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Detection of Inborn Errors of Metabolism: Galactosemia

Abstract. *Radioautography of cultured, human, galactosemic and nongalactosemic cells shows that, in the presence of 0.05*M D-*galactono-γ-lactone, the former incorporate much less galactose in acid-insoluble form than the latter. Presumably the lactone inhibits incorporation of the labeled galactose into pathways which do not require galactose-1-phosphate uridylyltransferase activity. Definite differences between the galactosemic and nongalactosemic condition can be demonstrated with as few as 100 to 1000 cells. This approach may be useful in facilitating prenatal detection of several kinds of inborn errors of metabolism.*

The detection of galactosemic and similar biochemical defects in utero requires growth in vitro of cells biopsied from the amniotic fluid of a patient. Obtaining a sufficient number of growing cells may require several weeks during which the patient may undergo emotional stress, and the difficulty of terminating a defective pregnancy may be increased. If a highly reliable radioautographic procedure could be devised, the number of cells needed might be materially decreased with consequent decrease in time required for cell growth and improvement in the usefulness of the diagnostic procedure.

Galactosemia was selected as a model system for study. There are four known pathways for the metabolism of galactose (Fig. 1).

1) Reduction of galactose to galactitol. This reaction occurs in several tissues and the product, galactitol, accumulates in urine and in various tissues, especially lens, in patients deficient in galactokinase or galactose-1-phosphate uridylyltransferase activity (*1*). The galactitol does not appear to be further metabolized.

2) Oxidation of galactose to galactono-γ-lactone. Galactose dehydrogenase has been reported to be present in rat liver (*2*). However, its significance in the metabolism of galactose has been questioned (*3*, *4*). Hydrolysis of the lactone would lead to galactonic acid which has been identified in large amounts in the urines of galactosemic patients (*5*). Srivastava and Beutler (*4*) suggest that the substrate for such a dehydrogenation might be galactose-6-phosphate.

3) Phosphorylation of galactose to galactose-1-phosphate and its conversion to uridine diphosphogalactose (UDPGal) by reaction with uridine triphosphate (UTP). Isselbacher first described UDPGal pyrophosphorylase and suggested that it might be involved in the metabolism of galactose by galactosemic patients (*6*). Recently, it has been suggested that this enzyme and uridine diphosphoglucose (UDPG) pyrophosphorylase are the same (*7*).

4) Reaction of galactose-1-phosphate with UDPG to form UDPGal and glucose-1-phosphate. (The enzyme galactose-1-phosphate uridylyltransferase is absent or greatly reduced in activity in galactosemia.) This route is probably the major route for galactose metabolism in normal cells. Further metabolism of UDPGal would lead the galactose backbone into the usual routes of glucose metabolism.

Galactose can be utilized by galactosemic cells since they incorporate [1-^{14}C]galactose into trichloroacetic acid–precipitable cell material at about one-half the rate of normal cells (*8*). Normal cells may use the same route or routes, but in addition they can utilize the transferase route. During short incubations normal cells produce $^{14}CO_2$ from [1-^{14}C]galactose, whereas galactosemic cells do not (*9*). The $^{14}CO_2$ produced by normal cells must come from the transferase pathway, probably through the pentose phosphate shunt. Galactosemic fibroblasts do produce $^{14}CO_2$ from [l-^{14}C]galactose, but this does not begin until after about 4 days of incubation (*10*). Since galactosemic cells display little lag in the conversion of [1-^{14}C]galactose into trichloroacetic acid–insoluble cell material (*8*), this $^{14}CO_2$ could come from indirect or secondary slowly acting pathways, from an increase in activity of the pyrophosphorylase, or from an increase in the transferase; the increase in the activity of the transferase seems unlikely.

The dehydrogenase route might be a major route for galactose metabolism in galactosemic cells from the following considerations: (i) Galactitol produced by reductase does not appear to be further metabolized in vivo (*1*).

