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TIME-DEPENDENT INCREASE IN URIDINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE ACTIVITY *IN VITRO*

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SUMMARY

UDPGlc pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) activity increases upon incubation of extracts prepared from a variety of sources including organ cultures of rat mammary glands. The increase of enzymatic activity is dependent upon temperature, concentration of extract, pH and urea concentration. The "activation" process does not alter the K_m for UTP or molecular weight of the enzyme. The present evidence indicates that the increase in activity is due to a process leading to a more active form of the enzyme.

INTRODUCTION

UDPGlc pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) catalyzes the following reaction:

$$UTP + Glc\text{-}1\text{-}P \rightleftharpoons UDPGlc + PP_i$$

The enzyme has been crystallized from bovine liver[1] and purified from bovine mammary[2] and rat mammary[3] tissue as well as from rabbit muscle[4], mung beans[5], peas[6] and *Escherichia coli*[7]. UDPGlc pyrophosphorylase is an enzyme associated with the biosynthesis of lactose in mammary tissue and was used as an indicator of mammary gland function in rat mammary gland explants cultivated *in vitro*. In these studies, there was a marked increase in the total enzymatic activity in the explant system which was independent of hormones and was not inhibited by puromycin. These results suggested a time-dependent increase in enzymatic activity and further experiments showed that similar results were obtained in a variety of tissue extracts.

Time-dependent activations of rat liver glycogen synthetase[8] and rat liver acetyl-CoA carboxylase[9] were previously observed.

EXPERIMENTAL PROCEDURE

Materials

UTP, phenylmethylsulfonylfluoride, glucose 1-phosphate (Glc-1-*P*), K-peni-

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cillin-G, UDPGlc and NAD^+ were obtained from Sigma Chemical Co., St. Louis, Mo. Medium 199 was from Baltimore Biological Lab.; puromycin from Nutritional Biochemicals; insulin and cortisol from Mann Biochemical; catalase (beef) from Worthington Biochemicals; Sephadex G-25, medium, from Pharmacia and prolactin was from the Endocrinology study section, National Institutes of Health. UDPGlc dehydrogenase was prepared from bovine liver through Step 5 (ref. 10). 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. Beans (Kentucky Wonder Pole) and peas (Miragreen) were from Ferry Morse, Calif. Mung Beans (Berken M-339) were obtained from the Agronomy Department. L-Cells were obtained from Dr. F. Leach of the Biochemistry Department.

Preparation of organ explants

Rat mammary gland explants were prepared and incubated as described for mouse mammary explants[11]. The cultures were maintained in Medium 199 supplemented with 50 μg/ml of penicillin G at 37° in an atmosphere of O_2–CO_2 (95:5, by vol.). About 50 mg of tissue were placed in 3 ml of the medium.

Preparation of enzymatic extracts

Rats or other animals were killed in a CO_2 chamber and 1 g of minced tissue was

TABLE I

INCREASE IN UDPGlc PYROPHOSPHORYLASE ACTIVITY IN A VARIETY OF TISSSUE HOMOGENATES AT 20°

Source	*Homogenate concn. (mg/ml)*	*Initial enzyme activity (units/g)*	*Time for maximum increase (h)*	*Increase at maximum value (-fold)*
Mammary glands (lactating)				
Rat	50	2.63	12.3	8.9
Rabbit	100	0.29	12.7	6.2
Opossum	50	7.44	5.4	3.4
Guinea pig	50	0.53	27.3	50.4
Bat	50	0.37	6.3	15.6
Hamster	100	0.18	11.7	2.7
Mouse	100	0.37	13.0	37.9
Bovine	50	1.33	4.7	2.1
Livers				
Rat	100	3.40	22.2	2.8
Rabbit	100	2.04	8.4	4.1
Opossum	50	7.59	12.5	2.8
Guinea pig	50	0.56	31.0	17.1
Bovine	100	0.50	4.5	2.1
Hamster	50	2.98	2.7	1.7
Chicken	200	0.11	9.7	5.1
Others				
Rat brain	50	0.003	3.5	55.6
Bean seeds	200	0.11	6.0	10.2
Pea seeds	200	0.65	6.0	2.3
Mung bean seeds	200	0.14	4.2	4.9
E. coli cells	200	0.25	2.6	1.3
L-cells	100	0.07	6.0	2.5

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 centrifuged at 10 000 × *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.

