ENZYMATIC EXPRESSION OF GENETIC UNITS OF FUNCTION CONCERNED WITH GALACTOSE METABOLISM IN *ESCHERICHIA COLI*

RICHARD L. SOFFER[1]

Department of Genetics, Stanford University, Palo Alto, California

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ABSTRACT

SOFFER, RICHARD L. (Stanford University, Palo Alto, Calif.). Enzymatic expression of genetic units of function concerned with galactose metabolism in *Escherichia coli*. J. Bacteriol. **82**:471–478. 1961.—A series of genetically characterized galactose-negative mutants of *Escherichia coli* strain K12 were studied with respect to the enzymes of the Leloir pathway for utilization of this sugar. The functions pertaining to the three genetically defined cistrons were found to relate, respectively, to the expression of galactokinase, galactose 1-phosphate uridyl transferase, and uridine diphosphate galactose 4-epimerase. Mutants defective in the latter enzyme represent a new biochemical class in this strain of *E. coli*. Most of the mutants defective in galactokinase were found to exhibit significantly elevated levels of galactose 1-phosphate uridyl transferase as compared with the wild type when grown under conditions of induction. A suppressed transferase-deficient mutant was found to have regained this enzymatic activity.

The concept that genetic material determines protein specificity (Beadle, 1945) has been supported by biochemical (Kurahashi, 1957) and genetic (Lederberg, 1960) studies of different galactose-negative mutants of *Escherichia coli* strain K12, where it has been shown that such mutants are defective in at least one of several enzymes required for galactose metabolism. These studies have also suggested that a functional genetic unit or cistron (Benzer, 1957) is associated with the production of a single enzyme.

[1] Present address: Department of Medicine, New York University Medical School, 550 First Avenue, New York 16, N. Y. The work reported here was done during the tenure of a Postdoctoral Fellowship under the auspices of the American Cancer Society.

Genetic classification of these strains has been facilitated by the fact that the temperate phage λ specifically transduces genetic material concerned with the utilization of galactose (Morse, Lederberg, and Lederberg, 1956*a*). The immediate product of such a transduction is a partially diploid cell termed a "syngenote," containing a fragment of donor chromosome called the "exogenote" as well as the entire genome of the recipient cell including the portion homologous to the donor piece and hence known as the "endogenote" (Morse, Lederberg, and Lederberg, 1956*b*). If donor and recipient chromosomes bear two different markers the syngenote is further classified as a "diheterogenote" and is said to be initially in the *trans* form because the different mutant markers are on different chromosomes. If such a cell ferments galactose then the two mutants are considered to be complementary and their genetic alterations to exist in different functional units (cistrons). Alternatively, if the cell is unable to ferment galactose until a rearrangement to the *cis* form occurs, whereby both mutant markers are found on one chromosome and both wild-type alleles on the other, then the functional discrepancy between the two arrangements is denoted by the term "position effect" and the mutants are said to be noncomplementary and to possess defects in the same cistron. Lederberg (1960) has utilized this system to classify galactose-negative mutants into five categories. Three of these, A, B, and D, may be said to define three corresponding cistrons, since members of each are able to complement those of the other two groups but display position effects with one another. A fourth group, C, is more complex and is characterized by the fact that its members complement neither each other nor those of groups A and B. This group, therefore, cannot be said to define a simple corresponding cistron. The final category, E, is composed of mutants which are not amenable to transduction by λ. Thus, the *cis-trans* test for functional com-

plementation is not applicable to this group when λ is used as the vector for transduction.