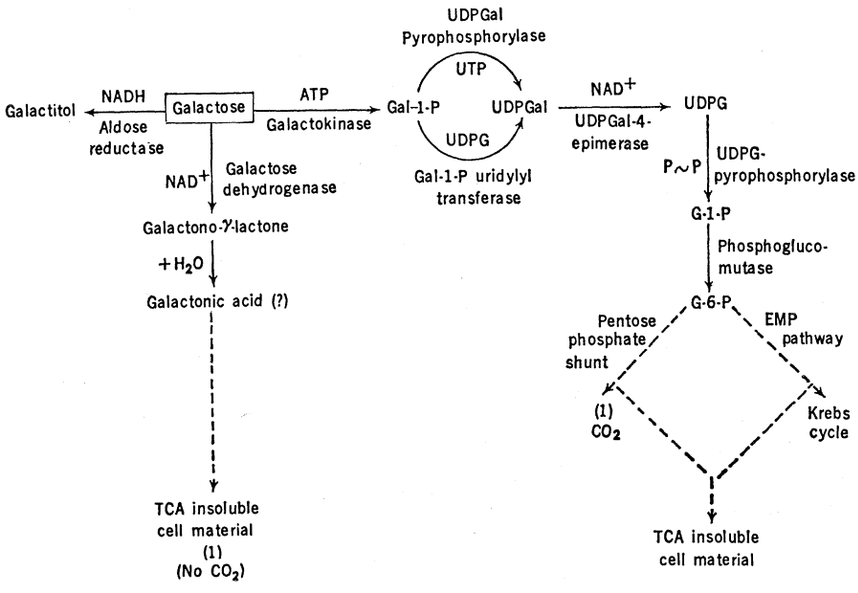

Fig. 1. Proposed scheme for galactose metabolism in cultured human cells. Abbreviations: UDPGal, uridine diphosphogalactose; UTP, uridine triphosphate; UDPG, uridine diphosphoglucose; Gal-1-P, galactose-1-phosphate; NADH, reduced form of nicotinamide adenine dinucleotide (NAD^+); ATP, adenosine triphosphate; EMP, Embden-Myerhof-Parnas pathway; and TCA, trichloroacetic acid. See (*14*).

(ii) $^{14}CO_2$ produced by normal cells from [1-^{14}C]galactose must come from the phosphorylation pathway since this pathway is blocked in galactosemic cells which do not produce $^{14}CO_2$ at least at early times. This also implies that the pyrophosphorylase reaction does not constitute a main route for galactosemic cells. (iii) The dehydrogenase route appears to be important in vivo (*5*).

If, therefore, galactosemic fibroblasts have one main route for galactose metabolism to trichloroacetic acid–insoluble material while normal fibroblasts have two, it should be possible to inhibit galactose metabolism of galactosemic cells materially or even completely while only partially inhibiting that of normal cells. Radioautography was chosen to test this approach because of its sensitivity and of the small number of cells it requires.

Most of the experiments described below utilized a culture of galactosemic fibroblasts (GSF) obtained by Dr. Arthur Robinson of the Department of Biophysics and Genetics from a skin biopsy of a male infant. Other galactosemic cultures were obtained from the American Type Culture Collection (JDU, Detroit 510, CHP3, and CHP4). Nongalactosemic human cells studied consisted of (i) normal cells grown from a sample of amniotic fluid cells obtained from Dr. Robinson (AF282) and (ii) fibroblasts from a normal female (C3), a male with cystic fibrosis (1033-79), a male with GM_1 gangliosidosis (315-33), and a male with argininosuccinic aciduria (237-13); the fibroblasts were supplied by Dr. Stephen Goodman of the Department of Pediatrics.

Table 1. Time course of incorporation of [1-^{3}H]galactose in the presence and in the absence of 0.05*M* α-D-galactono-γ-lactone. Cell lines used were GSF and AF282.

