MUTATION TO L-RHAMNOSE RESISTANCE AND TRANSDUCTION TO L-RHAMNOSE UTILIZATION IN *SALMONELLA TYPHOSA*[1, 2]

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Salmonella typhosa is unable to utilize L-rhamnose as a carbon and energy source for growth. When rhamnose is added to a nutrient agar medium (or other similar media), which otherwise supports growth of this organism, growth is reportedly partially inhibited, and rhamnose resistant papillae arise (Müller, 1911). These mutants, which are no longer inhibited by rhamnose, and which no longer papillate on rhamnose nutrient agar, are still similar to the parent strain in being unable to utilize rhamnose as a carbon and energy source, produce acid or gas from rhamnose, or oxidize rhamnose (Müller, 1911; Barkulis, 1949). Thus this mutational phenomenon differs from that of *Escherichia coli mutabile*, where papillae formed on lactose media produce acid and gas from lactose and utilize lactose as a carbon and energy source.

Salmonella typhimurium, on the other hand, is able to utilize rhamnose as a carbon and energy source, and it has been possible to transmit this ability to *S. typhosa* by transduction.

In this present work, by a comparative study of *S. typhosa* strain O-901, a rhamnose resistant variant isolated from O-901, and a rhamnose positive strain produced by transduction of O-901, the following problems have been investigated: (a) the mechanism of rhamnose sensitivity and resistance; (b) the basis for the inability of *S. typhosa* to utilize rhamnose as a carbon and energy source; (c) the phenotypic changes resulting from transduction to rhamnose utilization; and (d) possible linkage between gene loci involved in rhamnose utilization (Englesberg, 1957).

MATERIALS AND METHODS

Strains. S. typhosa strain O-901, which is Vi negative and nonmotile, was obtained from A. Felix. A streptomycin-resistant variant, derived from this strain, was employed for detailed study. For the sake of clarity, future reference to the streptomycin resistance of this strain and its derivatives shall be omitted since it is of no consequence in this work other than as a marker for identification purposes. This strain has a natural requirement for tryptophan and cystine. Besides being unable to utilize rhamnose as a carbon and energy source (rhamnose negative = rha$^-$), produce acid or gas from rhamnose, or oxidize rhamnose, it is inhibited by rhamnose (rhamnose sensitive = rha-s), and will be referred to simply as either prototroph or rha$^-$rha-s. This strain was employed in the isolation of a rhamnose resistant (rha-r) spontaneous mutant (rha$^-$ rha-r) and a rhamnose positive transductant (rha$^+$rha-s) i. e., a transductant that can use rhamnose as a carbon and energy source. Other strains of *S. typhosa* employed are as follows: H-901, a motile, Vi negative strain; Ty_2W, a Vi negative variant isolated from the Vi positive strain Ty_2 by one of the authors; R2, a Vi negative serologically

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rough strain; and 58Vi, the Vi positive strain currently used in the production of typhoid vaccine.

S. typhimurium strain LT 2, obtained from M. Demerec, can use rhamnose as a carbon and energy for growth and is amino acid independent. It was employed in transduction experiments as the donor of this ability to use rhamnose for growth with phage PLT 22 as the transmitting agent (Zinder and Lederberg, 1952).

Media. Endo's medium with L-rhamnose as the sole carbohydrate supplemented with L-tryptophan and L-cystine·HCl (40 μg/ml) was prepared as previously described (Englesberg, 1957).

A basal mineral and amino acid mixture, consisting of the following ingredients, was employed in preparation of various media as will be indicated: KH_2PO_4-K_2HPO_4, 1.0 per cent; $MgSO_4 \cdot 7H_2O$, 0.01 per cent; $(NH_4)_2SO_4$, 0.1 per cent; L-cystine·HCl, 0.004 per cent; and L-tryptophan, 0.004 per cent. This basal mixture shall henceforth be referred to as component M or simply M.

Component M, supplemented with 1 per cent casein hydrolyzate, 0.2 per cent glucose, and 1.5 per cent agar was employed to prepare slants for the inocula.

Cell extracts were prepared from cells grown in M plus 1.0 per cent casein hydrolyzate and 0.4 per cent L-rhamnose, 5.0 per cent L-rhamnose, 0.4 per cent glucose, or 0.25 per cent rhamnulose.

A medium devised to demonstrate the differences between the prototroph, rha$^-$rha-r, and rha$^+$rha-s consisted of M plus 0.05 per cent casein hydrolyzate and 0.1 per cent L-rhamnose.

Selection for cystine or tryptophan independent transductants was carried out in M plus 0.2 per cent glucose and 1.5 per cent agar with L-cystine or L-tryptophan omitted, respectively. Nutrient broth (Difco) was employed for growing LT 2 for phage production. Tubes of soft nutrient agar were employed to maintain stock cultures.

Transduction experiments. Phage PLT 22 stocks were maintained in T2 buffer (Hershey and Chase, 1952) and stored in the refrigerator. These stocks were prepared by infecting an aerated log phase culture of *S. typhimurium* containing about 2×10^8 cells per ml growing in nutrient broth at 37 C with PLT 22 at a multiplicity of 0.1 to 0.2. Lysis of the culture usually occurred after 3 to 4 hr additional incubation. The lysates were centrifuged for 20 min at low speed (3000 to 5000 rpm) to remove the remaining intact bacteria and bacterial debris, and the phage was centrifuged subsequently at 10,000 rpm (Servall SS-2) for 1 hr. The phage pellet was resuspended in T2 buffer and sterilized with chloroform treatment. Phage assays were made by the standard agar-layer technique (Adams, 1950).

Growth experiments. The growth from an overnight slant culture was scraped off and suspended in saline to a turbidity of 75 (see below). Two-tenths ml of this suspension was employed as the inoculum per 5 ml of medium in optically tared test tubes 18 by 150 mm. Washing the cell suspension had no significant effect on the results. The tubes were incubated at 37 C on their sides with a 5° angle from the horizontal on a shaker which rotated at a speed of 115 rpm with an eccentric of 9 mm in radius. The tubes were removed at intervals and turbidity measured directly using a Fisher electrophotometer modified to accept the growth tubes. A 425 filter was employed with the uninoculated medium as the blank. Results are recorded in Fisher units of optical density (OD) $\times$ 100, and are based on the average of duplicate tubes. With an exponentially growing culture of O-901 in M medium plus 0.05 per cent casein hydrolyzate, 1 unit on the Fisher equals 1.3×10^7 viable cells/ml.

