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GLYCOGEN SYNTHESIS IN MOUSE EHRLICH ASCITES CARCINOMA CELLS

THE PRESENCE OF AN ENDOGENOUS INHIBITOR FOR ACTIVATION OF GLYCOGEN SYNTHASE

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SUMMARY

1 In crude extracts of mouse Ehrlich ascites carcinoma cells, glycogen synthase (EC 2 4 1 11) is predominantly in the inactive D-form that is not convertible into the active I-form upon incubation at 30 °C An endogenous factor is responsible for this lack of conversion, since the synthase D recovered in the pellet fraction after high-speed centrifugation of the extracts was readily converted into the synthase I

2 The factor partially purified from the high-speed supernatant of crude extracts was found to possess an ability to inhibit the D- to I-form conversion of glycogen synthase catalyzed by glycogen synthase D phosphatase It is a heat-stable macromolecule presumably of protein nature, but differs from phosphorylase *a* known to inhibit the phosphatase reaction The action of the inhibitor can be reversed by its removal or by glucose 6-phosphate The inhibitor does not inhibit phosphorylase *a* phosphatase or glycogen synthase

3 The reasons for the apparent incapability of Ehrlich cells for glycogenesis in vivo as well as in vitro are discussed in the light of these studies

INTRODUCTION

Colorimetric as well as histochemical examinations revealed that mouse Ehrlich ascites carcinoma cells are devoid of glycogen Furthermore, these cells unlike other tumors previously studied [1–4], failed to synthesize glycogen when incubated in vitro in the presence of glucose While these findings may be interpreted to indicate that Ehrlich cells are incapable of glycogenesis, these cells were found to possess glycogen synthase (UDPglucose glycogen 4-α-glucosyltransferase, EC 2 4 1 11), the key enzyme, in an amount sufficient to conduct glycogenesis at a substantial rate It was, therefore, suspected that a unique mechanism might be operative in Ehrlich cells to suppress glycogen synthase activity, and for this reason, the present studies were undertaken

This paper reports that the glycogen synthase of Ehrlich cells, present almost

entirely in the inactive D-form, is inconvertible into the active I-form due to the presence of an endogenous macromolecular inhibitor The inhibitor blocks the conversion in a reversible and more or less specific manner

MATERIALS AND METHODS

Animals and tumor cells

Used in the present studies was a hypotetraploid Ehrlich ascites carcinoma strain maintained by serial intraperitoneal transplantation into male adult *dd* mice (30–40 g) 9–11 days after implantation, the tumor cells were harvested from the peritoneal cavity and washed twice with cold physiological saline

Preparation of crude extracts

All the preparative experiments described below were conducted at 0–4 °C Saline-washed tumor cells were washed with 50 mM Tris–HCl (pH 7 5)–5 mM EDTA, resuspended in 4 vol of the same buffer and subjected to ultrasonic disintegration at 10 kcycles for 2–3 min The sonicate was then centrifuged at 5000 × *g* for 10 min to obtain the crude extract

Preparation of the pellet fraction

The crude extract was centrifuged at 105 000 × *g* for 1 h The firmly packed pellet thus obtained was washed once and resuspended in one-fifth the original volume of the above buffer by gentle homogenization The resulting suspension was referred to as the pellet fraction

Assay of glycogen synthase

Glycogen synthase was assayed by the procedure previously described [5] The standard assay mixture contained 50 mM Tris–maleate buffer (pH 7 4), 1 mM UDP-[U-^{14}C]glucose, 10 mM glucose 6-phosphate (Glc-6-*P*) when indicated, 80 mM NaF, 0 6 mg of rabbit liver glycogen and enzyme in a final volume of 0 2 ml A unit of enzyme was defined as the amount which catalyzed the incorporation of 1 nmole of [^{14}C]glucose into glycogen per min The total activity (with Glc-6-*P*) minus synthase I activity (without Glc-6-*P*) was taken as the synthase D activity

Assay of glycogen synthase D phosphatase

Glycogen synthase D phosphatase activity of the pellet fraction was measured by the formation of synthase I from the pellet-bound synthase D The pellet fraction was incubated with indicated additions at 30 °C and at 0 and 60 min, aliquots were removed for measurement of glycogen synthase activity in the absence of Glc-6-*P* The difference in activity between the 0-min and 60-min samples was due to the formation of synthase I

