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PURIFICATION AND CHARACTERIZATION OF HUMAN ERYTHROCYTE URIDYLYL TRANSFERASE

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Summary

A new method for the purification of human erythrocyte uridylyl transferase (UDPglucose: α-D-galactose-1-phosphate uridylyltransferase EC 2.7.7.12) is described. It consists of a hydrophobic purification step associated with hydroxyapatite chromatography and provided for the first time a purification of more than 45 000-fold with a high specific activity (15 I.U./mg) and a yield of 32%.

We show that the enzyme is a dimer and has a molecular weight of 88 000. It can be resolved into three bands by isoelectric focusing with an apparent p*I* between 5.0 and 5.4.

It could be shown by steady-state initial rate measurements that the interconversion of the two substrates of human transferase (Gal-1-*P* and UDP-glucose) follows ping-pong bi-bi kinetics, with K_m values of 0.2 and 0.065 mM, respectively.

Introduction

Uridylyl transferase (UDPglucose: α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12) is one of the three enzymes which are involved in the Leloir pathway for the conversion of galactose to glucose 1-phosphate. Absence of the catalytic activity of the enzyme in man results in galactosemia, a genetic disease with an autosomal recessive transmittance [1]. This deficiency results in the accumulation of galactose, galactitol and galactose 1-phosphate [2,3] leading to

Abbreviations: Gal-1-*P*, galactose 1-phosphate; UDP-glucose, uridine diphosphoglucose; UDP-galactose, uridine diphosphogalactose. I.U., international unit of enzyme activity defined as the amount of enzyme which catalyzes the formation of 1 μmol NADPH/min at 37°C.

hepatosplenomegaly, mental retardation, cataracts and other severe disturbances. Several groups [4—7] have presented evidence that the disease is caused by a structural gene mutation. Genetic variants of the enzyme have been described on the basis of altered electrophoretic mobility and enzymatic activity [8—12]. Although many properties of the enzyme from several tissues and species have been studied [13—17], only a few laboratories have tried to purify it [5,6,18,19].

The shortcomings of the known procedures prompted us to design a new purification method for the human erythrocyte uridylyl transferase. The kinetic parameters as well as the molecular weight, subunit composition and p*I* of the purified enzyme were determined.

Materials and Methods

Materials. The substrates and auxiliary enzymes were supplied by Boehringer or Sigma, acrylamide and bisacrylamide by Eastman Kodak, ampholines by LKB. DEAE-cellulose (DE-52) was supplied by Whatman, Sephadex G-200 by Pharmacia Fine Chemicals; hydroxyapatite Bio-Gel HTP was obtained from Bio-Rad. Hexyl-agarose was produced by Miles Laboratories; Ultrogel ACA-202 was purchased from IBF-Pharmindustrie. Protein standards for calibration of gels were obtained from Boehringer; sodium dodecyl sulfate (SDS) and reagent used for protein determination were purchased from Bio-Rad.

Enzyme assays. Transferase activity was determined by a slight modification of the NADP-coupled system of Mayes and Hansen [20]. Standard assays were carried out at 37°C in glycine buffer (100 mM) containing: $MgCl_2$ (5 mM)/dithiothreitol (5 mM)/NADP (0.8 mM)/Gal-1-*P* (0.75 mM)/UDP-glucose (0.5 mM)/glucose 1-6-diphosphate (5 μM)/glucose-6-phosphate dehydrogenase (0.5 I.U./ml), phospho-glucomutase (0.5 I.U./ml) and 6-phosphogluconate dehydrogenase (0.5 I.U./ml). The pH of the mixture was 8.6. 1 enzyme unit is defined as the amount of enzyme which catalyzes the formation of 1 μmol NADPH/min at 37°C.

We have adopted the kinetic nomenclature and mathematical representation of Cleland [21—23]. Primary plots were constructed from a Lineweaver-Burk representation for determining kinetic patterns and parameters. Secondary plots were obtained by replotting intercepts against the reciprocal of the substrate concentration.

Thermal inactivation kinetics. A solution of transferase (about 0.2 mg/ml) was incubated in a water bath at the desired temperature and an aliquot was taken at intervals to test residual activity.