Utilization of galactose by *E. coli* K12 has been shown by Kurahashi (1957) and by Kalckar, Kurahashi, and Jordan (1959) to proceed virtually exclusively by the Leloir pathway:

1. Galactose + ATP[2] $\xrightarrow{\text{galactokinase}}$ galactose 1-phosphate + ADP
2. Galactose 1-phosphate + uridine diphosphate glucose $\xrightleftharpoons{\text{galactose 1-phosphate uridyl transferase}}$ uridine diphosphate galactose + glucose 1-phosphate
3. Uridine diphosphate galactose $\xrightleftharpoons{\text{uridine diphosphate galactose 4-epimerase}}$ uridine diphosphate glucose

Sum: Galactose + ATP → glucose 1-phosphate + ADP

The pathway of direct oxidation, dehydration, and cleavage, which is found in certain other microorganisms (Doudoroff et al., 1956), does not seem to play a role in this strain. Kurahashi (1957) has demonstrated the presence of the enzymes required for the Leloir scheme, as well as uridine diphosphate glucose pyrophosphorylase, the enzyme responsible for the production of uridine diphosphate glucose from uridine triphosphate and glucose 1-phosphate (Munch-Petersen et al., 1953), in cell-free preparations of galactose-positive organisms. In addition, Kurahashi first noted (1957) that extracts from mutants with defects in the A cistron failed to exhibit galactokinase activity, whereas preparations from organisms defective in the B cistron showed diminished galactose 1-phosphate uridyl transferase activity. Complementation between these two categories was illustrated at the cell-free level by the fact that intergroup hybrid extracts could incorporate radioactive galactose into uridine nucleotides, whereas intragroup mixtures could not.

Kalckar et al. (1959) showed that extracts from group C mutants had diminished activity for all three of the enzymes concerned with the Leloir pathway.

The present investigation was designed to test the correlation between functional genetic units and the expression of specific enzymes in additional mutants, including two which fell into a genetic category, D, not heretofore investigated at the biochemical level. It was found that the correlation applied almost uniformly and that the uncharacterized group represented a new biochemical class whose members exhibited diminished activity of uridine diphosphate galactose 4-epimerase.

[2] The abbreviations used in this report are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, dihydrotriphosphopyridine nucleotide; tris, tris(hydroxymethyl)aminomethane.

MATERIALS AND METHODS

All bacterial strains used in these experiments were derived from *E. coli* strain K12 and each was characterized by the inability to grow on galactose as a sole carbon source. All except strain no. 63 were isolated by Lederberg (1960) and are considered to represent different single point mutations on the basis of reversion rates and recombination patterns. Strain no. 63 was the gift of J. Adler and A. D. Kaiser. ATP, D-galactose substantially free of glucose, glucose 1-phosphate as the dipotassium salt, uridine diphosphate glucose, TPN, TPNH, DPN, and glucose 6-phosphate dehydrogenase (zwischenferment) were obtained from the Sigma Chemical Company. The barium salt of galactose 1-phosphate was the gift of H. A. Lardy. Glucose 1,6-diphosphate was derived microbiologically from glucose 1-phosphate as described by Leloir and associates (1949). Phosphoglucomutase was crystallized from rabbit muscle according to the method of Najjar (1955). Galactose 1-phosphate uridyl transferase was prepared from *E. coli* strain W3098 by the procedure of Kurahashi and Sugimura (1960). Uridine diphosphate glucose dehydrogenase was obtained from calf liver as outlined by Strominger et al. (1957); however, the fraction precipitated by acetone was taken up in 0.02 M potassium acetate buffer (pH 5.9) and the resulting suspension was used in the assays. This enzyme was also obtained from the Sigma Chemical Company. Uridine diphosphate galactose was synthesized from uridine diphosphate glucose and galactose 1-phosphate by an enzymatic technique similar to that of Anderson, Maxwell, and Burton (1959) except that the above described preparations of galactose 1-phosphate uridyl transferase, phosphoglucomutase, zwischenferment, and glucose 1,6-diphosphate were employed.