Assay of glycogen phosphorylase

Glycogen phosphorylase (EC 2 4 1 1) was assayed by the procedure described by Sato et al [6], except that the standard assay mixture contained 50 mM Tris–maleate buffer (pH 6 1), 25 mM [U-^{14}C]glucose 1-phosphate, 1 mM AMP when indicated, 80 mM NaF and 1 8 mg of rabbit liver glycogen in a final volume of 0 2 ml

A unit of enzyme was defined as the amount which catalyzed the incorporation of 1 nmole of [^{14}C]glucose into glycogen per min The total activity (with AMP) minus phosphorylase *a* activity (without AMP) was taken as the phosphorylase *b* activity

Preparation of glycogen synthase from liver and skeletal muscle

The D- and I-forms of glycogen synthase were prepared and partially purified from the liver and hind leg muscle of the mouse by the procedures essentially similar to those used for the rat liver and muscle enzymes [5, 7]

Other assay methods

Glycogen was determined by the anthrone method [3] Protein was determined with a phenol reagent [8]

Chemicals and commercial enzymes

UDP-[U-^{14}C]glucose and [U-^{14}C]glucose 1-phosphate were obtained from New England Nuclear Corp , Boston UDPglucose, Glc-6-*P*, glucose 1-phosphate, glycogen, ribonuclease, phospholipase D and lysozyme were the products of Boehringer Trypsin was purchased from Novo Industri, Copenhagen

RESULTS

Glycogen content and glycogenic activity

Freshly harvested Ehrlich ascites carcinoma cells were devoid of glycogen as revealed by examination with a highly sensitive anthrone method [3] Furthermore, these cells when incubated in a glucose-containing medium at 30 °C, failed to synthesize glycogen The latter finding was rather unexpected one as we had experienced that rat ascites hepatomas, even if they lack glycogen while growing in the peritoneal cavity, readily synthesize glycogen from glucose in vitro [1–4]

Enzyme levels

To find out the reasons why Ehrlich cells are incapable of glycogenesis, crude extracts were prepared and assayed for glycogen synthase and phosphorylase The results are given in Table I together with comparable values for a rat ascites hepatoma (AH-130 cells) that is glycogen-deficient in vivo but highly glycogenic in vitro [2–4] Contrary to our expectations, the level of the key glycogenic enzyme (glycogen synthase) was higher and the level of the key glycogenolytic enzyme (phosphorylase) was lower in Ehrlich cells than in the hepatoma cells Inherent activities of the rate-limiting enzymes are thus insufficient to explain why Ehrlich cells are incapable of glycogenesis

The lack of conversion of synthase D into synthase I in crude extracts

In crude extracts of Ehrlich and AH-130 cells, synthase activity was predominantly in the D-form as evidenced by an almost absolute requirement for Glc-6-*P* (Table I) Although the synthase D of AH-130 cells extracts is readily convertible into the synthase I upon incubation at 30 °C [4, 7], similar conditions failed to convert the Ehrlich enzyme into the I-form (Fig 1A) This failure of conversion could be due to the presence of an inhibitor Such an inhibitor, if proved to be present, should

TABLE I

ACTIVITIES OF GLYCOGEN SYNTHASE AND PHOSPHORYLASE IN CRUDE EXTRACTS OF MOUSE EHRLICH ASCITES CARCINOMA AND A RAT ASCITES HEPATOMA

The hepatoma studied was AH-130 maintained by serial intraperitoneal transplantation into male adult Donryu rats Its detailed biochemistry has been described [2–4, 7] The cells were harvested 4–5 days after implantation and crude extracts were prepared as described for Ehrlich cells Each value shown is the mean of four experiments ± the standard error of the mean

Enzymes	Activities (units/mg protein)	
	Ehrlich cells	AH-130 cells
Glycogen synthase		
without Glc-6-*P*	0 33 ± 0 04	0 51 ± 0 07
with G c-6-*P*	2 88 ± 0 14	2 56 ± 0 18
Phosphorylase		
without AMP	5 89 ± 0 13	22 7 ± 1 0
with AMP	6 60 ± 0 25	28 3 ± 0 9

