CONTROL MECHANISMS AND BIOCHEMICAL GENETICS:

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Metabolic Inhibition of Mammalian Uridine Diphosphate Galactose 4-Epimerase in Cell Cultures and in Tumor Cells*

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SUMMARY

The metabolism of galactose compounds, especially that of uridine diphosphate D-galactose, has been studied in intact as well as in broken L cells and HeLa cells. It has been shown that the incorporation of D-galactose 1-phosphate into UDP-galactose is rate-limiting in broken cell preparations, whereas the enzymatic 4-epimerization takes place at an appreciable rate. The latter reaction is strongly inhibited by reduced diphosphopyridine nucleotide, especially at a pH close to 7.

In intact L cells and HeLa cells, UDP-galactose 4-epimerase is rate-limiting. The intracellular epimerase activity constitutes only about 0.1% of that found under optimal conditions in broken cells. Addition of galactose to these cells brings about a block between the general carbon pool of metabolites and the UDP-hexose pool. This is borne out by the fact that administration to intact L cells or to HeLa cells of ^{14}C-galactose and ^{12}C-glucose in equimolar amounts brings about incorporation by ^{14}C into UDP-hexose without detectable dilution of nonradioactive glucose. This blockage of the UDP-hexose pool from the general metabolic pool as well as the blockage of UDP-galactose 4-epimerase may have biological implications, some of which are briefly discussed.

D-Galactose is a common component of the mammalian cell surface. It occurs in galactolipids, sialyl oligosaccharides, blood groups A and B, and other complex compounds. The galactose is usually derived from D-glucose 6-phosphate through uridine diphosphate D-glucose and uridine diphosphate D-galactose. The process is catalyzed by a series of specific enzymes which are crucial to the maintenance of the complex galactosyl compounds of the cell surface. This series of enzymes comprises phosphoglucomutase, uridine diphosphate glucose synthetase (uridine diphosphate D-glucose pyrophosphorylase), uridine diphosphate galactose 4-epimerase, and the enzymes which catalyze the transfer of the galactosyl residue to the complex polysaccharides. The activities of these enzymes are particularly important for the maintenance of cell surface polysaccharide patterns in rapidly growing cells.

Two independent groups of investigators have emphasized that epimerase[1] activity in rapidly growing L cell cultures (1, 2) is below the threshold of detection. A third group has reported a cessation of growth of L cells on media when galactose replaced glucose as the sole carbohydrate source (3). Likewise, established cultures of bovine mammary gland seem to have lost their epimerase activity as well as their ability to produce lactose, although those cultures did retain their capacity to produce β-lactoglobulin (2). Most of these studies were done with broken cell preparations. However, attempts to detect epimerase activity in intact L cells were also made. The levels here were likewise found to be below the threshold of detectability (1).

Since it is conceivable that defects in epimerase could alter surface immunological patterns (*cf.* studies on *Salmonella* mutants defective in epimerase (4)), it seemed of interest to investigate the problem further.

Our own observations are somewhat at variance with the above mentioned reports. In homogenates of L cells and HeLa cells at pH 8.7 (the pH optimum for epimerase) and with excess DPN present, we had no difficulty in detecting epimerase and determining the activity quantitatively. At a pH of 7 to 7.5 and without the addition of diphosphopyridine nucleotide we found only traces of epimerase activity. We have found that the previously described inhibition by DPNH (5) is much more pronounced at a higher hydrogen ion concentration. Thus, a DPNH:DPN ratio of 0.03 which gives only a barely detectable inhibition of epimerase at pH 8.7 gives a 67% inhibition at pH of 7.0.

These observations seem pertinent when galactose catabolism

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[1] The abbreviations used are: epimerase, uridine diphosphate galactose 4-epimerase; transferase, galactose 1-phosphate uridyltransferase; kinase, galactokinase.

and anabolism in rapidly growing tumor cells are considered. Since a high aerobic glycolysis is a predominant feature in most tumors, large amounts of acid are produced as compared with normal cells and give rise to relatively high hydrogen ion concentrations. Likewise, the DPNH:DPN ratio will tend to increase if glucose is supplied. Both factors could contribute toward the suppression of the epimerase activity of tumor cells. However, other factors may turn out to be much more decisive. Regardless of which of the intracellular constituents may exert competitive or feedback inhibition on epimerase, it seemed important to develop methods for the study of epimerase activity in intact cells.

It will appear from the subsequent data that, in the case of epimerase, the activity of that enzyme in intact cells, and especially in tumor cells showing aerobic glycolysis, operates with only a fraction of a percentage of its capacity as assessed in broken cell preparations.

It is well known that estimations of enzyme activities in broken cell preparations do not furnish reliable information about the activities encountered in the intact cell. If substrate concentrations in the cell are much below the K_m for the enzyme in question, and inhibitors (competitors or feedback inhibitors) are present in relatively large amounts, intracellular activity may constitute only a small fraction of that found in broken cells under optimum conditions. The rate-limiting step in a pathway under study in the intact cell may therefore be different from that found in broken cell preparations.

EXPERIMENTAL PROCEDURE

Enzymatic Basis and Prerequisites for Determination of Epimerase Activity in Intact Cells—Epimerase catalyzes the reversible reaction

$$\text{UDP-galactose} \rightleftharpoons \text{UDP-glucose}$$

UDP-galactose can be synthesized from UTP and glucose metabolites (glucose-6-P, glucose-1-P) through the mediation of epimerase. However, if galactose is administered to cells, UDP-galactose can also be synthesized through the mediation of two other highly specific enzymes. The first of these enzymes is galactokinase, which catalyzes the phosphorylation of galactose to galactose-1-P; the second enzyme, galactose-1-P uridyltransferase, catalyzes the following exchange reaction.

$$\text{Galactose-1-P} + \text{UDP-glucose} \rightleftharpoons \text{glucose-1-P} + \text{UDP-galactose}$$

If epimerase activity were not rate-limiting with respect to influx from galactose-1-P, then the UDP-hexose pool would contain 25 to 35% UDP-galactose and 70 to 75% UDP-glucose (6); *i.e.* the UDP-glucose to UDP-galactose ratio would range between 2.3 and 3.0. Hence a determination of this ratio in intact cells which have been incubated with galactose might give some information about rate-limiting steps in the galactose pathway in the intact cell.

