Enzyme Kinetics in Mammalian Cells

III. REGULATION OF ACTIVITIES OF GALACTOKINASE, GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE AND URIDINE DIPHOSPHOGALACTOSE-4-EPIMERASE IN HUMAN ERYTHROCYTES [1]

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ABSTRACT The sequential enzyme assay as previously described has been used to study various effects on the three enzymes in human red cells involved in the phosphorylation of galactose: galactokinase, galactose-1-phosphate uridyl transferase and uridine diphospho-galactose-4-epimerase.

1. Enzyme activities in undiluted lysates appear to reflect the respective activities in whole cells.

2. Added extracellular Gal-1-P, G-1-P, UDPGal and UDPG do not affect enzyme activities in whole cells.

3. The kinase and transferase enzymes do not appear to be associated with the membrane fraction of the red cells.

4. Galactokinase activity is inhibited by G-6-P and Gal-1-P, but not by glucose, G-1-P, UDPG, UDPGal, UTP or NAD^+. It is inhibited by ATP and ADP in high concentration.

5. Galactose-1-phosphate uridyl transferase activity is inhibited by G-1-P, G-6-P, UDPG, UDPGal, ATP, and ADP. It is not affected by UTP, NAD^+, or galactose.

6. Uridine diphospho-galactose-4-epimerase activity is inhibited by UDPG, ATP, ADP, UTP and NADH. It is stimulated by NAD^+ and possibly by Gal-1-P. It is unaffected by G-1-P, G-6-P.

7. The rates of the three reactions decrease with decreasing temperature. The activities of transferase and epimerase are inactivated at the same rate, the kinase activity is inactivated more slowly.

8. Dilution experiments indicate the presence in lysates of a pool of UDPG (or, possibly UDPGal) which regulates the activities transferase and the epimerase enzymes.

9. Results of dilution experiments suggest that the radioactive product of the transferase enzyme is different from commercially available UDPGal-u-^{14}C.

10. ATP, UTP and UDPG interact with some substance(s) in the red cell lysate to cause a time dependent inactivation of the epimerase. These interactions are the result of glucose metabolism.

The preceding communications in this series (Puck and Hill, '67; Hill and Puck, '70) describe a quantitative method for the simultaneous determination of three enzymes which catalyze a series of consecutive reactions in the galactose pathway as follows:

$$1.\ \text{Gal}^* + \text{ATP} \xrightarrow[k_1]{\text{Gal kinase}} \text{Gal}^*\text{-1-P} + \text{ADP}$$

$$2.\ \text{Gal}^*\text{-1-P} + \text{UDPG} \xrightarrow[k_2]{\text{Gal-1-P uridyl transferase}} \text{UDPGal}^* + \text{G-1-P}$$

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$$3.\ \text{UDPGal}^* \xrightarrow[k_3]{\text{UDPGal-4 epimerase NAD}^+} \text{UDPG}^* \qquad k_{3R}$$

The asterisks mark the flow of radioactivity in this system.

It is the purpose of this report to (1) determine conditions which permit reproducible determinations of the three rate constants independent of variations in the small molecule pools; (2) define the parametric variability of the system; and (3) devise a system which can be used in comparing cells of different tissues, in different parts of the life cycle, of different ploidy and different chromosomal constitution. Such a system would then be applicable to other genetic pathways in various cells and tissues.

In these studies, undiluted lysates of erythrocytes have been employed with the object of developing a system which would mimic the *in vivo* system as closely as possible. The rate constants (k_1, k_2 and k_3, respectively) as measured *in vitro* appear to be equivalent to the *in vivo* constants for the three enzymes with no additions to the incubation mixture but higher for the third enzyme if NAD^+ is added (k_3). The *in vitro* experiments indicate the presence in red cell lysates of at least one and possibly more inhibitors of the epimerase.

MATERIALS AND METHODS

The materials and methods are essentially those used in the preceding paper (Hill and Puck, '70), where it was shown that the nature of the medium in which the cells are washed before the enzymes are assayed is important in the quantitative determination of the three rate constants.

Erythrocyte lysates were prepared by two methods. In the glucose method, the erythrocytes were collected as previously described (Puck and Hill, '67; Hill and Puck, '70) and washed twice in a buffer containing glucose (Puck and Hill, '67). They were then resuspended in two volumes of the same buffer and incubated at 37°C for five minutes. The cells were separated by centrifugation and the packed cells, as free from buffer as possible, were lysed by freezing and thawing (Puck and Hill, '67). In the glucose-free method, the buffer contained an equimolar amount of sucrose instead of glucose (Hill and Puck, '70). The cells were washed two or three times in this buffer and lysates were prepared as above omitting the incubation of whole cells at 37°C.

Unless otherwise noted, the relative quantitative effects of the various inhibitors reported here are unchanged by the method of preparation. The flow of radioactivity was halted by the presence of either 2.7×10^{-3} M UDPGal, 1.82×10^{-4} M UTP or 2.7×10^{-5} M UDPG. As was shown in the preceding paper, the rate of removal of radioactive UDPG is slow enough to be neglected, so in some experiments no trapping agent for radioactivity is employed.