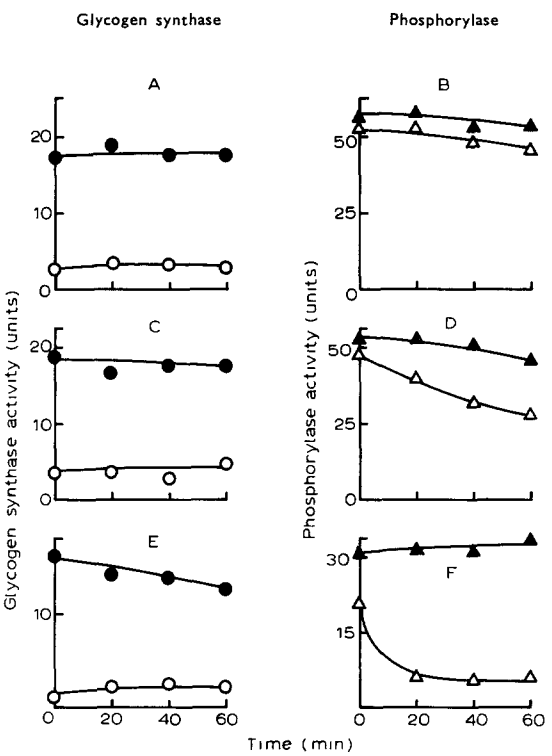


Fig 1 Effect of incubation of crude extract on glycogen synthase and phosphorylase activities Incubations were conducted at 30 °C and at times indicated, aliquots were removed for assay for glycogen synthase activity with (●) or without (○) Glc-6-*P* (A, C and E) or phosphorylase activity with (▲) or without (△) AMP (B, D and F) A and B, extract alone, C and D, extract added with 30 mM glucose, and E and F, extract previously passed through a column (1 5 cm × 20 cm) of Sephadex G-25

offer a reasonable explanation for the lack of glycogenesis in Ehrlich cells, since the above conversion represents the most important mechanism for the activation of glycogen synthase

Of interest in this respect was the finding that the phosphorylase activity of Ehrlich extracts was almost entirely in the *a*-form (as evidenced by the lack of activation by AMP, Table I) and remained to be in this form over the course of 1 h incubation at 30 °C (Fig 1B) Since phosphorylase *a* is known to inhibit the D- to I-form conversion of mouse liver synthase [9], efforts were first made to eliminate phosphorylase *a* from the extracts

Conversion of phosphorylase a into phosphorylase b

It was soon found that addition of glucose directly to the extract caused a substantial conversion of phosphorylase *a* into phosphorylase *b* as shown by the decrease of activity without AMP (Fig 1D) If the extract was previously passed through a column of Sephadex G-25, the conversion was more rapid and complete even without the addition of glucose (Fig 1F) (The reconversion of phosphorylase *b* into phosphorylase *a* occurred readily upon the addition of ATP and Mg^{2+} Ehrlich cells, therefore, possess both the inactivating (phosphorylase *a* phosphatase) and activating (phosphorylase *b* kinase) enzymes of phosphorylase) These findings are consistent with the view that the lack of the *a*- to *b*-form conversion of phosphorylase in crude extracts is due to the presence of a low level of AMP [10, 11] This action of AMP is known to be reversed by glucose [10, 11]

As shown in Fig 1E, however, the synthase activity of Ehrlich extracts continued to be in the D-form even after all the phosphorylase activity had been converted into the *b*-form, suggesting that the lack of the D- to I-form conversion of synthase was not related to the presence of phosphorylase *a* These results are also against the possibility that the lack of synthase activation might be due to endogenous ATP [12] If ATP were the inhibitory factor, then the gel filtration or glucose addition should have eliminated the inhibition [13]

Subcellular localization of synthase

Crude extract of Ehrlich cells was subsequently fractionated by centrifugation at 105 000 × *g* for 1 h and both the pellet and supernatant were assayed for synthase and phosphorylase activities (Table II) As might have been anticipated [14–16], the major part of the phosphorylase activity was recovered in the supernatant But over

TABLE II

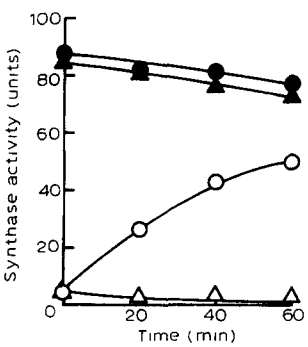
SUBCELLULAR DISTRIBUTION OF GLYCOGEN SYNTHASE AND PHOSPHORYLASE IN EHRLICH CELLS

Fractions	Units/ml of crude extract			
	Glycogen synthase		Phosphorylase	
	−Glc-6-*P*	+Glc-6-*P*	−AMP	+AMP
Crude extract	1 23	22 4	49 6	59 2
Supernatant	0 69	3 14	58 1	60 4
Pellet	0 23	18 5	12 3	12 3

