A BIOCHEMICAL AND GENETIC STUDY OF PATIENTS WITH GYRATE ATROPHY OF THE CHOROID AND RETINA

by

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(Chairman, Graduate Council)

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ABBREVIATIONS

CPM	counts per minute
DPM	disintegrations per minute
FCS	fetal calf serum
G6PD	glucose 6-phosphate dehydrogenase
HHH	hyperornithinemia, hperammonemia, homocitrullinuria
HP-MEM	medium with 10 mg/l pyridoxine
K_{eq}	equilibrium constant
K_m	michaelis affinity constant for substrate
LP-MEM	medium with no added pyridoxine
MEM	minimum essential medium
MW	molecular weight
NADH	reduced nicotinamide adenine dinucleotide
OKT	ornithine ketoacid transaminase
P5C	Δ^1-pyrroline-5-carboxylate
PEG	polyethylene glycol
Θ	probability of recombination of two genotypes
TCA	trichloroacetic acid
V_{max}	maximum velocity

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ABSTRACT

Fibroblasts from seven patients with hyperornithinemia and gyrate atrophy of the choroid and retina have been studied. This disorder is associated with deficient activity of ornithine ketoacid transaminase, (OKT), a pyridoxal 5'-phosphate (PLP) requiring enzyme. The seven patients in this thesis have previously been divided into two groups based on the change in their serum ornithine levels when given oral doses of vitamin B6.

Two direct methods for measuring OKT activity in fibroblast homogenates and one indirect method based on the incorporation of radioactivity from labelled ornithine into protein have been evaluated and compared. OKT activity from the four responsive patients was 14 to 48 nmol/mg protein/hr compared to 0 to 3 nmol/mg protein/hr in the three nonresponders (controls = 152 to 385 nmol/mg protein/hr). With the indirect measurement of OKT pathway activity the responders incorporated 199 to 274 DPM/µg protein compared to 62 to 69 DPM/µg protein in the nonresponders (control = 966±57 DPM/µg protein). Thus, all patients had severely deficient levels of OKT. Moreover, the enzyme activity in the responders was clearly higher than in the nonresponders. Furthermore, the four responders showed an increase in OKT pathway activity with increasing concentrations of pyridoxine in the culture medium, whereas neither the nonresponders nor the control showed increased activity related to pyridoxine concentration. Thus, this method provides excellent correlation between *in vivo* and *in vitro* response.

The mechanism of the response to vitamin B6 *in vitro* in the four

responders was investigated in several ways. The heat stability of patient enzyme was similar to normal in the presence and absence of PLP. The responders had a much higher K_m for PLP (0.421 - 1.617 mM) than the control (0.018 mM). The V_{max} in the responders ranged from 42 to 67 nmol/mg protein/hr compared to 223 nmol/mg protein/hr in the control. From these results, the mechanism of the vitamin B6 response in the four responsive patients appears to be related to a lowered enzyme affinity for PLP.

Complementation analysis was done in heterokaryons derived from fused crosses of fibroblasts from the seven patients by testing the resulting heterokaryons for their ability to incorporate radioactivity from labelled ornithine into protein. No complementation was observed in any of the crosses. Thus, the genetic heterogeneity observed in the two phenotypes i.e., vitamin B6 responder vs. nonresponder was shown in this group of patients to be the result of an allelic mutation.

To locate OKT on a specific chromosome, family studies of four of the patients with gyrate atrophy were done. With the addition of previous results in which eleven heterozygotes had been studied, and analysing the data on blood protein markers provided to me, linkage analysis was performed. The location of OKT on a particular chromosome was not feasible with the data gathered in this study. Further family studies combined with the above data will be required to place OKT on a particular chromosome.

INTRODUCTION

A. GYRATE ATROPHY: REVIEW OF THE LITERATURE.

1. Clinical Description

Gyrate atrophy of the choroid and retina was first described in 1895 by Cutler (1). Three affected sisters, whose parents were first cousins, had myopia, night blindness, cataracts and a peculiar form of choroidal atrophy. Gyrate atrophy is a descriptive term applying to the appearance of separate or clustered oval shaped areas devoid of choroid and retina, especially in the peripheral region of the fundus. Thus, "gyrate" describes the scalloped shape of these areas and "atrophy", the absence of choroidal and retinal components. This is a rare disease as evidenced by reports of only twelve cases from 1896 to 1941 (1-3). In the early 1930's it was proposed that the degenerative changes first seen in the peripheral retina are followed by invasion of the central zones and finally by a stage in which practically all the normal fundal structures disappear (2). The first long range follow-up of a patient in 1941 documented that the disorder is progressive (2). At the age of eleven, the patient had had no trouble with night vision but did have a slowly progressive myopia and bilateral peripheral retinitis. When he was seen at intervals of six months for a period of six years, the myopia

progressed slowly with slight loss of visual acuity. Next seen three years later, the patient reported increasing difficulty in seeing at night. The areas of choroidal atrophy had extended inward from the periphery of the fundus in each eye.

Although the symptoms vary from patient to patient, the first symptoms usually appear at five to nine years of age with night blindness and progressive myopia. The few patients diagnosed this early were usually ascertained because older siblings had been diagnosed previously. Cataracts requiring surgical correction may develop by the third decade. By age thirty to fifty years, most patients are practically blind and the progression of the disease appears to be faster in males (4).

Although the eye is the major organ affected in gyrate atrophy patients, muscle fiber degeneration is also reported (5), suggesting that this defect is a generalized disorder. Decreased capacity for fast muscular performance has been found in several patients indicating a disturbance in type 2 muscle fibers. Under electron microscopy type 2 fibers appeared atrophic and tubular aggregates were present (5-8). In one patient the ratio of type 1 to type 2 fibers was low, with a relative increase in type 2 fibers (6). With increasing age, the number of type 2 fibers decrease (5). The dilator muscle of an iris removed during an iridectomy of a patient with gyrate atrophy with cataracts was markedly atrophic with loss of muscle and derangement of fibrils. In some but not all areas crystalline tubular aggregates similar to those observed in skeletal muscle were seen (9).

Another eye disorder, termed choroideremia, has often been confused with gyrate atrophy. For many years several opthalmologists

(10-12) argued that gyrate atrophy was an early form of choroideremia since gyrate atrophy is usually diagnosed at a younger age. Usher (10) reviewed the literature and found that on average, gyrate atrophy cases are ten years younger than are those with choroideremia, seventeen years vs. twenty-seven in choroideremia. However, it is now known that choroideremia is inherited as an X-linked recessive disease while gyrate atrophy is autosomal recessive. Thus, a good pedigree should help to differentiate the two conditions. Furthermore, the biochemical findings in gyrate atrophy are quite distinct, as discussed below.

2. Inheritance

The inheritance of gyrate atrophy as an autosomal recessive trait was first documented by Kurstjens (13). The sex ratio of affected subjects is approximately 1:1 with five males and eight females in Kurstjen's study (13) and fifteen males and eleven female patients cited by Usher (10). A complete review of the literature shows that twenty-six females and twenty-two males have been reported (Table I) (1-3,6,7,13,14). Consanguinity has been reported in a total of fourteen out of twenty-six families. Only one generation is affected, i.e., siblings have gyrate atrophy but their parents or children are not affected. These observations are most compatible with autosomal recessive inheritance.

3. Biochemical Findings

The recognition of increased plasma levels of ornithine and decreased levels of lysine in subjects with gyrate atrophy did not come until 1973, 77 years after the disease was originally described. Simell and Takki (15) demonstrated the concentration of ornithine in

Table 1. Summary of gyrate atrophy families described in the literature.

Consanguinity	Number of Affected*		Number of Families	Total Number Of Sibs	References
	M	F			
+	12	15	14	83	1,3,10,11,13, 14
-	10	11	12	61	2,6,10,12,13, see Figure 1

* M = male, F = female

plasma, cerebrospinal fluid and aqueous humor of patients to be ten to twenty fold higher than in controls. Seventy-one relatives of nine patients with gyrate atrophy had normal plasma ornithine concentrations (14). However, parents of these patients had a mean fasting plasma ornithine level that slightly exceeded the normal mean concentration. When given oral ornithine loads obligate heterozygotes in families with known cases of gyrate atrophy were easily detected, suggesting that this would be a feasible method for heterozygote detection (14). However, in another study utilizing ornithine load tests, the obligate heterozygotes had serum ornithine levels that overlapped the normal range indicating that this method is not the optimal choice for heterozygote detection (7). Other biochemical findings in the patients include decreased plasma lysine levels (6, 16-18), decreased whole blood glutamate and glutamine levels (17,19), increased 3-amino-2-piperidone in urine (19) and increased serum uric acid (6).

Based on the above results the association of gyrate atrophy with ornithinemia was postulated to be due to a defect in ornithine ketoacid transaminase (OKT) (E.C. 2.6.1.13), since this is the major pathway for the disposal of ornithine (see Figure 1) (15). Deficient OKT has since been demonstrated in liver (20,21), transformed lymphocytes (22) and fibroblasts (7,23-25) from patients with gyrate atrophy. Pyridoxal 5'-phosphate (PLP) is a required coenzyme of OKT. High doses of vitamin B6 have resulted in clinical improvement in other inherited biochemical disorders of PLP-requiring enzymes such as cystathioninuria and homocystinuria (26-28). Recognizing the potential value of administering vitamin B6, Weleber et al. (29) have

Figure 1. Ornithine metabolic pathways in liver and cultured fibroblasts.

1 = ornithine ketoacid transaminase

2 = ornithine carbamyltransferase

3 = ornithine decarboxylase

4 = arginine-glycine transaminase

5 = Δ^1-pyrroline-5-carboxylate reductase

6 = Δ^1-pyrroline-5-carboxylate dehydrogenase

7 = Δ^1-pyrroline-5-carboxylate synthetase

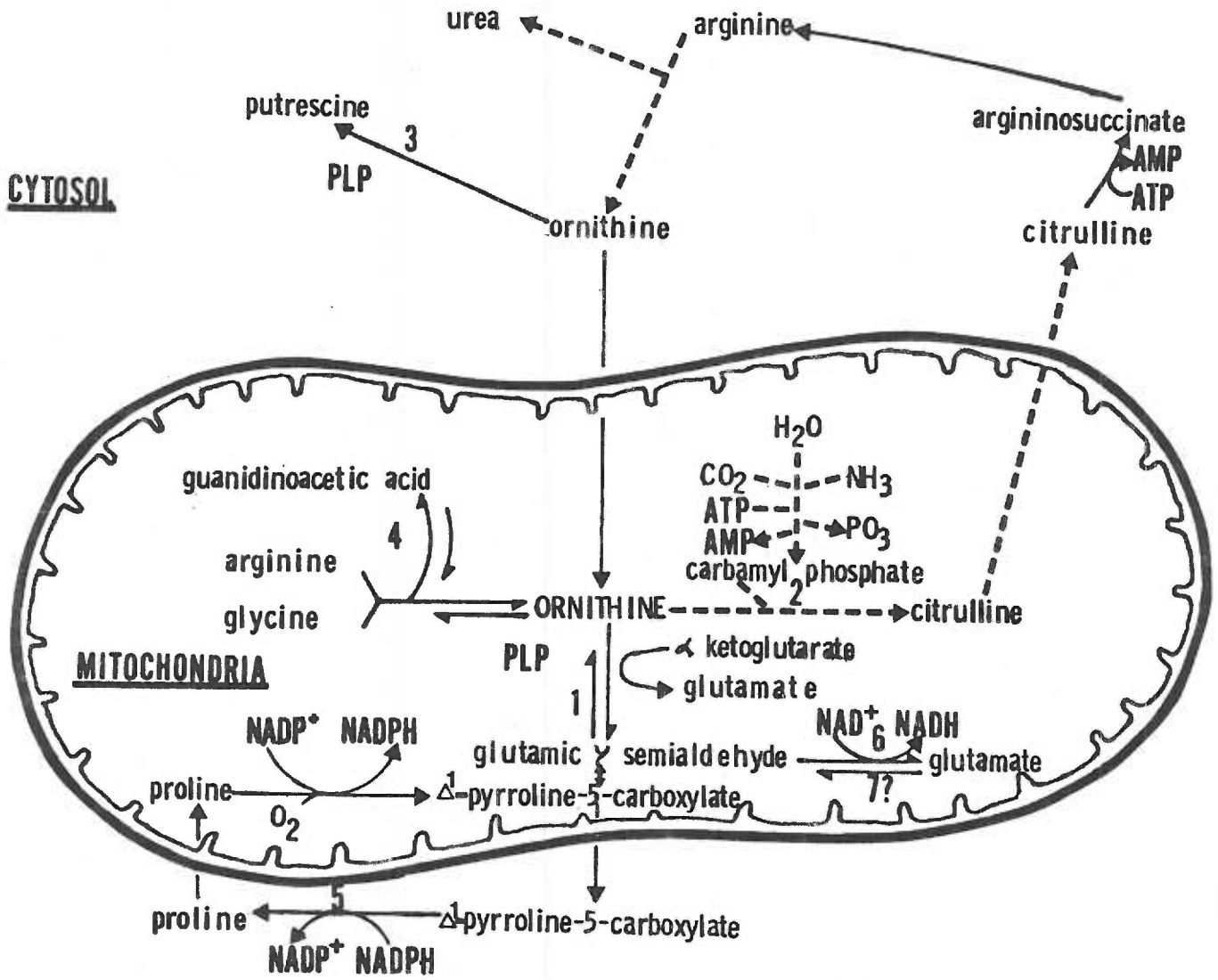
AMP = adenosine 5'-monophosphate

ATP = adenosine 5'-triphosphate

$NADP^+$ = oxidized nicotinamide adenine dinucleotide phosphate

NADPH = reduced nicotinamide adenine dinucleotide phosphate

------ pathways present only in liver


urea
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CYTOSOL
ornithine
argininosuccinate
AMP
ATP
citrulline
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arginine
glycine
MITOCHONDRIA
H2O
CO2
NH3
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carbamyl phosphate
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ORNITHINE
citrulline
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NAD+ NADH
6
glutamic γ semialdehyde
glutamate
7?
proline
O2
Δ1-pyrroline-5-carboxylate
5
proline
Δ1-pyrroline-5-carboxylate
NADP+ NADPH

demonstrated that three of his six patients respond in vivo to the vitamin with a reduction in serum ornithine levels, and improvement in electroretinographic and electrooculographic abnormalities. Berson et al. (30), Tada et al.(31) and Kaiser-Kupfer et al. (25) have reported three additional responsive patients, as evidenced by a reduction of blood ornithine levels by oral doses of vitamin B6. An increase in activity of OKT was demonstrated in skin fibroblasts with increasing PLP concentrations in five of these six responsive patients (7,24,31). Among over sixty patients reported in the literature, only the above six have been shown to respond to vitamin B6 in vivo.

4. Metabolism of Ornithine and Pathogenesis of Gyrate Atrophy

The metabolic interrelationship of proline, ornithine and glutamic acid, first proposed by Dakin in 1913 (32), was demonstrated by Krebs in 1939 (33) using mammalian kidney extracts. This relationship was further delineated by Stetten in 1951 (34) when she showed that ornithine specifically labelled at the αN or δN contributed the α-amino group extensively to proline and the δ-amino group to glutamic acid derived from α-ketoglutarate. From these results she hypothesized that glutamic γ-semialdehyde was the intermediate metabolite suggesting that there is a transaminase which specifically transaminates the δ position of ornithine. This is unusual in that most transaminases transfer the α amino group of amino acids. Glutamic γ-semialdehyde was demonstrated to be an intermediate in the conversion of glutamic acid to proline by Vogel et al. in 1952 (35) and Abelson et al. in 1953 (36).

OKT activity in animal tissues was first demonstrated in 1951 (37). The properties of purified rat liver, kidney and crude

intestine OKT appear to be identical according to Ouchterlony double diffusion, ultracentrifugal and electrophoretic behaviour, absorption spectra, pH optima and K_m values for substrates (38). These results suggest that the rat OKT enzyme of liver, kidney and small intestine is the same protein. Rat liver OKT is composed of four identical subunits, each weighing 43,000 daltons (39).

Four enzymes can utilize ornithine as a substrate; these include OKT, ornithine decarboxylase, ornithine transcarbamylase and arginine-glycine transamidinase (Figure 1). All are mitochondrial enzymes. Thus, a specific transport mechanism for ornithine is also involved, as most ornithine is produced in the cytoplasm by conversion of arginine to ornithine by arginase. Ornithine decarboxylase removes the carboxyl group from ornithine to synthesize putrescine and other polyamines. Ornithine transcarbamylase, one of the urea cycle enzymes, combines ornithine and carbamyl phosphate to form citrulline. Arginine-glycine transamidinase transfers an amidino group from arginine to glycine to form ornithine and guanidinoacetic acid. This reaction is reversible; thus, ornithine is a substrate as well as a product of arginine-glycine transamidinase.

Any of the pathways involved in ornithine metabolism i.e., polyamine synthesis, creatine synthesis or urea synthesis could potentially be affected by the large increase in ornithine concentrations that result from deficient OKT activity in gyrate atrophy. Polyamine synthesis is apparently normal in gyrate atrophy patients (7). However, low guanidinoacetic acid and creatine levels have been reported (40, 41). The regulating enzyme in creatine synthesis is arginine-glycine transamidinase. Ornithine is a product

of this reversible reaction. If enough product built up, this could reverse the reaction. In addition, Sipila has shown that ornithine is a potent inhibitor of transamidinase in homogenized rat kidney with an inhibitor constant for ornithine of 0.253±0.029 mM (41). This is clearly higher than the normal plasma ornithine concentration (0.058±0.014 mM). Thus, the inhibition of the transamidinase by ornithine is probably insignificant at physiological ornithine concentrations. However, the ornithine levels in gyrate atrophy patients are in the range or higher than the inhibition constant (0.2-1.0 mM) suggesting that the inhibition of transamidination by ornithine could occur. The low levels of creatine and phosphocreatine in gyrate atrophy patients lend further support to this hypothesis (41). Arginine-glycine transamidinase has its highest activity in kidney and pancreas, although it is also present in muscle, liver and brain (41). The major proportion of creatine is thought to be formed in the kidney and liver and transported to other tissues. Creatine reacts with ATP to form phosphocreatine, which serves as a store of high energy bonds. Phosphocreatine can then be reconverted to ATP as required. (41). On the assumption that the abnormalities seen in muscle biopsy from patients are due to low creatine levels, seven patients with gyrate atrophy were treated with 0.5 g creatine perorally three times daily over one year. Tubular aggregates disappeared in all but one of the patients (42). The retina and pigment epithelium also have a high ATP concentration which is presumably derived from creatine produced in the kidney and transported to these tissues (41). Thus, in gyrate atrophy patients the atrophies observed in the eye and muscle may have resulted from

inhibition of creatine synthesis by ornithine in the rate-limiting transamidinase reaction.

Ornithine transaminase transfers the δ amino group from ornithine to α-ketoglutarate to form glutamic acid and glutamic γ-semialdehyde, which spontaneously cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C). This compound can then be reduced to form proline. Alternatively, P5C can be converted to glutamic acid. OKT is a reversible enzyme but the equilibrium constant (K_{eq} = 71) is highly in favor of glutamic γ-semialdehyde formation. Some researchers have speculated that OKT may regulate turnover of the urea cycle by controlling the concentration of ornithine. OKT activity appears to be regulated by two factors: 1) the transport of ornithine into the mitochondria, and 2) the disappearance of glutamic γ-semialdehyde (one product of the OKT reaction). A specific carrier apparently transports ornithine across the mitochondrial membrane as shown by the specificity of the transport and the saturation kinetics of the process (43). Since glutamic γ-semialdehyde spontaneously cyclizes to P5C and P5C reductase is present in the mitochondria, the accumulation of enough glutamic γ-semialdehyde to overcome the high equilibrium of OKT in the direction of glutamic γ-semialdehyde is unlikely. It follows that OKT does not catalyse the net formation of ornithine and the suggestion that OKT functions primarily to increase the cellular concentration of urea cycle intermediates is unlikely (44, 45). Instead OKT probably regulates the urea cycle by maintaining a relatively constant level of ornithine.

Tissue levels of OKT are highest in kidney and specific areas of the eye including the iris, ciliary body, neuroretina and retinal pigment epithelium. In all of these tissues the activity of OKT has

been shown to be at least six times the activity in rat liver (46). The high activity in these tissues suggests that OKT serves some critical function in the eye. Therefore, lack of OKT activity in ciliary body and iris may be related to the incidence of myopia, cataract and lens dislocation found in gyrate atrophy patients (46). The deficiency in neuroretina and retinal pigment epithelium may be related to the degeneration found in the retina. This may account for the presentation of gyrate atrophy patients with primary involvement of the eye.

Proline biosynthesis from ornithine through OKT and P5C reductase may be the major source of this amino acid in the eye and brain. Proline penetrates the blood brain barrier very slowly and is essentially undetectable in the cerebrospinal fluid (47). In patients with gyrate atrophy proline may be an essential amino acid since the deficient level of OKT activity would severely limit the synthesis of proline. Thus, the loss of affected retinal cells in these patients could result from chronic proline deficiency. In contrast, cells outside the central nervous system can derive proline from the large circulating pool sustained by dietary sources.

As noted earlier, the progression of the disease is faster in males. OKT is an inducible enzyme with the kidney form being induced by estrogen in rats (44). The specific activity of the rat kidney OKT is five times higher than the liver enzyme (44). Thus, women with gyrate atrophy may have an advantage over men in that their kidney OKT protein may be elevated by their estrogen levels, especially during pregnancy.

High concentrations of ornithine *per se* cannot account for the

eye disorder in gyrate atrophy, since another metabolic disorder, hyperornithinemia, hyperammonemia and homocitrulluria (HHH) (48) also results in high serum ornithine concentrations and does not involve the eye. The primary defect in HHH appears to be related to the ornithine transport protein for the mitochondria (49). If this is so, then the high levels of ornithine would be confined to the cytoplasm in HHH patients and this may be the actual difference in the manifestation of HHH vs. gyrate atrophy. Potentially the high levels of ornithine in the mitochondria would inhibit arginine-glycine transamidinase as discussed above. Since in HHH the mitochondrial contents of ornithine would be low, this enzyme will not be inhibited and thus the energy metabolism in the eye, muscle and brain will be unaffected.

B. HISTORY OF PACIFIC NORTHWEST GYRATE ATROPHY PATIENTS

1. Family History

The diagnosis of gyrate atrophy in patients in this study was made by Dr. Richard G. Weleber; most of these patients are under his care. Six of the patients were born in the northwest and the seventh patient was from the midwest. Pedigrees of five of the patients are given in Figure 2. Patients 5 and 7 were adopted. Patients 1 and 2 are sisters. Interestingly, none of these patients is of Finnish background, although at least 50% of the patients described in the literature are Finnish. Instead, five of the northwest patients, four of whom are vitamin B6 responders, are of British ancestry, as is one of the responders described in the literature (25). Since the gene frequency of vitamin B6 responsive gyrate atrophy is rare ($q = 0.001$), and these patients have a common ethnic background, this would suggest

Figure 2. Pedigrees of the families of five patients with gyrate atrophy. The numbers given to the families correspond to the proband's number, i.e., patient 1's family is family 1.