If the galactose administered is labeled with ^{14}C, then the galactose-1-P will have the same isotope concentration as the galactose administered. UDP-galactose and UDP-glucose will also be radioactive. However, their isotope content will be diluted from the glucose pool unless the pathway from glucose-6-P to UDP-glucose is blocked.

An estimate of the rate of intracellular epimerase activity might be determined, provided the following conditions are fulfilled. (*a*) Transferase activity should be lower than galactokinase activity. (*b*) Epimerase activity should be lower than transferase activity. (*c*) The rate of influx of nonlabeled glucose constituents should be lower than the turnover rates of transferase and epimerase. It will appear from the present paper that conditions in L cells and tumor cells favor the fulfillment of all three requirements provided that exogenous galactose is administered. The low transferase and epimerase activities give rise to an accumulation of galactose-1-P which somehow (presumably by interference with phosphoglucomutase) brings about an almost complete block of the influx of glucose metabolites to the UDP-hexose pool. Hence if ^{14}C-galactose is administered, the galactose-1-P and UDP-hexose pool (UDP-galactose and UDP-glucose) within a short time acquires the same isotope concentration as the galactose administered. Therefore, the amount of ^{14}C found in the UDP-hexose pool gives an approximate estimate of the rate of transferase activity in the cells Since the UDP-glucose to UDP-galactose ratio (determined enzymatically as well as by a specific chromatography and scanning method to be described here) remains much below 2 (usually around 0.9 to 1.5), even after 1 hour of incubation in medium containing galactose, epimerase is apparently rate-limiting. In most cases the epimerase activity was one-half to one-third the transferase activity. Since the intracellular transferase activity was found to be very low, about 1 mμmole per 10^6 cells per hour, the epimerase activity found was usually well below 0.5 mμmole per 10^6 cells per hour.

Sources of Cells—Clone 929-L (7) was obtained from the laboratory of Dr. L. Siminovitch. These cells (referred to hereafter as L cells) were grown and made available by Dr. J. Littlefield according to the method of McLimans *et al.* (8). Cells of this clone, adapted to growth in chemically defined Medium NCTC 109, are designated NCTC strain 2071 (9).

Strain HeLa (10) cells obtained from Dr. J. Darnell were grown in spinner flasks in Eagle's medium (11). HeLa cells adapted to chemically defined Medium 109 (12) are designated NCTC strain 3952 and a clone of human skin cells as NCTC 3075 (12). All cells grown in chemically defined media were tested and found to be free of pleuropneumonia-like organisms (13).

Growth Assay Procedure—Growth responses of the cells were measured by procedures described (14). Cells were loosened from the floor of the flask without use of trypsin or EDTA by means of a cellophane-tipped spatula. They were suspended, sieved, and stirred mechanically in Earle's balanced salt solution containing 0.1% Methocel, to ensure viability of the inoculum (14). Suspensions of the epithelial cells were not sieved because of possible injury to the cells. In certain tests as indicated, cells were suspended and stirred in glucose-free balanced salt solution. Aliquots (0.5 ml of cell suspension) were dispensed into T-15 flasks containing 2.5 ml of culture fluid, and three samples were taken for measurement of inoculum size. Cultures were incubated at 37.5°. Three times a week, 2 ml of culture fluid were renewed. Cultures were then gassed with a humidified mixture of 10% CO_2 in air in order to adjust the pH to 7.3.

D-Glucose-free Medium 109 (lacking cysteine) was prepared as a 40% concentrated solution (12). This medium and stock solutions of glucose and D-galactose were individually filtered by pressure through Selas 03 filters (12) and were combined aseptically. No antibiotics were used. Experimental media were so constituted as to be isotonic with Medium 109.

The size of cell population at different intervals after planting

was determined by the procedure for enumeration of cell nuclei (16). Five replicate cultures were grown on each medium tested. Three cultures were used for gauging short term effects and two cultures were carried for studying long term effects.

Preparation of Extracts—L cells were harvested at densities ranging from 5 to 12 $\times$ 10^5 cells per ml by centrifuging at approximately 500 $\times$ g. The cells were washed twice with phosphate-buffered NaCl solution. Extracts were prepared by suspending the washed cells in 1 ml of deionized distilled water (60 to 80 $\times$ 10^6 cells per ml), freezing twice at $-80°$, and thawing and centrifuging at about 1500 $\times$ g at 0°. The protein concentration of the extracts ranged from 4 to 6 mg per ml.

The L cell growth studies were done with L cells adapted to unsupplemented NCTC Medium 109 designated "strain 2071." The D-galactose used was Sigma Lot 60 B-637-15, which contained less than 0.5% glucose. Cells were harvested at a density of 4 $\times$ 10^5 cells per ml in the same manner as were the L cells. However, a buffered NaCl solution described by Wu (17) was substituted for phosphate-buffered NaCl solution as the wash solution. Extracts were prepared as described for the L cells.

HeLa cells adapted to unsupplemented NCTC Medium 109, strain 3952, were used for growth studies.

Radiochemicals, Coenzymes, and Enzymes—Galactose-1-^{14}C and glucose-1-^{14}C preparations were supplied by New England Nuclear, and they showed specific activities of 2.5 to 3.0 mC per μmole.[2]

DPNH and DPN were Boehringer products. UDP-D-glucose dehydrogenase was purified from calf liver acetone powder through Step 5 as previously described (18).

Calf liver epimerase was purified according to Strominger *et al.* (18); a fraction with a specific activity of 1600 units per mg of protein was used in these studies. Purified yeast epimerase (19, 20) was kindly supplied by Dr. R. Darrow and Dr. C. R. Creveling of this laboratory. All other enzymes and chemicals were commercial products.

Enzymatic Assays in Broken Cells—Galactokinase was assayed by incubating cell-free extract with galactose-1-^{14}C, ATP, NaF, and $MgCl_2$ in Tris buffer of pH 7.5 for 30 min at 37°, after which the mixture was heated for 90 sec at 100° and centrifuged. A suitable aliquot was chromatographed and the galactose-1-P formed was determined as described in the next section.

Transferase, UDP-D-glucose dehydrogenase, and epimerase were assayed as described previously (21). UDP-glucose pyrophosphorylase was assayed by substituting inorganic pyrophosphate for galactose-1-P in the transferase assay (22). The amount of cell extract used in the assays corresponded to 4 to 8 $\times$ 10^6 cells.

