taken for a measurement of radioactivity, and another of 0.1 ml was taken for bioassay. Results are shown in Fig. 2. It is probable that methionine is a methyl donor in 1-methyladenine production, since the elution pattern of the radioactivity was in good agreement with those of the biological activity and absorbance at 260 nm of 1-methyladenine. The amount of 1-methyladenine was very reliably determined by the bioassay; this assay indicated that the MIS produced in the ovary in the presence of GSS and methionine was 1-methyladenine.

The remaining samples of the biologically active fractions were pooled, concentrated to about 1 ml, and applied to a Sephadex G-15 column (1.37 by 39 cm) equilibrated with 0.2*M* pyridine acetate buffer (*p*H 8.4), which was used as eluant. The flow rate was 40 ml/hour and the fraction size was 3 ml. The remainders of fractions with high radioactivity (a 0.5-ml sample from each fraction had been used to determine the radioactivity) were pooled, concentrated to dryness, dissolved in a small amount of deionized water, and applied to a thin-layer chromatographic plate of microcrystalline cellulose (*12*). The plate was developed with isopropanol : hydrochloric acid : water (65 : 16.7 : 18.3, by volume). A single spot was detected by an ultraviolet light lamp. Both the radioactivity, determined by a gas-flow thin-layer chromatography scanner (*13*), and the maturation-inducing activity were confined to this spot. From these experiments it is clear that the methyl radical of L-methionine is incorporated into 1-methyladenine synthesized in the starfish ovary under the influence of the gonad-stimulating hormonal peptide, GSS.

The precise biochemical pathway of 1-methyladenine synthesis in the starfish ovary is not known. Although 1-methyladenine is a minor base in nucleic acids, especially some transfer RNA's, the 1-methyladenine produced under the influence of GSS is not a breakdown product of nucleic acids but is newly synthesized (*14*). The fact that isolated ovary can produce 1-methyladenine from 1-methyladenosine by the action of 1-methyladenosine ribohydrolase (*15*) suggests that 1-methyladenosine is an immediate precursor of 1-methyladenine. 1-Methyladenosine monophosphate (1-methyl AMP) also served as a precursor of 1-methyladenine in isolated ovary (*14*). However, the production of 1-methyladenine from either 1-methyladenosine or 1-methyl AMP is unaffected by the presence of GSS.

On the other hand, the results we report clearly show that methionine enhances the production of 1-methyladenine in the presence of GSS. In the absence of GSS, production of 1-methyladenine is not detectable. These facts suggest that the hormonal peptide GSS, which is released from nervous tissue, stimulates methylation of the N-1 position of the purine nucleus of a precursor of 1-methyladenine. Neither the enzyme involved in this methylation nor the acceptor of the methyl radical is yet known.

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8. Wako Junyaku Co.
9. The GSS-water solution was prepared as follows: lyophilized radial nerve material was homogenized in deionized water (40 mg of nerve material per milliliter) and centrifuged for 1 hour at 30,000*g*. The supernatant was diluted with deionized water to an appropriate concentration.
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Galactose Utilization in Galactosemia

Abstract. *Cultures of human galactosemic fibroblasts without detectable transferase activity were able to convert [1-^{14}C]galactose to $^{14}CO_2$ to the same extent as normal cells, but did so at a significantly slower rate. The utilization of galactose in both normal and galactosemic cells was strongly inhibited by glucose at physiologic concentrations.*

The defect in galactosemia is generally accepted as an absence of activity of the enzyme galactose-1-phosphate uridyltransferase (transferase) (*1*). More recently it has been shown that a protein, immunologically identical with active transferase, is produced in cells derived from galactosemic patients; this finding suggests that the basic defect is a mutation in the structural gene which then results in an inactive enzyme (*2*).