Bean, pea and mung bean seeds were ground to a powder in a mortar and pestle. The powders were homogenized (see Table I for concentrations) in the same manner as were the rat tissues. After filtering through cheese cloth they were centrifuged at 30 000 × *g* for 20 minutes and the supernatant solution was filtered through glass wool. *E. coli* K-12 wild-type cells were grown on minimal media with glucose as the carbon source. Late log-phase cells were harvested by centrifugation. Cells were suspended (1 g/5 ml) in the homogenizing buffer and were disrupted for 15 min in a Raytheon Sonic Oscillator. The mixture was centrifuged at 30 000 × *g* for 20 min and the supernatant solution was used as the enzyme source. Extracts of L-cells (0.2 g/ml of homogenizing buffer) were prepared as the extracts from mammary explants.

Enzymatic assays

UDPGlc pyrophosphorylase was assayed normally by measuring UDPGlc, or in some cases, Glc-1-*P* formation as previously described[2]. A unit of enzyme is the amount which catalyzes the formation of 1 μmole of UDPGlc or Glc-1-*P* per min.

Other procedures

Sucrose density gradient experiments were performed as outlined by MARTIN AND AMES[12] in 5–20% linear sucrose gradients containing 0.15 M KCl, 0.005 M $MgCl_2$ and 0.005 M EDTA (pH 7.5). 0.1 ml of extract was layered onto the gradients which were centrifuged at 65 000 rev./min for 1.5 h in a Spinco L2-65 centrifuge. The K_m for UTP was determined as previously described[2].

RESULTS

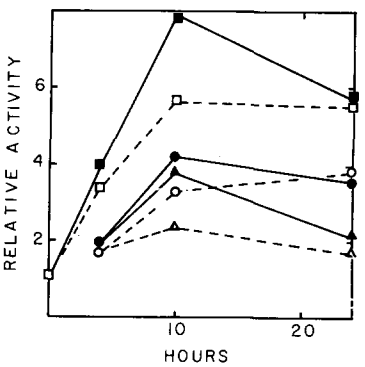
Increase in UDPGlc pyrophosphorylase activity in organ explants

The total UDPGlc pyrophosphorylase activity as a function of time in explants and the media of the explants from a 16-day lactating rat mammary gland in the absence or presence of hormones is shown in Fig. 1. The initial enzymatic value is the level found in freshly prepared explants just prior to incubation. Both explants and medium contained approximately similar levels of enzymatic activity which shows that considerable amount of the enzyme was leaking into the medium. The total amount of enzymatic activity increased in a linear manner for about 10 h and then decreased to a lower level at 24 h. The results of other experiments showed that there was a larger increase in total enzymatic activity in explants prepared from late lactating glands than those prepared from mid to late pregnancy glands. The experiment presented in Fig. 1 suggested that hormones (cortisol, prolactin and insulin) increased enzymatic activity but the results of other experiments have shown that the increase in enzymatic activity was independent of hormones. That is, the differences observed in Fig. 1 are not significant since there was considerable variation in the extent of enzymatic increase in the explants (6–10-fold for late lactating tissue). Other experiments similar

to that represented in Fig. 1 showed less increase in enzymatic activity in incubations containing hormones. Furthermore, the addition of 0.2 mM puromycin to the culture medium did not inhibit the time-dependent increase in enzymatic activity. In the mouse explant system there is no synthesis of casein until after 24 h in culture and this synthesis is dependent upon addition of hormones[11]. The increase in enzymatic activity which reached a maximum at 10 h is not due to enzyme-protein synthesis but due to some other process which results in the increase of the activity of the enzyme.

