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Comparative Studies on the Human Glutamate-Pyruvate Transaminase Phenotypes— GPT 1, GPT 2–1, GPT 2

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Summary. The quantitative differences between the activity of the 3 common phenotypes of human red cell GPT has been confirmed. In addition, the activity of red cell GPT 1 was found to be greater in young children than in adults. No such difference was found for the GPT 2 phenotype. The activity of the red cell GPT 1 was found to decrease with age, reaching the adult level at the age of 10 to 12 years. Red cell GPT of all the 3 common phenotypes in both adults and children was found to show a similar response to the addition of excess pyridoxal phosphate.

A method has been devised for the partial purification of human GPT (cytoplasmic) from liver. GPT 1 and GPT 2 have been purified, and very few significant differences were found amongst the physical and kinetic parameters tested.

Introduction

Glutamate-Pyruvate Transaminase (GPT), also known as Alanine Aminotransferase (E.C. 2.6.1.2), catalyses the reversible interconversion of L-alanine and α-ketoglutarate to L-glutamate and pyruvate. Chen and Giblett (1971) reported that the enzyme GPT from human erythrocytes exhibited genetic polymorphism. Three common electrophoretically distinguishable phenotypes (GPT 1, GPT 2—1 and GPT 2) were detected, and family studies indicated that the 3 phenotypes could be readily explained as the expression of 2 autosomal codominant alleles, GPT^1 and GPT^2 (Chen and Giblett, 1971; Chen *et al.*, 1972). In most Caucasian populations the frequencies of the 2 common alleles GPT^1 and GPT^2 are found to be approximately 0.5 (Chen *et al.*, 1972; Martin and Niebuhr, 1973; Scozzari *et al.*, 1975; Welch *et al.*, 1975). In all Negro populations so far reported, the frequency of the GPT^1 allele has been found to be much higher, in the range 0.80—0.92 (Chen *et al.*, 1972; Welch, 1972a; Welch *et al.*, 1975).

Population studies have revealed several rare alleles; GPT^3, GPT^4, GPT^5, GPT^6, and GPT^7 (Chen *et al.*, 1972; Olaissen, 1973a). In addition to these rare alleles giving rise to further electrophoretic variants of GPT, 2 examples of a silent allele GPT^0 have been reported (Spielmann *et al.*, 1973; Olaisen, 1973b). Kömpf *et al.* (1974) have described 2 further variants of red cell GPT. Both variant phenotypes differ from the normal GPT 2—1 electrophoretic pattern by showing a reduced activity for the GPT 1 isoenzyme.

When Chen and Giblett first described the polymorphism of red cell GPT (1971) they suggested that in view of the frequencies of the alleles GPT^1 and GPT^2, the

enzyme should be a useful additional genetic marker in man. Whilst this has been found to be true in the case of paternity testing (Welch and Dodd, 1974), the low activity of red cell GPT has meant that GPT has not been of any great value in the forensic field of blood stain identification (Welch, 1972b).

The 3 common phenotypes GPT 1, GPT 2—1 and GPT 2 can be readily distinguished by means of starch gel electrophoresis. The GPT 1 and GPT 2 phenotypes appear as a single band of enzyme activity, the latter migrating further towards the anode. Three isoenzymes can be seen in individuals of the GPT 2—1 phenotype, 2 of the isoenzymes having identical electrophoretic mobilities to the isoenzymes of the GPT 1 and GPT 2 phenotypes. The third isoenzyme has an intermediate electrophoretic mobility, indicative of an enzyme with a dimeric structure. The distribution of enzyme activity between the 3 isoenzymes of the GPT 2—1 phenotype is not that expected of a dimeric enzyme with randomly associating sub-units, assuming that both sub-units have approximately equal enzyme activity. In the case of the GPT 2—1 phenotype, the isoenzyme pattern that is normally seen is one where the GPT 1 isoenzyme is strongly stained, whereas the GPT 2 isoenzyme is often barely detectable. The reason for this has been shown to be due to a difference in activity between the products of the GPT 1 and GPT 2 alleles (Welch, 1972a; Chen *et al.*, 1972; Kömpf and Bissbort, 1974). Red cell GPT activity in individuals of the GPT 1 phenotype has been shown to be 2 to 3 times greater than that of the GPT 2 phenotype, the GPT 2—1 activity being intermediate between the two extremes. The reason for this quantitative difference between the 3 common GPT phenotypes is not yet known. No difference in heat stability could be detected (Welch, 1972a).

