GALACTOKINASE MUTANTS OF CHINESE HAMSTER SOMATIC CELLS RESISTANT TO 2-DEOXYGALACTOSE

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ABSTRACT

The growth of Chinese hamster somatic cells was inhibited by 0.2 mg/cc of 2-deoxygalactose. Mutants partially or fully resistant to 2-deoxygalactose were isolated in a single-step or two-step selection. Some of them did not grow as well as the wild type; one of them which lacked galactokinase (EC.2.7.1.6) activity did not grow at all in galactose medium. The galactokinase kinetic properties (Vmax & Kmax) of the other mutants and of the wild type were different. Therefore resistance resulted either from the possible absence of galactokinase synthesis or from a structural mutation, possibly a missence mutation, in the galactokinase gene.—A simple diagnostic test for juvenile cataract is proposed.

WE have isolated and characterized Chinese hamster somatic cell mutants resistant to 2-deoxygalactose and defective for galactokinase (EC.2.7.1.6) to study gene regulation of eukaryotes. AMES *et al.* (1972), ALPER and AMES (1975) and WILSON (private communication) have already isolated galactose$^-$ mutants of *Salmonella typhimurium* and *E. coli* that are resistant to 2-deoxygalactose. Although not known, it is possible that the 2-deoxygalactose phosphorylated derivatives were poisonous for these organisms. Similarly, mutagenized Chinese hamster somatic cells were therefore grown in the presence of 2-deoxygalactose. Survivors were isolated as fully or partially resistant to 2-deoxygalactose. Some of them did not grow as well as the wild type and one of them which lack galactokinase activity did not grow at all in galactose medium. The galactokinase kinetic properties (Vmax & Kmax) of the other mutants and the wild type were different. Therefore resistance resulted either from the possible absence of galactokinase synthesis or from a structural mutation, possibly a missence mutation, in the galactokinase gene.

These mutants, defective for the enzyme galactokinase which catalyzes the reaction galactose + ATP → ADP + galactose-1-phosphate are important for somatic cell genetics and the elucidation of the galactose pathways in mammalian cells (HILL and PUCK 1973; FRIEDMAN, YARKIN and MERRIL 1975).

MATERIALS AND METHODS

Cells, media and culture conditions:

Chinese hamster cells V79 were cloned three times in succession. V6, one of the clones, was kept. Cells from frozen stocks of V6 and the mutants were grown in air-CO_2 incubators at 37°

in petri dishes or in glass bottles. Dulbecco's medium was made as described by SMITH *et al.* (1960), but with 1 mg of glucose/cc or 1 mg of galactose/cc instead of 4.5 mg of glucose/cc, 8% fetal calf serum and without antibiotics. Dialyzed fetal calf serum was prepared at 4° by dialyzing one volume of fetal calf serum against 30 volumes of phosphate buffered saline (PBS) (DULBECCO and VOGT 1954) and by four successive replacements, at eight-hour intervals, of the PBS solution by a fresh solution. Growth curves were determined in Dulbecco's medium with glucose or galactose and 10% dialyzed fetal calf serum.

A line of A13, resistant to 8-azaguanine was derived from a mutagenized population of V6. The resistant line grew in 30 μg/ml of 8-azaguanine. It did not grow in HAT medium (5.5 μM amethopterin, 16 μM thymidine, 0.1 mM hypoxanthine, SZYBALSKI, SZYBALSKA and RAGNI 1962) and did not incorporate ^{14}C-hypoxanthine *in vivo*. Extracts of A13 lacked detectable hypoxanthine guanine phosphoribosyl transferase activity (EC.2.4.2.8) (SHARP, CAPECCHI and CAPPECHI 1973).