Preparation of enzyme extracts. Enzyme extracts were prepared from cells grown in supplemented M medium as previously indicated. The medium was dispensed in 250-ml amounts in 1-L Erlenmeyer flasks. Five ml of a suspension of cells of a turbidity of 75 Fisher units from an overnight agar slant were employed as an inoculum per L of medium. The flasks were agitated on a rotatory shaker at 37 C at 240 rpm with an eccentric of 1.3 cm in radius.

In initial experiments (tables 1 and 2, experiments A and B), subsequent to 16 hr of incubation additional rhamnose or glucose was added (0.2 per cent), as the case may be, and incubation was continued for an additional 2 hr before harvesting. Subsequently rha$^+$rha-s was harvested during exponential growth at a density indicating rhamnose utilization. Rha$^-$rha-r and rha$^-$rha-s were harvested at this time and at other periods during the growth cycle, as will be indicated, in attempts to produce cells with detectable isomerase and kinase activity. The cells were centrifuged in the cold and the super-

natant fluid decanted. The insides of the tubes were then washed with cold distilled water[5] or with 10^{-3} M Versene at pH 7.4. The cells were then washed with distilled water[5] or with 10^{-3} M Versene. The washed wet pellet was then resuspended in distilled water[5] or in 10^{-3} M Versene plus 10^{-3} M glutathione at pH 7.4 (Simpson *et al.*, 1958) to yield approximately a 15 per cent solution on a wet weight basis. The suspension was then treated in a Raytheon 10 kc sonic oscillator for 10 min. In early experiments a 9 kc oscillator was employed for 30 min.[5] The temperature of the treatment cup was kept cold by the rapid passage of water maintained at 3 C. After sonic treatment, the suspension was centrifuged in the cold for 30 min at 10,000 rpm in the 30 head of the Spinco ultracentrifuge and the supernatant collected and stored in the deep freeze. On occasion the washed pellet was stored in the deep freeze before treatment in the oscillator.

Kinase. Kinase activity was determined manometrically at 37 C by the method of Colowick and Kalckar (1943). Specific activity is recorded as the μmoles of CO_2 liberated per hr per mg extract protein after subtraction of adenosine triphosphatase activity.

Rhamnose isomerase. The reaction mixture was prepared in small test tubes held in an ice bath. One-tenth ml of the mixture was removed at zero time, i. e., just after the addition of the extract, and pipetted into 0.9 ml of 0.1 N HCl. The tubes containing the reaction mixture were then placed in a water bath at 30 C and 0.1-ml samples were removed to 0.9 ml of 0.1 N HCl at various time intervals. Assays for rhamnulose were conducted directly on these samples or on appropriate dilutions. Activity was calculated on the basis of the amount of rhamnulose produced during the 3- to 6-min interval of incubation in the case of very active extracts, and in extracts showing little or no activity longer time intervals were employed. Specific activity is recorded in terms of μmoles of rhamnulose produced per hr per mg extract protein.

Analytical methods. Rhamnulose was determined by the cysteine-carbazole test (Dische and Borenfreund, 1951). The color produced was determined after 2 hr incubation after the addition of the reagents using a Klett-Summerson colorimeter with a no. 54 filter. Ribulose-*o*-nitrophenylhydrazone was employed as a standard to determine the absolute amount of L-rhamnulose produced (Palleroni and Doudoroff, 1956; Englesberg, 1957).

Pie plate chromatrography was employed in the identification of rhamnulose in the presence of rhamnose. The paper was sprayed with alkaline triphenyltetrazolium and heated at 37 C in a moist chamber to develop the rhamnulose spot and then at 100 C to develop the rhamnose spot (Englesberg, 1957). Protein was estimated by the method of Stadtman *et al.* (1951). The absorption spectrum of the chromagen produced by the cysteine-carbazole test was determined with a spectrophotometer.

Chemicals. Rhamnose was obtained from Pfanstiehl Laboratories, Inc.; adenosine triphosphate from Sigma Chemical Company. Ribulose-*o*-nitrophenylhydrazone was obtained from M. Green and D. D. Wood. Casein hydrolyzate was casamino acid (Difco).

RESULTS

Isolation of a rhamnose resistant mutant (rha⁻rha-r). When *S. typhosa* O-901 is streaked on Endo's medium containing rhamnose as sole carbohydrate, growth is slightly inhibited (as compared to growth in the same medium in the absence of rhamnose), all colonies give a negative reaction on the plate (pink colonies) as compared to deep red colonies indicative of rhamnose utilization, and after a few days develop innumerable papillae. Restreaking of papillae results in the isolation of clones which continue to produce a negative reaction on Endo's medium, but no longer papillate on medium containing rhamnose (figures 1 and 2) and grow equally well on medium containing or lacking rhamnose. One such mutant which was investigated in detail will be referred to as rha⁻rha-r.

Attempted isolation of rhamnose positive mutant (rha⁺). Attempts to isolate rha⁺ spontaneous mutants were unsuccessful. Employing the plate method to determine mutation rate using Endo's rhamnose (Englesberg, 1957), it is calculated that mutation of the prototroph occurs at a rate less than 1×10^{-12}. Evidence such as this, indicating inability to isolate mutants, has been interpreted by other workers as indicative of a deletion. This does not seem to be the case in this organism, since spontaneous mutants of this

[5] Experiments conducted at Cold Spring Harbor.

