*ENZYME KINETICS IN MAMMALIAN CELLS, I. RATE CONSTANTS FOR GALACTOSE METABOLISM IN ERYTHROCYTES OF NORMAL, GALACTOSEMIC, AND HETEROZYGOUS SUBJECTS**

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Study of the relationship between genetic constitution and biochemical activities of mammalian cells arising from tissue culture and from various differentiated tissues requires rapid, quantitative means for determination of rate constants for enzyme activities in metabolic chains, on a cellular basis. Such measurements (*a*) facilitate study of protein synthesis regulation and feedback control mechanisms in cells taken from different tissues and tissue culture souces; (*b*) allow quantitative comparison of the behavior of normal cells with those possessing specific chromosomal monosomies, polysomies, deletions, and other aberrations, so making possible determination of the chromosomal location of at least those genes which exhibit simple dosage effects;[1] and (*c*) permit screening of populations for detection of heterozygous carriers of allelic genes. The present communication describes measurement in human erythrocytes of the cellular rate constants for galactokinase and galactose-1-phosphate uridyl transferase enzymes, whose actions have been previously studied in somewhat different and less quantitative fashion in this and other laboratories.[2, 3]

The method involves packing of washed and counted cells by centrifugation, discarding the supernatant fluid, and lysis of the resulting cell mass by freezing and thawing. Use of this undiluted material permits accurate cell quantitation, preserves the internal environment of the cell (at least as an over-all average situation), and allows use of substrates, trapping agents, and other metabolically active compounds, while eliminating complexities produced by active transport and other processes. A radioactive substrate plus a measured excess of a nonradioactive compound which occurs at the point in the metabolic chain where one wishes to stop the flow of radioactive label, are added to the system at zero time. The system is sampled after appropriate time intervals, the contents are resolved by chromatography or other means, and the radioactivity of each reaction product is measured. Calculation of specific reaction rate constants on a cellular basis can be carried out for each step from this data.

The first two steps of galactose metabolism in mammalian cells are:

$$\underset{\text{Galactose}}{A} \xrightarrow[K_1]{\text{ATP + kinase}} \underset{\text{Galactose-1-PO}_4}{B} \xrightarrow[K_2]{\text{UDP-glucose + transferase}} \underset{\text{UDP-galactose + glucose-1-PO}_4}{C},$$

where ATP represents adenosine triphosphate, and UDP-, the uridyl diphospho derivative of glucose or galactose, respectively. We shall designate the three compounds shown, by the letters *A*, *B*, and *C*, and the pseudo rate constants for the kinase and transferase by K_1 and K_2. Then, provided that (*a*) the galactose con-

centration is sufficiently far below the saturation level for the kinase enzyme so that the proportionality between reaction rate and substrate concentration is maintained, (*b*) the concentrations of ATP and UDP-glucose are constant, and (*c*) all the radioactive UDP-galactose formed is trapped by a constant excess of nonradioactive carrier, the following relationships hold (see *Appendix*).

$$A = A^\circ \exp(-K_1 t), \tag{1}$$

$$B = \frac{K_1 A^\circ}{K_2 - K_1} [\exp(-K_1 t) - \exp(-K_2 t)] \tag{2}$$

$$C = \frac{K_1 K_2 A^\circ}{K_2 - K_1} \left[\frac{\exp(-K_2 t) - 1}{K_2} - \frac{\exp(-K_1 t) - 1}{K_1} \right], \tag{3}$$

where K_1 and K_2 are parametric, pseudo first-order constants, t is the elapsed time, and A° is the initial concentration of radioactive substrate added to the tube.

These relationships predict a simple functional time dependence for the concentrations of galactose and its two derivatives in the system described.

