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Mutation Leading to Increased Sensitivity to Chromium in *Salmonella typhimurium*

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CORWIN, L. M. (Walter Reed Army Institute of Research, Washington, D.C.), G. R. FANNING, F. FELDMAN, AND P. MARGOLIN. Mutation leading to increased sensitivity to chromium in *Salmonella typhimurium*. J. Bacteriol. **91**:1509–1515. 1966.—Certain deletion mutants including the tryptophan operon in *Salmonella typhimurium* are unable to utilize several sugars as carbon sources in solid media, although they are able to grow in liquid media with these sugars. The addition of citrate or washing the agar with ethylenediaminetetraacetic acid permits growth on solid media. Analysis of the agar revealed that Fe^{3+} and Cr^{3+} were present at concentrations of 22 and 75 μM, respectively. The addition of Fe^{3+} to liquid media in 0.5 mM concentrations did not inhibit the wild type or the mutants. A similar concentration of Cr^{3+} did not inhibit the wild type, but concentrations as low as 0.01 to 0.05 mM inhibited the deletion mutants. Other metals were inhibitory at various concentrations, but none showed any significant differential effects on the mutants and the wild type. The increased sensitivity of the mutants to chromium may be due either to an increased permeability to Cr^{3+}, resulting in higher effective intracellular concentrations and inhibition of one or more metabolic functions, or to a binding of Cr^{3+} to an altered cell wall, resulting in decreased permeability of required substrates.

An investigation of deletion mutations of the LT2 strain of *Salmonella typhimurium*, involving the *cysteine B*, *su leu 500*, and *tryptophan* loci, revealed that some of the strains isolated were unable to grow in the absence of citrate, despite the presence of adequate quantities of glucose and required amino acids (10). Substitution of arabinose, galactose, maltose, mannose, sorbitol, or mannitol for glucose as a carbon source likewise did not permit growth of these mutants. When citrate was added to the agar medium, the mutants were able to grow. These mutations were always associated with tryptophan auxotrophy, but never when cysteine was the sole amino acid requirement. Therefore, the *car* locus was presumed to be close to the tryptophan cluster on the side opposite that occupied by the *cysteine B* and *su leu 500* markers. This paper will be concerned with the nature of this *car* mutation. It will be shown it is due to an increased sensitivity to Cr^{3+}, which exists in a surprisingly high concentration in agar.

MATERIALS AND METHODS

The bacteria used were all derivatives of strain LT2 of *S. typhimurium* and involve deletions through all or part of the tryptophan cluster as described by Mukai and Margolin (10). The growth-inhibition experiments were performed in test tubes containing 5 ml of medium to which 10^6 to 10^7 cells were added as an inoculum. They were incubated in a rotating test tube drum on an axis just enough off the horizontal to prevent spillage.

Preparation of cell-free extracts. The bacteria were grown overnight in nutrient broth (Difco). After harvesting and washing the cells twice with water, a concentrated cell suspension (ca. 10^{10} cells per milliliter) in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5) was subjected to sonic vibrations for 1 to 2 min at 3 amp in a Branson sonifier at 0 to 4 C. The suspension was then centrifuged at 8,000 × *g* to sediment unbroken cells and cell debris, and the supernatant fraction was used as the cell-free extract.

Determination of isocitric dehydrogenase. The method used, based on that of Ochoa (12), depends on the reduction of nicotinamide adenine dinucleotide phosphate. Units were expressed as millimicromoles of reduced nicotinamide adenine dinucleotide phosphate formed per milligram of protein per minute.

Aconitase. The method of Racker (13), based on increased light absorption at 240 mμ owing to *cis*-aconitate formation, was used. The activity was expressed as millimicromoles per milligram of protein per minute.

Citrate synthetase. The method of Dixon and Kornberg (1) for malate synthetase was used, with oxalo-

acetate as substrate instead of glyoxylate. Specific activity was defined as millimicromoles of acetyl coenzyme A reduced per milligram of protein per minute.