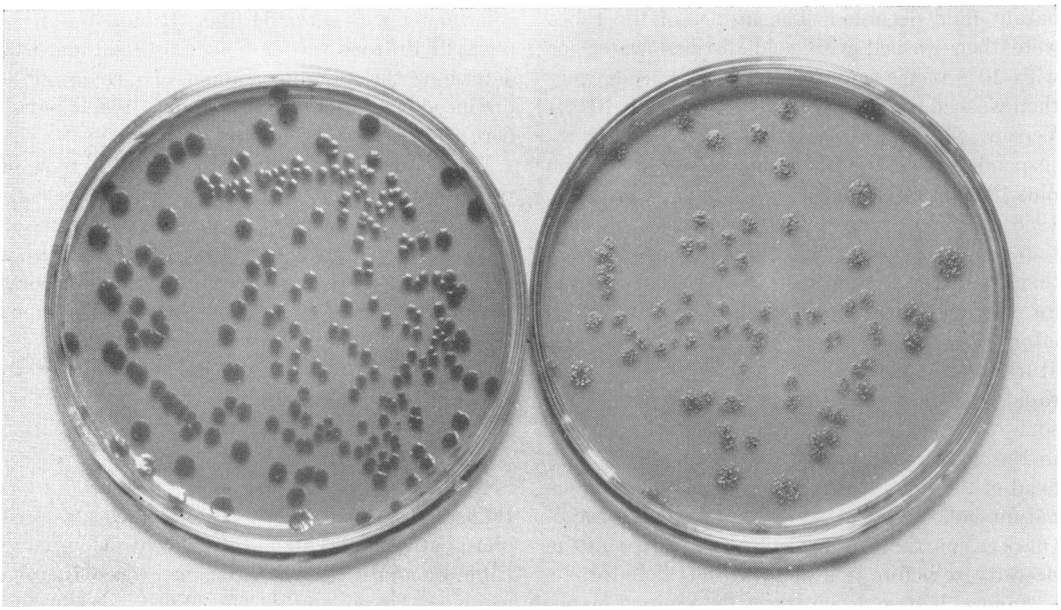

Figure 1. A comparison of 72-hr Endo's rhamnose agar cultures of *Salmonella typhosa* O-901, rha⁻rha-s, (*left*) and a rhamnose resistant spontaneous mutant, rha⁻rha-r, (*right*) isolated from rha⁻rha-s.

strain have been isolated previously in fermentation tubes with liquid media by one of the authors.

Transduction to the utilization of rhamnose as carbon and energy source (rha⁺). It was possible to transduce rhamnose utilization from *S. typhimurium* to *S. typhosa* employing phage PLT 22 previously grown on wild type *S. typhimurium.* Frequencies of transduction to rha⁺ of *S. typhosa* O-901 averaged about 20 per 10^8 cells plated with a multiplicity of infection of 20. Similarly it was possible to transduce rha⁻rha-r mutants to rhamnose utilization (rha⁺rha-s) at frequencies of about 7 in 10^8 cells plated.

In the same experiments both rha⁻rha-s and rha⁻rha-r were transduced to tryptophan independence with frequencies of 100 per 10^8 cells plated. Transduction to cystine independence occurred at high frequencies but showed much heterogeneity in size of the cystine independent transduced colonies produced. In any case, it was impossible to transduce both cystine and tryptophan independence simultaneously, as has been performed with certain cystine and tryptophan markers in *S. typhimurium* (Demerec and Hartman, 1956). Rha⁺ transductants were isolated in pure culture and one that was nonlysogenic was employed for future analysis.

Comparison of the growth of rha⁻rha-s, rha⁻rha-r, and rha⁺rha-s on casein hydrolyzate in the presence and absence of rhamnose. The differences between these three strains can best be seen in a 0.05 per cent casein hydrolyzate M medium in the presence and absence of 0.1 per cent rhamnose (figure 3). It will be noted that with casein hydrolyzate alone, all three strains behave exactly alike. Growth of rha⁻rha-r in the presence of rhamnose is the same as in the absence of this compound. Growth of rha⁻rha-s, on the other hand, follows that of growth in casein hydrolyzate alone initially for a little over 2 hr, before inhibition sets in, whereas growth of rha⁺rha-s is stimulated by rhamnose, enters a lag phase at approximately the density given by growth in casein hydrolyzate alone, and then resumes growth—a typical diauxie type of growth curve.

Analysis of these cultures after 14 and 24 hr of incubation by the cysteine-carbazole test for keto sugars and by paper chromatography for carbohydrates revealed the following. Rha⁻rha-s produces a keto sugar (later identified as rhamnulose) during incubation in the presence of rhamnose and not in its absence, with 0.19 μmole per ml and 0.48 μmole per ml accumulated at 14 and 24 hr of incubation, respectively. Only rhamnose and the keto sugar, rhamnulose, were

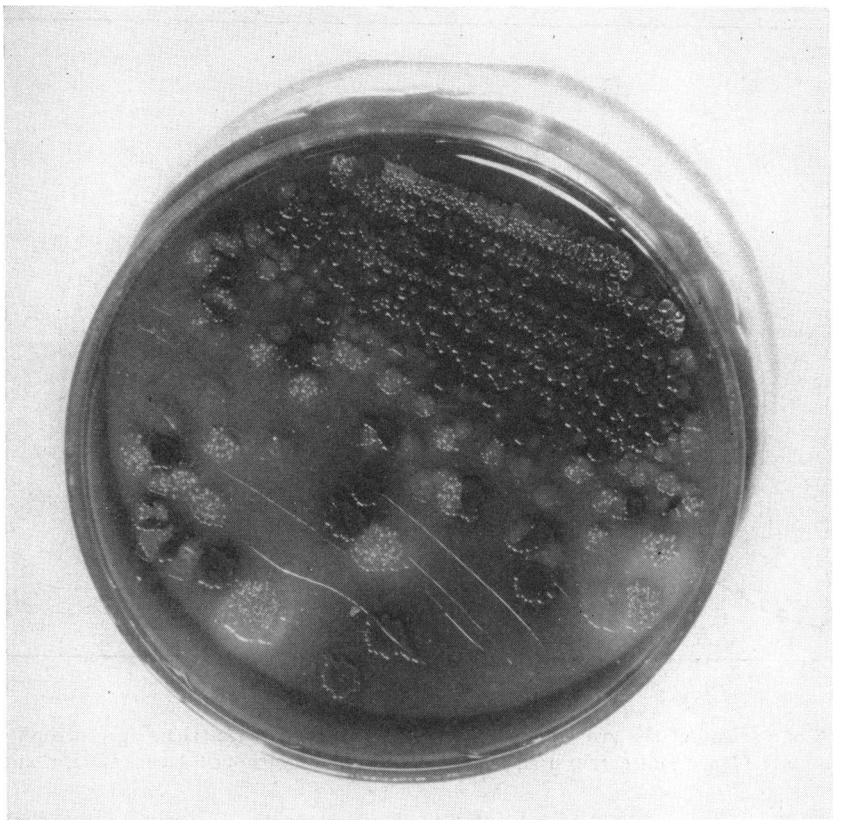

Figure 2. An artificially mixed culture of rha⁻rha-s (light in color and papillated), rha⁻rha-r (light in color, not papillated), and rha⁺rha-s (dark colonies, not papillated) on a 1-week-old Endo's rhamnose agar plate. Note that the rhamnose inhibition is not evident on old plates containing this mixed population.

demonstrable in the cultures by paper chromatography. Trace amounts of rhamnulose were detectable in similar cultures of rha⁺rha-s with the cysteine-carbazole test, and paper chromatography indicated complete disappearance of rhamnose after 14 hr. On the other hand, rha⁻rha-r showed neither keto sugar accumulation nor rhamnose disappearance.

