MUTANTS OF SALMONELLA DEFICIENT IN REDUCTOISOMERASE[1]

F. B. ARMSTRONG[2] AND R. P. WAGNER

The Genetics Laboratory of the Department of Zoology, University of Texas, Austin 12, Texas

Received July 3, 1962

RECENT studies of the biosynthesis of valine and isoleucine in microorganisms have described an enzyme, α-hydroxy-β-keto acid reductoisomerase, that catalyzes the isomerization and reduction of the α-hydroxy-β-keto acid precursors of the amino acids to produce the corresponding α, β-dihydroxy acids (UMBARGER, BROWN and EYRING 1960; RADHAKRISHNAN, WAGNER and SNELL 1960; and ARMSTRONG and WAGNER 1961a). This reaction is depicted in Figure 1. Another enzyme, α-keto-β-hydroxy acid reductase, indicated in Figure 1, also has been found to catalyze the production of the dihydroxy acid precursors, but through the reduction of the postulated intermediates in the reductoisomerase reaction, the α-keto-β-hydroxy acids (WAGNER, RADHAKRISHNAN and SNELL 1958; and RADHAKRISHNAN, WAGNER and SNELL 1960), rather than by the isomerization and reduction of the α-hydroxy-β-keto acids. Indeed, the reductase cannot act on the α-hydroxy-β-keto acid substrates. The reductoisomerase is considered to be the enzyme directly involved in the biosynthesis of the amino acids, and the reductase activities are currently thought to represent altered forms of the reductoisomerase and, therefore, to be considered as artifacts.

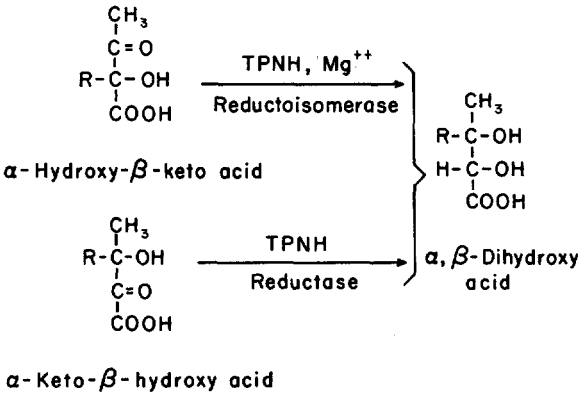

FIGURE 1.—Biosynthesis of valine and isoleucine: reactions involving the production of α, β-dihydroxy acids. R = CH_3 for valine biosynthesis, and CH_2CH_3 for isoleucine biosynthesis.

[1] This investigation was supported by Research Grant RG-6492 and Traineeship Grant 2G-337 from the National Institutes of Health, United States Public Health Service.

[2] Present address: Department of Genetics, North Carolina State College, Raleigh, N.C.

Interest in studies on the gene-enzyme relationship, involving the reductase and the reductoisomerase, was prompted by the genetic studies carried out by GLANVILLE and DEMEREC (1960) on the mutants of *Salmonella typhimurium* requiring both isoleucine and valine (*ilva*) for growth. These authors described linkage studies which indicated that four closely linked loci (labelled *A*, *D*, *B* and *C*) are involved in the biosynthesis of the amino acids. However, enzymatic studies on these mutants by WAGNER and BERGQUIST (1960) showed that, functionally, three loci are involved; each of which controls one of three separate and successive steps in the pathway. WAGNER and BERGQUIST reported that two of the four loci (*A* and *D*) proved to be similar in their low reductoisomerase activity. GLANVILLE and DEMEREC designated *A* and *D* as separate loci on the basis of complementation (abortive transduction) behavior and differences in growth requirements (see Discussion). The studies by the two groups showed, moreover, that the order of the loci, as determined genetically, corresponds to their action in the sequence of reactions.

Since both the reductase and reductoisomerase activities of the wild-type strain of Salmonella are fairly well understood (ARMSTRONG and WAGNER 1961b), an investigation of these activities in the *ilva* strains *A-8* and *D-27* and 22 additional *ilvaA* strains was initiated. Enzymatic, immunological and electrophoretic techniques were used in this effort to analyze the nature of biochemical changes brought about by mutations at the *ilvaA* locus.

MATERIALS AND METHODS

The strains of *S. typhimurium* used in this study were obtained from DR. M. DEMEREC, Brookhaven National Laboratory. In addition to the two wild-type strains, LT-2 and LT-7, the following mutant strains were used: *ilvaA-8, 12, 14, 19, 28, 29, 31, 33, 34, 35, 36, 37, 39, 42, 43, 44, 46, 48, 50, 51, 52, 54, 55, ilvaD-27, B-6* and *C-13*. Strain *D-27* is the only known member of the *D* group. The mutant strains were obtained from either LT-2 or LT-7; their origins are listed in Table 1.

The procedures used to grow the bacteria, prepare the cell-free extracts, and purify the reductoisomerase are given in previous publications (WAGNER and BERGQUIST 1960; and ARMSTRONG and WAGNER 1961a).