80% of the synthase activity was found to be contained in the pellet fraction In Ehrlich cells, therefore, synthase appears to be bound to particulate components other than glycogen, although the nature and significance of this association has remained to be elucidated

D- to I-form conversion of pellet synthase —presence of an endogenous inhibitor for the conversion

When the pellet fraction alone was incubated in a fresh medium at 30 °C, the synthase D was readily converted into synthase I (Fig 2) That this conversion was due to glycogen synthase D phosphatase was shown by its complete inhibition by 50 mM NaF (Fig 2) or 10 mg/ml of glycogen (Fig 3) Moreover, addition of ATP and Mg^{2+} caused a prompt reconversion of the I- to the D-form, presumably catalyzed by protein kinase (Fig 4) The rate of the reconversion was stimulated by cyclic AMP to the extent of 40% It is of interest that in addition to glycogen synthase, its



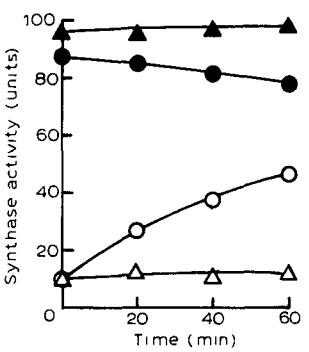


Fig 2 Conversion of pellet synthase D into synthase I and its abolishment by NaF The pellet fraction prepared as described in the text was incubated at 30 °C in the absence (○, ●) or presence (△, ▲) of 50 mM NaF The assays were made in the absence (○, △) or presence (●, ▲) of Glc-6-*P*

Fig 3 Effect of glycogen on the D- to I-form conversion of pellet synthase The pellet fraction was incubated in the absence (○, ●) or presence (△, ▲) of 10 mg/ml of glycogen The assays were made in the absence (○, △) or presence (●, ▲) of Glc-6-*P*

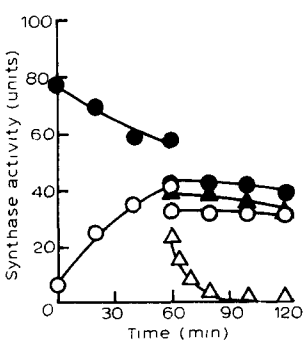


Fig 4 I- to D-form conversion of pellet synthase The pellet fraction was incubated at 30 °C for 1 h, after which 5 mM each of ATP and $MgCl_2$ (△, ▲) or water (○, ●) were added, and incubations were continued for an additional 1 h The assays were made in the absence (○, △) or presence (●, ▲) of Glc-6-*P*

activating (D phosphatase) and inactivating (protein kinase) enzymes are also present in the pellet fraction

These observations leave little doubt that the 105 000 × *g* supernatant of Ehrlich extracts contains a factor that is responsible for the lack of D phosphatase reaction in the extracts The action of this factor is reversible, as the D phosphatase activity readily emerged upon the removal of the supernatant fraction At the levels sufficient to suppress D phosphatase reaction completely, the factor caused no significant inhibition of the *a*- to *b*-form conversion of phosphorylase catalyzed by phosphorylase *a* phosphatase (see Figs 1D and 1F)

Characterization of the inhibitor

To examine the nature of this inhibitory factor, the 105 000 × *g* supernatant was heated at 100 °C for 3 min This treatment neither decreased or increased the inhibitory activity After removal of coagulated proteins by centrifugation, the supernatant was saturated with solid $(NH_4)_2SO_4$, the precipitate formed was collected by centrifugation, dissolved in a minimum volume of 50 mM Tris–HCl (pH 7 4) and dialyzed against several changes of the same buffer The inhibitory activity of the original 105 000 × *g* supernatant was almost quantitatively recovered in the final solution Although the inhibitor is non-dialyzable, a preliminary chromatographic study on Sephadex G-200 indicated that its molecular weight is not larger than 50 000

The inhibitor was quantitatively precipitated from the final solution by 1% trichloroacetic acid This behavior together with the precipitability by $(NH_4)_2SO_4$ implies that the inhibitor is a protein (or proteins) Consistent with this view is the finding that almost 70% of the activity was destroyed upon incubation with trypsin (0 4 mg/ml) for 1 h at 37 °C Other hydrolytic enzymes such as phospholipase D, ribonuclease and lysozyme (0 4 mg/ml, 1 h, 37 °C) were all without effect

