Further studies on the activity and subcellular distribution of alanine:glyoxylate aminotransferase in the livers of patients with primary hyperoxaluria type 1

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(Received 23 October 1987/1 February 1988; accepted 23 February 1988)

SUMMARY

1. The activity of alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44) has been measured in the unfractionated livers of 20 patients with primary hyperoxaluria type 1 (PH1), three patients with other forms of primary hyperoxaluria and one PH1 heterozygote. The subcellular distribution of AGT activity was examined in four of the PH1 livers and in the liver of the PH1 heterozygote.

2. The mean AGT activity in the unfractionated PH1 livers was 12.6% of the mean control value. The activities of other aminotransferases and the peroxisomal marker enzymes were normal. When corrected for cross-over from glutamate:glyoxylate aminotransferase (GGT; EC 2.6.1.4), the mean AGT activity in the PH1 livers was reduced to 3.3% of the control values.

3. The livers from a patient with primary hyperoxaluria type 2 (D-glycerate dehydrogenase deficiency) and one with an undefined form of primary hyperoxaluria (possibly oxalate hyperabsorption) had normal AGT levels. The livers of a very mild PH1-type variant and a PH1 heterozygote had intermediate levels of AGT activity.

4. Subcellular fractionation of four PH1 livers by sucrose gradient isopycnic centrifugation demonstrated a complete absence of peroxisomal AGT activity. The subcellular distribution of the residual AGT activity was very similar to that of GGT activity (i.e. mainly cytosolic with a small amount mitochondrial). There were no alterations in the subcellular distributions of any of the peroxisomal marker enzymes. The subcellular distribution of AGT activity in the PH1 heterozygote liver was similar to that of the control (i.e. mainly peroxisomal).

5. The residual AGT activity in two of the PH1 livers, which could be accounted for largely by cross-over from GGT, was only slightly dependent on substrate (glyoxylate and alanine) concentration and virtually independent of cofactor (pyridoxal phosphate) concentration.

6. These data confirm our previous findings (C. J. Danpure & P. R. Jennings, FEBS Letters, 1986, 201, 20-24), but on a much larger number of patients, that AGT deficiency is pathognomic for PH1, and is not found in other forms of hyperoxaluria.

Key words: alanine:glyoxylate aminotransferase, liver, peroxisomes, primary hyperoxaluria type 1, subcellular fractionation.

Abbreviations: AGT, alanine:glyoxylate aminotransferase; A1OT, alanine:2-oxoglutarate aminotransferase; AsOT, aspartate:2-oxoglutarate aminotransferase; CAT, catalase; CO, cytochrome oxidase; CRM+, presence of immunologically cross-reacting material; CRM−, absence of immunologically cross-reacting material; DAO, d-amino-acid oxidase; DHAPAT, dihydroxy-acetone phosphate acyltransferase; GGT, glutamate:glyoxylate aminotransferase; LDH, lactate dehydrogenase; LHO, l-2-hydroxy-acid oxidase; NAG, N-acetyl-β-glucosaminidase; PH1, primary hyperoxaluria type 1; SPT, serine:pyruvate aminotransferase.

INTRODUCTION

Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive inborn error of glyoxylate metabolism, characterized by increased synthesis and excretion of oxalate and glycolate. In a minority of patients the clinical condition can be improved by pharmacological doses of pyridoxine [1]. Recently, PH1 has been shown to be caused by a deficiency of hepatic peroxisomal alanine:gly-
oxylate aminotransferase (AGT) [2, 3] and not, as previously thought, by a deficiency of cytosolic glyoxylate:2-oxoglutarate carboxilase [4].

In our previous work, the livers from five patients with classical PH1 (hyperoxaluria plus hyperglycolic aciduria) were studied [2, 3], of which only one was subcellularly fractionated. In order to examine more fully the enzymic heterogeneity in PH1, we have extended our previous studies by examining the AGT activity in 20 PH1 livers, four of which have been subcellularly fractionated. In addition, these are compared with the livers from patients with other forms of primary hyperoxaluria and a PH1 heterozygote, whose liver was also subcellularly fractionated.