The enzyme assay system has been described in detail elsewhere (Puck and Hill, '67; Hill and Puck, '70). Therefore, it will only be briefly summarized here. The enzymes were assayed in three ways: (1) the simultaneous assay (Puck and Hill, '67; Hill and Puck, '70) was employed to measure the activities of the kinase, transferase and epimerase at the same time in the same reaction mixture. In this case, 2.9×10^{-6} M Gal-1-^{14}C was the initial substrate, 1.8×10^{-3} M ATP was added to saturate the kinase and 2.1×10^{-3} M NAD^+ was added to saturate the epimerase. It was not necessary to add UDPG to saturate the transferase as there is enough present in the lysates. Pseudo first order kinetics now apply for all three enzymes and the rate constants were calculated from the radioactivity in the products as previously described (Hill and Puck, '70). (2) The simultaneous assay was employed to measure the activities of the kinase and the transferase at the same time (Puck and Hill, '67). In this case, Gal-1-^{14}C was the initial substrate, 1.8×10^{-3} M ATP was added and 2.8×10^{-3} M UDPGal was also present to prevent the flow of radioactivity into any other intermediates. (3) Epimerase activity was assayed separately using 4.5×10^{-7} M UDPGal-u-^{14}C as the substrate in the presence of 2.1×10^{-3} M NAD^+; calculation of k_3 utilizes an analogous algorithm as in the simultaneous assay.

Rate constants on a per cell basis are calculated as follows: For lysed red cells, the pseudo-first order rate constants calcu-

lated by computer (Puck and Hill, '67; Hill and Puck, '70) are simply divided by the cell concentration in the lysate or red cell pack. Experiments involving whole cells, however, are complicated by the fact that the cells were suspended in two volumes of buffer. In this case, the concentration of gal-1-^{14}C is the same as that in the lysates, but the effective concentration of kinase is one third that of the lysates because of the dilution with two volumes of buffer. Thus k_1^c is obtained by dividing the observed k_1 by the cells/ml. Once the galactose has been converted to gal-1-P, however, the effective concentration of intracellular gal-1-P and transferase is the same as that in the lysates, *i.e.*, three times the concentration of cells/ml. The measured k_2, then, is divided by the (cells/ml) × the dilution factor to give $k_2 \cdot {}^c k_5{}^c$ is calculated in the same manner as $k_2{}^c$. This reasoning is based on the assumption that transport of galactose into the red cells is not rate limiting. Experiments to be described indicate that this is indeed the case.

EXPERIMENTAL RESULTS

A. *Studies with whole cells*

1. Measurement of rate constants in whole cells.

Table 1 shows a comparison of the pseudo-first order rate constants in lysates and whole cells.

The rate constants measured for the kinase (k_1) and transferase (k_2) in lysates reflect accurately the activity of these enzymes in the whole cell. The rate constant for epimerase (k_3), however, is lower in whole cells than in lysates. In lysates maximum activity is obtained by adding NAD^+. Apparently, then, the epimerase activity in whole cells is maintained at a submaximal level by lack of NAD^+ and by the presence of inhibitors (see below). Preliminary experiments with lysates in which k_3 was measured in the absence of added NAD^+ indicate a value of approximately 8.4×10^{-12} min^{-1} cell^{-1} ml^{-1}. This is close to the value found for whole cells. The transferase may also not be acting at maximum rate in whole cells or lysates as shown by dilution experiments reported below which indicate the presence of a transferase inhibitor in the lysates (probably UDPG). The fact that k_1, the rate constant for kinase, is the same in whole and lysed cells indicates that transport of Gal is not rate-limiting in this pathway.

2. Effect of sugar intermediates and metabolism of galactose in whole cells.

1.5×10^{-2} M Gal-1-P and 1.5×10^{-2} M G-6-P, concentrations which inhibit k_1 significantly in lysates, have no effect on k_1 of whole cells. Likewise, 2.8×10^{-3} M UDPGal, 2.8×10^{-3} M UDPG, 1.5×10^{-2} M G-1-P and 1.5×10^{-2} M G-6-P, concentrations which substantially inhibit k_2 in lysates have no effect on this rate constant in whole cells. Presumably, these intermediates do not freely diffuse into the cells. If the kinase enzyme were located in a region of the cell exposed to the external environment, such as on the cell surface, then one might expect it to be inhibited by Gal-1-P and G-6-P even if these compounds do not enter the cell. When red cell ghosts were sedimented by centrifugation and the resulting supernatant was assayed for kinase and transferase activity, there was no significant difference in k_1 assayed in the whole lysates and in supernatants suggesting that these enzymes are not tightly bound, if at all, to the membranes.

B. *Studies with lysates*

1. Inhibition by metabolites.

A series of experiments was performed to determine the effect of added interme-

TABLE 1

Rate constants in whole cells and lysates

	Whole cells	Lysates
k_1^c	$(9.7 \pm 0.95) \times 10^{-12}$ min^{-1} cell^{-1} ml^{-1} (3)	$(9.2 \pm 1.5\ ) \times 10^{-12}$ min^{-1} cell^{-1} ml^{-1} (10)
k_2^c	$(1.6 \pm 0.28) \times 10^{-10}$ (1)	$(1.4 \pm 0.18) \times 10^{-10}$ (10)
k_3^c	$(1.1 \pm 0.50) \times 10^{-11}$ (1)	$(6.2 \pm 1.1\ ) \times 10^{-11}$ (9)

Integers in parentheses are the number of blood samples. Whole cells were suspended in two volumes of glucose buffer. Incubation was for five minutes with 2.9×10^{-6} M Gal-1-^{14}C; final volume 0.315 ml. Lysates were prepared by the glucose buffer method. Incubation was for five minutes with 2.9×10^{-6} M Gal-1-^{14}C, 1.8×10^{-3} M ATP, 2.1×10^{-3} M NAD^+, 0.1 ml lysate; final volume 0.105 ml.

diates in the glucose and galactose pathways on the pseudo-first order rate constants k_1, k_2 and k_3. It should be noted that undiluted erythrocytes contain pools of some of the compounds studied. Until the substance added reaches a concentration on the order of that of its pool or greater, its effect will not be felt.

a. *Effects of sugars and sugar phosphate intermediates.* Figure 1 summarizes the results of a number of typical experiments in which the effects of various intermediates on the reaction rates were measured and compared on a molar basis. Glucose at concentrations as high as 9.3×10^{-3} M has no effect on any of the three rate constants. Galactose, at concentrations as high as 1.8×10^{-2} M, has no apparent effect on the course of the transferase and epimerase reactions as there is no change in the chromatograph pattern when either Gal-1-P-^{14}C or UDPGal-^{14}C is used as substrate, and varying concentrations of galactose are added to the reaction mixture.

