Nutritional Studies of Cysteineless Mutants of *Salmonella typhimurium*

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SUMMARY: Forty-three separately isolated cyst(e)ineless mutants of *Salmonella typhimurium* were investigated biochemically. Six main phenotypes were identified, accommodating thirty-nine of the mutants; the remaining four mutants were of intermediate phenotype. The phenotypes are suggested to result from a series of five metabolic blocks in a pathway of cyst(e)ine synthesis in which sulphate can be reduced by two alternative routes, in both of which most of the intermediates are inorganic.

In this paper the expression cyst(e)ine is used to represent cysteine or cystine. The pathway of cyst(e)ine biosynthesis has previously been studied in a variety of micro-organisms including *Escherichia coli* (Lampen, Roepke & Jones, 1947; Roberts *et al.* 1955; Simonds 1948); *Neurospora crassa* (Phinney, 1948; Horowitz, 1950, 1955); *Aspergillus nidulans* (Hockenhull, 1949; Shepherd, 1956); *A. niger* (Steinberg, 1941). In most of these studies, the data are consistent with an inorganic pathway of sulphate reduction through sulphite and thiosulphate, and only after passing through these inorganic intermediates is the reduced sulphur atom combined with a carbon chain to form cyst(e)ine. The carbon chain has been suggested by Roberts *et al.* (1955) to be provided by serine, which was also indicated by the data of Hockenhull (1949). The irreversibility of the cyst(e)ine-methionine pathway in *Escherichia coli* was apparent from the data of both Simonds (1948) and Roberts *et al.* (1955), although methionine was able to replace cysteine in the *E. coli* mutants studied by Lampen *et al.* (1947), and also in *Aspergillus* and *Neurospora*. In all cases, other inorganic sources of sulphur, wherever studied, gave analogous growth responses to either sulphate, sulphite or thiosulphate, and were thus considered to be rapidly converted to one of these compounds and not to be direct intermediates. Moreover, thiosulphate satisfied the growth of almost all mutants able to utilize cyst(e)ine. The data of Hockenhull (1949) and Horowitz (1955) showed that cysteic acid could replace sulphite. However, cysteine sulphinic acid could not replace thiosulphate, as would be expected if this were a direct intermediate, but had the same growth-promoting activity as the more oxidized cysteic acid. It was therefore suggested by these authors that the initial steps of the pathway involved only inorganic ions until the sulphur had been reduced to the state of thiosulphate. However, Shepherd (1956) isolated certain mutants of *Aspergillus nidulans* which were incapable of growth with

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sulphite and in some instances with thiosulphate, but which were able to utilize either cysteic acid or cysteine sulphinic acid. He therefore postulated alternative organic and inorganic pathways of sulphur reduction in which either serine, pyruvate or cysteic acid could act as alternative carbon sources.

This paper describes the differentiation of a series of cyst(e)ineless mutants of *Salmonella typhimurium* by biochemical tests, from which some indication of the mode of cyst(e)ine biosynthesis by this organism may be obtained.

METHODS

Media

Defined medium. The minimal medium of Davis (Lederberg, 1950) was used, supplemented as indicated with various sulphur sources, at a concentration of 10 µg. S/ml. (unless otherwise specified), or with amino acids (20 µg./ml.). It contained 240 µg. S/ml. as sulphate.

Nutrient agar (Difco) was used when a complete medium was required.

Sulphur-free (S-free) medium, used when a medium deficient in all sulphur sources was required, was made up as tris (2-amino-2-hydroxymethylpropane-1:3 diol) glucose medium (Hershey, 1955), with the omission of sulphate.

Enriched minimal agar, used in syntrophism experiments, consisted of minimal agar containing 0.01 % nutrient broth powder (Demerec & Cahn, 1953).

Organisms

The cyst(e)ineless mutants used in this study were auxotrophs derived from *Salmonella typhimurium*, strains LT-2 and LT-7 (Zinder & Lederberg, 1952), isolated by various members of the Department of Genetics, Carnegie Institution of Washington, and made available by Dr M. Demerec.

The wild-type strains grew well on the minimal medium in which sulphate was the sole sulphur source. A series of auxotrophic mutants were isolated from both u.v.-irradiated and non-irradiated cultures of the wild-type strains by penicillin screening (Lederberg, 1950). From the auxotrophs, detected by their ability to grow on nutrient agar but not minimal agar, the cyst(e)ineless mutants were identified by their growth when L-cysteine or L-cystine was added to the minimal medium. They were designated as described by Demerec (1956) by the prefix *cys*, followed by a figure denoting the sequence of isolation; e.g. *cys-1*. In some instances, previously isolated auxotrophic strains including tryptophanless (*try*) and prolineless (*pro*) mutants were used as parental strains for the isolation of double auxotrophs. The derivation of the mutants are listed in Table 1.

