*BIOCHEMICAL OBSERVATIONS ON E. COLI MUTANTS DEFECTIVE IN URIDINE DIPHOSPHOGLUCOSE**

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Recent studies on the cellular synthesis of various glucosides have taught us that in contrast to the catabolism of glucose the anabolic processes require the mediation of uridine diphosphoglucose (UDPG)‡ or related nucleotides.[1, 2]

The biosynthesis of the anabolic mediator, UDPG, proceeds via UTP and G-1-P by means of a specific enzyme UDPG pyrophosphorylase.[3, 4] We shall use the term UDPG synthetase instead of UDPG pyrophosphorylase in order to be more explicit about the problem with which we are dealing in this paper, i.e., cellular synthesis of UDPG and of polyglucoside compounds.

In the course of our studies on mutants of *E. coli* K-12, we have encountered a mutant, Gal 23,[5a, 5b] which by the token of microbiological assays on EMB galactose agar would be classified as a "galactose-negative" mutant. It was found to have only very low capacity to incorporate galactose into insoluble carbohydrate compounds. When cells of Gal 23 were grown on ammonia mineral medium with glycerol as a carbon source, addition of galactose to the medium induced a cessation of growth comparable to that seen in transferaseless mutants.[6] Surprisingly enough, enzymatic assays of the enzymes of galactose metabolism[7] revealed that this galactose-negative and galactose-sensitive strain contained all three enzymes, i.e., K, T, and E. All these observations focused our attention toward a genetic block in the pathway of synthesis of UDPG, especially the enzyme UDPG synthetase. This hypothesis has been fully substantiated as will appear from the present study.

Meantime Fukasawa *et al.*[8] described two mutants of *E. coli* K-12 which they found to be defective in UDPG synthetase. They also reported that these mutants contain no detectable amount of hexose in their cell walls, and that their major cell-wall components are heptoses and hexosamines. These observations will be discussed in conjunction with our own findings.

Materials and Methods.—The K-12 strains used stemmed from the collection of Drs. E. and J. Lederberg and were generously donated to us. The mating type was generally F^-. A few F^+ strains were also investigated with respect to their content of polysaccharides, but under the conditions used here, no differences were found. The chemical differences which may exist between the mating types will not be the subject of the present study. The strains used were not lysogenic with respect to λ or λ gal. The growth conditions used were in general those described previously[6] with main emphasis on ammonia mineral medium with glycerol as carbon source. Enzyme assays (see further) were carried out on cell sonicates. D-Glycero-L-manno-heptose used as a standard stemmed from the National Institutes of Health, as a generous gift from Dr. K. N. Richtmyer. D-Fucose was purchased from the K & K Company, and other sugars from Pfanstiehl Laboratories, Inc. Galactose (Sigma Chemical Company) was essentially free of glucose. 1-C-14 galactose stemmed from the National Bureau of Standards. The galactose-1-phosphate (Sigma Chemical Company) used was a dipotassium salt. UDPGal was prepared according to the principle described by Wiesmeyer and Jordan[9] with some modifications in the purification procedure, in which a Dowex formate column was used for fractionation; (the generous help of Dr. K. Ebisuzaki is acknowledged with thanks). Radioactive reaction products (Gal-1-P, UDPG, UDPGal), synthesized in the cell or in sonicates by input of 1-C-14 galactose, were spotted on

sheets of Whatman No. 1 paper. The solvent used for chromatography was a mixture of 30 ml 1 *M* sodium acetate buffer pH 3.5 with 75 ml absolute ethanol, and the chromatograms were scanned by means of a Vanguard low-background auto scanner. This same method (estimation of radioactive Gal-1-P formed) was used as a semiquantitative assay for galactokinase. A more quantitative estimation of galactokinase was worked out by Drs. K. Ebisuzaki and C. E. Lees (unpublished). In principle, it consisted in a separation of free galactose from Gal-1-P by means of a small Dowex-1 ammonium formate column.