Phosphoenolpyruvate carboxylase. The reaction mixture contained 4 μmoles of phosphoenolpyruvate (PEP), 5.0 μmoles of $MnCl_2$, 20 μmoles of reduced glutathione, 50 μmoles of Tris buffer (*p*H 7.5), 2 μmoles of $NaHC^{14}O_3$ (3.5×10^5 counts/min), and bacterial extract to a total volume of 1.0 ml. The $NaHC^{14}O_3$ was added after a 3-min preincubation period at 37 C, and the incubation was continued for 5 min in a Dubnoff shaker. The reaction was stopped with 0.1 ml of 90% trichloroacetic acid. The reaction mixture was centrifuged, and 0.1 ml of the supernatant fraction was placed in a counting vial and agitated in a vortex mixer for 2 min to release trapped $C^{14}O_2$. To the sample were added 2.4 ml of absolute ethyl alcohol and 12.5 ml of scintillation fluid. Care was taken to avoid any sodium ions, which were found to be inhibitory to the enzyme.

Protein determination. The biuret method of Gornall et al. (4) was used. When the protein content of whole cells was measured, 0.5 ml of the sample was boiled with 0.5 ml of 1.5 N NaOH for 5 min. After cooling, the biuret reagent was added.

Metal ion analyses. All chemicals used were of reagent grade. To wet-ash the agar, 2 g samples of a mixture of nitric, sulfuric, and perchloric acids (3:1:1) were used for digestion. Several 2-g agar samples were dry-ashed at 450 C in an electric furnace, and these samples were diluted to 20 ml.

Polarographic measurements were made with a polarograph, a Lingane hydrogen cell fitted with a saturated calomel reference electrode (SCE), and a saturated KCl-3% agar bridge was employed. The digested agar sample was placed in the cell and flushed with nitrogen gas for 10 min to remove oxygen and the resulting interfering oxygen wave.

All atomic absorption measurements were made with an atomic absorption spectrophotometer and a hydrogen-air flame. The 3,579 A line of chromium and the 2,483 A of iron were used in the determination (2). The determination involves the absorption of radiation being emitted from a hollow cathode lamp by neutral atoms of the element studied, which are aspirated into the hydrogen flame. The decrease in absorption is proportional to the concentration of metal present. A series of standards are run and a calibration curve is made.

RESULTS

The deletion mutants (C = cysteine; T = tryptophan) described here fall into five major types (Fig. 1) according to the extent of their deletions. The recombination evidence supporting this classification was described previously (8, 10).