Nutritional requirements of mutant strains were established auxanographically; 0.1 ml. samples of an overnight broth culture diluted 10^{-3} were spread over the surface of minimal agar plates. The agar was then removed at several places by a sterile cork-borer and drops of sterile solutions of the supplements placed into the holes. After incubation for 40 hr. at 37°, the presence or absence of haloes of growth around the holes was noted. Alternatively, the strains were spread, after appropriate dilutions, on minimal agar plates

supplemented with the growth factor. After incubation at 37° for 18 hr., the plates were examined for the presence or absence of colonies of *Salmonella typhimurium*.

Table 1. *Sources of stocks of cyst(e)ineless mutants of Salmonella typhimurium*

U.v.-irradiated*		Non-irradiated	
Source	Mutants	Source	Mutants
LT-2 (wild-type)	<i>cys-1, -2, -3, -4, -5, -6, -7, -8, -10, -11, -12, -13, -14, -15, -16, -17, -18, -20, -21, -22, -23, -24</i>	LT-2 (wild-type)	<i>cys-36, -37, -38</i>
		LT-2 try-8	<i>cys-40, -41, -42, -43, -44, -50, -51</i>
LT-2 pro-6	<i>cys-25, -27, -28, -29</i>	LT-2 try-2	<i>cys-45, -46</i>
LT-7 (wild-type)	<i>cys-30, -32</i>	LT-2 try-7	<i>cys-47, -48, -49</i>

* The designation 'U.v.-irradiated' indicates that the mutants listed were obtained from cultures which had been exposed to u.v. radiation. However, since auxotrophic mutants from such cultures were isolated with no greater frequency than from non-irradiated cultures, it is likely that the mutants from both sources are largely 'spontaneous' in origin.

Syntrophism between strains was tested in two ways:

(a) By the parallel-streak method between pairs of mutants. Overnight broth cultures of the two mutants were diluted and streaks were made in parallel about 0.5 cm. apart on enriched minimal agar. After incubation for 5 days at 37°, the growth was then compared with single-streak controls.

(b) By auxanograph testing of culture filtrates and disrupted organisms. Overnight broth cultures were washed, re-inoculated into liquid minimal medium containing 4 µg. L-cysteine/ml. to give about 5×10^8 bacteria/ml., and aerated for 18 hr. at 37°. By this method the required amino acid (cyst(e)ine) was exhausted from medium and organisms; no significant reversion to prototrophy occurred at this high density of inoculation. The organisms were then centrifuged down, washed, concentrated to 2×10^{10} /ml. in minimal medium, and 10 ml. samples disrupted for 2 min. in a 'Raytheon' sonic oscillator, after which time the viable count had fallen to 10^7 /ml. The supernatant fluids from the cultures in limiting cysteine-supplemented medium and the complete disruption products were then tested auxanographically for their ability to support the growth of other mutants.

Growth curves were plotted from cultures aerated at 37° in S-free media with various supplements, inoculated with about 2×10^8 washed organisms/ml. Growth was measured as optical density by means of a Klett-Summerson photoelectric colorimeter which was calibrated with a culture of the wild-type strain LT-2 in the late logarithmic phase of growth. Optical densities of serial twofold dilutions of this culture were plotted against the corresponding numbers of viable organisms/ml. obtained by counts of colonies appearing on plates of nutrient agar spread with appropriate dilutions of the culture and incubated at 37° for 18 hr. The optical density varied linearly with the number of organisms/ml. between the values of 5×10^7 and 2×10^9 , organisms/ml.

Isotopic competition studies used carrier-free ^{35}S -sulphate (Oak Ridge National Laboratory; kindly supplied by Dr A. D. Hershey).

RESULTS

Growth of mutant strains on solid media

The cyst(e)ineless mutants were first tested for ability to utilize sulphate, sulphite, thiosulphate or cysteine. All strains were unable to utilize sulphate but responded to added cysteine. However, when broth cultures of certain mutants were plated on minimal agar (*c.* 10^8 organisms/plate) in which sulphate was the sole sulphur source, at least several dozen prototrophic colonies were

Table 2. *Growth of cyst(e)ineless mutants of Salmonella typhimurium in the presence of various sulphur sources*

All sulphur sources provided to give 10 μg . S/ml. (except 'thiosulphate (a)' provided at 1 mg. S/ml.) incorporated into minimal agar. Plates were incubated for 18 hr. at 37° after inoculation.