Methods and Materials.—Freshly drawn human blood was heparinized and centrifuged. The buffy coat and plasma were discarded, the red cells were washed twice and resuspended in two volumes of a solution containing 4.4×10^{-3} *M* glucose, 1.36×10^{-1} *M* sucrose, 5.4×10^{-4} *M* Na_2HPO_4, 5.5×10^{-4} *M* KH_2PO_4, 5.0×10^{-4} *M* Tris, 2.7×10^{-3} *M* KCl, 3.0×10^{-4} *M* $MgSO_4$, and 5.5×10^{-5} *M* $CaCl_2$, at pH 7.3. Three-tenths ml of this cell suspension was delivered into each of a set of test tubes, incubated for 5.4 minutes at 37°C, and centrifuged. The purpose of this preliminary incubation is to allow glucose metabolism to furnish an amount of ATP and UDP-glucose sufficient to maintain these compounds at a constant concentration throughout the reaction. The supernatant buffer was removed, leaving about 0.1 ml of packed cells. Five μl of UDP-gal (60 μmoles/ml, Calbiochem) were added to the packed cells to act as a trap for radioactivity, and the cells were alternately quick-frozen in a CO_2 acetone bath and thawed three times to ensure complete lysis. The tubes were equilibrated for 5.0 minutes at 37°C, 2 μl of suitably diluted gal-1-C^{14} (0.215 μmole/ml, 34.9 mc/mmole, Nuclear-Chicago) were added, and incubation was continued for various times. The total red cell concentration in the reaction mixture was $(7.2 \pm 0.2) \times 10^9$/ml. The reaction was stopped by plunging the tubes into boiling water (94°C) for four minutes. The residue was extracted twice with 0.25 ml of distilled water and the extracts were combined. Less than 1 per cent of the radioactivity remained in the residue. Fifty μl from each tube were applied in a thin line to strips of Whatman DE81 anion exchange paper, 1.5 cm wide × 27 cm long. Ascending chromatography was carried out overnight in a cold room, using 0.015 *M* $Na_2HPO_4 \cdot 7H_2O$ and 0.015 *M* citric acid buffer at pH 3.8. The strips were dried, sectioned, and counted in a Nuclear-Chicago mark I liquid scintillation counter at an efficiency of approximately 75 per cent. Duplicate reaction vessels were always employed, and values obtained on the replicate chromatograms usually agreed within about 5 per cent. Figure 1 demonstrates the radioactivity distribution of a typical chromatogram.

When unlysed cells were used, a similar procedure was employed. The initial freezing and thawing step was omitted, and total volumes varying between 0.10 and 0.60 ml were used in different experiments, the diluent consisting of two parts of the previously described glucose buffer, to one part of packed cells.

Results.—(1) *Determination of the region of galactose saturation for the kinase enzyme:* Experiments were performed to determine the concentration limits for galactose lying sufficiently below the kinase saturation region for equations (1)–(3) to be applicable. Varying amounts of radioactive galactose (A°) were added to tubes containing standard human red cell lysates prepared as described, plus the carrier UDP-galactose trap. After 5.4 minutes incubation at 37°C, samples were taken and chromatographed, and the amounts of gal-1-PO_4 (*B*) and UDP-

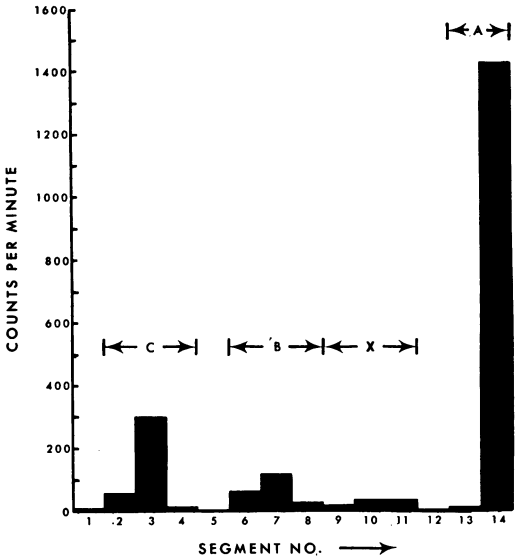

FIG. 1.—Typical plot of the distribution of radioactivity resulting from the chromatography of a sample obtained as described. *A*, *B*, and *C* represent the radioactive peaks corresponding to galactose, galactose-1-PO_4, and uridine diphosphogalactose, respectively. The region represented by *X* appears to be an impurity which is present in the original C^{14}-galactose, but which does not seem to interfere with the experiments here described.