Growth of bacteria and preparation of extracts. In

all instances where specific activities for galactose 1-phosphate uridyl transferase are presented, the following standardized procedure was adopted: a loopful of cells from a nutrient agar slant was transferred to 5 ml of the liquid mineral medium no. 56 of Monod, Cohen-Bazire, and Cohn (1951) which contained 10 ml of glycerol per liter as well as galactose in a concentration of 2×10^{-3} M. This was aerated on a horizontal shaker overnight at 37 C and a 0.2-ml aliquot was diluted into a tube containing 10 ml of fresh medium and again shaken at 37 C. The cells were harvested in the logarithmic phase of growth as determined by the optical density at 650 mμ measured in a Bausch and Lomb Spectronic colorimeter. A loopful was streaked on EMB agar (Lederberg, 1950) supplemented with 1% galactose to determine whether the population contained any galactose-positive organisms and the cells were then spun down for 5 min at 0 to 4 C using a Servall centrifuge at 5,000 rev/min. The supernatant was discarded and the cells were resuspended in 2 ml of 0.05 M potassium phosphate buffer (pH 7.4). They were then disrupted by treatment with a Mullard 20 kc/sec sonic disintegrator for two 30-sec intervals separated by a 60-sec interim to allow cooling. This process was carried out in a 7-ml centrifuge tube immersed in a water-ice mixture. Cellular debris was centrifuged off in the cold at 14,000 rev/min and the clear supernatant was suitable for analysis. If such preparations were stored at −20 C, the specific activity of their galactose 1-phosphate uridyl transferase was stable for at least 2 weeks.

Galactokinase was ordinarily sought in extracts of flask-grown cultures which were prepared by transferring a 5-ml inoculum from test tubes as above into 200 ml of fresh medium in a 1-liter flask which was then vigorously aerated on a horizontal shaker at 37 C. The cells were treated as above except that 4 ml of phosphate buffer were employed in the sonic treatment step and the final supernatant was ordinarily diluted 1 to 10 with distilled water.

Those mutants which failed to grow on the defined medium or whose pattern of growth suggested selection in favor of a second mutation (*see* Results) were treated alternatively as follows: 0.2-ml samples from Penassay (Difco antibiotic medium no. 3)-grown cultures were diluted into tubes containing 5 ml of the "complete medium" of Lederberg (1950) modified to contain 5 g of D-galactose and 1 g of D-glucose per liter. After overnight shaking at 37 C, they were inoculated into 200 ml of the same medium and extracts were obtained exactly as above except that the cells were first washed with 10 ml of Davis minimal medium (Lederberg, 1950) from which glucose had been omitted. If these extracts were kept at −20 C, kinase, transferase, and epimerase activity could be detected for at least 1 month.

It must be emphasized that quantitative data pertain only to mutants treated by the standardized procedure. Galactose-positive organisms were always assayed as controls for procedural modifications.

Proteins. Proteins were determined by the method of Lowry et al. (1951).

Enzyme assays. These measurements are patterned after those of Kalckar et al. (1959) and for a detailed discussion of their underlying principles the reader is referred to the original work.

Galactokinase. The following mixture was incubated for 30 min in a 37 C bath after ATP was added to start the reaction: water to make a total volume of 1.0 ml; tris buffer (1 M), pH 7.5, 0.10 ml; $MgCl_2$ (0.1 M), 0.05 ml; NaF (0.5 M), 0.05 ml; D-galactose (0.1 M), 0.05 ml; extract as required (ordinarily 0.1 to 0.2 mg protein); ATP (0.08 M), 0.03 ml. The reaction was halted by immersing in a boiling water bath for 60 sec using a marble to prevent evaporation. The tube was then cooled in ice and the precipitate was centrifuged off at 0 C.