Sulphur source	Phenotype				
	<i>cysA</i>	<i>cysB</i>	<i>cysC</i>	<i>cysD</i>	<i>cysE</i>
	Growth response				
Cysteine	+++	+++	+++	+++	+++
Sulphate ($\text{SO}_4^{''}$)	—	—	—	—	—
Sulphite ($\text{SO}_3^{''}$)	++	—	+++	—	—
Thiosulphate ($\text{S}_2\text{O}_3^{''}$)	—	—	+++	+++	—
Thiosulphate ($\text{S}_2\text{O}_3^{''}$) (a)	+++	+++	+++	+++	—
Lanthionine	+++	+++	+++	+++	+++
Cysteic acid	—	—	—	—	—
Dithionate ($\text{S}_2\text{O}_6^{''}$)	—	—	—	—	—
Cysteine sulphinic acid	++	—	+++	—	—
Metabisulphite ($\text{S}_2\text{O}_5^{''}$)	++	—	+++	—	—
Sulphur (S)	+	—	+	+	—
Sulphide ($\text{S}^{''}$)	+	—	+	+	—
Dithionite ($\text{S}_2\text{O}_4^{''}$)	++	—	+++	++	—

+++ = growth equivalent to that with cysteine supplement; ++ = growth slightly less than that with cysteine supplement; + = growth greatly decreased compared to that with cysteine supplement; — = no apparent growth.

produced. This high frequency of prototroph production made nutritional studies difficult and no further investigations were made with these strains (*cys*-9, -19, -26, -31, -35 and -39). The growth responses shown in Table 2 (lines 3 and 4) indicate that they can be separated into four classes exhibiting growth on:

- sulphite only, *cys*-1, -3, -5, -13, -20, -21, -22 and -32 (designated *cysA* mutants);
- on neither salt, *cys*-2, -4, -6, -8, -10, -11, -12, -14, -15, -16, -17, -18, -24, -25, -27, -30, -40, -41 and -45 (designated *cysB* or *cysE* mutants);
- sulphite or thiosulphate, *cys*-7, -38, -43 and -47 (designated *cysC* mutants); or
- thiosulphate only, *cys*-23, -28, -29, -36, -37, -42, -44, -46, -48, -49, -50 and -51 (designated *cysD* mutants).

In subsequent experiments the mutants were streaked on minimal agar + different concentrations of sulphite or thiosulphate at 20, 50, 100, 200, 500 and 1000 $\mu\text{g. S/ml.}$ The response to all concentrations of sulphite up to 500 $\mu\text{g./ml.}$ was the same as that at 10 $\mu\text{g./ml.}$, concentrations above 500 $\mu\text{g./ml.}$ being inhibitory. On thiosulphate supplemented media, the response at all concentrations was identical to that already reported at 10 $\mu\text{g./ml.}$, except at the 1 mg./ml. level, at which concentration in addition to those mutants growing at lower concentrations, a certain number of other strains unable to utilise these lower concentrations were able to grow. These mutants, which respond to thiosulphate only at a concentration of 1 mg./ml., are designated *cysB* mutants (*cys-4*, -10, -12, -14, -15, -16, -18, -24, -25, -27, -40, -41 and -45) and are differentiated by this criterion from those mutants which are unable to utilize thiosulphate at any concentration tested and which form the group designated *cysE* (*cys-2*, -6, -8, -11, -17 and -30). The mutants are thus differentiated into the five phenotypes *cysA*, *B*, *C*, *D* and *E*, as shown in Table 2 by the growth responses on sulphite and thiosulphate. The growth responses were identical when the supplements were added to minimal medium (containing sulphate) or to S-free medium and, with certain exceptions discussed later, prolonged incubation up to 5 days caused no detectable change.

The ability of other sulphur sources to support growth was then investigated. Lanthionine (β - β' -thiodialanine) allowed the growth of all mutants, but neither cysteic acid nor dithionate (S_2O_6) stimulated the growth of any mutant. Both cysteine sulphinic acid and metabisulphite (S_2O_5) supported the growth of the same mutants as did sulphite (*cysA* and *cysC*). Elementary sulphur and sulphide (S^0) gave no stimulation of some groups, and a slow and suboptimal growth with all other strains. Dithionite (S_2O_4), however, was a satisfactory source of sulphur for all *cysA*, *cysC* and *cysD* mutants.