2-deoxygalactose resistance test:

The dose responses of wild-type or mutant cells were determined by plating and cloning cells in 6 or 10 cm petri dishes at different cell concentrations and with different 2-deoxygalactose concentrations. After 8 to 16 days, the dishes were washed with PBS and fixed with methanol. The clones were stained with a 1:10 dilution of Giemsa in water and counted. The 2-deoxygalactose response of the strains was defined as the level of 2-deoxygalactose required for 50% inhibition of clone formation after 10 to 16 days and designated as R50. Sensitive, partially resistant and resistant strains were defined as strains whose R50 were greater than 0.1 mg/cc, 0.5 mg/cc and 2 mg/cc of 2-deoxygalactose respectively.

Mutagenesis and selection of fully resistant mutants:

About 5×10^6 cells were mutagenized for four hours in 10 ml of medium, 8% fetal calf serum with 10 μl of ethyl methanesulfonate (Eastman Kodak, lot no. A2A) which induces mainly base pair transitions and other DNA changes (see review by FREEZE 1971, MCCANN *et al.* 1975). About 20% of the cells survived. After mutagenesis, the cells were washed once with 10 ml of PBS, grown for 2 days to allow the mutations to be expressed. About 2×10^5 cells were then seeded in 10 cm petri dishes, with different 2-deoxygalactose concentrations (4 to 8 mg/cc). After 14 to 19 days the survivors were picked, tested for 2-deoxygalactose resistance, cloned twice at low cell density (5 to 10 cells/plate) in the presence of 5 mg/cc of 2-deoxygalactose, and stored in liquid nitrogen.

Enzyme assay and preparation of cellular extract:

Except when otherwise indicated, extracts of cells were prepared at 25° as follows: the medium of exponential phase or confluent cell layers (about 3×10^6 to 2×10^8 cells) was removed. The cells were washed twice with 10 ml of PBS without $CaCl_2$ and incubated for one minute in 5 ml of PBS without $CaCl_2$ and $MgCl_2$ but with 1.25 g/l of trypsin and 0.2 g/l of EDTA. The trypsin solution was then removed. The cells were detached by incubation for 10 min with 1 ml of PBS without $CaCl_2$. They were centrifuged twice in 7 ml of PBS without $CaCl_2$ to remove most of the trypsin, resuspended in 0.5 ml of a 5 mM $MgSO_4$, 5 mM Mg-EDTA (Mg-titriplex from Merk), 2 mM dithiothreitol, 50 mM Tris-HCl (pH 7.5) buffer and disrupted for 5 sec at 0° with an ultrasonic sonicator from Artex (Farmingdale, N.Y. 11735). Cell debris was removed at 4° by centrifugation in an RC-3 Sorval centrifuge with the HG-4 rotor at 7,000 g for 10 min. To determine the kinetic properties (Vmax & Kmax), 50 μl of cell extract (10 to 150 μg of protein) were incubated at 37° for different lengths of time (0 to 1 hour) with 25 μl of different ^{14}C-galactose (0.1 μC) or ^{3}H-2-deoxygalactose (0.5 μC) concentrations, 20 μl of 0.02M ATP and 5 μl of water. The enzymatic reaction was linear until at least 20% of substrate was consumed. Galactose was separated from galactose-1-phosphate by electrophoresis on Whatman 3MM paper in a 5% acetic acid-water solution (v/v) adjusted to pH 3.5 with ammonium hydroxide. The electrophoresis was carried on for 20 minutes at 50 volts/cm. Galactose remained at the origin while galactose-1-phosphate migrated between xylene cyanol blue FF and orange G,

two colour markers from BDH Co. The radioactive spots were detected on a Packard 7201 radiochromatogram strip scanner. They were cut and counted in a liquid scintillation counter.