The spectrophotometric assays for reductoisomerase and reductase activities have been described in a previous publication by ARMSTRONG and WAGNER (1961a). The abbreviations for the substrates used are: AHB = α-aceto-α-hydroxybutyrate; HKV = α-keto-β-hydroxyisovalerate (hydroxyketovaline); HP = hydroxypyruvate. These substrates were prepared by the methods described by RADHAKRISHNAN, WAGNER and SNELL (1960).

The procedures used for starch gel electrophoresis and for the detection of bands of reductoisomerase and reductase activities on starch gels following electrophoresis have been described elsewhere (ARMSTRONG and WAGNER 1961b).

Extraction of enzyme activities from starch gels: Cell-free preparations were subjected to starch gel electrophoresis. Triplicate gels were used in order to extract sufficient amounts of enzymatic activity. A guide strip was cut from each gel, and the bands of enzymatic activity located. The bands are labelled as

TABLE 1

Enzymatic and immunological survey of the ilvaA *strains of Salmonella*

	Origin of mutation	1 Test for CRM* reductoisomerase assay (AHB)	2 Activity neutralized	3 Specific activity (μM/hr/mg) reductoisomerase assay (AHB) with Mg^{++} (5mM)	4 without Mg^{++}
		Δ OD/min.†	Percent		
Standard assay		0.210	...	....	...
Assay + control serum		0.210	...	....	...
Assay + unabs. antibody		0.037	82.4	....	...
Strain‡					
LT-2(WT)		0.214§	0	13.10	0.78
LT-7(WT)		0.208	0	16.20	0.87
ilva B-6	Spontaneous, LT-2	0.214	0	66.60	2.02
ilva C-13	Spontaneous, LT-2	0.206	0	13.50	0.80
ilva A-8	Spontaneous, LT-2	0.040	81.0	0.36	0.36
A-12	Spontaneous, LT-2	*0.208*	0	1.37	0.98
A-14	Spontaneous, LT-2	0.036	82.9	0.42	0.48
A-19	Spontaneous, LT-2	0.039	81.4	0.33	0.33
D-27	Spontaneous, LT-7	0.043	79.5	0.64	0.54
A-28	Spontaneous, LT-2	*0.088*	*59.1*	0.60	0.60
A-29	Spontaneous, LT-7	0.037	82.4	0.41	0.41
A-31	2-aminopurine, LT-2	0.036	82.9	0.44	0.44
A-33	2-aminopurine, LT-2	*0.077*	*66.2*	0.44	0.44
A-34	2-aminopurine, LT-2	0.036	82.9	0.44	0.48
A-35	2-aminopurine, LT-2	*0.200*	*0*	0.60	0.48
A-36	2-aminopurine, LT-2	*0.200*	*0*	0.42	0.36
A-37	2-aminopurine, LT-2	*0.162*	*22.9*	0.46	0.46
A-39	2-aminopurine, LT-2	0.040	81.0	0.36	0.30
A-42	2-aminopurine, LT-2	*0.212*	*0*	0.35	0.35
A-43	2-aminopurine, LT-2	0.039	81.4	0.46	0.44
A-44	2-aminopurine, LT-2	0.041	80.5	0.18	0.18
A-46	2-aminopurine, LT-2	*0.208*	*0*	0.35	0.35
A-48	2-aminopurine, LT-2	*0.212*	*0*	0.24	0.20
A-50	2-aminopurine, LT-2	0.040	81.0	0.35	0.31
A-51	2-aminopurine, LT-2	0.038	81.9	0.60	0.50
A-52	UV, LT-2	0.034	83.8	0.41	0.39
A-54	UV, LT-7	0.040	81.0	0.48	0.37
A-55	UV, LT-7	0.037	82.4	0.46	0.40

* Antibody preparations (diluted one to three with 0.1 M KPO_4 buffer, pH 7.5) were absorbed with equivalent amounts (mg of protein) of cell-free extracts of the listed strains. One-tenth ml aliquots of the absorbed preparations were then tested against the reductoisomerase activity present in a cell-free extract of LT-2 (0.2 mg protein per assay).

† Measure of decrease in optical density at 340 mμ.

‡ All strains were grown in minimal medium under forced aeration 16 hr at 37°; the media for the *ilva* strains were supplemented with 10 μg L-isoleucine and 20 μg L-valine per ml.

§ Values within ±5 percent of the activity observed in the standard assay were taken to represent 100 percent activity.

follows: band I refers to the areas of the gel containing all reductoisomerase and a small amount of reductase activity; bands II and III refer to the areas containing reductase activity (ARMSTRONG and WAGNER 1961b). Both HKV and HP serve as substrates for the enzymatic activity of band II; whereas, only HP is appreciably active for band III. Each of the three bands of activity, as well as a control band (an area of the gel containing no enzymatic activity), was cut from the gels. The comparable bands from the three gels were pooled and squeezed

through a 5 ml syringe (no needle attached) into a 50 ml Lusteroid centrifuge tube containing 6 ml of 0.1 M potassium phosphate buffer at pH 7.5, 1 mM $MgSO_4$ and 1 mM β-mercaptoethanol. The four tubes were then kept in ice for one hour (with occasional stirring), after which they were centrifuged for 30 minutes at 18,400 $\times g$ in a refrigerated Lourdes centrifuge. The supernatants (extracts of the bands of enzymatic activity and the control band) were used without further treatment.

