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GLUCOSE-6-PHOSPHATE DEHYDROGENASE

PARTIAL CHARACTERIZATION OF THE RAT LIVER AND UTERINE ENZYMES

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Summary

Some properties of rat liver and uterine glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate NADP$^+$ oxidoreductase, EC 1.1.1.49) have been determined. A procedure has been used for the purification of rat liver glucose-6-phosphate dehydrogenase to homogeneity (spec. act. 210—225 units/mg protein) from large amounts of liver (0.5—2 kg) with yields of up to 30% Uterine glucose-6-phosphate dehydrogenase was obtained by immunoprecipitation methods and the properties of radioactively-labeled forms of this enzyme were then determined. The amino acid composition of the liver enzyme was found to be similar to that for the enzyme from other mammalian tissues. The liver and uterine enzymes have a subunit molecular weight of 57 000 and a p*I* of 6.5. The NH_2-terminal amino acid of both enzymes was found to be pyroglutamate.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate NADP$^+$ oxidoreductase, EC 1.1.1.49) of rat liver and uterus has been used as a model protein for studying the regulation of enzyme synthesis and degradation in mammalian tissues. Hepatic glucose-6-phosphate dehydrogenase levels fluctuate

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in response to changes in nutritional conditions of the animal [1,2] and to a number of hormonal regulators [3,4]. The level of the enzyme in the rat uterus is regulated by the differential secretion of ovarian steroids during the estrus cycle [5,6]. Administration of 17β-estradiol to ovariectomized rats causes both an 18-fold increase in the rate of synthesis and a nearly complete inhibition of the degradation of this enzyme [7,8].

Procedures have been developed for the isolation of large quantities of homogeneous rat liver glucose-6-phosphate dehydrogenase. The purified rat liver enzyme has been directly analyzed for its subunit molecular weight, its amino acid composition, its isoelectric point and its amino-terminal end group. Characterization of rat uterine glucose-6-phosphate dehydrogenase which was labeled in vivo with either [^{3}H]leucine or [^{3}H]glutamic acid revealed that it also has a molecular weight and amino-terminus which is the same as the rat liver enzyme

Experimental procedures

Chemicals and biochemicals Substrates for the assay of glucose-6-phosphate dehydrogenase activity, 17β-estradiol, *Streptomyces griseus* protease (Pronase), DEAE-cellulose, CM-cellulose and Sepharose 4B were obtained from Sigma Chemical Co. L-[U-^{3}H]leucine, L-[*methyl*-^{3}H]methionine, L-[U-^{3}H]glutamic acid (all with a spec. act. of 50 Ci/mol) were purchased from Amersham, Inc. L-[U-^{14}C]glutamic acid (14.3 Ci/mol) was from New England Nuclear. Components of the high carbohydrate fat-free diet were from Teklad, Inc. Pyroglutamate aminopeptidase, isolated from *Pseudomonas fluorescens*, was obtained from Dr. R F. Doolittle (University of California, La Jolla). Calf liver pyroglutamate amino peptidase was from Boehringer-Mannheim. Pyroglutamyl-L-alanine was purchased from Cyclo Chemical Co. NADP$^+$-Sepharose containing 2.65 μmol NADP$^+$ per ml Sepharose was prepared as described by Lamed et al. [9]. [^{14}C]Pyroglutamic acid was prepared from [^{14}C]glutamic acid by the method of Moav and Harris [10].

Animals. Mature female albino rats (Small Animal Supply Co., Omaha) weighing 160—180 g were ovariectomized three weeks prior to use. Animals were routinely fed Wayne Lab Blox and water ad libitum. Estradiol (10 μg per rat) was administered in some experiments by tail vein injection in 1.0 ml 5% ethanol/0.15 M NaCl. A non-surgical procedure previously described [11] was used for the transcervical application of radioactive amino acids.

Purification and assay of rat liver and uterine glucose-6-phosphate dehydrogenase Crude and fractionated preparations of liver and uterine glucose-6-phosphate dehydrogenase were assayed at 25°C as previously described [12]. 1 unit enzyme was the amount capable of reducing 1 μmol NADP$^+$/min under the conditions of the assay. Protein concentrations were determined by the method of Lowry et al. [13] using bovine serum albumin as standard. Purification of glucose-6-phosphate dehydrogenase from induced rat livers was by a five-step procedure adapted from earlier procedures [14—16]. To induce increased amounts of glucose-6-phosphate dehydrogenase in the rat livers, groups of rats (usually 60 per group) were fasted for 48 h and then refed a diet composed of sucrose (20% w/w), casein (30% w/w), corn starch (25% w/w), non-nutritive

