A BIOCHEMICAL GENETIC INVESTIGATION OF TRANSKETOLASE IN THE WERNICKE-KORSAKOFF SYNDROME

BY

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To my parents

Be it a fruit from a growing tree
of a seed you so well planted....

DECLARATION

I CERTIFY THAT THIS THESIS DOES NOT CONTAIN MATERIAL PREVIOUSLY PUBLISHED OR WRITTEN BY ANY OTHER PERSON, EXCEPT WHERE REFERRED TO IN THE TEXT AND THE RESULTS GIVEN HAVE NOT BEEN SUBMITTED FOR ANY OTHER DEGREE OR DIPLOMA.

Suzanne A. Al-Bustan

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LIST OF ABBREVIATIONS

Acety-CoA	acetylcholine
ApoTK	apotransketolase
ATP	adenosine triphosphate
ATZ	anilinothioxolinone
Au	absorbance unit
Bis	N,N^1-methylene bisacrylamide
BPB	bromophenyl blue
BSA	bovine serum albumin
CAPS	3-{cyclohexylamino}-1-propanesulfonic acid
CBB	coomassie brilliant blue
CM	carboxymethyl
DEAE	diethylaminoethyl
DHEBA	N,N^1-(1,2,dihydroxyethylene) Bis-acrylamide
DNFB	2,4-dinitro-fluorobenezene
DNPH	dinitro-phenyl-hydrochloride
DPTU	N_1-N^1-diphenylthiourea
DPU	N_1-N^1-diphenylurea
DTT	dithiothreitol
EC	enzyme classification
ETK	erythrocyte transketolase
ETKs	stimulated erythrocyte transketolase
FPLC	fast protein liquid chromatography
GDH	glycerol-3-phosphate dehydrogenase
G-3-P	glyceraldehyde-3-phosphate
GSH	cerebral glutathione
Hb	haemoglobin
HCl	hydrochloric acid
HiCn	Millimolar extinction coefficient
HoloTK	holotransketolase
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
kD	kiloDaltons
αKGDH	α-ketoglutarate dehydrogenase
Km	Michaele's constant
KP	Korsakoff's psychosis
MTT	methyl thiazolyl tetrazolium
MW	molecular weight
NBT	nitroblue tetrazolium
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NRC	National Research Council
OD	optical density
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PDH	pyruvate dehydrogenase

pI	isoelectric point
PITC	phenylisothiocynate
PMS	phenazine methasulphate
PTC	phenylthiocarbamyl
PTH	phenylthiohydantain
PVDF	polyvinylidene diflouride (Immobilon P)
RBC	red blood cells
RPM	revolutions per minute
R-5-P	ribose-5-phosphate
SAA	sulphosalicylic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	soduim dodecyl sulfate-polyacrylamide gel electrophoresis
S-5-P	sedoheptulose-5-phosphate
TA	transaldolase
TCA	trichlorocetic acid
TD	thiamine deficiency
TEMED	tetramethylethylendiamine
TFA	trifluoracetic acid
TK	transketolase
TPI	triosephosphate isomerase
TMP	thiamine monophosphate
Tris	trizma base
TPP	thiamine pyrophosphate
TPPE	thiamine pyrophosphate effect
TTP	thiamine triphosphate
X-5-P	xylulose-5-phosphate
WBC	white blood cells
WE	Wernicke's encephalopathy
WKS	Wernicke-Korsakoff syndrome

بسم الله الرحمن الرحيم

"وعسَىَ أن تَكرَهُوا شيئاً وهُو خَيرٌ
لَكُم وَعَسَى أنْ تُحبُوا شَيئاً وَهُو
شَرٌ لَكُم واللّه يَعلَمُ وأنتُم لاتَعلَمُون"

صدق الله العظيم
سورة البقرة الآية ٢١٦

"Yet, you may detest a thing, though it is good for you. And you might love a thing, though it is evil for you, for God knows and you do not know."

The Holy Koran
Chapter 2, Part 2, Verse 216

SUMMARY

The Wernicke-Korsakoff syndrome (WKS) is a severe neuropsychiatric disorder most commonly seen as a result of malnutrition associated with chronic alcohol abuse. In its acute form (Wernicke's encephalopathy, WE) it is characterised by ophthalmoparesis, nystagmus, ataxia and an apathetic confusional state. In those that survive this initial phase, over 70% will be left with a specific form of memory impairment with a varying degree of severity. In those most severely affected (~50% Korsakoff's psychosis, KP), the impairment will be such that they are incapable of independent living and many will require long-term institutional care.

In Scotland there is good evidence of an increase, over the past two decades, in the number of people suffering from alcohol-related brain damage, of which KP is the major form (Smith & McColl, 1992). As a result the absolute number of patients in long term psychiatric care and suffering from alcoholic KP has grown. They form a small but significant proportion of beds in the refractory wards of mental hospitals. One general argument that has fuelled interest in the possibility of genetic factors in alcoholic KP is the fact that only a small percentage of those who chronically abuse alcohol and become malnourished ever develop the characteristic neuropathology.