The examination for syntrophism by the parallel-streaking method gave positive results only after 3 days of incubation at 37°, the plates were incubated for 5 days before scoring, when it was seen that certain *cysD* mutants (designated *D*₁ mutants and comprising *cys-23*, -44, -46, -48, -50 and -51) supported the growth of all mutants in the *cysA* and *cysC* groups. Other *cysD* mutants (*D*₂ mutants, *cys-28*, -29, -36, -37, -42 and -49) did not feed any other mutant strain.

Auxanograph testing of disrupted cell suspensions showed that disrupted *cysA* cells fed *cysC* mutants, and that disrupted *cysD*₁ cells fed either *cysC* or *cysA* mutants. Furthermore, disrupted cells of *cysC*, *cysA*, *cysB* and *cysE* mutants fed *cysD*₁ mutants. However, this latter stimulation of *cysD*₁ mutants was also given both by disrupted wild-type cells and by the culture filtrates of both wild-type and of these mutants. Apart from this apparent non-specific stimulation of *cysD*₁ mutants, the supernatant fluids had no growth-promoting activity on any mutant.

Growth of wild-type Salmonella typhimurium in liquid culture

Overnight broth cultures of wild-type strains LT-2 and LT-7 were washed in S-free medium and inoculated into similar medium, either unsupplemented or

supplemented with a sulphur source at $10\text{ }\mu\text{g. S/ml.}$ (when stereoisomeric compounds were used, at $20\text{ }\mu\text{g. S/ml.}$). The cultures were incubated with aeration at 37° , and the optical densities recorded (Fig. 1). The growth rates in cysteine, sulphate, sulphite or thiosulphate media were the same (mean generation time *c.* 50 min.), attaining a maximum of 3×10^9 organisms/ml. which was determined by the glucose content of the medium (2 mg./ml.). The corresponding curves obtained with dithionite, DL-lanthionine or cysteine sulphinic acid

Fig. 1. Growth curves of wild-type *Salmonella typhimurium*, aerated at 37° in liquid medium with cysteine, sulphate (SO_4^{2-}), sulphite (SO_3^{2-}), thiosulphate ($\text{S}_2\text{O}_3^{2-}$), methionine or cysteine acid as a sole sulphur source at equiv. $10\text{ }\mu\text{g. S/ml.}$ medium. Each unit of optical density corresponds to about 10^8 bacteria/ml. O, Cysteine; x, SO_4^{2-} ; Δ , SO_3^{2-} ; $\bullet$, $\text{S}_2\text{O}_3^{2-}$; +, S-free; $\square$, methionine; ∇ , cysteine acid.

(not shown in Fig. 1) were similar. In S-free medium, the organisms grew initially at the optimal growth rate until a population corresponding approximately to a twofold to threefold increase had been obtained. There was little further growth. Analogous results have been described with *Escherichia coli* by Roberts *et al.* (1955) who showed that growth in S-free medium was associated with a utilization of the internal sulphur reserves of the organisms, mainly in the form of glutathione.

When L-methionine was the sole sulphur source, the organisms grew for a period about twice that obtained in S-free medium, after which time the growth virtually ceased. This suggests that methionine cannot act as a sole sulphur source for *Salmonellas*, the increase in growth over that observed in

S-free medium being due only to sparing of the internal sulphur reserves. Roberts *et al.* (1955) showed a similar phenomenon in *Escherichia coli*, and demonstrated that during growth 50% of the sulphur uptake was converted into methionine-containing protein, which would be consistent with these observations. When cysteic acid was the sole sulphur source, there was no growth beyond that obtained in S-free medium.

