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Genetics of Sulfate Transport by *Salmonella typhimurium*[1]

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Sixty-four mutants were isolated from the LT-2 wild-type strain of *Salmonella typhimurium* by selecting for chromate resistance. The majority of lesions were shown to lie in the *cysA* gene. (i) The mutants cannot take up sulfate, a finding which verifies the role of *cysA* in sulfate transport. In addition, 52 sulfate-transport mutants isolated without chromate selection were defective in the *cysA* gene. (ii) Most had less than 25% of the binding activity of the wild-type strain. (iii) Most had normal sulfite reductase (H_2S-nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.8.1.2) activity. (iv) Their sulfate-binding protein (binder) appears electrophoretically and immunologically normal. (v) Amber *cysA* mutants also make apparently normal binder in small amounts. (vi) All classical *cysA* mutants tested, including two with long deletions, had normal binding activity. From these observations, it is suggested that the *cysA* gene does not code for the binder. But many mutations in this gene reduce the binding activity in some unknown way. Other mutants, identified as *cysB* mutants, had neither binding nor uptake activities and their sulfite reductase activities were similarly reduced, thus confirming the regulatory role of the *cysB* gene. When binder was detectable, it had wild-type properties. No mutations in the binder gene were found among more than 100 mutants examined. Thus, sulfate binding has not been established as a part of sulfate transport. However, the production of binder is intimately connected with *cysA*, the established sulfate transport gene, and is regulated by the same mechanism that regulates both transport and the rest of the cysteine biosynthetic pathway.

This work was performed to gain genetic evidence regarding the parts of the sulfate-transport system of *Salmonella typhimurium*, and in particular to investigate the possible role in transport of a sulfate-binding protein or "binder" (9, 18-20). Indirect evidence implicates the binder in transport: the protein binds 1 mole of sulfate per mole of protein and this is its only activity detected so far (18); it is located near the cell surface (21); binding and transport are similarly affected by osmotic shock or spheroplast formation, inhibition by a series of anions, repression by growth on cysteine, and derepression by growth on djenkolic acid (20). More direct evidence regarding the role of binder in transport would be obtained from a study of a binder-negative mutant with a proven mutation in the structural gene for the binder. Such a mutant has not yet been isolated. The present paper reports a study of sulfate-transport mutants, which provides further indirect evidence relating binder to transport.

A large number of cysteine-requiring mutants of *S. typhimurium* have been isolated and studied by Demerec and his co-workers (2, 13). Genetic studies of those mutants were carried out by use of the P22-mediated transduction technique. Mutants were classified into nine genetic regions containing 14 complementation groups, according to genetic and nutritional studies. Two of these regions containing the *cysA* and *cysB* genes can each be further divided into three complementation groups. Five genes, *cysC*, *cysD*, *cysH*, *cysI*, and *cysJ*, are linked to one another, as shown by P22 transduction. The remaining four genes can be transduced independently of one another, and of the *cysC-D-H-I-J* cluster. After Hfr strains became available in *Salmonella*, the positions of the *cys* genes on its circular chromosome were determined (22).

All *cysA* mutants studied to date lack sulfate uptake activity. An earlier report indicated that

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some *cysA* mutants have normal binder (20). Evidence will be presented in this paper which supports the suggestion that *cysA* is not the structural gene for the binder. The low-binder mutants reported earlier will be shown to be *cysA* mutants, and therefore not structural gene mutants for the binder.

From previous information, one expects to find three kinds of mutations that affect sulfate transport: (i) in the *cysA* region that controls the structures of parts of the transport system (20); (ii) in the gene(s) that regulates the formation of the transport system, such as *cysB*; and (iii) in the structural gene for the binder, provided it is required for transport. The binding and transport properties of these mutants should provide indications of the parts of the entire transport system and the function of the parts.

MATERIALS AND METHODS

Bacterial strains and media. The *S. typhimurium* and *Escherichia coli* strains used are listed in Table 1. *S. typhimurium* prototrophic strain LT-2, classical cysteine auxotrophs, and transducing phage PLT-22 H1 were obtained from P. E. Hartman and K. E. Sanderson. *E. coli* strains and *S. typhimurium* strains carrying *E. coli* episomes were obtained from A. Newton. *S. typhimurium* was grown on E salts-glucose with cysteine, sulfate, or djenkolate as the sulfur source (20). An enriched medium for the isolation of temperature-sensitive cysteine auxotrophs was prepared by supplementing E salts and sulfur source with 10-fold diluted Cystine Assay Medium (Difco), L-methionine at 150 μg/ml, and thiamine·HCl and DL-thioctic acid, each at 5 μg/ml. *E. coli* was grown on Penassay Broth (Difco) supplemented with thiamine·HCl at 5 μg/ml. Bacteria were grown with swirling at 37 C unless otherwise indicated.