In 1930 Wagner and Weir reported on the association of the WKS with thiamine depletion. Thiamine exists in three forms, thiamine monophosphate, triphosphate and pyrophosphate(TPP): the last making up 80-90% of total thiamine in cells. TPP is a cofactor for many enzymes of carbohydrate metabolism including pyruvate dehydrogenases, α-ketoglutarate dehydrogenase and transketolase (TK). Several studies have implicated TK in the pathogenesis of the WKS.

The aim of this study was to investigate the role of TK in the WKS using quantitative and qualitative methods of analysis in patient and control groups.

Blood from 30 hospitalized patients with the WKS and 25 normal

controls was analysed for erythrocyte transketolase (ETK) activity and stimulated erythrocyte transketolase (ETKs) activity by the addition of TPP using an end point assay. By employing a modified TK assay (Smeets et al., 1971; Bayoumi & Rosalki, 1976), mean ETK activity in 10 normal controls was found to be 0.418 ± 0.193 IU/mg protein which was not significantly different ($p>0.1$) from the 20 patients (mean ETK activity 0.406 ± 0.155 IU/mg protein). The same was true for the ETKs activity in which normal controls had a mean of 0.738 ± 0.208 IU/mg protein and patients 0.680 ± 0.190 IU/mg protein. This difference was not statistically significant ($p>0.1$).

A report (Kaczmarek & Nixon, 1983) suggesting the existence of several species of TK was investigated by polyacrylamide gel electrophoresis (PAGE). TK activity was detected on PAG by specific TK enzyme stain. All normal controls and patient samples were run on PAG and then stained both specifically and generally (Coomassie-Brilliant Blue (CBB) R-250 and silver stain). They yielded two ETK bands which varied in intensity and were cathodal to haemoglobin. Although qualitative analysis strongly suggested ETK to be heterogeneous, it did not provide means of investigating WKS by biochemical analysis.

Because the analysis of ETK (quantitatively and qualitatively) showed no apparent differences between the two groups, it was further investigated following the purification of the enzyme. PAGE crosslinked with DHEBA, a form of crosslinker which allows gels to be solubilized under alkaline conditions, provided means of isolating pure fractions of TK from red blood cells of normal controls and patients. These extracted fractions were subjected to analysis using high performance liquid chromatography (HPLC), isoelectric focusing (IEF) and endpoint assay. Despite confirmation of TK heterogeneity (with respect to ionic strength, isoelectric point (pI) and ETK activity), no differences in electrophoretic profile between patients and controls were revealed. In all samples two distinct bands of varying intensity appeared at pI 7.35 and 6.55 on IEF gels.

Further studies on native TK were carried out after purification from white blood cells which had been collected as buffy coats from normal individuals (Mocali & Paoletti, 1989). Enzyme recovery declined as the steps of purification progressed, therefore another purification method was developed from the method of Takeuchi et. al.(1986) (where enzyme recovery was not sufficient for amino acid analysis). The fractions isolated by this method were subjected to fast protein liquid chromatography (FPLC) on Mono Q column which yielded two peaks containing TK activity. The fractions were also loaded on an SDS-PAGE where the protein band had a molecular weight (MW) of 68kD (kiloDaltons). These results suggested that TK exists in at least two forms varying in ionic strength as revealed by the chromatographic profile from FPLC. Amino acid analysis and amino acid sequencing of TK was carried out on enzyme purified by DHEBA-PAGE. The extracted fraction was loaded on SDS-PAGE and then transblotted on to an Immobilon-P transfer membrane. The membrane was then stained with CBB R-250 to detect presence of protein, namely TK. Two distinct bands appeared with estimated MW of 51 and 35kD, similar to the MW estimated from purified ETK by Warnock and Prudhomme (1982).

Amino acid analysis of the 5IkD band showed the presence of 16 amino acid residues with molar ratios(%): **D**=11.95, **E**=7.43, **S**=11.85, **G**=6.62, **H**=3.90, **R**=2.00, **T**=5.20, **A**=9.29, **P**=7.18, **Y**=3.32, **V**=7.12, **M**=0.73, **I**=4.45, **L**=9.58, **F**=3.99, **K**=5.39. As for the 35kD band, amino acid analysis revealed 15 residues with molar ratio(%): **D**=7.39, **E**=6.62, **S**=6.31, **G**=8.85, **H**=5.24, **R**=2.27, **T**=6.06, **A**=13.56, **P**=5.75, **Y**=2.17, **V**=11.60, **M**=0.73, **L**=13.90, **F**=4.98, **K**=4.58. The molar ratios(%) of amino acids of each protein band were compared statistically using non-parametric Kendall and Wilcoxon signed rank tests. Both statistical analyses showed the two protein bands to be positively correlated with significantly similar distribution of amino acids. Therefore statistical analysis strongly suggests that the two protein bands represent similar proteins. An amino acid sequence of 15 residues (**V-L-L-P-A-E-E-T-N-V-K-T-A-L-G**) was obtained for the 35kD peptide but sequencing was not possible for the 51kD peptide due to N-terminal blockage. The amino acid sequence for the smaller peptide was compared with other sequences in the Gen Bank Database for existing homologies. This verified that the sequence

obtained was unique.