fiber (21% w/w), mineral mix (Phillips Hart-3% w/w) and a vitamin fortification mixture (1% w/w) ad libitum for 60—72 h At the end of the refeeding period, the rats were killed by cervical dislocation, the livers were removed and frozen between two blocks of solid CO_2 for storage at —80° C. Purification of glucose-6-phosphate dehydrogenase was by a modification of our previously used procedure [7], to which was added an $NADP^+$-Sepharose step similar to that described by DeFlora et al. [15]. Specifically, the first CM-cellulose eluate [7] was precipitated at 50% satn. $(NH_4)_2SO_4$, dialyzed overnight against buffer (0.0125 M sodium phosphate, pH 7.0/0.025 M NaCl/0.025 mM EDTA/7 mM 2-mercaptoethanol) and applied to an $NADP^+$-Sepharose column. The $NADP^+$-Sepharose column was washed sequentially with the buffer containing 0.25 M NaCl, 0.5 M NaCl and then buffer without added NaCl and the enzyme was then eluted with buffer containing 0.1 M $NADP^+$. The enzyme was then dialyzed against storage buffer (0.05 M sodium phosphate, pH 7.0/0.1 M NaCl/0.1 mM EDTA/7 mM 2-mercaptoethanol/0.1 mM NADP) and 10% glycerol and stored at —20° C.

Characterization and immunoprecipitation of glucose-6-phosphate dehydrogenase. The amino acid content of purified rat liver glucose-6-phosphate dehydrogenase was determined using a Beckman-Spinco Model 120B amino acid analyzer with rapid point recorder and high sensitivity cuvette modifications. Beckman-Spinco custom resins PA-35 and PA-28 were used for the short and long columns, respectively. Every third analysis was Sigma AA-S-18 amino acid standards (0.05 μmol each amino acid). Enzyme (0.24 mg) with a specific activity of 225 units/mg was hydrolyzed in individually evacuated and sealed ampules in 6 M HCl at 110° C for 20, 40, 70 and 140 h. The samples were evaporated to dryness and aliquots in 0.2 M sodium citrate, pH 2 2 were applied to the analyzer columns. Threonine and serine were estimated by correcting to initial hydrolysis time, assuming first-order kinetics of their destruction during hydrolysis. Valine and isoleucine were estimated as the averages of samples hydrolyzed for 40, 70 and 140 h, and the other amino acid estimates were the averages of samples hydrolyzed for 20, 40, 70 and 140 h. Aspartic acid and glutamic acid values represent the estimates of these amino acids plus their amide derivatives, asparagine and glutamine. Tryptophan was estimated by the *N*-bromosuccinimide procedure of Patchornick et al [17] and the *p*-toluenesulfonic acid hydrolysis method of Liu [18] and the average result of these two methods which varied from each other by less than 2% are given. One-half cystine content was estimated using the performic acid oxidation method of Moore [19]. The amino acid values which are reported represent averages of three to five independent analyses made at each time of hydrolysis.

Amino-terminal analysis of glucose-6-phosphate dehydrogenase and other proteins were performed with the phenylisothiocyanate reagent of Edman [20] using the paper strip procedure described by Schroeder [21]. Enzymatic hydrolysis of carboxymethylated [22] glucose-6-phosphate dehydrogenase was performed using *S griseus* pronase in a buffer of 0.01 M Tris-HCl, pH 7 0 at a substrate to pronase ratio of either 50 : 1 or 25 · 1. Acidic peptides or amino acid derivatives with blocked amino groups were isolated from the pronase hydrolysate by adjusting the pH of the hydrolysate to pH 3.0 with formic acid and applying the mixture to a cation exchange column (Bio-Rad AG 50W-X2,

H^+ form) at 4°C The acidic peptides and/or amino acid derivatives, including pyroglutamate, were eluted from the column with 3—4 bed vol water and were then frozen, lyophilized and analyzed for composition by either amino acid analysis after hydrolysis in 6 M HCl at 110°C for 20 h, or by direct chromatography on Whatman 3MM paper strips in a *n*-butanol/acetic acid/water (2 : 1 · 1) solvent system.

Immunoprecipitation of radioactively-labeled uterine or liver glucose-6-phosphate dehydrogenase was performed as described by Smith and Barker [7], except that the immunoprecipitation reaction was in enzyme storage buffer to which was added 0.5% each of Triton X-100 and sodium deoxycholate. Sodium dodecyl sulphate(SDS)-polyacrylamide gel electrophoresis of purified glucose-6-phosphate dehydrogenase or immunoprecipitates of radioactively-labeled uterine glucose-6-phosphate dehydrogenase was performed as described by Weber and Osborn [23], except that samples were applied to the gels after denaturation in 0.1 ml of 0.1 M Tris-base/0.1 M dithiothreitol/1% SDS/6 M urea at 90°C for 15 min and 20° for 18 h. Isoelectric focusing of purified liver glucose-6-phosphate dehydrogenase or immunoprecipitated [^{3}H]methionine labeled uterine glucose-6-phosphate dehydrogenase was performed by the method of O'Farrell [24], using pH 3—10 and pH 5—7 ampholines in a ratio of 4 to 1, and with substitution of Triton X-100 for NP-40

Results

Purification

The purification procedure described above gave relatively high yields of purified liver glucose-6-phosphate dehydrogenase (up to 30% yield) from large amounts (0.5—2 0 kg) of fresh or frozen rat livers. Specific activities of 210 to 225 units/mg protein are obtained from starting crude rat liver supernatants having a specific activity of about 0.2 units/mg. When analyzed for homogeneity by SDS-polyacrylamide gel electrophoresis, the highly purified enzyme from this procedure migrated as a single protein band, which contained more than 99.8% of the total stained material on the gel and no significant individual impurities were detected on gels which had been overloaded by addition of up to 20 μg protein (four activity units) per gel.