**MATERIALS AND METHODS**

**Chemicals**

The inorganic chemicals were obtained from BDH and were of 'Analar' grade. TiOSO₄ for the catalase (CAT) assay was purchased from Pfaltz and Bauer. L-[U-¹⁴C]-glyoxylate 3-phosphate for the dihydroxy-acetone phosphate acetyltransferase (DHAPAT) assay was bought from New England Nuclear. All the organic biochemicals were obtained from Sigma, including the following: cytochrome c (type III from horse heart), lactate dehydrogenase (LDH) (type XI from rabbit muscle), glutamate dehydrogenase (type III from bovine liver), CAT (in powder form from bovine liver), malate dehydrogenase (from porcine heart) and glyoxylate reductase (from spinach leaves).

**Patients**

The PH1 patients were aged between 6 months and 36 years (mean 19 years) and were diagnosed on the basis of nephrolithiasis and/or nephrocalcinosis to varying extents. One patient was mildly pyridoxine-responsive, the rest being unresponsive. The non-PH1 hyperoxaluric patients were comprised of a patient (aged 30 years) with primary hyperoxaluria type 2 (hyperoxaluria plus hyperglycolic aciduria), one (aged 67 years) with hyperoxaluria alone (possibly oxalate hyperabsorption), and one very mild variant (aged 33 years) with mild hyperoxaluria but with extreme hyperglycolic aciduria, who was previously diagnosed as a PH1 (patient A in [3]). The controls were aged between 4 months and 80 years (mean 35 years). Most control specimens were obtained at autopsy from individuals who had died from diseases unrelated to peroxisome or aminotransferase function. There is no individual who had died from diseases unrelated to liver transplantation. One control liver was obtained from a road-accident victim, in whom intracerebral and intraventricular haemorrhage had been demonstrated by computed tomography. He had been on a life-support system for 38 h before removal of the liver. Percutaneous needle biopsies (typically 10–100 mg of tissue) were taken from three controls, 10 PH1 and two miscellaneous hyperoxaluric livers. Liver from two of the PH1 patients and the PH1 heterozygote was obtained by open biopsy, while under general anaesthetic. Where appropriate, informed consent was given. These investigations were approved by the Ethical Committee of the Harrow Health Authority.

For the enzyme assays on total unfractionated tissue, the liver specimens were frozen and stored at −20°C. After thawing, samples were sonicated in ice-cold potassium phosphate buffer (100 mmol/l, pH 7.4, containing 100 amol/l pyridoxal phosphate) to give a 10% (w/v) suspension. Any non-suspended fibrous material was removed by centrifugation at 500 g for 10 min. The supernatants were then assayed for various enzyme activities. The samples prepared for the experiments investigating the effect of changes in cofactor and substrate concentrations were treated in the same fashion, except that pyridoxal phosphate was omitted from the sonication buffer. Pyridoxal phosphate stabilizes AGT during sonication. Therefore in the latter experiments, the activities of AGT in the control are slightly lower than indicated in our normal range.

The livers for subcellular fractionation, all of which were obtained fresh, were immediately cooled in ice-cold sucrose (0.25 mol/l, containing 1 mmol/l ethylenediaminetetra-acetate pH 7.4). Homogenization was commenced within 2–4 h of the termination of blood supply.

**Subcellular fractionation**

Samples of liver (usually about 10 g) were minced and homogenized in a Potter–Elvejheim apparatus, using four up-and-down strokes of the Teflon pestle, to give a 10% (w/v) homogenate in ice-cold sucrose (0.25 mol/l, containing 1 mmol/l ethylenediaminetetra-acetate, pH 7.4). Fibrous material, unbroken cells and nuclei were removed by centrifugation at 600 g for 10 min. Samples of the postnuclear supernatants were fractionated on isopycnic sucrose gradients (1.05–1.30 g/ml), as described previously [4].