This present paper describes the results of experiments designed to investigate these qualitative differences. As well as experiments on the erythrocyte enzyme, a procedure has been devised for the partial purification of GPT from human liver. A number of physical and kinetic characteristics of the enzyme from individuals of the GPT 1 and GPT 2 phenotypes have been elucidated.

Materials and Methods

Tissue Extracts. Most experiments were carried out using red cell haemolysates prepared as follows. Blood samples were collected into EDTA. The red cells were washed three times in 0.9% saline, and the packed cells were diluted with an equal volume of distilled water. The cells were haemolysed by ultrasonic disintegration. Liver samples were obtained *post mortem*. A small piece of liver was homogenised in four volumes of chilled water, the debris was spun down, and the clear supernatant was used for electrophoretic analysis.

Electrophoresis. Horizontal starch gel electrophoresis was carried out at pH 6.8 using a Tris-Citrate buffer. The bridge buffer was 0.2 M Tris titrated to pH 6.8 with a saturated solution of Citric Acid. The gels were prepared containing 11% starch in a 1 in 30 dilution of the bridge buffer. The pH of the starch/buffer mixture was checked and adjusted to pH 6.8 using 0.1 M Tris. Electrophoresis was carried out for 16 hrs at 7 V/cm. The gels were sandwiched between cooling plates through which water at 10°C was circulated.

Staining. The GPT isoenzymes were detected using a modification of the procedure described by Chen and Giblett (1971). 200 mg DL alanine, 40 mg α-ketoglutarate, and 10 mg $NADH_2$ were dissolved in 10 ml of 0.3 M Tris/HCl buffer pH 8.0. The pH of the solution was checked and adjusted to pH 8.0 with dilute NaOH. 10 μ lof lactate dehydrogenase (Sigma Type 10—3000 units/ml) was added, and the stain was applied to the cut surface of the gel by soaking onto two layers of filter paper. The gels were incubated at 37°C, and inspected

under a U.V. lamp (366 nm) for the presence of areas of defluorescence indicating the position of the GPT isoenzymes. GPT in liver extracts were usually visible within 15 min, whereas the red cell GPT was not normally visible until after 2—3 hrs incubation.

Enzyme Assays. In view of the very low activity of red cell GPT in comparison to the enzyme from liver extracts, two different assay procedures were employed. Both were based on the same principle, namely the reduction of one of the products of GPT (pyruvate) by LDH and $NADH_2$, and the monitoring of the disappearance of $NADH_2$ in an ultraviolet spectrophotometer at 340 nm.

For the erythrocyte enzyme assay, a spectrophotometer cell (3 ml) was prepared as follows; 1.7 ml 0.3 M Tris/HCl pH 8.0, 1.0 ml DL alanine 20 mg/ml, 0.2 ml α-ketoglutarate 40 mg/ml (pH 7.0), 0.1 ml $NADH_2$ 3 mg/ml and 10 μl of the haemolysate (1 volume water: 1 volume red cells). The contents of the cell were mixed and incubated for 5 min at 45°C, after which the absorbance at 340 nm was measured. The cell was incubated for a further 60 min at 45°C and the absorbance at 340 nm measured. Finally, the absorbance at 540 nm was measured in order to determine the haemoglobin concentration. It had been found from previous experiments that it was not necessary to add LDH, there being sufficient of the enzyme in the haemolysate to reduce the pyruvate produced during the reaction. Enzyme activities are expressed as International Units/g haemoglobin at 45°C, a unit being the amount of GPT causing the production of 1 μmole of pyruvate (and hence the loss of 1 μmole of $NADH_2$) in 1 min.