Mode of the inhibitory action

Addition of the partially purified inhibitor to the pellet fraction incubated at

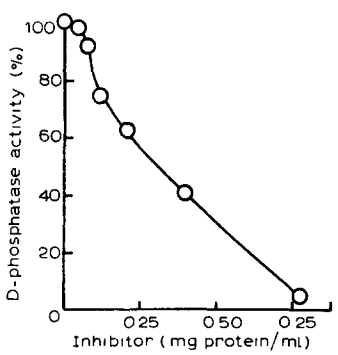
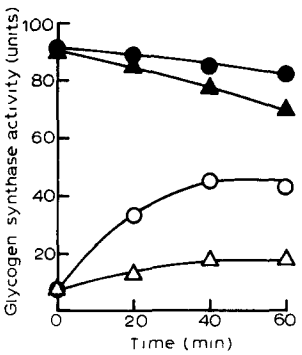


Fig 5 Effect of concentration of the inhibitor on D phosphatase activity of the pellet fraction D phosphatase activity was assayed as described in the text in the presence of varying concentration of the inhibitor The inhibitor partially purified from the 105 000 × *g* supernatant of crude extract by heating followed by $(NH_4)_2SO_4$ precipitation according to the procedure given in the text The purification was 147-fold with an almost quantitative recovery This inhibitor fraction was referred to as the partially purified inhibitor



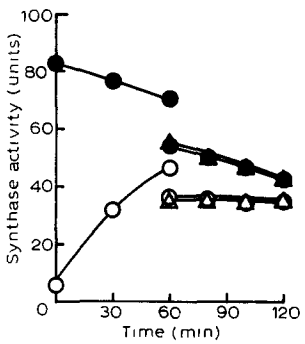
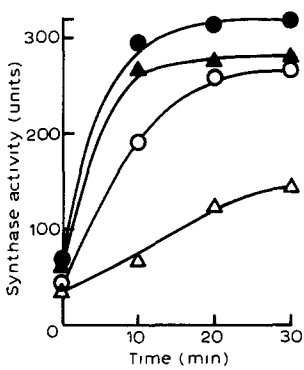


Fig 6 Inhibition of D phosphatase reaction by the partially purified inhibitor The pellet fraction was incubated at 30 °C in the absence (○, ●) or presence (△, ▲) of the inhibitor (0 84 mg protein/ml) The assays were made in the absence (○, △) or presence (●, ▲) of Glc-6-*P*

Fig 7 Effect of the partially purified inhibitor on the I- to D-form conversion of pellet synthase The pellet fraction was incubated at 30 °C for 1 h, after which the inhibitor (1 03 mg protein/ml, final concn) (△, ▲) or 50 mM Tris–HCl (pH 7 4) (○, ●) was added, and incubation was continued for an additional 1 h The assays were made in the absence (○, △) or presence (●, ▲) of Glc-6-*P*



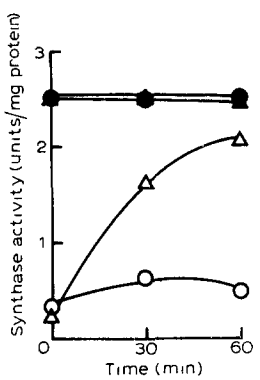


Fig 8 Effects of the partially purified inhibitor and Glc-6-*P* on rat liver D phosphatase reaction Rat liver was homogenized in 4 vol of 0 5 M sucrose containing 62 5 mM Tris–HCl (pH 7 4) and 5 mm EDTA, and after centrifugation at 5000 × *g*, the supernatant was further centrifuged at 50 000 × *g* for 1 h The pellet which contained both synthase D and D phosphatase was suspended in 50 mm glycylglycine (pH 7 4) containing 1 mM caffeine, 2 mN Na_2SO_4 and 4 mm $MgCl_2$, and incubated at 30 °C in the absence (○, ●) or presence (△, ▲) of the inhibitor (2 58 mg/ml) Glc-6-*P* (10 mm) was absent (○, △) or present (●, ▲) At times indicated, aliquots were removed for assayt for synthase activity with Glc-6-*P* The detailed procedures for the preparation of the glycogen pellet and assay for D phosphatase from rat liver were described previously [5, 17] Note that in the case of rat liver synthase, the activity with Glc-6-*P* also increases along with progress of D- to I-form conversion [5, 7]