□ ○ = Unaffected male/female

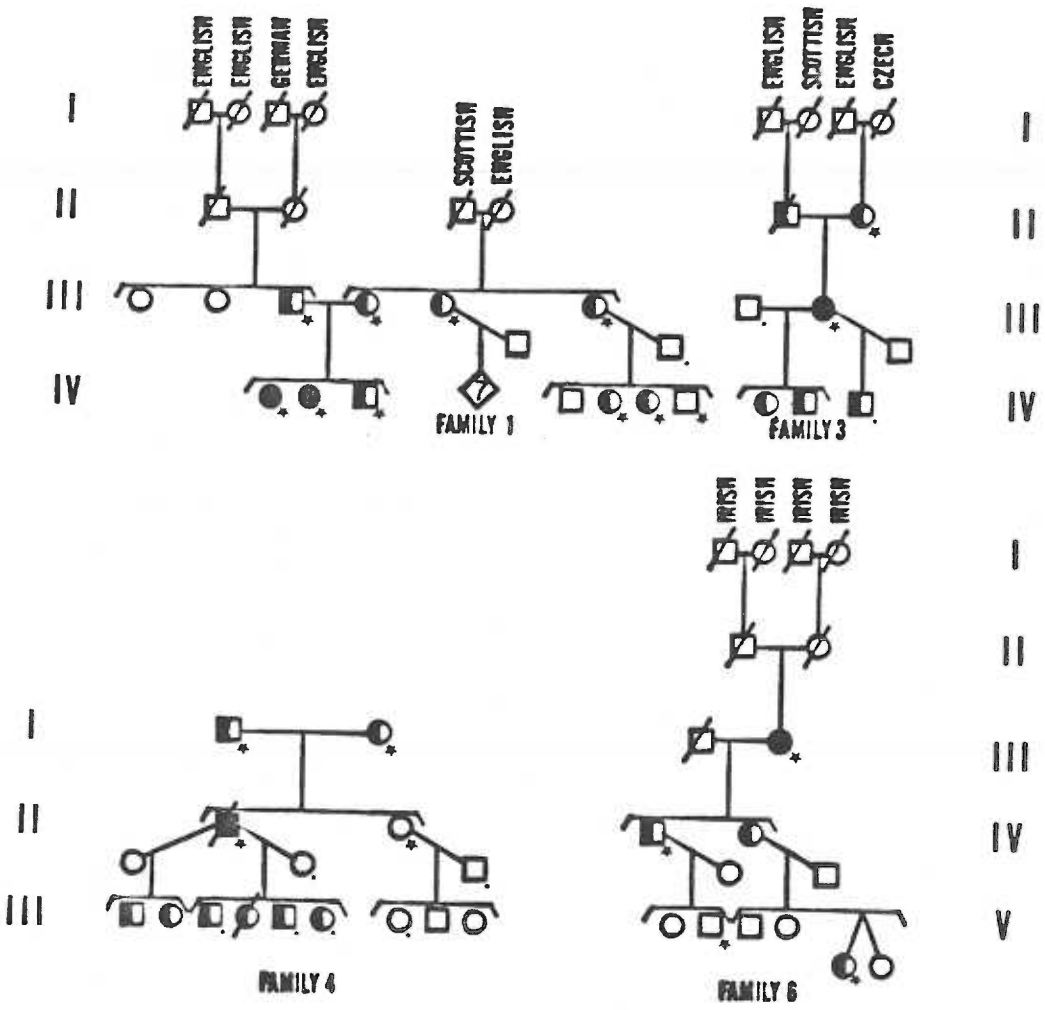
■ ● = Patients with gyrate atrophy, male/female

◧ ◐ = Heterozygote male/female

□. ○. = Blood polymorphisms measured, male/female

□★ ○★ = Blood polymorphisms measured and fibroblasts assayed for OKT activity, male/female

⊘ ∅ = Subject deceased, male/female


ENGLISH
ENGLISH
GERMAN
ENGLISH
SCOTTISH
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CZECH
I
II
III
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FAMILY 1
FAMILY 3
IRISH
IRISH
IRISH
IRISH
I
II
III
IV
V
FAMILY 4
FAMILY 6

that the original B6 responsive mutation occurred in the British Isles. No common ancestor has been found among the three families. Therefore, the question of a unique mutation being shared among the responder patients can only be answered by biochemical tests. However, it would be of academic interest to pursue the family names back to the British Isles to see if the mutation can be detected in collateral relatives.

2. Clinical History

All patients have the typical findings of gyrate atrophy as described above. The symptoms including myopia, night blindness and choroidal atrophy are milder in four of the seven patients (patients 1, 2, 3, and 6) when compared to the other patients in this study and to patients described in the literature (4, 20). Patients 1-3 have greater retention of visual field, better dark adaptometry and more retained ERG and EOG responses* compared to patients 4, 5 and 7. Early peripapillary and perimacular choroidoretinal atrophy, reported with some vitamin B6 nonresponsive patients (4, 20) was not found in any of the patients examined (patients 1-4 or 6). Patient 6, at age ninety, appears to have a greater retention of visual field than expected when compared to the other patients. However, she has been unavailable for ERG and EOG studies. Cataracts are present in

* ERG - electroretinogram, a test used to record the electrical response of the retina to stimulation by brief flashes of light. Responses can be separated into those mediated by rods versus cones and also by photoreceptors versus inner retinal neurons.

EOG - electrooculogram, a test, which measures the normal corneo-fundal or resting potential of the eye and the light-induced rise of this potential; as such, this is a measure of retinal pigment epithelium/photoreceptor physiology.

patients 3-7 and patient 1 has early coronary lens opacities which are not believed to be related to gyrate atrophy. Muscle biopsies from patients 2 and 4 showed atrophy of type 2 muscle fibers associated with subsarcolemmal inclusions, which on electron microscopy appear as tubular aggregates. Abnormal EEGs were found in patients 2 and 7; patient 2 also has suffered a seizure.

3. Biochemical Findings and Response to Vitamin B6 *in vivo*.

High concentrations of serum ornithine (0.25-1.14 mmol/l) are found in these patients (see Table 2) compared to control serum (0.057-0.132 mmol/l). After administration of vitamin B6, the ornithine level dropped in patients 1-3 (*in vivo* responders), but remained the same in patients 4, 5 and 7. This finding was consistent in patients 1 and 3. When they were taken off B6 for several weeks their serum ornithine level rose into the range of the nonresponders but returned to the previous lower levels after vitamin B6 administration was restarted. Patient 6 did not wish to take part in this study; however, her ornithine level (0.52 mmol/l) is below the range of the nonresponders (0.68-1.05 mmol/l), suggesting that she may be a responder. For the purpose of this thesis she will be referred to as a responder. When first treated with vitamin B6, patient 1 showed clear initial improvement in the ERG (29). After vitamin B6 was withdrawn, mild ERG worsening ensued, followed by mild improvement when B6 was resumed. Patient 3 showed smaller changes in the ERG with both discontinuation and resumption of vitamin B6 (29). Following these results patients 1-3 have continued taking vitamin B6, having been advised of the vitamin's possible therapeutic effect.

Table 2. Fasting serum ornithine in patients before and after oral pyridoxine supplementation.

Patient	Ornithine (mmol/l)*	
	Vitamin B6 supplementation	
Responders	0	> 50 mg+
1	0.64±0.17	0.34±0.10
2	0.57±0.02	0.25±0.01
3	0.77±0.08	0.34±0.07
6		0.52±0.08
Nonresponders		
4	0.68; 0.69	0.71±0.07
5	0.83; 1.00	0.87; 0.88
7	1.02±0.10	1.14
Controls	0.08±0.03 (0.06-0.13)	

* mean ± standard deviation except when less than three determinations are available.

+ The majority of these values were determined when patients were on 600 to 750 mg/day.

4. Enzyme Activity and Response to Vitamin B6 *in vitro*.

Four of the patients in this study have previously been shown to have deficient OKT activity in cultured skin fibroblasts (7). OKT activity with increasing PLP concentrations had also been determined in patients 1-4 (7). Fibroblasts from all four patients showed increasing activity with higher amounts of PLP, including patient 4, who was an *in vivo* nonresponder. The three *in vivo* responders reached a higher level of activity at 2 mM PLP (120, 170, and 200 nmol/mg protein/hr) than the *in vivo* nonresponder (100 nmol/mg protein/hr). However, the increase in activity from 0.1 mM to 2 mM was much more striking in the nonresponder, since his activity was lower than the responders at 0.1 mM PLP. The activity in the nonresponder increased seventeen fold while the activity in the responders increased 7.5 to 8 fold with increasing concentrations from 0.1 to 2.0 mM PLP.

C. GENETIC HETEROGENEITY IN INBORN ERRORS OF METABOLISM

1. General Review

Genetic heterogeneity has been described in almost every observed inherited disorder. A complex term, it covers different phenotypes resulting from allelic mutations (mutations at the same locus) and similar phenotypes arising from mutations at different loci. Point mutations affecting the same gene can give rise to proteins or enzymes with vastly different properties depending upon where the exact amino acid substitution occurs. If the change occurs at the catalytic site of the enzyme, the efficiency of the enzyme may be substantially reduced. On the other hand, if the substitution occurs at a neutral location, the enzyme may function normally. Within these two extremes, a wide range of possibilities exist. A classical example of

genetic heterogeneity resulting from allelic mutations is glucose 6-phosphate dehydrogenase deficiency (G6PD). In healthy black males one of two electrophoretically distinct forms is found, a slow migrating form, B, or a fast migrating form, A. G6PD deficient black males have fast migrating form A. Thus, three distinct classes in black males can be distinguished by enzyme activity and electrophoresis; these three phenotypes are referred to as Gd B, Gd A and Gd A-. According to family studies these phenotypes are determined by three distinct alleles occurring on the X chromosome. Male Caucasians from the Mediterranian area have another common type of G6PD deficiency, with the enzyme having the B electrophoretic mobility. The allele causing the Mediterranean type of deficiency is at the same locus as the common alleles occurring in black populations and it is referred to as Gd Mediterranean. Other variants of G6PD have similarly been ascertained, with more than 140 distinct forms of the enzyme being identified. Gd B is apparently the normal type since it is the commonest and occurs in all populations.

The second type of genetic heterogeneity is defined as similar phenotypes arising from mutations at different loci, the classical example being the mucopolysaccharide storage disorders, Hunter and Hurler syndromes. The phenotypes are similar, in particular the same mucopolysaccharides, heparan sulfate and dermatan sulfate, are excreted by patients with either disorder. But the mutant enzyme in Hunter Syndrome is sulphoiduronate sulphatase and in Hurler Syndrome is α-iduronidase. This heterogeneity, arising from mutations at loci determining different and quite distinct enzymes, is not totally surprising, considering that the enzymes are involved in a sequential

series of reactions in the same metabolic pathway.

To define genetic heterogeneity within a disorder, consistent laboratory methods must be used to ensure that the heterogeneity is a result of a distinct mutation rather than a difference in methodology. Using different buffers, different substrate or coenzyme concentrations or different pHs can all result in the same mutant enzyme appearing to have different properties. This problem has been recognized by workers studying the variety of mutations in human pyruvate kinase. As a result, a standard method has been drawn up and published for this enzyme, the ICSH Expert Panel on Red Cell Enzymes (50). Standardization for other mutant enzymes including ornithine ketoacid transaminase will also need to be established.

2. Vitamin Responsive Disorders

At least twenty-five different metabolic disorders have been described with an unequivocal biochemical and/or clinical response to a larger than physiological amount or a different than normal physiological route of administration of a single vitamin. Many of these vitamin-responsive disorders have only been recognized in the last fifteen years. The defect in vitamin-responsive conditions may include problems in intestinal absorption, plasma transport, cellular entry, intracellular compartmentation, conversion of vitamin to coenzyme or formation of holoenzyme. For a defect in any of the steps above, except the last one, the clinical picture will resemble vitamin deficiency resulting from a poor diet, since all of the enzymes requiring that coenzyme will be affected. However, if the formation of a specific holoenzyme is defective, a much more limited disorder will be observed. In order to prescribe an effective vitamin for the

latter type of disorder, the inherent deficient enzyme must be identified and the cofactor for that enzyme must be known. For example, in homocystinuria, the defective enzyme is cystathionine β-synthase, which requires PLP as coenzyme. Therefore, large doses of vitamin B6 might be beneficial for these patients. Genetic heterogeneity also exists within the vitamin-responsive disorders. For example, more than one type of homocystinuria exists with some patients responding to vitamin B6, while others do not. Thus, for any of the potential vitamin-responsive conditions, a therapeutic trial of vitamin supplementation is necessary to distinguish vitamin-responsive patients from the nonresponsive patients. Ideally, an *in vitro* biochemical test should be available to determine quickly and efficiently whether or not a patient is a responder. Cultured skin fibroblasts are frequently utilized to measure the enzyme activity or some end product of the deficient pathway. In order for the test to be of practical value, there must be correlation between the *in vivo* and the *in vitro* response of the patients. Unfortunately, this is not always the case, as seen in homocystinuria in which some clinically nonresponsive patients have increasing fibroblast enzyme activity with increasing vitamin levels, while some patients, who do respond *in vivo*, do not respond *in vitro* (51).

The heterogeneity seen in the patients with homocystinuria, for example, presumably reflects different mutations of the same enzyme (allelic or nonallelic). The responsive patients may possess a mutant enzyme with a defective binding site for the coenzyme and/or a mutant enzyme that requires high levels of the vitamin to prevent degradation (51-54). The nonresponder mutant enzymes may also have a defect at

the coenzyme binding site; however, their K_m for PLP may be so abnormal that not even huge amounts of the vitamin will result in sufficient binding of the coenzyme by the defective site. On the other hand, the nonresponder may have a defect at some other site than the cofactor binding site or the mutant enzyme may be synthesized at a slow rate if at all. To date, none of the mutant enzymes of the vitamin-responsive disorders have been purified, sequenced or mapped by recombination techniques. Therefore, the exact location of each mutation and its effect on the physiological functioning of the enzyme has yet to be determined for each of these disorders.

D. COMPLEMENTATION ANALYSIS

Complementation analysis is a unique tool used to study genetic heterogeneity. Fratantoni et al. (55) were the first to recognize complementation in human genetic disorders, when they grew fibroblasts from Hurler and Hunter patients together in the same flask and found that they cross-corrected each other's metabolic defect. Prior to this observation the defect in the two disorders was thought to be the same. When the Hunter and Hurler fibroblasts were grown together, the Hunter fibroblasts excreted a "corrective factor" that was taken up by the Hurler cells, while the Hurler cells excreted a different "corrective factor" that was taken up by the Hunter cells. In general, complementation analysis is used to determine if patients with similar phenotypes have different genotypes, as is the case in the Hunter and Hurler syndromes. The distinct genotypes can be at the level of different mutant loci (intergenic) or mutant alleles (intragenic or interallelic) at the same locus.

Three methods have been used to study complementation; these

include 1) mixed cultures of nonfused cells, 2) cell fusion to form heterokaryons and 3) formation of hybrid cells with fused nuclei. The study described above with Hunter and Hurler cells is an example of mixed cultures that are not fused. This is a special case since both enzymes are normally excreted out of the cells, processed and then reabsorbed. Thus, normal enzymes from both cell types are available to both mutant cells to be taken up and normal metabolism can then ensue. Most enzymes are not secreted into the extracellular environment. Thus, this type of method will not show complementation for most other mutant cells.

Hybrid cells can also be used to study complementation. They offer the advantage that they can be grown indefinitely. Therefore, they can be cultured to high densities for large scale biochemical analysis. Also, the parental genomes can interact freely within the same nucleus. However, the hybrids resulting from fusion of two different parental nuclei must first be isolated from all of the other cells. For every heterokaryon formed only a very small fraction (rarely more than one in 10,000) succeeds in forming a common nucleus and a growing cell. Another serious disadvantage is that the clones tend to be genetically unstable with one or more chromosomes being lost (56).

Complementation analysis is usually done with heterokaryons, i.e., cells with nuclei from more than one parent. Heterokaryons are derived from two parental cell lines that have been fused with polyethylene glycol (PEG) or inactivated Sendai virus. This method allows the mutant enzymes to interact within a cell, in contrast to growing cells together where one enzyme or metabolite must pass from one cell

to another before it can correct the defect. Studies on heterokaryons have two main advantages over using hybrid cells. First, all parental chromosomes may be assumed to be present. Second, the relatively high frequency of formation of heterokaryons makes it possible to assay various properties immediately after fusion. For these reasons heterokaryons are the usual method chosen for complementation analysis.

The complementation observed in methylmalonic acidemia represents one type of intergenic complementation. This is a heterogeneous group of enzyme defects resulting in deficient conversion of methylmalonyl CoA to succinyl CoA, which lead to the same clinical symptoms. The deficient enzymes include methylmalonyl CoA mutase and at least three enzymes involved in the synthesis of adenosylcobalamin, the active form of the coenzyme. When fibroblasts with different defects in the synthesis of adenosylcobalamin are fused with each other or with a mutase mutant, complementation occurs; the defects are intergenic (56). However, complementation does not occur within any group.

Complementation may also occur between cells containing a mutant enzyme with more than one type of subunit. The classical example is the complementation observed between Tay Sachs and Sandhoff cells (58, 59). In Tay Sachs disease the defective enzyme is hexosaminadase A, a tetramer composed of 2 α subunits and 2 β subunits. The mutant locus codes for the α subunits, with the β gene being normal. In Sandhoff disease two enzymes are deficient, hexosaminadase A and B (hexosaminadase B is composed of 4 β subunits). In Sandhoff disease the mutant locus codes for the β subunit. When Tay Sachs and Sandhoff

cells are fused, the normal β subunits synthesized from the Tay Sachs nucleus and the normal α subunit from the Sandhoff nucleus can combine to form normal hexosaminadase A and B. Thus, partial activity is restored.

The third situation in which complementation can occur involves cells with the same mutant enzyme or subunit but with different allelic mutations at that locus. Intragenic complementation occurs only rarely and even when it does, the complementation never reaches the same degree as obtained by the above two mechanisms. Catcheside's work (60) with the yeast Neurospora Crassa demonstrated that some allelic mutations will complement each other. Allelic mutations are defined by complementation mapping as 1) all pairs show no complementation or 2) most pairs show no complementation, but a minority show complementation with each other but not with any of the other mutants. The actual mechanism behind intragenic complementation is not known. The most likely explanation is that the mutant gene product has two centers of function such that a defect in one does not inactivate the other (61). This has been postulated to occur in the complementation of β-galactosidases of E. Coli (61). Intragenic complementation may also occur if the enzyme is composed of more than one identical subunit. If one mutant has a defect in one part of the subunit and the second mutant has a defect in a different part of the subunit, then complementation may also occur by the interaction of the two mutant subunits.

E. LINKAGE ANALYSIS

One objective in this thesis is to attempt to locate the OKT gene in relation to other genes and to establish on which of the

twenty-three chromosomes it resides. Identification of specific genes is made possible by the fact that most genetic characters have alternate forms, called alleles. For example, heterozygotes for OKT have one allele specifying normal activity and one allele specifying deficient activity. Thus, theoretically, heterozygotes have 50% of control activity. In the case of a number of blood proteins, allelism is more commonly detected by electrophoresis or immunological techniques.

Several approaches have been used to do linkage analysis, including hybridization, gene dosage and family studies. To analyze linkage using hybridization studies, mouse and human cells are hybridized. In successive generations of the hybrid cells, the human chromosomes gradually disappear. By examining a number of clones, or lines of cells, one notes what human phenotypes are present or absent and what human chromosomes remain. With a large enough sample a particular phenotype can be assigned to a specific human chromosome.

The use of gene dosage for linkage analysis utilizes the information gained from abnormal karyotypes, i.e., either part or all of a chromosome is missing or duplicated. The quantity of a particular phenotype is measured such as enzyme activity. When the enzyme activity is measured in cells from a person with a karyotype with a chromosomal deletion, the enzyme activity would be predicted to be 50% of normal if the gene for the enzyme is located on the missing chromosome piece. On the other hand, the enzyme activity would be predicted to be 150% of normal if the gene for the enzyme is located on a duplicated chromosome area. This method has some limitations since it is not directly measuring the location of the gene.

The most common method utilizes family studies for linkage analysis. This method is dependent upon following the pattern of inheritance of particular phenotypes through a family for several generations. To determine if the loci for two genes (coding for OKT and a blood protein) are located on the same chromosome the occurrence of recombination of the loci during meiosis of the parental cell is observed in the offspring. At meiosis the paired chromosomes line up parallel to each other and exchange segments, randomly. As a result, the offspring will possess a "recombination" of the traits possessed by the parent if there is an odd number of exchanges between the chromosomes. When two traits are involved, the farther apart the loci are on the chromosome (that is, the less closely linked), the greater is the probability that they will be separated onto opposite chromosomes by a crossover. The distance between the loci can be estimated from the relative frequency with which a switched combination of the observed traits occurs. The rarer the recombination, the closer the linkage of the loci being studied. In order to decide whether or not a given combination of traits was created by a recombination, we need to know how the traits were originally paired in the parent. Often no information is available on this question. Therefore, the problem has been approached with probability reasoning, which tests likelihoods in the light of *a priori* assumptions (62).

Various hypotheses are tested in order to arrive at an estimate of the relative likelihood that the observed traits are or are not linked on the same chromosome pair. In order to obtain information, two types of matings are informative: an intercross or a double

backcross mating. A double backcross means that one parent is heterozygous for both traits and one parent is homozygous for both traits. An intercross refers to both parents being doubly heterozygous. With an informative mating type one can ask - what is the likelihood that the observed pattern with which the traits are distributed in the offspring would occur if we assume that the traits are linked and recombine in a certain proportion of cases, as compared with the likelihood that the pattern would occur if the traits are not linked? That is, given the observed and computed data, what are the relative probabilities that recombination will occur with frequency a or b? This will give an estimate of the most likely distance between two loci. The probability of recombination or map distance is referred to as θ. The likelihood that the observed pattern of transmission would occur assuming a particular θ is then compared statistically to the likelihood in the absence of linkage. Testing the various sets of assumptions is a complex job. However, this can be done on a computer or by using a series of tables set up by Morton (63).

F. OUTLINE OF PROPOSAL

1. OKT Activity in Gyrate Atrophy and Control Fibroblasts. Comparison and Evaluation of Methods.

OKT activity has been shown to be deficient in patients with gyrate atrophy (7, 20-25). In order to measure the low activities in the patients' cells, a sensitive and reliable assay is needed. Two direct assays, a colorimetric and a radiochemical method will be compared and evaluated. In order to study the activity in growing cells, a situation which more closely resembles the *in vivo*

environment, an indirect method will be evaluated. This method measures the incorporation of radioactivity from labelled ornithine into protein. The optimal conditions for the radiochemical assay and the indirect method will be established. Patient and control enzyme activity will be compared with the radiochemical assay and the indirect method.