Molecular weight

The molecular weight of the monomeric form of purified rat liver glucose-6-phosphate dehydrogenase was assessed by SDS-polyacrylamide gel electrophoresis using bovine serum albumin (M_r 68 000), catalase (M_r 60 000), γ-globulin (heavy chain, M_r 50 000, light chain, M_r 23 500), ovalbumin (M_r 43 000) and myoglobin (M_r 17 200) as internal marker proteins which were coelectrophoresed on the same gel with the purified liver glucose-6-phosphate dehydrogenase. A density scan of an electrophoretic gel containing the enzyme and all marker proteins indicates that the enzyme has a subunit molecular weight of 57 000 by this method Published values for the molecular weight of glucose-6-phosphate dehydrogenase subunits vary from rat tissues from 58 000 to 64 000 [25—27], with the predominant active enzyme from rats being dimers with a molecular weight of 130 000 [28—30]. In our hands, glucose-6-phosphate

dehydrogenase prepared as described, clearly migrated further into the gel than the catalase marker and the 57 000 molecular weight estimate was determined from a plot of the log of M_r vs electrophoretic mobility of the density centers of both the marker proteins and glucose-6-phosphate dehydrogenase We have also observed that electrophoresis of dissociated antibody glucose-6-phosphate dehydrogenase complexes prepared with uterine cytosols, using specific rabbit antiserum prepared against rat liver glucose-6-phosphate dehydrogenase, yields only three stainable bands corresponding to the enzyme and the two subunits of rabbit γ-globulin, respectively. The glucose-6-phosphate dehydrogenase band from these preparations also had a molecular weight of 57 000 based on the relative position of the three proteins on the electrophoresis gels. These observations suggest that the molecular weight of 57 000 which we observed for the liver enzyme was not an artifact of the 'harsh' treatments used during routine enzyme purification and further indicated that the molecular weights of the liver and uterine enzymes were identical. Identical molecular weights of uterine and liver glucose-6-phosphate dehydrogenase were also suggested by the identical electrophoretic migration of the stained, purified liver enzyme with the major peak of [^{3}H]glucose-6-phosphate dehydrogenase which had been purified to a specific activity of 132 units per mg from [^{3}H]leucine labeled uterine cytosol proteins

Amino acid composition

The amino acid composition and an estimate of the number of amino acid

TABLE I

AMINO ACID COMPOSITION OF RAT LIVER GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Amino acid analysis was performed as described in Experimental procedures

Amino acid	Composition (%)	n	Residues per subunit (M_r 57 000)
Aspartic acid [c]	10 9 ± 0 4	(17)	54
Threonine [a]	4 9 ± 0 2	(16)	24
Serine [a]	4 7 ± 0 3	(13)	23
Glutamic acid [c]	11 7 ± 0 3	(16)	58
Proline [c]	4 9 ± 0 3	(15)	24
Glycine [c]	7 5 ± 0 4	(14)	37
Alanine [c]	5 9 ± 0 3	(14)	30
Valine [b]	6 7 ± 0 2	(10)	33
Methionine [c]	2 7 ± 0 1	(12)	13
Isoleucine [b]	5 4 ± 0 2	(10)	27
Leucine [c]	8 1 ± 0 2	(14)	40
Tyrosine [c]	4 2 ± 0 2	(16)	21
Phenylalanine [c]	5 1 ± 0 1	(17)	26
Lysine [c]	5 7 ± 0 3	(16)	28
Histidine [c]	2 5 ± 0 1	(16)	13
Arginine [c]	6 3 ± 0 3	(14)	32
Tryptophan [d]	1 5		7
½ Cystine [e]	1 5		7

[a] By extrapolation assuming a first-order rate of destruction

[b] Mean ± S E of values of samples hydrolyzed 40, 70 and 140 h

[c] Mean ± S E of values of samples hydrolyzed 20, 40, 70 and 140 h

[d] Bromosuccinimide and *p*-toluenesulfonic acid methods [17,18]

[e] Performic acid oxidation method [19]

residues per mol of the 57 000 molecular weight subunit of liver glucose-6-phosphate dehydrogenase is given in Table I. The composition of the rat liver enzyme we observed was very similar to that reported by others for the glucose-6-phosphate dehydrogenase from bovine adrenal, human erythrocyte and mouse liver [15,25,26,31—33].

