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TIME-DEPENDENT INCREASE IN RAT MAMMARY URIDINE DIPHOSPHATE GLUCOSE 4-EPIMERASE ACTIVITY *IN VITRO*

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SUMMARY

UDP-glucose 4-epimerase (EC 5.1.3.2) activity increases upon incubation of extracts prepared from lactating mammary glands. The increase in activity is enhanced by the addition of NAD^+, $NADP^+$, UTP, UDP, UMP and glycerol to the extracts. The increase in enzymatic activity is influenced by pH and urea concentration but not by the removal of low molecular weight material. The increase in activity is not influenced by 1.0 mM phenylmethylsulfonylfluoride or 1% sodium cholate. The "activation" process does not alter the K_m for UDP-galactose. The present evidence indicates that the increase in activity is due to a process leading to a more active form of enzyme.

INTRODUCTION

UDP-glucose 4-epimerase (EC 5.1.3.2) catalyzes the interconversion of UDP-D-glucose and UDP-D-galactose. The enzyme has been purified from a variety of sources[1-4] including the mammary gland[5] and is widely distributed in living organisms. Recent studies[6] have shown that the activity of UDP-glucose pyrophosphorylase (EC 2.7.7.9) increases upon incubation in a variety of tissue extracts and this time-dependent increase in enzymatic activity was most likely due to a process leading to a more active form of the enzyme[6]. Other studies[5] with purified UDP-glucose 4-epimerase isolated from bovine mammary gland acetone powders have shown that the enzyme is very labile but that it may be stabilized by NAD^+, $NADP^+$, NADH, UDP-galactose, UDP-glucose, UDP-gluconate, UDP, UTP and glycerol. It was on the basis of the above observations that the activity of UDP-glucose 4-epimerase as a function of time was measured in crude homogenates of mammary tissue. The results showed that there was a time-dependent increase in the activity of the enzyme which is enhanced by NAD^+ and its analogs, uridine nucleotides and glycerol. The increase in activity appears to involve a process that leads to a more active form of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

NAD^+, NADH, $NADP^+$, NADPH, UTP, UDP, UMP, UDP-glucose, UDP-

galactose, phenylmethylsulfonyl fluoride, K-penicillin-G, streptomycin, sodium cholate, glucose 6-phosphate and sodium pyruvate were from Sigma Chemical Co., St. Louis, Mo. Sephadex, G-25, medium was from Pharmacia. Rats were from the Holtzman Co., Wisc., guinea pigs, hamsters and mice were from Don B. Lab Animals, Calif. Other animals were obtained from local sources. The L-cells were from Dr. F. Leach of the Biochemistry Department. UDP-Glc dehydrogenase was prepared from bovine liver through Step 5 (ref. 7).

Preparation of enzymatic extracts

All animals were killed in a CO_2 chamber and 1 g of minced tissue was homogenized at 0° in a Sorvall Omnimixer for 60 sec at maximum speed with 10 or 20 ml of 4° buffer containing 0.15 M KCl, 0.005 M EDTA, 0.005 M $MgCl_2$ (pH 7.5). The homogenates were filtered at 10 000 $\times g$ for 20 min at 0° and the supernatant solution was filtered through glass wool. Extracts from organ cultures were prepared in a similar manner except that 50 mg of tissue were homogenized with 2 ml of the above buffer using the microattachment to the Sorvall Omnimixer. Extracts of L-cells (0.2 g/ml of homogenizing buffer) were prepared in a similar manner. Mammary tissue could be frozen at −10° for at least a week without any loss of enzymatic activity and the ability to exhibit the increase in activity upon incubation of crude extracts.