Preparation of antibody against purified reductoisomerase: The procedure used is similar to that described by SUSKIND (1957) and YANOFSKY (1959). The immunizing doses injected into the foot pad of a rabbit contained 1.5 to 2.0 mg of protein from a preparation of reductoisomerase (free of reductase and residual reductoisomerase activities), purified 50-fold to 75-fold from cell-free extracts of LT-2. Control serum was obtained prior to immunization. Both the control and immunized serum were (1) heated one hour at 56°, (2) subjected to 50 percent ammonium sulfate fractionation, and (3) dialyzed against 0.1 M potassium phosphate buffer, pH 7.5. The immune serum (referred to in the illustrations and, occasionally, in the text as antireductoisomerase or anti-RI for purposes of emphasis and clarity) was absorbed with cell-free extracts of *A-8* or *D-27*, containing no detectable Mg^{++}-dependent reductoisomerase activity. The absorption procedure was accomplished by adding an aliquot of the cell-free extracts, containing 0.1 mg of protein, to the antibody preparation. The mixture was incubated one hour at 37° then kept 48 hours at 4°. The precipitate that was present after this period of time was then removed by centrifugation. The above procedure was repeated with the supernatant until the formation of a precipitate was no longer detectable. Absorption with cell-free extracts of *A-8* and *D-27* showed no decrease in the enzyme neutralizing ability of the immune serum, indicating that no protein serologically related to wild-type reductoisomerase (cross-reacting material or CRM) was present in the extracts. The presence of CRM in other *ilva* strains used in this study was similarly determined.

The immunological studies presented in this report, with the exception of the data in Table 1, were carried out with a preparation of immune serum obtained from a single rabbit. A second batch of serum was prepared for the study presented in Table 1. Unless otherwise specified, all studies were carried out with absorbed immune serum diluted one to three with 0.1 M potassium phosphate buffer, pH 7.5.

Assay of antireductoisomerase activity: The antireductoisomerase preparation was added directly to the reaction mixture (reductoisomerase or reductase assay), and the mixture was incubated ten minutes at 35° prior to the spectrophotometric assay. Neither the presence of the substrate nor the length of incubation time (up to ten minutes) had an effect on the enzyme neutralizing ability of the immune serum. The effect that the addition of immune serum to the assay had on the observed decrease in optical density at 340 mμ (oxidation of TPNH to TPN) was noted and used to determine the amount of enzyme neutralized (calculated in μM of TPNH oxidized per minute).

RESULTS

Electrophoretic and enzymatic studies: Previous studies have shown that following starch gel electrophoresis of cell-free extracts and purified preparations of reductoisomerase from wild-type Salmonella, one band of reductoisomerase activity and three bands of reductase activity can be detected on the gel. These activities have been labelled I, I-A, II and III (see Figure 2). The various reductase activities have been classified according to substrate specificity; the order of specificity for HKV is I-A > II > III. A discussion of these activities is found in ARMSTRONG and WAGNER (1961b). When cell-free preparations of strains *A-8* and *D-27* are subjected to electrophoresis at pH 8.2 with borate buffer, the residual reductoisomerase activity can usually be detected in two faint bands that display electrophoretic mobilities different from that of wild-type activity (Figure 2). The reductase activities of these two mutant strains are detected as one major band, approximately the same distance from the origin as that of band II (Figure 2). However, when the electrophoretic procedure is carried out at pH 7.5 using potassium phosphate buffer, the major band of reductase activity of the mutant strains appears at a distance from the origin different from that of wild type (Figure 3). The residual reductoisomerase activities are again located in positions different from that of wild type. For the purposes of this report, the weaker reductase activities shown in Figures 2 and 3 will not be considered. Thus, the results of electrophoretic studies show that both

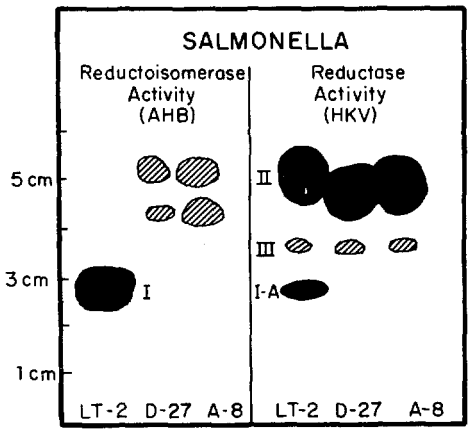

FIGURE 2.—Tracing of an assay for reductoisomerase and reductase activities on a starch gel following electrophoresis of cell-free extracts of wild type (LT-2), *ilvaA-8* and *D-27*. The electrophoresis was carried out with borate buffer, pH 8.2.