The work performed on the analysis of TK verifies the existence of at least two forms of the enzyme. They have different MW and pI values. No evidence was obtained to illustrate a difference in TK activity or its banding pattern between control and patient samples.

The amino acid sequence, described in this study, may be used as a template for oligonucleotide synthesis and then to clone the gene for the 35kD peptide or by cloning from a cDNA library generated using monoclonal antibodies. This would allow the role of TK in the aetiology of WKS to be investigated at the gene level.

CHAPTER 1: INTRODUCTION

1.1 WERNICKE-KORSAKOFF SYNDROME

1.1.1 General Background

The Wernicke-Korsakoff syndrome is a severe neuropsychiatric disorder which most commonly results from chronic alcohol abuse and malnutrition. The syndrome has a variable mode of onset but most typically presents with an acute phase (Wernicke's encephalopathy) that can evolve suddenly (hours to days) and may merge into the chronic phase (Korsakoff's psychosis) in some individuals. In a small number of cases, the chronic phase occurs without a previous history of Wernicke's encephalopathy.

Wernicke's encephalopathy (WE) is characterised by ophthalmoparesis, nystagmus, ataxia and an apathetic confusional state. This can result in recovery, death or a chronic Korsakoff's Psychosis (KP) of variable severity. KP is characterized by memory and learning impairment, anterograde and retrograde amnesia, sometimes coupled with confabulation. Historically, these two syndromes were described separately and many decades followed before the common pathology was recognized. It was the classic study of this condition (Victor et al., 1971) that coined the widely used term "Wernicke-Korsakoff syndrome" (WKS). Some controversy on whether KP can result independently from WE, or whether other coexisting pathology is essential, still continues (Freund, 1973; Freund, 1983; Lishman, 1990).

A current synonym for KP is alcohol amnestic disorders. However, non-alcoholic cases have been described (Hartman et al., 1987; Beatty et al., 1989; Becker et al., 1990; Parkin et al., 1991) and this synonym may therefore be misleading. The role of alcohol neurotoxicity in the aetiology of the syndrome is an issue of current contention (Freund, 1983; Harper, 1989; Lishman, 1990) and the most commonly held view is that the sole causative factor is thiamine deficiency (TD) (Victor et al., 1971; 1989). It is held by the proponents of this latter view that the early administration of thiamine completely reverses the syndrome and prevents a permanent KP stage. The severity of KP ranges from the mild learning difficulties

that do not impair normal memory but are detectable on psychological tests, through to severe memory impairment and apathetic states requiring constant nursing supervision.

The age of onset of the condition is variable but most often occurs in the fifth and sixth decades of life. The study of WKS by Victor and associates (1989) showed women tended to develop the condition a decade or two earlier than men and men outnumbered women. However, women were over-represented if the relative incidence of alcoholism in men as compared to women was considered.

The prevalence of WKS probably varies throughout the world, although the literature on epidemiology is sparse. Patients with KP often become long term residents in mental hospitals or alternative institutions. A survey of patients at the Boston State Hospital in 1954 showed that 6.4% of all patients had KP (Victor & Laureno, 1978). In Scotland data extracted from two surveys of longstay mental hospital patients (McCredie et al., 1983; 1990) revealed that patients with Korsakoff's psychosis or alcohol dementia formed a significant proportion of this population. Of those who had been in hospital between 1 and 6 years and were under 65 years at point of admission, 11.6% (66 out of 571) had this form of alcohol brain damage. Of those over 6 years in hospital and under 65 years at point of admission, 4.6% (121 out of 2606 individuals) had been diagnosed with this condition. (Smith & McColl, 1992). In addition, there is evidence of an accumulation of such cases in Scottish mental hospitals over the past two decades (Table 1.1). These patients are forming an increasing proportion of the long-stay mental hospital population (Smith & McColl, 1992). Given the disabling nature of this condition coupled with a near normal life expectancy these patients represent a substantial cost to the National Health Service.