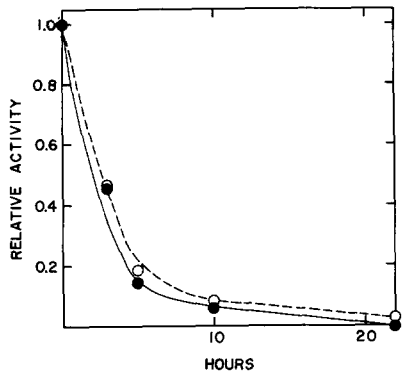
Enzymatic assays

UDP-glucose 4-epimerase was determined by measuring the formation of UDP-glucose[8]. Lactate dehydrogenase[9] and glucose-6-phosphate dehydrogenase[10] were assayed by standard procedures. A unit of enzyme is the amount of enzyme which catalyzes the formation of 1 μmole of product per min at 25°.

RESULTS

Initial experiments examining the UDP-glucose 4-epimerase activity in organ explants and tissue homogenates prepared from a 20-day lactating rat showed that there was a rapid loss of enzymatic activity (Fig. 1) in the absence or presence of the hormones, insulin, hydrocortisone and prolactin. Under similar conditions, there is a 5–7-fold increase in activity of UDP-glucose pyrophosphorylase[6]. These results show that UDP-glucose 4-epimerase activity is not a useful indicator of mammary gland function since it is highly unstable under these conditions. There was no detectable enzymatic activity in crude homogenates of rat mammary tissue after 1 h at 37° which shows that the enzyme is more labile in homogenates than in the explants. However, the enzyme is more stable at lower temperatures and in the presence of NAD^+.

The results presented in Fig. 2 show that there is a time-dependent increase of activity of UDP-glucose 4-epimerase in rat mammary gland homogenates at 0° in the absence and presence of 5 mM NAD^+. The effect of temperature on this process is presented in Fig. 3 which shows that the time required to reach maximum activity is decreased as the temperature is increased. The experiments presented in Figs. 2 and 3 are representative though the extent and time to reach maximum activity is somewhat variable and appears to be related to the initial activity of the extract. In the rat mammary gland, the highest activity of UDP-glucose 4-epimerase is found during



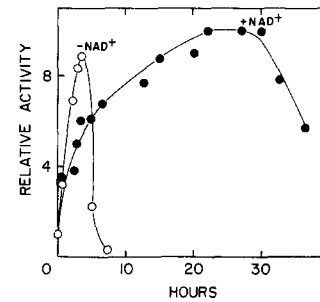


Fig. 1. Activity of UDP-glucose 4-epimerase in rat mammary explants as a function of time. Rat mammary explants were prepared from a 20-day lactating rat as previously described[6]. ●—●, enzymatic activity in explants incubated in Medium 199 containing insulin, hydrocortisone and prolactin (5 μg/ml each)[6]. ○— — —○, enzymatic activity in explants incubated in Medium 199. Relative activity: 1.00 = 0.60 unit/g tissue. The cultures were maintained at 37°.

Fig. 2. Time-dependent increase in UDP-glucose 4-epimerase activity in a rat mammary gland homogenate prepared from a 13-day lactating rat. ●—●, activity in the presence of 5 mM NAD^+. ○—○, activity in the homogenate. Relative activity 1.00 = 0.12 unit/g tissue. Incubations were at 0°.

the latter third (15–21 days) of the lactational period. Extracts prepared from this period are preferred to those prepared from earlier time periods. The activation will also occur in crude extracts prepared from frozen tissue stored at −10° for a period of about 1 month. The increase in activity was observed in other sources of mid to late

