4. F. Feyrter, *Über die Peripheren Endokrinen (Parakrinen) Drüsen des Menschen* (Maudrich, Vienna, 1954).
5. L.-I. Larsson *et al.*, *Lancet* **1977-I**, 666 (1977); O. P. Ganda *et al.*, *N. Engl. J. Med.* **296**, 963 (1977).
6. L. A. Sternberger, *Immunocytochemistry* (Prentice-Hall, Englewood Cliffs, N.J., 1974).
7. J. F. Rehfeld, *J. Biol. Chem.* **253**, 4016 (1978).
8. G. Tramu, A. Pillez, J. Leonardelli, *J. Histochem. Cytochem.* **26**, 322 (1978).
9. L.-I. Larsson and J. F. Rehfeld, *ibid.* **25**, 1317 (1977); *Nature (London)* **269**, 335 (1977).
10. L.-I. Larsson, *Histochemistry* **58**, 33 (1978).
11. _____ and J. F. Rehfeld, *ibid.*, p. 23.
12. R. E. Coggeshall, *Tissue Cell* **3**, 637 (1971).
13. A. G. E. Pearse, *Med. Biol.* **55**, 115 (1977).
14. We thank the Danish MRC for grant support and K. Siessing and J. B. Lauridsen for expert technical assistance.

28 February 1979; revised 1 May 1979

Galactosemia: Alterations in Sulfate Metabolism Secondary to Galactose-1-Phosphate Uridyltransferase Deficiency

Abstract. *Cultures of nonmutant as well as galactokinase-deficient fibroblasts incorporate 20 percent more [^{35}S]sulfate when galactose is substituted for glucose in the medium; galactose-1-phosphate uridyltransferase-deficient cells incorporate 65.5 percent less. In addition to incorporating less [^{35}S]sulfate, the uridyltransferase-deficient cells showed significant accumulation of intracellular galactose-1-phosphate within 4 hours after galactose exposure. Under the same conditions, no difference in [^{3}H]uridine incorporation was observed. This metabolic alteration, occurring in response to galactose exposure, may be related to the pathophysiology of classical galactosemia.*

Galactokinase and galactose-1-phosphate uridyltransferase deficiencies are well recognized autosomal recessive forms of galactosemia (*1*). Lenticular cataracts are a common symptom in both disorders. The conversion of excess galactose to galactitol by lens aldose reductase causes an osmotic imbalance, the underlying mechanism in cataract formation (*1*). Other symptoms of uridyltransferase deficiency include vomiting, diarrhea, hepatomegaly, and mental retardation. This form of galactosemia can even be fatal. The factors responsible for the more severe manifestations are not well understood (*1*).

Krooth and Weinberg (*2*) were probably the first to demonstrate that fibroblast cultures derived from patients with galactosemia failed to grow in a galactose medium. Uridyltransferase activity is not detectable in cells cultured from galactosemic patients (*3*), and Mayes and Miller (*4*) demonstrated that such cells accumulate galactose-1-phosphate within 2 hours after galactose exposure. The underlying toxic mechanisms that produce the internal organ and neurological pathology are probably secondary to the intracellular accumulation of galactose-1-phosphate or a manifestation of the absence of uridyltransferase activity. Therefore, such toxic mechanisms might be identified by searching for biochemical alterations in cultured mutant cells challenged with galactose.

Our report demonstrates that when challenged with galactose, cells deficient in uridyltransferase activity (GALT cells) exhibit significantly reduced incorporation of [^{35}S]sulfate into acid-insoluble macromolecules.

Five different strains of GALT cells, one galactokinase-deficient (GALK) strain, one Hurler strain, and eight control strains were used (*5*). Galactokinase and uridyltransferase activities were measured as in (*3*). Cells were grown in minimum essential medium (MEM; Gibco or KC Biologicals) containing 16.6 percent fetal calf serum (Gibco or KC Biologicals) for routine passage and maintenance, with feeding every 3 to 4 days. Hexose-free MEM (Gibco) was used to prepare all experimental media. Concentrates (100 times) of glucose (Mallinckrodt) or galactose (Pfansteil) were added through 0.22-μm Millipore filters to hexose-free MEM containing 16.6 percent dialyzed fetal calf serum to yield the desired final concentrations. All media contained penicillin (50 U/ml) and streptomycin (50 μg/ml).