galactose (*C*) produced were measured. The results are shown in Figure 2. They demonstrate that at the lowest substrate concentration utilized, the amount of *A* used in 5.4 minutes displays the proportionality to $A°$ required by the "below-

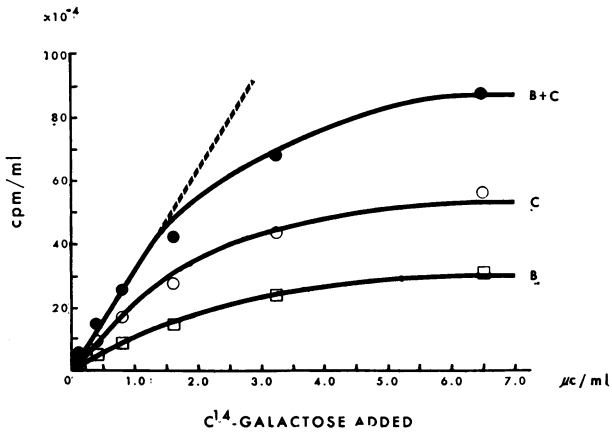

FIG. 2.—Determination of the saturation region of the kinase enzyme with respect to $A°$, the initial concentration of substrate, which in this case is C^{14}-galactose. Varying amounts of C^{14}-galactose were added to standard red cell lysates from a normal person. The total volume in the reaction mixture was 0.11 ml. The tubes were incubated for 5.4 min at 37°C, and then the total amounts of radioactive *A*, *B*, and *C* produced per ml of reaction mixture were determined. These are plotted against $A°$, the concentration of radioactivity added. The amount of C^{14}-galactose consumed, i.e., $A°-A$, is given by the sum of $B + C$. The lowest concentration used was selected as being sufficiently below the saturation region for eqs. (1), (2), and (3), to be valid. The $(B + C)$ curve drawn was calculated by means of eq. (8), while the solid circles indicate the actual experimental points.

saturation" formulation (eqs. (1)–(3)). The data of Figure 2 also demonstrate that even when the kinase enzyme is saturated by galactose, the transferase enzyme shows no indication of approaching saturation with respect to its substrate, for in that case the concentration of B would approach or even exceed that of C.

(2) *Determination of K_1, the rate constant for galactokinase, in normal red cells:* The time course of variation in concentration of A, B, and C was followed in normal subjects using the lowest concentration of C^{14}-galactose so that "below-saturation" kinetics apply. A typical set of data for A is presented in the semilogarithmic plot of Figure 3, demonstrating that the data obey the expected exponential relationship of equation (1). Determination of K_1 on a single person on six different days spread over an interval of several weeks yielded a value of 0.0602 ± 0.0014 per minute. Determination of K_1 on a series of four different persons on various days yielded virtually the same mean value for K_1, but with a standard deviation increased to ±0.007.

(3) *The rate constant for the transferase enzyme, K_2, in normal red cells.* In Figure 4 are presented data for the time course of B and C in the same experiment as shown in Figure 3. The value of K_2 was calculated from the equations (2) and (3). The curves shown in Figure 4 are the theoretical curves calculated from these equations while the circles and squares are the actual experimental points. It is obvious that the two constants K_1 and K_2 accurately determine the concentrations of all three reactants at any time throughout the period of study. The value for K_2 obtained for a single subject over a several-week interval is 0.487 ± 0.0148 minute^{-1}, and the mean for a group of four normal subjects was 0.55 ± 0.10 minute^{-1}.

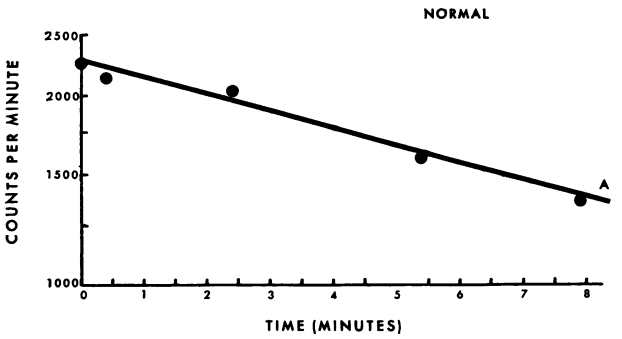

FIG. 3.—Typical semilogarithmic plot of the C^{14}-galactose (designated as A) disappearance at 37°C in a standard red cell lysate from a normal human subject under conditions far below the region of galactose saturation. This curve permits determination of K_1 from eq. (1).