Time (hours)	Silver grains per cell			
	Galactosemic cells		Nongalactosemic cells	
	Without lactone	With lactone	Without lactone	With lactone
1	2.2 ± 2.0	4.8 ± 2.6	22 ± 10	53 ± 26
2	12 ± 7.4	10 ± 8.5	187 ± 55	121 ± 28
4	75 ± 37	16 ± 8.5	> 500	171 ± 60
16	> 500	23 ± 9.0	> 1000	> 400

The cells were cultured on tissue culture eight-chamber slides (Lab-Tek). Galactosemic cells were placed in four chambers and nongalactosemic cells in the other four chambers of each slide. Inoculums of 500 to 10,000 cells per chamber were used. The cells were cultivated for 24 to 48 hours in Ham's F12 medium (*11*), supplemented with fetal calf serum (10 percent) and human cord serum (5 percent). The medium in each well was then changed to sugar- and pyruvate-free F12 supplemented with the macromolecular components of the fetal calf and human cord serums. Isotopes and inhibitors were added as indicated, and the cells were incubated at 37°C for various periods. Each well was then washed three times with growth medium containing 0.005*M* added galactose. The cells were incubated for 10 minutes at 37°C in the third wash solution, washed once with saline G (*12*) containing 0.005*M* added galactose, and then fixed with 100 percent methanol for 1 minute, and then with Carnoy's fixative for 2 minutes. Other fixatives such as Bouin's solution or 1 percent glutaraldehyde in saline G, *p*H 7.3, for 1 hour, gave similar results. After fixation, the plastic dividers were removed, the slides were rinsed several more times with saline G containing galactose, five times with 5 percent trichloroacetic acid, and finally with running distilled water for 1 hour. The slides were then coated with Kodak NTB2 or NTB3 nuclear track emul-