Protein determination. Proteins were determined by absorbance at 280 nm and by the Bio-Rad reagent procedure with bovine serum albumin as standard [24]. The color development of this reagent is not affected by the dithiothreitol added to all buffers.

Polyacrylamide gel electrophoresis. Disc electrophoresis was carried out as described by Davis [25] using 5% acrylamide gels. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn [26] using 7.5% acrylamide gels. Prior to electrophoresis, the samples were heated in the presence of 1% SDS at 65°C for 10 min, and then concentrated in

a collodion bag (sartorius Membran Filter). Proteins were detected by staining with Coomassie brilliant blue.

Polyacrylamide gradient slab gel electrophoresis in the presence of SDS. SDS-polyacrylamide gels (5—30% acrylamide, w/v) were prepared according to the method of Laemmli [27]. The samples and the marker proteins were heated at 65°C for 10 min in 1% SDS. Flat-bed gels were run at room temperature at a constant current of 10 mA/plate for 20 h. After staining and destaining gels, the subunit molecular weights were estimated from a calibration curve obtained from simultaneous electrophoresis with albumin (M_r 68 000), trypsin (M_r 21 000), aldolase (M_r 40 000) and RNA-polymerase (M_r 39 000, M_r 155 000, M_r 165 000) as protein markers.

Isoelectrofocusing on acrylamide gel. Isoelectrofocusing on acrylamide gel was performed on a Multiphor LKB, using Vesterberg's method [28] with modifications as described previously [29]. The gel contained a final concentration of 2.5% of ampholines, pH 4.0—7.0. After migration for 6 h at 750 V and 4°C, the gel was covered with an agar layer containing a specific staining mixture. The detection of transferase was performed according to Mathai and Beutler [30] with some modifications as previously described [10]. Fluorescent bands of NADPH corresponding to the enzyme activity were detectable under long-wave ultraviolet light.

Electrophoresis on starch gel. A crude hemolysate was submitted to horizontal gel electrophoresis in a phosphate/citrate buffer (pH 7.5) containing 10 mM β-mercaptoethanol in starch gel, at 3 V/cm for 16 h at 4°C. After migration, transferase activity was localized on the sliced gel by a specific staining mixture as described previously [29].

Determination of molecular weight by gel filtration. The molecular weight was determined by gel filtration on a Sephadex G-200 column, equilibrated with 10 mM phosphate buffer (pH 7.0)/2.5 mM dithiothreitol. The protein markers used for column calibration were: catalase (M_r 240 000), lactate dehydrogenase (M_r 140 000), galactose dehydrogenase (M_r 100 000), phosphoglucomutase (M_r 65 000) and creatine phosphokinase (M_r 81 000). The elution volume (V_e) of the protein was determined by monitoring the enzyme activity; Dextran blue and [^{3}H]leucine were used, respectively, to determine the void volume of the column (V_0) and the total volume of the column (V_t).

Determination of molecular weight by sucrose density gradient centrifugation. The sediment coefficient and the molecular weight were determined by sucrose density gradient centrifugation [31]. The linear gradient used was 5—20% (w/v) sucrose in 10 mM phosphate buffer (pH 7.0) containing 2.5 mM dithiothreitol. Centrifugation was performed at 4°C for 17 h at 35 000 rev./min. After centrifugation, the gradients were divided into 0.3 ml fractions, which were assayed for enzyme activity and protein determination.

Purification of erythrocyte transferase. All of the steps in the enzyme purification were performed at 4°C. Buffer 1 used was 10 mM phosphate buffer, pH 7.0/2.5 mM dithiothreitol. Buffer 2 had the same composition as buffer 1 except that it contained only 1 mM phosphate.

Step 1. Hemolysate preparation. Human blood was obtained from blood banks. The red cells were sedimented at 5000 × g for 10 min and were washed three times with 3 vol. 0.9% NaCl. Then they were lysed by the addition of