Specificity of the enzymatic reaction and identification of the reaction product:

The enzyme specificity was determined using a chromatographic system (DIEDRICH and ANDERSON 1961). A wild-type cell extract (3.5 mg of protein) was incubated with ^{14}C-galactose and ATP as described above. After one hour, at 37° about 35% of the ^{14}C-galactose was converted into a product which migrated by electrophoresis at pH 3.5 between xylene cyanol blue FF and orange G. This product was eluted with 0.1 cc of water, mixed with 15 μmoles of galactose, 15 μmoles of glucose-1-phosphate, 30 μmoles of galactose-1-phosphate and 20 μmoles of galactose-6-phosphate, loaded on a Dowex-1×8 column and chromatographed (DIEDRICH and ANDERSON 1961). Both the radioactivity and the carbohydrate elution patterns (Figure 1) were compared. They showed (1) that galactose-1-phosphate and not galactose-6-phosphate was the reaction product (2) that the ^{14}C-galactose commercial solution was most probably free of ^{14}C-mannose or ^{14}C-fructose (SRIVASTAVA and BEUTLER 1969) since none of their phosphorylated derivatives were detected in the column eluant.

Column chromatography on Sephadex G-100:

A 0.9 × 24 cm column was packed with Sephadex G-100, medium grade and equilibrated with buffer A (0.5 mM EDTA, 7 mM dithiothreitol, 10 mM potassium phosphate, pH 7.2). Cell extracts were prepared as described above, made 2% in glycerol, loaded on a Sephadex G-100 column and eluted at a flow rate of 5 ml/hr with buffer A. The elution volumes for blue Dextran 2000, bovine serum albumin (68,000 MW), galactokinase and cytochrome C (12,500 MW) were 6.5, 9, 10.75 and 14 ml respectively. The enzymatic activities were determined with fifty μl of each fraction which were mixed with 50μl of 7 mM dithiothreitol, 0.5 mM EDTA, 10 mM $MgCl_2$ and either 0.25 mM ^{14}C-galactose (0.025 μCi) or 2 mM ^{3}H-deoxygalactose (0.5 μCi) and incubated for 1 hour at 37°. The reaction was stopped at 0°. Fifty μl of the reaction mixture were applied on a dried Whatman DEAE-81 cellulose paper disc, 2.3 cm in diameter, previously washed with 5.5 mM galactose. The discs were dried quickly under an infrared lamp, washed slowly with 30 ml of a 1 mM $NaHCO_3$, 5.5 mM galactose solution, then with 3 ml of 95% ethanol, dried and counted in a scintillation counter.

Measurement of radioactivity and absorbance:

The radioactivity of the dried papers was measured in 5 ml of toluene with 8.25 g/l of 2,5 diphenyloxazole and 0.25 g/l of 1,4-bis-2 (5 phenyloxazole)-benzene in a scintillation counter. Absorbance was measured at 280 mμ or 440 mμ in a Gilford recording spectrophotometer.

Protein determination:

Protein determination was as described (LOWRY *et al* 1951) with bovine serum albumin as a standard.

RESULTS

Isolation and classification of 2-deoxygalactose resistant Chinese hamster cell mutants:

We performed preliminary experiments on growth and plating to find the best conditions for the selection of mutants. Table 1 shows that 2-deoxygalactose inhibits the growth of the wild-type cells. The addition of galactose to the medium reduces that inhibition as if 2-deoxygalactose, galactose and possibly their derivatives are the substrates of the same enzymes of the galactose pathway or followed the same permeation routes. Galactosamine is less efficient than 2-deoxygalactose in growth inhibition and was not used any further. Cells are also killed more

