*HEREDITARY DEFECTS IN GALACTOSE METABOLISM IN ESCHERICHIA COLI MUTANTS, I. DETERMINATION OF ENZYME ACTIVITIES**

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Communicated September 8, 1959

Lederberg and his associates have isolated a number of mutants of *Escherichia coli*, strain K-12. These mutants have defects in galactose metabolism and hence are unable to use this sugar as a carbon source for growth or energy metabolism.[1] Moreover, a virus called lambda which is characteristic for the K-12 strain was found to carry genes for synthesis of enzymes involved in galactose metabolism.[1] It was of primary interest to try to find out whether or not galactose metabolism in the K-12 strains (and in transduced K-12 strains) proceeds along an explored anabolic pathway[2–4] which we shall call the Leloir pathway.

The specific enzymatic methods performed as differential microspectrophotometry for the study of the Leloir pathway[4–6] were shown by one of us (K.K.) to be well applicable to sonicates of *E. coli* K-12 strains.[7] A number of galactose-negative K-12 mutants (isolated by E. and J. Lederberg) were classified according to which enzymes of the Leloir pathway showed a defect. Three different mutants were found to be blocked in galactokinase and four different mutants were blocked in galactose-1-phosphate uridyl transferase.[7]

The experiments to be presented here were performed for various purposes.

(1) A description of enzymatic methods with the intention of studying the physiological role of the Leloir pathway in the K-12 strains. The requirements for specificity made it necessary to develop elaborate and somewhat tedious methods. Once it has been proved that the Leloir pathway dominates the picture it should be possible to streamline the methods. These considerations are particularly relevant to mention in connection with the galactokinase test which is relatively complicated if the insistence on the specificity of the methods is maintained.

(2) To characterize the location of the hereditary block in the Leloir pathway; whether they be single or multiple defects.

(3) To describe the correlation between enzyme defects and the synthesis of other enzymes on a constitutive basis. The enzymes studied include mainly those of the Leloir pathway but also β-galactosidase.

(4) A study of physiological and pathological growth (unbalanced growth) and its dependence on specific hereditary enzyme defects. The phenomenon "galactose induced sensitivity" will be the entire topic of the second paper.

Materials.—*O*-nitrophenol galactoside, β-propyl galactoside, and thiomethyl galactoside were gifts from L. Herzenberg of the National Institute of Allergy and Infectious Diseases; α-galactose-1-phosphate (Gal-1-P)[8] as the barium salt or the dipotassium salt was kindly supplied by D. Broida of Sigma Chemical Company. Some of the Gal-1-P preparations were obtained from E. Maxwell of this Institute and other preparations we owe to the generosity of Z. Hassid and E. Putnam of the University of California and to K. Isselbacher of Massachusetts General Hospital. Uridine diphosphoglucose (UDPG) is a product of Sigma Chemical Company. Uridine diphosphogalactose (UPDGal) was prepared by enzy-

matic means from UDPG and Gal-1-P[6] (kindly prepared by E. Maxwell). Another sample of UDPGal was prepared by one of us (HMK) according to the method of Khorana *et al.*[9,10] Glucose-1,6-diphosphate was generously supplied by L. Leloir of the Institutio de Investigaciones Bioquimicas, Buenos Aires, and V. Najjar of the Vanderbilt University. Uridine diphosphoglucose dehydrogenase (UDPG dehydrogenase) was prepared from calf liver acetone powder according to the method described by Strominger *et al.*[11] Uridine diphosphogalactose-4-epimerase (UDPGal-4-epimerase) was purified from calf liver acetone powder according to the method described by E. Maxwell.[6] Phosphoglucomutase was prepared from rabbit muscle according to the method described by Najjar.[12] Galactose-1-phosphate uridyl transferase (Gal-1-P uridyl transferase) was prepared from calf liver acetone powder.[13] Glucose-6-phosphate dehydrogenase was purified from dried yeast by Kornberg's method.[14]