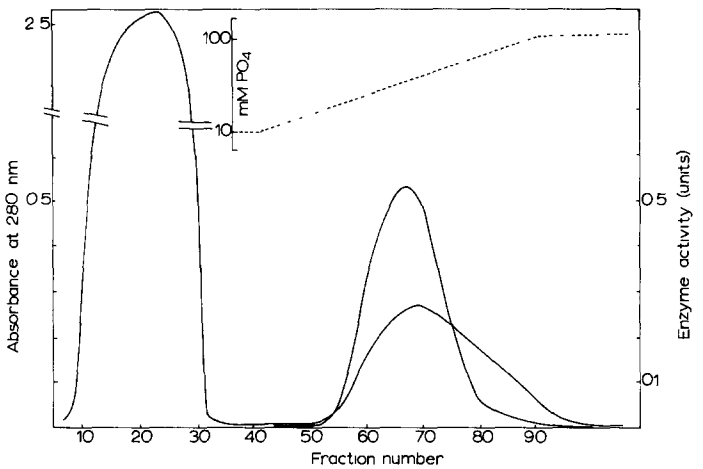

Fig. 1. Elution pattern of human erythrocyte transferase from hexyl-agarose chromatography. The enzyme was eluted with a linear gradient of 10—100 mM phosphate buffer (pH 7.0)/2.5 mM dithiothreitol. 4-ml fractions were collected. Absorbance at 280 nm (———); transferase activity (▬▬); gradient concentrations (- - - - -).

5 vol. cold water containing protease inhibitors: 1 mM diisopropyl phosphorofluoridate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM *N*-α-*p*-tosyllysine chloromethylketone, 0.5 mM *p*-tosylamido-2-phenylethyl chloromethylketone, 0.05 mM of the cathepsin D inhibitor, pepstatin and 2.5 μg/ml of the cathepsin B inhibitor, leupeptin.

Step 2. Batch treatment with DEAE-cellulose. After centrifugation the sediment was discarded and the supernatant was added to a suspension of DEAE-

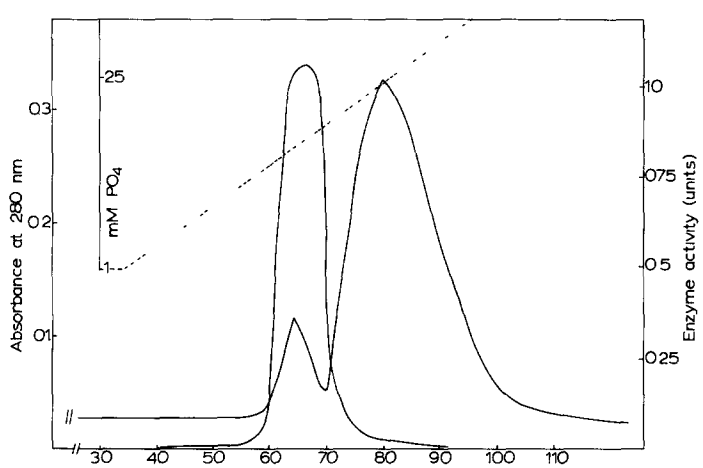

Fig. 2. Elution pattern of human erythrocyte transferase from hydroxyapatite chromatography. The enzyme was eluted with a linear gradient of 1—50 mM phosphate buffer (pH 7.0) containing 2.5 mM dithiothreitol. 1.0-ml fractions were collected. Fractions no. 60—70 were pooled and constitute the purified enzyme preparation as shown in Table I. Absorbance at 280 nm (———); transferase activity (▬▬); gradient concentrations (- - - - -).

cellulose. The mixture was stirred for 2 h and then washed in a scintered-glass funnel until the absorbance at 280 nm of the filtrate was the same as that of the buffer. The enzyme was eluted by 75 mM $(NH_4)_2SO_4$ in phosphate buffer.

Step 3. $(NH_4)_2SO_4$ precipitation. The eluate was fractionated with solid $(NH_4)_2SO_4$: the transferase was precipitated at 55% $(NH_4)_2SO_4$ saturation. The precipitate was collected and resuspended in buffer 1. The $(NH_4)_2SO_4$ was eliminated by filtration on an Ultrogel ACA-202.

Step 4. DEAE-cellulose chromatography. The enzyme partially purified by $(NH_4)_2SO_4$ precipitation was applied to a DEAE-cellulose column (2.5 × 40 cm) and washed with 400 ml buffer 1. The enzyme was then eluted with a linear gradient formed from 400 ml starting buffer and 400 ml 50 mM $(NH_4)_2SO_4$ in buffer 1. The enzyme was found to elute at approx. 20 mM $(NH_4)_2SO_4$.

