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Giorgio Battistuzzi, Carlo Santolamazza, Romano Petrucci, Maria Beretta, Rosaria Scozzari, Walter Frattaroli, Guido Modiano, Silvana Augusta Santachiara-Benerecetti

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Genetica. — *Dissection of the enzyme activity variation of human red cell glutamic pyruvic transaminase.* Nota di GIORGIO BATTISTUZZI (*), CARLO SANTOLAMAZZA (*), ROMANO PETRUCCI (*), MARIA BERETTA (**), ROSARIA SCOZZARI (*), WALTER FRATTAROLI (***), GUIDO MODIANO (*) e SILVANA AUGUSTA SANTACHIARA-BENERECETTI (**), presentata (****) dal Socio G. MONTALENTI.

RIASSUNTO. — Si presenta una scomposizione della variabilità dell'attività della glutamico-piruvico-transaminasi (GPT) riguardo al polimorfismo elettroforetico, al grado di saturazione con il cofattore piridossalfosfato (PLP) ed alla composizione per sesso della popolazione. Si è osservato che l'esistenza di tre fenotipi elettroforetici comuni con differente attività enzimatica media rende conto di circa il 25 % della variazione totale della popolazione maschile. L'aggiunta di PLP causa un aumento sia della varianza inter- che di quella intrafenotipica. Infatti circa il 5% della varianza interfenotipica è dovuta alla differenzialità dell'effetto del PLP sui vari fenotipi. Il sesso non influenza nè l'attività media dei diversi fenotipi nè la saturabilità con PLP, mentre ha un effetto cospicuo sulla varianza della attività, che nelle femmine è circa cinque volte maggiore di quella dei maschi.

INTRODUCTION

Glutamic pyruvic transaminase (GPT; alanine aminotransferase, E.C.2.6.1.2) has an important role in the interconversion of carbohydrates and amino acids catalysing the reversible transformation of alanine and α-ketoglutarate to glutamate and pyruvate. The enzyme exists in both cytoplasmic and mitochondrial form. The soluble form, the only one present in red cells, is known to be genetically polymorphic. It is coded by an autosomal locus with two codominant alleles, GPT^1 and GPT^2 [1], common in all populations so far studied although very unevenly distributed [2]. Furthermore different mean enzyme activities have been reported for the three common phenotypes: GPT 1 > GPT 2-1 > GPT 2 [3, 4, 5, 6].

The aim of this study was to dissect the quantitative variation of the enzyme activity of GPT in the general population by extending the analysis beyond the level of the variation associated with the electrophoretic polymorphism (which has been the subject of several studies [7, 8, 9]) to other components superimposed on it.

MATERIALS AND METHODS

The sample: This consisted of 235 healthy unrelated subjects 25-40 years old. Blood specimens were collected by venipuncture either into EDTA (subsample A, which includes both sexes) or into ACD (subsample B, only males).

(*) Institute of Genetics and CNR Unit for Evolutionary Genetics, University of Rome, Italy.

(**) CNR Laboratory for Biochemical and Evolutionary Genetics, Pavia, Italy.

(***) National Blood Transfusion Centre of the Italian Red Cross, Regina Elena Hospital, Rome, Italy.

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Preparation of RBC (red blood cells) *lysates*: Each blood sample was processed within four hours after collection, as follows. Each sample was percolated through a cotton wool column [10] to eliminate leucocytes and platelets. Erythrocytes were then washed three times with cold isotonic saline. Lysates were obtained by adding 3 volumes of cold distilled water and, after 15 minutes, 2 vols of 0.3 M Tris-HCl buffer pH 8.0. GPT activity is stable for at least 3 hours in an ice bath.

GPT activity assay: A two-step procedure has been used:

(1) Glutamate + Pyruvate $\xrightarrow{\text{GPT}}$ Alanine + α-ketoglutarate

(2) α-ketoglutarate + NADH + NH_4^+ $\xrightarrow{\text{GLDH}}$ Glutamate + NAD^+.