G-1-P (fig. 1) has a marked inhibitory effect on the transferase enzyme. In fact, this compound and UDPG are the most potent inhibitors of k_2 found, on a molar basis. However, G-1-P has no effect on k_1 at concentrations up to 1.4×10^{-2} M, and preliminary experiments indicate that it does not affect k_3 at concentration up to 2×10^{-3} M. G-6-P inhibits both k_1 and k_2, but, again, preliminary experiments indicate no effect on k_3 at concentrations of less than 6×10^{-3} M. Gal-1-P inhibits k_1. Preliminary work indicates it may actually stimulate k_3. In any case, it appears to stimulate the disappearance of UDPGal-^{14}C brought about by both the epimerase enzyme and by the reverse transferase reaction.

Ballard (Ballard, '66) has reported the inhibition of kinase in pig liver by Gal-1-P. Cuatrecasas and Segal (Cuatrecasas and Segal, '65) studying newborn and adult rat liver found that 1×10^{-3} M Gal-1-P gave 50% inhibition in the former and 5×10^{-3} M produced the same inhibition in the latter. Bertoli and Segal (Bertoli and Segal, '66) have observed that G-1-P inhibits the transferase in adult rat liver.

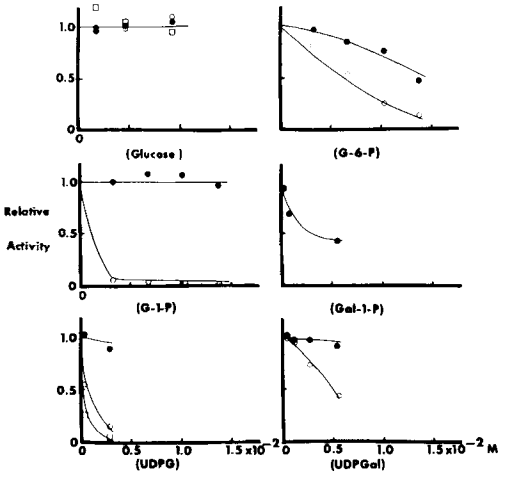

Fig. 1 The effect of various substances on k_1, k_2 and k_3. Erythrocytes were harvested, washed twice in glucose buffer, resuspended in two volumes of the same and incubated five minutes at 37°C. Cells were reharvested and lysed. Lysates were equilibrated five minutes at 37°C at which time the substrates were added. Each reaction mixture (except the bottom two) contained 0.1 ml lysed erythrocytes, 1.8×10^{-3} M ATP, 2.1×10^{-3} M NAD^+, 2.8×10^{-5} M UDPG, 3.9×10^{-6} M Gal-1-^{14}C and increasing amounts of inhibitor as indicated. In the bottom left, the other ingredients were the same but UDPG was added as indicated. In the bottom right, only 3.9×10^{-6} M Gal-1-^{14}C was present in all incubations, UDPGal was added as indicated. Total volume for each incubation was 0.11 ml. Incubations were for five minutes except for the Gal-1-P and UDPG curves where incubation was ten minutes. The initial rate constants for 0 inhibitor were as follows: Top left k_1, 0.078; k_2, 0.91; k_3, 0.17. Top right: k_1, 0.095; k_2, 1.12. Middle left: k_1, 0.070; k_2, 1.12. Middle right: k_1, 0.069. Bottom left: k_1, 0.067; k_2, 0.61; k_3, 0.18. Bottom right: k_1, 0.067; k_2, 0.88 min^{-1}. ● k_1, ○ k_2, □ k_3.

b. *Effect of nucleotide sugars.* UDPG inhibits both k_2 and k_3 but has no marked effect on k_1. It is most effective against k_3. UDPGal also inhibits k_2 but requires greater concentrations than UDPG for the same effect. The red cell lysates must contain a pool of UDPG since maximum activity is obtained when no UDPG is added. Since UDPG is a reactant in the transferase reaction, it must be present in concentra-

Abbreviations

ADP, ATP, adenosine di- and triphosphate, respectively, UDPG, UDPGal, uridine diphosphoglucose and -galactose, respectively, G-1-P, gal-1-P., glucose- and galactose-1-phosphate, respectively, G-6-P, glucose-6-phosphate, NAD^+, nicotinamide adenine dinucleotide, NADH, reduced nitotinamide adenine dinucleotide, UMP, UDP, UTP, uridine mono-, di-, and triphosphate, respectively, Gal, galactose.

tions high enough to furnish maximum activity or even to be on the inhibitory side (see below).