Bacterial cultures: The bacterial strains used were *E. coli* K-12 generously supplied by E. and J. Lederberg. Most of these mutants were unable to grow on galactose as a carbon source (gal−). The Lederbergs described strains like 3100 (gal+). 3092 (gal−), 3096 (gal−), 3099 (gal−) as isogenic strains differing only in the locus which is affected by a point mutation.[15,16]

Strains 3099, 3264, and 3265 were spontaneous mutants (Lederberg[17]). The other galactose negative K-12 strains were obtained by ultraviolet irradiation.[17] Some of the strains are lysogenic with respect to lambda (and transducing lambda). The lysogeny is subject for a special study by Wiesmeyer and Yarmolinsky (unpublished work). Strain 3104 was obtained through D. Kaiser from E. Lederberg. Strain ML 32,400 was obtained through B. L. Horecker from J. Monod, Institut Pasteur, Paris.[18] Strain C7M stems from Fukasawa and Nikaido, Keio University, Medical School, Tokyo, who have described the strain recently.[19,20]

Methods.—Culture conditions: The cultures were usually obtained as stab culture in tryptone dextrose agar and transferred to slants on tryptone dextrose yeast extract agar or bacto-nutrient agar. They were kept at 0–2°C and frequently transferred. In some cases, for instance, strain 3104 and C7M, slants were prepared from single colonies (derived from single cells). From the slants they were subcultured in a small volume (10 to 30 ml) of liquid medium. In most cases the liquid medium used was ammonium sulphate mineral medium (Medium 56 of Monod *et al.*[21] or Medium A of Hartman[22]). For strain C7M, Bactotryptone broth (Difco) was used. The carbon source used was glycerol in amounts between 0.5 to 1.5 per cent, usually 1 per cent (1×10^{-1} *M*). Glucose was occasionally used as a carbon source in the first subculture in order to speed up growth. However, because of the tendency of glucose to suppress the formation of adaptive enzymes[23] it was usually left out even in the first subculture unless otherwise specified. Galactose was used in rather low concentrations (1×10^{-2} *M* to 1×10^{-4} *M*) and the impurities of glucose (4–5%) which it usually contains were considered negligible. However, in some cases it was found preferable to use purified galactose. This was done by recrystallizing galactose from 70 per cent ethanol solution several times.[24] The cultures which were cultivated for the assay of enzymes were grown in volumes from 200 ml to 300 ml. Cultures of 200 ml or 400 ml medium were shaken at 37°C on a rotatory shaker with favorable aeration.

The cultures were usually harvested when growth had reached 2 to 3 gm. wet weight cells per liter medium. The generation time in a glycerol medium was usually between 60 and 100 minutes. Streaks of such cultures were taken on Eosin Methylene blue agar plates.[25] to ascertain whether the cells were still gal + or gal−. In the former case black colonies develop.[15]

Harvesting and disintegration of bacteria: Cultures of volumes ranging between 200 ml and 2 liters were spun at 0°C in polyethylene containers at 5,900 × g in a Lourdes centrifuge and washed with chilled water. For larger volumes a refrigerated Sharples centrifuge was used. For amounts ranging between 0.5 to 1.5 per cent wet weight of cells the Nossal bacterial cell disintegrator[26] was used. The glass beads (Minnesota Mining and Manufacturing Company Superbrite Glass Beads Type 150) added in amounts of 4 to 6 gm were cooled to −10°C. Phosphate buffer, 0.05 *M*, pH 7.0 was used in amounts of 4 to 6 ml Shaking in the disintegrator placed in an −10° room was stopped every 30 seconds to allow cooling. Usually two 30-second periods sufficed to break the cells. The mixture was subsequently spun for 5 minutes at 11,000 × *g* in the high speed attachment of the International Refrigerated Centrifuge. The pale yellow, slightly turbid supernatant fluid was stored at −20°C.

Determination of growth: Sterile aliquots were drawn from the cultures. Turbidity measurements were performed in a Beckman cuvette (1 cm depth) at 650 mμ. A reading of 0.3 corresponds to 1 × 10^9 cells per ml[27] which again corresponds to 1 mg wet weight of cells per ml.