TABLE 1. *Bacterial strains used*[a]

Strain	Characteristics
E. coli strains:	
E5014	F'lac Pro Sm^S
MNG590	F^-lac$^-$(z^-UGA), Sm^R
MX90	F^-lac$^-$(z^-UAA)
s4	F-lac$^-$(z^-X74); IGA suppressor
S. typhimurium strains:	
Pro25	*Deletion in pro gene*
Pro25:F'lac z^-X82	Amber mutation on the episome
Pro25/F'lac z^-U118	Ochre mutation on the episome
Pro25/F'lac z^- NG590	UGA mutation on the episome
Sp25	Low-binder mutant (*cysAa*)
Sp30	Low-binder mutant (*cysAb*)
TrpA8	Tryptophan auxotroph
TrpD10	Tryptophan auxotroph

[a] Cysteine auxotrophs (derivatives of *S. typhimurium* LT-2 (2, 13): *cysA1, 3, 13, 20* (deletion), *21, 32, 197, 201, 205, 265, 272, 533* (deletion); *cysB12, 15, 403, 482; cysC200, 428, 514; cysCD519* (deletion); *cysD23, 220, 313; cysE2, 17; cysH7, 75* (deletion), *271; cysI149, 279; cysJ266, 288, 299.*

Chemicals. All chemicals were of reagent-grade quality and were purchased from the sources previously cited (20, 21).

Isolation of mutants. Chromate-resistant mutants were isolated as described previously (20). Temperature-sensitive revertants were selected on plates as colonies that grew at 25 C but not at 41 C, after one of the original mutants had been spread.

Sulfate transport-negative mutants were also isolated without chromate selection. *S. typhimurium* LT-2 was mutated with *N*-methyl-*N'*nitro-*N*-nitrosguanidine (nitrosoguanidine) as previously described (20). Sulfate transport-negative mutants were selected on plates by virtue of their ability to use L-cysteine sulfinic acid and their inability to use either sulfate or thiosulfate as a sulfur source.

Temperature-sensitive cysteine auxotrophs were obtained after mutagenesis with nitrosoguanidine under Gesteland's conditions (4). These cells were separated into 12 cultures, which were then grown in rich medium with cysteine for 6 hr at 28 C followed by 20 hr at 41 C. The cells were then shifted to enriched medium with sulfate and were grown at 41 C for two generations, at which time they were treated with penicillin G according to the procedure described by Gorini and Kaufman (5). After removal of the penicillin and outgrowth in Penassay Broth at 37 C, temperature-sensitive cysteine auxotrophs were selected on plates, as colonies that grew on enriched medium with cysteine agar at 41 C, and on enriched medium with sulfate agar at 28 C but not at 41 C.

Transduction. Standard phage techniques were used for transduction and abortive transduction (1, 17). Nonsense mutants were identified by replica-plating their cys^+ revertants onto a lactose-minimal plate containing a lawn of a *S. typhimurium* strain that carries an *F'lac* episome with a nonsense mutation in the *lacZ* gene. Since *S. typhimurium* is unable to ferment lactose, these revertants cannot grow on the lactose plates unless they receive an *F'lac* episome from the cells on the lawn, and unless the supressor of the *cys* mutation also supresses the *lacZ* nonsense mutation. The growth of a revertant on the lactose plate constitutes evidence that the original mutation was a nonsense mutation (11).

Physiological and biochemical tests. Sulfate-binding activity was measured by the resin assay method (20). In later studies, the method was modified slightly to make it more rapid and accurate: the supernatant cell suspension or cell-free extract was passed through a small plug of glass wool in a Pasteur pipette; the glass wool traps fine resin particles but not the bacteria. Sulfate transport was assayed by the membrane filter technique as previously described (21). Osmotic shock into water was carried out according to Neu and Heppel (14). Acrylamide gel electrophoresis was at *p*H 8.6 [as before (18)], with a Canalco apparatus. Antibinder antibody was prepared as previously described (21). The immunological test was by the double-diffusion method according to Ouchterlony (16). Sulfite reductase [H_2S-nicotinamide adenine dinucleotide phosphate (NADP)

oxidoreductase, EC 1.8.1.2] was measured in cell-free extracts as sulfite-dependent reduced NADP oxidation by the method of Siegel et al. (23). Cell-free extracts were prepared as follows: derepressed cells were harvested in late log phase, washed with E salts, suspended at an optical density at 540 nm of 80 in 50 mM potassium phosphate buffer (*p*H 7.7), broken by sonic oscillation (Branson Sonifier), and centrifuged at 100,000 × *g* for 30 min at 4 C; the supernatant fluid (cell-free extract) was removed for storage at 2 C and assay within 24 hr. Protein in extracts was determined by the procedure of Lowry et al. (10), with bovine serum albumin as a standard.