All assays, other than the galactokinase assay, were done in a total volume of 1 ml with a Cary model 14 spectrophotometer. Protein concentrations were determined by the method of Lowry *et al.* (23). All activities are expressed in terms of micromoles of product formed per hour per mg of protein.

Determination of Epimerase in Intact Cells—Washed cells in amounts of 30 to 50 $\times$ 10^6 were added to 2 ml of wash solution to which galactose-1-^{14}C (6 mM) or glucose-1-^{14}C (7.5 mM) had been added. Incubation time was 60 min at 37°. CO_2 was trapped in 10% KOH. After incubation, the cells were centrifuged, washed, and then suspended in 70% ethanol at 70° for 5 min at a concentration of 10 $\times$ 10^6 cells per ml of ethanol mixture. The mixture was centrifuged and the ethanol-soluble extract was concentrated 10-fold by evaporation. Concentrated extract (10 μl) was chromatographed on Whatman No. 1 paper in the Paladini-Leloir ethanol-sodium acetate system at pH 3.5 (24). In this system, galactose and glucose have an R_F of about 0.85; galactose-1-P and glucose-1-P, an R_F of 0.32 to 0.39; and UDP-glucose and UDP-galactose, an R_F of 0.13 to 0.18. UDP-galactose and UDP-glucose cannot be separated in this chromatographic system. The UDP-glucose-UDP-galactose mixture is therefore treated with UDP-glucose dehydrogenase and DPN, and UDP-glucose is specifically converted to UDP-glucuronic acid.

$$\text{UDP-glucose} + 2\,\text{DPN} \rightarrow \text{UDP-glucuronic acid} + 2\,\text{DPNH} + 2\text{H}^+$$

A 30-μl aliquot of the ethanol extract was incubated with 10 μl of DPN (25 mM), 5 μl of 1 M glycylglycine buffer (pH 8.7), 10 μl of UDP-glucose dehydrogenase (300 units), and 5 μl of H_2O for 60 min at 25°, heated at 100° for 90 sec, and centrifuged; 20 μl of the supernatant solution were chromatographed as described above. UDP-glucuronic acid has an R_F of 0.04 to 0.08 and thus separates well from UDP-galactose.

In order to ascertain that the material remaining in the UDP-hexose spot after treatment with UDP-glucose dehydrogenase was UDP-galactose, another incubation of the ethanol extract with yeast epimerase, DPN, and UDP-galactose dehydrogenase was performed. Ethanol extract (30 μl) was incubated as above, but 5 μl of yeast epimerase (20,000 units per ml) were substituted for the H_2O. Supernatant fluid (20 μl) was chromatographed.

Unlabeled galactose-1-P or glucose-1-P and UDP-glucose were always chromatographed as markers. Galactose-1-P or glucose-1-P was located by dipping in ferric chloride-salicylsulfuric acid reagent (25). UDP-glucose was located by its quenching of ultraviolet light. The sample strips were scanned for radioactivity with a Vanguard 880 autoscanner at a setting of 300 cpm. The areas of the radioactive peaks were measured with a Keuffel and Esser polar planimeter and counts per min were determined from a standard curve of area as counts per min in a Packard Tri-Carb liquid scintillation counter. The standard curve was obtained by scanning one ^{14}C-glucose spot of known radioactivity. On this basis the number of millimicromoles of ^{4}C equivalent to a particular area could be determined.

$^{14}CO_2$ in KOH was counted in a solution described by Bray (26). Quenching due to KOH was 23%.

One experiment was performed in which galactose-1-^{14}C was injected into a normal rat which was on a glucose-rich diet. The rat was killed after 30 min, and various tissues and organs were minced in warm 70% ethanol. The ethanol-soluble extracts were analyzed as described above.

The ethanol-soluble extracts were also assayed spectrophotometrically for total, *i.e.* labeled and nonlabeled, UDP-galactose and UDP-glucose, by measurement of DPNH formation.

[2] 1-^{14}C-Labeled galactose and glucose from The National Bureau of Standards (NBS) were originally listed as containing 1 μC per μmole. Since this radiochemical contained various inert substances (dulcitol and other constituents), it had in fact, based on galactose, a specific 1-^{14}C content which was 2.5-fold larger. This must be the explanation of the puzzling fact that the UDP-galactose and UDP-glucose of L cells and HeLa cells incubated with 1-^{14}C-galactose from NBS had a specific radioactivity 2.5-fold higher than that of the NBS 1-^{14}C-galactose of the medium. However, such a discrepancy was not encountered if ^{14}C-galactose from New England Nuclear was used.

RESULTS

Growth Response to Galactose—Short term growth responses of L cells (strain 2071) and HeLa cells (strain 3952) to Medium 109, to Medium 109 containing galactose in place of glucose, and to Medium 109 lacking glucose are summarized in Table I.

Cells of strain 2071 grew slowly on galactose. After 16 weeks of culture (seven transplant generations), cells appeared healthy but tended to become detached from the floor of the flask. Those cultures to which no sugar was added were dead after 2 weeks; those grown on 5 mg of glucose per liter showed some healthy cells after 9 weeks, but their rate of proliferation appeared to be somewhat lower than that of cells in the galactose medium.

Efforts to grow HeLa cells (strain 3952) on galactose were unsuccessful. Cells were usually dead after 2 weeks or survived for as long as 10 weeks, but failed to proliferate.

Human skin cells (clone NCTC 3075 (12)) grew slowly on galactose and were still healthy and vigorous after 20 weeks (nine transplant generations). Cultures to which no glucose or 5 mg of glucose per liter were added failed to survive for more than 2 and 3 weeks, respectively.

TABLE I

Growth responses to galactose and glucose in Medium NCTC 109

Experiment	Strain or clone (NCTC)	Inoculum size	Days of culture	Final cell population[a] (× inoculum)			
				Glucose (100 mg/100 ml)	Galactose (100 mg/100 ml)	No sugar	Glucose (0.5 mg/100 ml)
		× 10^5 cells					
1	2071 (L cells)	2.6	13	18.7	3.5	0.04	
2	2071[b]	3.5	13	14.6	1.7	0.02	
3	2071[c]	3.3	7	4.5	1.2	0.4	
4	2071[c]	4.8	9	6.2		0.3	0.4
5	3952[c]	1.7	7	6.9	1.8	1.1	

[a] Averages of three replicate cultures for each determination with the exception of only two replicate cultures with glucose in Experiment 4.