Fig. 2. Growth curves of cyst(e)inless mutants of *Salmonella typhimurium* aerated at 37° in liquid medium with cysteine, sulphate (SO_4^{2-}), sulphite (SO_3^{2-}), thiosulphate ($\text{S}_2\text{O}_3^{2-}$), cysteine sulphinic acid (CSA), cysteic acid (CA), or dithionite ($\text{S}_2\text{O}_4^{2-}$) as a sole source of sulphur. —○—○— represents growth with cysteine (*cysA*, *B*, *C*, *D*, *E*); $\text{S}_2\text{O}_3^{2-}$ (*cysC*, *cysD*); SO_3^{2-} , $\text{S}_2\text{O}_4^{2-}$ (*cysC*). —×—×— represents growth with SO_3^{2-} (*cysA*); or with $\text{S}_2\text{O}_4^{2-}$ (*cysA* and *cysD*). —△—△— represents growth in either S-free medium or with SO_4^{2-} (*cysA*, *B*, *C*, *D*, *E*); SO_3^{2-} or CSA (*cysB*, *D*, *E*); $\text{S}_2\text{O}_3^{2-}$ (*cysA*, *B*, *E*); and $\text{S}_2\text{O}_4^{2-}$ (*cysB*, *E*), and —●—●— represents growth of all mutants in broth. All sulphur sources provided at 10 µg. S/ml. The interrupted line shows the effect of addition at time marked by arrow of a further 10 µg. S/ml. of either SO_3^{2-} or $\text{S}_2\text{O}_4^{2-}$ to the medium. Each unit of optical density corresponds to c. 10^8 bacteria/ml.

Growth of mutant strains in liquid culture

The growth of the cyst(e)inless mutants in cysteine-supplemented liquid media was, in general, rather less rapid than that of the wild-type strain (Fig. 2), giving a mean generation time of about 65 min., eventually attaining the same maximum of 3×10^9 organisms/ml. In S-free medium or in sulphate medium no mutant of any group was able to grow to any significant extent. The growth of *cysC* strains in media supplemented with sulphite, thiosulphate, dithionite

or cysteine sulphinic acid and that of *cysD* strains in thiosulphate-supplemented medium showed the same type of growth response as with the cysteine supplement. However, *cysA* strains in sulphite or cysteine sulphinic acid-supplemented media, and *cysA* and *cysD* strains in dithionite-supplemented medium, grew more slowly (mean generation time about 100 min.) and attained a lower maximum of *c.* 10^9 organisms/ml. No growth of *cysA* strains was observed in thiosulphate-supplemented medium, nor of *cysD* strains in sulphite or cysteine sulphinic acid-supplemented media. *CysB* and *cysE* strains could not grow on any of these sulphur sources except cysteine, in which case the growth response was similar to that given by other mutants with this sulphur source. All the mutant strains at first grew more rapidly in broth (mean generation time 35–40 min.) than in cysteine-supplemented defined medium, but there was little growth beyond 10^9 organisms/ml. Further growth to the normal (3×10^9) yield could be achieved by the addition of cysteine ($5 \mu\text{g./ml.}$). Since this apparent deficiency of cyst(e)ine in broth would tend to select any reversions to wild type, cyst(e)ineless mutants were grown in broth fortified with cysteine ($20 \mu\text{g./ml.}$).

Isotopic competition experiments

Wild-type organisms, after overnight growth in broth at 37° with aeration, were washed twice and inoculated into S-free medium + ^{35}S -sulphate at $0.05 \mu\text{C/ml.}$ + ^{32}S -sulphate at $3 \mu\text{g. S/ml.}$ to give 10^8 organisms/ml. Methionine, cysteine, sulphite, dithionite, cysteic acid or thiosulphate (all at $3 \mu\text{g. S/ml.}$) were added individually to parallel cultures. All cultures were incubated at

Table 3. *Suppression of ^{35}S -sulphate uptake by Salmonella typhimurium-LT2 in the presence of non-radioactive competitors*

Non-radioactive supplement (a)	Uptake of radiosulphate (% of control)	
	(b)	(c)
None (control)	100	100
L-Cysteine	1	1
Thiosulphate ($\text{S}_2\text{O}_3^{2-}$)	2	3
Dithionite ($\text{S}_2\text{O}_4^{2-}$)	17	60
Sulphite (SO_3^{2-})	37	—
DL-Methionine	40	—
Cysteic acid	100	—

(a) Sulphur concentration of non-radioactive compound, $3 \mu\text{g./ml.}$ medium.

(b) Total sulphate in each culture, $3 \mu\text{g./ml.}$ medium.

(c) Total sulphate in each culture, $30 \mu\text{g./ml.}$ medium.