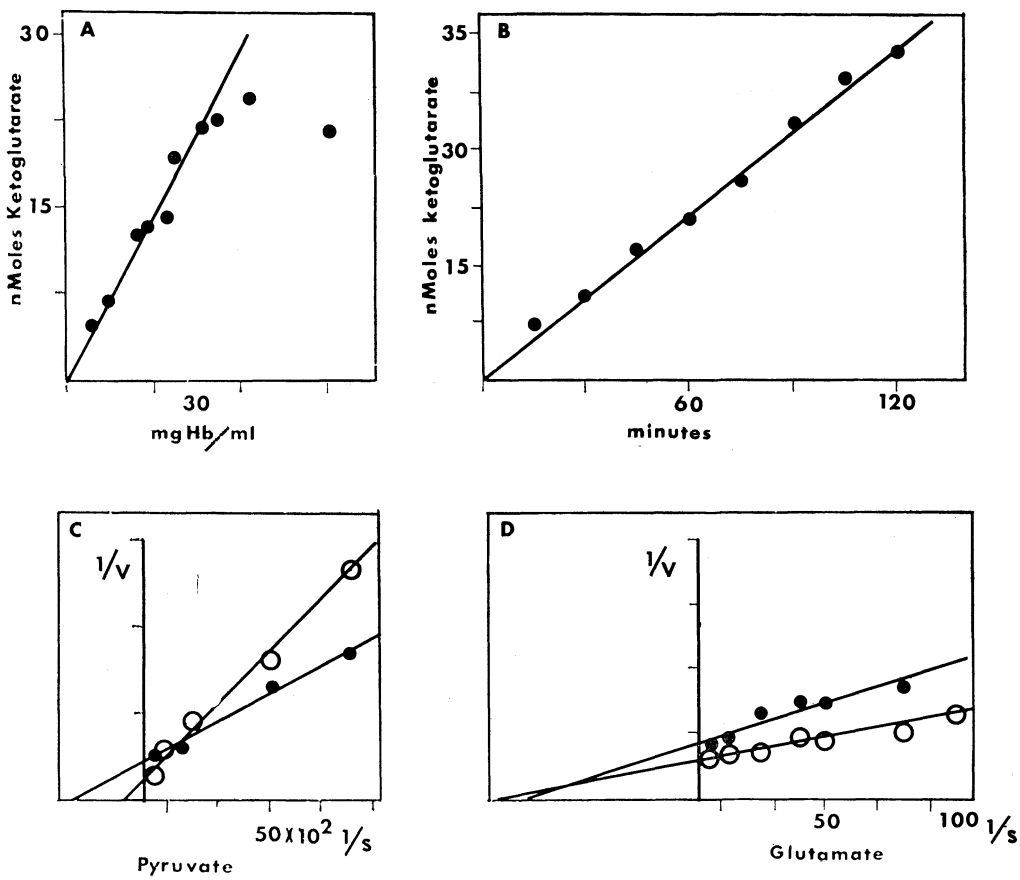

Fig. 1. – Relationship between GPT reaction and hemolysate concentration (A) (incubation time: 1 hr) and time (B). C and D. Lineaweaver and Burk plots for pyruvate and glutamate of GPT 1 (○) and GPT 2 (●) lysates. Glutamate concentrations ranging from .01 to .2 M were assayed at 10 mM pyruvate. Pyruvate concentrations ranging from .1 to 10 mM were assayed at .1 M glutamate. The observed K_m values were: $1.25 \cdot 10^{-2}$ M (GPT1) and $1.49 \cdot 10^{-2}$ M (GPT 2) for glutamate and $12.4 \cdot 10^{-4}$ M (GPT 1) and $3.5 \cdot 10^{-4}$ M (GPT2) for pyruvate.