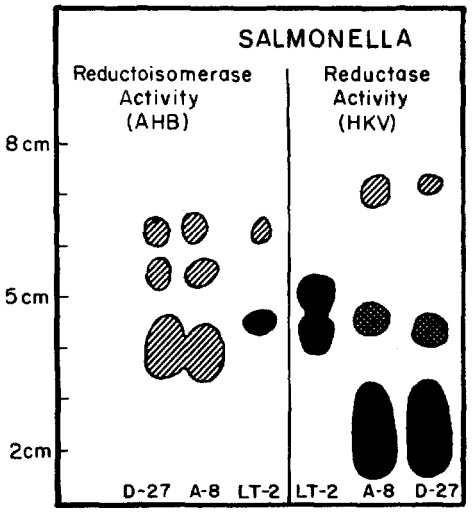

FIGURE 3.—Tracing of an assay for reductoisomerase and reductase activities on a starch gel following electrophoresis of cell-free extracts of wild type (LT-2), *ilvaA-8* and *D-27*. The electrophoresis was carried out with potassium phosphate buffer, pH 7.5.

the residual reductoisomerase and the major reductase activities of strains *A-8* and *D-27* display different electrophoretic mobilities from that of wild type; however, the two mutant activities bear a similar electrophoretic behavior to each other. Since the reductase activities are considered to represent altered forms of the reductoisomerase, it is not surprising to note differences between the mutant and wild-type reductase activities.

The study was extended to include a survey of the residual reductoisomerase activity observed in the remaining 22 *ilvaA* strains. Enzymatic assays show that all of the *ilvaA* strains possess the residual activity; however, unlike reductoisomerase activity, Mg^{++} is not required for activation (last two columns in Table 1). The electrophoretic data on the residual reductoisomerase activities of the mutant strains were not all satisfactory since repeated trials show that the very low activities are difficult to detect and that the residual activity is often unstable to electrophoresis. However, the electrophoretic mobilities that were obtained are similar to those found in *A-8* and *D-27* (Figure 2).

Although the reductoisomerase of the wild-type strains is dependent on the presence of Mg^{++}, a small amount of activity remains in the absence of the metal ion (Table 1). The cell-free extracts were dialyzed four hours at 4° against 200 volumes of 0.005 M potassium phosphate buffer, pH 7.5 to remove any traces of Mg^{++}, then reassayed. The same amount of residual activity was again observed; hence, the residual activity is found to be present in wild type in amounts comparable to that found in the mutant extracts. Under standard reductoisomerase assay conditions, the low level of residual activity is masked by the presence of high levels of reductoisomerase activity.

Immunological studies: The preparation of an immune serum for wild-type reductoisomerase (Methods) made possible an investigation of antigenic similarities among the various wild-type and mutant enzyme activities.

The effects of adding increasing amounts of immune serum to an assay containing a reductoisomerase preparation that had been purified 100-fold from cell-free extracts of wild type (LT-2) is shown in Figure 4. The reductoisomerase activity decreases linearly with increasing amounts of antibody preparation. One-tenth ml of diluted immune serum (antireductoisomerase preparation) neutralizes an amount of reductoisomerase that is capable of catalyzing the oxidation of about 0.02 M of TPNH per minute under standard assay conditions. When the assay was reversed, i.e. a fixed amount of immune serum was titrated against increasing amounts of 100-fold purified reductoisomerase, the inhibition of TPNH oxidation per volume of immune serum was the same as calculated for the preceding experiment.

When the immune serum was tested with cell-free extracts of wild type, the enzymatic neutralizing ability was identical with that observed with the preparation of purified reductoisomerase (Figure 5). Assays of immune serum with cell-free extracts of the *ilva* mutants *B-6* and *C-13*, blocked at the dehydrase and transaminase steps, respectively, show that the amount of enzyme activity neutralized per volume of immune serum is equivalent to that of wild type. These results show that the reductoisomerase activities in these mutant strains are