TABLE I

INCREASE IN UDP-GLUCOSE 4-EPIMERASE ACTIVITY IN A VARIETY OF TISSUE HOMOGENATES AT 20°

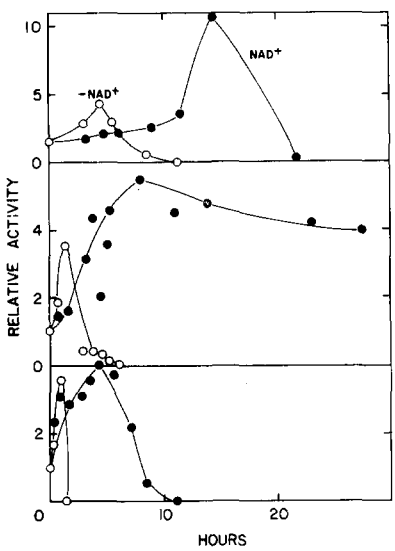
Source	*Homogenate concn. (mg/ml)*	*Initial enzyme activity (units/g tissue)*	*Time for maximum increase (h)**		*Increase at maximum value (-fold)*	
			−NAD+	*+NAD+*	*−NAD+*	*+NAD+*
(1) Mammary glands (lactating)						
Rat	100	0.61	2.2	3.9	3.7	4.0
Mouse	100	2.0	2.7**	4.5	—	2.2
Hamster	100	0.1	2.4**	5.9	—	1.7
Guinea pig	50	46.3	3.0**	12.7	—	2.9
Rabbit	100	0.4	2.6**	12.7	—	3.8
Opossum	50	2.8	4.1**	6.1	—	2.0
Bat	50	6.4	2.1**	5.0	—	1.2
(2) Other						
L-cells	200	0.05	5.1	3.5	2.6	2.4

* The time-dependent increases in activity were determined as outlined in Figs. 2 and 3.

** In these cases, a decrease in activity was observed the first time the homogenate was assayed after zero time. It is probable that the 1st assay was performed after the activation had occurred and only the decline in activity was observed, or the epimerase from these sources is more labile than that from the rat.

lactating mammary glands and L-cells (Table I). Again, the time required to reach maximum activity was somewhat variable as was the extent of the increase.

The effect of NAD^+ concentration from 0.5 to 5 mM on the extent of increase of UDP-glucose 4-epimerase activity at 15° is shown in Fig. 4. Similar increases in activity are also observed with 0.25 mM NAD^+. The effect of NAD^+ on the increase in activity obtained at other temperatures such as 0° and 24° are similar to those obtained at 15°. Temperatures between 10 and 15° are convenient for experiments since the time to reach maximum activation is usually between 4 and 6 h. The fact



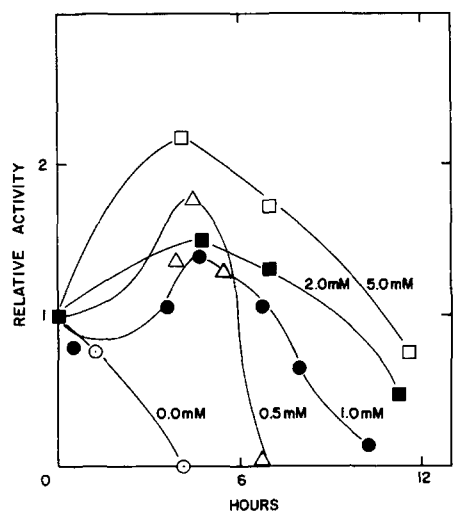


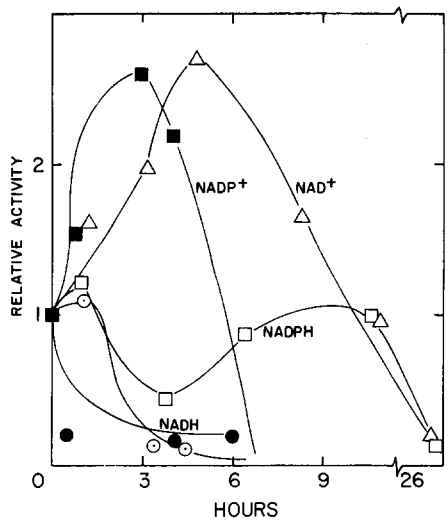
Fig. 3. Effect of temperature on the increase in activity of UDP-glucose 4-epimerase in an extract prepared from a 16-day lactating rat. ●—●, activity in the presence of 5 mM NAD^+; ○—○, activity in the absence of NAD^+. The temperatures of incubation were 0° (top), 15° (middle), and 24° (bottom). Relative activity 1.00 = 0.61 unit/g tissue.