Reaction (1) occurred at 37 °C in 1 ml mixture containing: 100 mM Tris-HCl buffer pH 8.0, 10 mM Pyruvate, 100 mM Glutamate and 0.5 ml lysate. When required Pyridoxalphosphate (PLP) was added to a 0.2 mM final concentration. Linearities of reaction rate and substrate saturation curves are shown in Fig. 1. Full activation was already observed at 10^{-5} M PLP and did not require preincubation with the cofactor. The reaction was stopped after 90 min. by adding 2 ml of 6 % cold perchloric acid. Protein precipitate was discarded by centrifugation and 1.8 ml of clear supernatant were neutralized with 0.25 ml of cold 5 M K OH. After 15 min. on ice, the sample was filtered and its α-ketoglutarate content assayed by recording the decay of absorbance at 3400 Å due to the disappearance

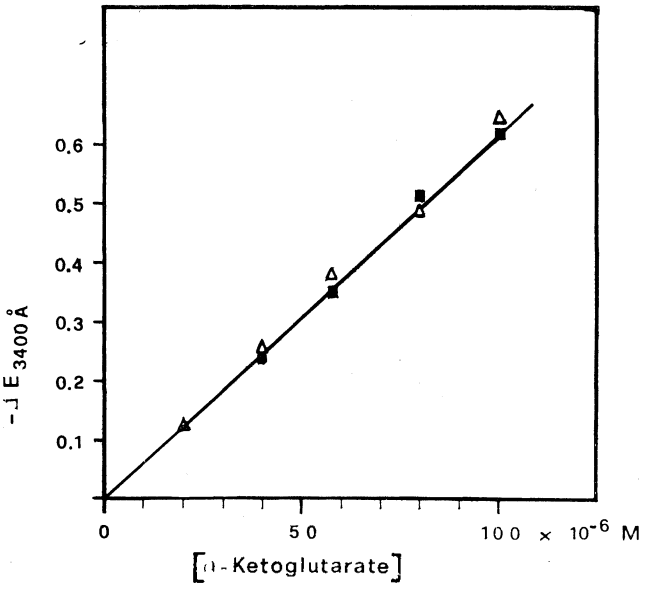

Fig. 2. – Molar equivalence between α-ketoglutarate and NADH disappearance.
△: Observed $E_{3400\,Å}$ when different amounts of α-ketoglutarate were added to the assay mixture.
▲: Observed $E_{3400\,Å}$ when deproteinized and neutralized assay solution containing different amounts of α-ketoglutarate and alanine (equimolar) were added to the assay mixture.
The line in the figure indicates the expected relationship.

of NADH in the following mixture: 0.71 ml of 1/15 M Sørensen phosphate buffer pH 7.4, 0.05 ml of 11.55% Ammonium acetate (*w* : *v*), 0.02 ml of 10 mg/ml NADH (grade I, Boehringer), 0.2 ml filtrate and 0.02 ml Glutamate dehydrogenase (GLDH) (1200 U/ml, Boehringer). Under these conditions α-Ketoglutarate is quantitatively transformed into Glutamate (Fig. 2). Allowance was made for the slight decrease of $E_{3400\,Å}$ due to GLDH-catalysed transformation of Pyruvate into Alanine. There are no appreciable amounts of α-Ketoglutarate in the lysate at time 0.

Haemoglobin concentration: This was determined according to [11].

Enzyme activity: This is expressed as μmoles of α-Ketoglutarate formed per hr per gram of haemoglobin at 37 °C. The method gives results reproducible within a 2% range (based on the examination of 10 subjects. For each of them two aliquots of the same specimen were examined). Each hemolysate has been tested in duplicate.

Electrophoresis: GPT electrophoretic phenotypes were determined on starch gel according to [1] with minor modifications [12].

RESULTS

A. GPT allele frequencies. GPT phenotype and allele frequencies of our sample are shown in Table I. The estimated frequencies of GPT^1, GPT^2 and GPT^3 alleles are 0.555, 0.443 and 0.002 respectively. A very good agreement with the Hardy-Weinberg equilibrium is evident on total sample and subsamples. However we noticed a significant unexplained difference between male and female gene frequencies ($\chi^2_{1df} = 6.293$; $P < 0.02$) consisting in a higher GPT^1 incidence among females. In spite of this relatively high level of significance we still tend to believe that it is a chance effect.