A number of investigators have studied galactose metabolism in galactosemia both in vivo and in vitro (*3, 4*), and some rapid screening procedures for transferase activity deficiency have been based on the demonstration in vitro of little or no $^{14}CO_2$ production from [1-^{14}C]galactose by fibroblasts, leukocytes, or whole blood of galactosemics (*5, 6*). Other reports, however, have shown that [1-^{14}C]galactose can be converted to $^{14}CO_2$ in vivo by galactosemic patients and that the ability to metabolize galactose is greatly stimulated by progesterone (*7*). In addition, the presence of low levels of transferase activity in some galactosemic fibroblasts has been described (*8*). We now report that fibroblast cultures derived from a galactosemic patient which have no detectable transferase activity when assayed with a radioactive substrate (*9*) can convert [1-^{14}C]galactose to $^{14}CO_2$, and that glucose inhibits galactose utilization in this system.

A diploid cell line, CCL-72, from a human galactosemic patient was obtained from the American Type Culture Collection, Rockville, Maryland, and the normal human diploid cell line, WI-38, was obtained from HEM Research, Inc., Rockville, Maryland. Cells were grown in monolayer culture to con-

fluency with medium MAB 87/3 (*10*) supplemented with 5 percent fetal bovine serum and penicillin (100 unit/ml); they were then maintained in the stationary phase by replacing the growth medium with MAB 87/3 without serum. Glass screw-top tubes containing approximately 0.2 × 10^6 cells in the stationary phase were used in all the $^{14}CO_2$ studies.

Normal human fibroblasts, WI-38, were capable of metabolizing galactose to CO_2 in the presence or absence of glucose, although a significant depression of galactose utilization was noted when glucose was present at a concentration 1000-fold higher (5 μmole/ml) than that of galactose (Fig. 1).

The galactosemic cells, CCL-72, also metabolized galactose to CO_2 to the same extent as normal cells but did so at a much slower rate (Fig. 2), and glucose caused severe depression of galactose utilization when present at a concentration 1000-fold higher than that of galactose. Very little CO_2 was produced by galactosemic cells during the first 24 hours, whereas normal cells produced easily detectable amounts within the first hour. Results similar to those for CCL-72 cells were obtained with another galactosemic cell line, JDU (*5, 6*).

The inhibitory effect of glucose on galactose utilization in both the normal and transferase-deficient cells was studied further by using thin-layer chromatography on lysates of cells incubated with [1-^{14}C]galactose in the presence or absence of glucose. Cells were treated as described for the $^{14}CO_2$ experiments, except that plastic 250-ml Falcon flasks containing about 2 × 10^6 cells in the stationary phase were used, and the incubation period was 20 hours.

As was expected, there was an accumulation of galactose-1-phosphate (Gal-1-P) by the galactosemic cells exposed to galactose while normal cells showed no Gal-1-P accumulation. The presence of glucose at 1000-fold higher concentration (5 μmole/ml) than galactose resulted in an 87 percent reduction in the accumulation of Gal-1-P in the galactosemic cells when compared with replicate cultures without added glucose.

The inhibition of galactose utilization by glucose may involve competition for a shared hexose-uptake system, as has been suggested for L cells and other mammalian cells (*11*). The lack of accumulation of Gal-1-P in galactosemic cells incubated with galactose and excess glucose is consistent with such a process, since phosphorylation of glucose and galactose involve different systems, and no competition by glucose has been demonstrated in the galactokinase reaction (*12*).