RESULTS

Characterization of chromate-resistant mutants. Since there was no direct way of selecting sulfate binding-negative mutants, transport-negative mutants were obtained by selecting chromate-resistant cells (20). These were then tested for their ability to transport and bind sulfate. Forty chromate-resistant mutants were induced by the mutagen nitrosoguanidine (strains *N1* to *N40*), and another 24 spontaneous mutants (strains *S1* to *S24*) were selected by four successive dilutions of overnight growth cultures in medium containing 0.5 mM chromate. All of the selected mutants were found to be cysteine auxotrophs, and lacked sulfate-transport activity.

The relative sulfate-binding activities of the mutants are shown in Table 2. Of 36 nitrosoguanidine-induced mutants tested, 9 showed nearly normal (above 50%) binding activities. Most of the remaining mutants had 10 to 25% of the control activity. *N38a* was the only mutant with near zero binding activity. Among the 24 spontaneous chromate-resistant mutants, only 4 had less than 50% of the normal activity.

The positions of the newly isolated mutations relative to known cysteine genes were studied by P22 phage-mediated transduction. The *cysA20* and *cysA533* mutations are long deletions in the *cysA* region; they cover all known *cysA* point mutations. The newly isolated mutations were first mapped by use of these deletions. At least 28 (of 40) nitrosoguanidine-induced mutants and all 24 spontaneous mutants did not produce any transductants after infection with phage grown on mutant *cysA20*. Those strains which were crossed with strain *cysA533* gave the same results obtained with strain *cysA20*. Therefore, these chromate-resistant mutants appeared to have lesions in the *cysA* region. Mutants *N15*, *N16*, *N37*, and *N38*, on the other hand, produced recombinants at high frequency after infection with phage from either of the long *cysA* deletions or from a point mutant, *cysA205* (Table 3). Their further examination will be discussed below. Transduction of mutants *N17* and *N19* gave small colonies; *N24* was temperature-sensitive.

TABLE 2. *Whole-cell binding activity of chromate-resistant mutants*

Activity[a]	Mutants
0–10	*N3, N12, N18, N32, N34, N38, N40*
11–25	*N1, N6, N7, N8, N10, N15, N16, N20, N27, N29, N35, N36, N37, N39, SP30, S14*
26–50	*N11, N19, N28, N33, SP25, S18, S23, S24*
>50	*N5, N13, N14, N21, N22, N23, N25, N26, N31;* all 22 other *S* mutants; all Demerec *cys* mutants (2) tested

[a] Percentage of wild-type activity (0.06 nmoles per mg of protein).

TABLE 3. *Transduction with cysA mutants*[a]

Recipient	Control	Donor			
		+	A20	A205	A533
N15	0	50	80	92	49
N16	0	41	190	88	76
N17	0	—	300	—	—
N18	0	82	0	1	0
N24	0	—	26	—	—
N37	0	36	88	38	37
N38	0	146	289	180	206
A288 ...	1	110	1	6	3

[a] In each experiment, 0.1 ml of overnight nutrient broth culture and about 10^{10} phage particles were plated out on a sulfate plate. Control plates received no phage. The number of recombinants (colonies per plate) was scored after 2 days of incubation at 37 C.

Sulfate-transport mutants isolated without chromate selection. Most of the mutations isolated by chromate selection were mapped in *cysA*. To obviate the possibility that chromate selection was eliminating the very class of mutants desired (i.e., with mutations in the structural gene for the binder), the penicillin selection method was used to obtain sulfate transport-negative mutants (see Materials and Methods). The 52 mutants obtained were transduced with phage grown on *cysA20;* no transductants capable of growing on sulfate plates were obtained. All 52 isolates are thus tentatively *cysA* mutants. They were not further studied.

Mutants in cysA. The *cysA* locus can be divided into three complementation regions, *cysAa*, *cysAb*, and *cysAc* (13). Chromate-resistant mutants of the "*cysA*" type recombined with all *cysA* point mutants only at low frequency (Table 4). The data from these two point crosses were insufficient for fine-structure mapping.

Classification of mutants into complementation groups was tried by abortive transduction (17), in the hope of finding a new group. A low-binder mutant should complement with every *cysA* point mutant, if the gene for the binder does not correspond to one of the three *cysA* cistrons.