TABLE I.

Distribution of GPT electrophoretic phenotypes in the present sample.

Phenotypes	GPT 1	GPT 2-1	GPT 2	GPT 3-1	GPT 3-2	Total
Subsample A: Males	5	10	11	—	1	27
Females	13	17	4	—	—	34
Total	18 (16.2)	27 (29.9)	15 (13.8)	— (0.5)	1 (0.5)	61
Subsample B: Males	55 (56.3)	88 (85.3)	31 (32.3)	—	—	174
Grand Total	73 (72.4)	115 (115.6)	46 (46.1)	— (0.5)	1 (0.4)	235

Comparison males *vs* females for gene frequencies: $\chi^2_{1df} = 6.293$; $P < 0.02$.

Estimates of the pooled (see text) gene frequencies: $GPT^1 = .555$, $GPT^2 = .443$, $GPT^3 = .002$.

B. Quantitative results. Analysis of enzyme activity distributions has been done on 60 subjects of subsample A and 138 subjects of subsample B. If not otherwise stated, statistical analyses refer only to subsample B. As reported under Methods, we called *UP* GPT activity that found in lysates without addition of PLP (unsaturated by PLP or *UP*) and *SP* GPT activity that observed by adding PLP (saturated by PLP or *SP*). A third variable ($R = UP/SP \times 100$) was derived for each individual.

Comparison of mean phenotypic enzyme activities. The distributions of GPT activities according to the electrophoretic phenotype and to PLP supplementation are represented in Fig. 3. Statistical indexes of GPT activity distributions

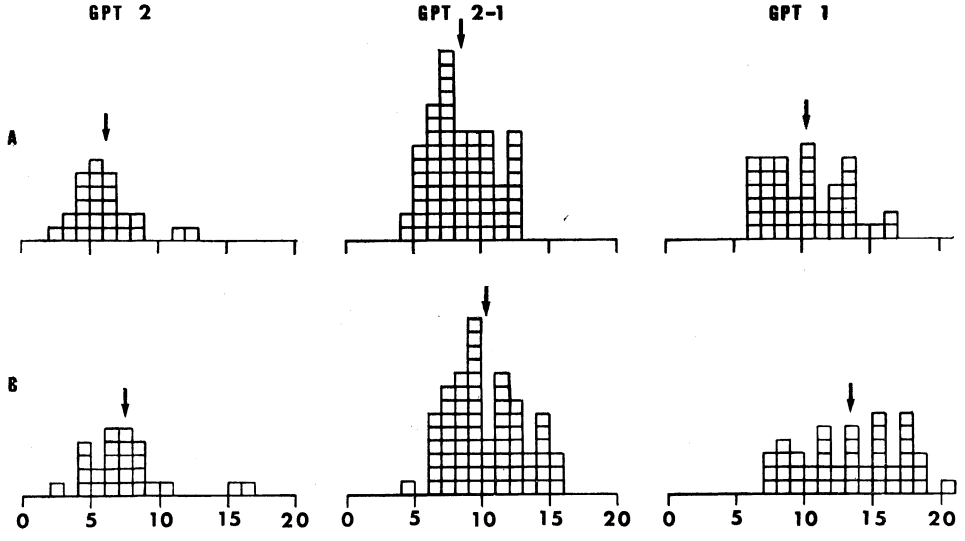

Fig. 3. - Enzyme activity distributions of the three common GPT electrophoretic phenotypes without (A) and with (B) PLP addition.
Arrows indicate means. Ordinate: number of individuals. Abscissa: GPT μMoles/hr/g Hb. One box symbolizes one subject.

are reported in Table II with respect to subsamples, PLP, sex and electrophoretic phenotype. As previously reported [3, 4, 5, 6] the mean activity of GPT 1 phenotype is higher than that of GPT 2-1, which in turn is higher than that of GPT 2. The differences are highly statistically significant ($P < 0.001$) as evaluated by the G statistics (1). The difference between the mean activities of males from