Fig 9 Effect of Glc-6-*P* on conversion of synthase D into synthase I in Ehrlich extract Crude extract was prepared as described in the text, except that 2 vol of the homogenizing medium were employed The extract was incubated at 30 °C in the absence (○, ●) or presence (△, ▲) of 5 mM Glc-6-*P* At times indicated, aliquots were removed, and after the removal of Glc-6-*P* by passing through a Sephadex G-25 column, assayed for synthase activity in the absence (○, △) or presence (●, ▲) of Glc-6-*P*

30 °C caused an inhibition of the D- to I-form conversion that was proportional to the amount of the inhibitor added (Fig 5) The inhibition occurred readily and persisted throughout the incubation period (Fig 6)

In the experiment shown in Fig 7, the pellet fraction was first incubated at 30 °C to effect the conversion of synthase D into synthase I (at the end of this incubation, a portion of the fraction was removed for centrifugation at 105 000 × *g* for 1 h The synthase was still bound to particulate) Subsequent addition of the inhibitor led to no decrease in synthase activity without Glc-6-*P*, thereby excluding the possibility that the inhibitor acted by reversal of the D- to I-form conversion

The inhibitor is also active against D phosphatase from other tissues as shown in Fig 8, the D- to I-form conversion of rat liver synthase was readily inhibited by the inhibitor Fig 8 also shows that the inhibition can be reversed by Glc-6-*P* Glc-6-*P* has previously been shown to activate D phosphatase reaction [18, 19] That Glc-6-*P* reverses the inhibition is also indicated from Fig 9, where it is shown that the synthase D of Ehrlich extracts was readily converted into synthase I upon addition of Glc-6-*P*

Kinetic properties of mouse tissue synthases

Figs 6 and 7 indicate that, while producing a marked change in D phosphatase activity, the above inhibitor may have no effect on the activities of the D- and I-forms of Ehrlich synthase Kinetic properties of the D-forms (the pellet fraction) and I-forms (the pellet fraction previously incubated at 30 °C for 1 h) of Ehrlich synthase were studied in Tris–maleate buffer of pH 7 4 and the results were compared with those obtained for mouse liver and skeletal muscle enzymes (Table III) It can be seen that the K_m of Ehrlich synthase D for Glc-6-*P* (1 72 mM) was many-fold greater than that of muscle synthase D The possibility that such a low affinity towards Glc-6-*P* may have resulted from contamination by the above inhibitor is unlikely, since addition of the partially purified inhibitor little affected the affinity (towards

TABLE III

KINETIC PARAMETERS OF GLYCOGEN SYNTHASE D AND SYNTHASE I OF MOUSE TISSUES IN TRIS–MALEATE BUFFER OF pH 7 4

K_m for UDPglucose, assays were made under the standard conditions in the presence of 10 mM Glc-6-*P*, except that varying concentrations of UDPglucose were employed The K_m values were obtained by plotting the data double reciprocally and expressed in mM K_m for Glc-6-*P*, assays were made under the standard conditions except that varying concentrations of Glc-6-*P* were employed The K_m values were obtained and expressed as described above

	Ehrlich carcinoma	Liver	Skeletal muscle
K_m for UDPglucose			
Synthase D	0 20	6 80	0 95
Synthase I	0 21	0 89	0 96
K_m for Glc-6-*P*			
Synthase D	1 72	1 13	0 25
Synthase I	0 23	0 08	0 06

Glc-6-*P*) of low-K_m (0 23 mM, ref 7) rat muscle synthase D as well as Ehrlich enzyme (Fig 10)

Table III further demonstrates that the K_m for UDPglucose of liver synthase D is much greater than that of muscle or tumor synthase D A similar pattern of difference has previously been observed with rat tissue enzymes [5, 7]