Beutler and Baluda (Beutler and Baluda, '66) found maximum activity for transferase in human erythrocyte hemolysates at 5×10^{-4} M UDPG. In these studies, this concentration gives about 50% inhibition. They find 50% inhibition with 5×10^{-3} M. Maxwell (Maxwell, '57) reported that calf-liver epimerase is not inhibited by concentrations of UDPG of less than 3.5×10^{-4} M, but Cohn and Segal (Cohn and Segal, '69) find considerable inhibition of the rat liver enzyme with 1×10^{-4} M.

c. *Effect of nucleotides.* ATP inhibits k_1 above 1×10^{-2} M (fig. 2). It is necessary to add ATP, however, in order to sustain pseudo-first order kinetics for more than four minutes. When 1.8×10^{-3} M ATP is added to the reaction mixture, the kinase reaction follows first order kinetics for at least 16 minutes. This concentration of ATP has little, if any, effect on the transferase reaction. However, higher concentrations are inhibitory. ATP also inhibits the epimerase reaction. In fact, on a molar basis it is more inhibitory to the epimerase reaction than to the other two reactions. ADP displays much the same effect as ATP with respect to k_1, k_2 and k_3. Cuatrecasas and Segal ('65) have reported that ATP inhibits the rat liver kinase at concentrations greater than 2×10^{-2} M. The human red cell enzyme would seem to behave similarly. Ballard ('66) has observed the inhibition of kinase by ADP in pig liver.

UTP (fig. 3) markedly inhibits k_3 with little or no effect on k_1 and k_2. Tsai (Tsai, Holmberg and Ebner, '70) observed that UTP, also UMP and UDP inhibit the epimerase in bovine mammary gland. UMP also inhibits rat liver epimerase (Cohn and Segal, '69).

Figure 4 shows that NAD^+ has little effect on k_1 or k_2 while stimulating k_3. This is in keeping with the findings of Ng et al. (Ng, Donnel, Hodgman and Bergren, '67) that the epimerase in erythrocytes of human adults requires added NAD^+. The figure also indicates that the pool of NAD^+ present in the erythrocytes is not sufficient to permit maximum activity of this enzyme, unless, of course, endogenous NAD^+ is destroyed during preparation of the lysate. NADH inhibits k_3 without affecting k_1 or k_2 (fig. 4). Maxwell ('57) found that 1×10^{-4} M NADH gave about 50% inhibition of calf liver epimerase. The results in this system are analogous.

d. *Effect of other substances.* Addition of Mg^{++} has no effect on k_1 or k_2 up to 2×10^{-3} M. 2×10^{-2} M $MgCl_2$ is inhibitory to both the kinase and the transferase. Reducing agents such as 2-mercaptoethanol and dithiothreitol have no effect on any of the three enzymes in our system al-

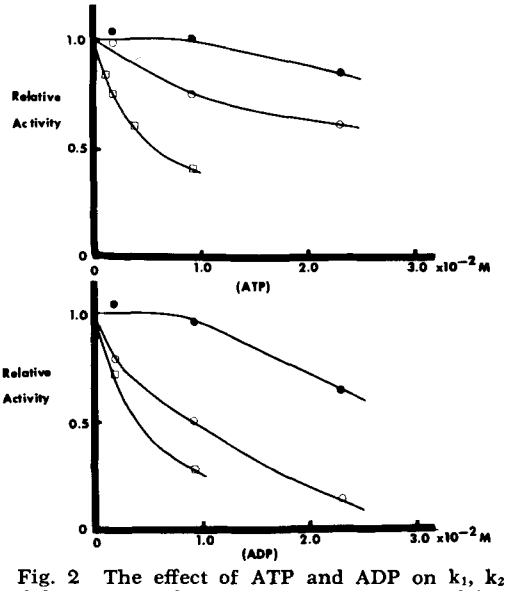

Fig. 2 The effect of ATP and ADP on k_1, k_2 and k_3. Top graph: Determination of k_1 and k_2: Lysates prepared and treated as in figure 1. Incubation mixtures contained 0.1 ml lysate, 2.1×10^{-3} M NAD^+, 2.8×10^{-5} M UDPG, 3.9×10^{-6} M Gal-1-^{14}C. Total volume 0.11 ml. Incubation with substrates was for five minutes. Determination of k_3: Erythrocytes were harvested and washed three times with glucose-free buffer. Lysates were prepared directly and preincubated at 37°C for 15 minutes. Incubation mixtures contained 2.1×10^{-3} M NAD^+ and 6.8×10^{-7} M UDPGal-^{14}C. Incubation with substrates was for two minutes. These results are comparable to those obtained when lysates were prepared as in figure 1 but the values for k_3 are correspondingly higher by this method. Bottom graph: Lysates prepared as in figure 1. Incubation mixtures contained 0.1 ml lysate, 2.1×10^{-3} M NAD^+, 2.8×10^{-4} M UTP and 3.9×10^{-6} M Gal-1-^{14}C. Total volume 0.11 ml. Incubation with substrates was for five minutes. Initial rate constants for 0 inhibitor were as follows: Top graph: k_1, 0.0748; k_2, 1.00; k_3, 0.554. Bottom graph: k_1, 0.0568; k_2, 1.55; k_3, 0.172 min^{-1}. ● k_1, ○ k_2, □ k_3.

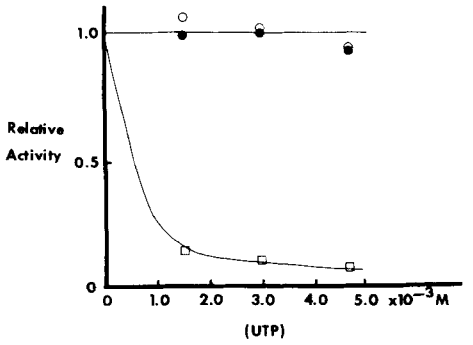

Fig. 3 The effect of UTP on k_1, k_2 and k_3. Erythrocyte lysates prepared and treated as described in figure 1. Incubation mixtures contained 0.1 ml lysate, 2.1×10^{-3} M NAD^+, 1.8×10^{-3} M ATP and 3.9×10^{-6} M Gal-1-^{14}C. Total volume 0.11 ml. Incubation with substrates was for five minutes. ● k_1, ○ k_2, □ k_3.

though both transferase and epimerase activities have required reducing agents for maximum activity in several mammalian systems (Bertoli and Segal, '66; Maxwell, '57; Kurahashi and Anderson, '58). Presumably the lysed erythrocyte suspension provides the necessary factors for the stability of these enzymes. Even in diluted erythrocyte lysates, dithiothreitol has no effect on any of the enzymes in our system.