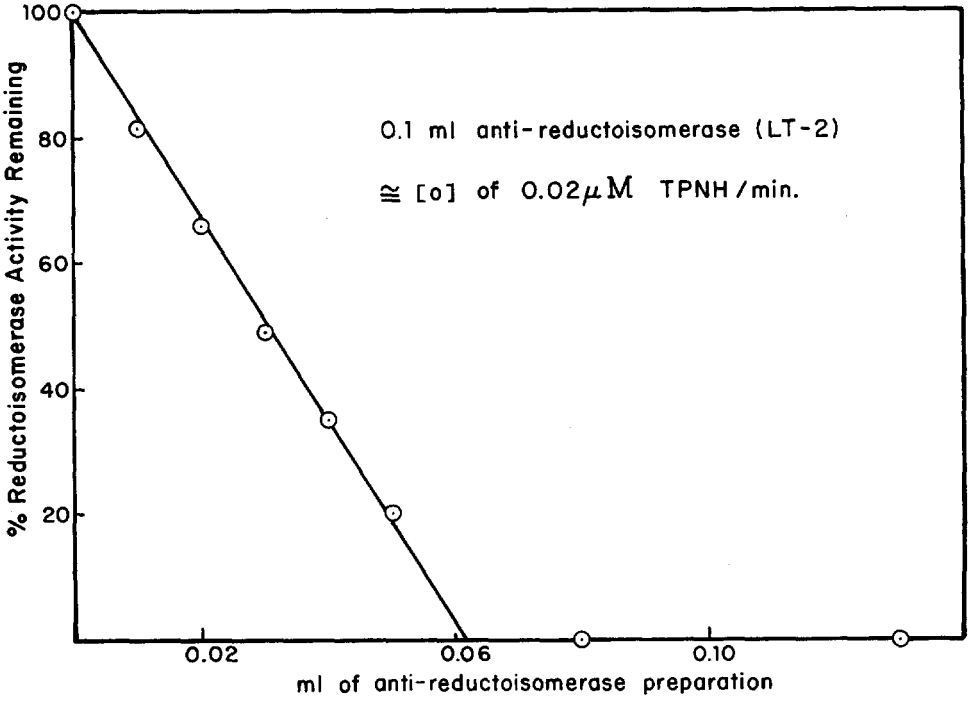

FIGURE 4.—The neutralization of purified reductoisomerase activity (LT-2) by homologous immune serum. 100 percent activity = △ OD of 0.086 per minute. Degree of enzyme neutralization is referred to as "≅ [O]."

antigenically related, indicating that the same enzymatically active protein is present in these strains as in wild type. The "trailing" effect noted on the neutralization curves of Figure 5 is observed frequently in assays of crude extracts. The linear relationship between the loss of reductoisomerase activity and the amount of immune serum does not hold after approximately 80 percent of the activity has been neutralized.

As depicted in Figure 6, the enzymatic neutralizing ability of the immune serum is the same when the antibody preparation is absorbed with cell-free extracts of either *A-8* or *D-27* (possessing no detectable Mg^{++}-dependent reductoisomerase). Since the effectiveness of the absorbed antibody preparation on inactivation of 60-fold purified wild-type reductoisomerase is the same as that of nonabsorbed antibody (not shown), it is assumed that the mutant extracts contain no cross reacting material (CRM). A cell-free preparation of *D-27*, displaying a residual reductoisomerase activity (one tenth the activity of wild type), was tested with immune serum that had been absorbed with cell-free extracts of *A-8* or *D-27*. In both assays, amounts of diluted antibody preparation up to 0.1 ml had no effect on the observed residual reductoisomerase activity. Similar results were obtained when immune serum was tested with cell-free extracts of *A-8*, displaying residual reductoisomerase activity (Figure 6), as well as with the residual activity in the remaining 22 *A* strains.

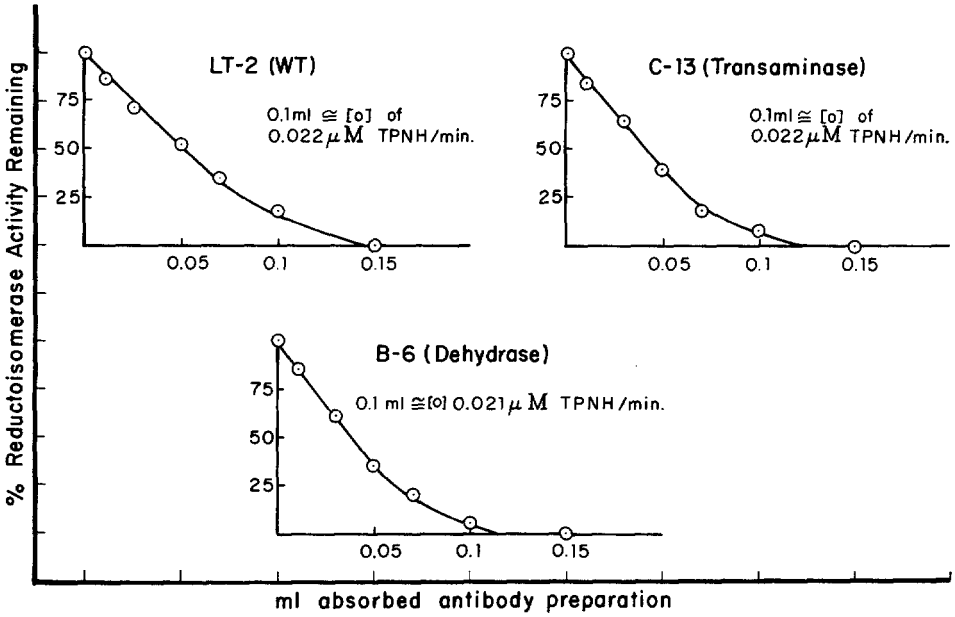

FIGURE 5.—The neutralization of reductoisomerase activity of cell-free preparations of LT-2, *ilvaC-13* and *B-6* by immune serum prepared against reductoisomerase of wild type (LT-2). 100 percent activity = △ OD of 0.142, 0.096 and 0.123 per minute LT-2, *B-6* and *C-13*, respectively.