2. Mechanism of Response to Vitamin B6 *in vitro*.

The mechanism of the response of gyrate atrophy patients to vitamin B6 has yet to be determined. Using skin fibroblasts, several approaches will be used to elucidate the mechanism of this *in vitro* response:

1) measurement of the level of patient and control OKT pathway activity with increasing medium pyridoxine levels,

2) assessment of holoenzyme and apoenzyme content in patient and control cells grown in medium with low and high levels of pyridoxine,

3) measurement of the K_m of responsive patient and control OKT for PLP and finally,

4) determination of the heat stability of patient and control OKT.

3. Genetic Heterogeneity and Correlation of Response *in vivo* and *in vitro* to Vitamin B6.

Patients with gyrate atrophy have been divided clinically into two groups: *in vivo* vitamin B6 responders and nonresponders. Responders are defined as those patients whose serum ornithine level drops when given oral doses of vitamin B6. The correlation between *in vivo* and *in vitro* response was not found in one patient, an *in vivo* nonresponder showing response *in vitro* (7). This patient will be

included in this study along with four *in vivo* responders and two additional *in vivo* nonresponders. Further evidence for genetic heterogeneity will be sought in the studies outlined in sections 1 and 2. The level of OKT activity will be measured both directly and indirectly as well as the response of patient cells to growth in different concentrations of pyridoxine. Additional evidence for genetic heterogeneity will be sought by determining the K_m for PLP, heat stability, and pH optimum of the four *in vivo* responders.

4. Complementation Studies

Complementation analysis is a classical tool used to predict if a particular phenotype shared by more than one person is the result of allelic or nonallelic mutations. In one study in which fibroblasts from one responsive and three nonresponsive gyrate atrophy patients were fused with each other, no complementation was observed (64). In this thesis, complementation analysis will be used to search for evidence of genetic heterogeneity in the two phenotypes observed in gyrate atrophy, i.e., responder and nonresponder. Fibroblasts from the four responders and three nonresponders will be fused with each other and assayed for activity. Evidence for complementation will be assessed by looking for increased incorporation of radioactivity from labelled ornithine into protein.

5. Linkage Analysis

To date the location of the OKT locus on a specific chromosome has not been identified. Linkage analysis is one of the classical studies used to determine the location of a particular gene. Four gyrate atrophy families with at least three generations are available for linkage analysis. OKT activity in fibroblasts from members of

these families will be measured. With data provided to me on blood marker proteins from the above relatives, I will examine dependent segregation of the deficient OKT gene with each of the blood marker proteins in the four families.

MATERIALS AND METHODS

A. SOURCE OF SKIN FIBROBLASTS

Dr. Frederick Miller, III (University of Washington, Seattle) kindly provided a skin biopsy from patient 5. All of the skin biopsies from the remaining gyrate atrophy patients and their relatives were obtained by Dr. Richard Weleber. Skin fibroblasts from a patient with hyperornithinemia, hyperammonemia and homocitrullinuria were kindly provided by Dr. Vivian Shih (Massachusetts General Hospital, Boston). A control cell line was purchased from the Human Genetic Mutant Cell Repository. Other control cells were obtained from relatives of patients with unrelated metabolic disorders. Most of these were from people ranging in age from twenty to seventy, corresponding to the age range of the gyrate atrophy patients.

B. CHEMICALS AND RADIOCHEMICALS

Polyethylene glycol (M.W. 6,000), pyridoxal 5'-phosphate, thiamine, nicotinic acid, pantothenate, choline, folic acid, L-inositol, riboflavin, o-aminobenzaldehyde, L-ornithine, and D,L-dithiothreitol were purchased from Sigma. Alpha-ketoglutarate-Na salt was purchased from Calbiochem and Sigma. Dialyzed fetal calf serum, (FCS), Eagle's minimum essential medium (10x), Eagle's basic salt solution, Hank's basic salt solution, essential vitamin solution

(10x), nonessential amino acids (100 x), essential amino acids (100x), glutamine, Dulbecco's phosphate buffered saline (10x), and penicillin/streptomycin (100x) were purchased from GIBCO. FCS was purchased from Flow and Rehico and gentamycin from Microbiogical Associates. Penicillin was purchased from the hospital pharmacy. Other chemicals used were all reagent grade. L-^{3}H [4,5]-leucine was purchased from Amersham and New England Nuclear. Alpha-[1-^{14}C]-ketoglutarate, L-[^{14}C-U] and ^{3}H-(G)-ornithine were purchased from New England Nuclear and glutamic acid decarboxylase from K & K. Toluene was scintillant grade. Trypsin (0.25%) and trypsin/EDTA (0.25%) were purchased from GIBCO. Crystalline trypsin (0.025%) was purchased from Worthington. Sephadex G-50 and G-25 were purchased from Pharmacia. Hyamine hydroxide was purchased from Sigma.

C. TISSUE CULTURE

1. Routine Culture Conditions.

Skin fibroblasts were routinely grown in Eagle's minimum essential medium (MEM) (15 ml/T250, 35-50 ml/roller bottle) with nonessential amino acids, 10% FCS, penicillin (100 u/ml) and gentamycin (50 mg/l) or streptomycin (100 mg/l). For the complementation studies and pyridoxine studies (following ^{14}C or ^{3}H-ornithine incorporation into protein) nonessential amino acids were omitted. Cell lines had been in culture for three to twenty-two passages at the time of study. All special media and serum were sterile-filtered through 0.22 μ Millipore filters. Corning or Falcon flasks and petri dishes were used.

2. Harvesting Cells for the Colorimetric or Radiochemical Assay.

Fibroblasts were harvested four to ten days past confluency. The flasks were rinsed with Hanks basic salt solution without calcium and

magnesium. Trypsin was added to the flasks (3 ml/T250 or 10 ml/roller bottle), which were shaken gently until the cells had detached. An equal amount of medium, containing 10% FCS, was added to stop the action of the trypsin. The solution was thoroughly mixed, transferred to a test tube and then centrifuged at 1,000 g for ten minutes. The supernatant was removed, the cell pellet was suspended in cold 0.9% NaCl and centrifuged at 1,000 g for ten minutes. The last step was repeated once. The final pellet was suspended in water or, if dialyzed, in the dialysis buffer. The extract was sonicated four times for fifteen seconds on ice using a Braunsonic sonicator 1510 at 50 watts. The sample was cooled on ice for 45 seconds after each burst.

3. Preparation of Fibroblast Extract for the Radiochemical Assay.

In order to reduce the concentration of amino acids and/or PLP in the fibroblast extract, it was dialyzed overnight or passed through a Sephadex column that had been equilibrated with water. The dialysis buffer routinely contained 62.5 mM potassium phosphate pH 7.8, 0.063 mM EDTA, 0.5 mM dithiothreitol and 0.09 mM PLP. Cell extract containing a maximum protein concentration of twelve mg/ml was dialysed against two l of buffer overnight. The dialysate was centrifuged at 1,000 g for ten minutes before assaying. If the extract was to be passed through a column, it was first centrifuged at 1,000 g for ten minutes or in a Beckman microfuge for one minute. In the experiments studying holoenzyme and apoenzyme activity, the fibroblast extract, containing one to five mg of protein in 0.5 ml of water, was passed through a ten ml Sephadex G-25 column and eluted with water. For the PLP kinetics and heat stability experiments

0.5-1 ml of extract containing up to ten mg of protein was passed through a ten ml Sephadex G-50 column and eluted with water.

4. Preparation of Medium with Different Pyridoxine Concentrations.

The routine medium used to grow fibroblasts contains one mg/l pyridoxal or 500 times the minimal required amount for essential growth of mouse fibroblasts and HeLa cells (64). Eagle has demonstrated that pyridoxal and pyridoxine are essentially equivalent in their growth-promoting activity (64). Standard medium used for growing human fibroblasts contains pyridoxal. However, pyridoxine is the form of B6 used for treating gyrate atrophy patients. For this reason, pyridoxine was used in the following experiments. Medium containing one mg/l pyridoxal will be referred to as standard medium. In order to study the effect of different medium concentrations of pyridoxine on patient and control OKT, special media that contain no pyridoxine, must be made to which different amounts of pyridoxine can then be added. FCS, which is added to the medium, is another source of vitamin B6, where it occurs largely in the form of pyridoxal and PLP (65). To lower the amount of vitamin B6, serum was dialysed against buffer or against hydroxylamine in buffer. Hydroxylamine reacts with pyridoxal and PLP to form a Schiff base, which can then be removed by dialysis.

a. Preparation of 50 x stock vitamin solution without pyridoxine.

A stock vitamin mix was prepared containing pantothenate 50 mg/l, choline 50 mg/l, folic acid 50 mg/l, L-inositol 100 mg/l, nicotinamide 50 mg/l, riboflavin 5 mg/l, and thiamine 50 mg/l. Folic acid was

rather insoluble and was therefore solubilized separately in water by adding 0.1 N NaOH until the final pH was approximately 6.0. The folic acid was then added to the rest of the vitamins and the stock was brought up to volume with freshly double distilled water. The solution was sterile-filtered and frozen.

b. Dialysed FCS

Dialysed FCS was either purchased from GIBCO or prepared by dialysing against 200 volumes of phosphate buffered saline, pH 7.4, with three changes of the buffer over a total period of at least thirty hours. The dialysed FCS contained 4.2 - 8.6 µg/l pyridoxine as determined by Dr. Miller (Oregon State University, Corvallis, Oregon).

c. Hydroxylamine-treated FCS

FCS was dialysed against 2 mM hydroxylamine in phosphate buffered saline, pH 7.6, for twenty-four hours. The serum was then dialysed against phosphate buffered saline with three changes over three days, then sterile-filtered and frozen.

d. Preparation of medium

Special medium containing only the vitamin B6 added by the FCS was prepared using Earle's basic salt solution (10x), essential amino acids (100 x), 7.5% sodium bicarbonate, glutamine (100x), penicillin (100 u/ml), gentamycin (50 mg/l) and the special vitamin solution without pyridoxine described above. This medium will be referred to as low pyridoxine medium or LP-MEM, which contains 10% dialysed FCS unless otherwise indicated. To look at the effect of different concentrations of pyridoxine on OKT activity, amounts of pyridoxine ranging from 0.01 to 100 mg/l were added to the LP-MEM. Medium containing 10 mg/l pyridoxine and 10% dialysed FCS will be referred to

as high pyridoxine medium or HP-MEM.

D. ACTIVITY OF ORNITHINE KETOACID TRANSAMINASE

1. Colorimetric Assay

The colorimetric assay has been the most commonly used assay. It was developed by Peraino et al. in 1957 (67) and is based upon the reaction of o-aminobenzaldehyde with the product P5C, which is formed spontaneously from glutamic γ-semialdehyde to form a dihydroquinazolinium that absorbs light at 440 nm.

In the experiments described in this study the incubation mixture contained 30 mM ornithine, 5 mM α-ketoglutarate, 0.04 mM PLP, 50 mM potassium phosphate, pH 7.6 and the fibroblast extract in a total volume of 0.25 ml. The blank contained no α-ketoglutarate. Both substrates and PLP were brought to a pH between 7 and 8 before adding to the incubation mixture. The reaction was stopped after sixty minutes with 0.5 ml of 10% TCA. Ortho-aminobenzaldehyde (5 mg/1) was then added and the test tubes were allowed to sit for twenty minutes. The incubation mixture was centrifuged in a Beckman microfuge for two minutes and the optical density of the supernatant was determined at 440 nm. The activity was calculated using a millimolar extinction coefficient of 2.71 (68).

2. Radiochemical Assay

The radiochemical assay using tissue homogenates was recently developed by Wong et al. (69). To date, measurement of OKT activity in patients with gyrate atrophy using this method has not been described. The assay is based on the conversion of α-[1-^{14}C]-ketoglutarate to α-[1-^{14}C]-glutamate, which is then decarboxylated by glutamic acid decarboxylase to $^{14}CO_2$.

The standard reaction mixture contained 5 mM α-ketoglutarate (0.05 μCi/assay), 30 mM ornithine and 0.06 ml of homogenate (0.15 to 5.6 mg/ml of protein) in a final volume of 0.12 ml. For the heat stability studies and the PLP affinity studies 60 mM ornithine was used. For samples purified on Sephadex G 25 or G 50, 50 mM potassium phosphate pH 7.8 and 0.04 mM or 2 mM PLP were included in the reaction mixture. The blanks contained no ornithine. The samples were incubated at 37°C for two hours. The reaction was stopped by lowering the pH with the addition of excess bacterial glutamic acid decarboxylase (0.063 mg/assay) dissolved in 0.25 ml of 0.1 M NaCl in 0.1 M pyridine:HCl, pH 4.0. For the experiments following OKT activity at different concentrations of PLP, the bacterial glutamic acid decarboxylase was dissolved in 0.1 M NaCl in 0.1 M pyridine:HCl, pH 4.0 with 0.05 mM PLP. A concentration of 0.125 mg/assay was used in the heat stability studies. The incubation tube was immediately placed in a scintillation vial with a gelatine capsule containing 0.1 ml hyamine hydroxide on Whatman #1 filter paper. The vial was sealed with a rubber stopper and the mixture further incubated at 37°C for thirty minutes to convert all glutamate to CO_2. Two-tenths of one ml of 1 M sulfuric acid was then added and the vials were left standing overnight. After removal of the incubation tube, ten ml of scintillant (0.4 g omnifluor/100 ml toluene:ethanol 4:1) was added to the scintillation vial and radioactivity was determined by liquid scintillation spectrometry on a Packard Model Tri CARB 460C scintillation counter using external standardization.

3. Incorporation of ^{3}H Ornithine into Protein *in situ* (Indirect OKT Pathway Activity)

This assay measures the metabolism of ^{3}H ornithine into proline and glutamate via OKT with the subsequent uptake of the labelled amino acids into protein. This method includes incubating the fibroblasts with radioactive ornithine for twenty-four hours, harvesting the cells and determining the radioactivity in the protein. Cells were grown in LP-MEM for one week before being subcultured into petri dishes for each experiment, unless otherwise indicated.

Procedure:

Cells were seeded into 35 mm petri dishes at a concentration of 8 x 10^{4} cells/dish to give a confluent monolayer. After two days medium was aspirated from each petri dish, and the cells were then washed with Hanks balanced salt solution. The dish was tilted to ensure that all of the salt solution was aspirated. Radioactive medium (1.5 ml) containing 2 mCi/l ^{3}H ornithine was then added and the dishes were incubated at 37^{o}C for twenty-four hours. The cells were harvested by washing three times with three ml of cold 0.9% NaCl. Cold 5% TCA was left on the dishes for thirty minutes, then they were rinsed three times with cold 5% TCA, washed three times with cold absolute EtOH and air dried. The cellular protein was solubilized by incubating the dishes with 0.7 ml of 2% Na_2CO_3 in 0.1N NaOH at 37^{o}C for thirty minutes. The dishes were allowed to cool, then mixed well and two aliquots of 0.2 ml were assayed for protein concentration using the method of Lowry et al. (66). A third aliquot of 0.2 ml was added to a seven ml scintillation vial containing five ml of Aquasol or Instagel, 0.06 ml of 1N HCl and 0.24 ml H_2O to gel the mixture. Addition of

water has previously been shown to prevent the slow decline of counts with time, presumably due to the precipitation of protein from solution. The radioactivity was counted in a Packard Tri CARB 460C scintillation counter with external standardization.

E. COMPLEMENTATION STUDIES

After growing the cells for a week in LP-MEM with 10% FCS, fibroblasts were subcultured and seeded in 35 mm Corning tissue culture dishes with inocula of 2.5×10^5 cells/dish. For complementation testing, equal numbers of cells from two cell lines were mixed well before plating. Three dishes were set up from each cross, one to be fused and one that was not fused for comparison. Simultaneously, with the test mixtures each cell line was plated separately in two dishes with one to be fused for comparison. Each cross was repeated at least once on a separate occasion. One day after seeding, cells were fused by a modification of the procedure of Willard et al. (56). Medium was removed from the cells and the dish was allowed to drain for thirty seconds. The remaining medium was removed and one ml of 50% PEG in serum-free MEM (without pyridoxine) was added. After exactly one minute the PEG solution was rapidly diluted with phosphate-buffered saline and aspirated. The dish was rinsed four times with serum-free MEM then once with LP-MEM (containing serum) and finally reincubated with LP-MEM containing 10% FCS. Twenty to twenty-four hours after fusion, intact cells were tested for their ability to incorporate ^{14}C-ornithine and ^{3}H-leucine into TCA-precipitable material. The cells were incubated for twenty-four hours at 37°C in LP-MEM with 10% FCS with added L-^{14}C(U) ornithine (0.223 mCi/l; 247-279 mCi/mmol, New England Nuclear) and

L-[4,5]-^{3}H-leucine (2 mCi/1; 422 mCi/mol, Amersham and New England Nuclear). Each dish was harvested and solubilized as above. The number of fused cells was determined after staining with Giemsa in additional dishes that had been set up and fused simultaneously with the test dishes. Twenty hours after fusion, these dishes were fixed with 10% formaldehyde in 0.5 M NaCl for ten minutes. The cells were rinsed two times with 5% TCA/0.15 M NaCl for ten minutes each, then rinsed several times in tap water and finally twice in distilled water. The dishes were then air dried. Giemsa stain was applied to the dishes and the number of nuclei in each cell was counted. At least thirty cells were counted in each dish.

F. LINKAGE ANALYSIS

OKT activity in relatives of gyrate atrophy patients was determined with the colorimetric assay. Blood marker proteins were analysed in the laboratory of Dr. E. Lovrien , Medical Genetics, Oregon Health Sciences University. Statistical techniques were applied to data on inheritance of the mutant OKT allele in relation to each blood marker. Morton's tables (62) were used to derive the relative likelihood that the observed inheritance pattern would occur assuming a particular map distance vs. the likelihood in the absence of linkage.

III. RESULTS

A. OKT ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH GYRATE ATROPHY AND CONTROLS. COMPARISON AND EVALUATION OF METHODS.

OKT activity in fibroblasts from patients with gyrate atrophy and controls was assayed using three different methods; the first two, the colorimetric and radiochemical assays, measure OKT activity in tissue homogenates. Method three is an indirect measure of OKT activity in growing fibroblasts in culture and depends upon the conversion of ^{14}C or ^{3}H ornithine into proline or glutamic acid, which are incorporated into protein. The advantages of the colorimetric assay are its simplicity, no radiochemicals are required and the parameters have been extensively worked out by several groups. The second assay is more sensitive than the colorimetric assay and therefore is more suitable for certain studies. The advantages of the indirect method include the measurement of OKT activity in live cells, which may better reflect the environment in vivo, and the requirement for less cells than the first two assays.

To determine the limits of the three assays, the parameters of substrate concentrations, linearity over several protein concentrations and linearity over time were studied. Previous data from this lab have been obtained with the colorimetric assay using

substrate concentrations of 30 mM ornithine and 5 mM α-ketoglutarate. However, Wong et al. (69) found that 5 mM α-ketoglutarate is inhibitory in the radiochemical assay and therefore used substrate concentrations of 2 mM α-ketoglutarate and 20 mM ornithine. However, these concentrations are not optimal for the colorimetric assay as determined in this lab (data not shown). Therefore these two conditions (30 mM ornithine and 5 mM α-ketoglutarate vs 20 mM ornithine and 2 mM α-ketoglutarate) were compared using the radiochemical assay on the same sample over several protein concentrations (Figure 3). In the radiochemical assay there was little difference between the two conditions. In order to be consistent with previous work done in this lab, 30 mM ornithine and 5 mM α-ketoglutarate were used in the following experiments, unless otherwise indicated. In addition, the second assay was linear in control cells up to a protein concentration of 1.5 mg/ml and in patient cells at least to a protein concentration of 5.7 mg/ml. However, since the radiochemical assay gave 45-77% higher activities than the colorimetric assay done with the same sample on the same day (data not shown), it may be a better method for measuring total OKT activity.

The linearity of the radiochemical assay with time was determined by measuring OKT activity of control fibroblasts over a period of two hours as shown in Figure 4. This assay was linear for at least two hours while previous data have shown that the colorimetric assay was linear for only one hour. Thus, the radiochemical assay has several advantages in comparison to the colorimetric assay, including higher activity with the same protein concentrations, linearity with time for

Figure 3. Determination of linearity of OKT activity with protein concentration with the radiochemical assay and comparison of substrate concentrations. The control fibroblast extract was incubated for one hour and blanks with no ornithine were used. Patient cells were assayed for two hours and boiled blanks were used. Each point is the average of triplicate determinations.