[b] Cells had been frozen and returned for several generations to culture.

[c] Cells stirred in glucose-free NaCl solution for dispensing.

TABLE II

Activity of enzymes of galactose metabolism in broken cell preparations[a]

Enzyme	L cell	HeLa cell
	mμmoles/mg protein/hr	
Kinase	170	120
Transferase	30	50
Epimerase	160	440
UDP-glucose dehydrogenase	210	50
UDP-glucose pyrophosphorylase	690	800
Phosphoglucomutase[b]		150

[a] In most cases analyses on two or more cell populations were made. In those cases the averages were calculated and expressed. In the case of L cell epimerase 12 determinations were made, and the distribution of values was plotted (see Fig. 1).

[b] Phosphoglucomutase was determined according to Najjar (27).

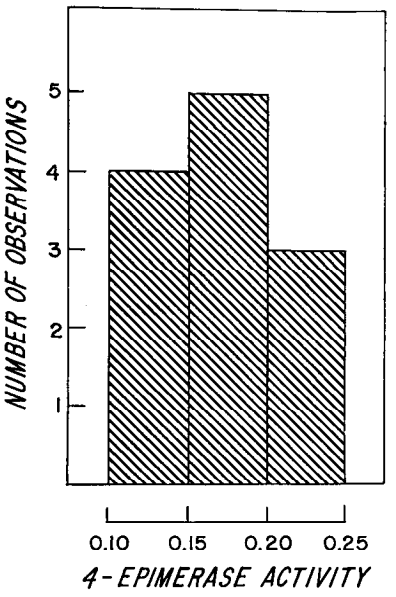


FIG. 1. Activity in micromoles of UDP-glucose formed per mg of protein per hour at 27°; epimerase activities measured in preparations represent independent cell lines.

TABLE III

Epimerase activity of broken L cell preparations as function of pH and DPN:DPNH ratios

The concentration of DPN was 0.3 mM; of DPNH, 0.09 mM. Whenever DPNH was present, the DPN:DPNH ratio was 4.2. Buffer mixtures were phosphate, Tris, and glycine, each in 0.1 M concentration and adjusted with HCl or NaOH according to the pH desired.

pH	Activity[a]	
	Control	+ DPNH
7.5	80.0	19.2
8.6	160.5	78.4
9.4	160.0	115.2

[a] Millimicromoles of UDP-glucose formed at 37° per mg of protein per hour.

Enzyme Activities—Table II shows the activities of the enzymes of galactose metabolism found in L cell and HeLa cell extracts at optimum pH and DPN concentrations. The galactokinase and epimerase activities are moderately well expressed, while the transferase activities are low in both cases; the UDP-glucose pyrophosphorylase levels are relatively high. The occurrence of UDP-glucose dehydrogenase activity in L cells is noteworthy.

Since the appearance of epimerase in L cell preparations has been subject to discussion (1, 2), numerous determinations were made in this study. From Fig. 1 it can be seen that the spreading among 12 samples is only moderate. Each independent preparation showed activity well above that of transferase.

Inhibition of Epimerase by DPNH—DPNH inhibits L cell epimerase more at pH 7 than at pH 9.5 (Table III). The effect of DPNH on liver epimerase activity at different pH values and different concentrations of DPNH and DPN is shown in Table IV, while Table V gives the results obtained with various DPN:DPNH ratios with L cell and HeLa cell extracts. As can be

seen, inhibition of epimerase activity by DPNH is strikingly increased as the pH is lowered; *i.e.* inhibition at pH 7.0 is much greater than at pH 8.7 or pH 10 in all cases observed. Epimerase activity at pH 7.5 is about 50%, and at pH 7.0, about 25% of that at pH 8.7. Change in absolute amounts of DPN with 0.01 μmole of DPNH gave as much inhibition as a mixture of 0.3 μmole of DPN and 0.1 μmole of DPNH. In other words, the DPN:DPNH ratio appeared to be the determining factor. In the assays of L cell epimerase, difficulty was encountered owing to the endogenous UDP-glucose dehydrogenase present in the extracts. When the first step of the two-step procedure (extract + DPN + UDP-galactose) was observed spectrophotometrically, it was found that small amounts of DPNH were being formed. Addition of larger amounts of DPN (up to 1.5 μmoles) to the control incubations (no added DPNH) seemed to overcome the effect of DPNH formed, but it is not certain whether a condition of true reversal of inhibition was reached, or merely a plateau.

When DPNH was kept at 7.5 for 30 min before addition to enzyme at pH 9.4, no reduction in enzyme activity as compared with enzyme plus DPNH at pH 9.4 was observed. Enzyme plus DPNH and DPN (with or without UDP-galactose) kept at pH 7.5 for 30 min and added to another enzyme sample at pH 9.4 similarly gave no reduction in enzyme activity. It appears, then, that increased inhibition of mammalian epimerase by DPNH at more acidic pH is due neither to a different form of DPNH at low pH nor to a product formed by enzyme plus DPNH, but instead to an effect on the enzyme itself, perhaps due to conformational changes.

Whole Cell Incubations—Chromatographic and spectrophotometric analyses of the ethanol-soluble extracts of incubations of intact HeLa cells were carried out with labeled and unlabeled galactose and glucose. Fig. 2 illustrates the chromatograms obtained with untreated ethanol extract, with extract plus UDP-glucose dehydrogenase, and with extract plus yeast epimerase plus UDP-glucose dehydrogenase. If some radioactive material still remained in the UDP-hexose area after treatment with yeast epimerase plus UDP-glucose dehydrogenase, this was not included in calculating the amount of UDP-galactose.

The principle of this resolution method is evident from Scan II, in which the usual UDP-hexose peak represents the remaining UDP-galactose fraction and the slow moving UDP-glucuronic acid peak represents the UDP-glucose fraction. In Scan III, in which both enzymes work together, all the UDP-hexose (sum of UDP-glucose and UDP-galactose) is converted to UDP-glucuronic acid. Scan III is essentially a qualitative as well as quantitative control.