For the liver GPT assay, because of the possibility of producing an extract with far greater enzyme activity than a red cell lysate, a continuous assay (340 nm) can be carried out. In contrast to the red cell GPT assay, LDH is added, since in the later stages of the liver GPT purification contaminating LDH will be removed. However, a complication arises due to the presence of ammonium ions in the liver extract, and also in the LDH added to the assay. The addition of α-ketoglutarate, $NADH_2$ and NH_4^+ provides both the substrates and coenzymes for the enzyme glutamate dehydrogenase. GDH is present in liver extracts, whereas it is not present in red cells. This means that for the assay of liver GPT, the assay is carried out initially in the absence of alanine, to measure GDH activity, and then in the presence of alanine to measure both GDH and GPT. The difference between the first and second assay is taken as a measure of the GPT activity. In the later stages of the liver GPT purification, once GDH activity has been removed, this problem does not arise.

A 3 ml spectrophotometer cell was prepared as follows; 1.5 ml 0.3 M Tris/HCl pH 8.0, 0.2 ml α-ketoglutarate 40 mg/ml (pH 7.0), 0.1 ml $NADH_2$ 3 mg/ml, 0.2 ml LDH (10 units/ml in 50% $(NH_4)_2SO_4$), and 10 μl of a suitably diluted liver extract. The decrease in absorbance at 340 nm (GDH activity) was measured at a temperature of 30°C. 1.0 ml DL alanine 20 mg/ml was added to the cell and the decrease in absorbance at 340 nm measured (GDH + GPT activity).

All other technical methods will be described in the relevant section of the Results.

Results

Red Cell GPT Experiments

Red Cell GPT Activity in Families

Genetically determined quantitative variants of red cell GPT have been reported, other than the more general observations that the activity of the GPT 1 is 2—3 times higher than the activity of the GPT 2. Two silent alleles GPT^0 have been reported (Spielmann *et al.*, 1973; Olaisen, 1973b). In addition 2 further variant alleles have been described whose gene products, whilst being electrophoretically indistinguishable from that of the GPT 1 allele, were shown to have reduced enzyme activity (Kömpf *et al.*, 1974). If these quantitative variant alleles were found to occur at any significant frequency, it would introduce a source of error in the use of GPT in paternity testing, since these variant alleles are often difficult to detect. With this in mind, a project was started with the aim of

measuring the frequency of quantitative variants of red cell GPT. Blood samples were collected from families and the GPT phenotypes, as shown by starch gel electrophoresis, were determined. The samples were assayed for red cell GPT.

It was soon apparent from the results that an analysis of the red cell GPT activity in families, with the aim of detecting the occurence and inheritance of variant alleles causing reduced activity, would prove to be extremely difficult. The reason being that superimposed upon the normal variation in GPT activity within and between each of the 3 common phenotypes, was an increased GPT activity in the children. 124 adults and 56 children were studied. The children's ages ranged from 4 weeks to 7 years, the average being 14 months. The mean red cell GPT activity of the children was found to be 0.738 units/g Hb, as compared to a mean value for the parents of 0.519 units/g Hb.

In view of the already known quantitative difference in GPT activity between the 3 common phenotypes, the activity of the GPT 1 being 2—3 times higher than the GPT 2, such a difference between the parents and children could be due to a greater proportion of GPT 1 phenotypes amongst the children. An analysis of the results from the starch gel phenotypes did not show any significant difference in gene frequency between the 2 groups (Table 1). Therefore the increased GPT activity in the group of children could not be explained by an increased proportion of GPT 1 phenotype.

The reason for the difference was discovered when the distribution of the red cell GPT activity was plotted for each of the 3 phenotypes, and the results of the adults and children then compared (Fig. 1). The results for the adults are similar to those already described (Welch, 1972a; Chen *et al.*, 1972; Kömpf and Bissbort, 1974) with the activity of the GPT 1 phenotypes being higher than the activity of the GPT 2 phenotypes. The same was found to be true in the case of the children. However, in the latter group, there seemed to be a higher activity of red cell GPT amongst those children of the GPT 1 phenotype. The difference is more clearly seen in Table 2, where the mean activity of red cell GPT of the 3 phenotypes in adults and children are compared.