Protein determination: Protein concentration of cell free extracts was determined by the phenol reagent method.[28]

Detection of sugar in complex polysaccharides: Detection of sugars in complex polysaccharides was performed according to the method of Westphal and Lüderritz.[29]

Quantitative determination of enzyme activity in broken cell preparations: (*1*) *Enzymes of the Leloir Pathway.*

(a) Galactokinase.

Principle of method: The enzyme catalyzes the reaction[1]: ATP + Gal → ADP + *α-Gal-1-P*. The italicized product is the constituent formed in a preincubation mixture which is being quantitatively determined. Fluoride is added in order to protect ATP and Gal-1-P from being dephosphorylated by phosphatase. Addition of magnesium ions serves to insure that the reaction which depends on these ions is not slowed down by a depletion of magnesium due to the presence of fluoride. The Gal-1-P formed is determined in a protein free filtrate by an analytical incubation mixture; the basis for the method rests on its ability to interact with UDPG in the presence of the specific enzyme, Gal-1-P uridyl transferase (used here as indicator enzyme)[10,16]. The sequence of enzymatic reactions used for the analysis of Gal-1-P is as follows:

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDP-Gal}$$

$$\text{G-1-P} \rightleftharpoons \text{G-6-P (catalyzed by phosphoglucomutase)}$$

$$\text{G-6-P} + \text{TPN} \rightarrow \text{6-phosphogluconate} + \textit{TPNH} \text{ (catalyzed by G-6-P dehydrogenase)}$$

The absolute molar amount of TPNH formed is used as a direct measure of the galactokinase activity in the preincubation mixture. This is provided that the substrates used in the analytical reactions are in considerable excess of the Gal-1-P stemming from the preincubation mixture.

The determination of the amount of Gal-1-P accumulated in a preincubation setup is based on a preincubation mixture of the following composition: Galactose (recrystallized), 25 μl of a stock solution containing 200 μmoles/ml, i.e., 5 μmoles galactose per ml incubation mixture; ATP (0.1 *M*), 20 μl corresponding to 2 μmoles; Tris buffer (1 *M*, pH 7.5), 100 μl; sodium fluoride (0.5 *M*), 50 μl; $MgCl_2$ (0.1 *M*), 50 μl; H_2O 655 μl; bacterial extract (galactokinase to be determined) 100 μl. Incubation was 0, 10, 20, and 30 minutes. The temperature was 37°C. The reaction was stopped by placing the incubation tube in a boiling water bath for 90 seconds. After spinning, part of the protein-free clear supernatant fluid was analyzed enzymatically for Gal-1-P. Usually a 100 μl filtrate was analyzed.

The *analysis mixture* was composed as follows: UDPG, 20 μl or 0.2 μmoles; G-6-P dehydrogenase, 10 μl of a solution containing 10 mg per ml; TPN, 20 μl of a solution containing 20 mg/ml; cysteine (33 mg/ml, pH 8.5), 100 μl; phosphoglucomutase,[30] 20 μl; $MgCl_2$ (1 *M*), 10 μl; glucose-1,6-diphosphate (0.5 μmole/ml), 5 μl; glycine (1 *M*, pH 8.7), 100 μl; H_20, 610 μl; fractionated Gal-1-P uridyl transferase from liver[13] or from *E. coli* K12 mutant 3092 (galactokinase-less mutant[31]). The amount of filtrate used for the assay was 100 μl. In order to calibrate the system, Gal-1-P in amounts of 0.05 to 0.075 μmoles was added. For each 0.1 μmoles of Gal-1-P per ml a density increase of 0.620 at 340 mμ took place. Usually the G-6-P dehydrogenase was added first in order to determine small amounts of G-1-P or G-6-P present in the filtrate. Subsequently the Gal-1-P uridyl transferase was added and the total density increase at 340 mμ minus the value obtained with a 0 time control of preincubation mixture[32] was a measure of the Gal-1-P which accumulated in the preincubation period and hence of the activity of the galactokinase.