2. Effect of temperature on the activities of the kinase, transferase and epimerase in lysates.

The effect of temperature on reaction rate is generally studied at saturating levels of substrate in order to determine the effect of temperature on dissociation of the enzyme substrate complex. The experiments reported here are performed at non-saturating substrate levels. Thus the interpretation of temperature curves is complicated by the fact that the overall rate of the reaction is a function not only of the rate of conversion of substrate to product but also of the affinity of the enzyme for its substrate. Nevertheless, it seemed of interest to determine what effect temperature has on the relative rates of the three reactions so that, in routine assays, the relative amounts of radioactive intermediates could be optimized. Figure 5 shows Arrhenius plots of the three pseudo first order rate constants vs. the reciprocal of the absolute temperature. Table 2 summarized the data in figure 5 and shows the relative rates at the different temperatures. The transferase and the epimerase are inactivated at very nearly the same rate while the kinase is inactivated more slowly with decreasing temperature.

3. Effect of dilution of lysates on the three enzyme activities.

When red cells are prepared by either the method utilizing glucose buffer or glucose free buffer, diluted in the same buffer and the simultaneous assay performed, k_1 is proportional to the lysate concentration but k_2 is higher than expected at higher lysates dilution. If a sufficiently large constant concentration of either UDPGal or UDPG is added, proportionality is now achieved for k_2 also (figs. 6, 7). These find-

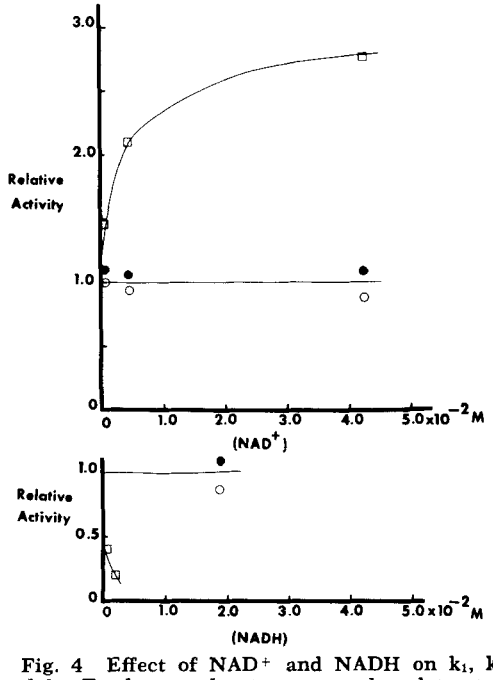

Fig. 4 Effect of NAD^+ and NADH on k_1, k_2 and k_3. Erythrocyte lysates prepared and treated as described in figure 1. Top graph: Incubation mixtures contained 0.1 ml lysate, 1.8×10^{-3} M ATP, 3.8×10^{-5} M UDPG and 3.9×10^{-6} M Gal-1-^{14}C. Total volume 0.11 ml. Incubation with substrate was for ten minutes. Bottom graph: k_1 and k_2 determined as in the top graph. For k_3, incubation mixtures contained 0.1 ml lysate, 2.8×10^{-5} M UDPG and 6.8×10^{-7} M UDPGal-1-^{14}C. Total volume 0.11 ml. Incubation was for ten minutes. Initial rate constants at 0 inhibitor: Top graph: k_1, 0.0569; k_2, 0.758; k_3, 0.0438. Bottom graph: k_1, 0.0569; k_2, 0.758; k_3, 0.060 min^{-1}. ● k_1, ○ k_2, □ k_3.

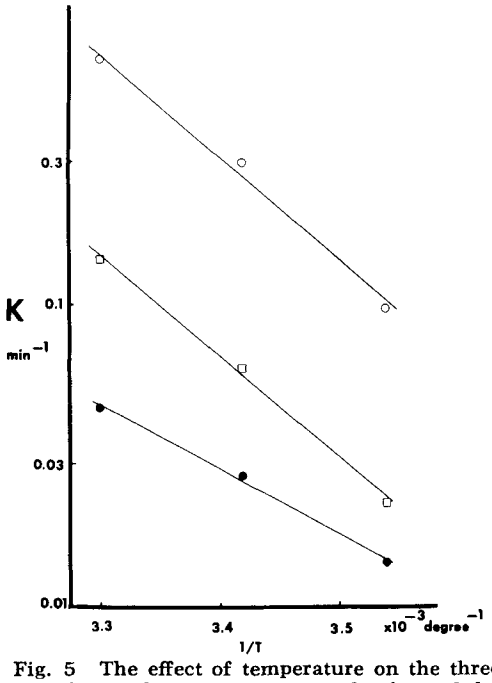

Fig. 5 The effect of temperature on the three pseudo-first order rate constants, k_1, k_2 and k_3. Lysates prepared and preincubated as in figure 2, k_3 curve. Substrates were added and incubation was continued at either 30°C, 20°C or 10°C. Incubation mixtures contained 0.1 ml lysate, 1.8×10^{-3} M ATP, 2.1×10^{-3} M NAD^+ and 3.9×10^{-6} M Gal-1-^{14}C in a total volume of 0.104 ml. Rate constants were calculated by averaging over duplicate samples at three time points at each temperature. ● k_1, ○ k_2, □ k_3.

ings suggest that either UDPGal or UDPG or both are present in the lysates and serve to regulate the activity of the transferase enzyme in the cells. Addition of enough of one or the other to cancel the effects of the diluting pool then brings about proportionality, thereby indicating no further inhibitors of these activities are present in the preparations.