Victor et al. (1989) regard alcoholic dementia as indistinguishable from alcoholic KP, both being attributed to TD and its resultant neurotoxicity. Others consider that the two entities are a spectrum of disease occurring from the interplay of alcohol neurotoxicity and thiamine depletion that results in cortical and sub-cortical brain pathology. The greater the former pathology, the more likely is the

Table 1.1: Accumulation of alcoholic Korsakoff's psychosis (291.1)* and alcoholic dementia (291.2)* in Scottish mental hospitals (1970-1990). (Reproduced from Smith & McColl, 1992).

YEAR	291.1	291.2	TOTAL
1970	33	-	33
1975	116	-	116
1980	148	10	158
1985	162	35	197
1990	189	58	247

* The Diagnostic and Statistical Manual of Mental Disorders (DSMIII) and the 9th edition of the International Classification of Diseases (ICD-9) classifies Korsakoff Psychosis as 291.1 and alcoholic dementia as 291.2 (Freund, 1983).

classification as a case of alcoholic dementia; the greater the latter, the more likely is classification as a case of alcoholic KP (Lishman, 1990).

The possibility of accurate cross-national comparison of the prevalence of the condition is limited due to a dearth of literature. Yellowlees (1986) reported that WKS was rare in the U.S.A. and U.K. while being very prevalent in Australia. However, many individuals with WKS do not show clinical symptoms and therefore are not diagnosed until post-mortem. For example, Harper (1983) found that of 131 cases of alcoholic WKS diagnosed at autopsy, only 26 had been suspected during life. Post mortem prevalence figures include 2.5% in Oslo, 2.2% in Cleveland and 1.7% in New York, U.S.A (Victor et al., 1989) but these may be under or overestimates as not all the deaths result in autopsies.

1.1.2 Historical Background

In 1881 Wernicke described three patients with similar symptoms of nystagmus, ophthalmoparesis, apathy and ataxia who subsequently died. Two of the patients were male alcoholics and the third was a 20 year old female who swallowed sulphuric acid that had led to uncontrollable vomiting. Wernicke concluded following postmortem studies of the brains that there were pathological changes in these cases representing an acute inflammatory disease of the oculo-motor nuclei ("polioencephalititis hemorrhagica superioris") leading to death (Victor et al., 1989).

In 1887 Korsakoff gave the first detailed account of the now eponymous amnestic disorder with a description of symptoms and pathology affecting both the central and peripheral parts of the nervous system. The description of the syndrome made by Korsakoff was given in a series of reports from 1887-1891. The first report was based on 20 alcoholic and non-alcoholic patients presenting with multiple neuritis for which he suggested the name "psychosis polyneuritica". With further studies, Korsakoff changed the name of this amnestic disorder to "Cerebropathia psychica toxaemica" after concluding that the disorder was a result of a toxin affecting the spinal cord and the brain. In 1897 Jolly suggested that KP should replace "Cerebropathia Psychica toxaemica". The clinical relationship between

WE and KP was not established until 1904 by Bonhoeffer et al.. In 1928 Gamper concluded that both disorders shared a characteristic neuropathology with similar anatomic distribution.

1.1.3 Pathology and Treatment

WKS is a focal syndrome with a characteristic histopathology affecting specific brain regions. The pathological changes in the WE are in the cortex, the cerebellum and in the midbrain and hypothalamus. Pathological changes in the brain cortex in KP were first reported in 1931 (Thomson et al., 1987). Such changes involve cerebral damage which exists over a wide range in the brain affecting the grey matter, third and fourth ventricles and Sylvian aqueduct involving the thalamus (Victor, 1971), hypothalamus, midbrain, floor of the fourth ventricle and the mammillary bodies with presence of cell necrosis (Hore, 1980). The brain ventricles tend to be larger in alcoholics with poor memory KP than normals making those alcoholics susceptible to cortical changes and possibly neuronal loss due to alcohol neurotoxicity (Lishman, 1990). However, the sequence of events leading to severe neurological dysfunction and irreversible histopathological changes of certain selective vulnerable parts of the brain remains unknown (Thomson et al., 1980).

Chronic alcohol abuse leads to nutritional deficiencies and, in some, organic brain damage. Thus chronic alcoholics can show neurologic disorders as a result of malnutrition. In addition, all clinical and pathological evidence presented by Neville et al. (1968) and Ron (1977) conclude that the major aetiological factor in most neurological disorders associated with chronic alcohol abuse is malnutrition (Thomson et al., 1980). The depletion of vitamins which are essential to cerebral function (thiamine, nicotinic acid, pyridoxine and vitamin B12) can result in neurologic damage.

The first report indicating the association of TD with WKS was made by Wagner and Weir (Victor et al., 1989). In 1978 Thomson concluded that vitamin deficiency may be a common cause of brain damage in alcoholics (Thomson et al., 1980). Long-term thiamine defeciency due to hyperemesis of pregnancy, chronic

gastrointestinal disease, malnutrition secondary to metastatic cancer and anorexia nervosa may all lead to the development of the WKS. Jollife and associates in 1941 followed by Phillips and associates in 1952 showed that TD causes the acute reversible phase of WE with or without the presence of alcohol (Freund, 1983). However, no evidence was available to show that TD causes KP rather than chronic alcohol abuse alone (Freund, 1973).