The method is highly specific but not highly sensitive. In its present design it can detect activities of 0.1 to 0.2 μmoles per hour per mg protein. The sensitivity can be increased by analyzing a larger fraction of the preincubation mixture. The latter can also be incubated for longer periods of time (1 and 2 hours). Fluoride has not been found to inhibit galactokinase significantly. Simpler and more sensitive methods for galactokinase can be devised. However, at the initiation of the present project it was essential to demonstrate that the hereditary blocks affected the steps of the Leloir pathway, hence the specific enzymatic methods were deemed most essential.

A new sensitive specific method for Gal-1-P determination has most recently been worked out.[33] In this method human hemolysate (which is free of epimerase) is used as transferase source. The incubation mixture is heat-inactivated and analyzed for consumption of UDPG by means of UDPG dehydrogenase.[11] This method is being modified with the purpose of assaying galactokinase in bacterial extracts.

(b) Gal-1-P uridyl transferase.

Principle of method: The enzyme catalyzes the reaction[4]: Gal-1-P + UDPG $\rightleftharpoons$ *G-1-P* + UDPGal.

The *rate of G-1-P formation* is in this case analyzed by adding the two indicator enzymes and coenzymes in excess, phosphoglucomutase (plus glucose-1,6-diphos-

phate) and G-6-P dehydrogenase plus TPN. The initial *rate of formation of TPNH* is used as a direct measure for the Gal-1-P uridyl transferase activity of the disrupted bacterial cell. Controls having either Gal-1-P or UDPG omitted from the incubation are always used. Reoxidation rate in the extracts is much slower than that of TPN reduction. Yet, for determination of low transferase activities it is necessary specifically to determine the rate of reoxidation of TPNH.

The enzyme activity was determined directly in the Beckman spectrophotometer (i.e., "generation incubation" and "analysis incubation" were combined) by the rate at which TPN was reduced. All constituents, substrates, indicator coenzymes as well as indicator enzymes were added in considerable excess over the transferase to be determined. An incubation mixture for the assay of Gal-1-P uridyl transferase had the following composition: cysteine solution (33 mg per ml pH 8.5), 30 μl; magnesium chloride (0.1 *M*), 10 μl; glycine (1 *M*, pH 8.7), 60 μl; phosphoglucomutase together with glucose-1,6-diphosphate; TPN (20 mg per ml), 10 μl; G-6-P dehydrogenase; UDPG (10 μmole per ml), 20 μl; water, 360 μl. To this mixture is added the K-12 extract in amounts of 50 to 100 μg protein and as the last component Gal-1-P (10 μmole per ml), 30 μl. The total volume is 620 μl. In order to ensure that the indicator enzymes are in excess it may be necessary to add more G-6-P dehydrogenase or phosphoglucomutase or glucose-1,6-diphosphate and ascertain that the rate of release of G-1-P from UDPG and Gal-1-P is not increased. Control mixtures with UDPG without Gal-1-P or Gal-1-P without UDPG should only give very slow release of G-1-P. The release of G-1-P is measured by the rate of increase in optical density change at 340 mμ, using 400 mμ as isobestic point.

Another complication which might obscure comparison on a quantitative basis is the fact that 6-phosphogluconate dehydrogenase present in the lysate (or in some preparations of indicator enzyme glucose-6-phosphate dehydrogenase) will alter the scale. That is, the activity of transferase will be overestimated because each mole of UDPG exchanged will give a deflection at 340 mμ which is double as high. It is therefore necessary always to calibrate the enzyme mixture with a known amount of glucose-1-phosphate or glucose-6-phosphate. A direct assay with 6-phosphogluconate is to be recommended too. In most cases the activity of phosphogluconate dehydrogenase is low. Hence the calibration with glucose-1-phosphate is most important in lysates in which transferase activity is moderate or low. These difficulties are less likely to be encountered if one is dealing with extracts having highly active transferase.

If several factors contribute to complicate the quantitative analysis it might be helpful to perform the assay by a two step procedure. The reaction mixture would in such a case consist of *E. coli* extract, phosphoglucomutase and glucose-1,6-diphosphate, cysteine, $MgCl_2$, Gal-1-P, and UDPG. After a brief incubation (for instance, 5 and 10 minutes) the reaction is stopped and the filtrate is analyzed for glucose-6-phosphate by means of TPN and glucose-6-phosphate dehydrogenase (which should be free of transferase).