(4) *Results on red cells of a galactosemic patient:* Similar studies on a patient with galactosemia were performed. The data for disappearance of A were virtually identical to those of the normal subject in Figure 3. The variation of B and C with time for the galactosemic patient are shown in Figure 5, in which no transferase activity whatever is detectable so that the concentration of B rises steadily, a

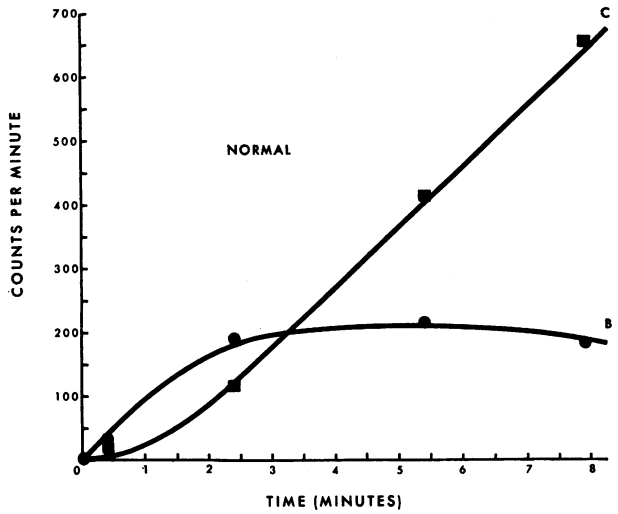

FIG. 4.—Typical plots of the time course exhibited by the concentrations of C^{14}-galactose-1-PO_4 (B) and C^{14}-UDP-galactose (C) at 37°C in a standard cell lysate from red cells of a normal person under conditions far below galactose saturation. The ordinate represents the mean number of counts per minute (cpm) in an aliquot containing one tenth of the reaction mixture. These curves permit calculation of K_1 and K_2 from eqs. (2) and (3). The points indicated are the actual experimental values, while the curves are computed theoretically.

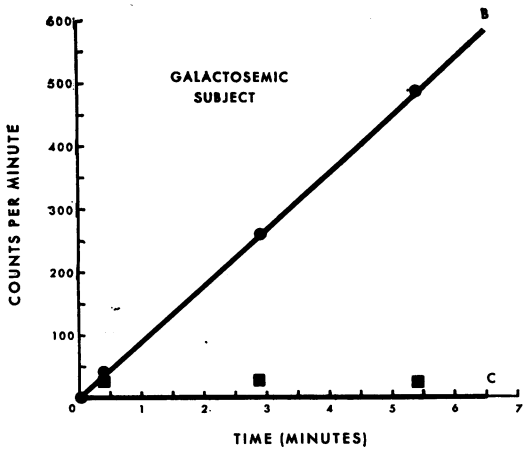

FIG. 5.—A typical experiment identical to that of Fig. 4, but using red cells of a galactosemic subject. There is no conversion of galactose-1-PO_4 (B) to UDP-galactose (C), so the concentration of B rises steadily.

result expected from earlier studies.[3] The values for the constants are presented in the summary of Table 1.

(5) *Results on heterozygous carriers:* The foregoing measurements lead to the expectation that persons heterozygous for the galactosemia defect should display a K_1 value similar to, and a K_2 value that is one half of, the corresponding values for normal subjects if a simple gene-dose relationship obtains. Previous work had demonstrated that the heterozygotes have transferase activity intermediate between

the normal and the homozygous defective but had not established the quantitative relationships of the constants.[3a, f] Measurements were conducted on the father and the mother of the galactosemic patient studied. The A curve was again virtually identical to that of Figure 3. The B and C curves are shown in Figure 6, from which it is evident that the concentration of B is always higher and that of C lower than the corresponding values from a normal patient. Calculation of K_2 for the heterozygous subjects yields a value which within experimental uncertainty is just half that of the normal value. Table 1 summarizes these data.