Since the *car* mutation appeared to involve the

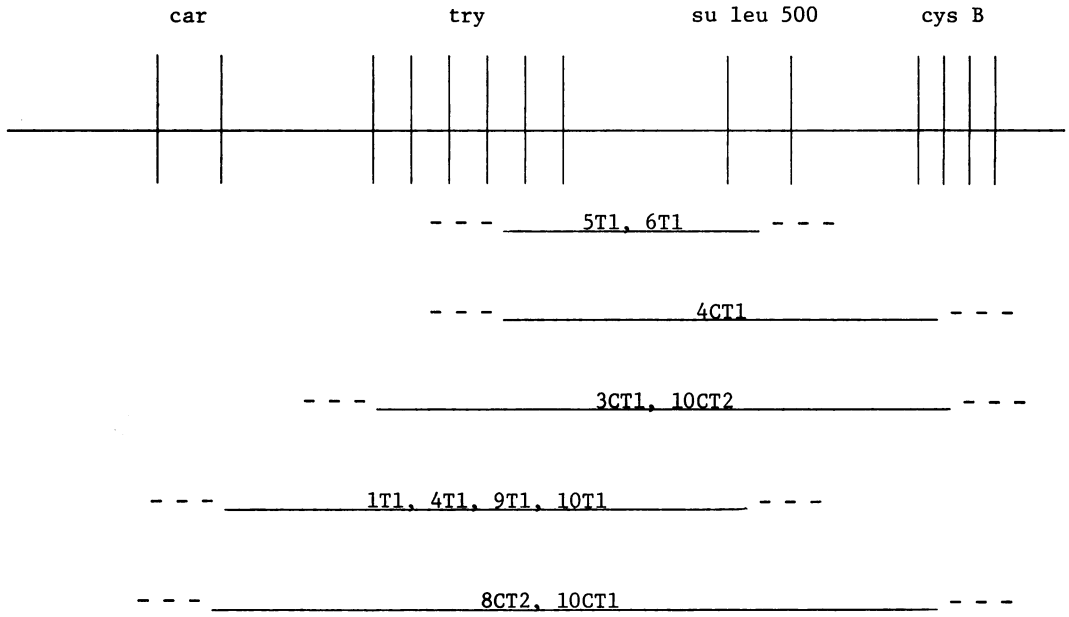

FIG. 1. *Diagram of the car-try-cys B portion of the Salmonella typhimurium chromosome. The lines below the chromosome represent the approximate extent of the deletions in the mutant strains listed above the line. The symbols used are: car = carbohydrate utilization; try = tryptophan; su leu 500 = suppressor of leu 500 mutation; and cys = cysteine.*

utilization of carbohydrates, the ability of strains 1T1, 4T1, 9T1, 8CT2, and 10T1 to oxidize a variety of substrates was tested and compared with a number of other related mutants and the wild-type LT2 strain which are able to grow on glucose. The strains were grown overnight (16 to 20 hr) in a meat-extract broth with no added carbohydrate, harvested, washed once in saline, and taken up in saline. There was very little difference in the ability of the *car* mutants to oxidize the substrates, compared with *car*$^+$ strains (Table 1). There were a few differences, such as the lowered oxidation of acetate by 4T1, 5T1, and 6T1, but this finding bears no relation to the nature of the *car* locus, since 5T1 and 6T1 are able to grow on glucose. In any event, the ability to oxidize glucose was unimpaired in any strain.

TABLE 1. *Oxygen uptake by car mutants and controls**

Strain	Substrate				
	Succinate	Acetate	Glucose	Glycerol	Pyruvate
1T1†	5.3	1.2	4.3	5.4	4.7
4T1†	5.3	0.6	4.6	4.5	2.4
9T1†	6.5	1.7	4.9	6.4	5.4
10T1†	5.6	1.7	5.2	4.0	5.1
5T1	3.7	0.9	3.8	2.7	2.5
6T1	4.8	1.1	3.4	3.5	2.0
8CT2†	3.9	2.6	4.4	3.4	3.5
Ara 9 Leu 500	3.2	2.1	3.4	4.1	4.4
Ara 9	2.9	3.3	3.8	3.7	4.2
LT2	5.5	2.5	4.0	4.0	3.5

* Strains were grown overnight in meat-extract broth. The incubation mixture contained 40 μmoles of phosphate buffer (*p*H 7.4), 300 μmoles of NaCl, 12 μmoles of KCl, 6 μmoles of $MgSO_4$, and 30 μmoles of substrate. The final volume was 3 ml. The incubation temperature was 37 C. The results are expressed as microatoms of oxygen per mg of protein per 30 min.

† A *car* mutant strain.

The observation that the presence of citrate in the agar medium allowed the *car* mutants to grow on glucose made it necessary to consider the possibility of a block in the tricarboxylic acid cycle or perhaps between the Embden-Meyerhof pathway and citrate. It has been shown that mutants deficient in PEP carboxylase are unable to grow on glucose (16). This inability is also found in mutants deficient in citrate synthetase (3). In *Salmonella*, which is permeable to citrate, the addition of citrate to the medium would promote growth of such mutants. Citrate should not overcome blocks in the Krebs cycle beyond citrate. In Table 2, the presence of both PEP carboxylase and citrate synthetase in sonic extracts of the *car* strains is indicated. Furthermore, the enzymes involved in the utilization of citrate, isocitric dehydrogenase, aconitase, and isocitratase were all present.