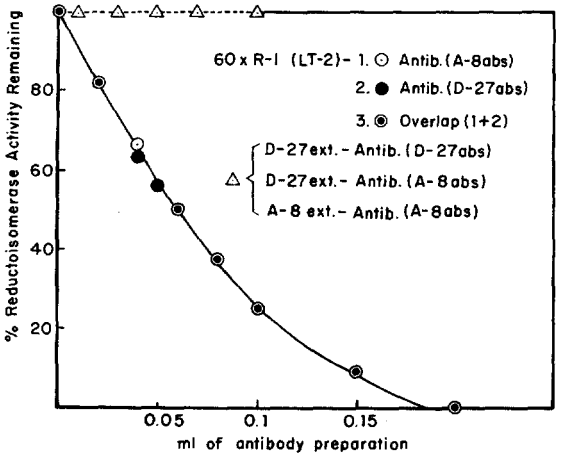

FIGURE 6.—The neutralization of a preparation of reductoisomerase purified 60-fold from LT-2 (60 × R-I) and of residual reductoisomerase activity in cell-free preparations (*ext.*) of *ilvaA-8* and *D-27* by the immune serum (*antib.*) prepared against the reductoisomerase activity of LT-2. The immune serums were absorbed (*abs.*) with cell-free extracts of *A-8* and *D-27*. 100 percent activity = △ OD per minute of 0.168, 0.095 and 0.165 for the purified reductoisomerase, *D-27* and *A-8*, respectively.

Columns 1 and 2 of Table 1 show the results obtained when the *ilvaA* strains, as well as both wild-type strains and *ilvaB-6* and *C-13*, were tested for ability to absorb an antireductoisomerase preparation. As expected, extracts of *B-6*, *C-13*, LT-2 and LT-7 are equally effective in absorbing the antibody preparation. Six *ilvaA* strains, *12*, *35*, *36*, *42*, *46* and *48*, possess CRM that is as effective as the homologous antigen. Three *A* strains, *37*, *28* and *33*, evidently possess CRM's in low amounts or with low activity. The remaining *A* strains are devoid of detectable CRM. Thus, it was found that six strains possess a CRM with the same activity as wild type; three strains produce reduced but significant activity, and 15 strains possess no CRM.

Extracts of enzymatic activities detected on starch gel following electrophoresis of a cell-free extract of wild type (LT-2) were used to test the effect of antireductoisomerase preparation on the reductases. When the extract containing reductoisomerase activity (band I of Figure 2) was assayed, only reductoisomerase activity was detected (Table 2). Band I-A (reductase activity) was not observed; the activity, if present, was too weak to be detected. The extract of Band II utilized both HKV and HP as substrates in the reductase assay, and no reductoisomerase activity was detected (Table 2). Band III also displayed reductase activity; however, only HP was active as a substrate (Table 2). The control extract showed none of the enzymatic activities. When the various activities detected in the spectrophotometric assays were tested with 0.05 ml of diluted immune serum, reductoisomerase activity was shown to be partially neutralized. The reductase activities, whether assayed with HKV or HP, were unaffected by the presence of the immune serum. Thus, the two reductase activities of wild type do not appear to be antigenically related to the reductoisomerase. Comparable studies carried out with extracts of the major reductase activity of both *A-8* and *D-27* show similar results.

One ml aliquots of the extracts shown in Table 2 were incubated in ice for three hours with 0.1 ml undiluted immune serum, then centrifuged. Controls of each extract were prepared with 0.1 ml potassium phosphate buffer instead of antibody preparation. Longer periods of incubation were not attempted since the

TABLE 2

Effect of antireductoisomerase on electrophoretically separated enzymatic activities

Band* extracted	Protein mg/ml	Δ OD per minute†					
		Reductoisomerase‡				Reductase	
		AHB		HKV		HP	
		None	Anti-RI§	None	Anti-RI	None	Anti-RI
I	0.11	0.095	0.030	0	....	0	....
II	0.13	0	....	0.030	0.028	0.060	0.060
III	0.18	0	....	0	....	0.025	0.023
Control	0.05	0	....	0	....	0	....

* See text for extraction procedure.
† Measure of decrease in optical density at 340 mμ.
‡ For reductoisomerase and reductase assays, 0.4 ml of the gel extract was used except for the reductoisomerase assays of Band I, in which 0.1 ml was used. See text for abbreviations.
§ Anti-RI = 0.05 ml diluted antireductoisomerase.

reductoisomerase is not stable under these conditions. Assay of the supernatants show that the reductoisomerase is partially neutralized; whereas, the reductase activities are unaffected by the incubation with immune serum (Table 3).

The studies with an antireductoisomerase preparation show that although the immune serum is capable of neutralizing wild-type reductoisomerase activity (and similar activities in *ilva* strains *B-6* and *C-13*), it has no effect on (1) the residual reductoisomerase observed in the *ilvaA* strains (2) the major reductase activities of mutant strains *A-8* and *D-27* and (3) the two reductase activities tested in the wild-type strain.