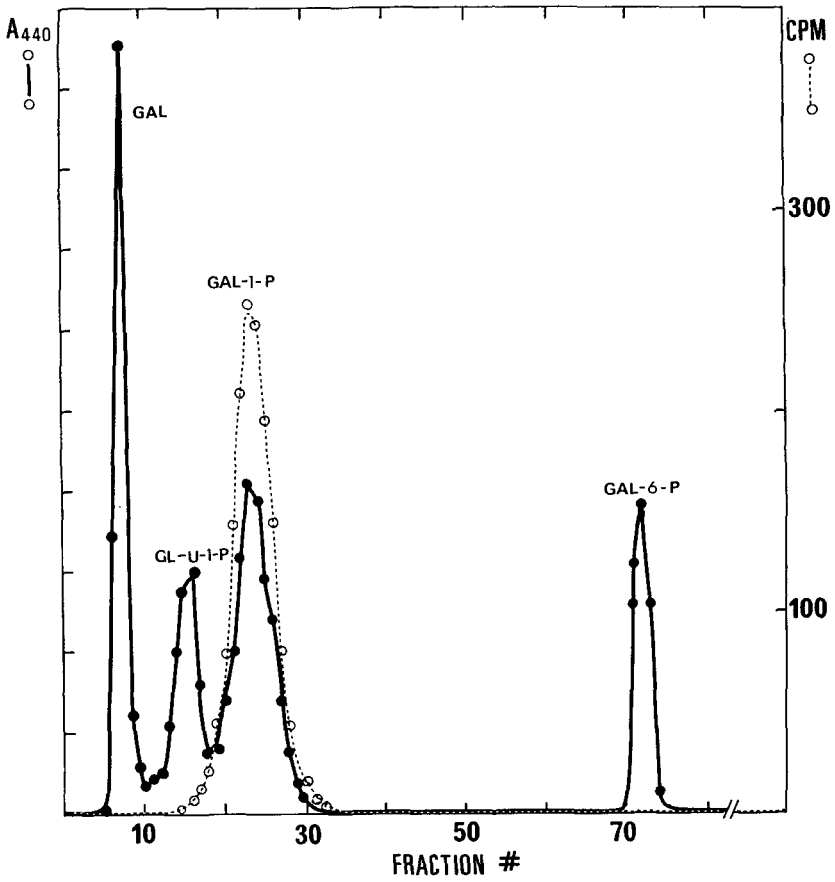

FIGURE 1.—*Column chromatography of the enzymatic reaction product.* Chromatography of the reaction product on a 0.7 × 12 cm Dowex-1×8 column. (200–400 mesh borate form). The column was washed first with 200 ml of 0.1M $Na_2B_4O_7$ (pH 9.3) to elute galactose (peak 1), glucose-1-phosphate (peak 2), galactose-1-phosphate (peak 3). Then a linear pH gradient of pH 9.3 to pH 8.5 of 0.1M $Na_2B_4O_7$ (200 ml total) was used to elute galactose-6-phosphate (peak 4). Finally the column was washed with 200 ml of 0.1M $Na_2B_4O_7$, pH 8.5. A flow rate of 20 ml/hr was maintained throughout. Fractions of 5 ml were collected. (O—O) C^{14}-radioactivity. About 1 ml of each column fraction was mixed with 1.6 ml of Triton X-100, 3.2 ml of toluene with 8.25 g/l of diphenyloxazole and 0.25 g/l of 1,4-bis-2 (5-phenyloxazole)-benzene and counted in a scintillation counter. (●—●) O.D. at 440 mμ representing the amount of carbohydrate as detected wtih cystein and sulfuric acid (DIEDRICH and ANDERSON 1961).

efficiently in Dulbecco's medium with 1 mg/cc of glucose instead of 4.5 mg/cc (data not shown).

Figure 2 shows the plating efficiency curves against drug concentrations for the mutagenized and nonmutagenized wild-type cells. Suggestive of a genetic modification is the increase of the number of resistant colonies with mutagenic treatment.

TABLE 1

Plating efficiency of wild type cells in the presence of different concentrations of galactose and 2-deoxygalactose

2-deoxygalactose	Sugar concentrations (mg/cc) in the medium galactose					
	0	0.125	0.25	0.75	2.5	10
0	138	125	135	114	107	82
0.25	0	31	79	99	93	82
0.5	0	16	59	90	54	74
1.25	0	4	23	64	—	80
2.5	0	0	8	32	55	55

About 170 wild-type cells were plated in 6 cm diameter petri dishes in Dulbecco's medium with 8% fetal calf serum, with 1 mg/cc of glucose and with different concentrations of galactose and 2-deoxygalactose as indicated. The plates were incubated until the clones were big enough (8–16 days), to be stained with Giemsa and enumerated.