TABLE 1

VALUES FOR THE GALACTOKINASE AND GAL-1-PO$_4$ URIDYL TRANSFERASE FOR RED CELLS OF HUMAN SUBJECTS OF VARIOUS GENETIC CONSTITUTIONS WITH RESPECT TO THE GALACTOSEMIC DEFECT

	K_1 (min^{-1})	K_2 (min^{-1})
Five normal subjects	0.059 ± 0.007	0.54 ± 0.09
1-Galactosemic patient	0.049 ± 0.003	0.0
1-Heterozygous carrier (father)	0.043 ± 0.004	0.23 ± 0.02
1-Heterozygous carrier (mother)	0.052 ± 0.004	0.25 ± 0.04

Where measurement on a single patient is given, the standard deviation is calculated on the basis of repeated determinations on the same subject. The cell density in the reaction vessels was 7.2 × 10^9/ml.

(6) *Results with higher substrate concentrations—calculation of the Michaelis-Menten equilibrium constant for galactokinase:* At galactose concentrations sufficiently high so that equations (1)–(3) are no longer valid, equation (8) of the *Appendix* can be used. From a series of observations at various initial concentrations of galactose like those presented in the data of Figure 2, one can solve equation (8) for K_1 and for K_M, the equilibrium constant for dissociation of the complex between galactose and its kinase. The value obtained for K_1 is 0.055 ± 0.003 minute^{-1} which agrees well with that in Table 1. The value for K_M obtained by using the value for K_1 from Table 1, is (3.76 ± 0.43) × 10^6 $\frac{\text{cpm}}{\text{ml}}$. Using the value for the specific activity of the C^{14}-galactose furnished by the supplier, K_M becomes 6.5 × 10^{-5} moles/liter. This value is lower than others previously reported for this enzyme in other systems.[2a–f] The values for the specific cellular pseudo rate constants, K_1^c and K_2^c, as defined in equation (10) are 8.2 × 10^{-12} min^{-1} cell^{-1} cm^3 and 75.0 × 10^{-12} min^{-1} cell^{-1} cm^3, respectively.

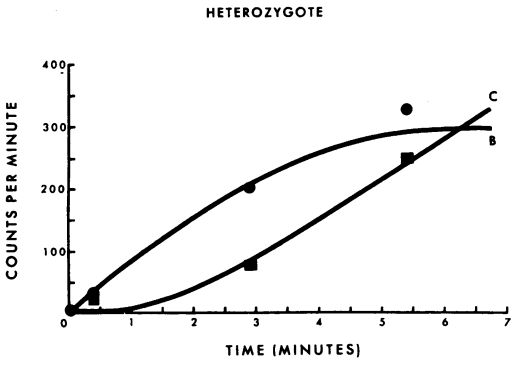

FIG. 6.—Change in concentrations of galactose-1-PO$_4$ (B) and UDP-galactose (C) in a standard red cell lysate taken from a heterozygous person, and treated with C^{14}-galactose at concentrations far below saturation as in Fig. 4. The constant K_2 has been calculated from eqs. (2) and (3), and K_1 was obtained from eq. (1). The points shown are the experimental measurements and the curves are the theoretical ones calculated from eqs. (2) and (3) using the averaged values of K_1 and K_2. The curves differ markedly from the normal shown in Fig. 4, in that B is higher, C is lower, and their intersection occurs at 6.4 instead of 3.2 min.

(7) *Comparison of cell lysates and whole cells:* Experiments were performed using the standard procedure but omitting the initial lysis of the cells in order to determine whether K_1 is altered thereby. The value obtained for K_1 in three different normal subjects was 0.050 ± 0.010 min^{-1}, a value not significantly different from that in Table 1. Therefore, it appears that for the kinase enzyme the activity of the cell lysates here described is the same as that of whole cells. Presumably then active transport of galactose is not important at the galactose concentrations here employed.