Step. 5. Hydrophobic chromatography. The dialyzed preparation from step 4 was further chromatographed on a hexyl-agarose column (1.5 × 25 cm) equilibrated with buffer 1 and the uridylyl transferase was eluted with a linear phosphate gradient. This gradient was formed from 100 ml each of 10 mM and 100 mM sodium phosphate buffers, pH 7.0 containing 2.5 mM dithiothreitol. A typical elution profile is shown in Fig. 1.

Step 6. Hydroxyapatite chromatography. The purified enzyme from step 5 was rechromatographed on an Ultrogel ACA-202 column equilibrated with buffer 2 in order to lower the ionic strength of the preparation. The active fractions were pooled and applied to a hydroxyapatite column (1.5 × 20 cm) equilibrated and washed with buffer 2. The enzyme was eluted with a linear gradient formed from 80 ml each of 1 mM and 50 mM sodium phosphate buffers (pH 7.0) containing 2.5 mM dithiothreitol (Fig. 2). Active fractions were assayed and then pooled.

Results

Purification and characterization of enzyme. A summary of the results of the purification procedure is given in Table I. The hydrophobic chromatography step on hexyl agarose resulted in a further 6-fold purification of the enzyme from the step above. The last step (hydroxyapatite chromatography) yielded an additional 5-fold purification of the enzyme fraction over the previous step. After this final step, the enzyme was purified about 45 000-fold with an overall recovery of 32%. The specific activity of the final preparation is about 15 units/mg protein.

Upon acrylamide gel electrophoresis at pH 8.6, the purified enzyme ran as a single protein band. The transferase activity staining indicated that this band corresponds to the active enzyme. Moreover, when the electrophoresis was carried out under denaturing conditions in the presence of SDS, a single band was obtained.

Molecular weight of the native enzyme and subunit composition. The molecular weight of the enzyme determined by a Sephadex G-200 gel filtration was estimated to be 88 000. The Sephadex column was calibrated with the following proteins: phosphoglucomutase (65 000), creatine phosphokinase (81 000), galactose dehydrogenase (100 000), lactate dehydrogenase (140 000) and catalase (240 000). Very similar results were obtained when the determination

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE URIDYLYL TRANSFERASE

Transferase was purified and assayed as described in the text.

Steps	Total protein (mg)	Spec. act. (I.U./mg)	Purification ratio (-fold)	Yield (%)
1. Hemolysate	315 000	0.00033	1	100
2. Batch treatment with DEAE-cellulose	4900	0.020	60	95
3. Fractionation with 55% $(NH_4)_2SO_4$	1210	0.062	200	70
4. DEAE-cellulose chromatography	135	0.50	1500	61
5. Hydrophobic chromatography on hexyl-agarose	19	2.9	8700	52
6. Hydroxyapatite chromatography	2.2	15	45 000	32

was carried out by sucrose density gradient centrifugation.

By SDS-polyacrylamide gradient slab gel electrophoresis, the purified enzyme migrated as a single band at a position corresponding to an apparent molecular weight of 46 000. Standard molecular weight proteins were aldolase subunit (40 000), RNA-polymerase subunit (39 000), trypsin (21 500) and serum albumin (68 000).

Electrophoresis and isoelectrofocusing of erythrocyte transferase. Fig. 3 shows the electrophoretic pattern on starch gel of erythrocyte transferase after specific staining; the sample runs as a single anodic band of activity.

Nevertheless, isoelectric focusing on polyacrylamide gel (Fig. 3B) indicates that the purified enzyme is microheterogeneous. Three erythrocyte transferase