TABLE 4. *Transductions among mutants which map in the* cysA *gene*[a]

Recipient	Control (selfing)	Donor								
		(+)	*N3*	*N6*	*N10*	*N19*	*N27*	*N28*	*N35*	*N39*
Aa205	0	66	6	12	6	24	12	26	12	26
Ab12	2	165	4	38	105	228	60	13	31	96
Ab21	0	101	5	35	40	137	64	17	8	72
Ab201	0	88	10	25	52	142	87	8	26	68
Ac22	0	91	28	46	32	156	97	85	122	9
N39	0	70	23	12	15	69	81	8	12	0
Sp30	0	138	7	33	106	316	23	47	2	0
H271	0	324	532	668	1,018	542	372	2,092	648	1,518

Recipient	Control	Donor			
		(+)	*Ab21*	*Ab201*	*Ac22*
N6	0	362	21	31	77
N10	1	371	72	17	20
N19	69	226	163	121	129
N27	0	223	63	34	20
N39	0	284	98	59	3
Ab21	0	514	0	1	16
Ac22	0	272	22	27	0
H271	1	2,166	2,844	1,451	1,206

[a] In each cross, 0.1 ml of an overnight nutrient broth culture and about 10^{10} phage particles were plated on a sulfate plate. The number of recombinants per two petri dishes is given in the body of the table. Some reciprocal crosses are given in the lower half of the table. The control represents the colonies obtained when phage grown on a mutant were used to transduce that same mutant (selfing).

However, each mutant failed to produce abortive transductants with mutants in one of the three cistrons (table 5). The absence of abortive transduction means by definition that the two mutational events exist in the same complementation group. Thus, low-binder mutations seem to be located in each of the three complementation groups of *cysA*.

Polar mutations could affect not only the product(s) of gene *cysA*, but also the expression of an adjacent gene in the operon that might code for the binder protein (15). Twenty-five nitrosoguanidine-induced mutants were examined for nonsense mutations by the method of Margolies and Goldberger (11). Three amber mutants, *N19*, *N27*, and *N18*, were found, and they contained lesions in genes *cysAa*, *cysAb*, and *cysAc*, respectively. By abortive transduction, complementation was found between each pair; therefore, the mutants do not have complete polar mutations. All produced low levels of binding activity (Table 2), as do many missense mutants.

TABLE 5. *Summary of complementation tests*[a]

Complementation group	Mutants
cysA a	*N10, N19, Sp25*
b	*N1, N3, N12, N20, N21, N26, N27, N28, N33, N35, N40, Sp30*
c	*N7, N18, N29, N33, N34, N36, N39*
Non-*cysA*	*N15, N16, N37, N38*
Unclassified	*N2, N5, N8, N9, N11, N13, N14, N17, N22, N23, N24, N25, N30, N31, N32*

[a] All chromate-resistant mutants tested are classified according to the results of complementation tests.

Many of the mutants could be reverted by nitrosoguanidine or 2-aminopurine, and so probably contain point mutations because reversion of "frameshifts" by nitrosoguanidine is rare (24). The lowered binding activity of these mutants thus does not seem to be caused by the polar effect of a *cysA* mutation on an adjacent binder gene.

Non-cysA mutants. Mutations in strains *N15*, *N16*, *N37*, and *N38*, which recombined with *cysA20*, were tested for their possible linkage to other known cysteine mutations. Their growth responses on agar plates containing medium E supplemented with various sulfur sources suggested that *N15* and *N16* are I or J mutants, *N37* is a C, D, or H mutant, and *N38* is an A, B, C, D, or H mutant.

Next, each was examined by cotransduction. Mutants *N15* and *N16* were transduced with phage grown on strain *cysH271* and plated on cysteine sulfinic acid-supplemented plates; there was 70% or more recombination of their lesions with the *cysH* locus (as shown by replica plating on sulfate-supplemented plates). Strains *N15* and *N16* probably have mutations in the *cysI* or *cysJ* genes, which are contransducible with *cysH*.

Mutant *N37* was similarly used as a donor in a cross with *cysI270*, and their lesions were found to be linked by transduction; mutation *N37* is likely to be in gene *cysC*, *cysD*, or *cysH*. Cotransduction of the lesion in strains *N38a* and *N38b* (large- and small-colony isolates of *N38* on cysteine sulfinic acid plates) with mutation *trpD10* was observed. These mutations are thus probably in *cysB*, a gene which cotransduces with *trp*.

Additional support was obtained with intracistronic transduction tests. Mutant *N15* recombined with phage obtained from known *cysI* and *cysJ* mutants at lower frequencies than with phage from *cysCD519*, consistent with the results obtained by nutritional and cotransduction tests. Mutations *N15* and *N16* did not recombine with each other; they are probably identical. Mutant *N37* did not recombine with phage from the deletion mutant *cysCD519*, and so it must be a *cysC* or *cysD* mutant. Mutation *N38a* recombined with *cysB* mutations only at low frequencies. Mutations *N38a* and *N38b* are probably at an identical point, since they did not recombine.