Isoelectric point

The isoelectric point of glucose-6-phosphate dehydrogenase subunits from both purified liver glucose-6-phosphate dehydrogenase and immunoprecipitated [^{3}H]methionine labeled uterine glucose-6-phosphate dehydrogenase was determined by polyacrylamide gel-isoelectric focusing under denaturing conditions by co-focusing 2 units of the liver enzyme with 0.1 units of the ^{3}H-labeled uterine enzyme (Fig. 1). The position of the stained liver glucose-6-phosphate dehydrogenase subunit, indicated by the arrow, was coincident with the major peak of radioactivity in slices prepared from the same gel and both proteins focused at a p*I* of 6.52. The p*I* of rat adipose and mammary glucose-6-phosphate dehydrogenase is reported to be 5.8 [29,34] and the p*I* of the two major forms of human erythrocyte glucose-6-phosphate dehydrogenase is reported to be 6.6 and 6 8 [35]. The differences between our value and those reported by others may be due to species or tissue differences as well as the fact that our value was for a urea-denatured, presumably subunit form, of the enzyme while other reported values were for catalytically active forms of the enzyme which were likely to be in a native dimer or tetramer form.

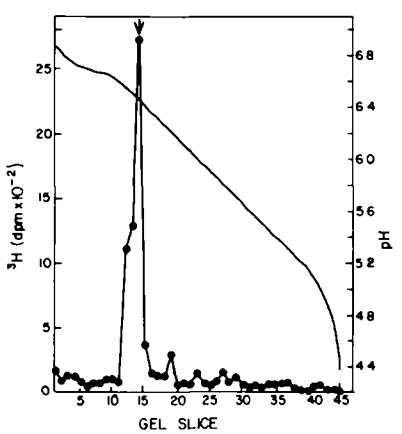

Fig 1 Isoelectric focusing of [^{3}H]methionine containing uterine glucose-6-phosphate dehydrogenase prepared by immunoprecipitation of the uterine cytosol enzyme [^{3}H]Methionine labeled uterine cytosols were prepared from a group of 60 rats which had been given 5 μg estradiol 12 h before the administration of 100 μCi [^{3}H]methionine Animals were killed 3 h after methionine administration Prior to isoelectric focusing, the immunoprecipitate containing about 0 1 units of the labeled uterine enzyme was incubated in the lysis buffer of O'Farrell [24] (9 5 M urea/2% Triton X-100/1 6% pH 5—7 ampholines/ 0 4% pH 3—10 ampholines/5% β-mercaptoethanol) in the presence of an additional 2 units of purified rat liver glucose-6-phosphate dehydrogenase After electrofocusing, the gel was stained, destained and then sliced in 2 mm gel slices The slice containing the densely stained liver glucose-6-phosphate dehydrogenase peak was noted (arrow) and radioactivity was determined for each gel slice The pH gradient of the gel was estimated by extracting gel slices prepared in identical gels in distilled water and measuring the pH of the solution

Amino-terminus

Amino-terminal analysis of liver glucose-6-phosphate dehydrogenase using the phenylisothiocyanate reagent of Edman indicated that this enzyme does not contain an amino-terminus capable of reacting with the reagent. Proteins with known amino-termini (phenylalanine and glycine for insulin and aspartic acid for bovine serum albumin) were simultaneously reacted with the reagent to serve as procedural controls and gave the expected phenylthiohydantoin derivatives. We conclude that the amino-terminus of liver glucose-6-phosphate dehydrogenase is blocked or otherwise unreactive with the phenylisothiocyanate reagent. Structural studies of glucose-6-phosphate dehydrogenase from bovine adrenal, human leucocytes and human erythrocytes also indicated the presence of blocked amino-terminal groups [15,31—33, 36,37]. The amino-terminus of the erythrocyte enzyme was found to be L-pyrrolidone-5-carboxylic acid (pyroglutamate) by Yoshida [37]. The following experiments were thus designed to determine whether the rat liver and uterine enzymes might also contain pyroglutamate at their amino-termini.

Purified liver enzyme (135 nmol) was carboxymethylated and exhaustively digested with *S griseus* pronase for 19 h at 37° C. The resulting hydrolysate was applied to a cation-exchange column (1.5 × 10 cm column of AG50W-X2) which retains amino acids or amino acid derivatives which contain free amino groups. The water eluate from this column, which contained amino acids or peptides which were devoid of free amino groups, was divided into two equal fractions (A and B) and lyophilized Fraction A was hydrolyzed in 6 M HCl at 110° C for 20 h Fraction B was incubated for 20 h with 75 μg calf liver pyroglutamate aminopeptidase, which is specific for cleavage of peptide bonds adjacent to pyroglutamate, and the hydrolysate was applied to a second cation-exchange column. Again the water eluate was lyophilized and hydrolyzed in acid which quantitatively converts pyroglutamate to glutamic acid. The hydrolyzed products from these two treatments (A and B) were then analyzed for their amino acid compositions. The results given in Table II indicate that frac-

TABLE II

AMINO ACID ANALYSIS OF AMINO GROUP-FREE PEPTIDES DERIVED FROM RAT LIVER GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY EXHAUSTIVE DIGESTION WITH PRONASE

Reduced and carboxymethylated rat liver glucose-6-phosphate dehydrogenase (G6PD) was digested with 4% (w/w) pronase The digest was fractionated by cation-exchange chromatography (Bio-Rad AG 50W-22, H^+-form) and the water eluate was lyophilized One-half of this fraction was hydrolyzed in 6 M HCl for 20 h at 110° C (Fraction A) The remaining fraction was incubated for 20 h at 30° C with 75 μg calf liver pyroglutamate aminopeptidase (PGA peptidase), chromatographed on another cation-exchange column, and then the water eluate was hydrolyzed (Fraction B) Fractions A and B were subjected to a complete amino acid analysis and only glutamic acid and glycine were present in significant amounts (>0 5 mol/mol of 57 000 M_r enzyme)