Increase in UDPGlc pyrophosphorylase in tissue extracts

An attempt was made to demonstrate the time-dependent increase of UDPGlc pyrophosphorylase in a variety of tissue extracts. The results presented in Fig. 2 show the time-dependent increase in UDPGlc pyrophosphorylase in rat mammary gland homogenates prepared from a 19-day lactating and an 18-day pregnant rat. The time course of activation was similar to that observed in the explant system (Fig. 1). There



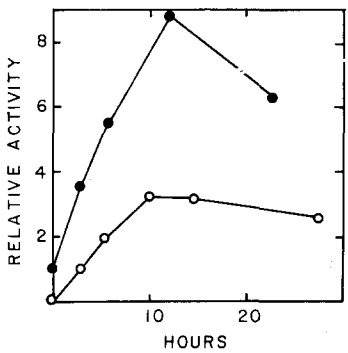


Fig. 1. Activity of UDPGlc pyrophosphorylase as a function of time in explants and medium from a 16-day lactating mammary gland in the absence (open figures) and in the presence of hormones (closed figures; prolactin, hydrocortisone and insulin, 5 μg/ml). ●—●, medium; ▲—▲, tissue; ■—■, total of medium and tissue with hormones. ○— — —○, medium; △— — —△, tissue; □— — —□, total medium and tissue with no hormones. Relative activity 1.0 = 0.15 units/g tissue.

Fig. 2. Time-dependent activation of UDPGlc pyrophosphorylase in rat mammary gland homogenates. ●—●, 19-day lactating rat, 1 g/20 ml homogenate; relative activity 1.0 = 2.6 units/g. ○—○, 18-day pregnant rat, 1 g/5 ml homogenate; relative activity 1.0 = 0.29 units/g. Incubations were at 24°.

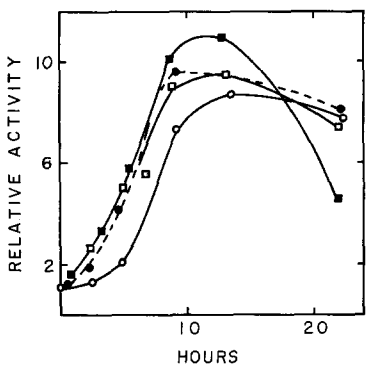
was a 9-fold increase in enzymatic activity in the homogenate prepared from the late pregnancy rat. In the rat mammary gland, UDPGlc pyrophosphorylase is most active in the late lactating gland which corresponds to the period where the extent of increase in enzymatic activity is the highest. A similar time-dependent increase in enzymatic activity was observed in a variety of other tissue homogenates and these results are presented in Table I. The extent of increase and the time required to reach the maximum increase varied somewhat but a similar general pattern of increase in enzymatic activity was observed.

The experiments reported in Table I are the results of single experiments carried out at a satisfactory homogenate concentration. Experiments with the rat mammary

gland (Fig. 4) have shown that the original concentration of the extract influences the extent of the observed increase. Further work with the rat mammary gland has shown that there is considerable variation in the extent of activation from tissues at comparable lactational states. The guinea pig and mouse mammary gland and the rat brain had the highest fold increase. However, the initial values of activity in the brain are very low and often undetectable until 3–4 h of incubation at 20°.