(1) Since the differences between intraphenotype variances were statistically significant the comparisons between the means were performed by the G statistics according to the formula:

$$G = \sum_{i=1}^{i=n} w_i \bar{x}_i^2 - (\Sigma w_i \bar{x}_i)^2 / \Sigma w_i ,$$

where $\bar{x}_i$ is the mean and w_i is the reciprocal of the variance of the mean ($w_i = n_i/s_i^2$) for the i^{nth} group. For high values of n_i, G is approximately distributed as $\chi^2_{(k-1)}$, where k is the number of groups. It is apparent from the formula that the value of G increases by increasing the differences between $\bar{x}_i$ (18).

TABLE II.

Statistical indexes of the distribution of variables UP, SP *and* R *in subsamples A and B subdivided according to electrophoretic phenotype and sex.*

Sub-sample		A									B								
PLP supplementation		*UP*			*SP*			*R*			*UP*			*SP*			*R*		
GPT phenotype		1	2–1	2	1	2–1	2	1	2–1	2	1	2–1	2	1	2–1	2	1	2–1	2
N	M		10	11		10	11		10	11	44	69	25	44	69	25	44	69	25
	F	13	17		13	17		13	17										
$\bar{X}$	M		10.41	7.10		12.85	8.23		81.12	86.55	10.36	8.57	6.15	13.36	10.36	7.48	78.25	83.04	83.56
	F	14.75	9.77		18.24	11.84		79.59	82.59										
S^2	M		3.50	6.39		5.07	9.30		23.53	16.88	7.92	4.99	5.57	12.97	6.67	9.21	87.22	75.72	128.8
	F	23.46	20.35		27.14	26.07		30.08	64.73										
SD	M		1.87	2.53		2.85	3.05		4.85	4.11	2.81	2.33	2.36	3.60	2.64	3.04	9.34	8.70	11.35
	F	4.84	4.51		5.21	5.11		5.48	8.04										
CV	M		0.18	0.36		0.18	0.37		0.06	0.05	0.27	0.27	0.38	0.27	0.25	0.41	0.12	0.10	0.14
	F	0.33	0.46		0.28	0.43		0.07	0.10										

Symbols: *UP* and *SP* = GPT activities without and with PLP supplementation; *R* = (UP/SP) × 100; N = number of individuals; M = Males; F = Females; $\bar{X}$ = mean; S^2 = variance; SD = standard deviation; CV = coefficient of variation (SD/$\bar{X}$).

subsamples A and B can be attributed to the different anticoagulant used. In fact the GPT activity was 10–15% higher if blood was collected into EDTA rather than into ACD (Tests performed on 5 GPT 1 and 5 GPT 2 subjects of both sexes).

Effect of PLP. PLP activation is evident in Fig. 3 concerning subsample B, but it was obvious in subsample A as well: out of a total of 189 individuals examined (51 of subsample A and 138 of subsample B, shown in Fig. 4) only 4 were not activated by PLP. The extent of the activation (ranging from 0 to 55 percent) is shown in Fig. 4 and Table II where the distribution and relevant

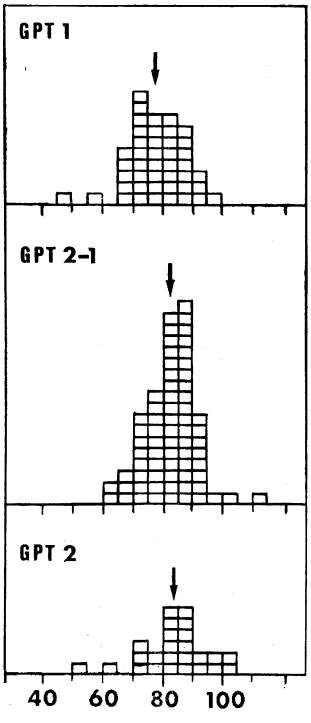

Fig. 4. – Distribution of the percent ratio between activities measured without and with added PLP in a sample of 138 unrelated subjects subdivided according to the electrophoretic phenotype.