Amino acid	Pronase digestion (A) (mol/mol G6PD)	Fraction A after PGA peptidase digestion (B) (mol/mol G6PD)	B/A
Glutamic acid	0 68	0 52	0 77
Glycine	0 76	0 19	0 25

tion A contained nearly equal amounts of glutamic acid and glycine. Fraction B contained nearly the same amount of glutamic acid as fraction A but the amount of glycine in fraction B was reduced. Since the product obtained from the initial Pronase treatment could be enzymatically hydrolyzed to a product which contained glycine, which could now bind to the cation exchange column following treatment with pyroglutamate aminopeptidase, and since this peptidase is specific for peptide bonds between pyroglutamate and the penultimate amino acid of a peptide, these results suggest that the Pronase digestion of purified rat liver glucose-6-phosphate dehydrogenase yielded a product which contained pyroglutamate and glycine. Since the amino group in pyroglutamate is not available to form a peptide bond with another amino acid, these results place the pyroglutamate at the amino-terminus of liver glucose-6-phosphate dehydrogenase.

A more direct approach to determining the amino-terminus of liver glucose-6-phosphate dehydrogenase was also employed. Purified liver glucose-6-phosphate dehydrogenase (175 nmol) was directly incubated at 30° C, for 24 h, with *Ps fluorescens* pyroglutamate aminopeptidase (an amount which could hydrolyze 25 nmol pyroglutamyl-L-alanine per h). After hydrolysis, the proteins were precipitated by addition of ethanol, the ethanol extract was lyophilized and subjected to cation-exchange chromatography as above. One-half of the water eluate from this column (Sample A) was hydrolyzed in 6 M HCl at 100° C for 2 h to quantitatively convert pyroglutamate, if it were present, to glutamic acid The second half (Sample B) was not acid hydrolyzed. A third fraction (Sample C) which was identical to fraction A, except that glucose-6-phosphate dehydrogenase was deleted, served as a reagent control. The acid-hydrolyzed and the unhydrolysed fractions were individually subjected to amino acid analysis (Table III). Sample B contained less than 2 nmol glutamic acid and Sample A contained 79 nmol glutamic acid. These results indicated that pyroglutamate

TABLE III

REMOVAL OF AMINO-TERMINAL PYROGLUTAMIC ACID FROM RAT LIVER GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY PYROGLUTAMATE AMINO PEPTIDASE

Purified liver glucose-6-phosphate dehydrogenase (G6PD) (175 nmol) was incubated with *Ps fluorescens* pyroglutamate aminopeptidase (PGA peptidase), 25 nmol units, in 2 ml 0 05 M potassium phosphate, pH 7 3/1 mM EDTA/10 mM 2-mercaptoethanol at 30° C for 24 h After ethanol precipitation of protein and the supernatant was dried and subjected to cation-exchange chromatography as described in Table II One-half of the water eluate from this column (Sample A) was hydrolyzed in 6 M HCl at 100° C for 2 h while the second-half (Sample B) was not acid hydrolyzed Sample C is a reagent control and was identical to Sample A except that glucose-6-phosphate dehydrogenase was deleted A pyroglutamated standard (156 nmol) subjected to the same procedures yielded 141 nmol glutamate (90 3% recovery) The net yield of pyroglutamate-derived glutamate obtained from glucose-6-phosphate dehydrogenase is the amount present in Sample A minus the sum of the amount in samples B and C

Sample	Treatment	Glutamic acid (nmol)	Recovery corrected glutamic acid (nmol)
A	G6PD + PGA peptidase (acid hydrolyzed)	79 0	87 4
B	G6PD + PGA peptidase (not acid hydrolyzed)	1 9	2 1
C	PGA peptidase (acid hydrolyzed)	8 0	8 8
		69 1 (net)	76 5 (net)

aminopeptidase action on 175 nmol glucose-6-phosphate dehydrogenase produced a total of 77 nmol pyroglutamate, of which 8 nmol were present as a contaminant of the peptidase (Sample C) When the 69 nmol derived from glucose-6-phosphate dehydrogenase were corrected for the 90.3% yield for the recovery and conversion of a pyroglutamate standard to glutamic acid, the amount of pyroglutamate produced from liver glucose-6-phosphate dehydrogenase by the peptidase was found to be 43.6% of the molar amount of glucose-6-phosphate dehydrogenase subunits added to the initial reaction. This relatively low yield of amino-terminal pyroglutamate was expected since pyroglutamate aminopeptidase is known to exhibit a low efficiency, but high degree of specificity for removal of pyroglutamate from large proteins [39,40]. These two independent experiments clearly indicate that the majority, if not all, of the blocked amino-terminal residues in purified rat liver glucose-6-phosphate dehydrogenase are pyroglutamate.

