is governed either by some independent marker which is deleted in both strains or by the combined effects of the deletion and some other mutation in SM16 and SM51.

The deletion strains were originally described as lacking phospho*enol*pyruvate carboxykinase due to deletion of the *pck* gene and this was considered responsible for their inability to use succinate as sole carbon source (Carrillo-Castaneda & Ortega, 1970). This conclusion seems unlikely since $C_4$-dicarboxylic acids could be converted to phospho*enol*pyruvate by an alternative route involving malic enzyme and phospho*enol*pyruvate synthase. A more plausible explanation for failure to use succinate (and acetate) would be the absence of the $\alpha$-keto acid dehydrogenase complex activities. Unfortunately, the previous observation that prototrophic transductants regained the ability to utilize succinate (Carrillo-Castandea & Ortega, 1970) could not be confirmed. In fact the present results suggest that the deletion strains contain one or two unlinked mutations preventing growth on succinate and yet a further mutation preventing growth on acetate. None of these mutations is likely to involve the *pck* gene since a functional carboxykinase is probably not obligatory for growth on succinate or acetate. If the strains have not changed since they were first examined, the reported lack of carboxykinase could be an indirect consequence of these lesions, a direct or indirect result of the deletions or it could be due to yet another mutation, presumably in the *pck* gene.

We wish to thank all those who have provided mutant strains used in this work. D.L. also acknowledges the receipt of a Research Studentship from the Science Research Council.

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