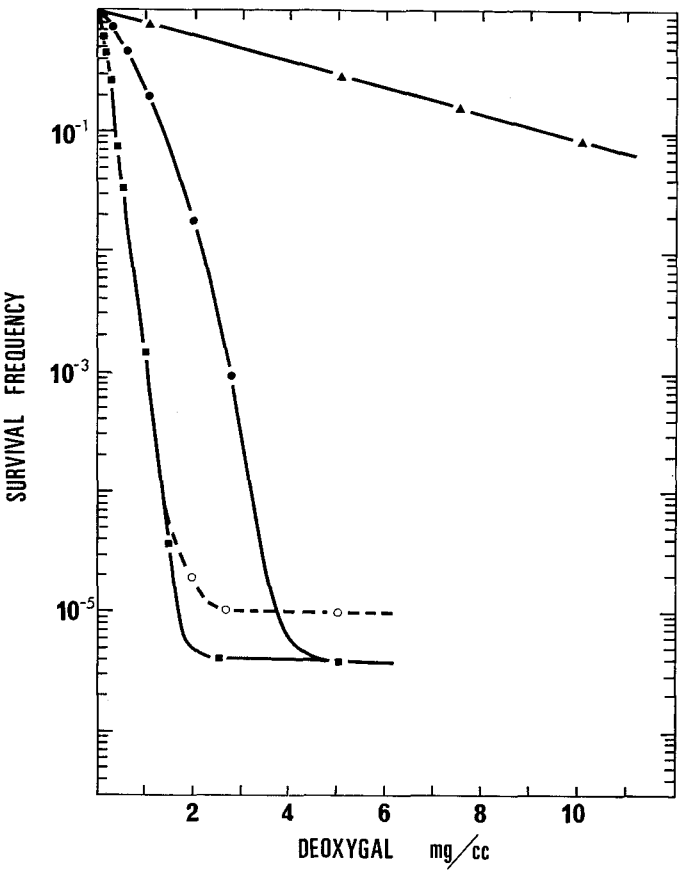

FIGURE 2.—Plating efficiency of wild type cells (■–■), wild type mutagenized cells (○—○), 2-deoxygalactose partially resistant DR2 subline (●–●) and DR2R7 subline (▲–▲), all plated in the presence of 2-deoxygalactose at the concentrations shown.

Mutants partially resistant to 2-deoxygalactose were selected among mutagenized A13 cells grown for 4 weeks in the presence of 1 mg/cc of 2-deoxygalactose. Killing was very slow. After 4 weeks, the survivors were then seeded at a concentration of 10^2 to 10^3 cells/petri dish in the presence of 1 mg/cc of 2-deoxygalactose. Two weeks later one or two clones appeared in each petri dish. Since they were not independent, only one clone DR2 was studied in detail. DR2 was purified by cloning twice in 0.5 mg/cc of 2-deoxygalactose. Figure 2 shows the inactivation curve of DR2. The R50 (see 2-deoxygalactose resistance test) of DR2 is about 0.5 mg of 2-deoxygalactose/cc while the R50 of the wild type is only 0.1 mg of 2-deoxygalactose/cc.

Twenty-four fully resistant, non-independent mutants were selected among mutagenized DR2 cells plated in different 2-deoxygalactose concentrations (4 to 8 mg/cc). Three of them DR2R7, DR2R22, DR2R1610 were studied in detail. Within the experimental errors, their inactivation curves were not significantly different from one another (Figure 2). Their R50 were about 2.5 mg/of 2-deoxygalactose/cc.

Clone DR11 was isolated in a single-step selection among mutagenized A13 cells plated in 5 mg/cc of 2-deoxygalactose. Its R50 and its inactivation curve were not different from those of DR2R7.