The *cysB* gene(s) is considered to be regulatory. The genes *cysC*, *cysD*, *cysH*, *cysI*, and *cysJ* are genes for known steps of the cysteine biosynthetic pathway. Also, strain *cysCD519*, which contains a deletion, makes the binder. Therefore, it is highly unlikely that these genes also code for the binder. Nevertheless, some mutations in these genes produce strains with low binding activities, as will be demonstrated later.

Biochemical characterization of cysA mutants. Various *cysA* mutants have different binding activities (Table 2). The diminished binding could result from a difference in the quantity of the binder protein, or from its state in the cell, or from its structural properties. The tests described below suggest that the quantity of the binder depends on mutations in the *cysA* gene.

An in vivo inhibitory interaction of gene *cysA* products with the binder could cause *cysA* mutants to have lower binding activities. To test this possibility, binding activities of intact cells and of shock fluids prepared from them were compared. The cells of all mutants lost 70 to 90% of their binding abilities after two successive osmotic shocks. No more activity was found in a shock fluid than that which was lost from the cells. Thus, the low-binding *cysA* mutants appear actually to make less binding material, or else the inhibited binder is also not released by osmotic shock.

If the binder leaks into the medium, lower binding activity would remain with the cells. Assay of media from each of four mutants, *N18*, *N19*, *N27*, and *cysCD519*, revealed about 5% leakage, far too little to account for the decreased binding activities of these cells.

As another possibility, low-binder *cysA* mutants might be unable to transfer binder into its proper location on the periplasmic side of the cell membrane. Then the binder should be present in the cytoplasm; it should not be released by osmotic shock. To test this possibility, binding assays were performed on cell-free extracts from control and mutant cells. In no case was more binding activity found in the extract than in the whole cells.

A difference between the structures of binder protein made by wild-type and *cysA* cells was sought by acrylamide gel electrophoresis of shock fluids concentrated two to five times. The many *cysA* mutants tested contained a binder that corresponded in position on the gel to that of the purified binder protein. The intensities of the bands corresponded roughly to binding activities. No alteration of the structure of the binder protein was detected by this method.

The immunological agar diffusion technique was used to examine the binder proteins made by the mutants. Antiserum against purified binder protein was placed in a center well about 5 mm distant from wells containing the 5 to 10 times concentrated shock fluids. Each *cysA* mutant tested, including the three amber *cysA* mutants, gave a precipitin band identical to that of the wild-type binder. This observation provides strong evidence that the *cysA* gene does not determine the structure of the protein.

Sulfite reductase activities of chromate-resistant cysA mutants. From the previous evidence, it appears that low-binder chromate-resistant *cysA* mutants are defective to varying degrees in the synthesis of binder. Thus, their mutations appear to have a regulatory effect on binder production. To determine whether this regulatory effect is specific or whether it extends to other genes in the cysteine biosynthetic pathway, five low binder-producing mutants and two controls (wild-type and *cysA20* bacteria) were assayed for sulfite reductase (Table 6). The five chromate-resistant low-binder *cysA* mutants, *N18*, *N19*, *N27*, *SP25*, and *SP30*, had sulfite reductase activities approximating that of the wild-type or *cysA20* values. Mutant *N18* had a somewhat reduced enzyme activity (53% of the *cysA20* value), but its binding activity was considerably more decreased (7% of the *cysA20* value). Thus, the apparent regulatory effect of these *cysA* mutations on binder production does not extend to sulfite reductase, and presumably not to other genes in the sulfate reduction pathway.

The genes *cysI* and *cysJ* are two structural genes for sulfite reductase. As expected, *cysI270* and *cysJ266* lacked reductase activity. The results obtained with the *cysB* mutants will be discussed

TABLE 6. *Sulfite reductase activities of various strains*[a]

Strain	Activity
Control strains:	
LT-2	108
cysA20	100
cysI270	0
cysJ266	2
Low-binder *cysA* strains:	
N18	53
N19	85
N27	94
SP25	95
SP30	78
cysB strains:	
cysB403	0
N38a	0
R108 (grown at 25 C)	134
R108 (grown at 42 C)	1

[a] The strains were grown in E medium with djenkolate as a sulfur source; assays were carried out on cell-free extracts as described in Materials and Methods. The values are given as a percentage of the value for *cysA20*, which was 261 enzyme activity units per mg of extract protein. One enzyme activity unit causes a reduction of 0.001 optical density unit at 360 nm/min.