Incubation of extract with dehydrogenase for 10 min or 60 min gave the same results. When UDP-hexose was eluted, hydrolyzed at pH 2 at 100° for 10 min, and then rechromatographed, all of the radioactivity had an R_F corresponding to galactose.

Comparison of radiochromatography data with spectrophotometric data showed good agreement in the ratios of UDP-glucose to UDP-galactose (except in the incubation with radioactive glucose and nonradioactive galactose, in which these ratios are bound to deviate greatly (see "Discussion"). The results are summarized in Table VI.

Incubation of HeLa cells with galactose alone gave a UDP-glucose to UDP-galactose ratio of 1.9 (Table VI, Experiment 2a) and, with glucose alone, an average ratio of 3.5 (Table VI, Experiments 1c and 2c). However, incubation with galactose plus glucose gave an average ratio of 1.0 (Table VI, Experiments 1a and 2b).

TABLE IV

Inhibition of calf liver epimerase by DPNH as function of pH

A semifractionated epimerase, catalyzing 3.5 μmoles of UDP-galactose per mg per hour, was used. Incubation times ranged from 5 to 15 min; 70 to 90 units were used for the assays.

pH	DPN:DPNH	Inhibition
		%
7.0	3:1	93
7.0	37:1	69
7.5	3:1	90
7.5	37:1	54
8.7	3:1	71
8.7	37:1	18
9.5	37:1	3

TABLE V

Inhibition of L cell and HeLa cell epimerase by DPNH as function of pH

Cells	pH	DPN:DPNH	Inhibition as compared with control with only DPNH
			%
L[a]	7.0	4:1	86[b]
	7.0	45:1[c]	36
	7.5	4:1	80
	7.5	30:1[c]	45
	8.6	4:1	61
	8.6	16:1	31
	9.4	4:1	28
HeLa[d]	7.0	4:1	89[e]
	7.5	4:1	86

[a] Incubation, 30 min; epimerase, 10 to 20 units.

[b] Incubation, 15 min; epimerase, 90 units.

[c] DPNH formed during incubation brought about by L cell UDP-glucose dehydrogenase.

[d] Control with only DPN (0.3 mM) at pH 7.0; 0.04 μmole per mg of protein per hour.

[e] Control with only DPN (0.3 mM) at pH 7.0; 0.1 μmole per mg of protein per hour.

The same analysis of L cells incubated with galactose-1-^{14}C revealed a UDP-glucose to UDP-galactose ratio of 0.76. However, the chromatogram of the original untreated ethanol extract revealed radioactive material with an R_F value corresponding to that of UDP-glucuronic acid, which suggests that the UDP-glucose dehydrogenase assayed in the cell-free extracts was active in this system *in vivo*. If the material is indeed UDP-glucuronic acid, formed by enzymatic conversion of UDP-glucose, the UDP-glucose to UDP-galactose ratio should be revised to 2.0. Incubation with galactose plus glucose gave a UDP-glucose to UDP-galactose ratio of 0.9 (again including the UDP-glucuronic acid in the untreated extract). Thus, with both HeLa cells and L cells, epimerase activity was markedly decreased when cells were exposed to a combination of galactose and glucose. The ethanol extract of rat brain cells incubated under similar conditions gave a UDP-glucose to UDP-galactose ratio of 2.5.