Arrows indicate means. Ordinate: number of individuals. Abscissa: percent ratio values. Each box symbolizes one individual.

statistical indexes of the variable R in the three GPT electrophoretic phenotypes are presented. Interphenotype comparisons of the mean R values show the following: GPT 1 $<$ GPT 2–1 $\simeq$ GPT 2. The differences are highly statistically significant ($G = 14.19$; $P < 0.001$ for subsample A and $G = 7.05$; $P < 0.03$ for subsample B).

Effect of sex: Direct comparison of GPT activity distributions of males *vs* females could be performed only for GPT 2–1 phenotype of subsample A (see Table II). The statistical significance of the difference between mean enzyme activities of males and females has been tested by the Cochran corrected t'. Observed t' values were 0.711 for UP and 0.520 for *SP* by far lower

than the values corresponding to the 5 % level: 2.143 and 2.145, respectively. On the other hand, intraphenotype variances are apparently much higher in females than in males. The $F_{17,11}$ values (V_F/V_M) are 5.814 and 5.142 ($P < 0.01$) for the two variables. The same result can be extended at least also to GPT 1 phenotype on the reasonable assumption that intraphenotype variances of males of subsamples A and B are similar, although this can be verified only for GPT2–1 and GPT 2.

DISCUSSION

Erythrocyte GPT activity behaves as a codominant character: the observed mean value of GPT 2–1 heterozygotes, 8.57, is in fact very close to the average of the mean activities of the two homozygotes, i.e. $(10.36 + 6.15)/2 = 8.26$. We can thus estimate the enzyme activity contributed by GPT^1 and GPT^2 alleles as $\overline{GPT^1} = \overline{GPT\ 1}/2 = 5.18$ and $\overline{GPT^2} = \overline{GPT\ 2}/2 = 3.07$. If one takes as a term of reference the $\overline{GPT^2}$ activity, the difference, d, between $\overline{GPT^1}$ and $\overline{GPT^2}$ is 0.68, which is much lower than those reported by [3] and [4], 1.5. and 2.5 respectively. However this is still one of the highest differences so far reported for the red cell polymorphic enzymes not involving pathological consequences [8, 13].

Modiano (1976) [8] has calculated that the portion of the total variance of an enzyme's activity which is associated with the occurrence of two codominant electrophoretic alleles for that enzyme can be estimated by the formula $V_E = 2pqd^2(1 + K^2_{ne})$ where K_{ne} is the mean coefficient of intraphenotype variation which is assumed to be the same for all the phenotypes (K_{ne} takes into account the lack of independence between mean and variance). In our sample $p = 0.56$; $q = 0.44$; $d = 0.68$ and $K_{ne} = 0.29$. Thus, if activities are expressed in terms of $\overline{GPT^2}$, $V_{TOTAL} = 0.894$ and $V_E = 0.247$. Therefore the estimate of the relative variance associated with the electrophoretic polymorphism of GPT is about 27% of the total enzyme activity variance. This estimate is in full agreement with that obtained directly by the analysis of variance (~25%) as shown in Table III, upper row. This value ranks among the largest contributions found in this type of analysis [8] due to the fact that GPT is both highly polymorphic and shows a large difference between allelic activities.