◊ - control; 30 mM ornithine, 5 mM α-ketoglutarate;
correlation coefficient = 0.995

♦ - control; 20 mM ornithine, 2 mM α-ketoglutarate;
correlation coefficient = 0.997

□ - patient 6; 30 mM ornithine, 5 mM α-ketoglutarate;
correlation coefficient = 0.999

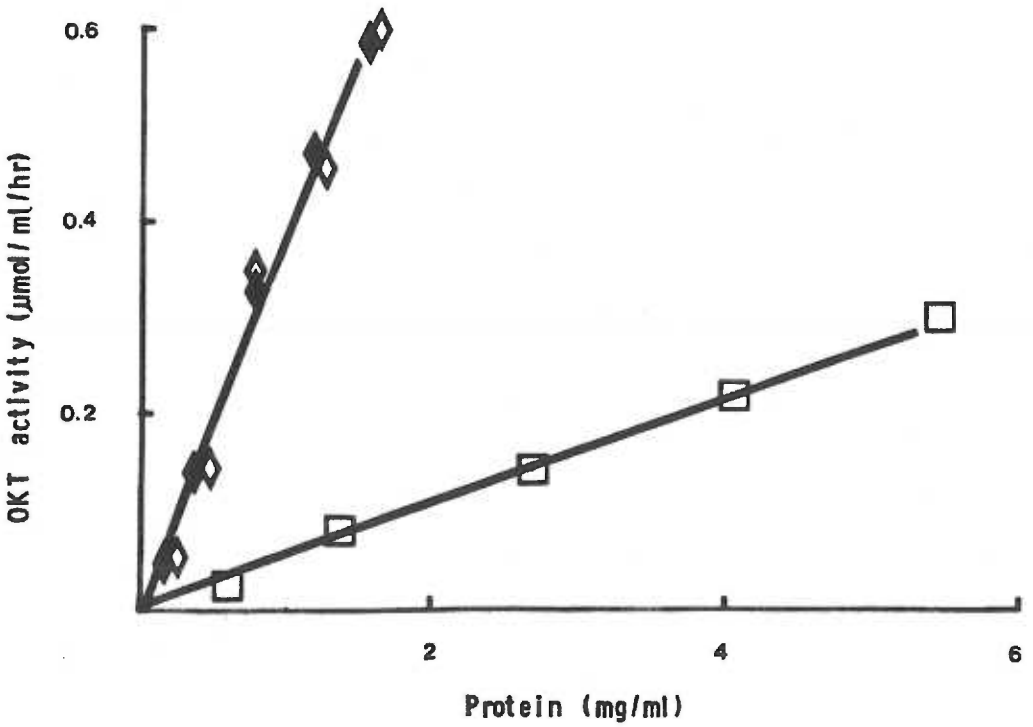
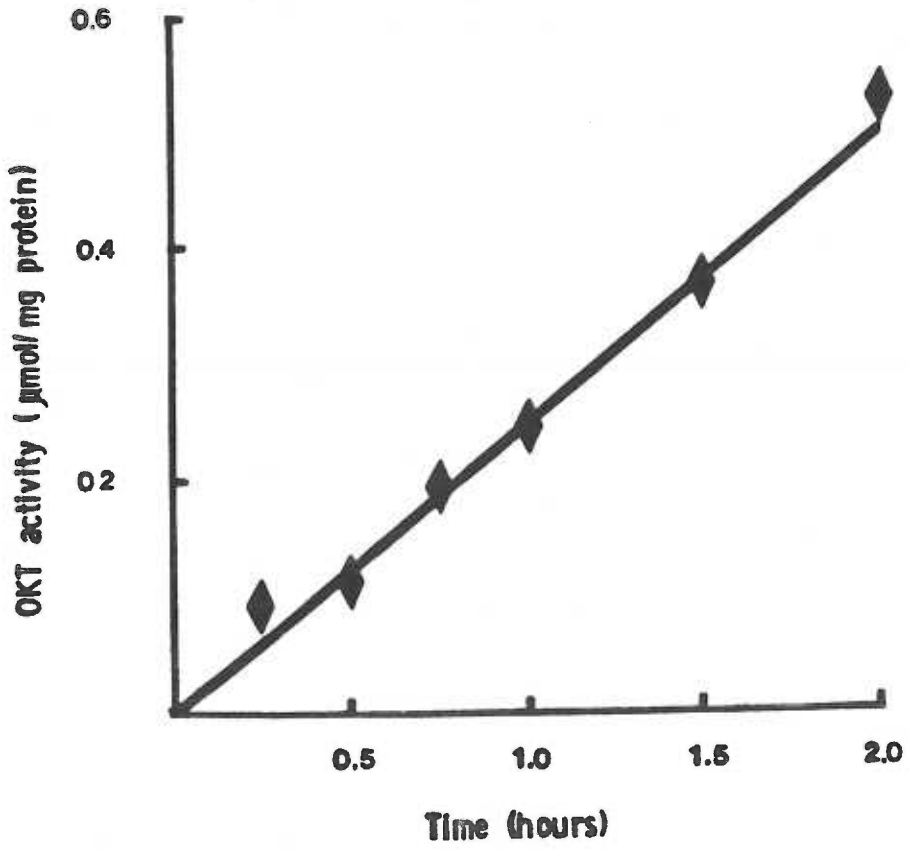

0.6
0.4
0.2
OKT activity (μmol/ml/hr)
2
4
6
Protein (mg/ml)

Figure 4. Determination of linearity with time in the radiochemical assay in control fibroblast extract. Each point is the average of triplicate determinations. The correlation coefficient is 0.996.


0.6
0.4
0.2
OKT activity (µmol/ mg protein)
0.5
1.0
1.5
2.0
Time (hours)

two hours vs. only one hour and higher sensitivity. Because of the properties outlined above, the radiochemical assay allows the measurement of low activities in patient cells that could not be easily measured with the colorimetric assay.

In order to determine conditions to be used in homogenates from patient cells in further experiments, the pH optimum was determined and the effect of increasing ornithine concentrations was examined in patient and control fibroblasts. As shown in Figure 5, control OKT has a broad pH optimum with a peak between 7.8 and 8.2. The responders also had a broad pH profile resembling that of the controls. From this data the appropriate pH appears to be 7.8 for both patients and controls and is the pH used in all following experiments unless otherwise indicated. To determine if responsive patient and control cells had similar OKT affinities for ornithine, fibroblast extracts were assayed with ornithine concentrations ranging from 10 to 500 mM. As shown in Figure 6, the optimal ornithine concentration was 30 mM for controls with the enzyme activity decreasing at higher concentrations, suggesting that high levels of ornithine are inhibitory. The three responders showed a similar decline in activity at high concentrations of ornithine. From this and other data obtained in this lab the most suitable range of ornithine concentration for the OKT assay is 30 to 60 mM. Under standard assay conditions less than 5% of ornithine and 21% of α-ketoglutarate are used by control cells in this assay.

For any enzyme assay the appropriate blank must be determined in order to correct for interfering chemical or enzyme reactions. For the OKT radiochemical reaction, interference could theoretically be

Figure 5. Determination of optimum pH in the radiochemical assay for patient 1 (■), patient 2 (●), patient 3 (▲), patient 6 (□), and controls (○, ◊). Fibroblast extracts were incubated for two hours with 2 mM PLP for the patients and 0.045 mM PLP for the controls. Each point is the average of three determinations.

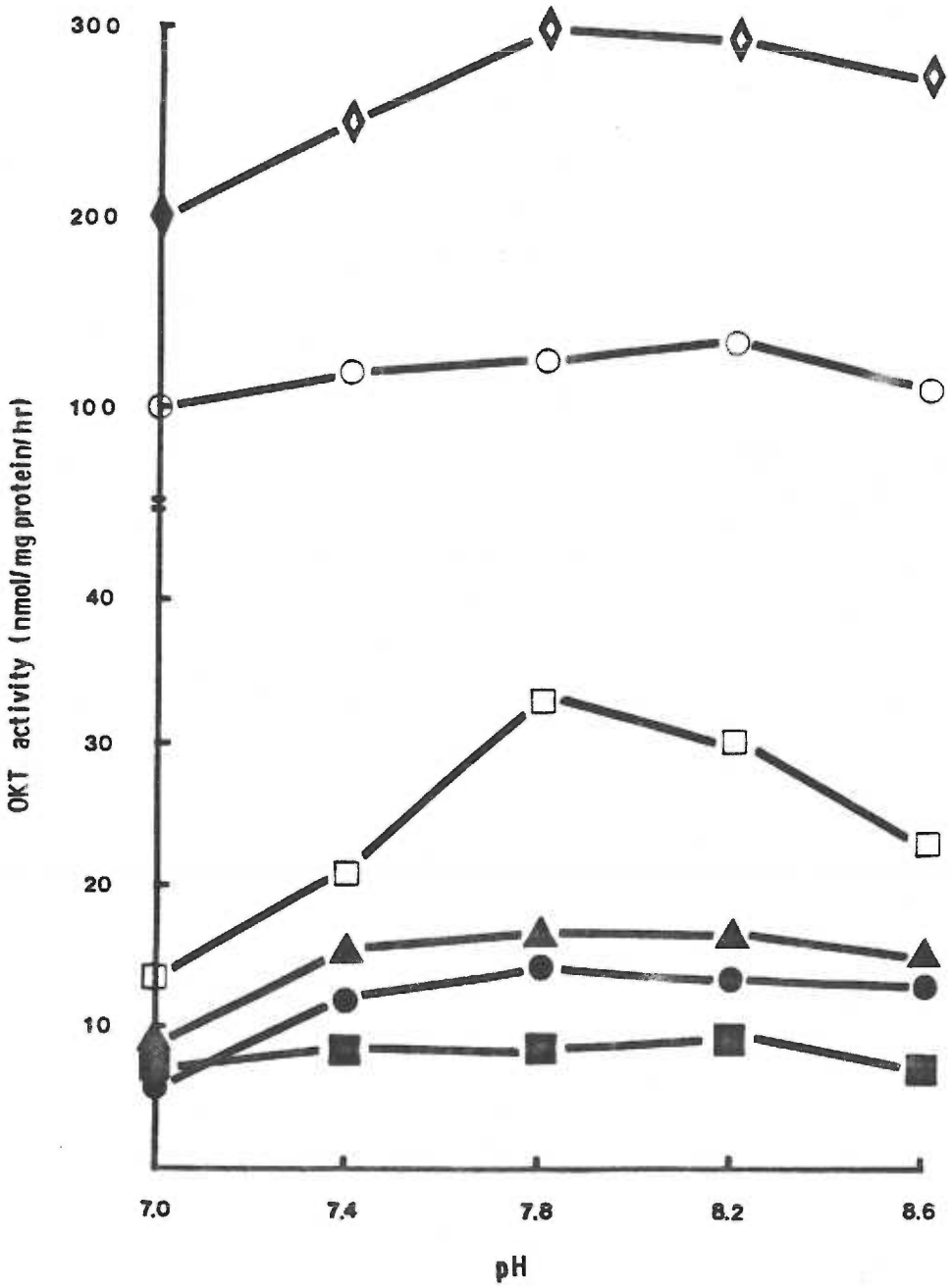
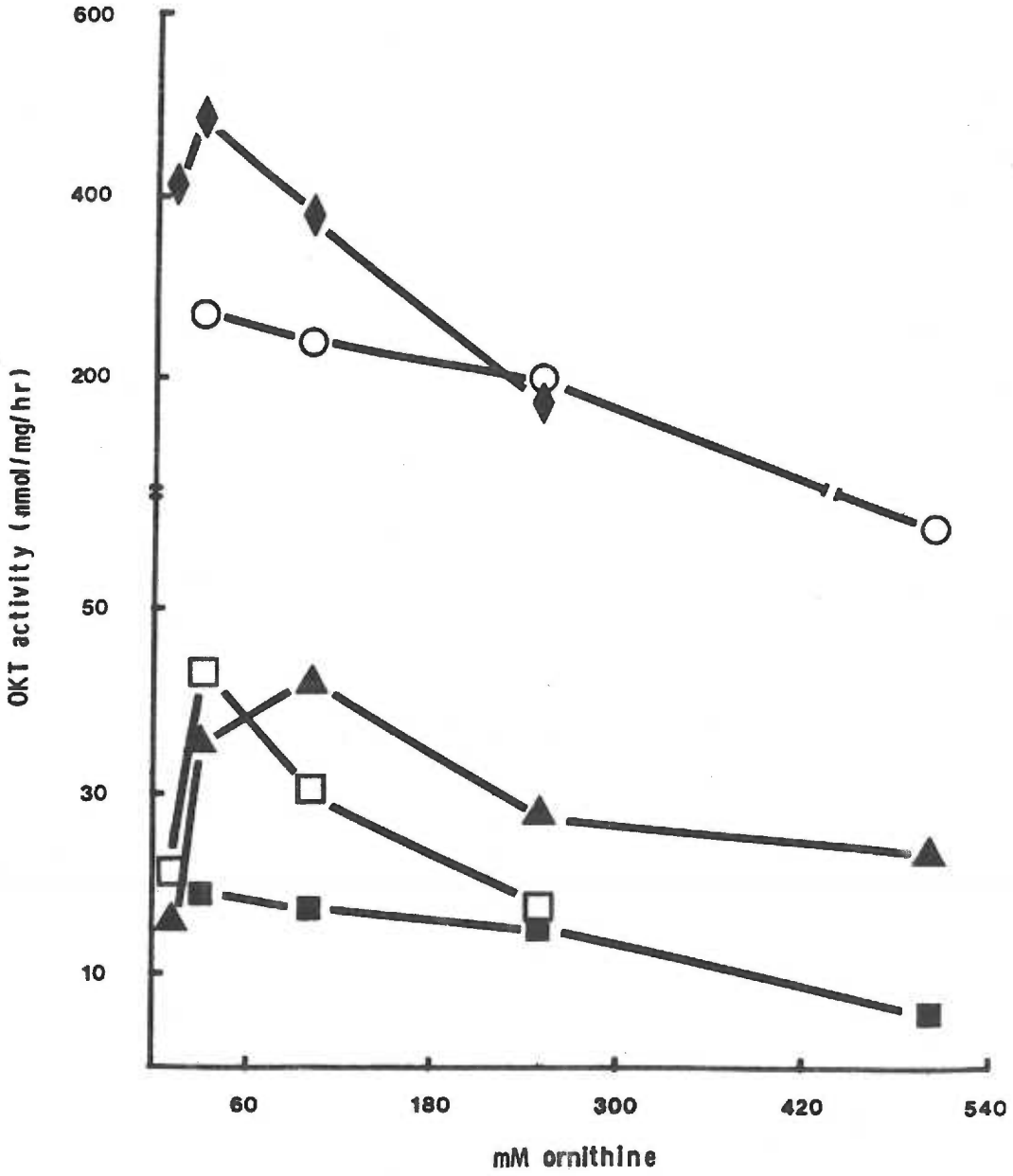

300
200
100
40
30
20
10
OKT activity (nmol/mg protein/hr)
7.0
7.4
7.8
8.2
8.6
pH

Figure 6. Determination of optimum ornithine concentrations for assaying patient and control fibroblasts with the radiochemical assay. Fibroblast extracts from patient 1 (■), patient 3 (▲), patient 6 (□), and controls (○, ♦) were incubated for two hours at 37°C with 2 mM PLP for the patients and 0.045 mM PLP for the controls. Each point represents triplicate determinations. Control (○) and patient 1 were assayed with 31.5 mM potassium phosphate pH 7.6, control (♦) and patient 3 were assayed with 81.5 mM potassium phosphate pH 7.6 and patient 6 was assayed with 81.5 mM potassium phosphate pH 7.8.


600
400
200
50
30
10
OKT activity (nmol/mg/hr)
60
180
300
420
540
mM ornithine

due to three factors: 1) other enzymatic transamination reactions, 2) glutamate dehydrogenase activity or 3) non-enzymatic decarboxylation of α-ketoglutarate. Three different blanks were compared including 1) a boiled blank, 2) addition of 0.01 mM canaline, a specific inhibitor of OKT (71), and 3) omission of ornithine from the assay mix. A boiled blank would not correct for interfering enzyme activities, since they would also be inactivated. Since samples were purified by dialysis or by a Sephadex column, the interference of other transaminases, which require other amino acids, or glutamate dehydrogenase, which requires NADH, should be minimal. A blank containing the appropriate concentration of canaline would inhibit only OKT and thus account for all three interferences described above. A blank in which ornithine is omitted should also correct for the three interfering reactions. When the three blanks were tried with several concentrations of control cell homogenate, similar activities were obtained. Two concentrations of canaline were also tried. Canaline is an analogue of ornithine and acts as an irreversible inhibitor of OKT. It will also inhibit other transaminases but a much higher concentration is required (71). A concentration of 0.01 mM canaline gave similar results as a blank containing no ornithine while a concentration of 0.1 μM canaline gave much higher blank reactions, presumably, due to incomplete inhibition of OKT activity. However, when the boiled blank was compared to the blank lacking ornithine using patient cells containing nine times more protein than the above control, boiled blanks resulted in consistently higher specific activity than the blanks lacking ornithine (Table 3). This is probably due to the inactivation of other enzymes in the boiled blank that would convert α-[1-^{14}C]- ketoglutarate to [1-^{14}C]-glutamate and

Table 3. Comparison of OKT activity in fibroblasts from patient 6 using boiled blanks and blanks with no added ornithine.

	OKT activity nmol/ml/hr	
Dilution	Boiled Blank	No ornithine added
None	302±35	248±4
1/2	147± 8	120±7

The mean and standard deviation are derived from three determinations.

implies that small amounts of substrates are still present in the sample. Thus, blanks containing 0.01 mM canaline or lacking ornithine are the most appropriate blanks to use.

The parameters of the third assay in regard to cell density, time and medium ornithine concentration were also studied. The effect of cell density was assessed to determine if growing or confluent cells had higher activity and/or less variability in activity. In addition, the activity at several pyridoxine concentrations in responsive patients was compared in confluent and growing cells to determine which condition best demonstrates the response to pyridoxine (Table 4). Less variability in activity and higher total activities were present in the confluent cells. Thus, a greater difference in activity could be discerned between the low and high pyridoxine levels in the confluent cells than in the growing cells. Therefore confluent cells were used in all subsequent experiments.

The linearity of the indirect method is illustrated in the results of two experiments using control and patient fibroblasts, respectively. The control fibroblasts were grown for seven days in LP-MEM then LP-MEM with ^{3}H-ornithine (2 mCi/l) was added. In a separate experiment the patient cells were grown for seven days in LP-MEM with 5 µg/l pyridoxine, then MEM with ^{3}H ornithine (2 mCi/l) and 1 mg/l pyridoxine was added (Figure 7). Under these circumstances the patient data was linear with a correlation coefficient of 0.997. The control cell activity was also approximately linear up to twenty-four hours, the correlation coefficient being 0.960. Therefore, an incubation period of twenty-four hours was chosen for future experiments as it is convenient and results in measurable responder cell activity.

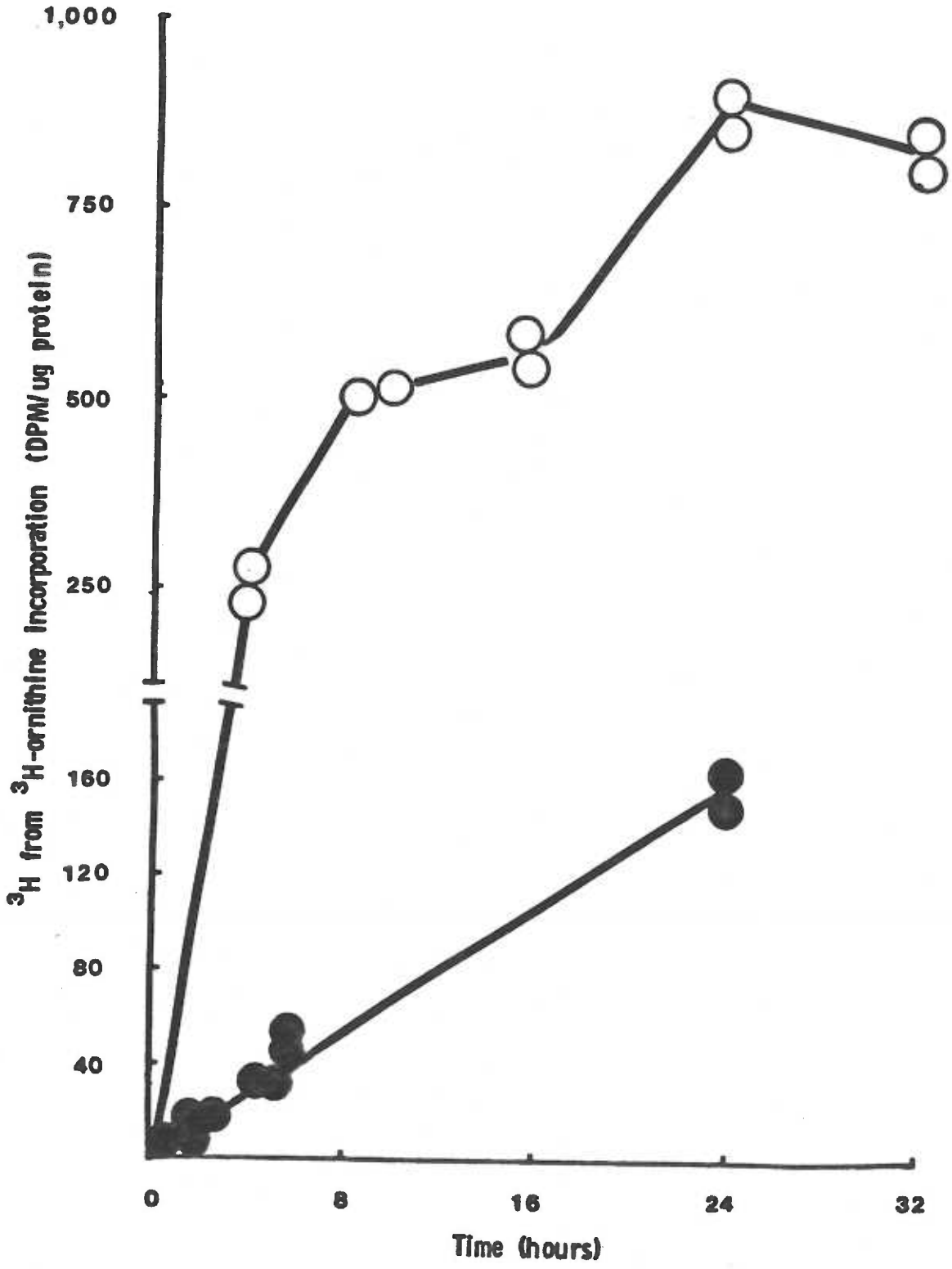
Table 4. Comparison of OKT activity in growing versus confluent fibroblasts using the indirect assay.

	Confluent		Growing	
Pyridoxine µg/l	DPM/µg protein	DPM/dish*	DPM/µg protein	DPM/dish*
		Patient 1		
2	41± 1	2340	77±16	847
5	72± 5	3456	138±20	828
10	50± 2	3250	86± 4	1118
20	51± 3	3060	75± 3	525
50	86± 2	5332	101±18	1313
100	123± 8	7380	147±23	2646
1000	123±15	8118	89±18	801
		Patient 3		
2	40± 2	2120	45±16	405
5	61± 5	4209	48± 2	528
10	45± 1	2610	39± 8	312
20	75±17	2400	73±16	146
50	105±31	3675	76	342
100	92± 5	5336	84±12	840
1000	142;152	9003;5168	107;105	867;756

The mean and standard deviation are derived from three determinations. In separate experiments, cells from patients 1 and 3 were grown for five days in LP-MEM with the specific concentration of pyridoxine as indicated. The cells were then subcultured into 35 mm petri dishes. On the next day, medium that contained ^{3}H-ornithine (2 µCi/ml) and the specific concentration of pyridoxine as indicated above was added.

* Since these data are calculated from the DPM/µg protein, the standard deviations are not given.

Figure 7. Determination of linearity with time in the indirect assay with control (○) and patient (●) cells.


1,000
750
500
250
160
120
80
40
^3H from ^3H-ornithine incorporation (DPM/ug protein)
0
8
16
24
32
Time (hours)

Control fibroblasts were assayed with medium ornithine concentrations ranging from 0.003 to 0.5 mM to determine the optimal conditions for converting radioactivity from labelled ornithine into protein (Table 5). Fibroblasts in duplicate dishes were allowed to grow for seven days in standard medium with 10% dialysed FCS. Two ml of medium containing ^{3}H-ornithine (1.8 mCi/l) with 10% dialysed FCS was added to the dishes which were incubated for twenty-four hours and then harvested as described in METHODS. Maximum activity was reached with the lowest concentration of ornithine (0.003 mM). Therefore, MEM containing 0.003 mM ornithine was used in all of the following experiments with the indirect assay.

OKT activity in patients and controls has been measured with the radiochemical and the indirect method. By either method, OKT activity is severely deficient in patients compared to controls as shown in Table 6. With the radiochemical assay the patients possess 0 to 14% of mean control activity, while with the indirect method the patients have 6 to 57% of mean control activity. The three nonresponder patients have considerably lower activity than the responders by either method. The responders have 1.5 to 2.5 times more activity than the nonresponders with the radiochemical assay and three to nine times more activity with the indirect assay. Both assays are of value in studying patient enzyme properties under different conditions as will be outlined below.

B. MECHANISM OF RESPONSE TO VITAMIN B6 IN VITRO

Four approaches were used to study the mechanism of response to vitamin B6 in vitro in the responsive patients' fibroblasts. These included determination of 1) the effect of medium pyridoxine

Table 5. Determination of optimum medium ornithine concentration for ^{3}H from ^{3}H-ornithine incorporation into protein in control fibroblasts.