The sensitivity of the direct transferase assay is of the order of magnitude of 0.01 μmole per hour per mg protein.

(c) UDPGal-4-epimerase.

Principle of method: The enzyme catalyzes the reaction[1,6]: UDPGal $\rightleftharpoons$ *UDPG*. UDPG formed is determined in a protein-free filtrate by means of a DPN dependent

specific dehydrogenase (UDPG dehydrogenase) which catalyzes the reaction[11]: UDPG + 2 DPN → Uridine Diphosphoglucoronic Acid + 2 DPNH. The latter is performed in an analytical incubation. The *absolute molar amount* of DPNH formed divided with 2 provides a direct measure for UDPGal-4-epimerase activity in the disrupted cells. Since the disrupted K-12 cells do not contain any enzymes which hydrolyze or oxidize UDPG (or UDPGal) it is possible by this method to determine quantitatively even very low activities of UDPGal-4-epimerase.

(a) The two step procedure was performed as follows: The reaction mixture contained glycine (1 *M*, pH 8.7), 30 μl; *E. coli* extract, 20 μl; UDPGal (2.2 μmole/ml), 50 μl; and H_2O, 150 μl. The mixture is incubated for 5 and 10 minutes at room temperature (25°C). At termination the mixture is heat inactivated by placing the tubes (with a marble on top) in a boiling water bath for one and a half minutes. It is then cooled on ice, spun down, and 200 μl of the clear supernatant is used for the analytical mixture. The latter contained glycine (1 *M*, pH 8.7), 70 μl; DPN (25 μmoles per ml), 20 μl; 200 μl supernatant of the preincubated sample; 50 μl water and finally 15 or 20 μl of UDPG dehydrogenase. The control contains the same mixture except that the supernatant stems from a reaction mixture to which UDPGal was added after boiling.

The sensitivity of the epimerase test is of the order of magnitude of 0.005 μmole per hour per mg protein.

(b) The one step direct procedure can be used occasionally for crude extracts but preferentially for fractionated UDPGal-4-epimerase preparations free of hydrogen donors and acceptors. This procedure is executed as follows: The reaction mixture contained glycine (1 *M*, pH 8.7), 50 μl; DPN (25 μmoles per ml), 20 μl; UDPGal (2.2 μmoles per ml.), 30 μl; water, 475 μl; UDPG dehydrogenase 25 μl and *E. coli* extract, 5 or 10 μl. Upon the addition of extract the rate of increase of optical density at 340 mμ is recorded (the isobestic region at 400 to 410 mμ is used for optical density at not too long time intervals). It is important that UDPG dehydrogenase is in excess. This can be ascertained by lowering the amount of *E. coli* extract and seeing if the rate decreases proportionally. The control tube contains the same ingredients with the exception of UDPGal. Reoxidation of DPNH interferes and a direct assay using DPNH as substrate should be performed. Each millimicromole UDPGal converted to UDPG corresponds to an optical density change of 0.020 at 340 mμ.

The sensitivity of the direct epimerase test is as high as that of the indirect.

(2) *β-Galactosidase:*

This enzyme was determined according to the method of Cohn and Monod,[34] using as substrate sodium orthonitrophenol galactoside.

The *rate of increase* of optical density at 420 mμ was followed directly in the Beckman cuvette at 25°C. While the reaction was still proceeding at a linear rate an excess of sodium carbonate (one-fifth volume of 2 *M* sodium carbonate) was added, bringing the pH close to 10. Under these conditions, the enzymatic reaction stops immediately. The optical density at 420 mμ increases by a factor of 1.5 to 2.

The determination of the cellular β-galactosidase was performed in small samples of toluenized bacteria.[35,36] Assays for the enzyme was also performed in the medium. In the assay for β-galactosidase, using toluenized cells or medium, the incubation

temperature was 37°C. The absorption was read at 420 mμ in a Beckman spectrophotometer after the addition of equal volumes of 1 *M* sodium carbonate.