Glucose, galactose, and galactose-1-phosphate were measured enzymatically (*6*), using a fluorometer (Aminco model SPF 125) with limits of detection around 0.1 μg/ml. Hexokinase, glucose-6-phosphate dehydrogenase, galactose dehydrogenase, alkaline phosphatase, oxidized nicotinamide adenine dinucleotide (NAD^+), and oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) were obtained from Boehringer Mannheim and the Sigma Chemical Company.

The cell cultures were labeled by adding $H_2{}^{35}SO_4$ (New England Nuclear; 10 μCi/ml) to the experimental medium in 25-cm^2 disposable Corning flasks containing a confluent cell sheet. The labeled cells were harvested by ethanol or trichloroacetic acid (TCA) precipitation. The ethanol precipitation method was described by Fratantoni *et al.* (*7*). The final washed precipitate was dissolved in 0.5 ml of 10 percent NaOH. Portions of 50 or 100 μl were counted in a Packard scintillation spectrometer (model 3385), 10 ml of Instagel (Packard) being used as the scintillation medium. Protein concentration was measured by the method of Lowry *et al.* (*8*); bovine serum albumin dissolved in 10 percent NaOH was used as the standard.

Alternatively, cultures were labeled with [^{35}S]sulfate or [5, 6-^{3}H]uridine (New

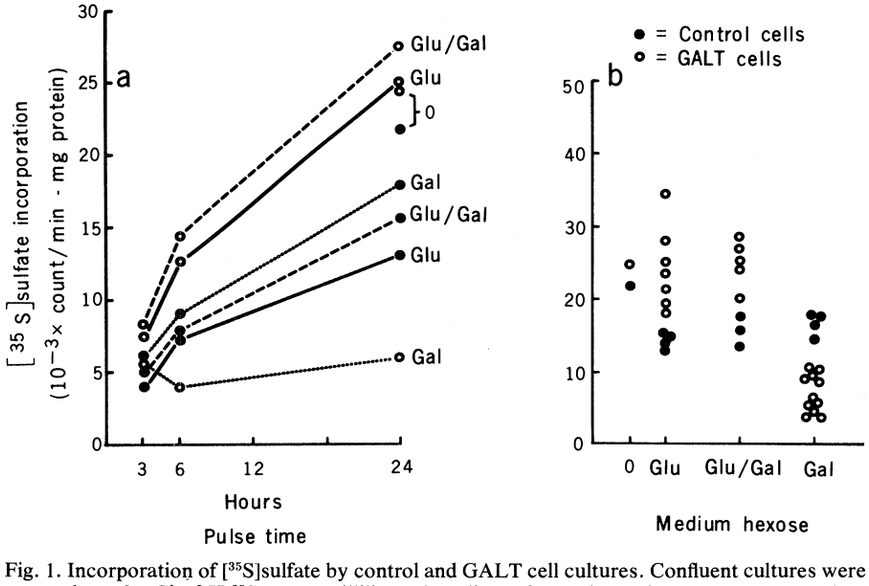

Fig. 1. Incorporation of [^{35}S]sulfate by control and GALT cell cultures. Confluent cultures were exposed to 10 μCi of $H_2{}^{35}SO_4$ per milliliter of medium after 6 days of exposure to a medium containing no hexose (*0*), 100 mg/dl glucose (*Glu*), 100 mg/dl galactose (*Gal*), or 50 mg/dl of hexose mixture (*Glu/Gal*). (a) The amount of isotope incorporated during 3-, 6-, and 24-hour exposures. (b) The amount of isotope incorporated by individual cultures during a 24-hour exposure.

0036-8075/79/0928-1395$00.50/0

Table 1. Intracellular galactose-1-phosphate of normal and galactose-1-phosphate uridyltransferase-deficient (GALT) cell cultures after various periods of galactose exposure; S.D. = standard deviation.