The rate of incorporation of ^{14}C into CO_2 was determined in

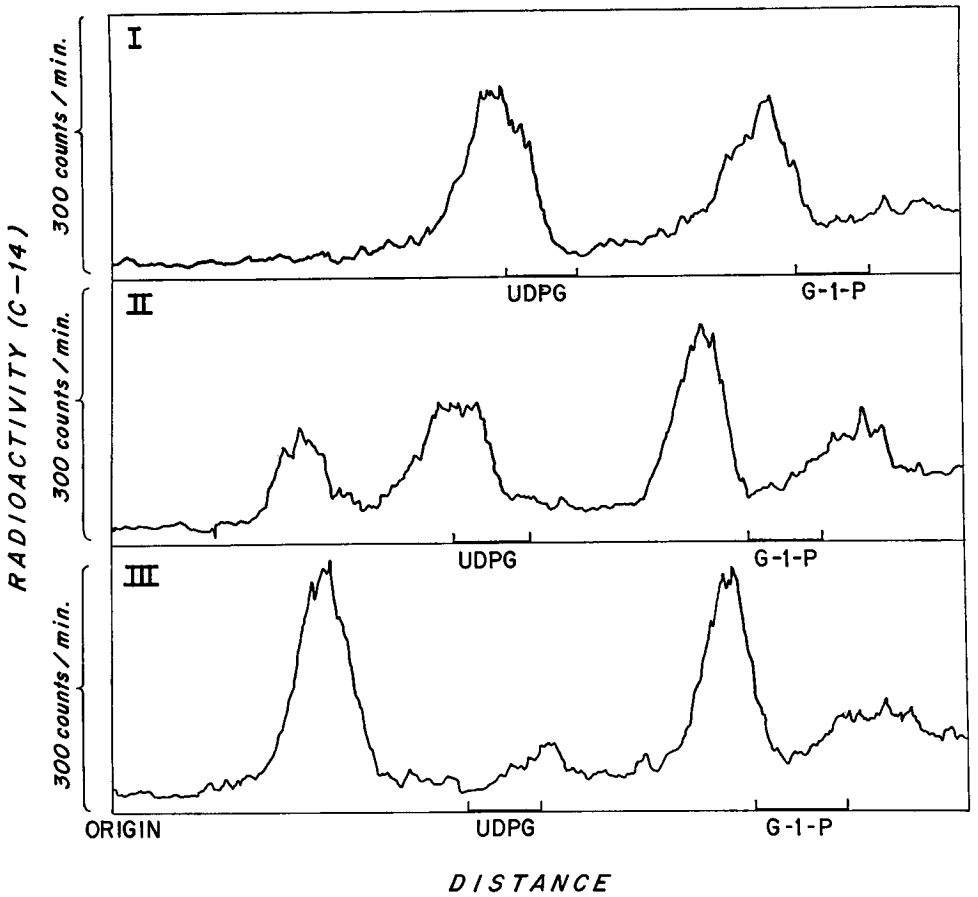


FIG. 2. UDP-hexose, UDP-glucose, and UDP-galactose levels in intact HeLa cells. Scanning of paper chromatograms (paper counted simultaneously on both sides by Vanguard scanner) from protein-free filtrates of HeLa cells labeled with 1-^{14}C-galactose-^{12}C-glucose for 60 min at 37°. *I*, scanning without addition of enzymes. Areas (in square centimeters): UDP-hexose peak (sum of UDP-glucose and UDP-galactose), 13.8; galactose-1-P peak, 11.9. *II*, scanning after incubation of filtrate with UDP-glucose dehydrogenase. Areas (in square centimeters): UDP-glucuronic acid (UDP-glucose fraction), 5.7; UDP-galactose, 11.1; galactose-1-P, 12.5. *III*, scanning after incubation of filtrate with UDP-glucose dehydrogenase and yeast 4-epimerase. Areas (in square centimeters): UDP-glucuronic acid, 14.1; UDP-galactose, 2.7; galactose-1-P, 12.3. Indicator spots for UDP-glucose and glucose-1-P chromatographed are indicated. The first peak in *I* represents cellular radioactive UDP-hexose (mixture of UDP-glucose and UDP-galactose). The second peak represents galactose-1-P. In *II* the slow moving first peak presents UDP-glucuronic acid stemming from UDP-glucose. In *III* the first slow moving peak represents UDP-glucuronic acid from UDP-hexose. *UDPG*, uridine diphosphate glucose; *G-1-P*, glucose 1-phosphate.

TABLE VI

Flux rates and UDP-glucose-UDP-galactose pools in intact HeLa cells

Experiment	Hexoses added	Cellular ^{14}C-UDP-hexoses (scanning of radioactive spots)			UDP-Glc*: UDP-Gal*	Cellular UDP-hexose pools (differential spectrophotometry)			UDP-Glc: UDP-Gal
		UDP-Glc*	UDP-Gal*	UDP-Hex (sum)		UDP-Glc	UDP-Gal	UDP-Hex (sum)	
		mμmole/10^6 cells/hr				*mμmole/10^6 cells/hr*			
1a	Gal* + Glc	0.33	0.46	0.79	0.71	0.29	0.44	0.73	0.66
1b	Gal + Glc*	0.04	0.003	0.043	13.0	0.29	0.29	0.58	1.00
1c	Glc*	0.13	0.026	0.16	5.1	0.33	0.33	0.15	2.20
2a[a]	Gal*	0.21	0.11	0.34	1.90	0.22	0.12	0.34	1.80
2b[a]	Gal* + Glc	0.19	0.18	0.37	1.06	0.16	0.18	0.34	0.88
2c[a]	Glc*	0.2	0.06	0.26	3.30	—[b]	—[b]	—[b]	—[b]

[a] Only traces of ^{14}C were found in glycogen (0.005 to 0.05 mμmole of ^{14}C-hexose per 10^6 cells per hour).

[b] Not measured.

two incubation experiments, with the use of HeLa cells and ^{14}C galactose or glucose. In one case, the amount of $^{14}CO_2$ generated from ^{14}C galactose definitely exceeded the rate of input into UDP-galactose and UDP-glucose. The existence of alternative pathways for galactose catabolism in HeLa cells must therefore be considered.

In the series of experiments in which glycogen was extracted according to Carroll, Longley, and Roe (28) and determined (see Table VI), it was found that only 0.005 to 0.05 mμmole of ^{14}C per 10^6 cells was incorporated per hour. Hence, no significant losses through this pathway occurred.

The lack of any detectable dilution of the UDP-hexose labeled by ^{14}C-galactose must be due to a failure of incorporation of endogenous glucose metabolites into the cellular UDP-hexose.

This is illustrated in another way by administering ^{14}C-labeled glucose and unlabeled galactose at the same time (Table VI, Experiment 1b). It can be seen that under these circumstances the extent of incorporation of ^{14}C-glucose into UDP-hexose amounts to only about 5% of the rate of incorporation of galactose (Experiment 1a). Such a small degree of dilution would not be detectable. It can also be seen that the rate of flow of ^{14}C-glucose into UDP-glucose in the absence of administration of galactose increases (Experiment 1c), although it still is lower than the rate of galactose-1-P uridyltransferase (about 20 to 25% of the rate of the latter enzyme). Administration of galactose brings about a lowering of the rate of glucose incorporation into the nucleotide to one-fifth, bringing it down to the rate of transferase activity.

Galactose administered to HeLa cells accumulates to a significant extent as galactose-1-P (see, for instance, chromatogram in Fig. 2). Such an accumulation may not be surprising since the galactokinase activity of broken L cells and HeLa cells was found to be considerably higher than that of transferase activity (*cf.* Table II).

UDP-glucose synthetase and other uridyltransferases are not inhibited by galactose-1-P, and UDP-glucose synthetase of HeLa cells is not inhibited by galactose-1-P.[3] Phosphoglucomutase is, however, readily inhibited by galactose-1-P (29, 30), presumably because of a competition between glucose 1,6-diphosphate and the corresponding galactose analogue (*cf.* other analogues (31, 32)). In any event this step seems, at least at present, the most likely one to be blocked by galactose-1-P. Epimerase is a further rate-limiting factor for incorporation of glucose metabolites into UDP-galactose. In the intact cell, epimerase activity may, in fact, not amount to more than about 20% of the activity of the transferase (*i.e.* approximately 0.1 mμmole of glucose metabolites can be converted to galactosyl compounds within 1 hour in a population of 10^6 cells). In order to make the results derived from Table VI more intelligible, Equation 1 is presented. The order of rate is given in millimicromoles per hour per 10^6 cells, as follows:

In order to simplify the interpretation, we shall assume that phosphoglucomutase (Step 2) is the one which is hindered by the galactose-1-P accumulation. This point, however, needs further experimental proof.

It seems clear that with the low epimerase, the ratio of radioactive UDP-glucose to radioactive UDP-galactose (UDP-Glc*: UDP-Gal*) would be much *above* the average equilibrium point of 2.5. Experiment 1b showed a radioactivity ratio of 13. The reason why the ratio of UDP-glucose (sum of ^{14}C and ^{12}C) to UDP-galactose (sum of ^{14}C and ^{12}C) is *below* the equilibrium point (*i.e.* only 1.00) is due to the fact that Steps 6 and 5 are operating, if not rapidly, then at least many times faster than Steps 4 and 2, and are, therefore, building up UDP-galactose from ^{12}C-galactose-1-P faster than UDP-glucose.