TABLE 7. *Binding and uptake activities of mutants in various cysteine genes*[a]

Strain	SO_4^{2-} bound	SO_4^{2-} taken up in 30 sec
+	.062	1.95
cysA20	.078	0
SP30	.018	0
N38a	<.001	0
cysBb12	0	—
cysBc15	0	—
cysB403	.003	0.004
cysB482	.001	0
cysCD519	.047; .059	0.64; 0.78
cysC200	.014	0.19
cysC428	.003	1.57
cysC514	.005	1.56
cysD23	.044	3.00
cysD220	.084	0.82
cysD313	.011	0.63
cysE2	.031	0.09
cysE17	.001	0.09
cysH7	.022	1.79
cysH75	.057	1.66
cysH271	.050	1.70
cysI149	.052	1.37
cysI270	.057	3.60
cysJ266	.058	3.81
cysJ299	.078	3.52

[a] All strains were grown to about 6×10^8 cells/ml on 0.15 mM djenkolate. Binding and uptake activities were assayed as described in Materials and Methods, and are expressed as nanomoles per milligram of protein.

below.

Mutants in cysB. Mutant *N38a*, the only chromate-resistant mutant which had almost no binding activity, appears from the genetic and nutritional tests to be a *cysB* mutant. Binding and transport activities of other *cysB* mutants (*Bb13*, *Bc15*, and *Bc482*) were also very low (Table 7). These mutants also had no sulfite reductase activity (Table 6), thus confirming the pleiotropic effect of *cysB* mutations (7; H. T. Spencer, J. Collins, and K. J. Monty, Fed. Proc. **26**:677, 1967). Binding, acrylamide gel, and immunological tests of their shock fluids failed to show appreciable amounts of the binding protein. Transductants to *cys*$^+$, obtained with phage from the donor strain *trpD10*, had normal binding activities. The gene *cysB* is a regulatory gene for the cysteine biosynthetic pathway. These results show that it also regulates the production of the binder protein.

Spontaneous revertants of strain *N38a* were selected on sulfate plates at 37 C. Eleven of 32 revertants (*R8*, *R14*, *R15*, and *R17* to *R24*) showed distinctly smaller colony sizes than the rest, but growth curves of all were similar in liquid media. That they might be "*cysA su*$^+$" type mutants (6) was not likely, because they grew quite normally in cysteine-supplemented medium, both in liquid and on agar plates.

These revertants of mutant *N38a* were grown on djenkolate and then tested for abilities to take up and bind sulfate (Table 8). It was found that the revertants can be divided into two classes. (i) Revertants which have uptake activity equal to or greater than that of the wild type: these had either 25% (*R1* and *R25*), or less than 10% (*R17*), of normal binding activity. (ii) Revertants which did not recover full uptake activity: these had very little (less than 5%) binding activity (*R8*, *R14*, and *R15*). In contrast, transductants of mutant *N38a* had normal transport and binding activities.

Derepression of binding and uptake activities of wild type and mutants *R1*, *R14*, and *R108* was observed (Table 8). However, the extent of derepression was different for different strains; furthermore, the changes in binding and uptake activities in a strain were not coordinated.

Revertants *R14* grown at 37 C and *R108* grown at 43 C did not make binder protein in a quantity detectable by the electrophoretic and immunological methods. Binder protein of mutant *R1* was detectable by both tests, and seemed to be identical to normal binder. One revertant isolated at 24 C (*R108*) was temperature-sensitive. It grew well on a sulfate plate at 24 C and poorly at 37 C; no growth was seen at 42 C. It grew well on a cysteine plate at 42 C. Similarly, this revertant had high sulfite reductase activity when grown at 24 C and very low activity when

TABLE 8. *Binding and uptake activities of the revertants of N38a*[a]

Strain	Growth temp (C)	Medium	SO_4^{2-} bound	SO_4^{2-} taken up in 30 sec
LT-2	37	Djenkolate	.046	0.84
LT-2	37	Sulfate	.003	1.19
cysCD519	37	Djenkolate	.046, .055	0.78, 0.64
N38a	37	Djenkolate	.002	0
R8	37	Djenkolate	.002	1.22
R15	37	Djenkolate	.002	0.17
R17	37	Djenkolate	.004	1.22
R25	37	Djenkolate	.013	1.92
LT-2	37	Djenkolate	.054	1.36
LT-2	37	Cysteine	0	0
R1	37	Djenkolate	.014, .011	1.48, 1.16
R1	37	Cysteine	.002	0.44
R14	37	Djenkolate	.001, .001	0.16, 0.12
R14	37	Cysteine	—	0
R108	24	Djenkolate	.032	2.99
R108	37	Cysteine	.012	0.17

[a] Cells were grown in medium E containing cysteine (repressed), djenkolate (derepressed), or sulfate. Binding and uptake activities were assayed as described in Materials and Methods, and are expressed as nanomoles per milligram of protein.

grown at 42 C (Table 6). When mutant *R108* was grown at 24 C on djenkolate, binding was 50 to 70% of the wild-type activity and uptake was normal. This strain grown at 43 C had absolutely no transport or binding activity. But cells grown at 24 C had quite stable transport and binding activities at 43 C for at least 60 min. Stability was confirmed by heat-inactivation curves of cell-free preparations. Shock fluids were prepared from *R108* and wild type, and were concentrated approximately 10 times by lyophilization and resuspension in small amounts of distilled water. Portions of each preparation were heated for 10 min at various temperatures and assayed immediately at 24 C. The inactivation curves were essentially the same; 50% inactivation occurred at about 63 C. Thus, heat sensitivity of the mutant strain is not attributable to a heat-sensitive binder, but to the synthesis of a protein that is temperature-sensitive.