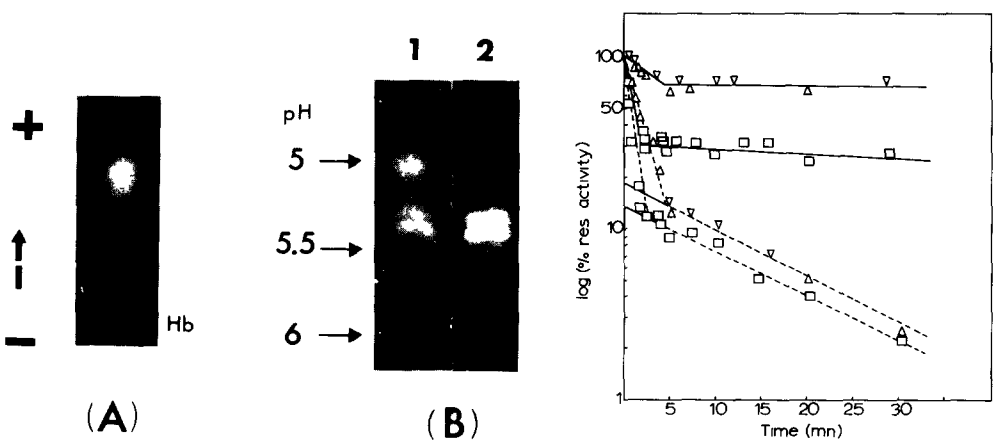

Fig. 3. Human erythrocyte transferase patterns: (A) starch-gel electrophoresis performed in a phosphate citrate buffer pH 7.5. (B) polyacrylamide gel isoelectrofocusing with ampholines in pH range 4.0—7.0 purified transferase (1) and crude hemolysate transferase (2) activities were detected by fluorescence of the NADPH formed.

Fig. 4. Thermal inactivation of erythrocyte transferase. Aliquots of purified enzyme were incubated for various periods of time at 44°C (△) and 48°C (□) in the presence of 2 mg/ml of serum albumin (———) or in the absence of this stabilizer (- - - - -). All values were expressed as percentage of the initial activity of the enzyme preparation, which was taken in all cases as 100%.

bands with p*I* values between 5.0 and 5.4 can be distinguished. This pattern is identical to that of the enzyme in crude preparations, which indicates that no modification takes place during the purification procedure described above.

Thermal inactivation. The inactivation of the enzyme was measured at 44 and 48°C as a function of incubation time. As can be seen from Fig. 4, the thermal inactivation curves were biphasic, that is to say there is a thermolabile fraction. The percentage of this fraction was obtained by extrapolating the final slope of the curve to the control value (zero-time heating). The percentage of labile fraction is about 80% at 44°C and 85% at 48°C. Moreover, the purified