Investigation on the increase of UDPGlc pyrophosphorylase activity in rat mammary gland homogenates

Further studies were carried out relating to the possible mechanism of the observed increase in UDPGlc pyrophosphorylase activity in rat mammary gland



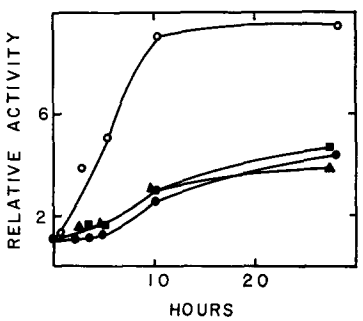


Fig. 3. Activation of UDPGlc pyrophosphorylase in 10-day lactating rat mammary gland extracts (50 mg per 2 ml homogenizing buffer) at 0° (○—○), 15° (●— — —●), 25° (□—□), and 37° (■—■). Relative activity 1.00 = 1.42 units/g tissue.

Fig. 4. Time-dependent activation of UDPGlc pyrophosphorylase in diluted extracts from a 16-day lactating rat, maintained at 24°. Relative activity 1.00 = 2.91 units/g tissue. ○—○, normal control, undiluted (100 mg tissue/2 ml homogenizing medium); ●—●, diluted 5-fold with the homogenizing buffer; ■—■, diluted 10-fold; ▲—▲, diluted 25-fold.

homogenates. The increase in activity occurred at temperatures between 0 and 37°. Fig. 3 shows the effect of temperature (0, 15, 25 and 37°) on the increase in activity. At 0° there was a 2–4 h lag which gradually decreased as the temperature was raised.

The extent of activation was dependent upon the concentration of the initial extract. Fig. 4 shows a 9-fold increase in activity in the control held at 24° whereas there was only a 3–4-fold increase when the control was diluted with the homogenizing buffer. Similar results were obtained at 0°.

Similar increases in activity were observed when extracts were prepared at 25° in different ways. A similar increase in activity was observed when extracts were prepared in 4% sucrose; 0.15 M KCl (pH 7.5); 0.1 M potassium phosphate (pH 7.6); or an extract (homogenizing buffer) of an acetone powder or in a 30–50% $(NH_4)_2SO_4$ fraction of an initial extract prepared in the homogenizing buffer. An increase in enzymatic activity was observed in all cases. The extent of increase varied but was related to the initial activity of the extract. A similar increase in enzymatic activity was observed in rat mammary gland homogenates when the enzyme was assayed for Glc-1-*P* formation rather than for UDPGlc.

The increase in UDPGlc pyrophosphorylase activity was not inhibited by 0.5 mM phenylmethylsulfonyl fluoride, a proteolytic inhibitor, or by 1% sodium cholate but was inhibited progressively by urea as shown in Fig. 5.

Increasing the concentration of urea reduced the extent of increase in activity and the time required to reach a maximum level. 4 M urea did not completely abolish enzymatic activity whereas 5 M urea prevented an increase in activity and resulted in a complete loss after 2 h incubation. Efforts to reverse the effects of urea were unsuccessful. For example, when an extract was made 4 M in urea and immediately placed on a Sephadex G-25 column to remove the urea, the enzymatic activity did not increase as the control but still retained a level of activity similar to the initial activity of the extract.