The colorimetric method is identical with that described above. Readings at an isobestic wavelength, for instance, 550 mμ, at the start and at the end of the reaction, so as to correct for dispersion differences due to varying amounts of cells, are to be recommended.

Molar absorbancy index of o-nitrophenol (Eastman Organic Chemical Department) in 0.2 *M* sodium carbonate at 420 mμ (maximum) was determined to be 3,500.

Results and Discussion.—Energy and biosynthetic metabolism of galactose: It is well known that galactose can be metabolized at least in catabolic metabolism in bacteria by another pathway than that of Leloir, namely by direct oxidation, dehydration, and cleavage.[37] Table 1 illustrates that galactose in K-12 *E. coli* is metab-

TABLE 1

PHENOTYPIC DESCRIPTION

Genotype	Medium	Galactokinase μM/mg protein/hr. non-induc.	Galactokinase μM/mg protein/hr. Gal-induc.	Gal-1-P Uridyltransf. μM/mg protein/hr. non-induc.	Gal-1-P Uridyltransf. μM/mg protein/hr. Gal-induc.	4-epimerase μM/mg protein/hr. non-induc.	4-epimerase μM/mg protein/hr. Gal-induc.
W 3100*	A	<0.05	40.0	0.6	5.5	5.0	40.0
W 3100	Br†			0.4	5.8		
W 3092	A	<0.05	<0.05	5.2	4.3	4.0	42.0
W 3078	56	<0.05	<0.05	2.4	2.5		
W 3142	56	<0.05	<0.05	1.4	4.0		+
W 3096	A	0.10	15.0	<0.01	<0.01	2.0	23.0
W 3096′	A	0.20	2.1	<0.1	<0.06		6.4
W 3104	A		+	<0.01	0.1 app.		+
W 3099	A		0.05 app.		0.05 app.	<0.01	<0.01
W 3264	A		0.10		0.35	0.60	0.90
W 3265	A		0.10		0.30	0.50	0.80
ML 32,400	A	<0.1	<0.1		<0.1		+
C7M	Br		+	1.8	9.0	<0.01	<0.01

* The letter W is usually added to the numbers of the K-12 strains. In the text it is left out for the sake of abbreviation.

† Br refers to tryptone broth.

olized practically solely through the Leloir pathway. As appears from the table there are genetic blocks in galactokinase, Gal-1-P uridyl transferase, and in 4-epimerase. A single block in each of these steps is sufficient to render the cells "galactose negative" i.e., unable to use galactose as a carbon source for growth or for acid formation. Some of the strains, namely those with single blocks in transferase are not only galactose negatives but also galactose sensitives presumably due to the accumulation of Gal-1-P and related products (see succeeding article[38]). It is mentioned here because this phenomenon likewise underlines the dominant role of the Leloir pathway. Comments on the doubly defective mutants 3096′ (isolated by selection from 3096 rendered static by galactose) and ML 32,400 will be found in the succeeding article. The effect of "lambda Gal" on enzyme synthesis is under study.

The importance of this pathway for *anabolic* metabolism (synthesis of cell material) is illustrated by the following facts. Mutant 3099 has no detectable amount of 4-epimerase. Consequently one should expect that if this mutant is grown on media which contain no galactose, such as an ammonia mineral medium with gly-

cerol as the sole carbon source, the cell should be devoid of galactose. A preliminary attempt to investigate this point was undertaken. Complex polysaccharides were isolated, hydrolyzed and analyzed. No detectable amount of galactose was found; if present, it must have been in amounts less than 2 per cent of that of glucose. In another epimerase-less strain, C7M, Fukasawa and Nikaido[20] likewise found glucose with no detectable amount of galactose. In contrast to this 3096 which has a block in transferase and a relatively high constitutive 4-epimerase (see Table 1) contains galactose in the ratio of 1:3 as compared with glucose;[39] this corresponds closely to that of the equilibrium of 4-epimerase (Leloir[1]). More work is underway with respect to a characterization of sugars from a number of K-12 mutants.