(8) *Other properties of the reaction:* If the reaction chain in lysed cells is permitted to continue beyond ten minutes at 37°C, a decrease in the rate of the initial kinase reaction occurs. The addition of ATP will prevent this decay or restore the original reaction velocity after the inhibition has been allowed to occur. K_2 as here defined, has been found to behave as an inverse function of the concentration of UDP-galactose concentration utilized in the trap, and can be doubled by a decrease in concentration of that compound. Lowering the temperature from 37 to about 4°C produces a large decrease in K_2, with less effect on K_1. The quantitative responses of these rate constants to changes in these and other parameters will be described later.

These enzymatic activities of whole human red cells, and particularly that of the transferase enzyme, decay on storage at 4–5°C. Differences in K_2 are detectable within 24 hours, regardless of whether the cells are stored as whole heparinized blood, or in the glucose-buffer here employed. However, the frozen lysates prepared as above preserve both enzyme activities unchanged for at least nine days and probably much longer. This ability of frozen lysates to be stored constitutes a great convenience in the study of large human populations.

Preliminary experiments on cells grown in tissue culture reveal these to possess kinase and transferase activities approximately 100 times greater than those of erythrocytes.

Discussion and Conclusions.—The data reveal that red cell lysates quantitatively obey expected theoretical relationships governing the rate of the first steps of galactose metabolism and that simple and reproducible determination of cellular rate constants is possible. It is of interest that the rate constant for the second step in the reaction chain is normally ten times that of the first, a situation which would preclude accumulation of the toxic intermediate, galactose-1-PO_4. The rate constants for the kinase enzyme have been shown to be the same for red cells from normal, galactosemic, and heterozygous subjects, and from whole and lysed cells. The transferase enzyme obeys a simple, gene-dosage relationship. The methodology appears useful for study of gene-enzyme relationships and regulatory processes in metabolic chains from different types of mammalian cells, and for heterozygote screening in large populations.

Appendix.— In these experiments the ATP and UDP-galactose are furnished by the cells. Their concentrations which are unknown but presumably constant, are absorbed in the values of the pseudo reaction rate constants so calculated. Justification of this procedure arises from the agreement of the data with theoretical expectation under the particular conditions specified. In the reaction sequence

$$A + E_1 \overset{K_M}{\rightleftarrows} AE_1 \overset{k_1}{\rightarrow} B \overset{K_2}{\rightarrow} C, \qquad (4)$$

E_1 is the first enzyme in the sequence; K_M is the Michaelis-Menten equilibrium constant; k_1 is the rate constant for reaction of the enzyme-substrate complex, AE_1; and K_2 is the over-all rate constant for the second step of the chain.

$$-\frac{dA}{dt} = k_1(AE_1) = \frac{k_1(E^\circ)(A)}{K_M + (A)}, \tag{5}$$

where E° is the original concentration of the enzyme. For low substrate concentrations, $A \ll K_M$, and can be neglected. If we define the over-all rate constant

$$K_1 = \frac{k_1 E^\circ}{K_M}, \tag{6}$$

equation (5) is integrated to yield

$$A = A^\circ \exp(-K_1 t) \tag{7}$$

as in (1), and the expressions for B and C in equations (2) and (3), respectively, are obtained in straightforward fashion from the corresponding pseudo first-order rate equations for the last two steps of (4).

If the concentration of substrate A is too large to be ignored in (5), that equation can be integrated as it stands to yield

$$A^\circ - A = \exp\left(\frac{A^\circ - A}{K_M} - K_1 t\right). \tag{8}$$

The corresponding equations for B and C can be obtained by graphical or machine integration. It is obvious that (5) also reduces to a simple form which permits ready solution of the appropriate differential equations when $A \gg K_M$, but such conditions have not been used in the experiments reported here.

The values for K_1 and K_2 have been defined in terms of the absolute concentrations of the respective enzymes. To convert to a cellular basis we rewrite (6) as

$$K_1 = \frac{k_1 E_1{}^c n}{K_M} \tag{9}$$

where $E_1{}^\circ$ is the concentration of enzyme per cell, and n the cell density. Therefore we can define the pseudo rate constant per unit cell density as

$$K_1{}^c = \frac{K_1}{n}. \tag{10}$$

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