To determine the amino-terminal residue of rat uterine glucose-6-phosphate dehydrogenase, 19 ovariectomized mature rats, treated 12 h previously with 5 μg estradiol, were each given 100 μCi [^{3}H]glutamic acid by the intrauterine route 2 h prior to sacrifice The uterine enzyme was isolated by immunoprecipitation and the immunoprecipitate was carboxymethylated Pronase digestion was carried out on the carboxymethylated immunoprecipitate for 18 h with subsequent fractionation of the hydrolyzed sample by AG 50W-X2 cation-exchange chromatography The material which did not bind to the column was then digested with pyroglutamate aminopeptidase, subjected to a second round of cation-exchange chromatography and after concentration was subjected to

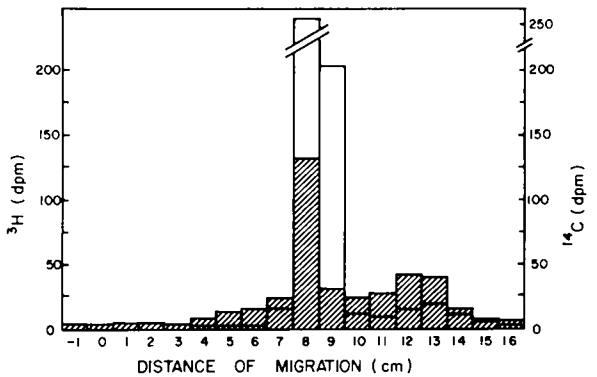

Fig 2. Distribution of radioactivity after paper chromatography of acidic peptide fragments derived by Pronase digestion and then pyroglutamate aminopeptidase digestion of immunoprecipitates of [^{3}H]glutamate labeled uterine glucose-6-phosphate dehydrogenase 20 ovariectomized mature rats were given 5 μg estradiol for 12 h prior to the intrauterine administration of 100 μCi [^{3}H]glutamic acid After 2 h, uteri were pooled and uterine glucose-6-phosphate dehydrogenase was obtained by immunoprecipitation The immunoprecipitate was carboxymethylated, incubated with Pronase and subjected to cation-exchange chromatography to separate the acidic peptides from the Pronase The acidic peptides present in the water eluate were digested with calf liver pyroglutamate aminopeptidase and then chromatographed on a second cation-exchange column The water eluate was lyophilized and subjected to paper chromatography on Whatman 3 MM paper strips in a butanol/acetic acid/water (2 1 1, v/v/v) solvent system A [^{14}C]-pyroglutamate standard, unlabeled carrier pyroglutamate and glutamic acid were chromatographed with the sample ^{3}H derived from glucose-6-phosphate dehydrogenase is given as crosshatched bars and the [^{14}C]pyroglutamate standard is given as open bars Free glutamate was localized 4—5 cm into the chromatogram

paper chromatography. The ^3H radioactivity peak migrated coincidentally with an internal [^{14}C]pyroglutamate standard which was also added to the sample (Fig. 2). This result identifies the ^3H-labeled compound, derived from [^3H]glutamate labeled uterine glucose-6-phosphate dehydrogenase, to be pyroglutamate based both on the chromatographic analysis of the products and the specificity of pyroglutamate aminopeptidase We therefore conclude that the amino-terminus of uterine glucose-6-phosphate dehydrogenase is also pyroglutamic acid. A comparison of the amount of [^3H]pyroglutamate relative to [^3H]glutamate in the immunoprecipitable uterine glucose-6-phosphate dehydrogenase revealed that about 1% of the total radioactivity was in the form of pyroglutamate. This is consistent with the presence of 58 glutamate residues per molecule if one assumes an approximate 60% recovery of [^3H]pyroglutamate following two steps of enzymatic digestion and chromatography.

The presence of pyroglutamate at the amino-terminus of human erythrocyte, rat liver and rat uterine glucose-6-phosphate dehydrogenase indicates that this enzyme is initially synthesized in a precursor form. Since methionine is the amino acid which is required to initiate the synthesis of eukaryotic proteins [38], the presence of pyroglutamate rather than methionine indicates that methionine or a larger peptide or peptides is removed from a precursor, as are many other cytosol proteins, to produce the predominant form which is present in the cytosols of these tissues. SDS-polyacrylamide gel electrophoresis of immunoprecipitates of glucose-6-phosphate dehydrogenase, from cytosols of uteri which had received in vivo [^3H]leucine for very short pulse intervals (1—2 min), reveals that radioactivity was initially incorporated into a glucose-6-phos-