Cell type	Medium	Galactose-1-phosphate (micrograms per milligram of protein)												Mean ± S.D.
		Hour						Day						
		0	1	2	4	6	24	2	4	5	6	7	12	
Normal	Galactose	2.6	7.3	8.6	7.3	5.3	7.7	6.0	7.5	8.8	7.3			6.84 ± 1.81
GALT	Galactose	4.0	9.6	9.6	18.6	21.6	23.0	18.1	17.7	26.8	16.0	17.5	38.5	18.42 ± 8.92
Normal	Glucose							6.5	6.1	3.5	5.2			5.33 ± 1.33
GALT	Glucose							10.2	9.0	13.5	10.2	11.8		10.94 ± 1.74

England Nuclear; 5 μCi/ml) or with both, and labeling was terminated by precipitation with TCA. The cell sheet was washed three times with cold isotonic saline (0° to 4°C). One milliliter of cold 10 percent TCA was then layered over the cell sheet, and the culture flasks were kept at 0° to 4°C for 30 minutes. Flasks were rinsed three times with cold 10 percent TCA and drained to dryness. Next, 1 ml of 2 percent Na_2CO_3 in 0.1*N* NaOH was layered over the cell sheet and incubated overnight at 37°C to completely dissolve the cell sheet. Equal portions were removed for scintillation counting and protein measurement as described above, except that protein standards were prepared in the same Na_2CO_3-NaOH solution that was used to dissolve the cell sheet.

Each experiment was performed with replicate cultures of normal and mutant cells grown to confluence on maintenance medium. To initiate each experiment, cultures were rinsed three times and cultured on experimental medium. All experimental media were identical except for the carbohydrate source of glucose, galactose, or their mixture.

Although normal cells showed no differences, GALT cells cultured with galactose exhibited noticeable changes in density after 1 week, obvious deterioration of the cell sheet in 1 to 2 weeks, and irreversible damage after 2 to 3 weeks. After 2 weeks of galactose exposure, damaged GALT cell sheets recovered when they were returned to the glucose medium; recovery was not evident after 3 weeks of exposure to galactose. The GALT cells cultured on mixtures of glucose and galactose did not show damage until glucose was no longer detectable in the medium. Thus, glucose appears to prevent cell sheet damage. When confluent cultures are exposed to a medium free of hexose, survival without noticeable deterioration continues for at least 1 to 2 weeks. Normal cells recover fully when cultured on glucose after 2 to 4 weeks on hexose-free medium.

After 7 days, measurements of glucose and galactose media showed that normal cells consumed 85 to 100 percent of the glucose and 55 to 65 percent of the galactose. The GALT cells consumed 45 to 85 percent of the glucose and only 5 to 8 percent of the galactose, which presumably was utilized through alternate pathways (*1*).

The data for intracellular galactose-1-phosphate content of normal and GALT cells cultured with glucose (100 mg/dl) or galactose (100 mg/dl) are presented in Table 1. These data demonstrate that GALT cells contain nearly twice as much galactose-1-phosphate as normal cells, even when galactose is absent from the medium. Within 4 hours after galactose exposure, galactose-1-phosphate in the mutant cells increased four- to fivefold and remained at that concentration for about 7 days. After 12 days, when deterioration of the cell sheet was obvious, galactose-1-phosphate increased five- to tenfold. The galactose-1-phosphate content of normal cells appears to be unaffected by hexose or length of exposure, and remains relatively constant.

To minimize the potential effects of carbohydrate starvation, we studied isotope incorporation between days 4 and 6 of exposure to an experimental medium (when no obvious morphological changes were evident). In the glucose medium, GALT cells consistently incorporated more [^{35}S]sulfate than the con-