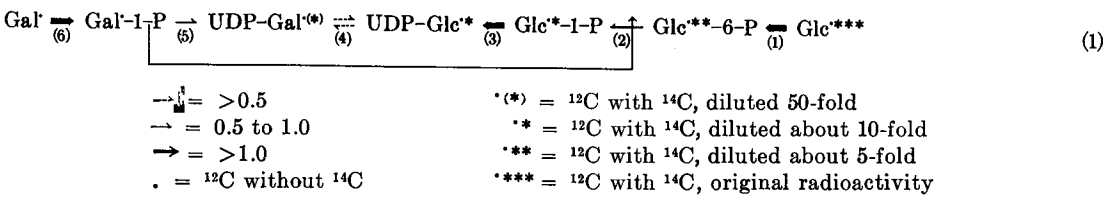
Experiment 1c shows that the rate of influx of radioactive glucose is appreciable, larger than 0.16 mμmole and smaller than 0.48 mμmole per hour per 10^6 cells. Since it is likely that there is a small pre-existing glucose or glucose-6-P pool, the rate must at least be estimated to be somewhat higher than 0.16 mμmole. It is noteworthy that the ^{14}C-glucose of UDP-glucose is only diluted by a factor of 2.5 to 3 (Experiment 1c, comparing UDP-Glc* with UDP-Glc). Hence the intracellular glucose or glucose phosphate pools must be of very modest size.

A somewhat clearer illustration of the state of the various pools as well as the flux rates is presented in Fig. 3, the data of which stem from Table VI.

The assessment of epimerase activity in intact HeLa cells as estimated by means of ^{14}C-galactose gave a value of 0.18 to 0.20 mμmole per 10^6 cells per hour. This is close to the estimate derived from labeling experiments with ^{14}C-glucose, in which the estimate amounted to 0.22 to 0.25 mμmole.

If L cells or HeLa cells were grown for many generations in a medium containing galactose as well as glucose, one might, on the basis of these observations, expect a gradual decrease of the UDP-hexose content unless some other transferase were present or when induced. The transferase required UDP-glucose in order to incorporate galactose-1-P. Hence, this enzyme is not able to bring about an increase of the UDP-galactose level in the cell. If the renewal of UDP-glucose from glucose is blocked by accumulated galactose-1-P, cellular levels should therefore decrease.

Most recently we have examined whether L cell cultures grown over several generations in glucose or in glucose-galactose growth media have different UDP-glucose levels. No differences were detectable. It may, however, be of some relevance to note that we found in both cultures appreciable activity of another transferase, UDP-galactose pyrophosphorylase. This enzyme, which catalyzes the biosynthesis of UDP-galactose from galactose-1-P and UTP, is in fact bringing about a synthesis *de novo* of UDP-galactose. If epimerase is operating, even at a very low rate, the UDP-glucose level should be at least partially sustained.



[3] H. Troedsson and H. M. Kalckar, unpublished data.

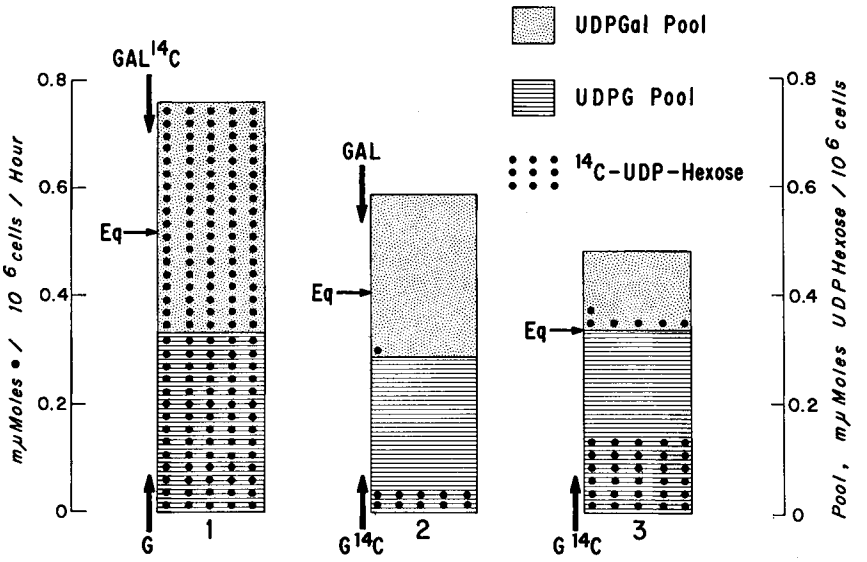


FIG. 3. Graphic illustration of flux velocities (*left ordinate*) as well as pool sizes (*right ordinate*) as summarized in Table VI. Each *dot* represents the smallest amount of ^{14}C-UDP-hexose detectable. This would amount to approximately 0.005 mμmole per 10^6 cells. *UDPG*, uridine diphosphate glucose.

Comments on Table VII will be presented in the subsequent section.

DISCUSSION

A comparison of enzyme activities in broken cells with those in intact cells has given us information which may be of some interest from the point of view of cell physiology. It will be noted that the transferase and epimerase activities found in intact L cells or HeLa cells are only a small percentage of those found in broken cell preparations. It is obvious that substrate concentrations in the cell are often far below the corresponding K_m values. This may be pertinent with respect to the substrates for transferase and epimerase. Moreover, concentrations of competitive or feedback inhibitors in the cell are also decisive for intracellular enzyme activity.

Transferase activity in intact cells is about 2% of that observed in broken cell preparations, when saturating concentrations of galactose-1-P and UDP-glucose and an optimal pH are used. The situation is even more extreme in the case of epimerase, in that one can detect only about 0.1% of the maximum activities found in broken cell preparations.

A few prerequisites are necessary in order to estimate cellular enzyme activities quantitatively. The estimation of transferase in the intact cell has been followed by a time curve, with the increase in the UDP-hexose radioactive peak as a measure. This would obviously be an inaccurate method and would not have been feasible as a quantitative method if it were not for the fact that the influx from the nonradioactive glucose metabolite pool had ceased after 20 to 30 min in incubation mixtures containing galactose. Determination after 1 hour can therefore be considered sufficiently reliable to give an approximate estimate of the capacity of transferase in the cell. The epimerase activity can be calculated from the transferase activity only if the latter is not rate-limiting. Otherwise equilibrium will be reached corresponding to a UDP-Glc*:UDP-Gal* ratio ranging from 2 to 3, or by an average of 2.5.