Ornithine mM	CPM/μg protein
0.003	99.6; 100.8
0.010	83.5; 96.7
0.100	63.1; 66.5
0.500	30.8; 28.7

Table 6. Comparison of patient and control OKT activity with the radiochemical and indirect methods.

	Radiochemical*	Indirect+
Cell Line	nmol/mg protein/hr	DPM/μg protein
Responder		
1	14± 5	248±61
2	18± 4	274±47
3	21± 8	199±25
6	48±12	547±62
Nonresponder		
4	3±0.6	62±22
5	ND	67±20
7	1±0.7	69±29
Controls		
1	152±14	966±57
2	215±149	
3	385±46	

* Responder and control activities represent the mean and standard deviation of at least three separate experiments. The nonresponder values are each from one experiment with triplicate determinations. Substrate concentrations were 30 mM ornithine, 5 mM α-ketoglutarate, and 2 mM PLP except for the controls in which 0.045 mM PLP was used. ND = not detectable. (The mean blank value was less than two standard deviations below the mean of the samples.)

+ Fibroblasts were grown in LP-MEM for seven days, then in MEM with 1 mg/l pyridoxine and 10% dialysed FCS for three days before assaying.

concentration on OKT activity, 2) OKT apoenzyme and holoenzyme activity in fibroblasts grown in low-pyridoxine vs. standard medium, 3) K_m for PLP and 4) heat stability in the presence and absence of PLP. These studies together may indicate whether the patients have an unstable enzyme, which is stabilized by the cofactor, or a lowered affinity for PLP or both.

1). The Effect of Medium Pyridoxine Concentration on OKT Activity.

The following three studies measure OKT activity with the indirect assay. These studies were designed to characterize the patient and control response to pyridoxine in culture. In order to be able to define the response, the cells must first be depleted of vitamin B6. Standard medium contains 500 times the minimum amount of pyridoxal required for viability of mouse fibroblasts (65). Human fibroblast requirements for pyridoxal may be different from mouse fibroblasts; however, an excess of 500 times for mouse cells probably also represents a large excess for human cells. First, the length of time necessary to deplete the cells of pyridoxal by growing in LP-MEM had to be established. Second, the length of time necessary to restore OKT activity in the depleted cells after adding pyridoxine was identified. Finally, the effect of different medium concentrations of pyridoxine on OKT activity in patient and control cells was determined. For these experiments LP-MEM containing 10% dialysed FCS was used unless otherwise indicated.

a). Length of time necessary to deplete fibroblasts of pyridoxine.

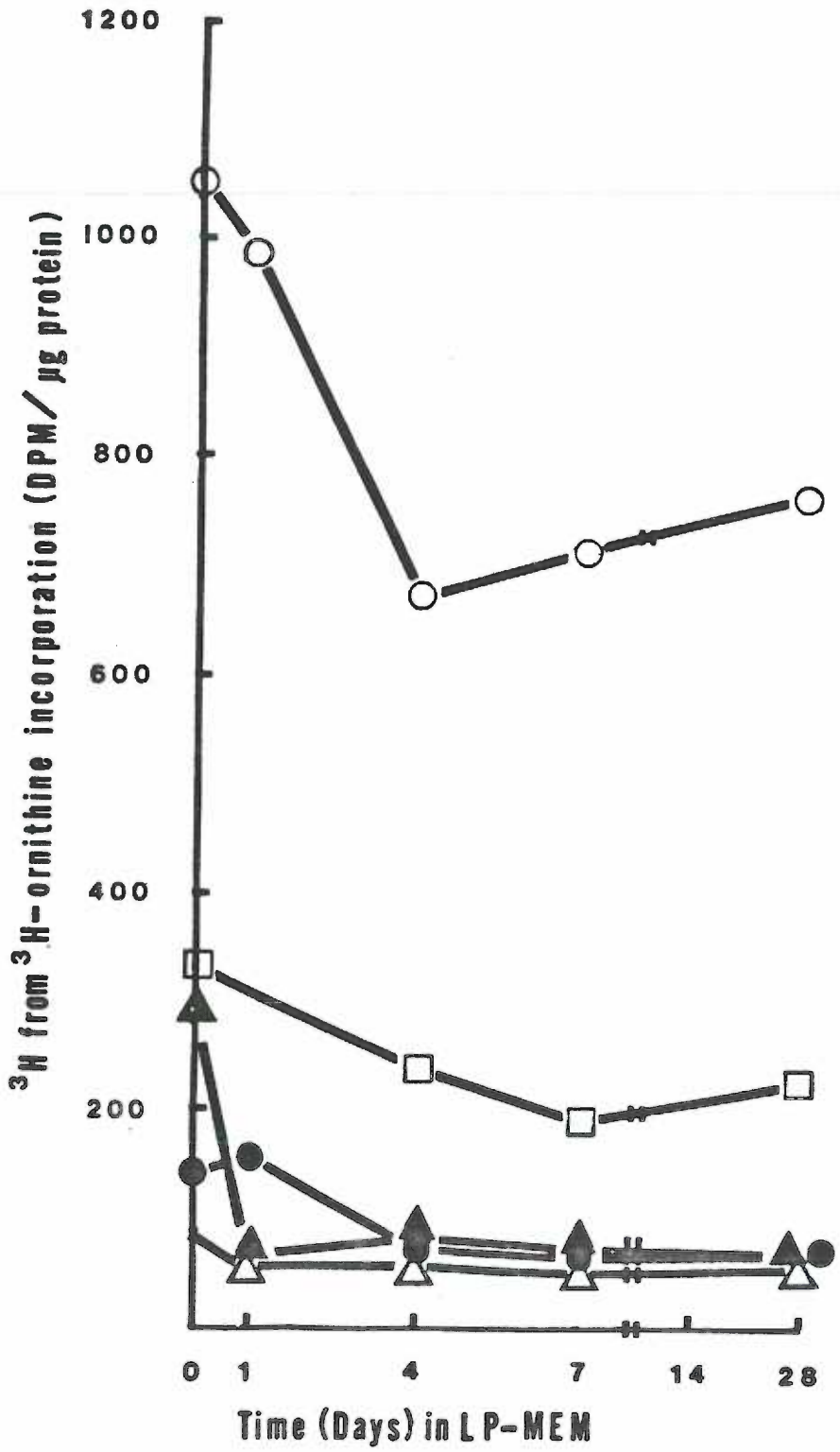
To determine how long cells must grow in LP-MEM before OKT

reaches minimum activity, fibroblasts from four patients and one control were grown in LP-MEM for varying times up to twenty-eight days and then assayed with the indirect method (Figure 8). All cell lines reached the lowest activity by the seventh day. Growing the patient cells for twenty-eight days and the control cells for fourteen days resulted in no further loss in activity. In subsequent studies with pyridoxine-depleted cells, all cell lines were grown in LP-MEM for at least one week before adding pyridoxine to the medium. The results in Figure 8 also show that after growing for seven or twenty-eight days in LP-MEM, patient 4, the nonresponder, had the lowest activity, the responders had only slightly higher activity (1/3 more) while patient 6 had three to four times as much residual activity as the other patients.

b). Time required to regain maximum OKT activity in cells transferred from low pyridoxine medium to high pyridoxine medium.

In order to study the response of patient cells to pyridoxine, the length of time required to regain maximum OKT activity in pyridoxine-depleted cells transferred to HP-MEM was determined. Patient and control fibroblasts were grown for seven to fifteen days in LP-MEM. A dish from each patient was then grown in HP-MEM for 0, 0.5, 1, 2, 4 or 7 days and then assayed for OKT activity by incubating in HP-MEM containing ^{3}H-ornithine for twenty-four hours. An additional dish was kept in LP-MEM and assayed with LP-MEM containing ^{3}H-ornithine. The radioactive LP-MEM or HP-MEM was added on the same day to all of the dishes, the initial transfer of cells from LP-MEM to HP-MEM having been timed so that all assays could be performed simultaneously. All cells attained maximum activity by the third day

Figure 8. Rate of loss of OKT activity (indirect assay) in patient and control fibroblasts after transfer from standard medium to LP-MEM. Each point represents the mean of duplicate determinations for patient 2 (●), patient 3 (▲), patient 4 (△), patient 6 (□), and control (○).


1200
1000
800
600
400
200
^3H from ^3H-ornithine incorporation (DPM/ µg protein)
0
1
4
7
14
28
Time (Days) in L P-MEM

in HP-MEM (Figure 9). All of the responders, except patient 1, were assayed simultaneously with a control. This experiment was repeated on three further occasions except that patient 1 was omitted from two of the experiments. Analysis of combined data (paired t-test, one-tailed) indicated that the increase in activity from cells grown in HP-MEM for three days was significant for patients 2 and 3 ($p<0.025$ and 0.01, respectively), but not for the control or any of the nonresponders. In patient 6 the difference showed significance between day 1 and 8 ($p<0.050$). After three days, activity in most of the cell lines decreased slightly, although activity assayed at either the fifth or eighth day was not statistically different from the third day in any of the patients or the control.

c). Effect of medium concentrations of pyridoxine on patient and control OKT activity in situ.

Preliminary experiments were done to work out the conditions for studying the in vitro response of patient cells to different concentrations of pyridoxine. A profile of responder, nonresponder and control activity was obtained over a range of 2 to 1,000 µg/l pyridoxine in MEM with 10% dialysed FCS as shown in Figure 10. Fibroblasts were grown in LP-MEM containing 2, 5, 10, 20, 50, 100 and 1000 µg/l of pyridoxine for six days except for patient 6 and control 3, which were grown in medium with 10 mg/l of pyridoxine for four days followed by 10, 100 or 1000 ug/l pyridoxine for two days. Medium containing ^{3}H ornithine (2 mCi/l) was then added, as described in METHODS. Patients 2 and 5 and control 2 were assayed in one experiment while patient 1 and control 1 were assayed in a second experiment. All of the responders had increasing activity

Figure 9. Time required to regain maximum OKT activity (indirect assay) in cells from patients and control after growing for seven to fifteen days in LP-MEM then in HP-MEM with 10 mg/l pyridoxine and 10% dialysed FCS for various times. Each point represents a single determination from one experiment including control (○), patient 2 (●), patient 3 (▲), patient 4 (△), patient 5 (+), patient 6 (□), and patient 7 (X). Patient 1 (■) was assayed in a separate experiment.

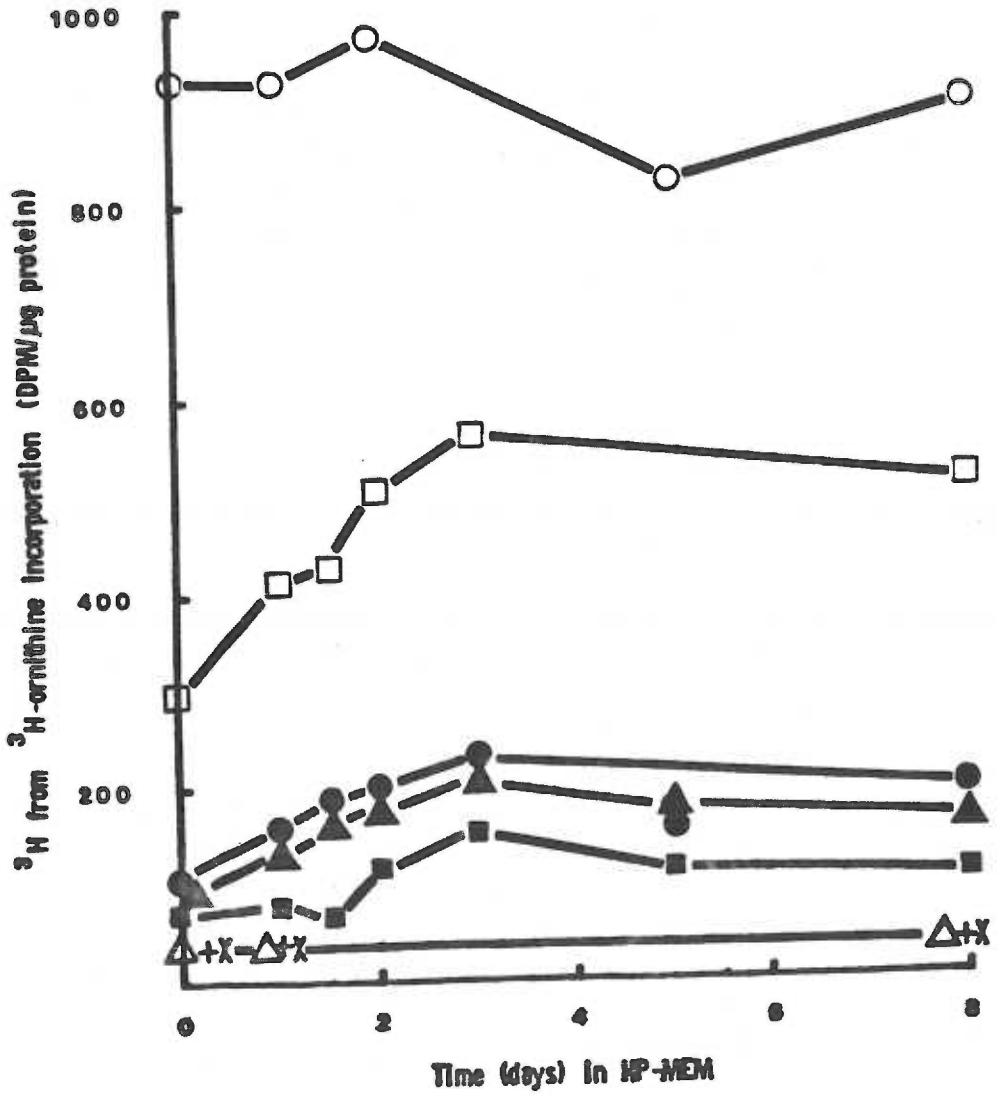

1000
800
600
400
200
^{3}H from ^{3}H-ornithine incorporation (DPM/µg protein)
0
2
4
6
8
Time (days) in HP-MEM
+X
-X

Figure 10. OKT activity (indirect assay) in control and patient cells grown in LP-MEM with 2 to 1,000 µg/l pyridoxine. Patient 1 (■), patient 2 (●), patient 3 (▲), and patient 6 (□) are responders; patient 5 (+) is a nonresponder. Controls are (○, ◊, ♦).

^{3}H from ^{3}H-ornithine incorporation (CPM/µg protein)
0
40
80
120
160
200
240
µg/l Pyridoxine added to LP-MEM
2
5
10
20
50
100
1000

corresponding to increasing pyridoxine levels, while the nonresponder and controls showed little change. The responder activities appeared to be increasing even at the highest concentration of pyridoxine tested. Therefore, further experiments were done to determine if higher activities could be attained with higher concentrations of pyridoxine. In addition, the effect of no added pyridoxine was also included. When concentrations of pyridoxine ranging from 0 to 100 mg/l were tested, maximum activity was reached between 0.1-100 mg/l pyridoxine as shown in Table 7. The difference in the responders' OKT activity between 0 and 0.01 mg/l of added pyridoxine was considerable, as seen in patients 1 and 2 ($p<0.01$), patient 3 ($p<0.1$) and patient 6 ($p<0.050$)(paired t-test; one-tailed).

In the above experiments, cells from B6 responsive patients retained approximately 50% of maximum activity even when no pyridoxine was added to the medium. This activity presumably depends upon the residual vitamin B6 contributed by the dialysed FCS. In order to better define the response of the patients' cells to pyridoxine, it was necessary to further deplete the cells of cofactor and this was achieved by growing patient and control cells for a week in LP-MEM with 10% hydroxylamine-treated FCS instead of dialysed FCS. The cells were then transferred into 35 mm petri dishes containing LP-MEM with 10% hydroxylamine-treated FCS, if they had been grown in this medium previously, or into dishes containing LP-MEM with 10% dialysed FCS with 0, 0.01 or 1 mg/l pyridoxine. As shown in Table 8, a large reduction in activity was observed in responder cells assayed in medium with no pyridoxine and 10% hydroxylamine-treated FCS compared to dialysed FCS. The OKT activity of the responders is also now seen

Table 7. Incorporation of radioactivity from ^{3}H-ornithine into protein in control and patient fibroblasts grown in medium with different concentrations of pyridoxine.

Cell Line	Medium Concentration of Pyridoxine (mg/l)					
	0	0.010	0.100	1.0	10.0	100
Responders			DPM/μg protein			
1	93±48	259±170	260; 218	248±61	316; 219	304; 196
2	158±12	240±24	277±39	274±47	284±73	228; 204
3	101±37	206±42	197±48	199±25	211±54	275; 126
6	310±32	413±246	596±143	547±62	667±191	740; 477
Nonresponders						
4	95±60	52; 50	65±14	62±22	68±22	60; 48
5	65±10	86; 57	66±13	67±20	66±16	53; 66
7	58±4	39; 53	53±15	69±29	60±23	35; 42
Control	829±205	941±40	954±77	966±57	952±198	992; 798

Fibroblasts were grown for five to seven days in LP-MEM; then subcultured into 35 mm petri dishes containing the concentration of pyridoxine as indicated. On the third day after subculture, medium containing ^{3}H-ornithine was added. The means and standard deviations represent the average of three or more separate experiments each with single determinations.

Table 8. Incorporation of radioactivity from ^{3}H-ornithine into protein with increasing medium concentrations of pyridoxine.

Serum	Hydroxylamine-treated FCS	Dialysed FCS		
Pyridoxine mg/l	0	0	0.010	1.0
	DPM/µg protein*			
Cell lines				
Responders				
1	54±20	70± 9	188±69	233±62
2	31±11	101±38	179±44	268±34
3	74±29	87±34	224±66	237±33
6	76± 9	227±46	415±109	482±91
Nonresponders				
4	54;54	58± 6	55;48	66±17
5	37;27	52±20	50;29	56±24
7	49;55	49± 9	46;38	57±16
Control	638±66	855±158	779±154	893±134

* The mean and standard deviation were obtained from at least three separate experiments.

to increase dramatically from LP-MEM with 10% hydroxylamine-treated FCS to LP-MEM with 1 mg/l pyridoxine as seen in patient 1 ($p<0.05$), patient 2 ($p<0.005$), patient 3 ($p<0.0005$) and patient 6 ($p<0.025$) (paired t-tests; one-tailed). The nonresponders showed no decrease in activity in medium with hydroxylamine-treated FCS as compared to medium with dialyzed FCS, while the control did show a small but insignificant decrease in activity.

From Table 7 a difference in activity between patient 6 and the other responders can also be observed. The higher activity in patient 6 compared to the other three responders is significant at all concentrations of pyridoxine tested - 0, 0.01, 1.0, and 100 mg/l ($p<0.001$) and 10 mg/l ($p<0.05$), (paired t-test, one-tailed) suggesting that patient 6 falls into a second class of responders with higher residual OKT activity. Patient 3 also had higher activities than patients 1 and 2 with significant differences at 0, 0.1 and 10 mg/l pyridoxine ($p<0.1$, $p<0.01$ and $p<0.05$, respectively; paired t-test, one-tailed).

2. Comparison of OKT Apoenzyme and Holoenzyme Activity in Patient and Control Fibroblasts Grown in Low Pyridoxine Medium and Standard Medium.

In order to clarify the mechanism underlying the increased incorporation of ^{3}H from ^{3}H ornithine into protein in cells grown in medium with increasing concentrations of pyridoxine, patient and control cells were assayed for OKT apoenzyme and holoenzyme activity after being grown for two passages in LP-MEM or standard MEM. Holoenzyme refers to the enzyme containing the bound cofactor PLP, and was determined by the radiochemical assay as described in METHODS, except that PLP was omitted. Total activity was measured by the

standard method including 2 mM PLP for patients and 0.04 mM PLP for controls. Two mM PLP results in optimal patient OKT activity but is inhibitory to control OKT activity. Therefore, 0.04 mM PLP was used for assaying control activity. Apoenzyme activity (enzyme free of PLP) was determined by subtracting the holoenzyme activity from total activity. These results are shown in Table 9. Both controls had higher total and holoenzyme activities in regular MEM. Except for patient 2, the patients' total activities were only measurable when the cells were grown in regular medium. The control holoenzyme activity ranged from 50 to 75% of total enzyme activity regardless of whether the cells were grown in standard MEM or LP-MEM. Holoenzyme activity was undetectable in cells from all patients grown in LP-MEM or standard MEM.

3. Kinetic Studies of Patient and Control OKT Affinity for Pyridoxal 5'-phosphate.

To obtain an accurate K_m for PLP, all of the OKT holoenzyme, ideally, should first be converted to apoenzyme. A previously studied method for removing bound PLP from the enzyme utilized hydroxylamine in the dialysis buffer and resulted in an apoenzyme content of 39%. In the present study, four additional methods were compared using control fibroblast homogenates. These included 1) dialysing sample overnight against dialysis buffer containing no PLP, 2) dialysing the sample against dialysis buffer containing 30 mM ornithine and no PLP, 3) growing the cells in LP-MEM for two passages prior to harvesting and 4) passing the sample through a Sephadex G-50 column. After dialysing the sample with no PLP, 58% of the OKT was in the apoenzyme form (Table 10). However, when homogenate of patient cells was

Table 9. Comparison of total enzyme activity vs. holoenzyme activity in patient and control fibroblasts grown in LP-MEM or standard MEM.

	OKT activity[+]			
Cell Line	LP-MEM		Standard MEM	
Patient	Total	Holo-enzyme	Total	Holo-enzyme
	nmol/mg protein/hr		nmol/mg protein/hr	
1	ND	ND	15*	ND
2	5	ND	21	ND
3	ND	ND	20	ND
6	ND	ND	34	ND
Controls				
1	239	114	287	147
2	157	131	213	158
2	157	104	305	185

[+] Cells were grown for two passages in LP-MEM with 10% hydroxylamine-treated FCS or in regular MEM with 10% FCS. The extracts were passed through a G-25 Sephadex column before assaying. Each point represents the mean of three determinations except * which indicates the mean of two determinations. ND - not detectable. (The mean blank value was less than two standard deviations below the mean of the samples.)

Table 10. Comparison of methods to convert OKT holoenzyme to apoenzyme in control fibroblasts.

Method	OKT activty nmol/mg protein/hr		
	+ PLP	- PLP	% apoenzyme activity
Dialysis with no ornithine or PLP	251	106	58
Dialysis with 30 mM ornithine*	255	29	89
Sephadex G-50 column	150	49	67
Prior growth of cells in LP-MEM	239	114	52

* canaline blanks were used

dialysed in this manner, 79% of the activity was lost (data not shown) Furthermore, dialysing the control sample against 30 mM ornithine resulted in a much more dramatic removal of bound PLP with 89% of the enzyme in the apoenzyme form. This method has one disadvantage in that the assay then requires a different blank, since blanks with no ornithine are routinely used. As discussed earlier, boiled blanks are not appropriate since they do not account for enzyme activity other than OKT that may add to the reaction. Canaline, a specific inhibitor of OKT may be used. The effect of canaline on patient enzyme is unknown and each patient may react differently with the inhibitor. Theoretically, inhibitors are less desirable to use in blanks since their effectiveness is unknown. The third method, growing the cells in LP-MEM, resulted in an apoenzyme content of 52%. This method was time-consuming and gave the poorest results. Passage through a Sephadex G-50 column resulted in 67% of the OKT being in the apoenzyme form. Thus, this method appears to be the most acceptable one.