**Enzyme assays**

AGT (EC 2.6.1.44) was assayed by a modification of a published method [5]. The standard reaction mixture contained 100 mmol/l potassium phosphate buffer pH 7.4, 50 mmol/l L-alanine, 50 mmol/l glyoxylate, 40 μmol/l pyridoxal phosphate and up to 1 mg of sample protein in a total volume of 300 μl. The mixture was incubated for 60 min at 37°C under nitrogen. The reaction was stopped by the addition of 50 μl of 50% (w/v) trichloroacetic acid. Blanks were run concurrently for each sample by omitting the glyoxylate until after the addition of the trichloroacetic acid. After cooling in ice, the precipitates were
removed by centrifugation at 2000 g for 10 min. The pyruvate formed was assayed by adding up to 300 μl of the supernatants to 2.7 ml of a solution containing 0.22 mmol/l nicotinamide–adenine dinucleotide (reduced) and 370 mmol/l Tris–HCl buffer pH 8.0. The drop in absorbance at 340 nm was measured after the addition of 100 μl of LDH (180 i.u./ml).

Alanine:2-oxoglutarate aminotransferase (AIOT; alanine aminotransferase; EC 2.6.1.2) was assayed similarly to AGT except that glyoxylate was replaced by 50 mmol/l L-glutamate [5]. The 2-oxoglutarate formed was assayed by adding up to 300 μl of a solution containing 0.22 mmol/l nicotinamide–adenine dinucleotide (reduced), 50 mmol/l ammonium chloride and 100 mmol/l potassium phosphate buffer pH 7.4. The drop in absorbance at 340 nm was measured after the addition of 100 μl of glutamate dehydrogenase (45 i.u./ml).

Serine:pyruvate aminotransferase (SPT, EC 2.6.1.51) was assayed similarly to AGT [7], except that the reaction mixture for the first assay contained 200 mmol/l Tris–HCl buffer pH 8.5, 50 mmol/l L-serine, 50 mmol/l pyruvate, 40 mmol/l pyridoxal phosphate and up to 1 mg of sample protein in a total volume of 300 μl. The hydroxypyruvate formed was assayed by mixing 200 μl of neutralized trichloroacetic acid supernatant with 800 μl of a solution containing 200 mmol/l potassium phosphate buffer (pH 7.4) and 0.22 mmol/l NADH. The absorbance drop at 340 nm was measured after the addition of 10 μl of glyoxylate reductase (27 i.u./ml).

Aspartate:2-oxoglutarate aminotransferase (AsOT; aspartate aminotransferase; EC 2.6.1.1) [8, 9] was measured as follows. Up to 10 μg of sample protein was added to a mixture containing 0.2 mmol/l nicotinamide–adenine dinucleotide (reduced), 30 mmol/l aspartate (neutralized), 40 μmol/l pyridoxal phosphate, 0.17 i.u. of malate dehydrogenase/ml and 100 mmol/l potassium phosphate buffer pH 7.5, all in a total volume of 3.0 ml. The rate of decrease in absorbance at 340 nm at 37°C was measured over 5 min and used as a blank. After the addition of 100 μl of 2-oxoglutarate (200 mmol/l, neutralized) the decrease in absorbance was measured for a further 5 min.

CAT [EC 1.11.1.6] was assayed by a modification of the method of Baudhuin et al. [10, 11]. One hundred microlitres of sample (containing up to 2 μg of protein) were added to 250 μl of a mixture containing 0.1% (w/v) bovine serum albumin, 20 mmol/l imidazole–HCl buffer pH 7.0, 0.25% (v/v) Triton X-100 and 0.15% (v/v) hydrogen peroxide (30%). The mixture was incubated for 10 min at 25°C and the reaction was stopped by the addition of 2 ml of a solution of titanium peroxysulphate in 1 mol/l sulphuric acid, prepared by a method similar to that of Leighton et al. [12]. The hydrogen peroxide remaining was determined by measuring the absorbance of its titanium peroxysulphate complex at 405 nm. For the blanks the sample was replaced by water.