Damage from malnutrition and direct ethanol toxicity are not mutually exclusive in KP; they may contribute or interact to various degrees in causing mental impairment. In addition, available morphological evidence in some patients with KP support the possibility that diencephalic and mesencephalic lesions are induced by TD (Freund, 1973). Lishman drew the following hypothesis on the cause-effect in the WKS. Increased susceptibility to alcohol neurotoxicity can lead to cerebral shrinkage, cognitive impairment tending to recede with abstinence. However, increased susceptibility to TD results in a mild and transient WKS. Finally, increased susceptibility to both alcohol neurotoxicity and thiamine depletion develops into the chronic resistant KP (Lishman, 1990).

Recovery and the reversal of symptoms in WE patients after treatment with thiamine was demonstrated by Phillips et al. in 1952 and Gould in 1954 (Thomson et al., 1980, Hunt, 1983). In a study of 245 patients, with WKS followed up for 10 years the ocular abnormalities were reversed hours after thiamine supplementation in 60% of patients whereas the horizontal nystagmus remained. There was an improvement of the confusional state in 15% of patients with a complete recovery after a week or two. Less than 20% of all cases showed a significant degree of recovery. Large doses (50mg) of thiamine were administered, yet as little as 2-3mg would modify the ocular signs. (Victor, 1971). However, KP does not respond to thiamine treatment, possibly, due to an accumulation of structural pathology over the years. This would also contribute to the gradual development of the syndrome. (Lishman, 1990).

1.2 THIAMINE: VITAMIN B1

1.2.1 Definition and Characteristics

Thiamine is an organic molecule of molecular weight 337.28kD and is required as an essential dietary factor for physiological function. Foods rich in thiamine include whole grain cereals, pork and legumes followed by other meats, fish, green vegetables, fruits and milk. Poor sources include polished rice, sugar, alcohol, fat and other refined foods.

There are three common forms of thiamin esters, thiamine monophosphate (TMP), thiamine triphosphate (TTP) and thiamine pyrophosphate (TPP) also widely known as thiamine diphosphate (TDP). Approximately 30mg of thiamine is stored in the body as 80% TPP, 10% TTP and 10% TMP and free thiamine. Almost half of the body store of thiamine is found in the skeletal muscle while the rest is distributed amongst the heart, liver, kidneys, nervous tissues including the brain which contains mostly TTP (Gibson, 1990). The normal human erythrocyte contains 50-150ng of TPP per 1ml packed cells (Sauberlich, 1984).

Thiamine is absorbed in the small intestine by an active transport process. The energy required for thiamine uptake at low (physiological) concentration is provided from the hydrolysis of ATP by Na^+K^+ ATPase system. As for higher (pharmacological) concentration absorption is carried out by a non saturable passive diffusion process.

1.2.2 Biochemical Function of Thiamine

The biochemical significance of TMP and TTP is obscure, however it has been suggested by Pincus in 1969 that TTP is essential in the central nervous system (Victor et al., 1989). TPP is the active coenzyme form of thiamine. Therefore ingested thiamine must be phosphorylated in order to become active and functional.

This mechanism is carried out in all cells by adenosine triphosphate (ATP) which acts as a phosphate donor.

In 1953, Horecker demonstrated the role of TPP as a cofactor for a range of enzymes involved in carbohydrate and amino acid metabolism (Butterworth, 1989). TPP is the prosthetic group requiring magnesium (Mg^{++}) in two general types of reactions. The first reaction is the oxidative decarboxylation of α-keto acids catalyzed by the dehydrogenase complexes including pyruvate dehydrogenases (PDH) and α-ketoglutarate dehydrogenase. In the second reaction, transketolase (TK) catalyzes the formation of α-ketols (ketoses). The multienzymatic dehydrogenase complexes, affecting decarboxylative conversion of α-keto acids to acetyl cholinesterase derivatives, are utilized in Kreb's cycle in the mitochondria. Transketolase requires TPP for carbohydrate metabolism in the hexose monophosphate shunt (pentose phosphate pathway), an alternative pathway for the oxidation of glucose, and is essential in the synthesis of ribose for nucleotide formation (McConachie and Haskew, 1988). The interrelationship of these TPP dependant enzymes is illustrated in Figure 1.1.

1.2.3 Thiamine Deficiency

The central role of thiamine is in carbohydrate metabolism. Hence, there will be a greater demand for it when the main source of energy is carbohydrate. (Figure 1.2). This applies to chronic alcohol abusers, as their only source of energy is alcohol which metabolizes entirely as a carbohydrate. The National Research Council (NRC) recommends a daily allowance of 1mg of thiamine and slightly more for pregnant and elder individuals. This amount is utilized daily by the healthy well nutritioned adult. Therefore it is inadequate for the treatment of thiamine deficient individuals who would require 100mg of thiamine. In order to completely saturate all their body tissues 50mg of thiamine is administered parenterally and 50mg orally (Victor et al., 1989).