With epimerase, the dilution kinetics are somewhat more complex. Consider first the assay for epimerase alone using UDPGal-^{14}C as substrate and NAD^+ as cofactor. When lysates are prepared in glucose buffer, k_3 is not proportional to the lysate concentration, indicating the presence of at least one inhibitor. Addition of 2.9×10^{-5} M UDPG to the diluted lysates alters the dilution profile by causing the values for k_3 to become more nearly proportional to the lysate concentration. These findings indicate that UDPG is present in the undiluted lysates in sufficient quantity to inhibit the epimerase. Since 2.9×10^{-5} M UDPG affects the dilution profile, but not enough to cancel the effects of diluting pools and bring about proportionality, the UDPG pool may be on the order of 10^{-5} M. On the other hand, it might be less than this and another inhibitor might also be present. 1.8×10^{-4} NADH, a concentration sufficient to inhibit 50%, does not affect the dilution profile of k_3 indicating that the pool of this compound in the lysates is not great enough to be inhibitory. Preparation of lysates using glucose-free buffer, dilution with the same and 15 minute pre-incubation produces proportionality with dilution for k_3 for most lysate preparations. This treatment does not eliminate all of the UDPG present in the lysates, however, since transferase, as measured by the simultaneous assay in such lysates is still active, and k_2 is proportional to the lysate concentration only in the presence of added UDPG under these conditions (fig. 7).

With glucose-free lysates, the dilution behavior of k_3 in the presence of UDPG depends upon which radioactive substrate is used (fig. 7). If Gal-1-^{14}C is the substrate, proportionality is obtained for k_1 and k_2, but k_3 acts as though an inhibitor is present. Under exactly the same conditions, however, if UDPGal-^{14}C is the substrate instead, k_3 is proportional to the lysate concentration. In other words, endogenously

TABLE 2

	Temp	k_1	k_2	k_3	$k_1:k_2:k_3$
	30°C	0.046 ± 0.0031	0.65 ± 0.063	0.14 ± 0.018	1:14:3
	20°C	0.027 ± 0.0025	0.30 ± 0.046	0.052 ± 0.010	1:11:23
	10°C	0.014 ± 0.00061	0.097 ± 0.019	0.022 ± 0.0088	1:7:1.6
Q_{10}	10°–20°	1.9	3.1	2.8	
	20°–30°	1.7	2.2	2.3	

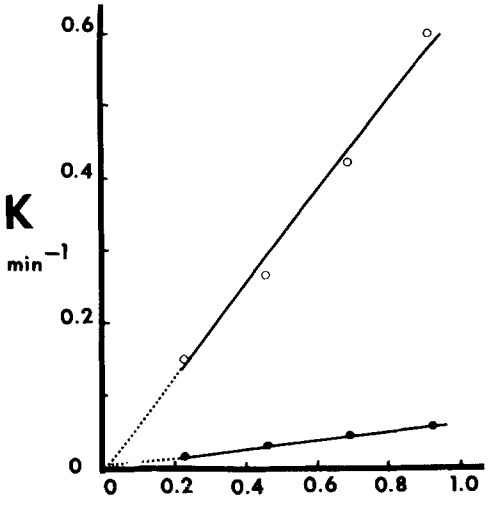

Fig. 6 Dilution of kinase and transferase activity. Lysates prepared as in figure 1. Lysates which were diluted with glucose buffer. Total volumes were 0.11 ml. Equilibration at 37°C for five minutes. Incubation with substrates for five minutes. Each incubation mixture contained 1.8×10^{-3} M ATP, 2.8×10^{-3} M UDPGal and 3.9×10^{-6} M Gal-1-^{14}C. ● k_1, ○ k_2.

produced UDPGal-^{14}C behaves differently from exogenously added UDPGal-^{14}C. These findings suggest that either the endogenous substrate is not UDPGal-^{14}C but is rapidly converted to it during preparations for chromatography or the transferase and epimerase activities are tightly coupled. Further, there may be present in the diluted lysates an inhibitor to which the altered substrate or coupled reaction is sensitive but to which the reaction driven by the exogenous substrate is insensitive. This could also be due to an isotope effect as the UDPGal produced by transferase activity would not be labelled in the four position of the galactose, while the commercial material would be.

4. Inactivation of k_3 by several metabolites.

The data presented thus far and in the preceding reports (Puck and Hill, '67; Hill and Puck, '70) suggest that glucose metabolism by whole erythrocytes results in the production of one or more inhibitors of the epimerase enzyme. One of these inhibitors would seem to be UDPG as indicated by the above dilution experiments. Further experiments show that more complicated interactions occur as a result of glucose metabolism.

Lysates were prepared by the glucose-free method and preincubated for 15 minutes at 37°C. Table 3 shows that further preincubation with either ATP, UTP or UDPG brings about inhibition of k_3 but there is no apparent time dependent effect of any of these substances. When lysates are prepared using glucose buffer, however, these compounds interact in some way with some substance or substances in the lysates to effect a time-dependent inactivation of k_3 (fig. 8). The figure shows that inactivation brought about by ATP can be further enhanced by UTP and UDPG.