Whilst the mean activity of the GPT 2 phenotypes was found to be very similar in adults and children, there is clearly an increased GPT activity in children of the GPT 1 phenotype compared with adults. It has been shown (Welch, 1972a) that the quantitative effects of the products of the GPT^1 and GPT^2 alleles are additive. The mean activity of the GPT 2—1 phenotype can be predicted from the results of the GPT 1 and GPT 2 phenotypes:

$$\tfrac{1}{2}\,\text{GPT 1} + \tfrac{1}{2}\,\text{GPT 2} = \text{GPT 2—1}$$

where GPT 1, GPT 2—1 and GPT 2 are the mean levels of activity.

Table 1. Red cell GPT phenotypes of the 124 adults and 56 children

	No. tested	Mean red cell GPT U/g Hb	GPT 1	GPT 2—1	GPT 2
Adults	124	0.519	46 (0.37)	53 (0.43)	25 (0.20)
Children	56	0.738	20 (0.36)	23 (0.41)	13 (0.23)

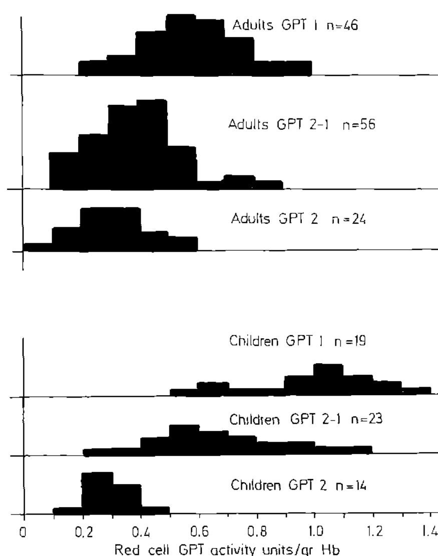

Fig. 1. Histogram showing the distribution of red cell GPT activity amongst adults and children of the 3 common phenotypes

Table 2. Mean levels of red cell GPT in the GPT 1, GPT 2—1 and GPT 2 phenotypes of the adults and children. Activities are expressed as Units/gram haemoglobin at 45°C

	GPT 1	GPT 2—1	GPT 2	Predicted result for the GPT 2—1 phenotype
Adults	0.617	0.488	0.406	0.511
Children	1.020	0.680	0.408	0.705

It can be seen from the results in Table 2 that there is a good agreement between the observed and predicted activities for the GPT 2—1 phenotype in both the adults and children.

The increased level of red cell GPT in children raises a number of questions, including:

(1) Why is the activity of red cell GPT higher in children?

(2) Why is this increased activity seen only in the product of the GPT^1 allele, and not in the product of the GPT^2 allele?

(3) At what age does the activity of the product of the GPT^1 allele decrease to the level seen in adults?

Red Cell Activity in Children of the GPT 1 Phenotype

The incidence of the GPT 1 phenotype in most European populations is about 25%, as compared to 80—90% in African populations. In order to obtain a sufficiently large number of examples of GPT 1 phenotype children, blood samples were collected from a village population (Keneba) in The Gambia, West Africa. Children below the age of 16 years, and of GPT 1 phenotype, were assayed for

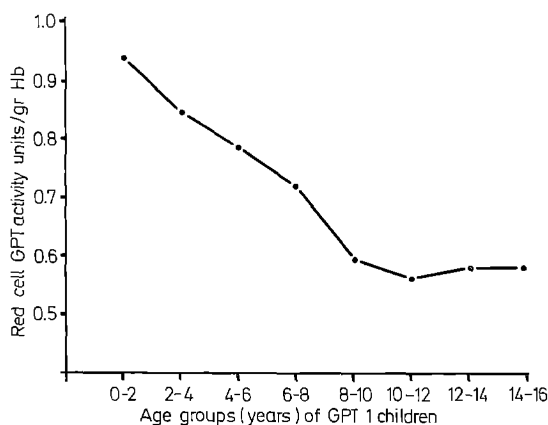

Fig. 2. Mean red cell GPT activity of children (2 years age groups) of GPT 1 phenotype

red cell GPT. 93 of the children were in this group. The results were divided into 2-year age groups and the mean red cell GPT activity of each of the 8 groups was determined. The results are shown in graphical form in Fig. 2. The results clearly show a decrease in red cell GPT activity with age, with the mean adult level of activity being reached by the age of 10—12 years. The results confirm the initial observation made during the family study, namely that children of GPT 1 phenotype and below the age of 2 years exhibit a red cell GPT activity 160% of that found in adults of the same phenotype.