There are several mechanisms which give rise to a TD state. These include inadequate intake of thiamine and decreased intestinal absorption as a result

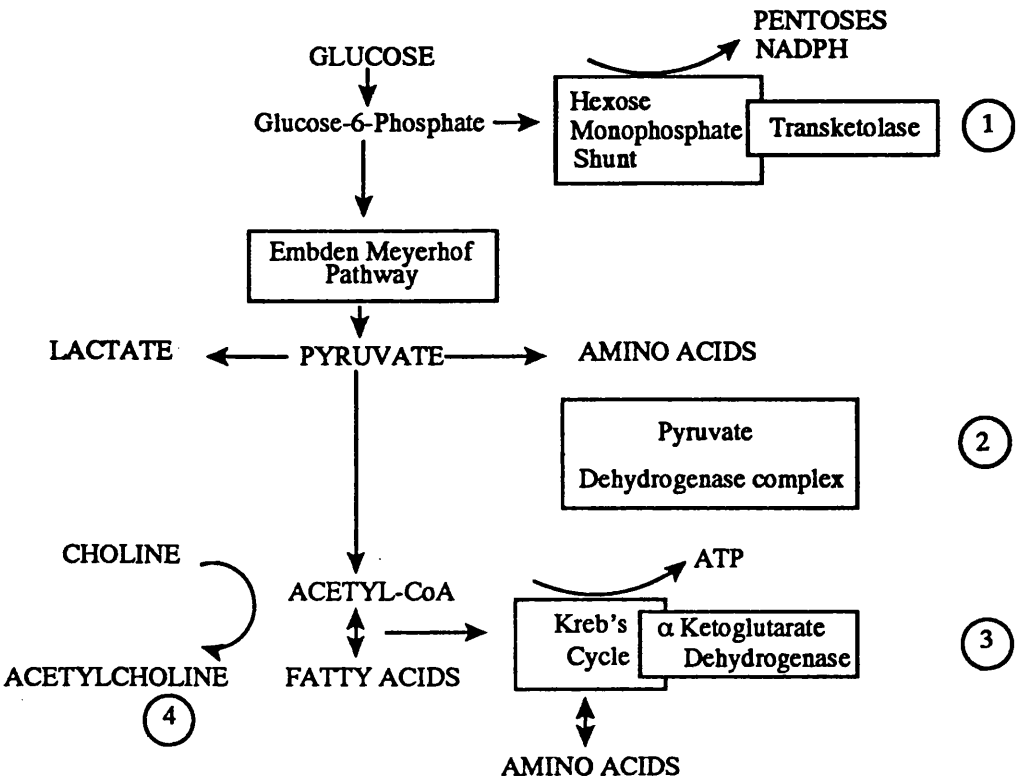


Figure 1.1: Enzyme activities impaired in thiamine deficiency. The enzymes effected are transketolase (1), pyruvate dehydrogenases (2), α-ketoglutrate dehydrogenase (3), and the synthesis of acetylcholine (4) (Schenker et al., 1980).

of chronic alcohol abuse (Thomson, 1980; Butterworth, 1989). Other effects of chronic alcohol abuse leading to TD, include reduction in phosphorylation of thiamine in the brain (Butterworth, 1989), increased metabolic demands ('empty' calories) (Figure 1.2), ethanol inhibition of Na^+K^+ ATPase system (Rosalki, 1984), and reduced storage capacity. Other mechanisms involve intake of food preparations which contain anti-thiamine factors, increased excretion of thiamine in the urine (Gradual et al., 1985) and finally presence of diarrhoea or infection. The latter will cause Mg^{++} deficiency which would impair the turnover of thiamine (Traviesa, 1974; Victor et al., 1989). The time required to produce a TD state is variable and may develop rapidly in a matter of hours, or may take weeks (Thomson et al.,1980; Victor et al., 1989). TD causes biochemical lesions in the brain as a secondary effect of chronic alcohol abuse (WKS) or as a direct effect.

1.2.3.1 Effect of Thiamine Deficiency on the Brain

Thiamine plays an important role in the overall function of the nervous system (Thomson et al., 1980; Rosalki, 1989). The brain becomes particularly vulnerable to TD as a result of the overall margin between the thiamine flux across the blood-brain barrier and the turnover rate of thiamine (Thomson et al., 1987). It is also known that diets lacking in thiamine result in alteration of brain metabolism. However, it is unknown at which point neurochemical transmission is affected, should the nerve-cell metabolism become susceptible, and at what level irreversible damage occurs (Thomson et al., 1980). Moreover, malfunction of blood-brain barrier (may be a result of direct ethanol toxicity) and failure of thymidine incorporation into brain DNA (reversible with thiamine treatment) have been illustrated (Thomson et al., 1980; Rosalki, 1984).