The assays for the enzymes T and E were performed as described previously.[7] Assays for UDPG and UDPG synthetase (UDPG pyrophosphorylase) were performed as described respectively by Kalckar *et al.*,[10] and by Munch-Petersen *et al.*[11]

Isolation and purification of bacterial lipopolysaccharides (LPS) was carried out according to the method described by Westphal and co-workers—by extracting freshly harvested cells with 45% phenol at 68°C.[12, 13] The aqueous extract, containing LPS and nucleic acids, was dialyzed and lyophilized. This crude extract (about 10% of the bacterial dry weight) was purified by 6 to 10 cycles of ultracentrifugation, in which the high molecular LPS is sedimented, while nucleic acids remain in the supernatant.[13] After disappearance of the absorption maximum at 260 mμ from both gel and supernatant, the LPS was dissolved in a small amount of water and lyophilized to give a fine, white powder. The yield of LPS from *E. coli* K-12 was 1–1.5% of the bacterial dry weight, irrespective of the strain used.

The LPS was hydrolyzed by heating for 4 hr at 100° with 1 *N* H_2SO_4. After removal of H_2SO_4, the hydrolyzates were evaporated and the sirupy residue used for paper chromatography and for colorimetric tests.

For the study of amino sugars, the hydrolysis was carried out by a 15-hr treatment with 6 *N* HCl at 100°C. The acid was removed *in vacuo* and the last traces eliminated by codistillation with absolute ethanol. The residue was used for paper chromatograms and for the Elson Morgan[14] determination.

Paper chromatograms[15] were done by the descending method, on Whatman No. 54 paper. The following solvent systems were used: (1) Ethyl acetate:pyridine:water (12:5:4),[16] which gives a fairly good separation of glucose and galactose. (2) *n*-Butanol:acetic acid:water (4:1:5) for separation of pentoses and methyl pentoses. (3) Ethyl acetate:pyridine:acetic acid:water (5:5:1:3) for amino sugars and sialic acid. (4) *n*-Butanol:*n*-propanol:0.1 *N* HCl (1:2:1)[17] for sialic acid.

Spots were made from 50 μg of reference sugars and from hydrolyzates of 0.5 mg LPS. Sugars were detected on paper chromatograms with the alkaline silver reagent of Trevelyan or by spraying with *p*-anisidine hydrochloride which gives different colors with various classes of sugars. Amino sugars were located by means of the ninhydrin spray.

The different categories of sugars making up the LPS were recognized by using various modifications of the cysteine-sulfuric acid reaction of Dische.[18] The absorption spectra obtained were recorded on a Cary spectrophotometer and compared with those given by standards.

Total carbohydrates were measured by the tryptophane-sulfuric acid reaction of Sheppard and Everett[19] and by the phenol-sulfuric acid reaction of Smith and co-workers.[20]

The results of the analysis of carbohydrates in the LPS of various strains of *E. coli* K-12 are recorded in Table 4.

Experimental Observations and Their Interpretation.—Gal 23 fails to produce detectable amounts of acid on an EMB galactose agar plate and is unable to use galactose as a carbon source for growth. On the contrary, if galactose is added to the growth medium, it induces a cessation of growth, "galactose-induced stasis" (cf. ref. 6) typical for gal negatives with a single block in T. Under such conditions, it is also possible to select K defective derivatives, i.e., double mutants (cf. 6 and 7). Such double mutants will be discussed below. As it appears from Figure 1, further addition of glucose to glycerol and galactose brings about a resumption of growth comparable to that found in transferaseless mutants.