37° with aeration. Samples were removed at intervals, optical densities recorded and radioactivity assays made of the washed organisms. At all times the uptake of isotope by the control culture (containing ^{32}S -sulphate) was directly proportional to the amount of growth as measured by the optical density. Table 3 shows the relative radioactive uptakes of cultures containing other sulphur sources after 2.5 hr. growth, and indicates that cysteine,

thiosulphate and dithionite were preferred sulphur sources, in that sulphate uptake was decreased in the presence of equimolar concentrations of these compounds. Methionine or sulphite decreased the uptake to a lesser extent, and cysteic acid had little effect. The results of similar experiments using sulphate at $30\text{ }\mu\text{g./ml.}$ and $0.5\text{ }\mu\text{C/ml.}$ and certain competitors at $3\text{ }\mu\text{g./ml.}$ are also shown in Table 3.

*Biochemical heterogeneity within *cysB* group*

From Table 2 it will be seen that the group of mutants designated *cysB* can be defined nutritionally by their inability to give detectable growth on low ($10\text{ }\mu\text{g. S/ml.}$) concentrations of either sulphite or thiosulphate, although they utilize thiosulphate at higher (1 mg. S/ml.) concentrations after incubation for 18 hr.

Fig. 3. Distribution of colony sizes obtained from a culture of *cysB-18* plated on minimal medium containing sulphite after the following treatments:

- (i) Growth in cysteine-supplemented minimal medium (cysteine grown). Selection of a colony (A) after above treatment having a maximal size on sulphite medium ($1\text{--}1.25\text{ mm.}$) and replating on minimal sulphite medium after:
 - (ii) Growth in cysteine-supplemented liquid medium (cysteine grown from A).
 - (iii) Suspension in saline (dilution from 'A').
 - (iv) Growth on sulphite-supplemented liquid medium (sulphite grown from A).
- Growth on cysteine-supplemented minimal medium was similar after all treatments and is illustrated in top right histogram.

However, when the duration of incubation on sulphite- or thiosulphate-containing ($10\text{ }\mu\text{g./ml.}$) medium was increased to 40 hr., certain of the mutants (*cysB-10*, *-18*, *-24* and *-25*) when spread at high densities, gave isolated colonies,

whereas no other mutants of this group gave any perceptible growth. Since there appeared to be no concomitant growth on sulphate to suggest 'leakiness', the phenomenon was further investigated as a result of which the *cysB* mutants are subdivided into three secondary phenotypes.

First phenotype. A single clone of *cysB-18* was grown to saturation in cysteine-supplemented medium, and after centrifugation and dilution, plated on sulphite-containing minimal medium. (In all experiments, the diluted samples plated on sulphite medium were also plated on cysteine medium to give a count of viable organisms, and on sulphate medium to give a control of reversion or leakiness.) After incubation for 48 hr. at 37° c. 0.5% of the organisms plated gave colonies with a diameter of about half (1.5 mm.) that formed on cysteine (3 mm.). Another 0.5% of the organisms produced colonies which were 0.8–1.5 mm. in diameter, and a further 50% gave colonies of diameters ranging from 50 to 800 μ . The total colonies, representing about 50% of the number of colonies on parallel platings on cysteine medium, appeared to show a continuous variation in size from a minimum of 50 μ . to a maximum of 1.5 mm. On further incubation up to 5 days the colonial sizes increased proportionally to a maximum diameter of 3 mm., but there was little increase in the total number of colonies, and the continuous variation in size was maintained. Several of the large colonies were subcultured on sulphite medium, either immediately after dilution in saline, or after an intermediate period of growth for 18 hr. at 37° from a small inoculum (c. 200 organisms/ml.) in either cysteine or sulphite-supplemented liquid minimal medium. The distributions of colonial sizes on sulphite-containing medium after these treatments are summarized in Fig. 3. In no cases were colonies produced on sulphate-supplemented medium. In a limited number of experiments, mutants *cys-24* and *-25* showed characteristics similar to those of *cys-18* described above, as did all three strains on thiosulphate medium. No increase in growth was detected when any of these strains was aerated for periods up to 18 hr. at 37° in liquid minimal media + sulphite or thiosulphate.

Second phenotype. This was exhibited by *cysB-10*. Cultures grown in cysteine liquid minimal medium gave colonies of uniform size when plated on either sulphite- or thiosulphate-supplemented minimal media, although the colony diameter was only a quarter of that resulting after growth under the same conditions on cysteine medium. The number of colonies was similar on all three media. Growth curves in sulphite-supplemented medium showed no increase in lag and a slow steady growth with a mean generation time c. 10 hr.