Inducibility of β-galactosidase and the enzymes of Leloir pathway: The absence of 4-epimerase, as well as of cellular galactose in the 3099 mutant, is contrasted by the ability of administered galactose to induce the formation of β-galactosidase. There is scarcely any induction of galactokinase and transferase. What we want to stress here is that a suspected "galactose-free" cell is nonconstitutive but inducible with respect to β-galactosidase and that galactose can serve as inducer. This poses questions as to the nature of the postulated β-galactosidase repressor,[40] if a specific repressor plays a role here.

The epimerase-less C7M strain behaves vastly differently, almost in the reverse way, from the 3099 epimerase-less strain with respect to inducibility by galactose. In the C7M strain galactose induces the synthesis of galactokinase[20] and of transferase (Table 1; see also succeeding article[38] on galactose induced bacteriolysis) but scarcely, if at all, β-galactosidase. The latter is, however, induced by thiomethylgalactoside (TMG). These results are summarized in Table 2. It

TABLE 2

INDUCIBILITY OF GAL-1-P URIDYL TRANSFERASE, 4-EPIMERASE, AND β-GALACTOSIDASE

Strain	Phenotypic defects (single or triple)	Inducer used	Gal-1-P uridyl transferase Ratio of activities*, induced to non-induced	4-Epimerase Ratio of activities*, induced to non-induced	β-Galactosidase Activity†	β-Galactosidase Ratio of activities induced to non-induced
W 3100	No defect	...	..	...	100	...
		Gal	9	8	1,000	10
W 3092	Single	...	..	...	3	...
		Gal	1‡	10	220	73
W 3099	Triple	...	..	...	2.5	...
		Gal	..	...	550	220
W 3264	Triple	...	..	...	3	...
		Gal	..	1.5	500	167
		...	..	...	100‖	...
W 3265	Triple	Gal	..	1.6§	3,000‖	30
		β-PG	..	(1)§	50,000‖	500
C7M	Single	...	..	...	30‖	...
		Gal	5	...	45‖	1.5§
		TMG	..	...	1,400‖	47

* Calculated from the values in Table 1.
† mμmoles ONP liberated per min. per mg protein.
‡ Fully constitutive.
§ Only traces of enzyme activity. The ratio is an approximate value.
‖ Determination on toluenized cells. Activities converted from mμmoles per mg dry weight to protein by assuming that soluble protein constitutes about 50% of the dry weight.

should be added that the poor induction of β-galactosidase by galactose could be due merely to the fact that it is delayed as compared to that of the enzymes of the Le-

loir pathway. The induction of the latter brings about lysis which would prevent any delayed induction of β-galactosidase from manifesting itself. It should also be noted from Tables 1 and 2 that the constitutive level of transferase in C7M is high. Moreover, the induced levels of transferase might have increased further if not interrupted by lysis. The value presented was obtained from an aliquot taken 1 hour after the addition of galactose, shortly before lysis ensued.

Single step mutants with triply defective phenotypes: According to E. Lederberg,[17] the single step spontaneous mutants 3264, 3265, and 3099 are readily reversible and render gal-positive recombinants when intercrossed in a number of combinations (recombinations with single galactokinase-less, transferase-less mutants). Whether 3099 really is a single step can still be debated. If it can be induced to synthesize any epimerase at all the levels are much lower than those of 3264 and 3265. Moreover the results of intercrosses are at the present time less conclusive for this strain since no single epimerase-less K-12 mutant has been identified so far.

The main argument is now the following: How can a genotype representing a single step mutation like 3264 and 3265 manifest itself by a triply defective phenotype? The induced levels of the 3 enzymes of the Leloir pathway are greatly below (only 2 to 5%) those of wild type or single mutants. It should be noted that the constitutive epimerase levels of the same two mutants are definitely lower than those of the other K-12 strains.

The easiest way out would be to assume the existence of a single permease block for galactose. Such a block would have to be a severe one because addition of high amounts of galactose (5% galactose in the glycerol ammonia medium) still does not induce the galactose enzymes nor does galactose in high concentration as the sole carbon source permit even slow growth. The latter observation supports the idea of a permease block. Moreover, the enzyme levels although low should be high enough to permit slow growth with galactose as a sole carbon source. The fact that growth stops completely after the exhaustion of added traces of glucose and of internal carbon reserves speaks in favor of a permease block. The situation would therefore seem fairly clear if it were not for the reason that galactose even in small concentrations elicits a considerable induced synthesis of β-galactosidase in the same strains (e.g., 3264, 3265, and also in 3099).