In order to determine the length of time needed for maximum binding of the cofactor by the apoenzyme, control homogenate was incubated at 37°C with 0.063 mM PLP for 0 to 60 minutes prior to assay of OKT activity. As shown in Table 11, there was no difference in activity at any of the time periods suggesting that the binding is very rapid. In all of the following PLP kinetic experiments the fibroblast homogenates were passed through a 10 ml Sephadex G-50 column and a preincubation period with PLP of at least fifteen minutes was used.

In order to determine the range of concentrations of coenzyme appropriate for kinetic studies, control and patient OKT activity was

Table 11. Determination of the length of time necessary to restore holoenzyme activity in control homogenate that has been dialyzed overnight against buffer containing no pyridoxal 5'-phosphate.

Minutes preincubated with PLP	OKT activity nmol/mg protein/hr
0	120±4.2
15	122±10.1
30	127±4.9
45	115±8.0
60	139; 129

The mean and standard deviation were obtained from three determinations.

measured over a range of 0 to 2 mM PLP. No patient activity was measurable with 0 mM PLP, while the control had 17% holoenzyme activity at 0 mM PLP. As shown in a plot of velocity vs PLP concentration (Figure 11), the control reached maximum activity between 0.1 to 0.5 mM PLP with the activity appearing to be inhibited at higher concentrations. The four responsive patients reached maximum activity at 1 mM PLP. For determination of K_m values activities were determined over a range of 0.2 to 1.0 mM PLP for patients and 0.01 to 0.05 mM PLP for the control. K_m values were calculated from a Lineweaver-Burke graph (Figure 12). The control has a K_m of 0.018 mM and a V_{max} of 223 nmol/mg protein/hr (Figure 12 and Table 12). K_m values of the patients ranged from 0.421 to 2.709 mM and the V_{max} ranged from 42 to 67 nmol/mg protein/hr (Figure 13 and Table 12). Thus, all four patients clearly have a higher K_m than the control, being greater than twenty-two times the control value. The V_{max} of the patients is approximately 1/5 to 1/3 that of the control.

4. Heat Stability

One method for determining the stability of an enzyme is to follow the decline in activity after heating for various periods of time. If the enzyme requires a coenzyme, such as PLP, then the coenzyme may have a stabilizing effect on the enzyme, especially during heating. Several preliminary heat stability studies were carried out. Fibroblast extracts from control and patient 2 were heated at 50°C for varying times in the presence of 0.09 mM PLP. Total activity was retained in both the control and patient extracts. Higher temperatures were tested with control in the presence and absence of PLP. When heated at 65°C for 30 minutes, control activity

Figure 11. Effects of increasing concentrations of PLP on OKT activity in fibroblast extracts from patient 1 (■), patient 2 (●), patient 3 (▲), patient 6 (□), and controls (○, ♦). The ornithine concentration was 60 mM.

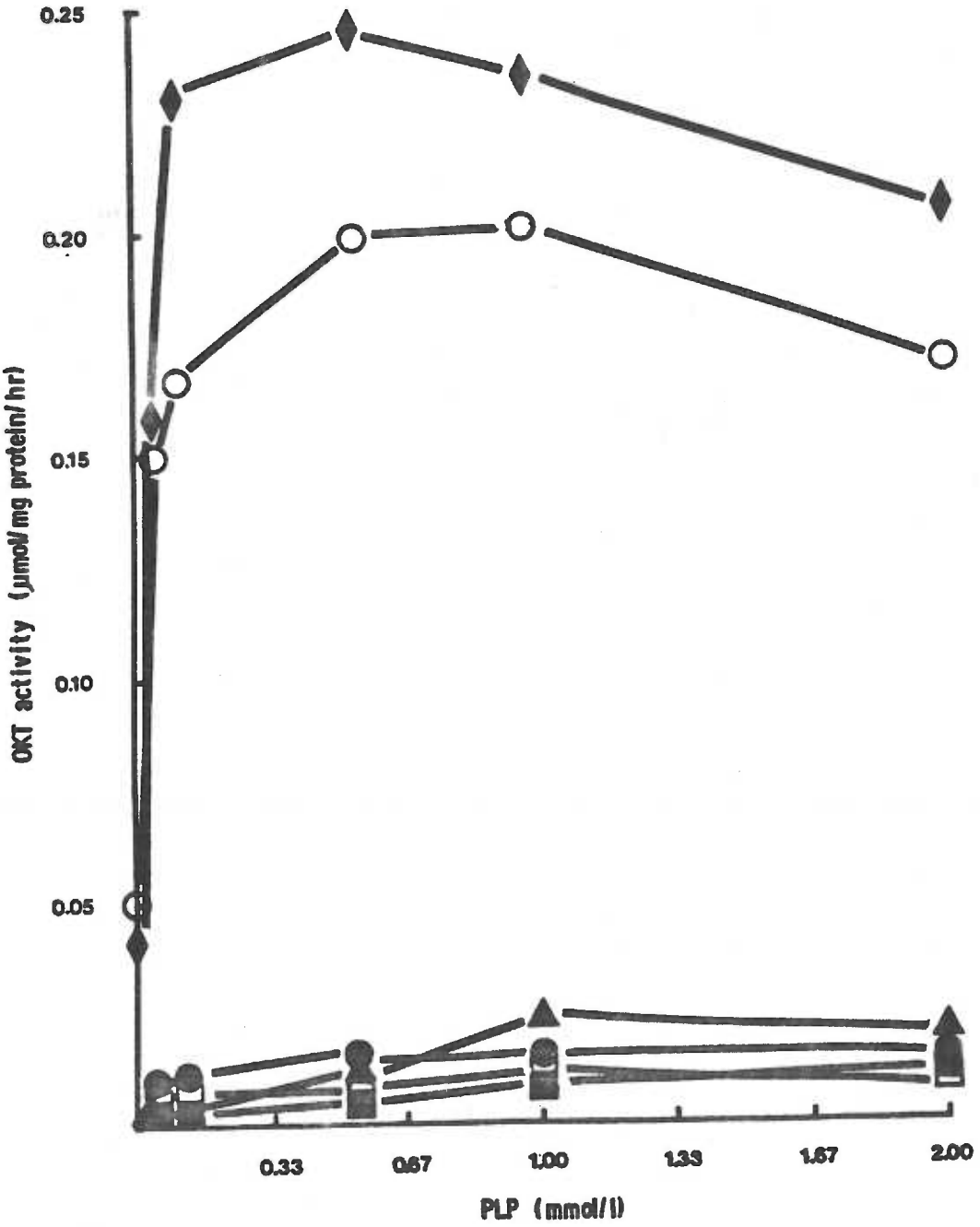

0.25
0.20
0.15
0.10
0.05
OKT activity (µmol/mg protein/hr)
0.33
0.67
1.00
1.33
1.67
2.00
PLP (mmol/l)

Figure 12. Lineweaver-Burke plot of OKT activity over increasing concentrations of PLP in fibroblast extracts from a control. The ornithine concentration was 60 mM.

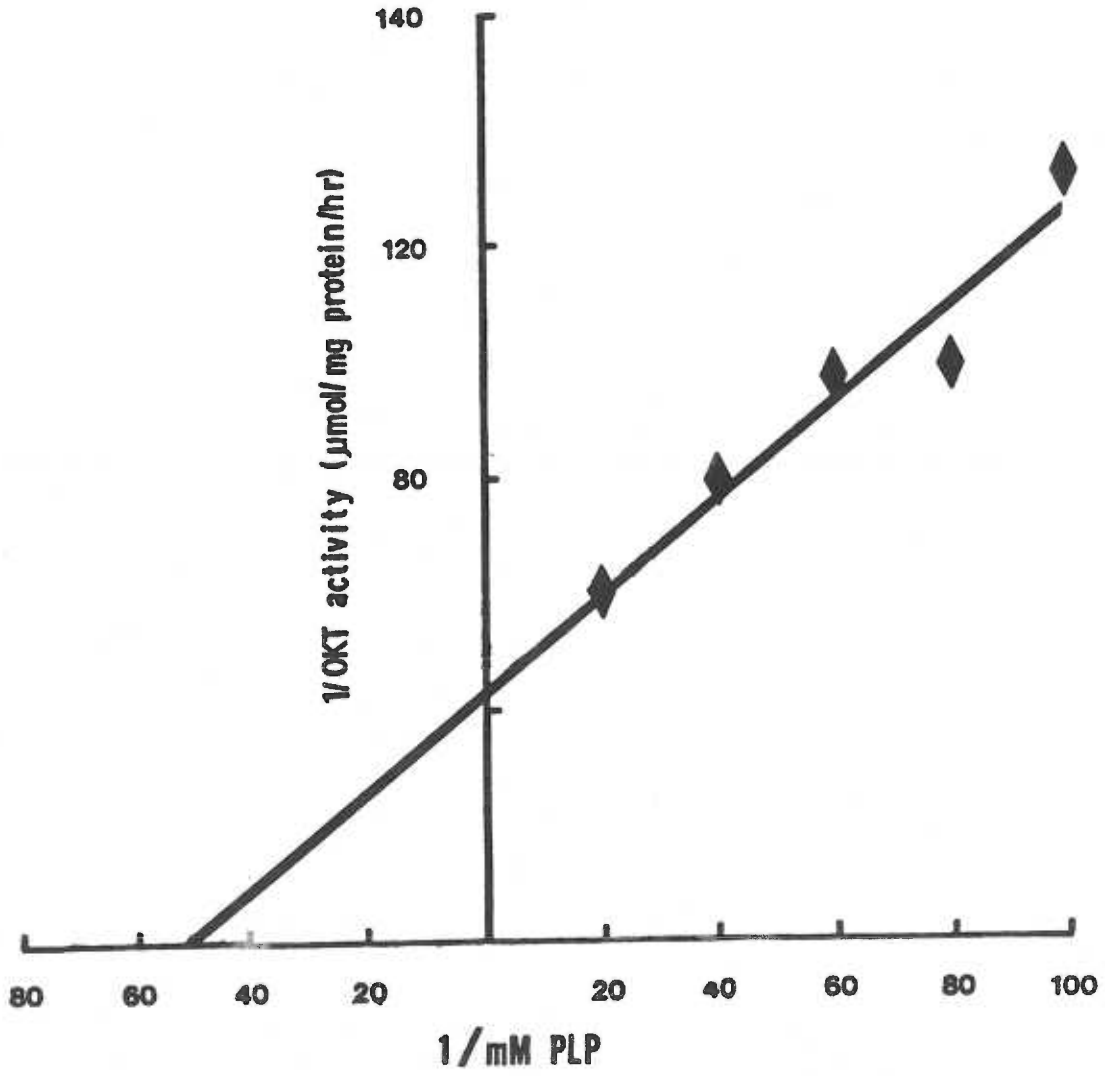
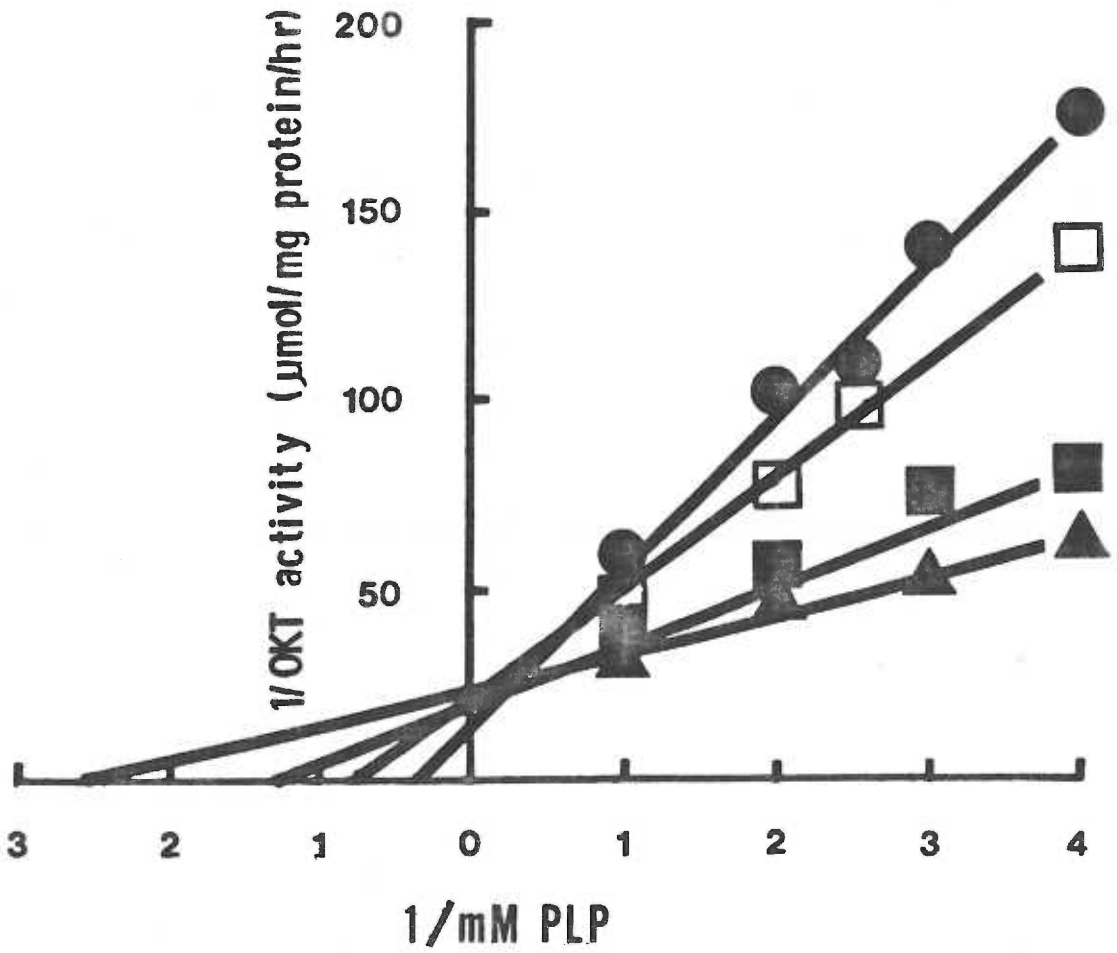

140
120
80
1/OKT activity (µmol/mg protein/hr)
80
60
40
20
20
40
60
80
100
1/mM PLP

Table 12. Affinity of patient and control ornithine ketoacid transaminase for pyridoxal 5'-phosphate.

Subject	K_m	V_{max}	Correlation Coefficient
Patients	mmol/l	nmol/mg protein/hr	
1	0.841	50	0.992
2	2.709	67	0.992
3	0.421	42	0.996
6	1.617	53	0.992
Control	0.018	223	0.979

Figure 13. Lineweaver-Burke plot of OKT activity over increasing concentrations of PLP in fibroblast extracts from patient 1 (■), patient 2 (●), patient 3 (▲), and patient 6 (□). The ornithine concentration was 60 mM.


200
150
100
50
1/OKT activity (µmol/mg protein/hr)
3
2
1
0
1
2
3
4
1/mM PLP

was retained in the presence of PLP and lost in the absence of the coenzyme. From these preliminary studies, a temperature of 45°C for the heat stability test of patient and control cells was decided upon and a time period of up to one hour with and without 1.67 mM PLP. Under these conditions control OKT retained full activity after heating for one hour with 1.67 mM PLP (Figure 14). However, less than 60% of control activity remained after heating in the absence of PLP (Figure 15). The stability of patient OKT activity resembled that of the controls (Figures 14 and 15). From the overall slopes there was no obvious difference between the stability of the control and the patient enzymes.

C. COMPLEMENTATION STUDIES

The incorporation of radioactivity from ^{14}C ornithine into protein can be used to study complementation in heterokaryons derived from the patients' fibroblasts to determine if the two classes of patients (B6 responsive and non-responsive) represent allelic or non-allelic mutations.

1. The Effect of the Fusion Process on OKT Activity in Control and Patient Fibroblasts.

The effect of the fusion process on OKT activity was examined in control and patient cells (Table 13). There appeared to be no difference between PEG-treated and untreated cells, regardless of whether the results were expressed as ^{14}C dpm/µg protein or $^{14}C/^{3}H$ ratio. Possible differences in the rate of protein synthesis are reflected by the ^{3}H-leucine incorporation into protein in the cells and therefore can be corrected by the ratio of $^{14}C/^{3}H$.

Figure 14. Thermostability of OKT activity when heated in the presence of 1.67 mM PLP. The data for patient 1 (■), patient 2 (●), patient 3 (▲), patient 6 (□), and controls (◊ ,♦) are the mean of two or more determinations. Remaining OKT activity of the heated samples (ordinate) is expressed as the per cent of OKT activity determined in paired samples not subjected to 45°C incubation. The final assay mix contained 60 mM ornithine.

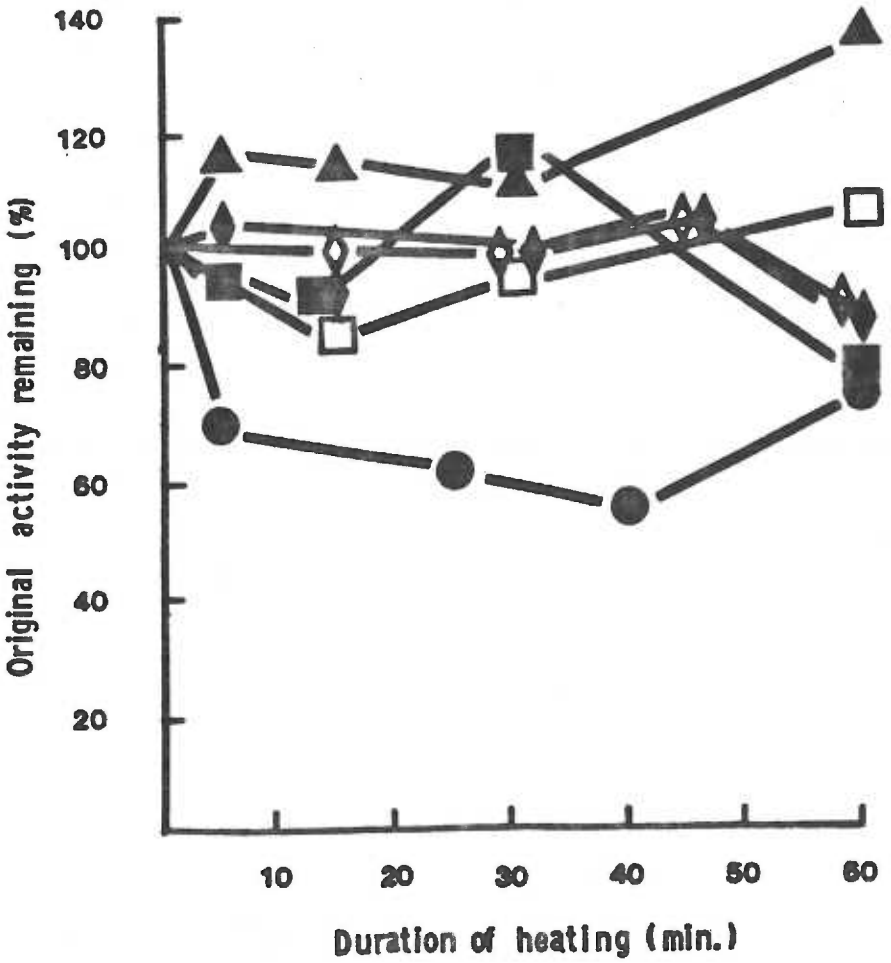

140
120
100
80
60
40
20
Original activity remaining (%)
10
20
30
40
50
60
Duration of heating (min.)

Figure 15. Thermostability of OKT activity when heated in the absence of PLP. The data for patient 1 (■), patient 2 (●), patient 3 (▲), patient 6 (□), and controls (◊ , ♦) are the mean of two or more determinations. Remaining OKT activity of the heated samples (ordinate) is expressed as the per cent of OKT activity determined in paired samples not subjected to 45°C incubation. The final assay mix contained 60 mM ornithine.

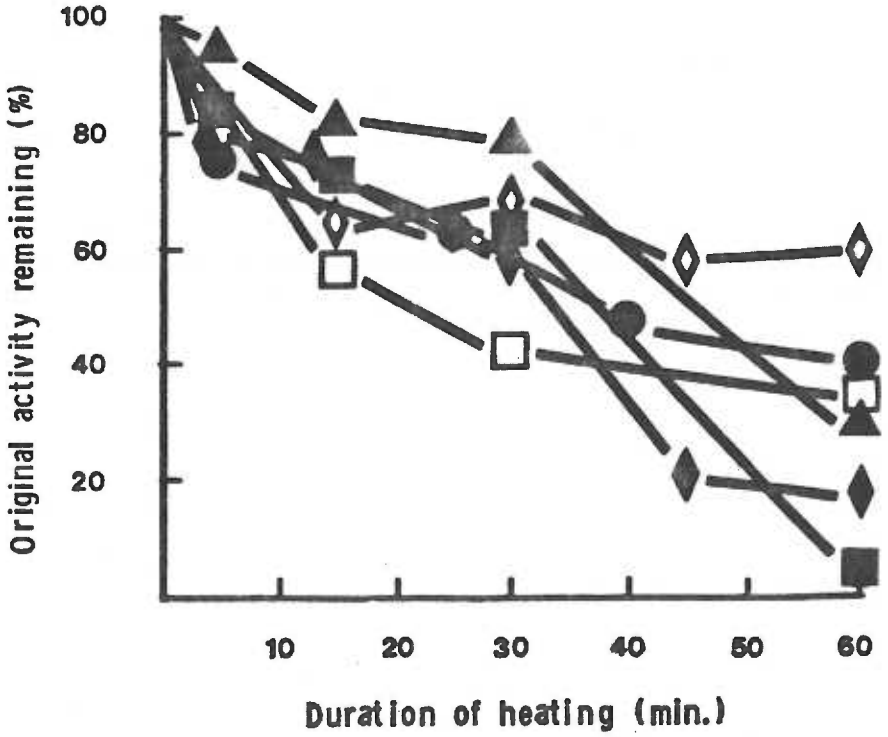

100
80
60
40
20
Original activity remaining (%)
10
20
30
40
50
60
Duration of heating (min.)

Table 13. Incorporation of radioactivity from ^{14}C-ornithine and ^{3}H-leucine into protein in fibroblasts.

Cell Line*	^{14}C DPM/μg protein	$^{14}C/^{3}H$ x 1,000	
	Nonfused	Fused	Nonfused
Patients			
Responders			
1	16	7	20
2	19	20	20
3	17	19	21
6**	67	53	72
Nonresponders			
4	1	1	1
5	1	1	1
7	1	2	2
HHH	3	4	4
Controls (n=6)	138±42	152±38	160±58

* All data obtained in one experiment except those indicated ** which were obtained in a second experiment.