In the 1960's, Dreyfus and coworkers studied the enzymes affected by thiamine depletion in the nervous system. The mechanism of TD encephalopathy had been suggested by Parker and colleagues to be an impairment of PDH activity which results in a decline of cerebral ATP synthesis and acetyl-CoA production from pyruvate (Figure 1.1). However, Dreyfus and Hauser (1965) demonstrated that PDH was less susceptible to thiamine depletion and concluded that the "biochemical

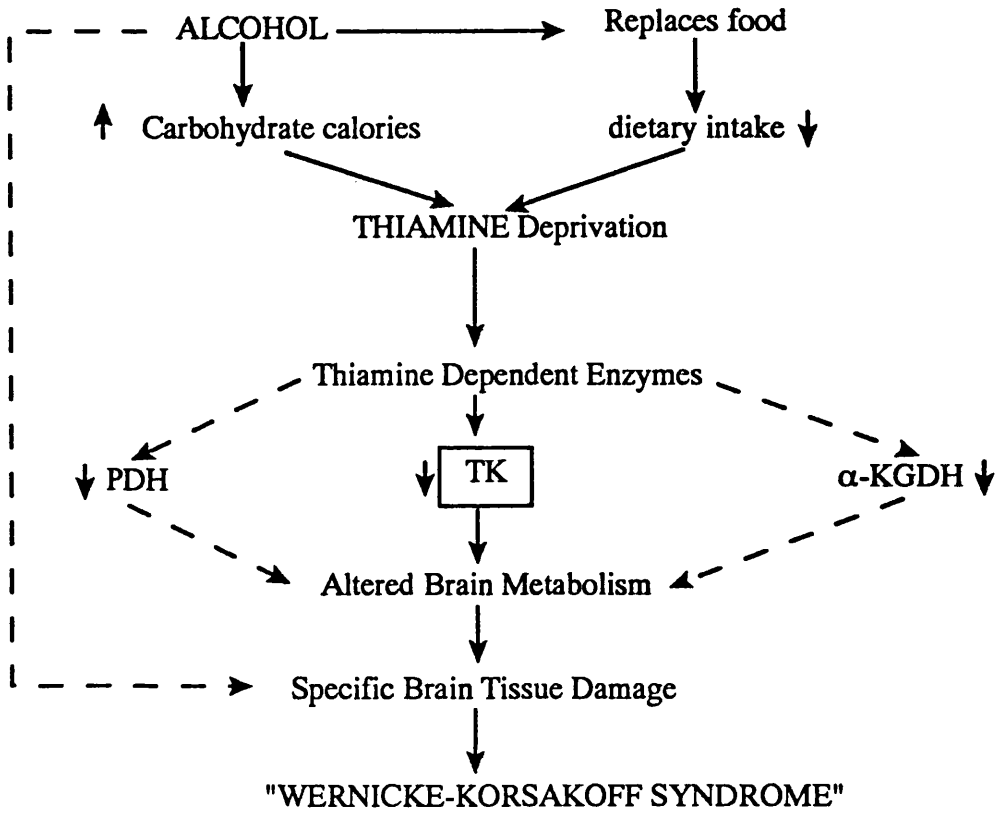


Figure 1.2: The role of chronic alcohol abuse and malnutrition in Wernicke-Korsakoff syndrome.

TK - Transketolase
PDH - Pyruvate dehydrogenase
α-KGDH - α-Ketoglutarate dehydrogenase

lesion" incurred by TD was a failure in TK activity (Figure 1.2). McCandless and Schenker (1968) demonstrated a greater decline of tissue TK activity as compared with PDH activity during induced TD. They also demonstrated that, in addition to the reversal of several neurologic signs following thiamine treatment, PDH activity returned to normal whereas TK activity increased slightly. Gibson et al., (1988) confirmed low TK activity (30-32% of normal) despite thiamine supplementation while α-KGDH and glucose oxidation returned to normal. Several other studies have been performed to investigate the effect of induced TD on PDH, α-KGDH and TK in peripheral and central nervous system of mammals (rats, monkeys) and other species (pigeons) (Gibson et al., 1988; Rindi, 1989; and Witt, 1985). Other works on α-KGDH and PDH in brains of rats and other animals were reviewed in 1989 by Victor and associates.

1.2.3.2 Beri Beri

Beri beri is the classical syndrome of TD. It has a high prevalence in the Orient where the main source of food is polished rice. Due to improvement in nutritional status the incidence of beri beri has declined but sporadic cases have been reported (Kuriyama et al., 1980). The major clinical manifestation of beri beri are of cardiac and neuropathetic origin.