Growth curves of the mutants and the wild type in the presence of glucose or galactose:

Some Chinese hamster cell lines have the characteristic of growing and cloning in Dulbecco's medium with galactose instead of glucose (THIRION 1972). Figure 3 shows the growth curves of the wild type and the mutants in glucose or galactose medium. The wild type and the mutants grow equally well in glucose with a generation time of about 18 hours. Mutants DR2 and DR11 grow as fast as wild type in galactose with a generation time of 26–28 hours. Mutants DR2R7 and DR2R22 grow in galactose with a generation time of 36 and 44 hours, respectively, while mutant DR2R1610 does not grow at all in galactose. These data indicate that 2-dexoygalactose resistance mutations are specific for the enzymes of the galactose pathway since their growth patterns are altered in galactose but not in glucose medium.

Galactokinase activities in cell free extracts:

The growth curves in galactose medium were correlated with the galactokinase activities of the cell-free crude extracts. The fastest growing cells had the highest galactokinase specific activity. Table 2 shows values ranging from about 50% of the wild type for mutants DR11 and DR2 to no detectable activity for mutant DR2R1610. The enzyme activities of the cellular extract of the mutants, of the wild type and a mixture of both the wild type and each mutant showed that these changes of specific activity did not result from the presence of inhibitors in the mutants.

To determine whether the resistance to 2-deoxygalactose was due to an altered form of galactokinase, the Kmax was determined with crude cell extracts for the

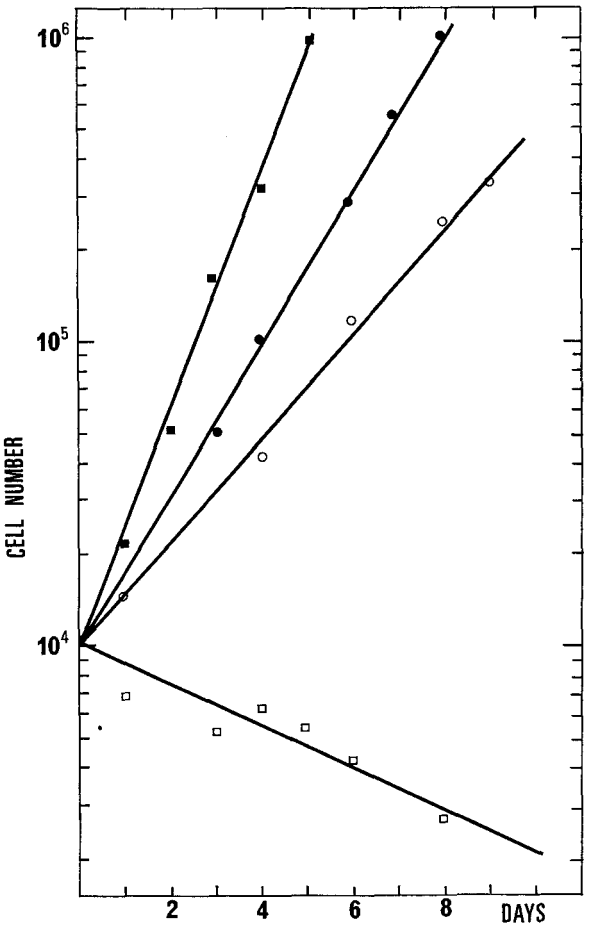

FIGURE 3.—Growth curves of wild type cells in glucose (■-■), of wild type cells in galactose (●-●); of DR2R22 cells in galactose (○-○) and of DR2R1610 in galactose (□-□).

TABLE 2

*Specific activity and kmax of the wild type and mutant strains**

Strains	Substrates: Galactose Kmax (mM)	Galactose Specific activity (nmole/min/mg of protein)	2-deoxygalactose Kmax (mM)	2-deoxygalactose Specific activity (nmole/min/mg of protein)
V6	0.040	4.1	1.1	20.
DR2	0.04	2.6	1.70	14.0
DR2R7	0.030	1.2	0.4	2.8
DR2R22	0.085	0.65	0.75	6.0
DR2R1610	—	<0.1	—	<0.1
DR11	0.06	2.3	1.30	16.