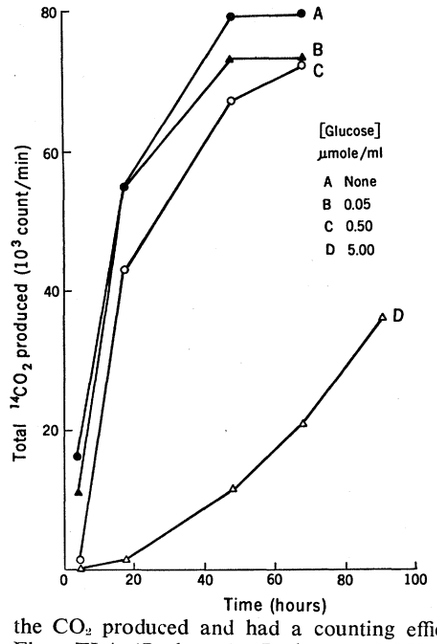

Fig. 1. Production of $^{14}CO_2$ in human cell line WI-38 incubated with [1-^{14}C]galactose and unlabeled glucose. Replicate tubes of cells in the stationary phase were rinsed three times with 2 ml of phosphate-buffered saline before the addition of 1.0 ml of medium MAB 87/3 modified to contain [1-^{14}C]galactose (5 nmole/ml; 48.1 mc/mmole; New England Nuclear) but without serum, glucose, or antibiotics. At time 0 various amounts of unlabeled glucose were added to appropriate tubes which were then sealed with rubber stoppers (Neogrene) and incubated at 37°C. Serial samples of the gas contained in each tube were obtained through a 25-gauge needle puncture of the stopper and removing 1.0 cm^3 of gas anaerobically in a glass syringe with the plunger coated with paraffin oil to prevent leaking. Then 1.0 cm^3 of air was added to the flask to maintain normal pressure. The collected gas was passed through a column CO_2 trap containing 0.48 ml of phenethylamine and 1 g of Chromosorb W (*13*). This system was capable of trapping 99.0 percent of the CO_2 produced and had a counting efficiency of 65 percent in 10 ml of toluene–Fluor TLA (Beckman) (Beckman LS 250 scintillation counter).

The production of $^{14}CO_2$ and the identification of Gal-1-P by chromatography depend on the purity of the [1-^{14}C]galactose used, since volatile ^{14}C products, other hexose contaminants, or radiodecomposition products could give spurious results. There was no measurable radioactive volatile product from the lot of galactose used in this study which could be trapped in the phenethylamine column (*13*) even after incubation for 6 days at 37°C in medium MAB 87/3. Thin-layer chromatography on cellulose with a system consisting of ethyl acetate, pyridine, and H_2O (2:1:2) revealed no hexose other than galactose, and no significant radioactivity in other areas of the chromatogram. That the ability to distinguish transferase-deficient cells from normal cells (Figs. 1 and 2) is most probably based on galactose utilization and not other [^{14}C]hexose contaminants or one- and two-carbon radioactive decomposition products of [1-^{14}C]galactose is supported by the fact that the addition of unlabeled galactose (11 μmole/ml) to the medium effectively stopped $^{14}CO_2$ liberation in both normal and galactosemic cell cultures.

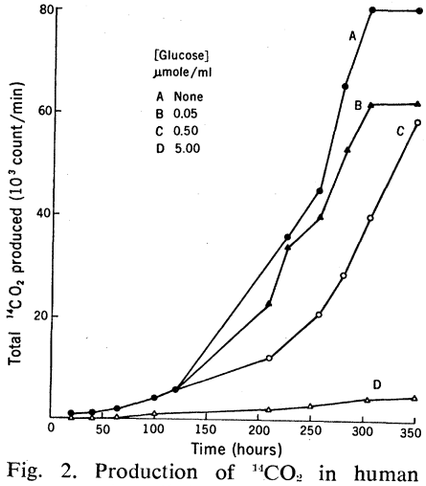

Fig. 2. Production of $^{14}CO_2$ in human galactosemic cell line CCL-72 incubated with [1-^{14}C]galactose and unlabeled glucose. Conditions are as described in the legend of Fig. 1.

The possibility that contaminating organisms were present and utilizing the galactose is remote because no organisms were detected in cultures of the spent medium in thioglycollate and Sabouraud's media and the cells were periodically shown to be free of mycoplasma.

The utilization of galactose by the galactosemic cell line, CCL-72, is consistent with observations that (i) liver cells from one galactosemic patient were found to catabolize [1-^{14}C]galactose with an efficiency of 75 percent that of the normal controls (*14*), and (ii) some galactosemic individuals are able to convert the carbon-1 of intravenously administered galactose into CO_2 (*7*). On the other hand, Krooth and Wein-

berg (*5*) were unable to demonstrate conversion of [1-^{14}C]galactose to $^{14}CO_2$ by galactosemic fibroblasts under conditions where normal cells performed this conversion. However, the incubation period in their system was only 90 minutes, and there was an excess of glucose present (*4*) which would have effectively inhibited any galactose utilization.