Apparently, the effect of the addition of citrate in promoting growth cannot be explained by invoking a block in the formation of citrate. The results of an investigation into the stimulation of growth in solid media by citrate are presented in Table 3. Several things should be noted: first, the *car* mutants (1T1, 4T1, 9T1, and 10T1) grew very poorly on minimal glucose, but there was some growth. If a required amino acid were omitted, there would be no growth. Second, the *car* mutants did not utilize citrate instead of glucose, since except for 10T1 there was little if any growth on citrate alone. There was thus real synergism between glucose and citrate.

Experiments in liquid media (Table 4) gave a

TABLE 2. *Enzymes in car mutants and controls**

Strains	Isocitric dehydrogenase	Aconitase	Isocitratase	Citrate synthetase	Phosphoenolpyruvate carboxylase
1T1†	332	32.7	12.8	109	22
4T1†	425	32.5	19.7	176	57
9T1†	366	34.8	15.8	115	24
10T1†	406	20.3	16.4	188	21
5T1	279	14.6	15.7	81	11
8CT2†	277	32.7	21.0	101	35
Ara 9 Leu 500	210	15.2	16.5	107.5	11
Ara	309	21.4	28.2	77	11
LT2	350	17.4	33.8	75	13

* Cells were grown overnight in meat-extract broth, washed, suspended in Tris buffer, (*p*H 7.4) and subjected to sonic treatment in ice for 90 sec. The treated preparation was centrifuged for 15 min at 8,000 $\times$ *g*, and the supernatant fraction was used as the extract. The results are expressed as millimicromoles of product per milligram of protein per minute.

† A *car* mutant strain.

TABLE 3. *Effect of citrate on growth of car mutants on solid media**

Strain	Glucose	Glucose + citrate	Citrate
LT2	4+	4+	3+
1T1†	1+	3+	0
4T1†	1+	3+	0
9T1†	1+	3+	1+
10T1†	2+	3+	2+
5T1	4+	4+	3+
6T1	4+	4+	3+

* Carbon sources were at a level of 0.2%. Incubation was for 24 hr. Media contained leucine and tryptophan at 20 μg/ml. Ratings of 1+ to 4+ are relative amounts of growth compared with the wild type, LT2, which is assigned a value of 4+.

† A *car* mutant strain.

TABLE 4. *Effect of citrate and EDTA on growth of car mutants in liquid media**

Medium	LT2	1T1	4T1	10T1	9T1	5T1
Glucose	580	241	200	362	225	320
Glucose + citrate	810	275	170	445	342	345
Citrate	445	37	20	218	46	182
Glucose + 10^{-5} M EDTA	590	200	215	375	215	340
Glucose + 10^{-6} M EDTA	585	240	170	350	170	318
Glucose + 10^{-7} M EDTA	580	225	182	350	181	325

* Carbon sources were at a level of 0.2%, and the media contained 20 μg/ml of tryptophan and leucine. EDTA was added as the disodium salt. The incubation period was 22 hr. The results are optical density readings (at 420 mμ) × 1,000 in a Coleman spectrophotometer.

clue to the role of citrate; there was definite growth in glucose without the addition of citrate. The addition of citrate had much less stimulatory effect than in the solid medium. Addition of ethylenediaminetetraacetic acid (EDTA) did not increase growth in the liquid. The growth of the *car* mutants was still less than that of the *car*$^+$ mutant 5T1 and wild-type LT2. Except for 10T1, which is intermediate, they were unable to utilize citrate.

The fact that citrate promoted growth on solid media, and not in liquid, indicated the possibility that citrate was acting as a chelating agent and binding a metal (in the agar) to which these mutants were sensitive. This was tested by washing agar with EDTA and then exhaustively washing the agar with distilled water. A comparison of growth on EDTA-washed agar compared with untreated agar is given in Table 5. The growth of the *car* mutants on the untreated agar was limited. The growth increased slightly when the agar was washed with EDTA. Since it might be expected that the EDTA removed required ions, such as Fe^{3+} and Mn^{2+}, these were added back to the media. As expected, this addition had no effect in the untreated agar, but restored growth of the *car* mutants almost to that of the *car*$^+$ strains. No effect on *car*$^+$ mutants was observed. Other commercial agar preparations were examined for their ability to support growth (Table 6). Special Agar (Noble) was no improvement over plain agar (Difco), but Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) allowed somewhat better growth. Ionagar did not approach the EDTA-washed agar, however.