Third phenotype. This is as originally described: there is no perceptible growth on sulphite or thiosulphate, except when the concentration of the latter salt is raised to 1 mg. S/ml. This phenotype is characteristic of the remaining members of the *cysB* group (*cysB-4*, *-12*, *-14*, *-15*, *-16*, *-27*, *-40*, *-41* and *-45*).

When *cysC* mutants were plated on the sulphite- or thiosulphate-supplemented media used in these experiments, colonies of the same number and size as those found on cysteine-supplemented medium resulted.

DISCUSSION

From the present studies the existence of a class of mutants (*cysA*) which use sulphite equiv. $10\mu\text{g. S/ml.}$ but not thiosulphate (except at a hundred-fold concentration) makes it seem unlikely that in *Salmonella*, cyst(e)ine is formed from sulphate through an unbranched reductive pathway. Hockenhull (1949) suggested that sulphite may be reduced in one of several ways. The simplest route is its direct reduction through the metabisulphite ($\text{S}_2\text{O}_5^{2-}$) and dithionite ($\text{S}_2\text{O}_4^{2-}$) to thiosulphate, but it may alternatively be reduced to the unstable sulphyxylate ($\text{SO}_2^{\cdot -}$). This latter compound may then either dimerize to form thiosulphate, or react non-enzymically with further sulphite to give dithionite, which is then spontaneously reduced by more sulphyxylate to thiosulphate.

Fig. 4. Schematic diagram to represent possible pathway of sulphur metabolism in *Salmonella typhimurium*. Interrupted lines represent reactions which may occur in the absence of enzymes. R_a , R_b , etc., represent binding sites or non-diffusible organic radicals holding diffusible ions within organisms. A, B, C, D and E represent the location of metabolic blocks. Flattened circles denote that the participation of the two reactants is necessary for the reaction to proceed in the direction of the arrows.

Thus sulphyxylate, once formed, may be converted to thiosulphate in one of two ways without enzyme participation or further reduction. These interconversions, included in Fig. 4, appear to offer a satisfactory basis for summarizing the growth responses and other data obtained with the cyst(e)ineless *Salmonella* mutants if one assumption be made, namely, that dithionite may be reduced by sulphyxylate to give, not thiosulphate, but rather a subsequent intermediate denoted as 'X'. In this compound the sulphur atom would not have already been incorporated into the cyst(e)ine carbon chain, and since one of the sulphur atoms of the thiosulphate molecule is in the lowest reduction state, the conversion of thiosulphate to 'X' need not necessarily be by reduction. The interrupted lines in Fig. 4 denote those reactions which, according to the evidence summarized by Hockenhull, may proceed in the absence of enzymes, and which would correspond to those steps which do not appear to be under genetic control, since no mutants have been isolated with a phenotype indicating a metabolic block at these points.

According to this scheme (Fig. 4), *cysC* mutants, blocked between sulphate and sulphite, would be expected to grow at a rate equivalent to wild-type on

either sulphite or thiosulphate, by utilizing either pathway. *CysD* mutants could also utilize thiosulphate by the direct pathway, but could not utilize sulphite, being unable to convert this compound to sulphonylate. On dithionite, *cysD* mutants would be required to make use of the shunt pathway. *CysA* mutants could not utilize thiosulphate, but could utilize dithionite via the auxiliary pathway, and sulphite also by this pathway, since reduction of sulphite to sulphonylate is possible, because of the integrity in *cysA* mutants of the step which is shown blocked at 'D' in *cysD* mutants (Fig. 4). *CysB* and *cysE* mutants would not be expected to utilize any of these inorganic salts as a source of sulphur. These metabolic potentialities are equivalent to those found by experiment and summarized in Figs. 1 and 2, and constitute the main evidence in favour of the scheme represented in Fig. 4.

The ability of *cysC* mutants to grow at the wild-type rate on dithionite may be due to its conversion to sulphite and sulphonylate, which, according to Newton-Friend (1947), proceeds at slightly acid pH values. That *cysD* mutants do not appear to be able to utilize this pathway of dithionite metabolism may be due to the accumulation of sulphite which cannot be further reduced in this direction.

Syntrophism by certain *cysD* mutants (D_1) mutants could presumably be due to the accumulation of sulphite-containing intermediates which can be utilized by either *cysC* or *cysA* mutants, and similarly the ability of *cysA* mutants to feed *cysC* mutants might well be due to the accumulation of thiosulphate-like intermediates. This accumulation should also support the growth of *cysD* mutants, but this is unable to be demonstrated in the presence of non-specific syntrophism of these mutants with the wild-type strain and all other mutants. The inability of the other *cysD* mutants (D_2 mutants) to support growth of either *cysC* or *cysA* mutants is not readily explained from the biochemical data.