Assays of dehydrase and transaminase activities of cell-free extracts of wild type show that the addition of antireductoisomerase does not affect the observed activity of either assay. The antibody preparation is specific for the reductoisomerase reaction.

TABLE 3

Enzymatic activities in supernatant of gel extracts following incubation with antireductoisomerase

Supernatant assayed	Anti-RI†	Δ OD per minute*		
		Reductoisomerase		Reductase
		AHB	HKV	HP
I	+	0.038	....	....
I	—	0.086	....	....
II	+	....	0.032	0.053
II	—	....	0.030	0.054
III	+	....	....	0.020
III	—	....	....	0.020

* See Table 1 for details of enzymatic assays and abbreviations.
† See text for incubation procedure.

DISCUSSION

Electrophoretic data provide further evidence that a relationship exists between the reductoisomerase and the reductase. A mutation involving the reductoisomerase step would be expected to affect the physical properties of the reductoisomerase but not those of the reductase if no relationship existed between the two activities. This is not the case since both enzymes are apparently affected by a mutation at the *A* locus. The difference in the reductases is shown by the electrophoretic mobility of the major reductase activity detected in the *ilva* strains *A-8* and *D-27* (Figure 3). The results, however, fail to show any detectable electrophoretic differences between the enzymatic activities observed in the two mutant strains. This is an interesting point for consideration. As mentioned previously, transduction and enzymatic studies carried out by GLANVILLE and DEMEREC (1960) and WAGNER and BERGQUIST (1960) describe two closely linked loci, *A* and *D*, that control the reductoisomerase step. These loci may represent, in reality, two sites within the same locus, using the term as defined by DEMEREC (1956), and the possibility exists that each of the sites controls one of the two reactions catalyzed by the reductoisomerase. This difference in their

action could possibly be detected by biochemical methods. An analogy can be found in the tryptophan synthetase system studied by YANOFSKY (1960). As interesting as this possibility appears, there is no biochemical evidence to date to support this contention. As with the electrophoretic data, the single *D* mutant consistently tests as an *A* mutant with the one exception the *D-27* grows suboptimally when valine only is added to the minimal medium. Thus, until evidence is produced to the contrary, the single *D* mutant should be considered another strain of the *ilvaA* series.

Although a relationship between the reductoisomerase and reductases has been shown by electrophoretic and other biochemical data (ARMSTRONG and WAGNER 1961a,b). attempts to show an immunological similarity between the enzymatic activities have been unsuccessful. These data imply that the alterations (detected as reductases) the reductoisomerase molecule undergoes not only affects the enzymatic properties but also the immunological properties. A change in secondary or tertiary structure could account for these results since it has been shown by BERSON and YALOW (1961) that insulins with identical amino acid sequences (primary structure) can display different immunological properties. To date attempts to test one of the three reductase activities (band I-A) have been unsuccessful.

The available information on the activity described as the residual reductoisomerase activity in this and previous reports can be summarized as follows:

1. The residual activity has been observed in wild type, as well as in 24 reductoisomerase-deficient strains.
2. The residual activity resembles the known reductases in that no Mg^{++} is required for activation and the immune serum prepared against wild-type reductoisomerase does not neutralize the activity observed in the spectrophotometric assay.
3. The residual activity, unlike the reductases, is capable of utilizing AHB as a substrate and is unstable to purification (unpublished observations) and to electrophoresis.
4. Electrophoretic studies indicate that the residual activities in the various *ilvaA* strains are similar in electrophoretic mobility. However, as mentioned previously, it is difficult to obtain conclusive results in those strains possessing very low activity.

The conclusion may be drawn from the above evidence that the residual reductoisomerase activity is not the result of a mutation at the *A* locus since its presence has been detected in wild-type and all *ilvaA* strains. The evidence further suggests that the residual activity is not a true reductoisomerase but rather a fourth reductase and possibly an additional altered form(s) of the reductoisomerase. Whether the residual activity and the described reductases occur *in situ* or as a result of the treatment the cells receive is as yet undetermined.

The data, therefore, show that in the available *ilvaA* strains, the mutations have resulted in a total loss of a specific reductoisomerase activity. Since transduction data obtained by GLANVILLE and DEMEREC (1960) and in this laboratory

(unpublished data) show that a series of different mutation sites are involved in the 24 *ilvaA* strains tested, it is evident that the structural configuration of the reductoisomerase molecule is of extreme importance to its enzymatic activity and, possibly, its immunological properties.