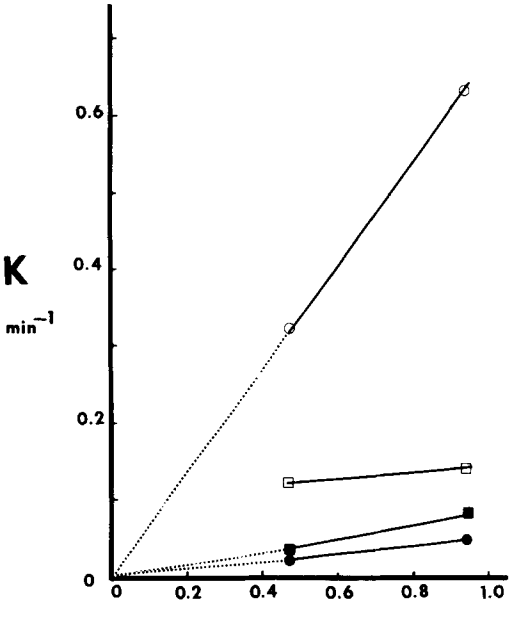

Fig. 7 Dilution of kinase, transferase and epimerase activity. Erythrocyte lysates prepared and preincubated as in figure 5. Diluted lysates were diluted with glucose-free buffer. For simultaneous assay incubation with substrates was for five minutes. Each incubation mixture contained 1.8×10^{-3} M ATP, 2.1×10^{-3} M NAD^+, 2.9×10^{-4} M UDPG, 2.9×10^{-6} M Gal-1-^{14}C. Total volume was 0.105 ml. ● k_1, ○, k_2, □ k_3, simultaneous assay. For independent assay of k_3, incubation with substrates was for two minutes and each incubation mixture was similar to the above except that 4.5×10^{-7} M UDPGal-^{14}C was present in place of Gal-1-^{14}C. ■, k_3 assayed independently.

TABLE 3

Effect of k_3 on preincubation with ATP, UTP and UDPG in glucose-free lysates

Preincubation time	Lysates preincubated with			Control
	ATP	UTP	UDPG	
minutes				
0	0.25 ± 0.029	0.32 ± 0.015	0.041 ± 0.0040	0.41 ± 0.037
3	0.26 ± 0.017	0.29 ± 0.010	0.047 ± 0.0059	
6	0.25 ± 0.018	0.29 ± .0067	0.040 ± 0.0017	

Erythrocytes were harvested and washed three times in glucose-free buffer. Lysates were made and preincubated at 37° for 15 minutes. 3.9×10^{-3} M ATP, 9.7×10^{-4} M UTP or 3.5×10^{-4} M UDPG was added and preincubation continued for 0, 3 or 6 minutes at which time 6.8×10^{-7} M UDPGal-^{14}C and 2.2×10^{-3} M NAD^+ were added and incubated for two minutes.

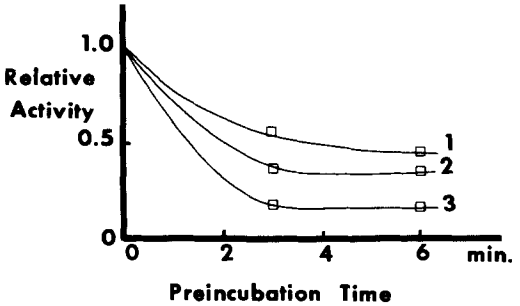

Fig. 8 The inactivation of k_3 by ATP, UDPG and UTP. Lysates were prepared as in figure 1. Lysates were equilibrated for five minutes at 37°C and then preincubated with 1.8×10^{-3} M ATP, 2.1×10^{-3} M NAD^+ and 4.5×10^{-7} M UDPGal (curve 1) or with the same plus 2.7×10^{-5} M UDPG (curve 2) or as in curve 1 plus 2.3×10^{-4} M UTP (curve 3). After 0, 3 or 6 minutes of preincubation, 4.5×10^{-7} M UDPGal-^{14}C was added and incubated for two minutes. The presence of UDPGal and NAD^+ in the preincubation have no effect and NAD^+ is present in sufficient concentration on addition of substrate so that the reaction proceeds at the maximum rate. The total volume of each final incubation mixture was 0.11 ml containing 0.1 ml of lysate.

In the earlier report (Hill and Puck, '70), it was shown that lysates prepared from cells washed in glucose-free buffer must be preincubated at 37°C for about ten minutes to achieve maximum activity. Lysates prepared from cells washed and incubated in glucose buffer can achieve the same maximum activity if preincubated for a sufficiently long period also. Presumably the difference between lysates is a quantitative one, the glucose-prepared lysates containing more inhibitor(s) than the lysates prepared in the absence of glucose. In order to gain further insight into the nature of the interactions of ATP, UTP and UDPG with the substances in the lysates, the experiment described in table 4 was performed. The table shows that the fold changes in the controls whether prepared by the glucose method or the glucose-free method, do not differ significantly from each other. This indicates that the same interacting substances are disappearing from both types of lysate. ATP interacts with something in both types of lysate and to the same extent, thus ATP probably interacts with the same substance in both types of lysate. UTP interacts with both types of lysate but in a different manner with each. This suggests that UTP may interact with more than one substance and that a possible second substance is present in much lower concentration in the glucose-free lysates. Similarly, the behavior of UDPG suggests more complex interactions since UDPG significantly affects the fold change in glucose prepared lysates but not in glucose-free lysates.

In these crude preparations, it is not possible to identify the interacting substances in the lysates. UDPG is probably not one of them, or at least not the only one, since addition of UDPG to glucose-free lysates preincubated for 15 minutes, (table 3) does not produce a time dependent interaction, although this could be because some other substance has now been used up. Furthermore, the interacting substances are being metabolized during preincubation (table 4) but addition of UDPG produces inactivation whereas, if it were being metabolized, it would be expected to have a decreasing effect with time. In any event, it is apparent that the metabolism of glucose by erythrocytes produces regulatory substances which tend to stem the catabolism of galactose via the Leloir pathway.