L-Rhamnose isomerase. With the definite indication that the prototroph (rha⁻rha-s) attacks rhamnose, producing a keto sugar, whereas the resistant mutant (rha⁻rha-r) fails to do so, it was of interest to compare the enzymatic activity of these strains toward rhamnose with that of the rhamnose positive transductant. Cell-free extracts of these strains grown in a 1 per cent casein hydrolyzate supplemented M medium with rhamnose or glucose as sole carbohydrate were tested for L-rhamnose isomerase activity. Extracts of rha⁻rha-s and rha⁺rha-s grown in the absence of rhamnose (i. e., in the presence of glucose) and rha⁻rha-r grown in the presence or absence of rhamnose, showed no isomerase activity, whereas extracts of rha⁺rha-s, as well as rha⁻rha-s, showed significant isomerase activity (table 1). These differences were verified with several independently produced extracts assayed under a variety of conditions (table 1). The isomerase activity of rha⁻rha-s was not due to mutation to rha⁺rha-s as verified by the absence of rha⁺ mutants on Endo's rhamnose medium streaked with the cell suspension used in preparing extracts prior to sonic treatment.

So as to give full expression of any rhamnose isomerase activity present in the extracts, optimum conditions for assaying for this enzyme were determined with extracts of rha⁺rha-s (table 1, experiment D). Under the conditions established, there is proportionality between the reaction rate and the quantity of rha⁺rha-s extract

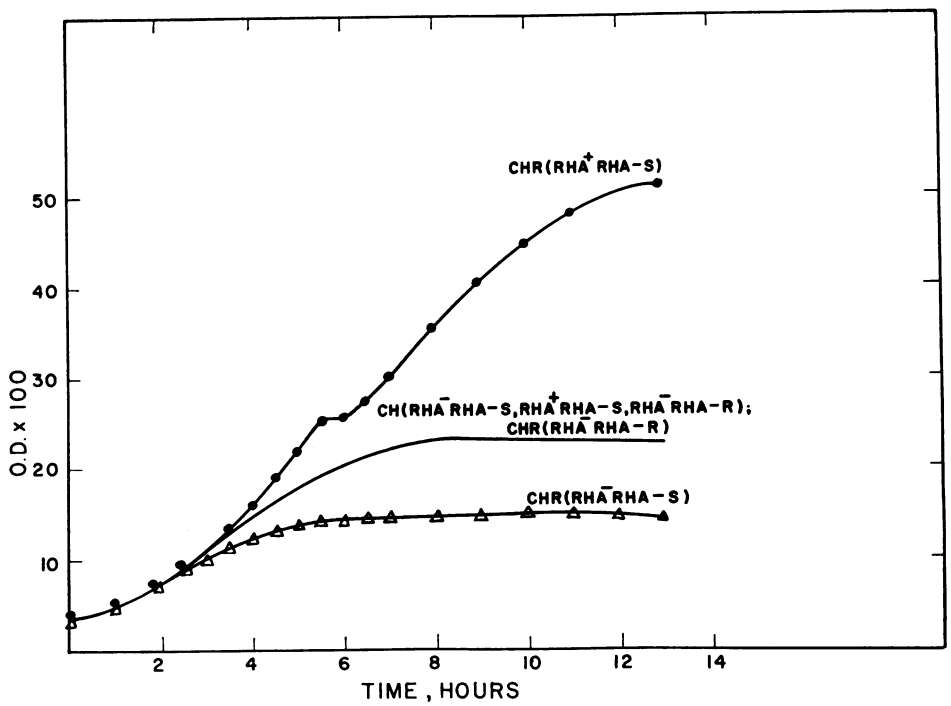

Figure 3. Comparison of the growth of *Salmonella typhosa* O-901 rha⁻rha-s (prototroph), rha⁻rha-r, and rha⁺rha-s on 0.05 per cent casein hydrolyzate (*CH*) with or without 0.1 per cent L-rhamnose (*R*).

tested from 100 μg to over 500 μg of extract protein. Both rhamnose and $MgCl_2$ are in large excess. Experiments indicate that optimum conditions for assay for the isomerase of rha⁻rha-s are similar.

Although the isomerase activity of rha⁻rha-s was initially low, extracts of higher activity were subsequently prepared by harvesting the cells only 2 hr after the complete inhibition by rhamnose when significant amounts of rhamnulose could be detected in the culture medium (10 hr of incubation) (table 1, experiments C and D), whereas extracts prepared from rha⁻rha-s harvested at shorter periods of incubation before rhamnulose could be detected in the medium, or after 16 hr of incubation, were low in activity.

Identification of the product of rhamnose isomerase. Isomerase reaction mixtures allowed to go to equilibrium were chromatographed. The keto sugar spot of *Salmonella typhosa* was found to coincide with rhamnulose prepared with an L-rhamnose isomerase preparation from *Pasteurella pestis*. The absorption spectrum of the chromagen produced by the cysteine-carbazole test coincides exactly with that of true rhamnulose, with a maximum peak of 550 mμ (Englesberg, 1957). Time for maximum color development is about 2 hr, which is the same as that for true rhamnulose. The aldo sugar detected by paper chromatography in the equilibrium mixture coincided with pure rhamnose. No other aldo or keto sugars were detected.