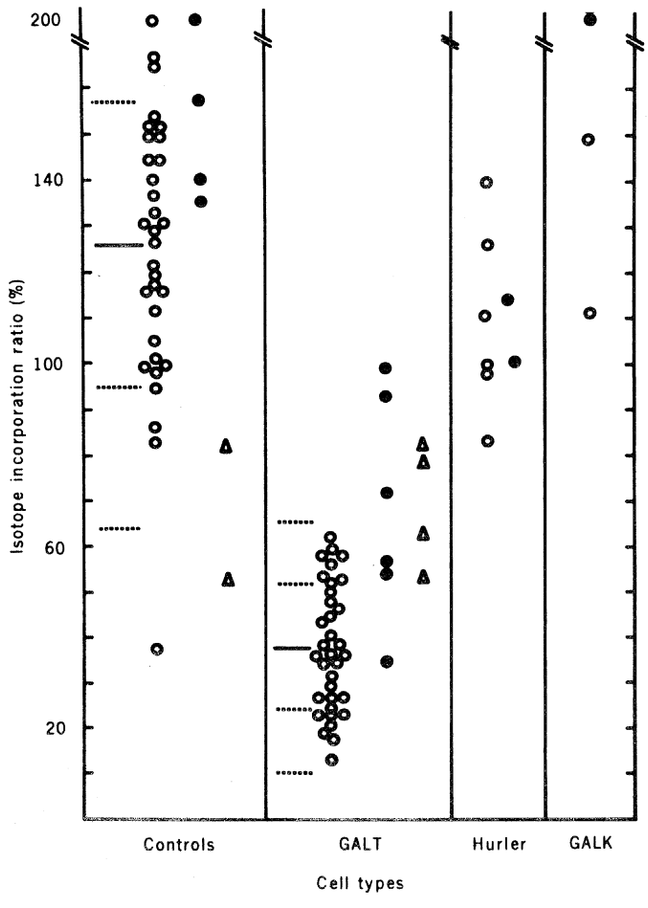

Fig. 2. Comparison of the effects of galactose and glucose on the incorporation of isotope into the acid-insoluble fraction of control and mutant cell cultures. Each symbol represents the ratio (percent) of isotope incorporated on a galactose medium to that incorporated on a glucose medium by paired cultures from the same passage exposed to an experimental medium for the same duration (4 to 6 days); ○, 24-hour [^{35}S]sulfate exposure terminated by TCA precipitation (the mean and standard deviations for these values are indicated by solid and dotted lines, respectively). The statistical difference between these data from eight different controls and five different GALT cell strains is highly significant ($P < .0005$ Student's *t*-test); ●, 24-hour [^{35}S]sulfate pulse followed by ethanol precipitation (*7*); △, a 24-hour-[^{3}H]uridine exposure terminated by TCA precipitation.

trols did, but much less than was incorporated by a Hurler cell strain. When exposed to an experimental medium for 6 days prior to labeling with [^{35}S]sulfate at 3, 6, and 24 hours, GALT cells incorporated twice as much of the isotope as did control cells when glucose was present (by itself or mixed with galactose), and only half as much when exposed to a galactose medium. In contrast, control cells incorporated slightly more [^{35}S]-sulfate when galactose was present (see Fig. 1a).

Exposure to media containing glucose, galactose, or both for 4 to 6 days and then to [^{35}S]sulfate for 24 hours has little effect on control cells, whereas GALT cells consistently incorporate much less isotope when exposed to a galactose medium (see Fig. 1b).

Decreased sulfate incorporation by GALT cells on a galactose medium is not an effect of carbohydrate starvation. Control and GALT cultures exposed to a hexose-free medium for 4 to 6 days and to [^{35}S]sulfate for 24 hours incorporated amounts of isotope into the acid-insoluble fraction that were similar to those incorporated by replicate cultures on a glucose medium (see Fig. 1). Thus it is more probable that the decreased sulfate incorporation by GALT cells that results from exposure to galactose is a consequence of galactose toxicity.

The incorporation of [^{3}H]uridine into the acid-insoluble fraction did not distinguish control and GALT cells, although on a galactose medium, both kinds of cell slightly decreased their incorporation of [^{3}H]uridine (see Fig. 2, triangles). Thus the two different isotopes are incorporated differently in the presence of galactose.

The isotope incorporation results are presented in Fig. 2. These data were obtained by calculating the ratio of isotope incorporation by cells on the galactose medium to that incorporated by the same cells on the glucose medium and expressing this ratio as a percentage. The Hurler cell strain incorporated excessive amounts of [^{35}S]sulfate on both the glucose and galactose media. The GALK cell cultures behaved like control cells, demonstrating that the differences observed for the GALT mutants are not obvious when galactose cannot undergo phosphorylation. The solid circles in Fig. 2 represent data obtained with the ethanol precipitation technique (routinely used to detect excess sulfate uptake by cultured cells deficient in hydrolytic enzymes, which degrade glycosaminoglycans). Although these values are higher than those obtained with the TCA precipitation method (Fig. 2, open circles), the difference between control and GALT cells is similar.

These data demonstrate that the absence of galactose-1-uridyltransferase activity or the accumulation of intracellular galactose-1-phosphate can produce secondary metabolic alterations in sulfate metabolism. Furthermore, the decreased incorporation of sulfate into acid-insoluble macromolecules occurs at a time when neither gross cellular morphology nor another biochemical pathway ([^{3}H]uridine incorporation) appears to be affected. Although the alterations reflected by reduced [^{35}S]sulfate incorporation remain to be defined in molecular terms, such changes could be related to the toxic mechanisms responsible for the tissue pathology associated with classical galactosemia.