Fig. 4. Effect of NAD^+ concentration on the time-dependent increase of UDP-glucose 4-epimerase in a homogenate prepared from tissue from an 18-day lactating rat frozen for 4 days. NAD^+ concentrations were: ○—○, 0; △—△, 0.5 mM; ●—●, 1.0 mM; ■—■, 2.0 mM; □—□, 5 mM. Incubation was at 15°. Relative activity 1.0 = 1.6 units/g.

that NAD^+ analogs, uridine nucleotides and glycerol stabilized purified bovine mammary gland UDP-glucose 4-epimerase[5] prompted the investigation of these compounds on the extent of the increase in activity observed in rat mammary gland homogenates. The effect of NAD^+, NADH, $NADP^+$ and NADPH on the increase of UDP-glucose 4-epimerase is shown in Fig. 5; the effect of UTP, UDP and UMP is shown in Fig. 6 and the effect of glycerol is shown in Fig. 7. In all cases, these compounds extended the degree of activation and the time required to reach maximum activity.

Investigation on the increase of UDP-glucose 4-epimerase in rat mammary gland homogenates

Further studies were carried out relating to the possible mechanism of the



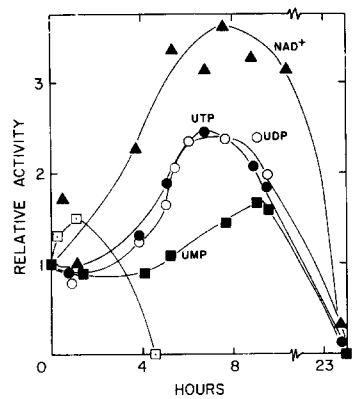
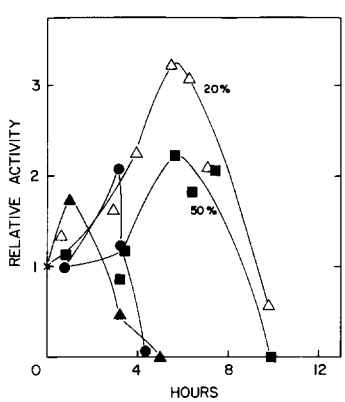


Fig. 5. Effect of NAD+ and its analogs on the time-dependent increase of UDP-glucose 4-epimerase in a homogenate prepared from tissue from a 18-day lactating rat frozen 7 days at −10°. ○—○, control; △—△, 5 mM NAD+; ■—■, 5 mM NADP+; □—□, 5 mM NADPH; ●—●, 5 mM NADH. Incubations were at 18°. Relative activity 1.00 = 1.14 units/g tissue.

Fig. 6. Effect of uridine nucleotides on the time-dependent increase of UDP-glucose 4-epimerase activity in a homogenate prepared from a 15-day lactating rat. ⊡—⊡, control; ▲—▲, 5 mM NAD+; ●—●, 5 mM UTP, ○—○, 5 mM UDP; ■—■, 5 mM UMP. Incubations were at 16°. Relative activity 1.00 = 0.89 unit/g tissue.



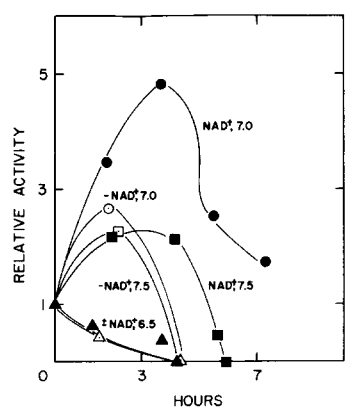


Fig. 7. Effect of glycerol on the time-dependent increase of UDP-glucose 4-epimerase activity in a homogenate prepared from tissue frozen for 4 days from a 15-day lactating rat. ●—●, 20% and ▲—▲, 50% control diluted with homogenizing buffer; △—△, 20% (v/v) glycerol; ■—■, 50% (v/v) glycerol. Incubations were at 15°. Relative activity 1.0 = 0.71 unit/g tissue.