* The values in the table are the average of at least 3 experiments. The relative error was 10%.

wild type and the mutants with 2-deoxygalactose and galactose as substrates. Table 2 shows that except for DR11, the Vmax and possibly the Kmax of the mutant enzymes are different from those of the wild type. This suggests that DR2, DR2R7 and DR2R22 are probably mutated in their galactokinase structural gene and that the mutations are not regulatory mutations. The kinetics properties of DR11 and the wild type are not different enough to conclude that DR11 is a galactokinase mutant for sure. Further work is needed to show that DR11 is not for instance a transport mutant.

Gel chromatography on Sephadex G-100:

Figure 4 shows the elution patterns of the wild-type and mutant cell-free extracts as analyzed by column chromatography on Sephadex G-100. Except for mutant DR2R1610 where no activity is detectable, the two galactokinase activities with galactose and 2-deoxygalactose as substrates, chromatograph together for the wild type and the mutants. These results suggest (1) the presence of one enzyme with 2 substrates and (2) about the same molecular weight of about 60,000 daltons for the mutant and the wild-type enzymes. The mutant polypeptides are probably not grossly shorter or longer than the wild-type polypeptide.

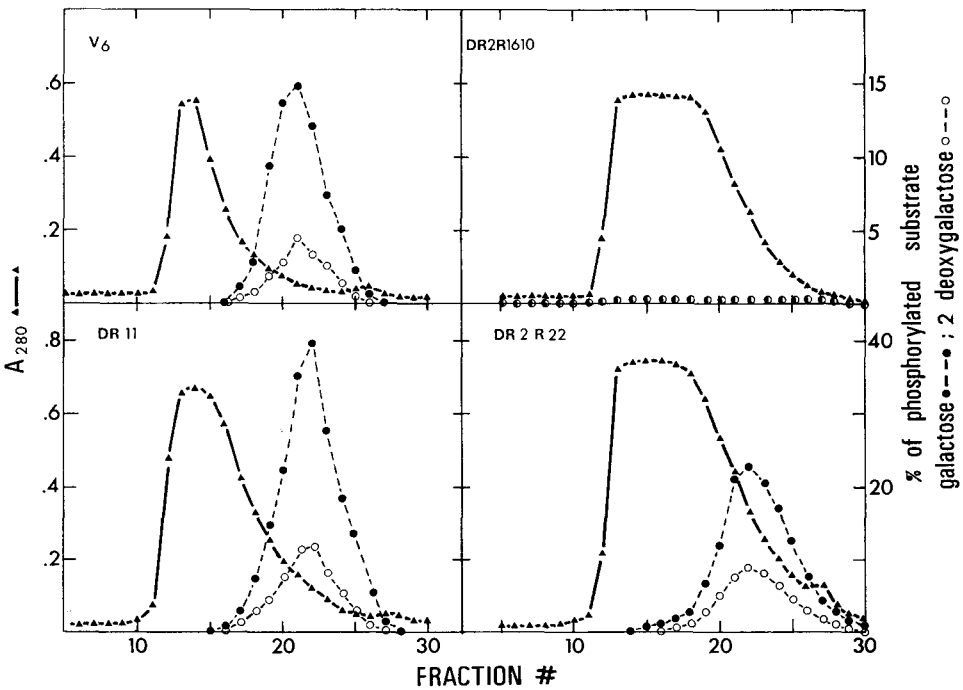

FIGURE 4.—*Column chromatography on Sephadex G-100.* Chromatography of 0.25 ml of cell extracts of V6 (0.52 mg of protein), DR11 (1.18 mg of protein), DR2R22 (2.56 mg of protein), DR2R1610 (4.26 mg of protein) on Sephadex G-100. Fractions of 0.5 ml were collected. (-▲-) optical density at 280 mμ. (-●-) ^{14}C-radioactivity. (-○-) ^{3}H-radioactivity.