In vitro stimulation of erythrocyte GPT by PLP was first recognized by Cinnamon and Beaton [14] and Woodring and Storvick [15]. These authors found that the percent stimulation is a function of the vitamin B_6 intake since " well nourished " individuals show little, if any, effect. These data suggest that part of the intraphenotype variance of GPT activity may be due to individual variations of the ratio between free apoenzyme and total enzyme. Thus, if saturation by PLP is provided, an increase of mean phenotype activities and a reduction of intraphenotype variation would be expected. Our data, however, suggest that PLP saturation elicits more complex consequences since it causes,

as expected, an increase of the mean GPT activity, but also an increase of the inter- and intraphenotype variances (Table III, middle and lower rows). PLP supplementation affects differentially the three phenotypes thereby increasing the amount of S_E^2 which is 31 % of the total variance as compared to the previous value of 25%. This increase is comparable with the relative amount of interphenotype variance of *R* (Table III), namely of the variable which directly estimates the effect of PLP on phenotypes. The reason why PLP supplementation increases also the intraphenotype variance is not clear. The possibility that this is simply the consequence of a positive correlation between enzyme activity level and percent increase can be excluded because such a correlation was not found within the phenotypic classes.

TABLE III.

Components of the variance of the three GPT quantitative variables.

Variable	Source	SS*q*	*df*	Variance	$\hat{S}^2$ *
GPT activity without addition of PLP (*UP*)	Intraphenotype	813.4	135	6.025	6.025
	Interphenotype	284.9	2	142.450	1.992
	Total	1098.3	137	8.017	8.017
GPT activity with addition of PLP (*SP*)	Intraphenotype	1252.6	135	9.279	9.279
	Interphenotype	579.1	2	289.550	4.092
	Total	1831.7	137	13.370	13.372
Percent Ratio *R* between the two activities	Intraphenotype	11989.9	135	88.814	88.814
	Interphenotype	732.6	2	366.314	4.051
	Total	12722.5	137	92.866	92.866

* Estimated population values.

When the effect of sex is considered, a very peculiar situation is found. In fact sex directly affects the variance of the distribution (the ratio between female and male variances is 5) leaving the means unaltered. The significance and the causes of this phenomenon are not clear. We were able however to exclude some obvious factors as possible causes, namely menstrual cycle and oral contraceptives, which are suspected to alter the GPT activity levels [16]. Moreover the range of temporal variation of GPT activity found in 5 males was about the same as that found in the females mentioned above (intraindividual variances

of males and females were not significantly different: $F_{24,16} = 1.265$ (2)). Altogether these negative findings are rather against the hypothesis that the females show an *inter*individual variability higher than that of males because they have a larger *intra*individual variability.

Taking into account all the sources of variation described above we can now dissect the variation of GPT activity as observed in the general population (Table IV). The genetic polymorphism, with its three common phenotypes having different mean enzyme activities, accounts for 12.4% of the total variation of the enzyme activity. Sex dramatically affects the intraphenotype variance which is much larger in females than in males while it has no effect on the interphenotype variance. Therefore the relative share of the latter is only 8.4% in females as compared with 24.8% of males.

TABLE IV.

Analysis of the variability of red cell GPT enzyme activity in the general population with respect to electrophoretic phenotype, sex and PLP supplementation.

	V_{TOTAL}	V_E (interphenotype)		V_{NE} (intraphenotype)
		absolute	percent	
UP:				
General Population	16.018	1.992	12.4	14.026
Males	8.017	1.992	24.8	6.025
Females	23.674	1.992	8.4	21.683
SP:				
General Population	22.188	4.092	18.0	18.096
Males	13.372	4.092	30.6	9.279
Females	30.626	4.092	13.4	26.534

The composition of the population is as described in the legend to Figure 5.

The effect of PLP consists in a preferential increase of the interphenotype variance since it differentially affects the three phenotypic means. It follows that the relative share of the variance associated with the electrophoretic polymorphism rises from 24.8 to 30.6% in males and from 8.4 to 13.4% in females.

A considerable portion of the residual non-electrophoretic variance (some 10%) is certainly explained by the variability of MCH [17] and very likely a further portion is due to the variability of the mean age of the red cell population since GPT is an age dependent enzyme (G.B., unp. results) in humans.

(2) The experiment was performed by following every 2nd week for two months the GTP activity levels in 9 females and 5 males.