The results described here suggest that only a little binding activity may be necessary for full uptake activity, and that the two activities are not proportional. If binder is involved in uptake, only a small fraction of the wild-type depressed level is required.

Temperature-sensitive cysteine auxotrophs. In a continuing search for a binder-structural gene mutant, temperature-sensitive cysteine auxotrophs were isolated as described in Materials and Methods. Twenty-four isolates were grown at 41 C on enriched medium with djenkolate as a sulfur source; whole-cell binding activities were determined at 41 C. Two of the 24 had binding activities that were only 4 and 7% of the control (*cysCD519*) activity. When grown at 28 C, these mutants had about 40% of the control activity, measured at 28 C. However, the binding activity of these mutant cells grown at 28 C had the same heat-sensitivity profile as that of the control cells. Thus, these mutants are temperature-sensitive for synthesis of the binder and are probably temperature-sensitive *cysB* mutants, like mutant *N38a-R108*. A temperature-sensitive binder gene mutant was not found.

Mutations in other cysteine cistrons. *N15*, *N16*, and *N37* are chromate-resistant low-binder mutants that have lesions in genes for cysteine biosynthetic enzymes. Also, they grow very poorly on djenkolate medium. Several mutants defective in other *cys* genes, picked randomly, were assayed for their sulfate-binding abilities. Rather unexpectedly, it was found that mutants *N15*, *N16*, and *N37* are not exceptional; mutants having lower sulfate-transport ability were found with quite high frequency in any *cys* cistron (Table 7; *cysG* was not tested). The following observations were made: (i) all *cysA* mutants, including the new mutants, were transport-negative and may or may not have high binding activity; (ii) *cysB* mutants almost completely lacked both binding and transport activity; (iii) *cysE* mutants also were defective in their uptake ability (*cysEa2* had high binding activity); (iv) other mutants in the cysteine pathway had varying degrees of transport activity, ranging from 10 to 100% or higher. Although mutants with low binding activity were found more frequently when lesions were in the *cysC* and *cysD* genes than in *cysH*, *cysI*, and *cysJ*, at least one mutant con-

taining a low level of binder was found in each of the cysteine genes, if strain *N37* which is a *cysI* or *cysJ* mutant is included in the list.

Binding and transport activity of wild-type strain. Sulfate transport by the djenkolate-grown strain LT-2, from which the mutants were derived, was linear for at least 3 min, and the overshoot observed with mutant *cysCD519* (3) was not seen. The rate for both strains was the same within the first 30 sec.

Binding and transport activities of strain LT-2 grown on different sulfur sources were compared (Table 8). Both activities were nearly completely absent in bacteria grown on cysteine and were high in djenkolate-grown bacteria. However, the binding activity of sulfate-grown bacteria was less than 10% of the value for djenkolate-grown cells, whereas the transport activity was about 50%. Furthermore, when the djenkolate-grown cells were transferred to sulfate or cysteine-containing medium and grown for an additional two generations, binding activity was lost much more rapidly (reduced to 5%) than would be expected from simple dilution during growth (Table 9). This lost binding activity was not found in the medium. These data, with those obtained with *cysB* mutants, suggest that the binder is in excess over the requirements for transport. All data comparing binding and transport are summarized in Fig. 1.

DISCUSSION

To clarify further the significance of the binding step in the active transport of sulfate, we isolated a number of additional low-binding mutants. By genetic analysis, these were classified into two groups, those with lesions in the *cysA* region (the majority) and several with lesions in other genes including the *cysB* gene.

A main aim was not realized: the isolation of mutants that are defective in the gene that determines the structure of the binding protein and also defective in transport. The reason no mutant was obtained that made a defective binding protein is obscure. But this negative finding does not prove that the binding protein is not part of the transport system, since, for example, unexpected selective conditions might have eliminated true binder-negative mutants during the isolation. It should also be noted that in this study no *cysE* mutants and only one *cysB* mutant were found, although these genes must be active to permit transport, owing to their control functions (7, 8; Spencer et al., Fed. Proc. **26**:677, 1967).