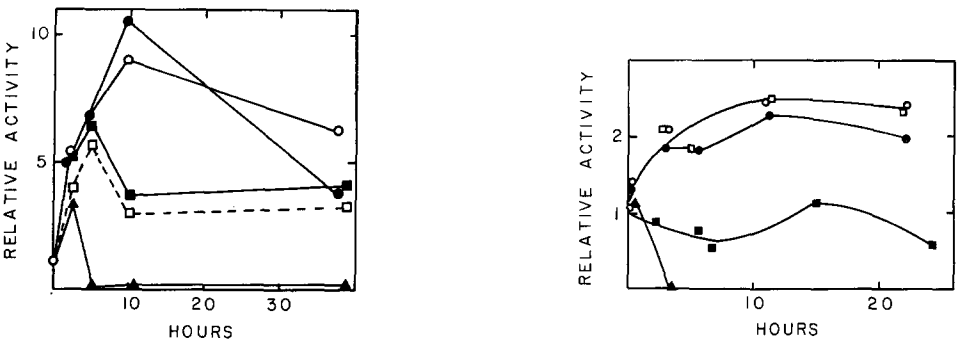


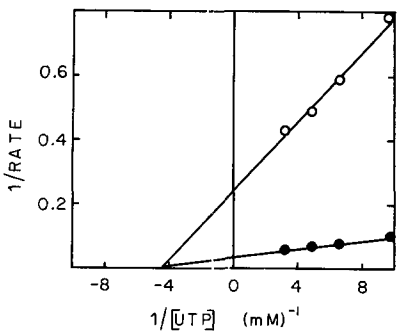
Fig. 5. Activation of UDPGlc pyrophosphorylase as a function of urea concentration in the extract. ●—●, control, no urea added; ○—○, 0.5 M urea; ■—■, 1 M urea; □— — —□, 2.5 M urea; ▲—▲, 4 M urea. The extract (1 g per 20 ml of the homogenizing buffer) was prepared from mammary tissue of an 11-day lactating rat. The extracts were maintained at 24°, and relative activity 1.0 = 5.91 units/g tissue.

Fig. 6. Activation of UDPGlc pyrophosphorylase as a function of pH. An aliquot of the extract was diluted with an equal volume of the indicated buffer and maintained at 24°; 0.2 M glycine (pH 9.0) (○—○); 0.2 M Tris–HCl (pH 8.0) (●—●); 0.15 M KCl, 0.005 M $MgCl_2$, 0.005 M EDTA, (pH 7.5) (homogenizing buffer) (□—□); 0.2 M potassium phosphate (pH 6.2) (■—■); and 0.2 M sodium acetate (pH 5.0) (▲—▲). The extract at pH 6.2 was 1 g tissue/20 ml homogenizing buffer from a 19-day lactating rat (relative activity 1.00 = 2.63 units/g tissue); the extract at the other pH's was 1 g tissue/10 ml homogenizing buffer from a rat lactating 7 days (relative activity 1.00 = 0.322 units/g tissue).

Low molecular weight materials were not responsible for the 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.

The increase in UDPGlc pyrophosphorylase activity as a function of pH is presented in Fig. 6. The extent of increase was similar between pH 7.5 and 9.0 whereas at pH 6.2 the activity remained fairly constant. At pH 5.0 there was a slight initial increase in activity followed by a rapid decline. Other experiments have shown that enzymatic activity may slightly increase or remain relatively constant at pH's between 5.5 and 6.0 though this is somewhat variable. Efforts to reverse the pH inhibition of the increase in enzymatic activity were not successful.

The K_m for UTP was determined in the initial extract and after 10 h of incubation at 25°. The results presented in Fig. 7 show that the K_m for UTP did not change



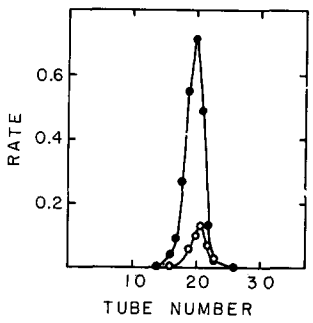


Fig. 7. Lineweaver–Burk plot of UDPGlc pyrophosphorylase activity at zero time and after activation at 24°. ○—○, zero-time values; ●—●, values obtained after incubating for 10 h. Homogenate (0.5 g mammary tissue/10 ml homogenizing buffer) was prepared from a 14-day lactating rat. The K_m at zero time was 227 μM and at 10 h was 192 μM. Rate is in nmoles UDPGlc/min.

Fig. 8. UDPGlc-pyrophosphorylase activity found in 5–20% sucrose density gradient centrifuged at 65 000 rev./min for 1.5 h at 4°. 0.1 ml of homogenate (1 g/10 ml) of 12-day lactating rat mammary gland was layered on the sucrose gradient which also contained 0.15 M KCl, 0.005 M $MgCl_2$ 0.005 M EDTA. ○—○, profile of extract at zero time, 1.03 units/g tissue; ●—●, profile of extract after incubation at 24° for 13 h, 2.52 units/g tissue. Rate is in nmoles UDPGlc/min.

upon an increase in activity of the enzyme. Other experiments showed that the K_m remained constant at intermediate levels of enzymatic increase.