If tracer amounts of 1-C-14 galactose are added to a Gal 23 culture which has been induced into stasis by ordinary galactose, a considerable proportion of the

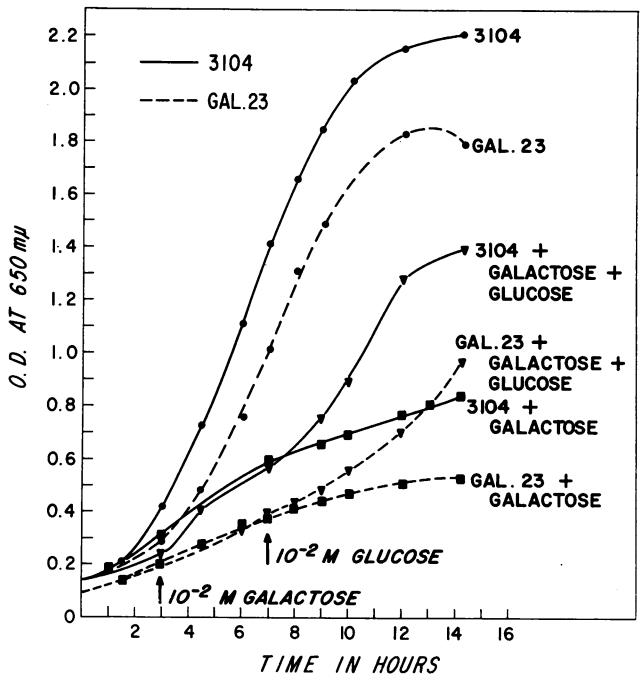

FIG. 1.—Galactose-induced stasis and effect of glucose, in Gal 23 and 3104. Shake cultures at 37° C of *E. coli* K-12, strains 3104 and Gal 23. Each organism was inoculated into 3 flasks containing ammonia minimum medium A with 1 per cent glycerol as carbon source. After 3 hr, galactose (10^{-2} *M*) was added to 2 flasks, and the third left unaltered. Four hours after addition of galactose, glucose (10^{-2} *M*) was added to one of the cultures in galactose stasis.

radioactivity appears in the soluble filtrates of the cellular material. The soluble radioactive material is probably mainly gal-1-P by the token of paper chromatography and subsequent scanning. There is no radioactivity in the region corresponding to UDPG or UDPGal, and the number of counts in the insoluble residue is very small as compared with that obtained from wild type. The gal-1-P accumulation was substantiated by specific enzymatic methods and found to amount to 9 to 10 μmoles per gram dry weight. All the biochemical studies on the cells clearly pointed towards a defect in T.

The picture of a T defect vanished entirely, however, as the result of a study of the enzymes of galactose metabolism in sonicates. It first became evident that 1-C-14 galactose added to sonicates in the presence of ATP, Mg^{++}, and UDPG was incorporated not only into gal-1-P but also into UDP-hexose. A possible defect in epimerase was ruled out by the results of a quantitative enzymatic assay of the three enzymes (see Table 1). It can be seen that all three enzymes (K, T, and E) are present and are well induced, especially by D-fucose.

Since UDPG is mandatory for galactose metabolism,[21] it was logical to make an inquiry into the status of this nucleotide both with respect to steady-state levels and with respect to the capacity of the synthesizing enzyme system. It will appear from Table 2 that the steady-state levels of UDPG in Gal 23 are markedly lowered as compared with those in a T^- mutant or in the wild type. It should be pointed

out that the UDPG levels in Gal 23 were always determined in filtrates from cells in logarithmic growth in the *absence* of galactose. We emphasize this because it has been found that T-defective mutants (such as W 3104) in the state of galactose-induced stasis show reduced steady-state levels of UDPG (Spyrides and Kalckar, 1960, unpublished). The accumulation of Gal-l-P in the UDPG-defective strain was also observed by Fukasawa *et al.*[8]

It appears from Table 3 that the level of UDPG synthetase is greatly reduced in Gal 23 as compared with wild type. This obviously also applies to the K^- derivative of Gal 23. The level of UDPG synthetase is seen to be also very low in strain 3142. This galactose-negative strain, which had previously been incompletely described as kinase-defective,[7] was recognized by Fukasawa *et al.*[8] to be a double mutant with a defect in UDPG synthetase as well as a kinase defect.