Pyridoxal Phosphate and Red Cell GPT Activity

Transaminase enzymes use Pyridoxal Phosphate as a coenzyme. The coenzyme is involved in the transfer of NH_2 groups from an amino acid to a keto acid, the reaction going via one or more Schiff's bases.

One of the possible explanations that could account for either the difference in activity between the products of the GPT 1 and GPT 2 alleles, or the difference in activity of the product of the GPT 1 allele in adults and young children, is that the enzyme from the various phenotypes and age groups shows a difference in saturation with Pyridoxal Phosphate. A red cell lysate was prepared from an adult of GPT 1 phenotype. The enzyme was assayed in the presence of various concentrations of Pyridoxal Phosphate, ranging from 0.001 to 1.0 mM. The results are shown in Fig. 3. Clearly the enzyme in the human erythrocyte is not fully saturated with coenzymes. The addition of extra Pyridoxal Phosphate causes an increase in activity up to a level of 150—160%. Further addition of coenzyme, above the level of 0.1 mM does not cause any significant rise in red cell GPT activity. Assuming that 0.1 mM Pyridoxal Phosphate causes maximal activation of GPT, the addition of this concentration was tried on red cell lysates from adults and children of all 3 common GPT phenotypes. The samples tested were from the same group as shown in Tables 1 and 2. The results, calculated as the percentage activity in the presence of 0.1 mM coenzyme, are shown in Table 3. The results suggest that differences in the saturation of red cell GPT by Pyridoxal Phosphate do not explain either the differences in activity of the products of the GPT 1

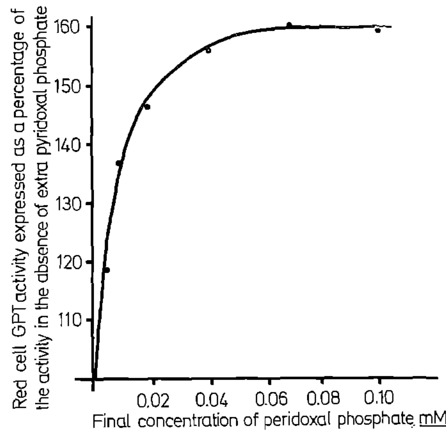

Fig. 3. The effect of pyridoxal phosphate on red cell GPT activity

Table 3. The effect of 0.1 mM Pyridoxal Phosphate on red cell GPT activity. Results expressed as $\frac{\text{Activity in presence of Pyr P}}{\text{Activity in absence of Pyr P}} \times 100$

	GPT phenotype		
	GPT 1	GPT 2—1	GPT 2
Adults	163	165	161
Children	162	165	155

and GPT 2 alleles in adults, or the difference in activity of the GPT 1 product between adults and young children.

In a second series of experiments the activity and coenzyme binding of GPT from red cell of different ages was measured. Erythrocytes of different ages were obtained by centrifugation. Older cells from both the GPT 1 and GPT 2 phenotype had lower levels of enzyme activity than younger cells. These differences could be partially rectified by the addition of Pyridoxal Phosphate. Similar results have been described for human red cell Glutamate Oxaloacetate Transaminase (Fischer and Walter, 1971).

Liver GPT Experiments

Purification of Liver GPT

The reason for the quantitative difference between the gene products of the GPT 1 and GPT 2 alleles was not found from the experiments on red cell lysates. A more detailed comparison of the kinetic and physical parameters of the enzyme from haemolysates would prove to be difficult because of the low activity of GPT in red cells. Human liver contains a far higher level of GPT activity. It was decided that a preliminary purification of the GPT from liver should be attempted prior to carrying out comparative experiments designed to investigate differences in the gene products of the GPT 1 and GPT 2 alleles.

GPT has been purified from rat liver (Matsuzuwa and Segal, 1968) and pig heart (Saier and Jenkins, 1967). The procedure to be described incorporates modi-

fications of some of the steps in both these published procedures, plus other steps arrived at as a result of many pilot experiments on human liver GPT. Human liver samples (approximately 250 g) were collected at *post mortem*. The GPT phenotype was determined as described in the Methods Section. Liver samples from individuals of either the GPT 1 or GPT 2 phenotype were stored at —20°C.