The general distributions of the GPT enzyme activity expected to result from the interplay of the various factors affecting mean and/or variance are shown graphically in Fig. 5. An interesting consequence of the very large intraphenotype variance occurring among females is that a small but appreciable

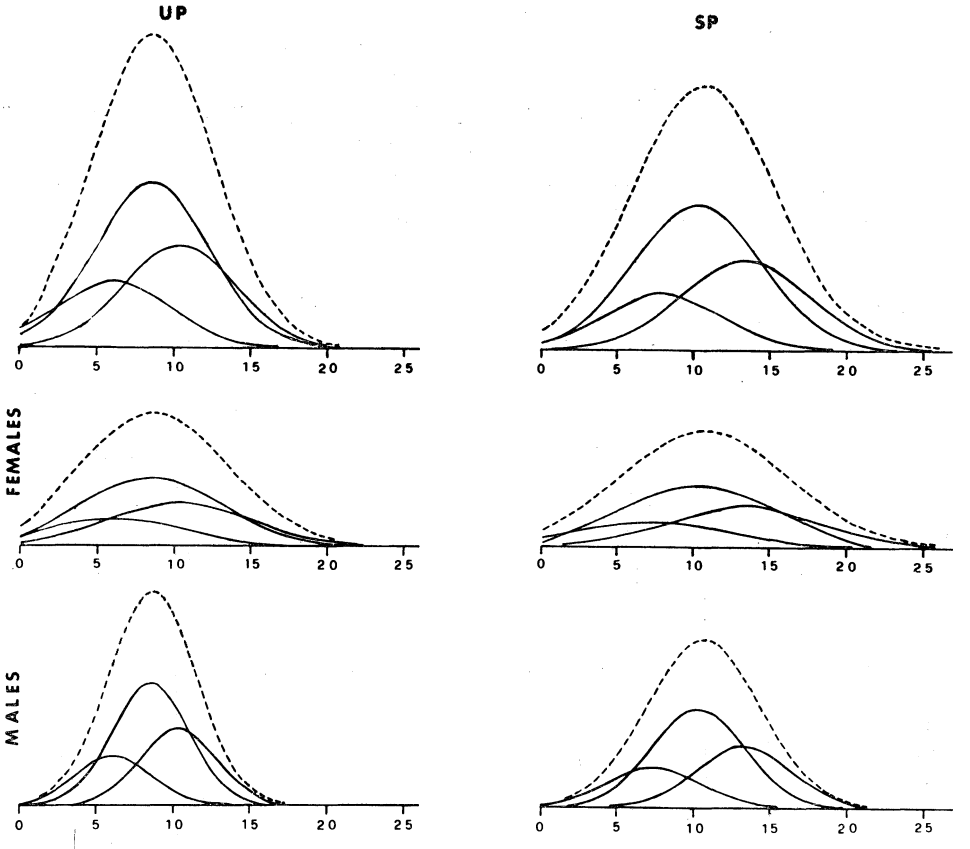

Fig. 5. – Effect of electrophoretic phenotype, sex and PLP supplementation on the distribution of GPT enzyme activity.

Dotted lines describe the general distributions. Continuous lines refer to the three phenotype distributions. Each general distribution was calculated by assuming that it was normal and using the general mean and variance as found in the corresponding sample (see Table II and III). The model refers to an Italian population of 20–35 year old subjects where the males/females ratio is 0.489/0.511 [19]. The effects of the three sources of variability discussed above are shown: the electrophoretic polymorphism (the three partial distributions in each of the six diagrams), the sex (2nd *vs* 3rd row) and the addition of PLP (2nd *vs* 1st column).

proportion of females is expected to show very little GPT activity. Thus some heterozygous 2–1 females may look like homozygotes and, owing to the higher activity associated with GPT^1, preferentially GPT 1. This may simulate a deviation from Hardy Weinberg equilibrium in females. It may also be worth pointing out that the general (theoretical) distribution, shown as dotted line in

each case of Fig. 5, is almost identical to that obtained by summing up the corresponding partial distributions. This confirms that genetic heterogeneity (excluding deficiencies) is practically undetectable from the shape of the overall enzyme activity curves [7, 8].

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