Phenotypic stability:

Finally, the partially and fully resistant mutants were cloned and grown in the absence of 2-deoxygalactose for about 70 generations. They were found to retain their 2-deoxygalactose-resistant phenotype, galactokinase specific activity and ability or inability to grow in the presence of galactose. This indicates that resistance is a stable hereditary alteration of normal cells. It is not an adaptation induced by the presence of 2-deoxygalactose and readily reversible in its absence.

DISCUSSION

Chinese hamster cell mutants resistant to 2-deoxygalactose were isolated in a single-step or two-step selection. The resistant phenotypes of the wild type and the mutants range from sensitive (R50 = 0.1 mg/cc of 2-deoxygalactose) to partially resistant (R50 = 0.5 mg/cc) to fully resistant (R50 = 2.5 mg/cc). These mutants when compared with the wild type have altered growth properties in galactose medium but not in glucose medium. The levels of galactokinase in the mutants range from undetectable level to about 60% of the wild-type level.

Particularly relevant to our studies of somatic cell genetics is the demonstration that resistance to 2-deoxygalactose is the result of a genotypic change and not of a phenotypic or epigenic change (HARRIS 1971; MEZGER-FREED 1972). This is usually not easy to demonstrate in the absence of recombination and segregation of genetic markers. Therefore, we have used instead the criteria of MANKOVITZ, BUCHWALD and BAKER (1974) and PATTERSON, KAO and PUCK (1974). The properties of the clones resistant to 2-deoxygalactose suggest to us that these clones are mutants rather than variants: First, ethyl methanesulfonate, a mutagenic agent, increases the number of clones resistant to 2-deoxygalactose 5-fold. Second, the 2-deoxygalactose-resistant phenotype is preserved from generation to generation in the absence of 2-deoxygalactose, the selective agent. Third, there is a specific functional alteration. Galactokinase activity is not detectable in DR2R1610. The galactokinase kinetic properties of the other resistant clones and of the wild type with 2-deoxygalactose or galactose as substrates are probably different. This justifies *a posteriori*, the utilization of the word "mutant" instead of the word "variant" to designate our clones resistant to 2-deoxygalactose. In summary then, we conclude from these data that we have isolated a variety of hereditary mutants of Chinese hamster cells and that the nature of the primary change remains to be determined.

SUN, CHANG and CHU (1975) have reported that none of their 67 Gal$^-$ Chinese hamster mutants are defective for any of the three enzymes of the galactose pathway. Our results show it is possible to isolate galactokinase$^-$ clones of the DR2R1610 type by selection of clones resistant to 2-deoxygalactose, an analogue of galactose. However, if we do not know yet the nature of the DR2R1610 and the DR11 phenotypes, the other ethyl methanesulfonate-induced resistant clones have galactokinases with about the same molecular weight as the wild-type enzyme but with different kinetic parameters. These results suggest, but do not demonstrate, a structural mutation in the galactokinase gene of the genome of the DR2,

DR2R7, and DR2R22 clones. Confirmation of this will require further characterization of the genetic change and the nature of the modified enzyme.

The existence of a pathway from D-galactose to γ-galactonolactone has been postulated by COHN and SEGAL (1973), CUATRECASAS and SEGAL (1966) HILL and PUCK (1973) and HSIA (1967). If mutation DR2R1610 is a structural mutation in the galactokinase gene (i.e. if CRM^+ material can be detected) and not a regulatory mutation, then either this pathway does not exist in Chinese hamster cells or is a minor one since mutant DR2R1610 is unable to grow on galactose.

In man, juvenile cataract is associated with galactokinase deficiency (GITZELMANN 1965). Our results suggest that defective homozygous or heterozygous human cells that have either no galactokinase activity or half of the wild-type amount may be fully or partially resistant to 2-deoxygalactose. This could be a fast and simple diagnostic test for prenatal screening of affected children and detection of heterozygosity in the parents since the number of cells needed for such assay would be small. RAPPAPORT and DEMARS (1973) have developed a similar test for human fibroblasts with diaminopurine.

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