Fig. 8. Effect of pH on the time-dependent increase in activity of UDP-glucose 4-epimerase in a homogenate prepared from tissue frozen for 4 days from a 14-day lactating rat. The pH was adjusted to the desired value on a pH meter. ●—●, 0.25 mM NAD+, pH 7.0; ○—○, pH 7.0; ■—■, 0.25 mM NAD+, pH 7.5; ⊡—⊡, 0.25 mM NAD+, pH 6.5; △—△, pH 6.5. Incubations were at 12°. Relative activity 1.00 = 0.30 unit/g tissue.

observed increase in UDP-glucose 4-epimerase activity in rat mammary gland homogenates. The effect of pH is presented in Fig. 8 and shows that the observed activity is dependent on pH. The optimum pH appears to be 7.0 and a higher or lower pH leads to a more rapid loss of activity.

The increase in UDP-glucose 4-epimerase activity was not inhibited by 1.0 mM phenylmethylsulfonyl fluoride, or penicillin G (50 μg/ml) and streptomycin (50 μg/ml) did not prevent the observed increase in activity which would argue against microbial activity as being the causative agent. The increase in activity was observed also in extracts prepared from rat mammary gland acetone powders though the extent of increase was only 2–3 fold at 15°.

The activity of two other enzymes was also measured as a function of time in the crude homogenates to determine if activation occurred with other enzymes. 85% of the activity of lactate dehydrogenase remained after 24 h at 0° and only a slow decrease in activity was observed during this period. At the end of 4 h at 0° there was no loss in activity of glucose-6-phosphate dehydrogenase; at the end of 10 h 52% remained; and at 24 h only 18% of the original activity was present. Other experiments have shown that the activity of lactose synthetase does not increase under similar conditions.

The K_m for UDP-galactose was determined in the initial extract and after 4–5 h of incubation at 0°. The results presented in Fig. 9 show that the K_m for UDP-galactose did not change with an increase in activity of the enzyme. The initial velocity was proportional to enzyme concentration (extract) at both zero time and at 4–5 h of incubation.

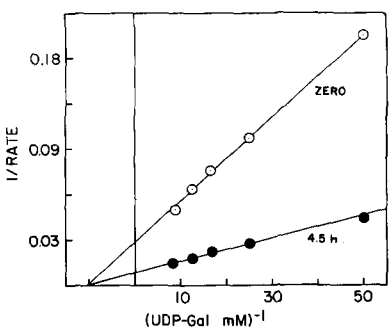


Fig. 9. Lineweaver–Burk plot of UDP-glucose 4-epimerase at zero time and after activation (4–5 h) at 14°. The homogenate was prepared from a 14-day lactating rat. ○—○, zero time; ●—●, 4–5 h. The K_m was $1 \cdot 10^{-4}$ M at both times.

Low molecular weight materials were not apparently responsible for the observed increase in activity since comparable increases to those of controls were observed in extracts which had been passed through a Sephadex G-25 column equilibrated in the homogenizing buffer.

Studies on the effect of urea on the increase in activity in the presence of 0.25–5 mM NAD showed that there is little loss in activation up to about 2 M urea. Concentrations of urea up to 2 M allowed the increase in activity to occur sooner than the controls. When the urea concentration was greater than 3 M there was no increase in

activity. When the concentration of urea was above 4 M, there was a rapid loss in activity.

DISCUSSION

Time-dependent increases in the activity of several enzymes have been previously reported. GOLD AND SEGAL[11] have observed such an increase in rat liver glycogen synthetase though the mechanism of activation was not ascertained. A time-dependent increase in 5'-nucleotidase from *Escherichia coli* is related to the presence of an endogenous inhibitor[12]. A slow time-dependent activation was also observed with rat liver acetyl-CoA carboxylase[13].