2. Complementation Between Gyrate Atrophy and Hyperornithinemia, Hyperammonemia and Homocitrullinuria Fibroblasts: a Positive Control.

A positive control was used to demonstrate that complementation, if it occurred, would be detected under the experimental conditions used. HHH fibroblasts provide such a control since they also show reduced ability to incorporate ^{14}C-ornithine into protein; this is thought to result from deficiency of a specific mitochondrial transport protein for ornithine (49). Two comparisons can be made with the fused cross of HHH x gyrate atrophy cells. The first compares the fused cross with the unfused mixture of cells, not treated with PEG. The second comparison is between the fused cross and the predicted value, that is, the calculated mean of the two parental lines, each treated separately with PEG. These values are derived from Table 13. In all crosses of HHH x gyrate atrophy cells, there was a clear increase in the $^{14}C/^{3}H$ ratio in the PEG treated compared to the untreated mixture or to the predicted values (Table 14). When the data for all crosses were combined, these differences were highly significant ($p<0.001$, paired t-test). The mean $^{14}C/^{3}H$ ratio in the PEG treated mixtures was 28±14, which is 20% of the mean control value (Table 13). The expected value for positive complementation can be calculated from the data given in Appendix Ia. In this appendix, the efficiency of the fusion process is calculated from the number of cells counted with different numbers of nuclei. From this data, the overall percentage of heterokaryons is calculated to be 10.4%. Thus, the expected activity, assuming positive

Table 14. Positive complementation in gyrate atrophy x HHH fibroblasts.

	$^{14}C/^{3}H$ x 1,000		
Cross*	Fused	Nonfused	Predicted
HHH x responders			
HHH x 1**	16	9	9
HHH x 2	35	13	12
HHH x 3	33	10	12
HHH x 6**	53	35	30
HHH x nonresponders			
HHH x 4	18	3	3
HHH x 5	28	2	3
HHH x 7	15	3	3

*all data obtained in one experiment except those indicated ** which were obtained in a second experiment.

complementation in all crosses, can be calculated as follows:

Expected activity = Mean of non-fused activity
+ 0.104 x mean control activity
- 0.104 x mean of non-fused activity
= 10.7 + (0.104 x 152) - (0.104 x 10.7)
= 25.4

This is close to the mean observed value after fusion of 28.2. Thus, this method is adequate to detect positive complementation if it occurs between the various gyrate atrophy cell lines.

3. Complementation Between Gyrate Atrophy Fibroblasts.

All possible paired combinations of gyrate atrophy cell lines have been fused and examined for complementation (Table 15). There was no increase in either the ^{14}C-dpm/μg protein (data not shown) or the $^{14}C/^{3}H$ ratio in any of the fused compared to the non-fused mixtures. Unexpectedly, there were considerable decreases in several crosses suggesting a possible toxic effect of PEG in the fused mixtures. For this reason, the comparison between the fused mixtures and the predicted values from the self-fused parental lines may be more valid. These also showed no increase or only very small increases in the fused mixtures, either as ^{14}C dpm/μg protein or $^{14}C/^{3}H$ ratio, again indicating a lack of positive complementation in these cells. The calculated per cent of heterokaryons in these crosses are similar to that described for the gyrate atrophy x HHH crosses (11.9% vs. 10.4%, see Appendix Ib). Thus, positive complementation should have been observed if it had occurred.

D. LINKAGE ANALYSIS

In order to do linkage analysis with family studies, one must be

Table 15. Complementation analysis in fibroblasts from patients with gyrate atrophy.

Cross*	$^{14}C/^{3}H$ x 1,000		
	Fused	Nonfused	Predicted
Responder x nonresponder			
1 x 4**	3	7	6
1 x 5	5	12	4
1 x 7	5	13	4
2 x 4	6	13	11
2 x 5	6	7	11
2 x 7	4	8	11
3 x 4	3	8	10
3 x 5	5	5	10
3 x 7	3	6	10
6 x 4**	15	36	27
6 x 5**	17	22	27
6 x 7**	11	32	28
Responder x responder			
1 x 2	11	20	14
1 x 3	8	24	13
1 x 6**	23	36	32
2 x 3	16	18	20
2 x 6**	31	46	41
3 x 6**	37	67	36
Nonresponder x nonresponder			
4 x 5	1	1	1
4 x 7	1	1	1
5 x 7	1	1	1

* All data obtained in one experiment except those indicated ** which were obtained in a second experiment.

able to detect alleles of each gene, i.e., the different phenotypes must be demonstratable by different enzyme activities or be distinguishable by electrophoretic or immunologic techniques. Heterozygosity for OKT can be determined by measuring OKT activity in fibroblasts. As shown in Table 16, the control activity for OKT ranges from 292 to 882 nmol/mg protein/hr, while the activity in the obligate heterozygotes ranges from 136 to 283 nmol/mg protein/hr. Six relatives of patients were assayed for OKT activity. Four were found to be heterozygotes and two had normal activity. In Figure 2 the inheritance of the deficient gene for OKT in gyrate atrophy families is outlined. These informative families were used for linkage analysis; also the results of the blood proteins and/or enzymes analysed for each member are depicted in Appendix II. The probability of recombination or the map distance between the OKT gene and each marker is referred to as θ. The likelihood that the observed pattern of transmission would occur assuming a particular θ is then compared statistically to the likelihood in the absence of linkage. The log of the ratio of the two likelihoods is referred to as a lod score. A lod score of three is considered good evidence for linkage, while a lod score of one is considered as a trend, indicating possible linkage. For each marker tested, only one family, if any, was informative. Thus, none of the lod scores are high enough to suggest linkage with the OKT gene (Table 17).

Table 16. OKT activity in controls, obligate heterozygotes and relatives of patients with gyrate atrophy.

Family Number**	Generation Number**	OKT activity* nmol/mg protein/hr	Diagnosis
1	III - 6	164; 218	Heterozygote
1	IV - 12	130; 167	Heterozygote
1	IV - 13	77; 47	Heterozygote
1	IV - 14	347; 394	Normal
6	V - 2	428; 354	Normal
6	V - 5	173; 145	Heterozygote
Controls n = 10		466±204 (292-882)	
Obligate Heterozygotes n = 4		203±71 (136-283)	

* The colorimetric assay was used to determine OKT activity.
** This number refers to the pedigrees shown in Figure 2.

Table 17. Linkage Analysis

	Θ - The Probability of Recombination of Two Genotypes				
	0.05	0.10	0.20	0.30	0.40
Marker*					
Jk	0.5353	0.4654	0.3181	0.1703	0.0492
Gc	0.2577	0.2148	0.1335	0.0645	0.0170
AK	0.2577	0.2148	0.1335	0.0645	0.0170
6PGD	0	0	0	0	0
AMY2	0	0	0	0	0
ESD	0	0	0	0	0
PI	0	0	0	0	0
E1	-0.7212	-0.4437	-0.1938	-0.0757	-0.0177
PGM	-0.7212	-0.4437	-0.1938	-0.0757	-0.0177
ADA	-0.7212	-0.4437	-0.1938	-0.0757	-0.0177
ABO	-0.1859	-0.0217	0.1243	0.0946	0.0315
Fy	0	0	0	0	0
Kell	0	0	0	0	0
Hp	0	0	0	0	0

* Markers are defined as:
Jk = Kidd blood group
GC = Group specific component
AK = Adenylate kinase
AMY2 = Amylase, pancreatic
ESD = Esterase D
PI = Protease Inhibitor
E1 = Pseudocholinesterase 1
PGM = Phosphoglucomutase
ADA = Adenosine deaminase
ABO = ABO blood group
6PGD = 6-phosphogluconate dehydrogenase
Fy = Duffy blood group
Kell = Kell blood group
Hp = Haptoglobin

DISCUSSION

A. GENERAL

Gyrate atrophy of the choroid and retina is the only eye disorder with a known biochemical defect that can be treated with a specific vitamin, vitamin B6. This disorder usually begins in childhood with constriction of visual fields, night blindness and progressive myopia. Blindness occurs between the third and fifth decade of life. High levels of plasma ornithine are associated with gyrate atrophy of the choroid and retina. Deficient levels of OKT, a PLP dependent enzyme, have been found in all gyrate atrophy patients studied. Obligate heterozygotes (parents of these patients) have 50% of normal levels of OKT suggesting that this lesion is the primary genetic defect in these patients. At least sixty patients with gyrate atrophy have been described, most of whom are of Finnish background. None of the Finnish patients has shown a response to vitamin B6 _in vivo_. Excluding the patients described in this thesis, only three patients have demonstrated a response to vitamin B6 _in vivo_. I have had the opportunity to study fibroblasts from seven patients. Clinical and biochemical studies in four of these patients have been previously reported (7). Three are _in vivo_ responders and patient 4 was an _in vivo_ nonresponder. In the patients who respond to vitamin B6 the mechanism of the response is not known.

In this study several questions concerning gyrate atrophy were investigated. These included:

1) What is the best method for measurement of OKT activity both under saturating conditions and under conditions which more clearly resemble those *in vivo*?

2) What is the mechanism of the vitamin B6 response?

3) Is there genetic heterogeneity among the responders and the nonresponders?

4) Do the responders represent a mutation at the same locus or at a different one than the nonresponders?

5) Can the location of the OKT gene be determined from family studies?

B. DETERMINATION OF THE BEST METHOD FOR MEASUREMENT OF OKT ACTIVITY BOTH UNDER SATURATING CONDITIONS AND UNDER CONDITIONS WHICH MORE CLEARLY RESEMBLE THOSE *IN VIVO*.

Three methods for measuring OKT activity in fibroblasts were evaluated. These included a colorimetric method, a radiochemical method and an indirect method for measuring OKT activity. The first two methods measure OKT activity in tissue homogenates under optimum substrate conditions, while the third method measures the OKT pathway activity in growing cells under conditions, which may more clearly resemble those *in vivo*. In this thesis, a sensitive method for measuring OKT activity was required in order to measure the low activities in fibroblasts from patients with gyrate atrophy.

When the colorimetric and radiochemical assays were compared

several differences were found between the two assays. First, the radiochemical assay gave 45-77% higher activities than the colorimetric assay. Second, substrate concentrations of 2 mM α-ketoglutarate and 20 mM ornithine were optimal for the radiochemical assay but not for the colorimetric assay. Third, the radiochemical assay was linear for two hours while the colorimetric method was linear for one hour. The basis of the discrepancy between the activities determined by the two assays has not been established. Wong et al. (69) have also documented that the colorimetric assay gives lower total activities than the radiochemical assay. Possibly, the former may be measuring a fraction of the total OKT activity due to the lability of the product P5C, since P5C is known to undergo polymerization especially at high concentrations (72). Moreover, loss of product may occur via the conversion of P5C to proline or glutamate; both of the enzymes necessary for the synthesis of proline or glutamic acid from P5C are present in cultured fibroblasts (see Figure 1). Therefore,the radiochemical assay appears to be a better method for measuring total OKT activity than the colorimetric assay. It, also, has the advantage of being considerably more sensitive.

I have also investigated an indirect measurement of OKT pathway activity. This method is dependent upon the uptake of radioactive ornithine from the medium into the cell's mitochondria where it is eventually metabolized to glutamate and proline, which are then incorporated into protein. The enzymes required for converting ornithine into proline or glutamate are present in cultured skin fibroblasts (see Figure 1) (47). Ornithine, itself, is not incorporated into protein. This assay then is dependent upon several

steps: 1) uptake of radioactive ornithine into the cytoplasm, 2) transport of ornithine into the mitochondria, 3) conversion of ornithine into glutamic γ-semialdehyde in the presence of OKT, 4) synthesis of radioactive proline or glutamic acid from the glutamic γ-semialdehyde and 5) synthesis of protein containing the radioactive proline or glutamic acid.

With the indirect method the patients have 21% to 57% of control activity, while with the radiochemical assay the patients possess 0 to 14% of control activity. The closer range of activity between the responders and the controls with the indirect method may reflect other limiting steps in the transfer of the radioactive label from ornithine to proline or glutamate. The most likely limiting step would be the transport of ornithine into the cells and then into the mitochondria. Patients with HHH have a presumed transport defect of ornithine into the mitochondria. They also have low activity with the indirect method. By either method the responsive patients with gyrate atrophy have higher activities then the nonresponders. The higher activity in the responders probably accounts for their lower serum ornithine levels, which in turn may lead to a slower progression of the disease (4, 25).

C. MECHANISM OF VITAMIN B6 RESPONSE.

Clinically, patients with gyrate atrophy can be divided into two groups, vitamin B6 responders and nonresponders. Presumably, this response is due to the deficient enzyme, OKT, requiring PLP as a coenzyme. The response of patient OKT to vitamin B6 may result from the alteration of any one of a complex series of reactions: apoenzyme synthesis, coenzyme formation and transport, apoenzyme-coenzyme

association and degradative processes. Presumably, coenzyme formation and transport are not the primary problem as other PLP-requiring enzymes would be affected. The approaches used in this thesis explored which of the two reactions, apoenzyme-coenzyme association or degradative processes, might be responsible for the deficient enzyme activity in the responsive patients. These included:

1) measuring enzyme activity with the indirect method in cells grown in different concentrations of pyridoxine,

2) assessing apoenzyme vs. holoenzyme content in cells grown in LP-MEM vs. standard medium,

3) measuring the OKT kinetics in patient fibroblasts with different concentrations of PLP and finally,

4) determining the stability of the enzyme exposed to heat in the presence and absence of PLP.

The first study (Figure 10) explored the effect of different medium concentrations of pyridoxine on OKT pathway activity in the growing fibroblasts. Controls and nonresponders showed no increase in activity at any of the concentrations of pyridoxine used. Increasing activity was found with higher amounts of pyridoxine in all of the responders. To investigate the optimum concentrations of pyridoxine needed to obtain maximum activity in the responders, concentrations of pyridoxine ranging from 0 to 100 mg/l were tested. Maximum activity was reached between 0.1 to 100 mg/l pyridoxine (Table 7). The increase in responder activity between 0 and 0.01 mg/l pyridoxine was significant as seen in patients 1 and 2 ($p<0.01$), patient 3 (<0.1) and patient 6 ($p<0.05$) (paired t-test, one-tailed). This difference in activity was even more striking when cells were grown in medium

supplemented with hydroxylamine-treated FCS (Table 8). The OKT pathway activity of the responders increased dramatically when transferred to LP-MEM with 1.0 mg/l pyridoxine as shown in patient 1 ($p<0.05$), patient 2 ($p<0.005$), patient 3 ($p<0.0005$) and patient 6 ($p<0.025$) (paired t-test, one-tailed). Both the control and the nonresponders showed little increase in activity. Thus, the responders and the nonresponders can be distinguished by their response to pyridoxine.

To further investigate the mechanism of response to pyridoxine, the apoenzyme and holoenzyme content of cells grown in LP-MEM and standard medium was determined. As shown in Table 9, both control and patient cells had higher total activities when grown in standard medium. However, the increase in total activity in patient cells from undetectable levels to 15-34 nmol/mg protein/hr was much more apparent than in the controls (157-239 to 213-305 nmol/mg protein/hr). This would be compatible with a mutant enzyme with a reduced affinity for the coenzyme leading to a greater percent of enzyme in the apoenzyme form, which may be more susceptible to degradation than the holoenzyme. Other enzymes that require PLP as a coenzyme have been shown to be more susceptible to degradation in the absence of PLP (73, 74). Litwack et al. (74) have proposed that dissociation of coenzyme may be the rate-limiting step, conditioning the apoenzyme for degradation. In addition, the half-life of OKT has been demonstrated to be shorter in vitamin-B6 depleted rats (75). Thus, the stability of OKT may be dependent upon the binding affinity of PLP and on the availability of PLP. Alternatively, the higher total enzyme activity in standard medium could reflect stabilization of an unusually labile

enzyme. Holoenzyme activities in cells from patients were so low that an accurate estimation of the per cent of apoenzyme could not be made, although it appears to be higher than the controls suggesting a decreased binding of coenzyme in mutant cells. When transferred from LP-MEM to standard medium, the levels of holoenzyme in cells from the controls increased slightly and in patients remained undetectable. Therefore, the total enzyme increase in patients appears to reflect an increase in apoenzyme and not holoenzyme content. This is somewhat surprising since if decreased binding or increased stability was the mechanism of response in patients, higher holoenzyme activity in standard medium would have been expected. Possibly, these results suggest that more bound PLP may be removed from patient OKT than from the control enzyme during passage through the Sephadex column. This would be compatible with a reduced affinity of patient OKT for PLP. This question was further studied by kinetic analysis.

The affinity of patient and control OKT for PLP was tested more directly by assaying cell homogenates with concentrations of PLP ranging from 0 to 2 mM PLP. As shown in a plot of velocity vs. substrate concentration (Figure 11), the patients reach maximal activity around 1 mM PLP while the control activity plateaus between 0.1 and 0.5 mM PLP. The apparent K_m in the patients ranged from 0.42 to 2.71 mM as shown in Table 12. The apparent K_m for the control was 0.018 mM, at least twenty times lower than those of the patients. This is only an estimate of the K_m since the kinetics were done with crude cell homogenates. However, this is an adequate comparison for demonstrating that the responders have a high K_m in comparison to the control. From these results, one can deduce that the reduced affinity

of the responsive patient enzyme must play a large role in each patient's response to vitamin B6 *in vivo*. The apparent K_m of the control is close to that found in partially purified OKT from bovine ciliary body and iris (0.010 mM) (76). The control and patient V_{max} were also strikingly different (Table 12). The control value was 223 nmol/mg protein/hr while the patients' values ranged from 42 to 67 nmol/mg protein/hr.

One method of examining an enzyme's susceptibility to degradation is to measure the activity after heating in the presence and absence of its coenzyme. In the heat stability studies described in this thesis (Figure 14 and 15), the controls showed no decline in OKT activity when the homogenate was heated in the presence of 1.67 mM PLP for one hour. However, when the coenzyme was not present, the controls lost at least 40% of their original activity, indicating that the coenzyme does have a protective effect on OKT. The patients' OKT response resembled the controls both in the presence and absence of PLP. Thus, the mutation in each of the responsive patients, presumably, does not affect the stability of the enzyme. However, a small difference in mutant enzyme stability that is not detectable by this method could have an effect *in vivo*.

The retention of 100% of OKT activity in cell extracts from control and patients in the presence of PLP during heating suggests that OKT is a very stable enzyme in the holoenzyme form. Interestingly, the patients and controls show a similar rate of decline even though as discussed earlier, their ratio of apoenzyme to holoenzyme appears to be different. Thus, some additional factor may be involved in the stabilization of this enzyme.

From the results discussed in this thesis, a prelimiinary biochemical model of the enzyme defect in the responsive patients can be proposed. All four patients have measurable but low OKT activity that is increased by high concentrations of vitamin B6. An abnormally low affinity for PLP has been demonstrated in these patients. However, the heat stability of patient OKT is normal. Thus, it would appear that the patient OKT has an abnormal apoenzyme-coenzyme association that does not affect the stability of this mutant enzyme. Theoretically, if the concentration of B6 in the cells is low, the enzyme is predominantly in the apoenzyme form; thus, it is more readily susceptible to degradation. Administration of pyridoxine leads to increased cellular PLP, which enhances enzyme activity modestly by increasing holoenzyme formation. In turn, less apoenzyme is present to be degraded. The clinical response, observed in the patients, is a lowering of plasma ornithine. Other studies that support this hypothesis include 1) reduced hepatic apoenzyme content of several PLP-dependent enzymes in pyridoxine-deficient rats (73, 77), and 2) the impressive positive correlation between the relative rates of coenzyme dissociation and degradative half-lifes of several PLP-dependent enzymes including OKT (74, 75).

It is of interest to compare these conclusions on the mechanism of response to vitamin B6 in patients with gyrate atrophy with similar studies on another B6 responsive condition, homocystinuria associated with a defect of cystathionine β-synthase, a PLP-requiring enzyme. Fowler et al. (51) divided a group of seven patients into three general classes according to the level of synthase activity in their fibroblasts: 1) no residual cystathionine β-synthase, 2) reduced activity,

normal K_m for PLP and normal heat stability and 3) reduced activity, reduced affinity for PLP and a more heat labile enzyme than the controls. Kim et al. (52) studied three responders with homocystinuria, who had an increased K_m for PLP, increased K_m for L-homocystine, a decreased affinity for L-serine, decreased V_{max} and an enzyme that was more heat labile than the control enzyme. Fowler et al. (51) proposed that the *in vivo* pyridoxine responsiveness in patients whose synthase has a reduced affinity for PLP and/or reduced stability can be accounted for by a partial, perhaps only modest, enhancement of catalytic activity brought about by the vitamin supplement. However, this hypothesis does not explain responsiveness in patients whose synthase exhibits essentially normal kinetics for PLP and near normal stability or nonresponsiveness in patients whose cystathionine β-synthase properties are still indistinguishable from those found in the cells of responsive patients. Lipson et al. (78) have shown that growth conditions of the fibroblasts may obscure important aspects of enzyme-coenzyme interactions by saturating most synthase molecules with their cofactor PLP. When patient and control cells were grown in pyridoxal-free medium, the enzyme of the responsive patients had an affinity for PLP two to four times less than the control. In contrast, the *in vivo* nonresponsive patients had a markedly reduced enzyme affinity for PLP from sixteen to sixty-three times less than normal (79). Lipson et al. (79) hypothesize that *in vivo* responsiveness or lack therof depends on at least three factors: 1) the presence or absence of residual synthase activity in mutant cells; 2) the affinity of mutant synthase for its cofactor, PLP; and 3) the cell's ability to accumulate PLP. Those patients whose cells contain a mutant synthase with a moderately reduced affinity for PLP are

able to increase cellular PLP content sufficiently after pyridoxine supplementation so that holosynthase activity rises above that critical value needed to prevent accumulation of homocystine and methionine. On the other hand, pyridoxine nonresponsiveness may be observed for one of two general reasons: either because the cell contains no residual synthase activity or because the cell contains a mutant synthase whose affinity for PLP is so reduced that, despite any feasible pyridoxine supplements, the cell is unable to increase PLP content enough to stimulate appreciable formation of synthase holoenzyme.

The response in the patients with gyrate atrophy differ in several major respects from the response observed in the patients with homocystinuria. First, the heat stability of OKT in the responsive patients is normal while the mutant synthase is more heat labile than normal in the responsive patients with homocystinuria. Second, the difference between the enzyme affinity for PLP in patients with gyrate atrophy is twenty to one hundred fold less than that of the controls, while the patients with homocystinuria have an affinity that is only two to four times less than the controls. Indeed the difference in the gyrate atrophy responsive patients more closely resembles the nonresponsive patients with homocystinuria whose enzyme affinity for PLP is sixteen to sixty-three times less than the controls. Finally, in the responsive homocystinuria patients the biochemical deficiency is corrected by administration of pyridoxine with homocystine and methionine levels returning to normal. When pyridoxine is administered to the responsive patients with gyrate atrophy, the serum levels of ornithine are lowered but still remain much higher than the controls (three to four times, see Table 2). This suggests that there may be

another class of patients with gyrate atrophy similar to the responders with homocystinuria, who have a reduced enzyme affinity for PLP only two to four times less than controls and whose symptoms are so mild or nonexistent, if their vitamin B6 intake is adequate, that they have yet to come to the attention of an ophthalmologist.