The galactose 1-phosphate which had been generated was measured by coupling the transferase, phosphoglucomutase, and zwischenferment reactions using an analytic mixture composed as follows: glycine (1 M), pH 8.7, 0.12 ml; supernatant, 0.30 ml; water to make total volume 1.0 ml; cysteine freshly neutralized to pH 8.7 (0.12 M), 0.10 ml; uridine diphosphate glucose (0.01 M), 0.02 ml; TPN (0.03 M), 0.015 ml; glucose 1,6-diphosphate (6×10^{-5} M), 0.005 ml; phosphoglucomutase, 0.015 ml (a total of 0.20 Najjar units (Najjar, 1955)); zwischenferment, 0.010 ml (a total of 0.14 Kornberg units (Kornberg, 1950)); galactose 1-phosphate uridyl transferase dissolved in 1% bovine albumin, 0.050 ml (a total of 0.02 Kurahashi units (Kurahashi and Sugimura, 1960)). The purified transferase was actually added after 5 min equilibration to

initiate the reaction. To determine the amount of TPNH produced the optical density at 340 mμ was followed in a 1-ml cuvette in a Beckman model DU spectrophotometer with a 1-cm light path.

Galactose 1-phosphate was not found when ATP, galactose, or extract was omitted from the primary incubation mixture. Known amounts of galactose 1-phosphate added to the incubation mixture could be measured quantitatively. The amount of galactose 1-phosphate generated increased linearly with time for at least 30 min and with enzyme concentration up to the formation of about 0.2 μmole. The indicator system was frequently checked with known amounts of galactose 1-phosphate; for example, it was found that 0.15 μmole yielded an optical density change of 0.947 (expected, 0.933). It was noted on several occasions that stoichiometric determinations of galactose 1-phosphate required considerably more time for supernatants than for equivalent amounts from a stock solution. The explanation could not be ascertained. One batch of purified transferase which had been prepared from strain no. 3092 was significantly contaminated with a TPNH oxidase and this must be regarded as a potential source of inaccuracy in the assay.

Galactose 1-phosphate uridyl transferase. The transferase, phosphoglucomutase, and zwischenferment reactions were again coupled. Since all substrates, indicator enzymes, and coenzymes were used in considerable excess, the rate of TPNH formation was a function of the amount of transferase in the sonic extract. The reaction was followed at 340 mμ and was run at room temperature in a cuvette containing: glycine (1 M) (pH 8.7), 0.06 ml; water to make a total volume of 0.62 ml; $MgCl_2$ (0.1 M), 0.01 ml; cysteine freshly neutralized to pH 8.7 (0.13 M), 0.03 ml; uridine diphosphate glucose (0.01 M), 0.03 ml; TPN (0.03 M), 0.015 ml; glucose 1,6-diphosphate (6×10^{-5} M), 0.005 ml; phosphoglucomutase, 0.015 ml (0.2 Najjar units); zwischenferment, 0.010 ml (0.14 Kornberg units); extract as required (ordinarily about 0.02 mg protein); galactose 1-phosphate dipotassium salt (0.015 M), 0.03 ml.

The galactose 1-phosphate was added to start the reaction after 10 min of equilibration. The uridine diphosphate glucose could also be used for this purpose but a lag before obtaining a maximal rate was noted under these circumstances, presumably because of inhibition of phosphoglucomutase when incubated with galactose 1-phosphate (Sidbury, 1957; Ginsburg, 1957).

The reaction depended on the presence of uridine diphosphate glucose, galactose 1-phosphate, and enzyme. The rate of TPNH production was constant for 20 min or until 0.06 μmole had been generated. The rate of the reaction was proportional to enzyme concentration up to an optical density change of 0.05 per min. Extracts from galactose-positive organisms were found to manifest neither TPNH oxidase nor 6-phosphogluconate dehydrogenase activity under these conditions. The latter was assayed using known amounts of glucose 6-phosphate in the above system from which uridine diphosphate glucose was omitted. Frequent readings taken at 400 mμ indicated that nonspecific optical density changes were not a significant factor in the assay.