α-Amino-acid oxidase (DAO; EC 1.4.3.3) was assayed by a method modified from that of Wanders et al. [13, 14]. One hundred microlitres of sample (containing up to 1 mg of sample protein) were added to 200 μl of a mixture containing 50 mmol/l α-alanine, 20 μmol/l flavin–adenine dinucleotide and 0.1% Triton X-100 and incubated for 120 min at 37°C. The reaction was stopped by the addition of 50 μl of 50% (w/v) trichloroacetic acid and the pyruvate measured as described for AGT, except that samples of the trichloroacetic acid supernatants (up to 300 μl) were added to only 900 μl of second reaction mixture, the Tris–HCl concentration being increased to 1.11 mol/l.

L-2-Hydroxy-acid oxidase [LHO; (S)-2-hydroxy-acid oxidase; EC 1.9.3.1] [17], LDH (EC 1.1.1.27) [18] and N-acetyl-β-glucosaminidase (NAG; EC 3.2.1.30) [19] were assayed by standard methods.

Protein was measured by the method of Lowry et al. [20].

RESULTS

The data in Table 1 demonstrate that the AGT activity in the PH1 livers was much reduced compared with that found in the controls. There were no differences in the activities of a number of other aminotransferases, such as GGT, AIOT and AsOT, nor a number of peroxisomal marker enzymes, such as CAT, DAO and LHO. Purified GGT also shows reactivity towards alanine [21], being about two-thirds as active with alanine as it is with glutamate. The AGT activity can be corrected for this 66% cross-over from GGT, giving much lower residual AGT activities in the PH1 livers (Table 1). SPT activity, which is due to the same gene product as AGT [22], was also deficient in the PH1 livers, as expected.

Considerable quantitative heterogeneity was found in the residual AGT activity in the PH1 livers (Fig. 1), which varied between 5.4 and 26.4% when uncorrected for GGT cross-over and between 0 and 14.2% when corrected. A patient with primary hyperoxaluria type 2 (glyceraldehyde dehydrogenase deficiency) [23] and one with an undefined type of primary hyperoxaluria (possibly oxalate hyperabsorption) had normal levels of AGT (98.7% and 90.3%, respectively) (Fig. 1). A patient with a very mild form of hyperoxaluria (previously diagnosed as PH1) had AGT levels only just below the bottom of the normal range (49.5%) (Fig. 1), while the liver of a PH1 heterozygote had AGT levels intermediate between those of the controls and the homozygotes (39.3%) (Fig. 1).

The effect of cofactor and substrate concentration on the activity of AGT in the homogenates from a control liver and two PH1 livers was investigated (Figs. 2 and 3).
Table 1. Aminotransferase and peroxisomal enzyme activities in the livers of controls and PH1 homozygotes

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<th>Controls</th>
<th>PH1</th>
<th>% of controls</th>
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<tr>
<td></td>
<td>Activity (μmol h⁻¹ mg⁻¹ of protein or units)</td>
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<td></td>
<td>Mean</td>
<td>Range</td>
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<tr>
<td>AGT*</td>
<td>5.00-0.89</td>
<td>16</td>
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</tr>
<tr>
<td>AGT</td>
<td>4.50-2.75</td>
<td>12</td>
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<tr>
<td>SPT</td>
<td>0.12-0.08</td>
<td>5</td>
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<tr>
<td>GGT</td>
<td>0.65-0.38</td>
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<tr>
<td>AlOT</td>
<td>3.50-1.09</td>
<td>7</td>
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<tr>
<td>AsOT</td>
<td>54.1-43.4</td>
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<tr>
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<td>1.98-1.06</td>
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<td>DAO</td>
<td>0.16-0.07</td>
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<td>LHO</td>
<td>0.029-0.014</td>
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* Corrected for 66% cross-over from GGT. Activity is expressed as μmol h⁻¹ mg⁻¹ of protein, except for catalase where activity is expressed as units [lo].

Fig. 1. AGT and GGT activities in the livers from various hyperoxaluric patients compared with controls. ●, Controls; ○, PH1 homozygotes; ▲, PH1 heterozygote; ■, other forms of primary hyperoxaluria (including a type 2 and a putative oxalate hyperabsorber); □, a very mild PH1-type variant. (a) AGT activity. (b) GGT activity. (c) AGT activity corrected for 66% cross-over from GGT. (d) AGT activity and AGT activity corrected for 66% cross-over from GGT (AGT*) in the PH1 patients on an expanded scale.