Kinase activity of extracts. Kinase activity was determined using rhamnose, rhamnulose, or a rhamnose isomerase equilibrium mixture of the two as substrate. The latter was prepared as follows. The reaction mixture consisted of H_2O, 25 ml; 0.058 M $NaHCO_3$, 25 ml; 0.1 M $MgCl_2$, 10 ml; 0.1 M cysteine, 10 ml; and rha⁺rha-s cell-free extract (59 mg of extract protein) 2.5 ml, in equilibrium with an atmosphere of 5 per cent CO_2 and 95 per cent N_2 and was incubated at 37 C for 12 hr. An equilibrium mixture of 25 per cent rhamnulose and 75 per cent rhamnose was produced. The mixture was inactivated by being placed in a boiling water bath for 5 min. The precipitate formed was centrifuged, the supernatant acidified with HCl to pH 4, and evaporated to dryness at 45 C under a stream of pure

TABLE 1

Rhamnose isomerase activity of cell extracts of Salmonella typhosa rha⁻rha-s, rha⁻rha-r, and rha⁺rha-s

Experiments A to C were performed at Cold Spring Harbor. *Reaction mixture for experiment A:* Tris(hydroxymethyl)aminomethane (Tris), 90 μmoles, pH 7.5; $MgCl_2$, 20 μmoles; L-rhamnose, 50 μmoles; cell extract, 0.5 to 1.0 ml (15 to 37 mg of extract protein) in a total volume of 2 ml. *Reaction mixture for experiments B and C:* Tris, 90 μmoles, pH 8.2; $MgCl_2$, 40 μmoles; L-rhamnose, 200 μmoles; and cell extract, 0.5 to 1.0 ml, in a total volume of 2.0 ml. In experiments C to D cells of rha⁻rha-s were harvested for extract preparation 2 hr. subsequent to the complete inhibition of growth (increase in optical density) by rhamnose. In experiments E through I, rha⁻rha-r were harvested at turbidities of 220, 232, 310, and 415 respectively, as determined on the Klett Summerson colorimeter with a no. 42 filter.

Experiments D to J were conducted at the University of Pittsburgh. Optimum conditions for assaying for rhamnose isomerase were employed in these experiments and consist of: Tris, 150 μmoles, pH 8.5; $MgCl_2$, 10 μmoles; L-rhamnose, 50 μmoles; cysteine·HCl, 10 μmoles; and cell extract in a total volume of 1.0 ml and final pH of 8.2. The amount of extract employed depended upon the activity of the extract, and in experiment D, for instance, this varied from approximately 0.35 mg of protein in the case of extracts of rha⁺rha-s to 30 mg of extract protein for rha⁻rha-r.

Activity given is based upon the amount of rhamnulose produced between 3 and 6 min of incubation, except in the case of extracts showing no isomerase activity during this interval. In this case, results given are based upon assays after 1 hr of incubation.

Expt*	Strain from Which Extract Was Prepared	Carbohydrate in Medium†	Rhamnulose Produced/hr/mg Protein
			μmoles
A	rha⁻rha-s	Rhamnose	0.56
	rha⁺rha-s	Rhamnose	4.86
	rha⁻rha-r	Rhamnose	—‡
	rha⁻rha-s	Glucose	—
	rha⁺rha-s	Glucose	—
	rha⁻rha-r	Glucose	—
B	rha⁻rha-s	Rhamnose	0.48
	rha⁺rha-s	Rhamnose	7.70
	rha⁻rha-r	Rhamnose	—
C	rha⁻rha-s	Rhamnose	4.48
D	rha⁻rha-s	Rhamnose	5.84
	rha⁺rha-s	Rhamnose	24.6
	rha⁻rha-r	Rhamnose	—
E	rha⁺rha-s	Rhamnose (5%)	21.2
	rha⁻rha-r	Rhamnose (5%)	—
F	rha⁺rha-s	Rhamnulose (0.25%)	11.7
	rha⁻rha-r	Rhamnulose (0.25%)	—
G	rha⁻rha-r	Rhamnulose (0.25%)	—
H	rha⁻rha-r	Rhamnulose (0.25%)	—
I	rha⁻rha-r	Rhamnulose (0.25%)	—
J	rha⁻rha-s	No carbohydrate	0.56

* Each experiment represents results obtained with a different extract preparation.

† Medium: 1 per cent casein hydrolyzate supplemented M medium with the addition of 0.4 per cent carbohydrate unless otherwise indicated.

‡ — = No detectable activity (less than 0.004 μmole/hr/mg protein).

nitrogen. The residue was taken up in a small volume of water, the pH adjusted to 7.4, and the insoluble residue removed by centrifugation. The supernatant was tested for rhamnulose and then adjusted to 0.2 M with respect to this compound.

Results of kinase assays are shown in table 2. Both rhamnose grown rha⁻rha-s and rha⁺rha-s show considerable kinase activity, whereas no activity was detectable with glucose grown rha⁻rha-s, rha⁺rha-s, rha⁻rha-r, or rhamnose grown rha⁻rha-r. Near optimum conditions established for kinase activity are indicated in experiment D, table 2. No actual requirement for glutathione or Versene has been demonstrated. Doubling the amount of adenosine triphosphate or substrate concentration did not increase activity. Where activity is indicated, increasing the amount of extract employed resulted in a proportionate increase in enzyme activity. Rhamnose, rhamnulose, or the isomerase equi-

TABLE 2

Kinase activity of cell extracts of Salmonella typhosa rha⁻rha-s, rha⁻rha-r, and rha⁺rha-s

Kinase activity was determined manometrically (Colowick and Kalckar, 1943). Experiments A to C were performed at Cold Spring Harbor. *Reaction mixture for experiment A:* $KHCO_3$, 81 μmoles; sodium iodoacetate, 6 μmoles; KF, 150 μmoles; $MgCl_2$, 20 μmoles; rhamnulose, 5 μmoles; Na adenosine triphosphate, 20 μmoles; and cell extract in a total volume of 2.1 ml; atmosphere 95 per cent N_2 and 5 per cent CO_2; final pH 7.6. For particular conditions employed in growing cells, see table 1. The cell extracts employed in each experiment are the same as employed in the corresponding experiment for isomerase activity.