Uridine diphosphate galactose 4-epimerase. A reaction mixture of the following constituents was incubated for 10 min at room temperature: glycine (1 M) (pH 8.7), 0.015 ml; water to make a total volume of 0.125 ml; extract as required (ordinarily approximately 0.02 mg protein); uridine diphosphate galactose (0.002 M), 0.05 ml. The reaction was stopped by immersion for 60 sec in a boiling water bath with a marble to prevent evaporation. If a precipitate formed it was removed by centrifugation at 0 C. The uridine diphosphate glucose which had been produced was measured with uridine diphosphate glucose dehydrogenase in a mixture containing: glycine (1 M) (pH 8.7), 0.20 ml; supernatant, 0.05 ml; water to make a total volume of 1.0 ml; DPN, (0.02 M), 0.015 ml; uridine diphosphate glucose dehydrogenase, 0.020 ml (200 enzyme units (Strominger et al., 1957)).

The dehydrogenase was added after 5 min of equilibration to start the reaction which was followed at 340 mμ. Controls without sonic extract were always run since the uridine diphosphate galactose was contaminated by trace amounts of uridine diphosphate glucose and since one batch of uridine diphosphate glucose dehydrogenase was noted to have slight epimerase activity.

The indicator system was checked with known amounts of uridine diphosphate glucose. For example, 0.05 μmole gave an optical density change of 0.654 (expected, 0.622). The production of uridine diphosphate glucose was dependent upon the presence of uridine diphosphate galactose and extract. The amount of uridine di-

phosphate glucose generated was proportional to the time of preincubation up to 15 min or the consumption of about 15% of the uridine diphosphate galactose. It was also proportional to protein concentration within approximately the same limits of substrate utilization.

RESULTS

The data are presented in Table 1. The genetic classification has been reported previously by Lederberg (1960). More biochemical data for mutants 1 to 9 are to be found in the publications of Kurahashi (1957) and of Kalckar et al. (1959). The results of the present study are in qualitative accord with those of the latter two authors in the incidences where the same mutants have been investigated. Numerical values are expressed in μmoles of substrate converted per hr per mg protein under the conditions which have been specified. Mutants reported as defective in galactokinase failed to yield any detectable activity under conditions where galactose-positive organisms, assayed in exactly the same way, gave values of about 2 to 3 μmoles:hr:mg protein. Those classified as deficient in galactose 1-phosphate uridyl transferase exhibited specific activities of less than 0.2 μmole:hr:mg, whereas the group with decreased activity of uridine diphosphate galactose 4-epimerase yielded values of 0.0 to 0.2 μmole:hr:mg as contrasted with a value of greater than 10 of the wild type.

Quantitative data are not presented for galactokinase because almost every mutant which produced this enzyme had to be grown on enriched medium to prevent selection. The reproducibility of values for specific activities of organisms grown on this medium was not investigated.

All quantitative data for galactose 1-phosphate uridyl transferase refer to experiments utilizing the standardized procedure for this enzyme described under Materials and Methods. The enzyme assay was reproducible within 10% for a given extract. For one experiment, eight separate inocula from the same galactose-positive organism gave a mean value of 5.8 with a standard deviation of 0.56 and a range from 4.9 to 6.5. In other experiments, however, such extracts yielded somewhat lower values despite ostensibly identical preparations. Specific activities were highest in the early logarithmic phase of growth and the data represent these conditions. In several hundred assays on the wild type, no value greater than 6.5 was ever obtained, so that the

TABLE 1. *Genetic and biochemical classification of galactose-negative mutants*

Genetic classification	Gal locus	Biochemical classification*		
		K	T	E
Wild type:				
W3100	Galactose-positive	+	3.6–6.5	+
Group A:				
W3092	2	−	14.7	+
W3098	8	−	18.6	
W4670	10	−	+	
W3848	12	−	+	
W3965	14	−	14.6	
Group B:				
W3091	1	+	−	+
W3094	4	+	−	
W3096	6	+	−	
W3097	7	+	−	+
W4677	11	−	−	
W4242	15	+	−	
Group C:				
W3265	3	−	0.3	
W3099	9	−	−	−
Group D:				
W4221	16	+	+	−
W3805	22	+	10.2	−
Group E:				
W3142	19	−	+	
W4600	21	−	+	
W4597	23	+	−	
Unclassified:				
W3798	13	−	12.5	
W4252	20	−	6.9	
W4247	24	−	+	+
W4244	25	−	17.7	
W4216†	26	−	−	
W4593	27	−	19.8	
W4683	28	−	−	
W4667		−	+	
63		−	+	+

* K = galactokinase; T = galactose 1-phosphate uridyl transferase; E = uridine diphosphate galactose 4-epimerase.