Two explanations can be offered for the absence of CRM in 15 of the *ilvaA* strains. The first is that in these strains the reductoisomerase molecule is not produced; hence, there is an absence of both enzymatic activity and CRM. The second explanation is that an altered reductoisomerase molecule is produced and that it possesses not only altered enzymatic activity but also altered immunological properties (no CRM). Although it is likely that both reasons are valid in this case, the second explanation deserves attention since it is favored by data presented in this and previous reports. As stated previously, biochemical data establish a relationship between the reductase and reductoisomerase activities; however, the enzyme activities are not related immunologically. These results have been interpreted to mean that altered immunological properties are associated with altered enzymatic activity. This reasoning can also be applied to the data obtained with the mutant strains *A-8* and *D-27*. In this case, the detection of a reductase with physical properties different from that of an equivalent reductase of wild type (Figure 3) is indirect evidence that an altered reductoisomerase is being produced in two mutant strains that lack both Mg^{++}-dependent reductoisomerase activity and CRM. Too, the ability of *D-27* to grow suboptimally at times on a valine supplement suggests that under certain conditions (as yet undetermined) the pathway in this mutant strain is operative for the biosynthesis of isoleucine and, therefore, does contain an altered reductoisomerase capable of catalyzing the production of sufficient amounts of the dihydroxy acid precursor of isoleucine. Since the enzymatic data show that the precursor of isoleucine is eightfold to tenfold more active in the *in vitro* reductoisomerase assay than the precursor of valine (WAGNER and BERGQUIST 1960), a reductoisomerase molecule with minimal enzymatic activity would be expected to favor the production of isoleucine. This point is currently being investigated.

This report has been concerned with the various enzymatic activities that are associated with the *ilvaA* locus. The survey of the available *A* strains have resulted in a better understanding of the nature of these enzymes and of the mutations that occur in the *ilvaA* locus. Studies are being continued on these strains in an effort to clarify further the results that have been presented in this report.

SUMMARY

Electrophoretic studies on LT-2 (wild type) and the *ilva* strains *A-8* and *D-27* of *Salmonella typhimurium* provide further evidence of a relationship between the reductase and the reductoisomerase since the electrophoretic mobility of the major reductase activity in the mutant strains (deficient in reductoisomerase) is different from that of wild type. No difference, however, is noted between the activities of the mutant strains.

Immune serum prepared against the wild-type reductoisomerase, is capable of neutralizing the wild-type activity but is ineffective against (1) the residual

reductoisomerase activity present in the *ilvaA* strains, (2) two of the reductase activities of LT-2 and (3) the major reductase activity of *ilva* strains *A-8* and *D-27*.

The residual reductoisomerase activity, observed in 24 *ilvaA* strains, has been detected in the wild-type strain.

The implication of the results of this study are discussed with respect to the nature of the residual reductoisomerase activity and of the mutations occurring at the *ilvaA* locus.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to MISS MARY LYNN GORDON and MRS. THERESA BARBEE for their technical assistance and to DRS. KAZUYOSHI KIRITANI and ROBERT RINEHART, whose aid in the preparation of the antibody is greatly appreciated.

LITERATURE CITED

ARMSTRONG, F. B., and R. P. WAGNER, 1961a Biosynthesis of valine and isoleucine. IV. α-hydroxy-β-keto acid reductoisomerase of Salmonella. J. Biol. Chem. **236**: 2027–2032.
1961b Electrophoretic studies on the reductoisomerase and reductase of Salmonella. J. Biol. Chem. **236**: 3252–3256.

BERSON, S. A., and R. S. YALOW, 1961 Immunochemical distinction between insulins with identical amino acid sequences. Nature **191**: 1392–1393.

DEMEREC, M., 1956 Structure and arrangement of gene loci. pp. 20–31. (Cytologia, Suppl. Vol.) Proc. Intern. Genet. Symp. Tokyo Kyoto.

GLANVILLE, E. V., and M. DEMEREC, 1960 Threonine, isoleucine, and isoleucine-valine mutants of *Salmonella typhimurium*. Genetics **45**: 1359–1374.

RADHAKRISHNAN, A. N., R. P. WAGNER, and E. E. SNELL, 1960 Biosynthesis of valine and isoleucine. III. α-keto-β-hydroxy acid reductase and α-hydroxy-β-keto acid reductoisomerase. J. Biol. Chem. **235**: 2322–2331.

SUSKIND, S. R., 1957 Properties of a protein antigenically related to tryptophan synthetase in *Neurospora crassa*. J. Bacteriol. **74**: 308–318.

UMBARGER, H. E., B. BROWN, and E. J. EYRING, 1960 Isoleucine and valine metabolism in *Escherichia coli*. IX. Utilization of acetolactate and acetohydroxybutyrate. J. Biol. Chem. **235**: 1425–1432.

WAGNER, R. P., and A. BERGQUIST, 1960 Nature of the genetic blocks in the isoleucine-valine mutants of Salmonella. Genetics **45**: 1375–1386.

WAGNER, R. P., A. N. RADHAKRISHNAN, and E. E. SNELL, 1958 The biosynthesis of isoleucine and valine in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **44**: 1047–1053.

YANOFSKY, C., 1959 A second reaction catalyzed by the tryptophan synthetase of *Escherichia coli*. Biochim. Biophys. Acta. **31**: 408–416.
1960 The tryptophan synthetase system. Bacteriol. Rev. **24**: 221–245.