D. GENETIC HETEROGENEITY.

Genetic heterogeneity has proved to be the rule rather than the exception in genetic biochemical disorders. This heterogeneity may arise from allelic mutations in the same structural gene or from mutations affecting different loci, if the enzyme is composed of subunits coded by different loci. Evidence suggests that OKT is comprised of identical subunits (39). Thus, genetic heterogeneity in gyrate atrophy is probably due to mutations at different sites in the OKT gene. With 300 or more nucleotides present in this gene it is unlikely that two heterozygotes would carry an identical mutation unless they were related by a common ancestor. Instead, it is more probable that each patient is a compound heterozygote, bearing two different mutant alleles. A mutation at one site, for example, may affect the binding of the substrate ornithine, while the mutation in the second allele may be in a separate location resulting in lower binding of the cofactor PLP. Interestingly, each of the responders had a preponderance of British ancestors (Figure 2) and one of the other three reported responders is also British. If a common ancestor was found for all of the responders, they would most likely share the same mutation of the OKT gene. Most of the reported patients with gyrate atrophy are from Finland and are nonresponders. In a majority of these families there is inbreeding. Thus, most of these patients

probably carry the same structural gene defect.

Clinically, the seven patients in this study can be divided into two groups, responders and nonresponders. Several studies in this thesis have further defined the genetic heterogeneity in the seven patients. These included measuring OKT activity both directly and indirectly, measuring the response of patients' cells to different concentrations of pyridoxine and complementation studies discussed below. The OKT activity of the patients was one parameter that clearly separated them into two if not three groups (Table 6). With both the direct and indirect assays the nonresponders had less activity than the responders and the difference in activity with the indirect method was highly significant ($p<0.001$). When the patients were studied using the indirect method with increasing concentrations of pyridoxine, the responders all clearly responded while the nonresponders did not.

Patient 6 has not been clinically characterized as a responder or a nonresponder. However, her clinical findings are much milder than those of the nonresponders and her serum ornithine levels are lower. In addition, patient 6 has higher levels of OKT activity than those of both the responders and nonresponders, especially evident with the indirect assay. Her K_m for PLP and heat stability results were similar to those of the responders, suggesting that she is also a responder. She was different from the other three responders in that her activity with the indirect radiochemical assay was significantly higher as shown in Table 6. There is no clear explanation for this difference. Possibly the high activity in the indirect assay is reflecting some limiting step at which patient 6 is more efficient

than the other responders such as transportation of ornithine into the mitochondria. A second possiblity would be that the mutation(s) in the OKT gene of patient 6 is different than that of the other responders, resulting in a milder phenotype. This could be investigated by other studies including α-ketoglutarate and ornithine kinetic studies and DNA recombinant techniques.

Constancy among siblings in their ability to respond is further evidence that this response is genetically controlled. Takki (14) has found several families with more than one sib affected with gyrate atrophy; all of the affected siblings were nonresponders. In this study two sisters, who both respond to vitamin B6, have been studied. In all experiments in which they were studied together, the results have been strikingly similar.

One problem that has been encountered in several of the vitamin-responsive disorders is predicting with a biochemical test whether a patient is a responder or not, since the response in vitro has not always correlated with the in vivo response (7). For example, in homocystinuria, due to cystathionine β-synthase deficiency, patients who respond to vitamin B6 generally have higher in vitro activity than the nonresponders. However, cells from one responsive patient had no residual activity and cells from two nonresponsive patients contained measurable activity (51). This inconsistency may be due to the difficulty of obtaining accurate values for very low activities, or may be due to the fact that the enzyme is generally assayed at a concentration of PLP, which does not reflect the intracellular concentrations attained in vivo during pyridoxine supplementation. Lipson et al. (78) have shown that growth of cells

in standard medium which contains a large excess of pyridoxal (1 mg/l) obscured various aspects of enzyme-coenzyme interaction. When patient and control fibroblasts were grown in medium with low pyridoxal concentrations, both the responders and nonresponders had higher K_m's than the controls. However, the nonresponders K_m was much higher than the responders.

Most reported gyrate atrophy patients have shown a correlation between *in vivo* and *in vitro* response. The biochemical test commonly used to determine *in vitro* responsiveness measures the deficient enzyme activity in fibroblast homogenates. I have investigated an alternative and perhaps more reliable indicator of *in vivo* responsiveness with the indirect method for measuring OKT pathway activity. This method measures activity in live cells and may be a better measure of the effect of B6 on OKT pathway activity in the patients than measurement of specific enzyme activity. Moreover, the concentration of B6 that the cells are growing in can be controlled. Therefore, the concentration may more likely reflect the intracellular level attained during pyridoxine supplementation, and the direct effect of B6 on the overall pathway can be ascertained. The activity measured by the indirect method is greatly reduced in all patients with gyrate atrophy as shown in Table 6. However, this activity is clearly greater in our B6 responsive patients than in the nonresponders (including patient 4), suggesting that this technique is a reliable indicator of B6 response *in vivo*.

A similar type of *in situ* assay has been used to study the *mut* mutants in methylmalonic acidemia. These cells have deficient methylmalonyl coenzyme A mutase activity, which requires

adenosylcobalmin as a cofactor. When they are grown in medium supplemented with hydroxocobalmin, the patient fibroblasts can be divided into two distinct groups: responders and nonresponders corresponding to the in vivo situation (80). The nonresponders show no increase in activity at any of the concentrations of hydroxocobalmin used. The responders can be divided into two groups with one group showing an increase in activity from one to three fold with increasing concentrations of coenzyme, while the second group's activity increases ten to twenty fold within the same concentrations of hydroxocobalmin. Neither group reaches control activity at their highest level of activation. Thus, as demonstrated in patients with methylmalonic acidemia and gyrate atrophy, the in situ method, when available, appears to be the best method for determining if a patient with a vitamin-responsive disorder is an in vivo responder.

E. COMPLEMENTATION

Complementation analysis is a classical tool used to determine if a particular phenotype shared by two or more organisms of the same species is the result of allelic or nonallelic mutations. In other words, complementation analysis can differentiate between the two different causes of genetic heterogeneity described by Harris (81). These are 1) nonallelic mutations with mutant alleles at different loci that affect different subunits of the same enzyme (or protein) or affect different enzymes or proteins or 2) allelic mutations with different mutant alleles at a single locus. Genetic heterogeneity of both allelic and nonallelic origin has been demonstrated with discrete inherited disorders including methylmalonic acidemia (57), propionic acidemia (82), and Tay Sachs with Sandhoff's disease (58, 59), the

mucopolysaccharidoses (55), maple syrup urine disease (83, 84) and galactosemia (85).

In order to demonstrate that intergenic complementation, that is complementation between different mutant loci, would be observed in the system used in this thesis, a positive control was used. Fibroblasts from patients with HHH have been shown to complement fibroblasts from gyrate atrophy patients (64). Neither of these cell types can metabolize ornithine normally either alone or when grown in the same dish (Table 13). HHH fibroblasts have normal OKT activity (data not shown) but have decreased transport of ornithine across the mitochondrial membrane (49). Presumably, positive complementation occurs between HHH x gyrate atrophy fused cells because the heterokaryons allow a rapid exchange of mitochondrial proteins resulting in mitochondria containing both normal and mutant OKT and normal and mutant transport protein. In these heterokaryons ornithine metabolism will occur much more efficiently than in either mutant alone. Positive complementation was demonstrated in all crosses of HHH and gyrate atrophy cells (Table 14). Therefore, the fusion technique was adequate to demonstrate complementation if it occurred in patients with gyrate atrophy.

Complementation analysis was used in this thesis to further define the genetic heterogeneity in the two phenotypes observed in gyrate atrophy, i.e., pyridoxine responder and nonresponder. This study extends the findings of Shih et al. (64) in which no complementation was found when fibroblasts from one responsive and three nonresponsive patients were fused with each other. The effect of the fusion process on the OKT pathway was first examined in

control and patient cells (Table 13). Most parental lines had similar activities when fused as compared to nonfused cells. Occasionally, a fused cell line had less activity than the mixture of nonfused cells, suggesting some toxic effect of PEG on the OKT pathway activity. For this reason a predicted value using the mean of the fused parental cell lines was compared to the fused crosses (Table 15). No increase occurred in the fused value vs. the nonfused or predicted value in any of the patient crosses. Therefore, no positive complementation occurred. However, there was a decrease of activity in some of the fused crosses suggesting a possible negative complementation. Negative complementation might be expected if two of the subunits have mutations at different sites, which cause a further decline in activity when combined together in one enzyme molecule. This decline might be due to 1) steric hindrance, 2), a combination of lowered affinity for both PLP and substrate or 3) an enzyme which is more labile and unable to bind substrate or coenzyme sufficiently. The significance of any one cross of gyrate atrophy cell lines is hard to evaluate but when all of the crosses were combined, there was no significant difference between fused and nonfused crosses. In conclusion, since no positive complementation occurred in any of the gyrate atrophy fibroblast crosses, the nonresponder and responder variants of gyrate aytrophy represent mutations in the same structural gene. This is consistent with the observation that OKT is a homopolymer.

F. LOCATION OF OKT GENE.

The location of the OKT gene on a particular chromosome has not been established. In order to map the OKT gene, family studies of the

patients with gyrate atrophy were used for linkage analysis. Blood samples from relatives and four patients were analysed for thirty markers with known chromosome locations in the laboratory of Dr. E. Lovrien, Medical Genetics, Oregon Health Sciences University.

Heterozygote detection is essential for using this methodology to locate the gene for OKT. Several methods are available for assessing if a person is a heterozygote. These include 1) measuring serum ornithine levels, 2) giving oral loads of ornithine and measuring the resulting increase in serum ornithine levels, and 3) measuring culture skin fibroblast OKT activity. The first method would be the quickest and easiest method for detecting heterozygotes. However, the obligate heterozygote serum ornithine levels overlap the control levels (7). This problem is also evident in the second method (7). Therefore, the most satisfactory method is measurement of _in vitro_ OKT activity. In this thesis, fibroblast OKT activity was measured with the colorimetric assay. As shown in Table 16, the range of control and heterozygote activity is distinct although the low end of the control range and the high end of the heterozygote range are close. Family studies were done on four of the families of the patients in this study (Figure 2). Six relatives were tested for OKT heterozygosity, eleven had previously been tested by this lab. Using this data and that provided in Appendix 2, lod scores were calculated for each of the blood marker proteins and OKT. The lod score is the log of the ratio of the likelihood that the observed pattern of transmission would occur compared to the likelihood in the absence of linkage. No significant lod scores were ascertained (Table 17). Therefore, the assignment of the OKT gene to a particular chromosome is still not

possible. Of all of the blood proteins studied, the ABO blood group was the only one that showed a positive score with a peak at 0.12 lod score with a θ value of 0.2. This group is located on chromosome 9. In spite of the fact that the location of the gene has not been identified, this information is still useful, since with further studies (forty-four relatives of Dr. Weleber's patients may be informative) future assignment of the OKT gene is feasible.

SUMMARY AND CONCLUSIONS

1. All patients with gyrate atrophy have severely deficient OKT activity as measured by the enzyme activity in fibroblast homogenates or by the incorporation of radioactivity from labelled ornithine into protein in growing cells.
2. By either method patients who did not respond to vitamin B6 *in vivo* clearly have lower levels of activity than those who did respond.
3. Studies on the mechanism of response to vitamin B6 have demonstrated that the responsive patients have a lowered affinity for PLP.
4. The mutation(s) in the OKT gene of the vitamin B6 responsive patients appeared to have no effect on the stability of the mutant OKT enzyme.
5. Further evidence for genetic heterogeneity between the pyridoxine responders and nonresponders was shown with the responders having consistently higher residual enzyme activity in fibroblast homogenates and higher levels of incorporation of radioactivity from labelled ornithine into protein than the nonresponders.
6. Additional evidence for genetic heterogeneity included the finding that the responders incorporate higher levels of radioactivity from labelled ornithine into protein when pyridoxine was added to the medium, while the activities of the nonresponders remained the same. Thus, this method provides good correlaion between *in vitro* and *in vivo* response.

7. The mutation(s) in the responsive patients could not be distinguished from each other by the results from the PLP kinetics, heat stability, or pH optimum.
8. Complementation analysis suggests that B6 responsive and nonresponsive variants of gyrate atrophy are due to non-complementing mutations in the same structural gene.
9. Linkage analysis was done with three informative pedigrees. Four carriers were detected. A positive lod score indicating linkage was not established; however, the ABO blood group on chromosome 9 showed a small correlation to the OKT gene.

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Appendix 1a - Counting the number of nuclei in fused gyrate atrophy x HHH fibroblasts in order to calculate the per cent of heterokaryons present. Additional dishes were set up and fused simultaneously with the test dishes. Twenty hours after fusion, these dishes were fixed. Giemsa stain was applied to the dishes and the number of nuclei in each cell was counted. At least thirty cells were counted in each dish.

Cell Crosses	Number of Nuclei in Each Cell								Cells			Nuclei		
	1	2	3	4	5	6	7	8 or more	# multi-nucleate cells	Total # cells	% cells with multi-nuclei	# nuclei in multi-nucleated cells	Total # nuclei	% nuclei in multi-nucleated cells
7 x HHH	87	12	5	4	2		1		24	111	22	72	159	45
3 x HHH	124	22	11	2	2			3	40	164	24	119	243	49
2 x HHH	119	15	7	5	2	2	1	1	33	152	22	108	227	48
5 x HHH	97	18	6	1	1		2	2	30	127	24	97	194	50
3 x HHH	51	14	8	8	2	1	3		36	87	41	121	172	70
4 x HHH	89	12	7	4					23	112	21	61	150	41
2 x HHH	191	33	5	5	5	4	1	2	55	246	22	176	367	48
5 x HHH	192	33	10	4	1	2	2	1	53	245	22	153	345	44
7 x HHH	55	5	3	1					9	64	14	23	78	29
Total # cells	1005	164	62	34	15	9	10	9						
% total cells	77.0	12.5	4.7	2.6	1.1	0.7	0.8	0.7						
Expected % hetero-karyons*	0	4.2	2.4	1.6	0.7	0.5	0.5	0.5	Total % heterokaryons = 10.4					

* % of heterokaryons = (% of cells with n number of nuclei) (n-1)/(n+1)

Appendix 1b. Counting the number of nuclei in fused gyrate atrophy fibroblasts in order to calculate the per cent of heterokaryons present, as described in Appendix 1a.

Cell Crosses	Number of Nuclei in Each Cell							Cells			Nuclei		
	1	2	3	4	5	6	7 or more	# multi-nucleate cells	Total # of cells	% cells with multi-nuclei	# nuclei in multi-nucleate cells	Total # nuclei	% nuclei in multi-nucleate cells
3 x 6	77	19	5		2	2	6	34	111	31	158	235	67
5 x 6	270	20	10	6	2			38	308	12	104	374	28
6 x 7	82	16	9	5	7	6	10	53	135	39	263	345	76
3 x 7	130	31	5	4	1	2	2	45	175	26	124	254	49
1 x 7	52	14	6	8	3	3	2	36	88	41	128	180	71
2 x 7	66	15	6	2	4	1	3	31	97	32	106	172	62
7 x 5	81	19	6	5			1	31	112	28	83	164	51
3 x 4	121	18	5	1	1			25	146	17	60	181	33
1 x 3	45	11	10	2		2	1	26	71	37	80	125	64
2 x 3	100	25	10	5	6		5	51	151	34	173	273	63
3 x 5	90	15	8	5	9	4	6	47	137	34	194	284	68
3 x 7	73	13	3	2		1	1	20	93	22	56	129	43
2 x 4	50	10	8	5	2	1		26	76	34	80	130	62
4 x 5	180	28	7	2	1		2	40	220	18	106	286	37
4 x 7	24	3	4	1	1			9	33	27	27	51	53
1 x 5	42	16	5	8	1	2	7	39	81	48	160	202	79
1 x 7	60	18	6	3				27	87	31	66	126	52
2 x 5	224	26	6	2	3		2	39	263	15	108	332	33
2 x 7	72	9	6					15	87	17	36	108	33
5 x 7	134	26	9	7	4		2	48	182	26	143	277	52
Total # cells	1973	352	134	73	49	24	50						
% total cells	74.3	13.3	5.1	2.8	1.8	0.9	1.9						
Expected % hetero-karyons*	0	4.4	2.6	1.7	1.2	0.6	1.4	Total % heterokaryons = 11.9					

* % of heterokaryons = (% of cells with n number of nuclei)(n-1)/(n+1)

Appendix II. Linkage analysis

			Red Cell Proteins*						
Number	Family Number	Pedigree Number	ABO	Rhesus	MNSs	Kell	Fy	Jk	Se
1	4	I-1	O	DcEe	MN s	-k-	b	a	Se
2	4	I-2	A	DCecE	NSs	Kk-	ba	a	Se
3	4	II-1	A	DcEe	MN s	-k-	b	a	Se
4	4	II-3	A	cde	MN s	-k-	bb	aa	Se
5	4	II-4	O	DCce	MN s	-k-	ba	a	Se
6	4	III-7	O	DCce	N s	-k-	ba	a	Se
7	4	II-2	O	DCce	MNS	-k-	b		Se
8	4	III-5	A	DCce	MNSs	-k-	b		Se
9	4	III-3	O	dce	M Ss	-k-	b		se
10	4	III-6	A	DCce	MNSs	-k-	ba		Se
11	1	III-3	A	DCce	M s	-k-	ba	a	Se
12	1	III-4	O	cde	MNS		ba	a	se
13	1	IV-1	O	cde	MNSs	-k-	ba	b	Se
14	1	IV-2	O	cde	M Ss	-k-	ba	b	Se
15	1	IV-3	A	DCce	MNSs	-k-	ba	a	
16	1	III-7	O	cde	MNSs	-k-	ba	ba	
17	1	III-8	AB	DCEce	M Ss	-k-	b	ba	
18	1	IV-12	A	DCce	MN s	-k-	b	a	
19	1	IV-13	B	DEce	M Ss	-k-	a	ba	
20	1	IV-14	A	DEce	M Ss	-k-	ba	ba	
21	6	III-2	O	cde	M s	-k-	a	a	
22	6	IV-1	O	cde	M Ss	-k-	a	a	
23	6	V-2	A	cde	M Ss	-k-	ba	ba	
24	6	IV-2	A	DcEe	M S	-k-	b	b	
25	6	V-5	A	DcEe	N s	-k-	ba	b	
26	3	III-2	A	DCce	NSs	-k-	a	a	Se
27	3	IV-3	O	cde	MNS	-k-	ba	ba	se
28	3	IV-1	O	cde	MNSs	-k-	a	ba	
29	3	11-2	A	cde	NSs	-k-	ba	a	Se
30	3	III-1	A	cde	MNSs	-k-	b	a	

* All red cell proteins were analysed by using specific antiserum.
ABO = the red blood antigen group
Rhesus = the Rhesus antigen group
MNSs = the MNSs antigen group
Kell = the Kell antigen group
Fy = the Duffy antigen group
Jk = the Kidd antigen group
Se = the Secretor antigen group
All of the following symbols refer to a specific reaction with a particular antiserum: A, B, O, D, C, E, c, e, M, N, S, s, -k-, K, b, a, Se.
No reaction to D antisera is indicated as "d". No reaction to secretor is indicated as "se".

Appendix II (cont.)

	Plasma Proteins*								
Number	Tf	Hp	E2	E1	Gc	Amy	Pi	C3	Bf
1	c	2	C5	U1	2	A		F	
2	c	2	C5	U1	1	A		S	
3	c	2	C5	U1	21	A		FS	
4	c	2	C5	U1	21	A		FS	
5	c	21	C5	U1		A		FS	
6	c	21	C5	U1		A		FS	
7	c							S	
8	c							S	
9	c							FS	
10	c							S	
11	c	21	C5	U1	1	A		S	
12	c	21	C5	U1	21	A		FS	
13	c	21	C5	U1	21	A		S	
14	c	21	C5	U1	21	A		FS	
15	c	21	C5	U1		A		S	
16	c	2		U1	1	A	M1	S	S
17	c	21		U	1	A	M1	FS	FS
18	c	2		U	21	A	M1	F	FS
19	c	21		U1	1	A	M1	F	FS
20	c	2		U	1	A	M	F	S
21	c	1		U1				S	
22	c	21		U1		A		FS	
23	c	2		U1		A		S	
24	c	2		U1		A		S	
25	c	b1		U1	2	A	M	FS	
26	c	21			1	A		F	
27	c	21		U1	1	A	M1	F	FS
28	c	21		A	1	A	M	F	FS
29	c	1			1			FS	
30	c							FS	

* All plasma proteins were analysed by gel electrophoresis.
Tf = transferrin
Hp = haptoglobin
E2 = pseudocholinesterase 2
E1 = pseudocholinesterase 1
Gc = group specific complement
Amy = amylase
Pi = protease inhibitor
C3 = complement component 3
Bf = Properdin factor B
All of the following symbols refer to a specific migration band: c, 2, 1, C5, U1, U, A, F, S,

Appendix II (cont.)

Red Cell Enzymes*

Number	GPT	GALT	GLO	EsD	ADA	6PGD	PGM	ACP	AK
1	21			1	1	A	21	BA	1
2	21			1	1	A	21	BA	21
3	1			1	1	A	21	BA	21
4	1			1	1	A	21	BA	1
5	21			1	1	A	1	B	1
6	21			1	1	A	1	BA	1
7	21		1	1	1	A	1	B	1
8	21		21	1	1	A	21	B	21
9	21		21	1	1	A	21	BA	1
10	21		21	1	1	A	21	BA	21
11	2	N		1	1	A	1	BA	1
12	21	N		1	21	A	21	BA	1
13	2		1	1	1	A	21	B	1
14	21	N		1	1	A	1	B	1
15	21			1	1	A	1	A	1
16		DN	21	1	21	A	2		1
17		N	21	2	1	A	21	BA	1
18			1	21	1	A	21	BA	1
19		N	1	21	1	A	2		1
20			21	21	1	A	2	BA	1
21		N	21	1	1	A	21	BA	1
22		N	21	1	21	A	1	BA	1
23		DN	21	1	1	A	1	BA	1
24		DN	21	1	1	A	1	A	1
25			21	21		A	1	B	1
26	1			1	1	A	1	BA	1
27			1	1	1	A	21	BA	1
28		N	1	1	1	A	1	A	1
29	21			1	1	A	21	CB	1
30	21			21	1	A	1	BA	1

*All red cell enzymes were analysed by gel electrophoresis.

GPT = glutamate pyruvate transaminase
GALT = galactose-1-phosphate uridyltransferase
GLO = glyoxylase
EsD = esterase D
ADA = adenosine deaminase
6PGD = 6 phosphogluconate dehydrogenase
PGM = phosphoglucomutase
ACP = acid phosphatase
AK = adenylate kinase
All of the following symbols refer to a specific migration band: 1, 2, A, B, DN, N.