† This mutant was originally classified in group A but its genetic status is now unclear since it has been found not to complement mutants 4, 6, and 15 (E. Lederberg, *personal communication*).

The genetic data in this table are taken from Lederberg (1960). Groups A, B, and D are mutually complementary and define corresponding simple cistrons. Group C is more complex and is characterized by the fact that its members complement neither each other nor those of groups A and B. Group E is distinguished by the fact that its constituents are not amenable to transduction by λ.

markedly elevated values exhibited by many of the mutants are considered to be significant.

Quantitative data for uridine diphosphate galactose 4-epimerase are not reported because detailed reproducibility studies were not carried out and because the range of linearity with enzyme concentration is quite narrow so that considerable effort must be expended to ascertain the proper amount of an active extract which must be used to fall within this range.

Kurahashi and Wahba (1958) have reported on the inhibition of growth of certain of the transferase-defective mutants by galactose and its partial reversal by yeast extract. In addition Kalckar et al. (1959) have described the isolation of a new mutant from a transferase-deficient organism grown on chemically defined medium, with galactose as inducer, which had diminished galactokinase activity. Spyrides and Kalckar (1960) have also shown that the so-called "M mutant" (Fukasawa and Nikaido, 1959), an enteric bacterium with a different genetic background than the organisms studied in the present investigation and defective in uridine diphosphate galactose 4-epimerase (Fukasawa and Nikaido, 1959; Kalckar et al., 1959), accumulates uridine diphosphate galactose and galactose 1-phosphate when grown in the presence of galactose. The same strain lyses in chemically defined media when galactose is added (Yarmolinsky et al., 1959). There is a considerable additional body of evidence supporting the conclusion that galactose 1-phosphate is responsible, at least in part, for the deleterious effects of galactose in these systems (Kalckar, 1958).

These observations suggest that, in mutants accumulating galactose 1-phosphate in synthetic media containing galactose, there may be a strong selective pressure for new mutants with a defective galactose-phosphorylating mechanism. In the present study every mutant with a single transferase defect, as well as those with deficient epimerase activity, often gave evidence of this phenomenon. When grown on chemically defined media with 2×10^{-3} M galactose, there was often no turbidity for several days followed by a sudden rapid burst of growth. Extracts from such organisms invariably failed to yield galactokinase activity. For this reason it was often necessary to grow these mutants in an enriched medium supplemented with galactose. Selection occurred much less readily under these circumstances and galactokinase was usually easily demonstrable, although the medium also contained glucose which suppresses the formation of adaptive enzymes (Monod, 1947).

DISCUSSION

It will be noted that there is a high degree of correlation between genetic and biochemical classifications for groups A, B, C, and D. Thus, all members of group A fail to yield detectable galactokinase activity but possess galactose 1-phosphate uridyl transferase. Group B, on the other hand, is characterized by the fact that its members uniformly exhibit a decrease in transferase, whereas galactokinase can be shown for all except Gal 11. As previously noted, selection for a galactokinase-deficient mutant is very prominent in this group. The mutant in question grew without any lag in synthetic medium, suggesting that accumulation of a second mutation must have occurred prior to this stage if at all. It is possible that this selection mechanism is operative even on a slant, since agar contains galactose residues. As an alternative to using an enriched medium, the use of D-fucose (6-deoxygalactose) as a gratuitous inducer for enzymes concerned with galactose metabolism (Buttin, Jacob, and Monod, 1960; Yarmolinsky and Wiesmeyer, 1960) may circumvent the problem of accumulating double mutants during growth.