If lysates to which 1.9×10^{-3} M ATP have been added are preincubated for 30

TABLE 4

Effect of ATP, UTP and UDPG on the increase in k_3 during preincubation. Lysates prepared with and without glucose

Preincubation time	Control		ATP		Lysates preincubated with UTP		UDPG	
	Glu	Glu-free	Glu	Glu-free	Glu	Glu-free	Glu	Glu-free
0 minutes	0.051 ± 0.0015	0.11 ± 0.0046	0.047 ± 0.0075	0.091 ± 0.016	0.022 ± 0.0045	0.045 ± 0.0040	0.044 ± 0.00089	0.076 ± 0.075
5 minutes	0.24 ± 0.0013	0.47 ± 0.016	0.054 ± 0.0029	0.14 ± 0.0075	0.012 ± 0.0018	0.14 ± 0.011	0.099 ± 0.034	0.38 ± 0.046
Fold change in 5 minutes	4.8 ± 0.24	4.2 ± 0.34	1.2 ± 0.24	1.6 ± 0.37	0.56 ± 0.20	3.2 ± 0.52	2.3 ± 0.72	5.1 ± 1.1
Diff. signif. by t test Glu vs. Glu-free	No		No		Yes		Yes	
Diff. of chg. in glu lysate signif. rel. to control	—		Yes		Yes		Yes	
Diff. of chg. in glu-free lysates signif. rel. to control	—		Yes		Yes		No	

Erythrocytes were harvested and either (1) washed twice in glucose buffer and incubated five minutes at 37°C in the same, or (2) washed three times in glucose-free buffer. Lysates were prepared and preincubated five minutes at 37°C 1.8×10^{-3} M ATP, 4.7×10^{-4} M UTP or 2.9×10^{-5} M UDPG was added and preincubation was continued for 0 or 5 minutes. At that time 1×10^{-7} M UDPGal-^{14}C and 2.2×10^{-3} M NAD^+ were added and incubated for two minutes. Student's t test was used to compare the fold changes under various conditions ($\alpha = 0.05$).

minutes, k_3 regains its full activity. Most of the added ATP has been consumed by 30 minutes judging by the low activity of kinase at this time which can be restored by the addition of more ATP. Thus the ATP effect on the epimerase, at least, is reversible.

DISCUSSION

Simultaneous assay of at least three enzymes in a metabolic chain is found to be feasible. Variations in the individual enzymes can be detected under these conditions. Such an assay can be performed using a minimum of material, a desirable situation for the study of mammalian cells where large quantities of extracts are obtained with difficulty. Furthermore, comparative results will be more meaningful if several enzymes can be assayed under precisely the same conditions. Since radiochemical materials are employed, the substrate for all but the first reaction is a product of the preceding reaction and is more likely to be pure. The sequential assay is suitable for whole cells when the rate constant for an enzyme in a particular chain is desired and the cell is not permeable to the substrate for the enzyme but often is permeable to the substrate of the first enzyme in the chain. Transport need only be a complicating consideration when assaying whole cells for the first enzyme in such a chain.

The sequential assay as employed here, utilizes a very low concentration of the initial substrate, galactose. Thus pseudo-first order kinetics apply to the study of this enzyme and the other two as well. Such an assay system could provide an opportunity for the detection of variations in the affinity of the enzyme for its substrate, and variations in the rate of dissociation of the enzyme-substrate complex, as well as variations in enzyme concentration. Since most assays used for screening human populations employ saturating amounts of substrate, variations in enzyme-substrate affinity are usually undetectible.

Since the red cell is devoid of a nucleus and of protein synthesizing machinery, control of enzyme activity must be exercised at the enzyme level. In this respect, it is interesting that the enzymes in the galactose pathway especially the epimerase appear to be more sensitive to inhibition by metabolites which arise from glucose utilization. This inhibition might be important in management of humans with deranged galactose metabolism. For example, an individual doubly heterozygous for the Durate variant of the transferase enzyme and galactosemia would have only 25% of the normal transferase activity. Thus, the intracellular concentration of Gal-1-P might be quite high. This could be further elevated by a high glucose diet, as the presence of higher concentrations of G-1-P, G-6-P and UDPG in the cells would inhibit the transferase and the epimerase thus causing greater accumulation of Gal-1-P, which is presumed to be toxic to cells.

Catabolite repression by intermediates in the glucose pathway is well known in bacteria and has also been shown for serine dehydratase in mammalian liver (Jost, Khairallah and Pitot, '68). In the red cell, however, such repression can only be exhibited at the level of enzyme activity. The epimerase enzyme seems to play a key role in this regulation, being quite sensitive to inhibition by UDPG, UTP, and surprisingly, to ATP. Regulation in the red cell may be effected by small molecule pools. Thus, the red cell apparently contains a pool of UDPG which regulates both the transferase and the epimerase. Other factors are also present which interact with added small molecules to inactivate the epimerase. Undiluted extracts may be useful for the detection not only of variations in enzyme amount and activity, but also of variations in the small molecule pools. Much information can be gained from the study of purified enzymes. However in order to understand the intricate behavior of cells in vivo, it is necessary to study systems which behave in a manner more closely analogous to the living cell, such as the one employed here.

The sequential assay accurately reflects the intracellular activity of the three enzymes in the galactose pathway, and thereby provides a means of studying *in vivo* interactions involved in enzyme rate control.

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