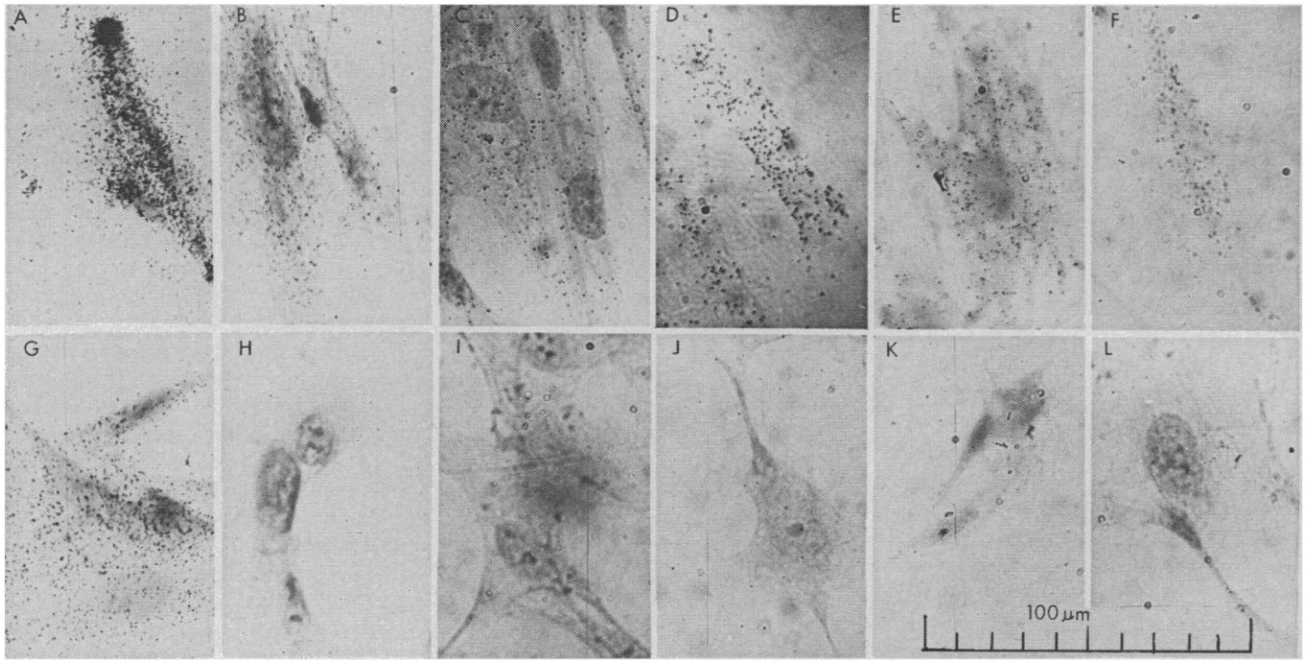

Fig. 2. Incorporation of [1-^{3}H]galactose (1 μc per well) into galactosemic and nongalactosemic cells during an overnight incubation; (A and G) No inhibitor; all other panels, 0.05*M* α-D-galactono-γ-lactone. (A and B) Normal fibroblasts AF282; (C) C3; (D) gangliosidosis; (E) cystic fibrosis; and (F) argininosuccinic aciduria. (G to L) Galactosemic lines: (G and H) GSF; (I) CHP3; (J) CHP4; (K) JDU; and (L) D510.

sion diluted 1 to 2 with distilled water. Use of 3 μc of [1-^{3}H]galactose (Nuclear-Chicago) per well and an overnight exposure of the emulsion, or 1.0 μc per well and a 3-day exposure, resulted in good radioautographs. Use of other forms of radioactive galactose, such as [1-^{14}C]galactose, uniformly labeled [^{14}C]galactose, [6-^{3}H]galactose and generally labeled [^{3}H]galactose (all New England Nuclear), gave similar results. These labeled compounds ranged in specific activity from 6.9 c/mmole for [1-^{3}H]galactose to 34.9 mc/mmole for the [1-^{14}C]galactose. After development, the slides were stained with crystal violet.

Overnight exposure to radioactive galactose causes both galactosemic and normal human fibroblasts to incorporate a considerable amount of radioactivity into cell material that is not removed by fixation and exhaustive washing (Fig. 2). This substantially confirms the finding of Russell and DeMars (*8*) that galactosemic cells can incorporate radioactive galactose into trichloroacetic acid–insoluble material.

The effect of a number of sugars and sugar derivatives on the incorporation of [1-^{3}H]galactose and [1-^{14}C]-galactose into normal and galactosemic cells was studied in order to determine whether an agent could be found which would specifically inhibit incorporation of radioactivity by the extraphosphorylation pathways, thereby producing differential inhibition of galactosemic and normal cells.

The product of the reductase reaction, galactitol, at 0.05*M*, had no effect on incorporation in either cell type. Other sugars and sugar derivatives were also tried at 0.05*M*. No effect on incorporation in either cell type was observed with the following compounds: mannitol, UDPG, *N*-acetylgalactosamine, *N*-acetylglucosamine, L-fucose, D-arabinose, cellobiose, and mannoheptulose. D-Galacturonic acid, D-galactosamine, and D-glucosamine were toxic at this concentration and caused the cells to become detached. Uridine diphosphogalactose and 2-deoxy-D-galactose were somewhat less toxic. Isotope incorporation was inhibited to about the same extent in both cell types by these latter and by maltose, L-xylose, D-xylose, galactose-1-phosphate, and galactose-6-phosphate. Incorporation was inhibited to a greater extent in galactosemic than in normal cells by D-glucose, D-mannose, D-fucose, D-tagatose, and D-galactono-γ-lactone. Of these, by far the most effective was the lactone. Galactosemic cells showed almost no incorporation while normal cells incorporated appreciable amounts of the label (Fig. 2). Galactonic acid was not available for testing.

The time course of incorporation of [1-^{3}H]galactose in the presence and absence of galactono-γ-lactone was followed (Table 1). Galactosemic cells exhibit about a 1½-hour lag before significant incorporation begins in the absence of lactone. This lag can be overcome by starving the cells for carbohydrate before exposing them to labeled galactose. Furthermore, the lactone does not appear to be effective in inhibiting galactosemic cells during the first 2 hours. If the lactone is added 1 hour before the radioactive galactose, its effect is more marked. In fact, with freshly prepared lactone and a 1-hour preliminary incubation, it is possible to reduce the number of silver grains in galactosemic cells to an average of 3 or 4, which is not demonstrably different from background. With the high-dry microscope objective, then, the difference between galactosemic and normal cells is essentially all or none.