The high specific activities for galactose 1-phosphate uridyl transferase in mutants defective in galactokinase, as contrasted with the wild type, are striking. The phenomenon does not seem to apply in all such mutants, since Gal 12, which had to be grown in complete medium, yielded a value of 1.1 compared with 2.5 for the wild type grown under similar conditions. Kalckar et al. (1959) have shown that this enzyme is inducible in the wild type but constitutive in some galactokinase-defective mutants. However, it is of considerable interest that Yarmolinsky and Wiesmeyer (1960) noted that such constitutive strains possessed enzyme levels somewhat less than the wild type if comparisons were made under derepressed conditions. From such a mutant these investigators isolated a secondary mutant which was no longer constitutive but which, in the presence of inducer, yielded a considerably higher specific activity than the wild type under similar circumstances. Such findings are not in accord with the predictions of a

simple repressor hypothesis (Pardee, Jacob, and Monod, 1959). It must be noted that the values reported in the present investigation were all determined from cells grown in the presence of inducer, so generalizations concerning the mechanism of induction and repression cannot be drawn.

The relationship between an apparent single point mutation and a triply defective phenotype as illustrated by group C remains unexplained. Although a galactose transport system has recently been described for *E. coli* (Horecker, Thomas, and Monod, 1960), members of this group apparently possess a permease mechanism as shown by the fact that galactose induces them to form β-galactosidase (Kalckar et al., 1959). The concept of an operator gene (Jacob et al., 1960) controlling the expression of this group of related enzymes is difficult to reconcile with the observation that the C and D groups complement one another (Lederberg, 1960) even though both possess diminished epimerase activity. It would be interesting to determine whether this complementation could be detected at the cell-free level. That induction λ of results in derepressed synthesis for all three of the enzymes (Buttin et al., 1960; Yarmolinsky and Wiesmeyer, 1960) represents additional evidence that their determinants are integrated with one another at some level of genetic organization.

Although the data very strongly suggest that any galactose-negative mutant is defective in one or more of the enzymes of the Leloir pathway, it must be noted that the assay procedures employed were designed for wild-type extracts. If a mutant occurred in which a new substrate-splitting enzyme had been unmasked it would be recorded as negative for the enzyme in which assay the particular substrate was used. For example, a mutant possessing an extremely active and heat-resistant enzyme capable of cleaving uridine diphosphate glucose to uridine diphosphate and glucose would be unable to ferment galactose by the Leloir pathway and would be classified as negative for all three of the enzymes in question. In this way a single genetic change would be associated with a triple biochemical aberration on a completely artifactitious basis. Since various simpler and more plausible examples can be imagined it is felt that formally, at least, the techniques are somewhat biased and that it is safest to construe negative data as representing "apparent" enzymatic defects. In this context it must also be noted that the possibility of more conventional inhibitors in the extracts preventing the expression of the enzymes has not been excluded.

The discovery that group D represents a new biochemical class of mutants in this series illustrates the usefulness of the genetic test of functional complementation by which these mutants were also found to be unique. It can be concluded that the three cistrons defined by the mutants of the A, B, and D groups correspond to the three enzymes of the Leloir pathway.

It is also of interest that group E is a heterogeneous biochemical entity with members lacking expression of either galactokinase or galactose 1-phosphate uridyl transferase. This group is susceptible to infection but not amenable to transduction by λ; thus, it appears that either part of the galactose locus is not transducible by λ or there exist other sites capable of influencing the expression of these two enzymes. Consistent with these possibilities is the fact that a suppressed mutant derived from Gal 4 (J. Lederberg, *unpublished data*) was found to produce an active transferase, yet λ grown on this suppressed mutant could not transduce Gal 4 to Gal$^+$.

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