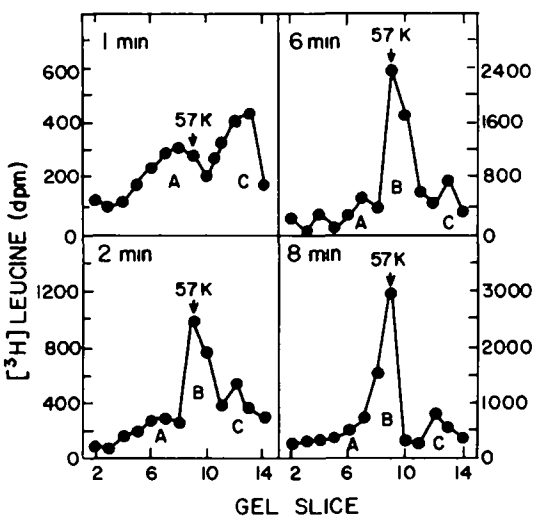

Fig 3 Effect of [^3H]leucine pulse time on the SDS-polyacrylamide gel electrophoretic profiles of radioactivity in uterine glucose-6-phosphate dehydrogenase immunoprecipitates. Groups of 10 ovariectomized rats which had received 5 μg estradiol for 12 h were given 20 μCi [^3H]leucine by the intrauterine route for 1, 2, 6 or 8 min prior to killing the animals Immunoprecipitates of uterine cytosols were prepared and electrophoresed as indicated in Experimental procedures The arrow indicates the position of the 57 000 M_r subunit of the predominant form of glucose-6-phosphate dehydrogenase which is normally found in the uterine cytosol Peaks A, B and C have been tentatively identified as a precursor-form, processed-form and nascent-chain of glucose-6-phosphate dehydrogenase, respectively

phate dehydrogenase-like protein (Peak A) with a molecular weight of 60 000—65 000 (Fig 3) and a glucose-6-phosphate dehydrogenase-like protein (Peak C) of a lower molecular weight (about 40 000) Only after longer pulse intervals (approx. 4 min) was the radioactivity converted to and found in an immunoprecipitable species (Peak B) with a molecular weight of 57 000. Immunoprecipitation of both Peaks A and B can be inhibited by addition of an excess of purified rat liver glucose-6-phosphate dehydrogenase and preliminary tryptic peptide map comparison studies using [^{3}H]methionine or [^{35}S]methionine labeled peaks A, B and C indicate considerable sequence homology between all three peaks The kinetics of labeling of peaks A, B and C suggest that they may be a precursor-form, processed-form and nascent-chain form of uterine glucose-6-phosphate dehydrogenase, respectively. Although we have found [^{3}H]pyroglutamate in immunoprecipitates of [^{3}H]glutamate labeled uterine cytosols (Fig. 2)., we have not yet determined whether it is specific for any one of the three peaks of radioactivity evident when these very short labeling times are employed.

Discussion

Uterine glucose-6-phosphate dehydrogenase is present in relatively small amounts in comparison to the amount of the enzyme which is present in the rat liver. Direct purification of the enzyme from the uterus is impractical if one needs a large amount of the enzyme to do analytical studies Studies of the uterine enzyme are, therefore, limited to measurements involving either partially purified enzyme or immunoprecipitates of labeled uterine glucose-6-phosphate dehydrogenase using antisera prepared against the purified liver enzyme. Our approach to characterization of uterine glucose-6-phosphate dehydrogenase has relied on making direct measurements of the properties of the purified liver enzyme and making comparisons of the properties found for the liver enzyme to those of the immunoprecipitable radioactively-labeled uterine enzyme. The present study indicates that glucose-6-phosphate dehydrogenase from rat liver and uterus have many properties in common, and that these properties are similar to those reported for this enzyme derived from other animal sources [25,26] Although the amino acid composition of the uterine enzyme could not be measured directly, the fact that the uterine enzyme has the same molecular weight and isoelectric point as liver glucose-6-phosphate dehydrogenase, and the fact that all amino acid determinations of the enzyme from mammalian sources are very similar, suggests that within the same species, the amino acid composition of uterine glucose-6-phosphate dehydrogenase is probably identical to that of the rat liver enzyme.

Several reports suggest that glucose-6-phosphate dehydrogenase obtained from a variety of animal sources has a blocked amino-terminus [15,31,36,37] and one report by Yoshida [37] identified this to be pyroglutamate It has been suggested that the presence of pyroglutamate at the amino-terminus of the human erythrocyte enzyme may have been an artifact which was generated by proteolytic cleavage or other modification reactions such as cyclization of glutamate or glutamine during the rigorous purification procedures used by that author In our studies, care was taken to use conditions during Pronase diges-

tion and fractionation of the Pronase digestion products which minimize non-enzymatic cyclization of glutamine. Specifically, the pH of the digestion was maintained at 7 or lower and cation-exchange chromatography was conducted at 4°C [41]. In one experiment, [^{14}C]glutamine was added during the Pronase digestion of the purified liver enzyme. After Pronase hydrolysis, less than 0.36% of the [^{14}C]glutamine was found in the water eluate of the cation-exchange column, indicating only minimal artifactual cyclization of free glutamine to pyroglutamate had occurred Others found that as much as 8% of glutamine added in this manner would be converted to pyroglutamate if the pH during digestion was maintained at 8 [42]. Additional indications that pyroglutamate at the amino-terminus of glucose-6-phosphate dehydrogenase is not an experimental artifact are (1) radioactively-labeled pyroglutamate was found at the amino-terminus of [^{3}H]glutamate labeled uterine cytosol glucose-6-phosphate dehydrogenase which was isolated by immunoprecipitation of the enzyme, which minimizes the likelihood that pyroglutamate was produced by the rigorous purification procedure which was used to prepare the rat liver enzyme. (2) The direct removal of pyroglutamate from purified liver glucose-6-phosphate dehydrogenase by pyroglutamate aminopeptidase without prior inactivation of the enzyme by carboxymethylation, indicates that pyroglutamate was not an artifact of either the alkylation reaction or the actions of Pronase.