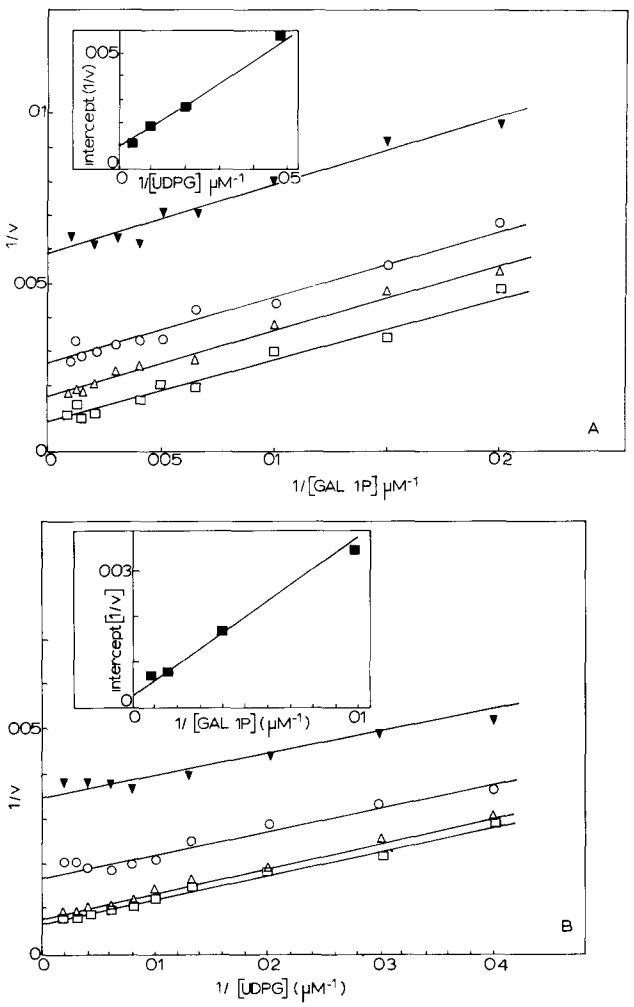

Fig. 5. (A) Double-reciprocal plots of initial velocities of Glu-1-*P* formation at varying (Gal-1-*P*) and several fixed (UDP-glucose): □——□, 0.002 mM; △——△, 0.005 mM; ○——○, 0.02 mM; ▲——▲, 0.025 mM. The inset shows replots of the intercepts against the reciprocal concentrations of UDP-glucose. (B) Double-reciprocal plots of initial velocities of glucose-1-*P* formation at varying (UDP-glucose) and several fixed (Gal-1-*P*): □——□, 0.01 mM; △——△, 0.025 mM; ○——○, 0.066 mM; ▲——▲, 0.1 mM. The inset shows replots of the intercepts against the reciprocal concentrations of Gal-1-*P*. The velocity units are μmol of Glu-1-*P* per min.

enzyme was very thermolabile. The half-life of the fast phase was very short: about 1 min at 48°C and 2 min at 44°C. The addition of 2 mg/ml serum albumin considerably stabilized enzyme.

Stability of the enzyme. The instability of purified transferase led us to investigate possible ways to stabilize it. Activity was completely destroyed when the diluted enzyme solutions were frozen. On the other hand, any attempt to concentrate the preparation led to a considerable loss of activity. The purified enzyme (hydroxyapatite fractions) was stored in an ice bath (it lost its activity at a rate of about 10% per week).

Kinetic studies. The effect of substrate concentration on enzyme activity was studied. The kinetics were Michaelian. The apparent K_m of purified uridylyl transferase estimated by the Lineweaver-Burk method was 0.2 mM for Gal-1-*P* and 0.065 mM for UDP-glucose.

Experiments with different concentrations of Gal-1-*P* and UDP-glucose are shown in Fig. 5. In each figure the points represent mean experimental rates from triplicate determinations. The Lineweaver-Burk curves are parallel lines. Moreover, the secondary plots of intercept against 1/[Gal-1-*P*] and 1/[UDP-glucose] are straight lines.

Discussion

In the present study, we describe a method which yields better than a 45 000-fold purification of active uridylyl transferase obtained from human red cells. The very low amount of transferase in erythrocytes explains why the different authors (Tedesco, Dale and Popjak, and Williams) [5,6,18] did not succeed before. In addition, marked instability of catalytic activity in vitro after purification may account for the scarcity of studies on partially purified enzyme.

In fact, several attempts have been previously made to purify and to study human transferase. Tedesco [5] partly purified it (1000-fold) by a DEAE-cellulose chromatography followed by a Sephadex G-200 gel filtration. On the other hand, Dale and Popjak [6] described a rapid method for the purification of erythrocyte transferase by using DEAE-cellulose chromatography followed by two affinity chromatography steps on a 'uridine-aminohexyl'-agarose column. However, the preparation obtained by these authors was not homogeneous, since it resolved on an acrylamide gel into one major and several minor components, one of which exhibited transferase activity while the major band was inactive. Williams [18] purified the enzyme by DEAE-cellulose chromatography, heat treatment and hydroxyapatite chromatography. This preparation had a relatively high specific activity but showed several protein bands after migration on acrylamide gel, only one protein band being active.

The addition of the hexyl-agarose chromatography step and the modification of several other steps enabled us to achieve for the first time a reproducible isolation of erythrocyte transferase. Using this procedure, we succeeded in extensively purifying the enzyme (45 000-fold) with a yield of 32% and a mean specific activity of 15 I.U./mg. Molecular weight studies suggested that it is a dimer with a molecular weight of 88 000.

Purified uridylyl transferase was very unstable and very thermolabile. The thermal inactivation curves were non-linear; this suggests the coexistence of

more than one form of transferase of different thermostability.

Our kinetic studies, described in detail above, strongly suggest that the human enzyme utilizes a ping-pong bi-bi mechanism. The same kinetic patterns have been reported for purified transferase from *E. coli* [32,33], with formation of an uridylyl-enzyme intermediate.

The apparent p*I* of the purified erythrocyte transferase was between pH 5.0 and 5.4. As shown in the preceding section a charge microheterogeneity of the enzyme was observed. Since similar patterns were obtained both with purified uridylyl transferase and with crude hemolysate enzyme, it follows that this microheterogeneity is not an artefact but an intrinsic property of the enzyme. Studies are in progress in order to determine what kinds of modifications occurred and whether this is due to post-translational or post-transcriptional events.

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