Most of the steps in the purification were carried out at 4°C. Protein concentration was measured by the Biuret procedure.

Step 1. 300 g of liver was homogenised in 1 l of 0.02 M Tris/0.1 M KCl/0.01 M alanine/10^{-4} M mercaptoethanol: pH 8.0. The homogenate was raised to a temperature of 52°C in a boiling water bath, and then transferred to a water bath at 52°C and held at this temperature for 5 min. The homogenate was cooled at 15°C and the pH adjusted to 6.3 with glacial acetic acid. The denatured protein and cell debris was removed by centrifugation at 5000 g for 30 min.

Step 2. Solid $(NH_4)_2SO_4$ was added to the supernatant from Step 1 to bring the final concentration to 33%. The precipitated protein was removed by centrifugation at 10000 g for 30 min, and the supernatant was brought to 55% satruation by the addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10000 g for 30 min and the supernatant was discarded.

Step 3. The pellet from Step 2 was suspended in 50 ml 0.005 M sodium phosphate buffer pH 6.0 containing 10^{-4} M mercaptoethanol, and dialysed against 10 l of the same buffer. The dialysate was applied to a CM cellulose column (50 × 2.5 cm) equilibrated against the same buffer. The column was eluted with 1 l of the phosphate buffer pH 6.0, then the pH of the buffer was adjusted to pH 6.6. The protein fraction eluted at this pH contained the GPT activity, and the protein was precipitated by the addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitated protein was collected by centrifugation at 10000 g for 30 min.

Step 4. The pellet from Step 3 was dissolved in 50 ml 0.01 M Tris/10^{-4} M mercaptoethanol: pH 6.9, and dialysed against 2 l of the same buffer. The sample was applied to a DEAE cellulose column (50 × 2.5 cm), and eluted with 200 ml of the same buffer, during which time the GPT activity remained bound to the column. The column was eluted with 500 ml 0.05 Tris/10^{-4} M mercaptoethanol/ 10 mM alanine/1 mM α-ketoglutarate: pH 6.4. The fractions containing GPT activity were pooled and precipitated with solid $(NH_4)_2SO_4$ to 75% saturation.

Step 5. The pellet from Step 4 was dissolved in 10 ml 0.02 M Tris/0.1 M KCl/ 10^{-4} M mercaptoethanol: pH 8.0, and dialysed against 1 l of the same buffer. The sample was applied to a Sephadex G-200 column (50 × 1.5 cm) and eluted with the same buffer.

Step 6. The fractions from Step 5 containing GPT activity were pooled and concentrated by pressure dialysis to a volume of 2 ml. The sample was re-chromatographed on a Sephadex G-200 column using the same conditions as in Step 5. The fractions containing GPT activity were pooled, assayed for enzyme and protein, and stored in 50% $(NH_4)_2SO_4$ at 4°C.

Using the protocol described above, 5.2 mg of GPT 1 was prepared from 300 g of liver. The purification procedure and results are summarised in Table 4. A similar procedure was carried out using 270 g of liver of a GPT 2 phenotype. In both cases the preparations were not homogeneous as shown by polyacrylamide

Table 4. Purification of GPT 1 from 300 g liver

Step	Protein (mg)	GPT activity (units 30°C)	Specific activity (units/mg)	Recovery (%)	Purification
1. Tris/KCl extract after heat denaturation	17230	4100	0.238	100	—
2. 33—50% $(NH_4)_2SO_4$	3600	3150	0.875	77	3.7
3. CM cellulose column	390	1930	4.95	47	20.8
4. DEAE cellulose column	47	1022	21.7	25	91.2
5. Sephadex G-200 column 1	14.6	720	49.3	18	207
6. Sephadex G-200 column 2	5.2	580	112	14	470

gel electrophoresis or ultracentrifugation, nevertheless they were considered sufficiently pure for comparative experiments.