Table 1 also shows that, for a 2-hour labeling in the absence of lactone, there is a significant difference between normal and galactosemic cells. However, this difference is smaller and depends in large part on the cells being well fed, so that use of this method as a test in the absence of lactone might tend to be less reliable.

The effect of the lactone in an overnight exposure to isotope and of a 2-hour exposure to [1-^{3}H]galactose alone was observed for four additional unstarved galactosemic and four additional nongalactosemic cell lines. Both methods proved effective in distinguishing the galactosemic lines. Figure 2 shows the radioautographs obtained with all ten lines.

One of these cell lines (CHP4) was of special interest because it arose from a patient who was asymptotic, and who produced almost normal amounts of $^{14}CO_2$ from intravenous administration of [1-^{14}C]galactose, but whose red cells exhibited the biochemical defect of galactosemia. His brother (whose cell line is CHP3) had both the typical syndrome and the biochemical defect (*13*). Fibroblasts from both brothers exhibited the behavior characteristic of galactosemic cells by the procedure described here.

The lactone is not effective if the *p*H of the medium is too high. The best results are obtained with freshly made lactone brought to a *p*H of about 7 with NaOH before addition to the medium. High *p*H may hydrolyze lactone to galactonic acid which should also be inhibitory. Possibly galactonic acid is not on the direct pathway of galactose utilization. It does not act as a substrate in the reverse reaction with galactose dehydrogenase to form galactose and nicotinamide adenine dinucleotide (NAD^+) although the lactone does (*2*).

The method described which distinguishes galactosemic cells from nongalactosemic cells is simple, rapid, reliable, and requires small numbers and very little manipulation of the cells. The method would appear to be adaptable for use in the detection of galactosemia in cultured cells from amniotic fluids.

Galactono-γ-lactone inhibits galactose utilization by galactosemic cells almost completely and by normal cells by about 50 percent. This provides support for, but does not establish, the dehydrogenation route as a major one for galactose metabolism. The lactone itself or a metabolic product could be inhibiting some other as yet undescribed enzyme system. Partial inhibition of normal cells by lactone is probably due to almost complete inhibition of the oxidative route with little or no inhibition of the phosphorylation route. Higher concentrations of lactone do almost completely inhibit incorporation in normal cells as well.

Of the tested compounds which had substrate activity in the galactose dehydrogenase system (*2*), 2-deoxy-D-galactose, D- and L-xylose, and maltose inhibited both galactosemic and normal cells to the same extent: *N*-acetylgalactosamine, D-arabinose, D-mannoheptulose, and cellobiose had no effect; and only D-tagatose had a differential effect. By contrast, of those compounds which had no substrate activity for galactose dehydrogenase, L-fucose had no effect in our system while D-mannose, D-glucose, and D-fucose showed a differential effect. Therefore, except for the lactone and possibly D-tagatose, there is no apparent correlation between our system and that of Cuatrecasas and Segal.

In the study of Petricciani *et al.* (*10*), glucose had a more inhibitory effect on the production of $^{14}CO_2$ by galactosemic cells as compared to normal cells. Since glucose also had a differential effect on incorporation of [1-^{3}H]galactose and [1-^{14}C]galactose in these studies, this supports the suggestion that $^{14}CO_2$ produced in their system was the result of turnover.

The use of lactone inhibition may

prove useful in the detection of enzymatic blocks in any of the first three steps of the phosphorylation pathway of galactose in a variety of cells. The rationale employed here of inhibiting alternate routes of metabolism in order to isolate the route of interest should be generally applicable to the study of other inborn errors of metabolism.