Experiments D to I were performed at the University of Pittsburgh. *Reaction mixture for experiments D to I:* $NaHCO_3$, 47 μmoles; glutathione at pH 7.5, 5 μmoles; Versene, 10 μmoles at pH 7.5; $MgCl_2$, 100 μmoles; NaF, 50 μmoles; Na adenosine triphosphate at pH 7.5, 20 μmoles; rhamnose plus rhamnulose equilibrium mixture containing 20 μmoles rhamnulose at pH 7.5; and cell extract in a total volume of 2.0 ml, final pH 7.5; atmosphere 95 per cent N_2 and 5 per cent CO_2. Similar results were obtained with rhamnose or rhamnulose as substrate.

Adenosine triphosphate and substrate were added after equilibration to the main chamber of the Warburg vessel which contained the other ingredients. Adenosine triphosphatase activity was determined in a separate vessel lacking rhamnulose. Activity given is determined on the basis of the amount of CO_2 evolved during the second 5 min of incubation after tipping in substrate and adenosine triphosphate minus CO_2 evolved as a result of adenosine triphosphatase activity.

Expt*	Strain from Which Extract Was Prepared	Carbohydrate in Medium†	Rhamnulose Phosphate Produced/hr/mg Protein
			μmoles
A	rha⁻rha-s	Rhamnose	0.91
	rha⁺rha-s	Rhamnose	0.28
	rha⁻rha-r	Rhamnose	—‡
	rha⁻rha-s	Glucose	—
	rha⁺rha-s	Glucose	—
	rha⁻rha-r	Glucose	—
B	rha⁻rha-s	Rhamnose	0.68
	rha⁺rha-s	Rhamnose	0.28
	rha⁻rha-r	Rhamnose	—
C	rha⁻rha-s	Rhamnose	0.68
D	rha⁻rha-s	Rhamnose	1.01
	rha⁺rha-s	Rhamnose	0.70
	rha⁻rha-r	Rhamnose	—
E	rha⁺rha-s	Rhamnose (5%)	1.10
	rha⁻rha-r	Rhamnose (5%)	—
F	rha⁺rha-s	Rhamnulose (0.25%)	1.06
	rha⁻rha-r	Rhamnulose (0.25%)	—
G	rha⁻rha-r	Rhamnulose (0.25%)	—
H	rha⁻rha-r	Rhamnulose (0.25%)	—
I	rha⁻rha-r	Rhamnulose (0.25%)	—

* Each experiment represents results obtained with a different extract preparation.

† Medium: 1 per cent casein hydrolyzate supplemented M medium with the addition of 0.4 per cent carbohydrate unless otherwise indicated.

‡ — = No detectable activity (less than 0.02 μmole/hr/mg protein).

librium mixture of these compounds gave similar results under these conditions. Evidence indicates, however, that extracts contain a rhamnulokinase which does not attack rhamnose, and no rhamnokinase. This was shown in the following manner. The contents of two Warburg vessels similar to that employed in assaying for kinase activity of rha⁻rha-s (experiment D, table 2) were collected after activity had proceeded for 2 hr. The reaction mixture was deproteinized with an equal volume of cold 10 per cent trichloroacetic acid. The precipitate was centrifuged and washed with 5 per cent trichloroacetic acid and the washings pooled with the original supernatant. The water soluble, alcohol insoluble barium salts were separated (Umbreit *et al.*, 1949) and washed with cold 75 per cent ethanol. The precipitate was dissolved in 0.1 N HCl and the barium precipitated as $BaSO_4$. The barium-free filtrate was neutralized and found to contain 13.5 μmoles of rhamnulose (or rhamnulose phosphate) by the cysteine-carbazole test. (The test

does not distinguish between the free sugar and the phosphorylated ester.) A sample of the filtrate was then deionized with a mixture of Amberlite IR-120 (H^+) and IR-45 (OH^-). The effluent was concentrated to dryness by lyophilization, made up to the original volume, assayed for rhamnulose, and chromatographed on paper as previously described. One sample was hydrolyzed at 100 C in 1 N HCl for 10 min and another for 3 hr, deionized, concentrated, and assayed as above. Deionization before acid hydrolysis removed all traces of the keto sugar, whereas 41 per cent of the keto sugar originally present was recovered in the sample hydrolyzed for 10 min before deionization. There was no detectable keto sugar present after 3 hr of hydrolysis, indicating a destruction of this compound as a result of prolonged treatment. There was no trace of rhamnose in the unhydrolyzed or hydrolyzed samples as shown by paper chromatographic analysis. The keto sugar was identified as rhamnulose by paper chromatography, by the absorption spectrum of the color produced by the cysteine-carbazole test, and by the 2-hr incubation period required for maximum color development (see above).

Loss of ability to produce two enzymes as a result of mutation to rhamnose resistance. On the basis that the failure of rha⁻rha-r to produce a detectable amount of rhamnose isomerase and rhamnulokinase is due to a mutation resulting in a defective permease (Rickenberg *et al.*, 1956), an attempt was made to by-pass the permease by forcing rhamnose into the cells by growing the cells in high concentrations of rhamnose. Both rha⁺rha-s and rha⁻rha-r were grown in component M supplemented with 1 per cent casein hydrolyzate and 5 per cent L-rhamnose. Extracts prepared from rha⁺rha-s cells grown in this manner showed normal isomerase and kinase activity, whereas there was no detectable activity in extracts prepared from rha⁻rha-r (tables 1 and 2).

The possibility that mutation to rha-r resulted directly in a deficiency in isomerase activity, while the potentiality to produce the kinase was still intact, but phenotypically not expressed since it might be sequentially induced, requiring rhamnulose as inducer, was next considered. To test this hypothesis, quantities of rhamnulose were required for growing large cultures of rha⁻rha-r for enzymatic analysis.