Experiments were carried out to determine if there were changes in molecular weight during the observed increase in enzymatic activity. Sucrose density gradient experiments showed that there was no significant change in molecular weight of the enzyme when the enzyme was fully activated (Fig. 8). When beef liver catalase was used as a marker (mol. wt., 250 000), the molecular weight of the rat mammary UDPGlc pyrophosphorylase was calculated to be about 450 000 (ref. 12) which is similar to the value reported for the beef liver enzyme[1].

DISCUSSION

A time-dependent increase in rat liver glycogen synthetase was reported by GOLD AND SEGAL[8] though the mechanism of activation was not ascertained. The time-dependent increase in 5'-nucleotidase from *E. coli* is related to the presence of an-endogenous inhibitor[13]. A slow time-dependent activation was observed with rat liver acetyl-CoA carboxylase which is increased by citrate and reaches a maximum in about 5 h (ref. 9). Short incubations with trypsin will also activate the enzyme.

The present results support the view that the increase in UDPGlc pyrophosphorylase activity represents an increase in activity of existing enzyme without any marked change in molecular weight. This view is supported by the observations that there is no alteration in the K_m for UTP but there is a marked increase in the maximum velocity. The observations that the extent of increase of enzymatic activity depended upon the concentration of the extract and the initial level of enzyme, and that the increase in activity is inhibited progressively by urea, exhibits a temperature dependence and is markedly dependent upon pH, support the view that there may be a slow time-dependent alteration of the enzyme structure allowing for the formation

of a more active form of the enzyme. The failure of phenylmethylsulfonyl fluoride to prevent the increase in activity 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 material by chromatography on Sephadex G-25 did not prevent the increase in activity and the increase in activity occurred in an acetone-powder extract and in a homogenate prepared in 0.25 M sucrose. These results would argue against possible ionic effects or interactions with low molecular weight compounds. The data presented are consistent with the view that the enzyme undergoes a structural change by some process that leads to a more active form of the enzyme.

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REFERENCES

1 G. J. ALBRECHT, S. T. BASS, L. L. SEIFERT AND R. G. HANSEN, *J. Biol. Chem.*, 241 (1966) 2968
2 V. S. STEELMAN AND K. E. EBNER, *Biochim. Biophys. Acta*, 128 (1966) 92.
3 R. S. EMERY AND R. L. BALDWIN, *Biochim. Biophys. Acta*, 136 (1967) 223.
4 C. VILLAR-PALASI AND J. LARNER, *Arch. Biochem. Biophys.*, 86 (1960) 61.
5 V. GINSBURG, *J. Biol. Chem.*, 232 (1958) 55.
6 D. H. TURNER AND J. F. TURNER, *Biochem. J.*, 69 (1958) 448.
7 A. KAMOGAWA AND K. KURAHASHI, *J. Biochem.*, 57 (1967) 758.
8 A. H. GOLD AND H. L. SEGAL, *Arch. Biochem. Biophys.*, 120 (1967) 359.
9 R. F. SWANSON, W. M. CURRY AND H. S. ANKER, *Biochim. Biophys. Acta*, 159 (1968) 390.
10 J. L. STROMINGER, E. S. MAXWELL, J. AXELROD AND H. M. KALCKAR, *J. Biol. Chem.*, 224 (1957) 79.
11 W. F. JUERGENS, F. E. STOCKDALE, Y. J. TOPPER AND J. J. ELIAS, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 629.
12 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
13 H. F. DVORAK, Y. ANRAKU AND L. A. HEPPEL, *Biochem. Biophys. Res. Commun.*, 24 (1966) 628.