T. A. TEDESCO
K. L. MILLER

Genetics Laboratories,
Department of Pediatrics,
University of South Florida,
College of Medicine, Tampa 33612

References and Notes

1. S. Segal, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, Eds. (McGraw-Hill, New York, ed. 4, 1978), p. 160; H. M. Kalckar, J. H. Kinoshita, G. N. Donnell, in *Biology of Brain Dysfunction*, G. E. Gaull, Ed. (Plenum, New York, 1973), pp. 31-88.
2. R. S. Krooth and A. N. Weinberg, *J. Exp. Med.* **113,** 1155 (1961).
3. T. A. Tedesco and W. J. Mellman, *J. Clin. Invest.* **48,** 2390 (1969).
4. J. S. Mayes and L. R. Miller, *Biochim. Biophys. Acta* **313,** 9 (1973).
5. All control cell cultures were derived from skin biopsies and initiated in our laboratory. The Hurler cells (GM34) were acquired from the National Cell Repository, Camden, N.J. The galactokinase-deficient cells (GM334) and the galactose-1-phosphate uridyltransferase-deficient cells (GM52, GM53, and GM54) were from patients previously studied by us [(*3*) and *Am. J. Hum. Genet.* **29,** 240 (1977)] and were recovered either from the National Cell Repository as identified by the GM numbers or from the stocks of Dr. W. J. Mellman, Department of Human Genetics, University of Pennsylvania, Philadelphia.
6. H. U. Bergmeyer, Ed., *Methods of Enzymatic Analysis* (Academic Press, New York, 1974), pp. 1196, 1279, and 1291.
7. J. C. Frantantoni, C. W. Hall, E. F. Neufeld, *Proc. Natl. Acad. Sci. U.S.A.* **60,** 699 (1968).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).
9. Supported by NIH grant 5-Rol-AM18719. We thank L. A. Barness and J. I. Malone for critical reviews of the manuscript.

11 April 1979; revised 29 May 1979

Meiotic Maturation in *Xenopus laevis* Oocytes Initiated by Insulin

Abstract. *Insulin can induce meiotic division in* Xenopus laevis *oocytes. This effect shows the specificity expected of a receptor-mediated mechanism. It is potentiated by ethynylestradiol, a steroid antagonist of progesterone (the natural hormone that provokes meiosis). The* Xenopus laevis *oocytes may serve as a model for the study of the poorly understood effect of insulin on cell division.*

Progesterone and other steroids can induce meiotic maturation of amphibian oocytes in vitro. They act at surface membrane sites to trigger meiosis (*1*), possibly by calcium movements or translocation from membrane stores, or both (*2-4*). Either cholera toxin, which increases adenosine 3′,5′-monophosphate (cyclic AMP) in oocytes, or cyclic AMP derivatives injected into *Xenopus laevis* oocytes hinders progesterone-induced meiosis in vitro (*5*) by interfering with the formation or activity of the maturation promoting factor (MPF). This factor develops after exposure of oocytes to progesterone and, in turn, initiates germinal vesicle breakdown (GVBD) and related phenomena of oocyte maturation (*1, 4, 6*). It is now reported that insulin can mimic the effect of progesterone on oocyte maturation.

Full-grown oocytes free of follicle cells were obtained from ovaries treated with collagenase (*6*), and were incubated at room temperature in Barth medium containing insulin (monocomponent, pork insulin, Novo). Groups of 50 oocytes were scored for GVBD after various periods of incubation.

Studies with 35 nM to 7 μM insulin (5 mU/ml to 1 U/ml) showed a dose-effect relationship with an apparent ED_{50} (50 percent effective dose) of 2 μM (Fig. 1A). The efficacy of the highest concentrations of insulin (up to 70 μM) remained less pronounced than that of progesterone used at lower concentrations, even when the oocytes were obtained under various conditions of collagenase treatment, or when the ovaries were dissected manually (data not shown). It is possible that the polysaccharide vitelline membrane impaired access of insulin to the oocyte surface or that insulin was progressively degraded during incubation. We therefore added fractions of insulin at different times of incubation, such as at 0, 2, 4, and 6 hours, and found the percentage of GVBD to be ~ 50 percent larger than when the total dose of hormone was introduced at time 0. This suggested a progressive time-dependent destruction of the hormone. The efficacy of insulin still remained lower than that

0036-8075/79/0928-1397$00.50/0