The history of the recognition of the UDPG defectives is worth recalling. Fukasawa, Jokura, and Kurahashi[8] in their elegant analysis of a situation identical with ours point out that the UDPG defective mutants were genotypically described as mutants with a defect outside the lambda region, and which therefore failed to become fermenters of galactose even if transduced with an all-positive λ gal.[5, 8]

Phenotypic studies showed that some of these strains were galactose negatives in EMB galactose plate tests, and galactose sensitives in growth tests (minimum medium with added galactose). Subsequently, a selection of galactose negatives without galactose sensitivity ensued. The main population before selection (i.e., the galactose negative-galactose sensitive strains) represents the mutant with a single defect in the gene for UDPG synthetase (UDPG pyrophosphorylase) which, as mentioned, is located outside the lambda region. In a way, this mutant could be called "pseudo galactose negative" since all the three enzymes specific for galactose metabolism are present. Selection of galactose negatives without galactose

TABLE 1

ENZYME LEVELS IN GAL 23

Inducer	Kinase μm gal-1-P/mg protein/hr		Transferase μmTPNH/mg protein/hr		Epimerase μm UDPG/mg protein/hr	
	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
D-fucose 10^{-3} *M*	0.7	11	1.05	14.7	5	37
D-galactose 10^{-2} *M*		10		7.5		30

Cells were grown in minimal medium-glycerol with fucose 10^{-3} *M* as inducer, or in 1 per cent broth, with galactose as inducer.

TABLE 2

UDPG LEVELS

	μmoles per gm dry weight cells
W 3100	0.5
W 3104	0.9*
Gal 23	0.05

* Spyrides and Kalckar, 1960, unpublished.

TABLE 3

UDPG SYNTHETASE

	μmoles TPNH formed per mg protein, per hr	
W 3100	0.60	0.90
Gal 23	0.08	
Gal 23K^-	0.10	
W 3142		0.05

sensitivity results in a population largely composed of double mutants. Some of these have a defect in galactokinase in addition to the defect in UDPG synthetase. Strain 3142 was a result of such a selection, albeit unintentional, whereas strain Gal 23 K^- was a result of a planned selection from Gal 23.

Among other mutants selected from Gal 23 should be mentioned a strain in which all three enzymes of the galactose pathway were found to be defective. This may represent an operator mutant, in which selective advantages are due to the low levels of galactokinase.

All these observations demonstrate the importance of assaying at least all the four enzymes of the galactose-glucose pathway, which had already been emphasized previously in the case of mammalian systems (cf. Table 1 in Kalckar[22]).

With regard to the study of the LPS (see Table 4), the most striking characteristic of the UDPG defective strains is the lack of detectable glucose and galactose. This is in agreement with the observations of Fukasawa *et al.*[8] Moreover, the absence of detectable 6-deoxyhexose (rhamnose or 6-deoxyglucose) is also notable.

The presence of galactose in the E defective Gal 22 is presumably due to a certain "leakiness" in epimerase; this may also apply to the presence of 6-deoxyhexose. Moreover, it should be pointed out that the "leakiness" is presumably more apt to manifest itself in the cell-wall composition under conditions of slow growth, such as in the minimum medium used.

A number of other sugar constituents were detected, such as ribose, mannose, and

TABLE 4

	W 3100 Gal +	W 3092 Gal⁻, K def.	W 3104 Gal⁻, T def.	Gal 22 Gal⁻, E def.	Gal 23 UDPG-def. Gal∓	Gal 23K⁻ UDPG def. Gal⁻, K def.
Glucose	+ / +	+ / +	+ / +	+ / +	n.d. / n.d.	n.d. / n.d.
Galactose	+ / +	+ / +	+ / +	+ / +	n.d. / n.d.	n.d. / n.d.
Amino-sugars (±acetyl)	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
Heptose (D-glycero-L-manno-heptose used as standard)	/ +	/ +	/ +	/ +	+ / +	+ / +
Rhamnose or other 6-deoxy-hexoses*	+ / +	+ / +	+ / +	+ / +	n.d. / +	n.d. / +
Ribose	n.d. / n.d.	n.d. / n.d.	n.d. / n.d.	n.d. / n.d.	+ / +	n.d. / n.d.
Fast-moving sugars	+	+	+	+	+	+
Neutral sugars in LPS (measured as glucose by phenol-H_2SO_4 method)	20%	23%	22%	20%	13%	11%