The occurrence of a transiently labeled immunoprecipitable form from rat uterine cytosol which has a heterogenous molecular weight of 60 000—65 000 suggests that the predominant form of the enzyme present in the rat uterus may be derived in this tissue by the action of an enzyme capable of processing the putative precursor form into a lower molecular weight form. We are presently evaluating the possibility that pyroglutamate is uniquely present in the 'processes' 57 000 molecular weight form of the enzyme and, if so, we propose to use the time course of the incorporation of [^{3}H]glutamate into [^{3}H]pyroglutamate of glucose-6-phosphate dehydrogenase immunoprecipitates to evaluate the effects of estrogen on this 'processing' event in the rat uterus.

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References

1 Tepperman, H M and Tepperman, J (1963) Adv Enzyme Regul 1, 121—136
2 Winberry, L. and Holten, D. (1977) J Biol Chem 252, 7796—7801
3 Tepperman, H M and Tepperman, J. (1964) Am J Physiol 206, 357—361
4 Garcia, D R. and Holten, D (1975) J Biol Chem. 250, 3960—3965
5 Pepe, G and Yochim, J M (1971) Biol Reprod. 5, 161—171
6 Pepe, G and Yochim, J M (1971) Endocrinology 89, 366—377
7 Smith, E R and Barker, K L (1974) J Biol Chem. 249, 6541—6547
8 Smith, E R and Barker, K L (1977) J Biol Chem 252, 3709—3714
9 Lamed, R , Levin, Y and Wilchek, M (1973) Biochim Biophys Acta 304, 231—235
10 Moav, B and Harris, T N. (1967) Biochem Biophys Res Commun 29, 773—776
11 Barker, K L (1967) Endocrinology 81, 791—797
12 Moulton, B C and Barker, K L (1971) Endocrinology 89, 1131—1136

13 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265—275
14 Kirkman, H N (1962) J Biol Chem. 237, 2364—2370
15 DeFlora, A , Morelli, A , Frascio, M , Corte, G , Curt, G , Galliano, M , Gozzer, C , Minchiott, C , Mareni, C and Goelani, G (1977) Biochim Biophys Acta 500, 109—123
16 Matsuda, T and Yugari, Y (1967) J. Biochem 61, 535—540
17 Patchornik, A , Larson, W B , Gross, E and Witkop, B (1960) J Am Chem Soc 82, 5923—5927
18 Liu, T Y (1972) Methods Enzymol 25B, 44—55
19 Moore, S (1963) J Biol Chem. 238, 235—237
20 Laman, P (1950) Acta Chem. Scand. 4, 283—293
21 Schroeder, W A (1972) Methods Enzymol 25, 298—313
22 Crestfield, A , Moore, S and Stein, W H. (1963) J Biol Chem 238, 622—627
23 Weber, K. and Osborn, M (1969) J Biol Chem 244, 4406—4412
24 O'Farrell, P.H (1975) J Biol Chem. 250, 4007—4021
25 Bonsignore, A. and DeFlora, A. (1972) Curr Top Cell Regul 6, 21—61
26 Levy, H.R (1979) Adv Enzymol 48, 97—192
27 Watanabe, A and Taketa, K (1973) Arch Biochem Biophys. 158, 43—52
28 Holten, D (1972) Biochim. Biophys Acta 268, 4—12
29 Geisler, R W , Roggeveen, A.E and Hansen, R J (1978) Biochim Biophys Acta 544, 284—293
30 Dao, M L , Watson, J J , Delaney, R. and Johnson, B C (1979) J Biol Chem. 254, 9441—9447
31 Singh, D and Squire, P G (1975) Int J Peptide Protein Res 7, 185—188
32 Yoshida, A (1972) J Biol. Chem. 241, 4966—4976
33 Hizi, A and Yagil, G (1974) Eur J Biochem 45, 201—209
34 Nevaldine, B H , Hyde, C M and Levy, H R (1974) Arch Biochem Biophys 165, 398—406
35 Kaloustian, V M D , Idriss-Daouk, S H , Hallal, R T. and Awdeh, Z L (1974) Biochem Genet 12, 51—58
36 Kahn, A , Bertrand, O., Cottreau, D , Biovin, P and Dreyfus, J C (1976) Biochim Biophys Acta 445, 537—548
37 Yoshida, A (1972) Anal Biochem 49, 320—325
38 Lucas-Lenard, J and Lipmann, F (1971) Ann Rev Biochem 40, 409—448
39 Doolittle, R F and Armentrout, R W (1968) Biochemistry 7, 516—521
40 Doolittle, R F (1972) Methods Enzymol 25, 231—244
41 Melville, J (1935) Biochem J 29, 179—186
42 Twardzik, P and Peterfofsky, A (1972) Proc Natl Acad Sci U S A 69, 274—277