In addition, there appears to be a by-pass reaction not normally operative, but one in which the thiosulphate may be converted to the unknown intermediate 'Y' further along the pathway, allowing *cysA* and *cysB* mutants to utilize thiosulphate when this is supplied at a concentration a hundred times greater than that normally utilizable. Certain mutants of *Aspergillus nidulans* have also been found, by Hockenhull (personal communication) to require concentrations of thiosulphate of 50–100 μ g. S/ml. for growth.

An alternative explanation for the inability of *cysA* mutants to be accommodated within a simple unbranched pathway of sulphate reduction through sulphite and thiosulphate respectively, by reason of their inability to utilize thiosulphate (at 10 μ g./ml.) might be supposed to be an impermeability of these mutants to thiosulphate, since growth can be demonstrated at the 1 mg./ml. level. However, this would imply that the purported single mutational event was capable of producing impermeability to thiosulphate, together with either an impermeability to sulphate or a metabolic block between sulphate and cyst(e)ine, since there is no growth on sulphate. The immediate uptake of radiosulphate by wild-type and mutant in the absence of growth, measured by the technique of Roberts *et al.* (1955) corresponded to between 65 and 75 %

of the volume of the organism, and no difference in this uptake could be detected between *cysA* mutants and those of other mutants or of wild-type *Salmonella*, suggesting that there is little difference in the permeability of any of these strains to sulphate. An unbranched sequence of sulphate reduction would therefore require that all *cysA* mutants are impermeable to thiosulphate, and in addition have a metabolic block in the pathway of reduction of sulphate to cyst(e)ine.

Previously reported experiments with cysteine sulphinic acid support the concept of an inorganic pathway at least as far as thiosulphate in all organisms except *Aspergillus nidulans* (Shepherd, 1956). In this latter study, alternative inorganic and organic pathways of sulphur reduction were suggested, but the proposed scheme required one of the mutants to have simultaneous metabolic blocks in two of the pathways concerned. Moreover, in *Escherichia coli* the ability of pyruvate to supply the carbon source for cyst(e)ine synthesis, as postulated by Shepherd, was considered unlikely by Roberts *et al.* (1955) because of its inability to compete with glucose for this function, whereas under the same conditions serine was able almost entirely to suppress the contribution of carbon from glucose to cyst(e)ine.

The concept of an inorganic pathway of reduction is supported by the present studies, since no accumulation of either cysteic acid or cysteine sulphinic acid was detected in the supernatant fluid or disrupted cells of representative mutants or wild-type grown in the presence of radiosulphate and tested chromatographically by the methods of Chapeville & Fromageot (1954), even though these disrupted cell preparations were active in stimulating the growth of other mutants as detailed previously. The inability of sulphide and free sulphur to give adequate growth responses supports similar findings by Hockenhull and by Steinberg, who suggested that these compounds were unlikely to be direct intermediates. The isotopic competition experiments suggest that thiosulphate in particular is a direct intermediate in view of its ability almost completely to exclude sulphate uptake on an equimolar basis. The ability of this compound to act as a competitor to sulphate was even greater in the experiments of Roberts *et al.* (1955) with *Escherichia coli*. However, it would appear that the demonstration by these same authors of the ability of sulphite to suppress sulphate uptake to a much greater extent in *E. coli* than was found in *Salmonella* suggests a different mechanism of participation of sulphite in the two systems. Moreover, *Salmonella typhimurium* appears to differ from previously studied organisms by its inability to utilize cysteic acid as a sulphur source.

The lack of apparent syntrophism except after 3 days of incubation at 37° with growing organisms and the presence of growth-promoting substances within the cells even after a dilution of 1/500, compared with the inactivity of the corresponding supernatant fluids suggests that these intermediates are not diffusible, and are normally liberated only after autolysis. It would appear, therefore, that if they are inorganic ions they do not exist in a free state but may be bound by some large non-diffusible organic radicals, at sites within the cell (Roberts *et al.* 1955), which are denoted as R_a , R_b etc. in Fig. 4.

The mutants *cys-10*, *-18*, *-24* and *-25*, classified provisionally as *cysB* mutants on the basis of primary nutrition in Table 2, appear on further investigation to be intermediate in phenotype to *cysB* and *cysC*, and cannot be easily accommodated within the proposed pathway.

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