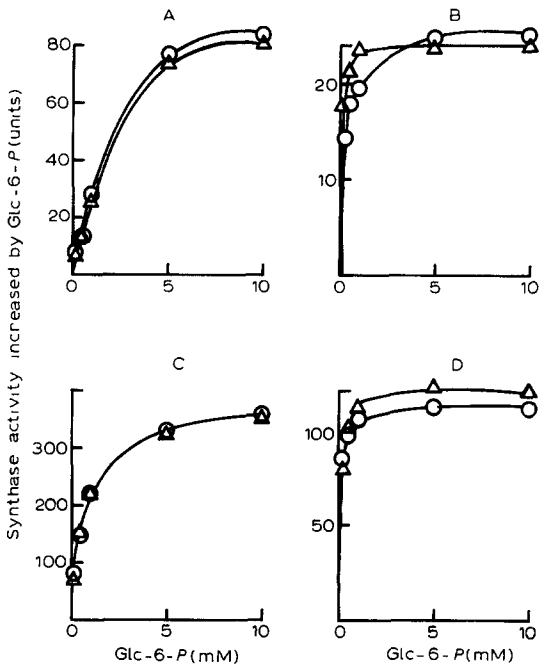


Fig 10 Effect of the partially purified inhibitor on Glc-6-*P* stimulation of glycogen synthase Synthase activity was assayed under the standard conditions, except that varying concentrations of Glc-6-*P* were employed The inhibitor was absent (○), or present at a final concentration of 1 24 mg protein/ml (△) (A) Ehrlich synthase D, (B) Ehrlich synthase I, (C) rat muscle synthase D, and (D) rat muscle synthase I Rat muscle synthase was prepared as described previously [7]

DISCUSSION

Glycogen synthase exists in tissues in two interconvertible forms, inactive synthase D and active synthase I The present studies leave little doubt that there is a factor present in mouse Ehrlich ascites carcinoma cells, which effectively blocks the D- to I-form conversion, thereby preserving the synthase activity entirely in the D-form

The factor is a macromolecule, but differs from either of phosphorylase *a* [9] and glycogen [19–24], which are known to inhibit the D- to I-form conversion Available data suggest that the factor is a heat-stable protein (or proteins) and reversibly and more or less specifically inhibits the reaction catalyzed by glycogen synthase D phosphatase

While the presence of the inhibitor in Ehrlich cells can perfectly account for their apparent incapability for glycogenesis, there is evidence which suggests that

additional tissue factors may be present to regulate the activity of the above inhibitor For instance, we have found in preliminary experiments that even glycogenic AH-130 cells contain a small but nevertheless significant amount of the inhibitor Furthermore, the inhibitor is counteracted by Glc-6-*P* Since the Glc-6-*P* stimulation of synthase D phosphatase reaction [18, 19] is known to be caused by interaction of Glc-6-*P* with the substrate (synthase D) [19], it is possible that the action of the inhibitor may also be to alter the conformation of the substrate rather than to inhibit the phosphatase itself Evidence that the inhibitor directly interacts with glycogen synthase, however, is lacking

Previous studies from this [5, 7] and other laboratories [6, 25–27] demonstrated that liver synthase differs from the synthase of skeletal muscle The present work confirmed these previous observations by showing that the K_m for UDPglucose of mouse liver synthase D was much greater than that of mouse muscle synthase D (Table III) In this respect, the synthase of Ehrlich tumor behaved rather closely to the muscle enzyme (Table III) as was the case with rat hepatoma enzyme [7] Ehrlich synthase, however, is readily distinguished from the muscle enzyme by the markedly low affinity of the D form towards Glc-6-*P* This may reflect the Ehrlich enzyme being a third form of glycogen synthase It might be worthwhile to note that the major phosphorylase form of advanced rat hepatomas was recently shown to be distinct from either form of liver and muscle [6, 28]

The subcellular localization of enzymes concerned with glycogen metabolism has been studied mainly in mammalian liver [14, 15] and skeletal muscle [16] The widely held view is that these enzymes are complexed with glycogen particles, thereby sedimentable when tissue homogenate are subjected to high-speed centrifugation If the tissues are devoid of glycogen, however, the enzymes remain in the supernatant [14–16] For the phosphorylase of Ehrlich cells, this was indeed the case, but the glycogen synthase almost totally sedimented by centrifugation at 105 000 $\times$ *g*, irrespective of the form of the enzyme This finding may be interpreted to indicate that the glycogen synthase of Ehrlich cells is bound to endoplasmic reticulum The occurrence of such binding has previously been suggested by Andersson-Cedergren and Muscatello [29], who found that in frog skeletal muscle, synthase but not phosphorylase sedimented with a microsomal fraction poor in glycogen

The present work further demonstrated that glycogen synthase D phosphatase and protein kinase were co-precipitated from crude extracts with glycogen synthase (Figs 2 and 4) It thus appears that an enzyme complex may exist in Ehrlich cells, which represents a functional unit for glycogenesis and is closely related to endoplasmic reticulum but not necessarily to particulate glycogen

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