A time-dependent increase in activity of UDP-glucose pyrophosphorylase was observed in a variety of tissue extracts including the mammary gland[6]. In rat mammary gland homogenates, the evidence indicated that the increase in activity was due to a process leading to a more active form of the enzyme. In general it would appear that the present evidence would support a similar conclusion with rat mammary UDP-glucose 4-epimerase except that compounds known to stabilize the enzyme[5] often prolong the extent and time to reach maximum activation. The increase in activity in the absence of NAD^+ is variable but in general best activity is observed with preparations made from late lactating glands. The extent and time to reach maximum activity is also dependent upon the concentration of NAD^+ (Fig. 4). The extent of increase in activity and the retention of activity is greatest at the higher concentrations. This is probably in part due to the ability of NAD^+ to stabilize the enzyme since it was shown that the purified bovine enzyme is stabilized by NAD^+ (ref. 5). Exogenous NAD^+ is required for maximum activity of the purified bovine enzyme ($K_m = 5 \cdot 10^{-7}$ M). This requirement for exogenous NAD^+ has not been shown for the rat enzyme. $NADP^+$ is also effective in stimulating an increase in activity (Fig. 5) but the effect of NADPH tends to cause an increase in activity after an initial decrease. It is possible that the late increase may reflect a conversion of NADPH to $NADP^+$. NADH is a strong inhibitor of epimerase reactions and hence it is difficult to determine if it may be effective in causing an increase in activity since the concentration in the assay may be inhibitory. UTP, UDP and UMP will cause an increase in activity though they are not as effective as NAD^+.

These compounds are linear competitive inhibitors of purified bovine mammary epimerase; UMP, $K_i = 5 \cdot 10^{-5}$ M UDP; $K_i = 3.5 \cdot 10^{-5}$ M and for UTP the $K_i = 2.2 \cdot 10^{-4}$ M (ref. 5). All of these compounds are effective in stabilizing the bovine mammary epimerase as is glycerol. Indeed the effect of glycerol is similar to that of NAD^+ (Fig. 2). Hence compounds which are effective in stabilizing bovine mammary epimerase are also effective in causing an increase in the activity of UDP-glucose 4-epimerase in rat mammary gland homogenates.

Increasing temperature (Fig. 3) causes the increase in activity to occur sooner than at the lower temperatures. In fact the increase and subsequent decrease in activity at higher temperatures in the absence of NAD^+ may occur during the first hour of incubation.

The present results support the view that the increase in UDP-glucose 4-epimerase activity represents an increase in activity of existing enzyme. This view is also supported by the fact that there is no change in the K_m for UDP-galactose but there

is an increase in the maximum velocity. The observations that the increase in enzymatic activity was inhibited by urea, exhibits a temperature dependence and has a pH optimum support the proposal that there is an alteration in enzyme structure which permitted the formation of a more active form of the enzyme. It is also possible that the "activated" form of the enzyme is more unstable than the "unactivated" enzyme since there is a rapid loss of activity after the maximum activity is obtained. NAD^+, uridine nucleotides, and glycerol all stabilize dilute solutions of bovine mammary UDP-galactose 4-epimerase and it is probable that they are acting in a similar manner with the rat mammary gland epimerase.

Phenylmethysulfonylfluoride failed to prevent the increase in activity which would tend to reduce the likelihood that limited proteolysis of the enzyme to a more active form was occurring.

The removal of low molecular weight materials by chromatography on Sephadex G-25 did not prevent the increase in activity and this result would argue against possible interactions with low molecular weight compounds.

The data presented are consistent with the proposal that the enzyme undergoes a structural change by a process that leads to a more active form of the enzyme. The effect of NAD^+ and other compounds may be related to the fact that these compounds are able to stabilize the enzyme.

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