Recent studies (*15*) have shown that the rate and total amount of $^{14}CO_2$ produced by cells in culture is a function of the extracellular concentration of [1-^{14}C]galactose, and that the difference in the extent of conversion of galactose to CO_2 between galactosemic and normal cells disappears as the concentration of galactose is reduced to those used in our experiments. This suggests that alternate metabolic pathways for galactose which lead to CO_2 production are present in galactosemic cells, but such alternate pathways become saturated with galactose at much lower levels than occur in the normal system. Of the three alternate pathways for galactose utilization that have been proposed and investigated (*3, 16*), the only route leading to CO_2 production for which there is substantial evidence is through the pyrophosphorylase reaction. Support for an alternate metabolic pathway beyond the galactokinase reaction which would lead to CO_2 production comes from an experiment in which we incubated human galactokinase-deficient cells (KIN) (*17*) with [1-^{14}C]galactose and in which we could not detect any $^{14}CO_2$ production. This finding is consistent with the absence of [1-^{14}C]galactose decomposition products giving rise to the $^{14}CO_2$ results in the transferase-deficient cells; it also suggests that the pyrophosphorylase reaction is the only significant one of the three proposed alternate pathways for galactose utilization.

The demonstration of significant galactose metabolism by galactosemic cells in culture indicates that effective alternate metabolic pathways that do not involve dead-end products are operative in vitro and may be functional in this disease in vivo as well. The inhibitory effect of glucose on galactose utilization in vitro suggests that glucose may play a protective role in galactosemia in vivo and is consistent with observations that galactosemic cells have nearly normal growth characteristics in medium containing 95 mg of galactose and 5 mg of glucose per 100 ml of cell culture medium (*5, 6*).

In addition to the activation or stabilization of a malfunctioning enzyme (*2*), two additional indirect approaches to therapy in genetic diseases might be considered: (i) to activate or make more efficient any alternate metabolic pathways that exist; and (ii) to inhibit utilization of the defective pathway if it leads to the accumulation of cytotoxic products. On the basis of the findings reported here, it should now be possible to explore each of these alternatives in galactosemia.

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Axons: Isolation from Mammalian Central Nervous System

Abstract. *Centrifugation of a homogenate of white matter, in a solution of buffered sucrose containing salt, produces a floating layer of myelinated axons. When these are suspended in hypotonic buffer, the myelin swells and strips away from the axon. Axons are then separated from the myelin by centrifugation. The resulting preparation consists of a variable population of processes with lengths up to 200 micrometers and diameters between 0.3 and 5.0 micrometers. The axons contain neurofilaments and mitochondria, although no axolemma or neurotubules are evident. The preparation contains cerebroside and sulfatide, yet is essentially free of myelin.*

Biochemical characterization of axons has been limited to large, and easily dissected, fibers (*1*). Fractions that are enriched in nonmyelinated axons have been isolated but they have been studied morphologically only (*2*). We now report the details of our method for the isolation of axons from the mammalian central nervous system in quantities that are sufficient for biochemical characterization (*3*).

All operations were carried out at 0° to 4°C. Brainstem, or centrum semiovale, from fresh bovine brain was finely minced and homogenized in 100 volumes of medium A (0.85*M* sucrose, 0.10*M* NaCl, and 0.05*M* potassium phosphate buffer; *p*H 6.0) with six strokes of a loose pestle and four strokes of a tight pestle in a Dounce homogenizer (Kontes). Centrifugation of this homogenate at 7000 rev/min (9500*g*) for 30 minutes in the 150-ml tubes of the Sorvall HS-4 rotor produces a white floating layer and a pellet; the pellet is discarded. The floating layer from each tube is suspended, with the use of the homogenizer, in 60 ml of medium A, and centrifuged at 25,000 rev/min (75,500*g*) for 15 minutes (Spinco SW 25.2 rotor). The floating layer obtained by this procedure is again suspended in medium A and centrifuged as before to ensure more complete removal of any trapped nuclei and capillaries.

The final floating layer, containing myelinated axons and myelin, is then suspended in 0.05*M* potassium phosphate buffer, *p*H 6.0 (5 ml of buffer