It should be noted that the ^{14}C-glucose of UDP-glucose is not diluted more than 3-fold even after 1 hour (see Experiment 1c in Table VI and Fig. 3). The relatively higher dilution of UDP-galactose in Experiment 1c furnished, in fact, another illustration of the low rates of cellular epimerase activity. This is the main reason why the ratio UDP-Glc*:UDP-Gal* is higher than the ratio UDP-Glc:UDP-Gal. The latter ratio is within the range of the ratio of 2.5, which corresponds to equilibrium. In any case, the nonlabeled UDP-glucose stems from an endogenous glucose-6-P pool which is fed by a variety of carbon compounds in the cell (glycogen, pyruvate, lactic acid, carbon skeleton of amino acids, etc.). Because of the varying isotope dilutions of the ^{14}C-glucose taken up in the cell, the rate of influx of glucose into UDP-hexose cannot be evaluated except, at best, on a rough relative basis. For instance, it seems reasonable to conclude that the greatly lowered rate of incorporation of 1-^{14}C-glucose in the experiment in which nonlabeled galactose is also present (Experiment 1b in Table VI) is real. If it were due merely to a dilution of the radioactive glucose at the stage of glucose-6-P or glucose-1-P, one would not expect a UDP-Glc*:UDP-Gal* ratio of 13. The modest dilution of glucose phosphate is probably due to the fact that glucose-6-P is converted to lactic acid at a rapid rate and hence does not accumulate.

The patterns described here have been found in a large number of mammalian cell cultures and in tumor cells. The biological significance of the low epimerase levels in rapidly growing cells deserves attention (33) but it cannot as yet be assessed. The same applies to the block between glucose-6-P and UDP-glucose seen in cells incubated with glucose as well as with galactose. We can therefore only state that various types of cells manifesting high aerobic glycolysis, such as L cells, HeLa cells, C3H mammary carcinoma, and Ehrlich ascites tumor, have a defective galactose metabolism.

Some unpublished observations[4] made on the UDP-glucose-UDP-galactose steady state levels in the cells of the regenerating liver may be appropriate to this discussion. The pool of UDP-hexose (UDP-glucose as well as UDP-galactose) in regenerating liver was found to be of an order of magnitude 50-fold larger than that found in L cells. Although one finds the nonlabeled UDP-glucose-UDP-galactose in equilibrium (the ratio was close to 3), the ^{14}C-labeled UDP-glucose-UDP-galactose is far from equilibrated. This is due to the fact that the labeled UDP-galactose

[4] H. M. Kalckar and N. Bucher, unpublished data.

TABLE VII

Estimates of transferase and epimerase capacities in intact normal tissue and tumor cells from mice

Mice were treated by intravenous injection with 1-^{14}C-galactose in amounts corresponding to 5 to 10 μmoles (containing 1 μC per μmole). Tissue specimens were frozen after 30 to 60 min. Cell suspensions were incubated for 30 to 60 min with 1-^{14}C-galactose (7.5 to 15 μmoles per ml of incubation medium) and nonlabeled glucose. Aliquots from protein-free filtrates were subjected to paper chromatography (ethanol-acetate mixture, pH 3.5) and scanned. Other aliquots were subjected to the same treatment after a prior incubation with UDP-glucose dehydrogenase (see Fig. 2). Radioactive standard peaks were calibrated in advance in the liquid scintillation counter. The quantitative epimerase method was based on quantitative estimate of transferase.[a, b] The latter was expressed as the amount of radioactive galactose incorporated into UDP-hexose. Incubation temperature was 37°.

Tissue and tumor cells	Transferase	Epimerase	Reference
	mμmoles converted/10⁶ cells/hr		
L cells	0.6	0.3	
HeLa cells	0.9	0.4	
Ehrlich ascites tumor	0.4	0.1	4
Mammary carcinoma (CH_3)	0.1	0.2[a]	4
Lactating mammary gland	>3.0	>3.0[a]	4
Normal brain		≧1.5[a]	4
Regenerating liver	0.2	≧1.0[b]	

[a] The UDP-galactose to UDP-glucose ratio had reached equilibrium; hence epimerase activity must be higher than transferase activity. Experiments with 1-^{14}C-glucose indicated, however, that the epimerase activity in C3H mammary carcinoma could scarcely exceed 0.5 μmole per 10^6 cells per hour.

[b] Calculations from transferase rate of a large UDP-galactose-UDP-glucose pool in regenerating liver permitted an approximate estimate of the epimerase rate.

is mixed with a pool of pre-existing UDP-galactose approximately 20 times greater; the dilution factor for UDP-Glc* was found to be as high as 60. Hence, the epimerase activity, although seemingly lower than that of transferase, is in fact much larger because of an appreciable UDP-hexose pool. In contrast, the cells did not have any detectable pools for galactose or galactose-1-P.

Table VII summarizes in general figures for the enzyme levels of transferase and epimerase in intact cells, normal as well as malignant, obtained from previous and present studies. As will appear in Table VII, transferase levels are very low in C3H mammary carcinoma as contrasted with those in normal lactating mammary glands. Regenerating liver shows appreciable epimerase activity, while this enzyme is very low in a variety of tumors. The latter may, as mentioned, be ascribed to the powerful inhibition brought about by the high levels of hydrogen ions and DPNH presumably present in tumor cells.

In assessing the findings reported here from a physiological point of view, a number of features deserve special attention. It seems clear that, in fast growing cells like L cells and HeLa cells, both transferase and epimerase operate on only a minute fraction of their capacities. As mentioned, competitive inhibitors or feedback inhibitors present in the cell constitute some of the vectors. The concerted inhibitory action of DPNH and hydrogen ions observed in broken cell preparations may well apply to the intact cell. The predominance of aerobic glycolysis in these cells used may lead one to emphasize such factors. If aerobic glycolysis were the main factor in the inhibition of cellular epimerase, minimum deviation hepatomas (34), which do not manifest aerobic glycolysis, should be able to use a larger fraction of their epimerase capacity. However, this awaits experimental proof.

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