It had previously been shown [24] that, although both of these PH1 livers were deficient in AGT activity, one possessed normal amounts of AGT protein (CRM+), whereas the other had no detectable AGT protein (CRM−). Unlike the control liver, the residual AGT activity in these PH1 livers was only slightly dependent on substrate concentration and virtually independent of pyridoxal phosphate concentration. Increasing the pyridoxal phosphate concentration by 100 times, compared with the standard assay conditions, failed to increase the AGT activity in the PH1 livers (Fig. 2). These data are compatible with the suggestion that the residual AGT activity is mainly due to cross-over from GGT. Although both AGT and GGT need pyridoxal phosphate as a cofactor, GGT (unlike AGT) is not dependent on the addition of exogenous pyridoxal phosphate [21, 25, 26].

The livers from four of the more severely affected and AGT-deficient PH1 homozygotes and the PH1 hetero-
zygote were subcellularly fractionated on isopycnic sucrose gradients. In the PH1 heterozygote the distribution of AGT was mainly peroxisomal, as found previously for normal human liver [2, 27] (Fig. 4). There was a considerable smearing of activity up the gradient that was difficult to relate to any of the marker enzymes. A small minority of AGT activity was found at the top of the gradients in fractions containing the cytosolic marker enzyme LDH. In all the PH1 homozygotes there was complete absence of peroxisomal AGT activity, as shown previously [2]. Most of the activity was restricted to the cytosolic fractions, with a small percentage (5–12%) co-fractionating with the mitochondrial marker CO (data not shown). In all four of the PH1 livers the AGT distribution was almost identical with that of GGT activity. The distribution of the other aminotransferases and peroxisomal markers were unaltered (data not shown). When corrected for the amount of protein loaded on to the sucrose gradients, it can be seen that, despite the overall differences in total AGT activity, activities associated with the cytosolic fractions remain fairly constant between the control, the PH1 heterozygote and the PH1 homozygotes (Fig. 5). The partial loss of activity in the heterozygote and the much greater loss in the homozygotes is confined to the peroxisomal fractions (1.19–1.26 g/ml) and fractions, as yet undefined, in the density range 1.11–1.19 g/ml. The very

Fig. 2. Effect of pyridoxal phosphate concentration on AGT activity. •, Control; ■, CRM– PH1 patient; ○, CRM+ PH1 patient. The assays were carried out as described in the Materials and methods section, except that the pyridoxal phosphate concentration was varied between 2 μmol/l and 4 mmol/l.

Fig. 3. Effect of glyoxylate (a) and alanine (b) concentration on AGT activity. •, Control; ■, CRM– PH1 patient; ○, CRM+ PH1 patient. The assays were carried out as described in the Materials and methods section, except that the glyoxylate and alanine concentrations were varied independently between 1 and 100 μmol/l.
Fig. 4. Isopycnic subcellular fractionation of a PH1 heterozygote liver on a sucrose gradient. ---, Density as determined by refractometry. Fraction 16 is the top and fraction 1 is the bottom of the gradient. Except for AGT and SPT, the distribution of the marker enzymes was qualitatively similar with those found in a control liver and the four PH1 livers.

similar profiles of the residual AGT activity in the PH1 homozygotes and GGT activity (i.e. mainly cytosolic with a minority mitochondrial) is compatible with these activities being due to the same protein [21]. In addition, in these patients without peroxisomal AGT activity, the residual AGT activity varied between 66 and 67% of the GGT activity, confirming the validity of the factor used to correct the AGT activity in unfractionated liver sonicates for cross-over from GGT (see above).

Based on the particulate CAT and DAO distribution in the sucrose gradients, the mean density of peroxisomes from the PH1 livers (range 1.223-1.237 g/ml) appeared to be slightly less than that found in the control liver (1.245 g/ml). However, in the PH1 heterozygote liver the density was also lower (1.233 g/ml). These differences may not be significant but are of interest after our observations that hepatic peroxisomes in PH1 patients might be slightly smaller than normal [28].