Molecular Weight Determination

The M. W. was determined by means of Sephadex G-200 filtration (Andrews, 1965). The following M. W. markers were used: Lactate Dehydrogenase, Haemoglobin, Albumin, Carbonic Anhydrase and Cytochrome c. A Sephadex G-200 column (100 × 1.5 cm) was equilibrated with 0.02 M Tris/0.1 M KCl pH 8.0. The sample, containing approximately 30 units of GPT, was applied to the column in a volume of 0.5 ml and eluted at a flow rate of 9 ml/hr. 3.6 ml fractions were collected. The results of 3 experiments on each isoenzyme did not show any significant difference between the molecular weight of GPT 1 (103000) and GPT 2 (106000).

Isoelectric Point

The isoelectric point of the 2 isoenzymes was determined by means of isoelectric focusing using an LKB column. The ampholyte range used in all the experiments was from pH 5.0 to 8.0. The focusing was carried out in a sucrose gradient for 48 hrs at a maximal voltage of 710 volts. The contents of the column were pumped into a fraction collector and 1.5 ml fractions were collected. The pH of each fraction was measured using a micro electrode, and the GPT activity was assayed. The isoelectric point of GPT 1 was found to be 6.08, and the isoelectric point of GPT 2, 5.68. Results in this range are to be expected in view of the behaviour of the GPT 1 and GPT 2 isoenzymes during starch gel electrophoresis at pH 6.8.

pH Optima

In order to determine the pH optima of the transaminase reaction catalysed by GPT 1 and GPT 2, the standard assay was modified to incorporate Tris/HCl buffers at a range of pH values (6.8—9.3). At the end of each assay, the pH in the spectrophotometer cell was measured using a micro electrode. Both isoenzymes had sharp pH optima between pH 8.1 and 8.2.

Heat and Urea Inactivation

The denaturation of enzymes by heat and high concentrations of urea has often been used as a means of comparing the stability characteristics of genetically determined variant forms of enzymes, for example human phosphoglucose isomerase (Welch, 1971). For the heat inactivation of human liver GPT, the enzyme

Table 5. Inhibition experiments on partially purified GPT 1 and GPT 2 from human liver

	Results expressed as percentage inhibition	
	GPT 1	GPT 2
1. Urea 1.8 *M*	59	62
2. Heat 10 min at 58°C	50	52
3. Amino-oxyacetate 3.3×10^{-7} *M*	89	74
4. Maleate 0.2 *M*	36	32

was incubated in 0.3 M Tris pH 8.0 for 10 min at 58°C. The residual activity was measured and compared to the activity of unheated controls. The results were expressed as percentage inhibition and are shown in Table 5. A similar procedure was adopted for the urea inhibition experiments. GPT was incubated for 1 hr at 30°C in a spectrophotometer cell containing all the ingredients for the assay with the exception of LDH and $NADH_2$, and the results, expressed as percentage inhibition are shown in Table 5. It was found that there was no significant difference in the stability of GPT 1 and GPT 2 to either heat or urea. Different temperatures and urea concentrations failed to show any significant differences between the two isoenzymes.

Michaelis Constants

Substrate affinity, as measured by the Michaelis Constant, has often been used for the comparison of genetically determined variant forms of enzymes. The lower activity of the product of the GPT^2 allele may be partly due to a reduced substrate affinity (increased Km), if the concentrations of substrates used in the red cell GPT assay are not sufficient to saturate the enzyme.

Transaminase reactions have equilibrium constants of approximately 1.0, which means that the reactions are freely reversible. In this work GPT has been assayed in the direction of pyruvate and glutamate production. In order to determine the Km for alanine and α-ketoglutarate, a more complex approach is required than for an enzyme with one substrate. The standard assay procedure was modified to allow the enzyme to be assayed at 5 concentrations of alanine (1.86—37.2 mM) and 5 concentrations of α-ketoglutarate (0.39—15.85 mM), resulting in 25 different assay conditions. The graphical determination of the Km values for an enzyme with two substrates is described by Dixon and Webb (1965). Six separate determinations of Km alanine and Km α-ketoglutarate were carried out on the GPT 1 and GPT 2 enzymes, and the mean values are given in Table 6. The only significant difference appeared to be an increased Km alanine for the GPT 1 enzyme. Such a difference (GPT 1 16.9 mM: GPT 2 11.5 mM) does not account for the lower activity of GPT 2 in erythrocytes. Even if the concentrations of alanine used for the red cell GPT assay were not saturating, the higher Km for GPT 1 would be expected to result in a proportionately lower velocity of reaction.