The mutants mainly contained lesions in the three cistrons of the *cysA* region. Most of the chromate-resistant nitrosoguanidine mutants produced less binder by at least half than did the original strain. Their binding proteins appeared to be identical in structure to that of the parent strain, as tested by acrylamide gel electrophoresis, immunodiffusion, and heat stability. Also, various genetic tests strongly suggested that the *cysA* region cannot determine the structure of the binder. For instance, two mutants with long deletions covering all known *cysA* mutations produced the binder in normal quantity, as did those

TABLE 9. *Effect of a changed sulfur source on the binding and transport activities*[a]

Fresh medium	SO_4^{2-} bound	SO_4^{2-} taken up in 30 sec
Djenkolate .	.025, .026, .022	1.86, 0.77, 1.36
Cysteine ...	.001, .002, .001	0.01, 0.15, 0.06
SO_4^{2-}	.003, .003, —	0.68, 0.57, —

[a] An overnight culture of strain LT-2 grown on djenkolate was diluted into three kinds of fresh medium after washing, and was allowed to grow for two generations. Then the cells were harvested and assayed for activities as described in Materials and Methods. Results of three experiments are given as nanomoles per milligram of protein.

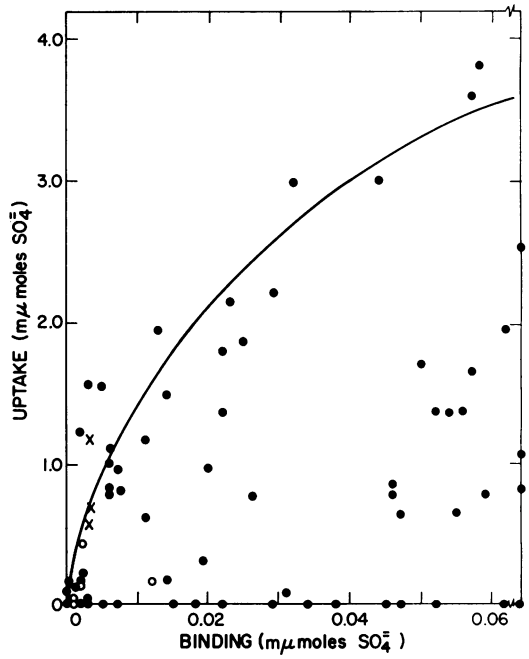

FIG. 1. *Correlation between binding and uptake. All available data on binding and uptake activities are plotted. Each point represents a result with a single culture for which transport rate is plotted against binding activity. The line is an adsorption isotherm that indicates roughly the average values for maximal rates of transport at each level of binding. Growth media: ●, djenkolate; O, cysteine; ×, sulfate.*

with nonsense mutations in each of the three cistrons. We conclude that *cysA* mutations have a regulatory effect on production of the binder. This regulation does not extend to sulfite reductase nor presumably to other steps of the cysteine pathway. These *cysA* mutants do not accumulate binder inside the cells or in the medium when it it is not found in its normal location—the periplasmic space. Some close relation therefore exists between *cysA*, the gene that determines sulfate transport, and the production of binding protein. Possibly an aggregation between defective *cysA* products and the binder could inactivate the latter.

Lesions in the *cysB* gene, which involves regulation by positive control of the cysteine pathway (7, 12; Spencer et al., Fed. Proc. **26**:677, 1967), also modify binder production. A *cysB* mutant picked during the selection and other *cysB* mutants isolated by Mizobuchi et al. (13) produced no detectable binder. A temperature-sensitive *cysB* mutant was shown to produce the binder only at the lower temperature, but, once produced, the binder protein was normally heat-stable. The present results demonstrate a close connection between this control system and production of the binder protein. Hence, they imply a connection between binder and the cysteine pathway.

Curiously, mutations affecting other steps of the pathway also modify production of the binder protein. Not every *cys* mutation has this effect; some mutations in each gene do and some do not. Again, a relation is suggested between the pathway and the binder; the basis of this relation is also obscure; it probably will not be elucidated until the control of the pathway is better understood.

Finally, when all available data are plotted (Fig. 1), one sees that there are no strains or conditions that give high transport with low binding activity. (Many mutants have less than maximal transport activity but make various amounts of binder; as an extreme example, *cysA* mutants have no transport activity. Some other necessary part of the transport system must be defective in these mutants.)

From the maximal rates, we conclude that binder appears necessary for transport, although the quantities of the two are not proportional. The line is an adsorption isotherm, adjusted by eye to indicate maximal values of the transport rates. This curve suggests that the binder might be a "cofactor" for the transport system. It appears to saturate the system well before it is produced in the maximal quantity, as in derepressed cells. As a consequence, cells grown with sulfate as a sulfur source make only about 10% of the maximal amount of binder but have about 50% the maximal transport rate. Similarly, two revertants of the *cysB* mutant *N38a* have transport rates 100 and 20% of normal, and binding levels about 25% and barely detectable, respectively. The correlation between the (maximal) rate of transport and the amount of binder thus again suggests a role of the protein in the transport process.

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