Fig. 5. Relative AGT activities in various subcellular compartments (as separated by isopycnic sucrose gradient centrifugation) in a control, PH1 heterozygote and PH1 homozygote liver. The AGT activity is corrected for 1 mg of protein loaded on to the gradient. Perox, Mito and Cytosol, positions of the peroxisomal, mitochondrial and cytosolic markers, respectively. ---, Normal; ——, PH1 heterozygote; ...., PH1 homozygote. The normal histogram is taken from [2] and is included for comparative purposes. The subcellular distributions of AGT activity in the other three PH1 homozygotes were very similar to the one shown.

DISCUSSION

These studies confirm our original findings [2] that PH1 is caused by a deficiency of peroxisomal AGT in the liver. They also show that hepatic AGT deficiency is pathognomonic for PH1 and is not found in other forms of primary hyperoxaluria. Despite considerable quantitative enzymic heterogeneity, PH1 patients can be clearly separated from controls. From the study of one individual, it would also appear possible that heterozygotes may be able to be separated from both controls and homozygotes. This quantitative heterogeneity is, to some extent, paralleled by disease severity, in so far as those patients with most residual AGT activity are the least severely affected ([3]; C. J. Danpure & P. R. Jennings, unpublished work). This is to be expected if the hypothesis concerning the causal nexus between peroxisomal AGT deficiency and the hyperoxaluria and hyperglycolic aciduria in PH1 is correct [2].

Various routes for the metabolism of glyoxylate and the synthesis of oxalate have been suggested (for review see [1]). The catastrophic consequences of peroxisomal AGT deficiency demonstrate the quantitative importance of peroxisomal transamination in the metabolism of glyoxylate. Failure of this innocuous metabolic route in PH1 presumably allows peroxisomal glyoxylate to pass into the cytosol. Although some will be transaminated by cytosolic GGT [29], significant amounts will be oxidized to oxalate by lactate dehydrogenase and reduced to glycolate by
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lactate dehydrogenase and/or glyoxylate reductase [23, 30–35].

AGT is a pyridoxal-phosphate-requiring enzyme, and the discovery of its importance in the pathogenesis of PH1 explains the earlier observations that a minority of patients can be improved clinically by administration of pharmacological doses of pyridoxine [36]. Wise et al. [24] have recently found that nine out of 12 PH1 patients were CRM− (i.e. had no immunologically detectable AGT protein). Of the remaining three who were CRM+, only one had significant residual AGT enzyme activity. It is likely that pyridoxine-responsive PH1 patients are co-factor−/Km mutants. Logically these patients will be CRM+. However, the mere presence of immunologically detectable AGT protein would be no indication of pyridoxine responsiveness. In the present study, the residual AGT enzyme activity in both a CRM− and a CRM+ pyridoxine-resistant PH1 patient could not be enhanced in vitro even when the cofactor concentration was increased 100 times.

The data in the present study provide a reference range for hepatic AGT activities in PH1. We have already found this useful in confirming diagnoses in patients in whom renal failure has made urinary or plasma metabolite measurements impossible or unreliable.

REFERENCES


amine oxidase, aspartate aminotransferase, alanine aminotransferase, ß-aminocorn acid oxidase and catalase in rat liver tissue. Biochemical Journal, 92, 179–184.


12. Leighten, F., Poole, B., Beaufay, H., Coffey, J.W., Fowler, S. & De Duve, C. (1986) The large scale separation of peroxi-

somes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation pro-

cedures, automated analysis, biochemical and morphologi-


ficiency of acyl-CoA:dihydroxyacetone phosphate acyl-

transferase in patients with Zellweger (cerebro-hepato-


17. Cooperstein, S.J. & Lazarow, A. (1951) Microspectro-

photometric method for determination of cytochrome ox-


23. Wise, P.J., Danpure, C.J. & Jennings, P.R. (1987) Immuno-