If we, therefore, were to assume that strains 3264 and 3265 were single mutants, permease-less for galactose, then we would arrive to the paradoxical situation that these cells are permeable for galactose as inducer for β-galactosidase but not for galactose as inducer for the enzymes of the Leloir pathway. One is almost forced into the assumption that the locus for induction of β-galactosidase is in a part of the cell which is accessible to galactose. In contrast the locus which can elicit induction of enzymes of the Leloir pathway is fixed in a less accessible spot for penetration of galactose, at least in the cells with the triple defects.

Degree of constitutivity of the enzymes of the Leloir pathway: As appears from Table 1, the constitutive levels of epimerase are in general high, about 10 per cent of the fully induced levels, whereas those of galactokinase are low, in most cases 1 per cent or less of that of fully induced levels. Constitutivity of transferase varies in an interesting fashion. In the 3100, wild type (gal +) the constitutive levels amount to between 7 and 10 per cent of those of the induced. However, in two of the galactokinase-less mutants the constitutive transferase levels are 100 per

cent or even a little higher than those of the "induced." The constitutivity of epimerase is not affected. The constitutivity of transferase in the galactokinase-less mutants cannot be accounted for by any obvious changes in galactose metabolism (cf. Table 1). True pleiotropism should therefore be considered.

Summary.—A number of mutants of *Escherichia coli* K-12 strains which are defective in galactose metabolism have been identified as having single or multiple defects in the Leloir pathway. Enzyme assays for each step of this pathway have been described. Among the singly defective mutants found can be cited the galactokinase-less and galactose-1-phosphate uridyl transferase-less. A singly defective mutant of uridine diphosphogalactose-4-epimerase of an *E. coli* strain different from K-12 (so called M strain) has also been described. The triply defective mutants (all K-12 strains) are genotypically single mutants (Lederberg).

An attempt to describe the triple defects as a result of a single defect of galactsoe permease is complicated by the fact that galactose although unable to promote induction of any of the enzymes of the Leloir pathway is able to elicit induction of β-galactosidase.

A hereditary block of galactokinase in two mutants of K-12 was found to be correlated with a synthesis of constitutive galactose-1-phosphate uridyl transferse. It seems, however, not possible to derive any correlation between the full constitutivity of galactose-1-phosphate uridyl transferase and the rate of formation of galactose-1-phosphate. Pleiotropism should be considered.

* Contribution No. 268 from the McCollum-Pratt Institute. Supported by grants from National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, and the National Science Foundation.

† Predoctoral Fellow of National Institutes of Health.

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[30] This enzyme was purified, crystallized, and kept in this form:[12] a small part of the crystalline suspension was dissolved in acetate buffer (0.15 *M*, pH 5.5) shortly before use. Such a solution remained active without the addition of glucose-1,6-diphosphate for about one week.

[31] Unpublished methods by K. Kurahashi and A. Sugimura.

[32] It is necessary to set up a zero time control for galactokinase activity and the value for Gal-1-P formed was corrected for the zero time control. This is especially important if liver transferase was utilized as indicator enzyme, since it is contaminated by small amounts of galactokinase.[13]

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*HEREDITARY DEFECTS IN GALACTOSE METABOLISM IN ESCHERICHIA COLI MUTANTS, II. GALACTOSE-INDUCED SENSITIVITY**

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Communicated September 8, 1959

The present study of hereditary galactose sensitivity in bacteria was prompted by the existence of such a pathological state in humans, so-called congenital galactosemia (more correctly, hereditary galactosemia). As is well known, the latter state manifests itself by disease symptoms if the subject receives galactose; later permanent tissue lesions ensue. It was shown [1,2] that the basis for abnormal sensitivity toward galactose is due to a defect in a single enzyme, galactose-1-phosphate