TABLE 5. *Growth of car mutants on EDTA-washed agar**

Strain	Untreated agar	Untreated agar + Mn^{2+} + Fe^{3+}	EDTA-washed agar	EDTA-washed agar + Mn^{2+} + Fe^{3+}
LT2	4+	4+	4+	4+
1T1†	½+	½+	1+	3+
4T1†	½+	½+	½+	2+
9T1†	½+	½+	1½+	3+
10T1†	1½+	1½+	1½+	3+
10CT1†	½+	½+	1+	3+
10CT2	3+	3+	2+	3+
3CT1	3+	3+	3+	3+
4CT1	3+	3+	3+	3+
5T1	4+	4+	4+	4+
6T1	4+	4+	4+	4+

* Glucose (0.2%) was the carbon source, with 20 μg/ml of tryptophan, leucine, and cysteine added. $MnCl_2$ and $FeCl_3$, when added, were at a level of 10^{-6} M. Incubation was for 24 hr.

† A *car* mutant strain.

TABLE 6. *Growth on other purified agars**

Strain	Ionagar No. 2	Ionagar No. 2 + Mn^{2+} + Fe^{3+}	Noble agar	Noble agar + Mn^{2+} + Fe^{3+}
LT2	4+	4+	4+	4+
1T1†	1+	1+	½+	½+
4T1†	1+	1+	½+	½+
9T1†	1½+	2+	1+	1+
10T1†	1½+	2+	1½+	1+
10CT1†	1½+	2+	1½+	—
5T1	3+	4+	4+	4+
6T1	4+	4+	4+	4+

* Glucose (0.2%) was used as the carbon source, with 20 μg/ml of tryptophan and leucine added.

† A *car* mutant strain.

From the preceding experiments, it appeared that some cation(s) in agar was responsible for the lack of growth of the *car* mutants on solid media. A polarographic analysis on ashed agar was therefore performed. A cathodic current was observed at the potential of the mercury dissolution wave, indicating the presence of a substance reducible at a more positive potential. The potential of the Fe^{3+} wave is more positive than the anodic dissolution potential of mercury, and a cathodic current would be observed. The ferricyanide and thiocyanate spot tests for iron were performed on the sample, and both gave positive tests, i.e., a blue and red color, respectively (9). A semiquantitative test with appropriate standards gave an iron concentration of ca. 1 mM in both the EDTA-washed and unwashed media.

A well-defined cathodic wave was also observed at −0.98 v versus the SCE, after which came the discharge of hydrogen. The addition of sodium hydroxide to the solution caused a voluminous precipitate to form, and the polarogram was essentially flat with no observable waves. The addition of ammonia to another sample caused a shift in the initial wave, and an additional wave was observed. The waves were at −1.44 and 1.75 v versus the SCE. The polarographic characteristics of these waves and the wave at −0.98 v in acid solution were very similar to that of Cr^{3+}. In acid solution, Cr^{3+} undergoes a one-electron reduction to Cr^{2+} at −0.91 v versus the SCE. In ammoniacal solution, chromium gives rise to two waves: a one-electron reduction to Cr^{2+} (−1.42 v) and a two-electron reduction to Cr^{2+} (−1.42 v), and a two-electron reduction to the metal at 1.70 v versus the SCE. A "log plot" (7) of the initial wave at −0.98 v versus the SCE gave a value of 0.98, which corresponds to a one-electron reduction. From the polarographic diffusion currents, the concentrations of chromium in EDTA-washed and unwashed agar were determined (Table 7).