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Fluid Shifts Associated with Gas-Induced Osmosis

Abstract. *It has been proposed that equilibration of nitrous oxide with blood plasma increases osmotic pressure and thereby causes hemodilution. However, calculations show that the 250-torr osmotic gradient produced by 0.7 atmosphere of nitrous oxide dissolved in blood plasma (separated from the other body fluids by a membrane permeable only to water) would be eliminated by a water shift which would dilute the plasma by only 4 percent. Permeability of the membrane to nitrous oxide would further reduce the shift. In vivo measurements confirmed the smallness of any osmotically induced shifts by demonstrating no significant transient changes in hematocrit value when 0.7 atmosphere of nitrous oxide was added to or removed from an inhaled anesthetic mixture in man. These results cast doubt on the suggestions that gas-induced osmosis is an important factor in dysbarism or in clinical anesthesia.*

Kylstra *et al.* (*1*) proposed that exposure to hyperbaric breathing mixtures containing helium or nitrogen causes transient osmotic gradients in man during the period of uptake or output of these gases. Such gradients have been implicated as causing hemoconcentration, capillary stasis, and impaired exchange of gases in tissues (*1*); dry joints (*2, 3*); aseptic bone necrosis (*2*); and urticaria (*4*). Further, Hills (*2, 5*) has suggested that gas-induced osmosis is an initiating mechanism for anesthesia. Because of the high solubility of nitrous oxide relative to both nitrogen and helium, the increase in osmolarity of an aqueous phase caused by 1 atm of nitrous oxide is approximately equal to that caused by 30 atm of nitrogen or 50 atm of helium. Thus, it has been inferred that large osmotic gradients result from nitrous oxide administration during clinical anesthesia (*2*).

However, these suggestions appear to ignore the background of osmotic pressures due to proteins and salts in physiological fluids. The osmotic pressures due to dissolved gases are superimposed on this background and form only a small fraction of the total osmotic pressure. This is particularly important when fluid shifts are considered which would remove the potential osmotic gradients. We report here the calculation of such shifts and the in vivo measurement of transient changes in hematocrit value when 0.7 atm of nitrous oxide was added to or removed from an anesthetic mixture inhaled by man. We calculated a maximum theoretical 4 percent fluid shift in the blood plasma and we could not demonstrate any significant change in hematocrit value.

Table 1. Effect on hematocrit values of adding and of eliminating 70 percent nitrous oxide. The changes are given as mean percentages of the control hematocrit value; S.E.M., standard error of the mean.

Time (min)	Change (%) ± S.E.M.	
	N_2O added	N_2O removed
1	100 ± 0.6	100 ± 0.3
2	100.2 ± .5	100.4 ± .5
3	99.1 ± .4	101.1 ± .4
4	99.4 ± .3	99.7 ± .5
5	99.8 ± .4	99.3 ± .5
10	98.7 ± .6	98.3 ± .3
15	96.5 ± .9	99.1 ± .7

We calculated the theoretical maximum change in blood volume produced by solution of nitrous oxide at 0.7 atm, assuming a membrane between the cardiovascular system and the other body fluids permeable only to water. Since the solubilities of nitrous oxide in blood and in water are similar (*6*), we assumed that the osmolarity of nitrous oxide in blood equals its molarity. Therefore 0.7 atm of nitrous oxide increases the osmolarity by 13 milliosmoles per liter (which from the van't Hoff equation is equivalent to an osmotic pressure of 250 torr). Addition of 13 milliosmoles per liter to the 310 already present in plasma and other body fluids (*7*) produces only a 4.2 percent increase in osmolarity. Since plasma contains 7 percent of the total body water, a shift from the remaining water stores (interstitial and intracellular fluid) sufficient to increase the plasma volume by 4 percent eliminates the osmotic gradient between plasma and other body water. That is, a 4 percent increase in plasma water content, resulting from the transfer of water from interstitial and cell fluid, would eliminate any osmotic gradient induced by the presence of nitrous oxide in plasma. In this calculation we assume that no nitrous oxide diffuses (or leaks) from plasma to interstitial or cell fluid, and find the maximum transient fluid shift that could occur. However, since nitrous oxide has a high permeability, such diffusion does occur and thereby decreases the osmotic gradient and fluid shifts. The calculation of osmotic pressures across a "leaky membrane" involves a correction factor in the van't Hoff equation called the Staverman reflection coefficient, σ ($0 \leq \sigma \leq 1$) (*8*). In the above calculation we have assumed that σ equals 1 for both nitrous oxide and the solutes in the blood. Hills (*2*) found that for nitrous oxide σ is less than .04, which indicates a 25-fold