Rhamnulose originally employed in kinase determinations was prepared with extracts of a rhamnose utilizing mutant of *P. pestis* (Englesberg, 1957). On the basis of the finding that rhamnulose accumulated in the culture fluid during the growth of *S. typhosa* O-901 (prototroph), a more efficient and simpler method was devised for producing this substance. This strain was grown in 3 L of M component, plus 1 per cent casein hydrolyzate plus 0.4 per cent L-rhamnose distributed in 12 1-L Erlenmeyer flasks. The flasks were incubated with constant agitation, harvested in the cold by centrifugation, and resuspended in 120 ml of 0.6 M borate buffer at pH 9.0. Rhamnose (3.8 mmoles) was added and the suspension was incubated at 37 C for 24 hr. The cells were removed by centrifugation, and the supernatant frozen. The cells were then employed again in the same manner. By this procedure, 85 to 100 per cent of the rhamnose added was recovered as keto sugar in the supernatant. The supernatants from several experiments were pooled, heated at 80 C for 10 min, cooled, and passed through two columns (20 by 350 mm) of Amberlite IR-120(H^+) to convert sodium borate to boric acid. The effluent and washings were evaporated to dryness *in vacuo* (maximum temperature, 45 C), and the borate was removed as methyl borate (Mitsuhashi and Lampen, 1953). The final syrup was taken up in a small volume of water, neutralized with NaOH, and clarified and sterilized by filtration. The keto sugar isolated was identified as rhamnulose by the criteria previously employed. Several grams of rhamnulose were prepared in this manner, having approximately 4 per cent rhamnose as determined by paper chromatographic analysis. These preparations were employed in growing rhamnulose adapted cells for enzyme preparations.

Rha⁺rha-s and rha⁻rha-r were grown in 500 ml of M component, supplemented with 0.5 per cent casein hydrolyzate and 0.25 per cent rhamnulose distributed in 2 1-L flasks. Cells were harvested 2 hr after detection of rhamnulose utilization by rha⁺rha-s. Rhamnulose was still present in the medium at this time. Extracts prepared from rha⁺rha-s had about ½ the usual isomerase activity and somewhat higher kinase activity, whereas extracts from rha⁻rha-r showed no isomerase or kinase activity. Several other attempts were made to produce rha⁻rha-r with detectable activity. Rha⁻rha-r harvested subsequent to full growth in the casein hydrolyzate

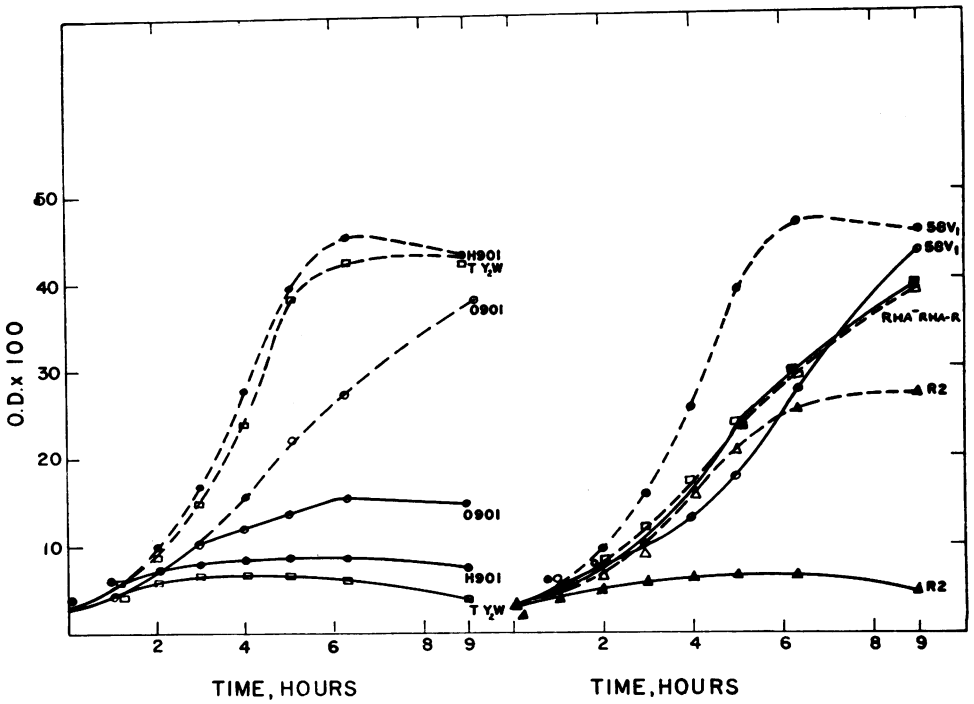

Figure 4. The effect of L-rhamnose on the growth of different strains of *Salmonella typhosa* on casein hydrolyzate. Rha⁻rha-r was employed as a control. This growth experiment was performed as previously described, except that 0.1 per cent casein hydrolyzate was employed. — — — = growth in 0.1 per cent casein hydrolyzate M medium; —— = growth in the above medium plus 0.4 per cent L-rhamnose.

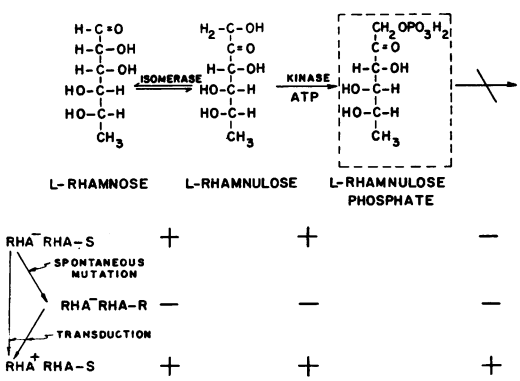

Figure 5. Summary of the enzymatic differences between rha⁻rha-s (prototroph), rha⁻rha-r, and rha⁺rha-s. + = Ability to produce enzyme indicated; − = lack of ability to produce enzyme indicated.

rhamnulose medium were completely inactive (turbidity of 415). Similarly, cells harvested at Klett readings of 232 and 310 showed no activity (tables 1 and 2). In no case was there evidence of rhamnulose disappearance in the rha⁻rha-r cultures.

To determine whether mutation to rha-r has led to the production of some substance that inhibits both isomerase and kinase activity, the activity of rha⁻rha-s and rha⁺rha-s extracts was determined in the presence and absence of equal amounts and 20 times the amount of rha⁻rha-r extract. No inhibition of kinase or isomerase activity was detected.

Comparison of several typhosa strains as to rhamnulose produced and rhamnose inhibition. Figure 4 demonstrates that the growth on casein hydrolyzate of all strains of *S. typhosa* tested (except rha⁻rha-r which was employed as a control) was inhibited by the addition of L-rhamnose. Furthermore, assays of the culture medium subsequent to full growth revealed that all strains (except rha⁻rha-r) accumulated large quantities of L-rhamnulose.