Table 6. Michaelis constants of GPT 1 and GPT 2 from human liver

	GPT 1	GPT 2
Alanine	16.9×10^{-3} *M*	11.5×10^{-3} *M*
α-ketoglutarate	1.3×10^{-3} *M*	1.2×10^{-3} *M*

Inhibition by Amino-oxyacetate and Maleate

In their studies on rat liver GPT, Hopper and Segal (1962) demonstrated that amino-oxyacetate and maleate both acted as competitive inhibitors, the former being far more affective. Since it has been shown that GPT 2 has a higher affinity for the substrate alanine, one might expect that the 2 isoenzymes would show differences in inhibition when the assays are carried out in the presence of either amino-oxyacetate or maleate. The activity of GPT was measured in the presence and absence of amino-oxyacetate ($3.3 \times 10^{-7} M$) and maleate ($0.2 M$). The results are shown in Table 5, confirming that at the same concentration of inhibitor, the GPT 2 isoenzyme is inhibited to a lesser degree than the GPT 1 isoenzyme.

The kinetic experiments on the partially purified GPT 1 and GPT 2, whilst failing to explain the reason for the lower activity of the GPT^2 allele product in red cells, has provided some useful data showing differences in substrate affinity and inhibition.

Experiments are in progress to prepare GPT 1 and GPT 2 in a sufficiently pure state to allow the structural difference between the two isoenzymes to be determined.

Discussion

Many of the red cell enzyme polymorphisms detected by means of starch gel electrophoresis have subsequently been found to show quantitative differences in enzyme activity. In a review of polymorphism in man, Harris (1971) pointed out that of 23 loci giving rise to electrophoretic variation, some form of quantitative variation had been found in 16 of these loci. Such a common occurence of quantitative variation was thought by Harris to favour the view that these are the consequences of natural selection rather than random drift. Human red cell GPT seems to be no exception. The quantitative difference in activity of the product of the GPT^1 and GPT^2 allele has been well documented (Welch, 1972; Chen *et al.*, 1972; Kömpf and Bissbort, 1974) and has been confirmed in this paper. The work described in the present paper has demonstrated a further complexity, namely that the product of the GPT^1 allele is more active in young children than in adults, whereas the activity of the product of the GPT^2 allele does not vary with age. For example children of the GPT 1 phenotype and below the age of 2 years were found to have levels of red cell GPT activity 160% of that of adults, and this activity was found to decrease with age and reach the adult level by 10—12 years. These differences, not seen in the case of the GPT 2 phenotype, were not found to be due to an increased saturation of the enzyme from young children by the cofactor pyridoxal phosphate.

The role of GPT in human red cell metabolism is unlikely to be of any great significance, the activity of the enzyme being so low in comparison to tissues like the liver and heart. It remains to be seen whether human liver cytoplasmic GPT shows the same quantitative differences as the erythrocyte enzyme. Liver GPT is known to show variation in activity dependant upon age, hormone and diet. Chen *et al.* (1973) in a study of mouse liver GPT showed that the enzyme activity in liver increased from 10 units to 300 units/g from the age of 12 to 26 days, the latter age coinciding with the onset of weaning. Rat liver GPT has been shown to increase in activity under the influence of a variety of hormones including cortisol

and thyroxine, and also as a result of a high protein diet (Snell and Walker, 1972). One or more of the above factors could be implicated in the increased activity of human GPT 1 in young children.

Experiments carried out on the partially purified GPT from livers of a GPT 1 and GPT 2 phenotype did not provide the information necessary to explain the quantitative differences between the red cell GPT activity of adults of the 3 common GPT phenotypes. Most of the kinetic and physical parameters of the GPT 1 and GPT 2 enzymes were found to be the same, the only exception being a small difference in substrate affinity and competitive inhibition. Experiments designed to elucidate the nature of the molecular basis for the electrophoretic differences between the products of the GPT^1 and GPT^2 alleles are in progress and await the preparation of the 2 enzymes in a pure state.

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