In support of the conclusion that chromium was responsible for the wave, a qualitative test was run of the reaction between *S*-diphenylcarbazide and Cr^{6+} formed after oxidation of Cr^{3+} with potassium persulfate in the presence of a silver catalyst. The deep red color formed indicated the presence of chromium. Since the oxidizing agent was required, the chromium was assigned to be Cr^{3+} and not Cr^{6+}.

TABLE 7. *Concentration of chromium and iron in agar**

Medium	Metal	Atomic absorption	Polarography
Agar (Difco)	Cr	75	50
	Fe	22	
EDTA-washed agar...	Cr	3	1.5
	Fe	16	

* Concentrations (μM) are those in a 1.5% medium.

For further identification and quantitation, the highly specific and sensitive method of atomic absorption spectroscopy was utilized. The results (Table 7) closely agree with the polarographic method.

Having established that agar contains substantial quantities of chromium and iron, it was necessary to determine whether or not the *car* mutants are more susceptible to growth inhibition by these and other metal ions at concentrations found in the agar. These experiments were done in minimal liquid media where growth occurs. The *car*$^+$ organisms, 5T1 and ara 9 leu 500, were quite resistant to $CrCl_3$ at 500 μM, whereas the *car* organisms were sensitive at 10 to 50 μM (Table 8). The atomic absorption data indicate that there was about 75 μM Cr^{+3} in the agar, which was enough to inhibit all the *car* mutants. They can all grow at a concentration of 5 μM Cr^{3+}, and this probably explains why they can grow in EDTA-washed agar, which has only 3 μM Cr^{3+}. The bacteria were not inhibited by 100 μM Fe^{3+}, whether they were *car* or *car*$^+$; of the two major metal contaminants of agar, chromium thus seems to be the one responsible for the difference in growth in agar.

In Tables 8 and 9, the results of testing the inhibitory effects of other metal ions are presented. At high concentrations, Fe^{3+}, Pb^{2+}, and Zn^{2+} were not inhibitory to *car* mutants or to the *car*$^+$ control. Hg^{2+} and Ag^+, which are potent sulfhydryl inhibitors, inhibited the *car* mutants at very low concentrations, but this inhibition was also true of the *car*$^+$ organism. The *car* mutant is two to four times as susceptible to Cu^{2+}, Ni^{2+}, and Co^{2+} as the *car*$^+$ organism. Polarographic analyses failed to detect Zn^{2+}, Cu^{2+}, or Pb^{2+}. A qualitative test for Ni^{2+} was negative. From the polarographic analysis, it is not possible to state whether Co^{2+}, Ag^+, or Hg^{2+} is in agar in sufficient quantity to cause inhibition, but the differences in susceptibility of the *Salmonella* strains to these cations are relatively minor.

DISCUSSION

The data presented give reasonably clear-cut evidence for a mutation which involves a lowered resistance to a specific trace element. Although the contribution of other, more toxic, trace elements has not been ruled out, there can be no doubt that the concentration of chromium in agar is sufficient to account for the inhibition of growth of the *car* mutants on solid media. The other trace

TABLE 8. *Effect of Cr^{3+} and Co^{2+} on growth in liquid medium**

Salt	Concn	1T1†	4T1†	9T1	10T1†	5T1	Ara 9 Leu 500
	μM						
No addition		345	341	306	287	440	465
$CrCl_3$	500	—	8	9	2	335	211
	200	—	5	6	2	340	320
	100	—	0	15	2	360	278
	50	2	149	28	0	370	410
	20	30	295	135	0	410	425
	10	8	331	312	102	—	425
	5	307	—	290	220	—	—
	2	340	—	305	—	—	—
$Co(NO_3)_2$	40	0	0	—	0	0	0
	20	0	0	—	19	258	60
	10	0	305	—	200	385	470
	5	200	370	—	329	390	500

* Medium contained 0.2% glucose and 20 μg/ml of tryptophan and leucine. The results are expressed as optical density units (at 420 mμ) × 1,000.
† A *car* mutant strain.