DISCUSSION

A portion of the findings presented in this paper are summarized in figure 5. Evidence presented has demonstrated that although all strains of *S. typhosa* tested are unable to utilize

rhamnose as a carbon source and all are inhibited more or less by rhamnose, all strains produce rhamnulose during growth in a casein hydrolyzate rhamnose medium. *S. typhosa* strain O-901, studied in detail, was shown to possess the two inducible enzymes involved in rhamnose utilization (Englesberg, 1957) when grown in such a medium: a rhamnose isomerase, which converts rhamnose to the keto sugar rhamnulose, and a kinase which phosphorylates rhamnulose probably to rhamnulose-1-phosphate. Although all strains employed were not examined enzymatically, the fact that they all accumulated rhamnulose as strain O-901, indicates that they at least possess the rhamnose isomerase. Thus there is selection in the natural environment for *S. typhosa* strains which cannot utilize rhamnose for carbon and energy, are inhibited by rhamnose, and yet apparently have the genetic information to synthesize one or two enzymes involved in the initial breakdown of this compound.

Mutation to rhamnose resistance leads to the complete loss in ability to produce both enzymes. Experiments have shown that this is not the result of the genetic loss in ability to produce the isomerase and phenotypic loss of the kinase as a result of the absence of the inducing substrate rhamnulose when rhamnose is employed as the carbon source. Nor is this phenomenon due to the production of a soluble inhibitor of isomerase and kinase activity. Moreover it cannot be simply explained on the basis of loss of ability to produce a permease (Rickenberg *et al.*, 1956), active for both rhamnose and rhamnulose. Rickenberg *et al.* (1956) have shown that it is possible to fully induce β-galactosidase in cryptic mutants of *Escherichia coli* by growing these cells in the presence of high concentrations of the inducer. Experiments to force induction of both the isomerase and kinase by growing rha$^-$ rha-r in large concentrations of rhamnose (5 per cent) were to no avail. Extracts produced from such cells had no trace of either rhamnose isomerase or rhamnulokinase. Furthermore, the fact that rha$^-$rha-s grown on casein hydrolyzate alone possesses a low basal level of isomerase activity (table 1, experiment J), whereas rha$^-$ rha-r lacks even this basal level of activity when grown on casein hydrolyzate plus rhamnose, is directly opposed to the permease hypothesis, since if rha$^-$rha-r were only permease deficient one would expect it to possess the same basal level of rhamnose isomerase activity exhibited by the prototroph.

The rha$^-$rha-r appears similar to wild type *P. pestis* with respect to rhamnose (Englesberg, 1957). The latter is rhamnose negative and rhamnose resistant, and as a result of a single mutational event gains the ability to produce both rhamnose isomerase and rhamnulokinase. The high mutation rate of rha$^-$rha-s to rha$^-$rha-r, as evident by the number of papillae appearing on colonies of rha$^-$rha-s on rhamnose media (figure 1), suggests that mutation to the loss of both enzymes may also occur in this case as a single mutational event.

Since *S. typhosa* strain O-901 utilizes glucose as a carbon source, the deficiency in rhamnose metabolism occurs probably before conversion into intermediates common with glucose. It seems probable that rhamnulose phosphate may be cleaved by an aldolase type of reaction to dihydroxyacetone phosphate and lactic aldehyde. Since it is a safe assumption to make that this organism can utilize the former compound, the enzymatic deficiency in the prototroph may be precisely at the cleavage step. The rha$^+$rha-s transduced strain then may have gained the ability to produce this enzyme.

As for the mechanism of the rhamnose inhibition, the information gathered so far helps very little in the elucidation of this phenomenon. If one presumes that rhamnose or a product of rhamnose is the inhibitory agent, and this is not the only, or for that matter the most likely, hypothesis (Neidhardt and Magasanik, 1957), the most one can say is that, because of the probable deficiency in rhamnose utilization exhibited by rha$^-$rha-s, as discussed above, the inhibitor cannot be very far removed from rhamnose and may therefore be rhamnose itself, rhamnulose, or rhamnulose phosphate.

Since the opposite phenotype of rhamnose resistance (rha-r) is the ability to produce both rhamnose isomerase and rhamnulokinase, it is obvious that the prototroph and the rhamnose utilizing transduced variant must be considered rha-s (rhamnose sensitive). The resistant mutant, therefore, has two deficiencies: (a) the deficiency of the prototroph, and (b) the deficiency gained as a result of mutation to resistance. The fact that transduction of rha$^-$rha-r occurs at frequencies similar to that for the transduction of rha$^-$rha-s to rha$^+$rha-s is an indication that the rha$^-$ and the rha-r markers are closely linked. The low efficiency of transduction of the rhamnose markers is not due to incompatability of phage PLT 22 with *S. typhosa*, but probably has some connec-

tion with the rhamnose loci themselves, since transduction to tryptophan and cystine utilization occurs at the usual frequency found in *S. typhimurium*.

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SUMMARY

All strains of *Salmonella typhosa* tested are unable to use rhamnose as a carbon and energy source, and growth in a casein hydrolyzate medium is inhibited by the addition of rhamnose.

Growth of these strains in this casein hydrolyzate rhamnose medium results in the accumulation of rhamnulose (keto rhamnose). Employing this fact, a procedure was devised for the production of large quantities of rhamnulose using intact cells of *S. typhosa* strain O-901.

Detailed enzymatic studies of *S. typhosa* O-901, rhamnose negative and rhamnose sensitive (rha$^-$ rha-s), have demonstrated that it possesses two inducible enzymes involved in the initial metabolism of rhamnose, a rhamnose isomerase which converts rhamnose to rhamnulose, and a kinase which in the presence of adenosine triphosphate phosphorylates rhamnulose probably to rhamnulose-1-phosphate.

Mutation to rhamnose resistance (rha$^-$rha-r) results in the loss of the ability to synthesize both enzymes. This loss cannot be explained on the basis of sequential induction, formation of a soluble inhibitor of isomerase and kinase activity, or a defective permease.

The prototroph (rha$^-$rha-s) can be transduced to rha$^+$rha-s with phage PLT 22 previously grown on *Salmonella typhimurium*, the latter being able to utilize rhamnose as a carbon and energy source.

The fact that rha$^-$rha-s and rha$^-$rha-r are transduced to rha$^+$rha-s at similar frequencies suggests that rha$^-$ and rha-r are closely linked.

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