Upper left: Observations from paper chromatography.
Lower right: Observations from Dische absorption spectra (or Elson Morgan reaction for amino sugars).
+ means present.
n.d. means non-detectable.
T def. means transferase defective.
K def. means galactokinase defective.
E def. means epimerase defective.
UDPG def. means UDPG synthetase defective, synonomous here with "UDPG defective."
Gal ∓ means "pseudo galactose negative."
Gal 23K^- means a galactokinase defective derivative of Gal 23.
Mating types not specified but most of them were F^- (see *Materials and Methods*).
* Identified chromatographically as rhamnose in strain W3100.

a component giving a positive reaction in the thiobarbituric acid test of Warren.[23] Concerning the ribose, it can be briefly mentioned here that it does not stem from nucleic acids since it is not associated with ultraviolet-absorbing material. The sugar moves with an R_f identical with that of ribose in solvents (1) and (2) described above and gives the pink color of pentoses with the *p*-anisidine spray. Hydrolyzates in which this spot was detected also exhibit the characteristic pentose maximum in the cysteine-sulfuric acid reaction of Dische. This pentose has so far been found only in certain cases in Gal 23 and never in the other strains, not even in the K-defective Gal 23. Its relevance to the UDPG defect is therefore still doubtful.

The material giving a positive Warren reaction was obtained under the same conditions as used for extraction of sialic acid:[24, 25] 45-min hydrolysis with 0.1 *N* H_2SO_4 followed by adsorption on a Dowex-2-acetate column and elution with pH 4.6 acetate buffer. This material was negative in the orcinol,[26] the Ehrlich,[27] and diphenylamine[28] reactions. It has been found in all the K-12 strains investigated so far.

The biological aspects of the alterations in the chemical makeup of the LPS may be far reaching. This has also been emphasized to a certain extent by Fukasawa *et al.*[8]

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‡ Abbreviations: ATP = Adenosine triphosphate; UDPG, UDPGal = Uridine diphosphoglucose, Uridine diphosphogalactose; UTP = Uridine triphosphate; TPNH = Reduced triphosphopyridine nucleotide; PP = Pyrophosphate; G-1-P = Glucose-1-phosphate; Gal-1-P = Galactose-1-phosphate; K = Kinase; T = Transferase; E = Epimerase; EMB agar = Eosine-Methylene Blue agar; LPS = Lipopolysaccharide.

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DISSOCIATION OF MACROMOLECULAR SYNTHETIC PROCESSES IN T2-INFECTED E. COLI

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The process of infection and replication of bacteriophage has played a central role in establishing modern concepts of the genetic function of DNA,[1] the intermediate role of a "messenger" RNA[2, 3] in the translation of genetic information, and the mechanism of RNA action in the synthesis of specific protein.[4] In part, this is due to the fact that the phage-bacteria system is particularly adaptable to operational techniques which can isolate one kind of macromolecular synthesis from the others. Thus, Hershey and Melechen,[5] by blocking protein synthesis of T2-infected *Escherichia coli* B with chloramphenicol, were able to demonstrate the accumulation of large amounts of genetically competent phage DNA that subsequently could be incorporated into mature phage. Experiments with a mutant, *E. coli* B94, as host revealed that T2-specific RNA, produced in the absence of net protein and DNA synthesis, retains its capacity to function biologically in a subsequent synthesis of infectious T2 particles.[6]

Experiments reported here describe an unexpected block in the conversion of bacterial DNA to phage DNA by the analog, 5-fluorodeoxyuridine (FUdR). In the presence of this inhibitor, the synthesis of phage-specific RNA and protein proceeds at about a normal rate, but only DNA from multiply infecting parental phage contributes to the infectious progeny. By combining FUdR with the B94 mutant as host, it is possible to demonstrate that a stepwise dissociation of T2-