TABLE 9. *Effect of various metals on growth in liquid cultures**

Salt	1T1†	10T1†	5T1
$CuCl_2$	20	50	50
$NiCl_2$	5	10	5
$HgCl_2$	0.5	1.0	0.2
$Pb(NO_3)_2$	>100	>100	>100
$FeCl_3$	>100	>100	>100
$ZnSO_4$	>100	>100	>100
$AgNO_3$	0.3	0.2	0.2

* Results are expressed as the highest concentration (μM) permitting 50% growth.
† A *car* mutant strain.

elements studied had various levels of toxicity, but few, if any, differences in susceptibility of car^+ and *car* organisms could be demonstrated. The polarograph did not reveal the presence of trace elements other than iron and chromium. However, it is quite probable that other metal ions present in much smaller concentrations might have been masked by the large waves of chromium, iron, and the decomposition of hydrogen ion.

Richmond and John (14) demonstrated the genetic linkage of Hg^{2+} resistance and penicillinase production in staphylococci; subsequently, Novick and Richmond (11) showed that these genetic determinants reside in the plasmid, an extrachromosomal element. They found a 10-fold difference in sensitivity to Hg^{2+} between susceptible and nonsusceptible strains. Resistant strains can grow on 20 μM Hg^{2+}, and sensitive strains cannot grow on 2 μM Hg^{2+}. The *Salmonella* strains would all be sensitive to Hg^{2+} by this criterion. The *car* locus, whose alleles should probably be renamed chr^+ and *chr*, is located on the chromosome near the tryptophan operon. So far, the sensitive mutants have been obtained only in deletions including all or part of the *try* region. With *Salmonella*, resistance to chromium seems to be absolute rather than relative as in Hg^{2+}-resistant staphylococci. At a chromium concentration of 500 μM, where $CrPO_4$ is beginning to precipitate out of solution, the resistant strains still grow. Some sensitive strains will grow only at chromium concentrations of less than 10 μM.

The chromium-sensitivity locus on the *Salmonella* chromosome appears to be near the coliphage T1 resistance (V_1^R) locus on the *Escherichia coli* K-12 map. Charles Yanofsky has informed us that some of his V_1^R mutants are slow-growing and respond to the addition of citrate in the medium. He has kindly sent us some of his mutants, and we have found that, similar to the *chr* mutants in *Salmonella*, a deletion mutation including the *try* A gene and V_1^R is much more sensitive to chromium than is a mutation in the *try* A gene. A detailed report of these experiments is in preparation.

The nature of the change which causes chromium sensitivity is still unknown. Two alternative mechanisms leading to sensitivity seem plausible. (i) The regulation of chromium transport is eliminated by the deletion of all or part of the *chr* locus. The *chr* cells, being more permeable to Cr^{3+}, contain concentrations which inhibit one or more metabolic functions. This notion parallels

(in an inverted sense) the findings of Suskind and Kurek (15), who reported a mutant form of tryptophan synthetase which appeared to be inhibited by the normal concentration of zinc in the cells. They could select suppressor mutations which appeared to work by lowering the level of active inhibitory zinc, in some cases perhaps by decreasing the cell's permeability to this ion. (ii) The deletions of all or part of the *chr* locus may cause a cell wall alteration which increases the binding of Cr^{3+}, thereby decreasing permeability and excluding substrates normally obtained from the medium. This appears to be related to the observation of Leive (6) that EDTA breaks down the barrier for a variety of compounds to which *E. coli* is normally impermeable. She reported that EDTA renders *E. coli* more permeable to a variety of substrates, as well as making the bacterium actinomycin-sensitive, a property usually associated with gram-positive organisms. Presumably, the EDTA acts by chelation of trace metals which normally inhibit permeability to a variety of compounds.

The *chr* mutant, 10T1, differs from the other *chr* mutants by being able to utilize citrate, although not so well as chr^+ strains. It appears (Margolin and Corwin, *in preparation*) that this property of 1T1, 4T1, and 9T1 is due to deletion of the region between *cys B* and *su leu 500*, which affects citrate utilization and is thus unrelated to the *chr* locus.

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