The apoenzymes PDH, α-KGDH and TK have been studied in beri beri patients. The levels of PDH and α-KGDH have been found to be elevated whereas TK activity was reduced (Victor et al., 1989). Direct assessment of thiamine status has been widely applied for diagnosis of beri beri followed by analysis of TK activity (Kuriyama et al., 1980).

1.2.4 Assessment of Thiamine Status

Several biochemical parameters can be used to measure levels of thiamine (Sauberlich, 1967; Victor et al., 1971). However, direct measurements of thiamine levels in serum, erythrocytes, whole blood and urine have not proved satisfactory due to the limitation of the methodology, and the fact that the decrease

in blood thiamine levels during deficiency is not great (Sauberlich, 1984). Also, daily fluctuations in thiamine levels in normal individuals may mimic the low levels associated with TD (Kuriyama et al., 1980).

Biochemical tests based on levels of TPP, that is measuring coenzyme activation of erythrocyte enzymes rather than thiamine, may reflect more accurately the status of the individual. One such test used measures the ratio of lactic acid to pyruvic acid in blood after administering glucose. These metabolites would be elevated in TD (Victor et al., 1989). A more recent and widely used biochemical test is measuring erythrocyte transketolase (ETK) activity before and after addition of TPP in vitro (Rosalki, 1984).

1.3. TRANSKETOLASE (E.C. 2.2.1.1)

1.3.1 Definition and Function

TK (E.C. 2.2.1.1.) is a conjugated enzyme consisting of a protein moiety, the apoenzyme, and a prosthetic group, the coenzyme, joined by a metal ion, the cofactor. The prosthetic group serves as an intermediate carrier of specific functional group. In 1976, Conn and Stumpf reported that TK is made up of two identical subunits utilizing both TPP as its prosthetic group and Mg^{++} as its cofactor. In the presence of these two factors, the transketolase holoenzyme is formed and activated. Horecker and Smyrniotis (1953) and Baker et al. (1953) demonstrated the requirement for TPP. The native enzyme TK had been shown to contain Mg^{++} in its molecular structure (Victor et al., 1989). The positive cooperative interaction between TPP binding sites and Mg^{++} was demonstrated by Kochetov et al (1975). Moreover, Egon and Sable suggested the function of Mg^{++} to be related to the modification of the enzyme structure in the complex formation with TPP (Jung et al., 1988).

TK is a cytoplasmic enzyme creating, like transaldolase (TA), a reversible link between the hexose monophosphate shunt and glycolysis (Figure 1.3). The reactions catalyzed by these enzymes yield the formation of two hexoses and one

triose from three pentoses (Reactions 1, 2 and 3 in Figure 1.3). That is, TK catalyzes the transfer of a 2-carbon unit, activated aldehyde group, from a 5-carbon sugar ketose (ketol donor) to an aldose (aldehyde acceptor). The specific reaction catalyzed by TK is illustrated in Figure 1.4. In addition, TK participates in recycling ribose-5-phosphate into glucose-5-phosphate for glycolysis.

1.3.2 Brain Transketolase and Thiamine Deficiency Related Disorders

The work by Peters and colleagues on thiamine deficient brain tissue, in the 1930's, demonstrated the basis for the organic brain disorder to be a failure of some thiamine dependent metabolic process. The discovery of TK in the hexose monophosphate shunt (1950's) provided the basis of the biochemical lesion in the central nervous system of TD. It was also illustrated that TK was significantly reduced at the early stages of TD (Pratt et al., 1985). The findings of Dreyfus and Hauser in 1965 have also confirmed that the the failure in the TK system might be responsible for initiating clinical and pathological changes. Their work has also indicated that TK activity varies from one part of the nervous system to another. It was lowest in the cerebral cortex, highest in the spinal cord and greater in heavily myelinated structures than in neuronal masses. This may be related to the disproportion of effected myelin structures comprising both nerve cells and myelinated fibres (e.g. mammillary bodies). (Victor et al., 1989)

Despite severe reduction of TK activity in the brain, McCandless and coworkers in 1981 and 1982 reported the hexose monophosphate shunt to be unaffected in induced TD (Victor et al., 1989). Giguere and Butterworth (1987) also found a 25% reduction of TK in the lateral vestibular nucleus and surrounding pons prior to onset of neurological symptoms in chronically thiamine-deprived rats. This had persisted in the brain stem and cerebral regions despite treatment of thiamine. Adding TPP in vitro did not normalize TK activity suggesting loss of the apoenzyme (Giguere & Butterworth, 1987; Gibson et al., 1989; Butterworth, 1989; Thomson et al., 1980). Finally, Mukherjee et al. (1987) found that ribose-5-phosphate (R-5-P), produced from reversed reaction of TK (Figure 1.3), was essential in maintaining

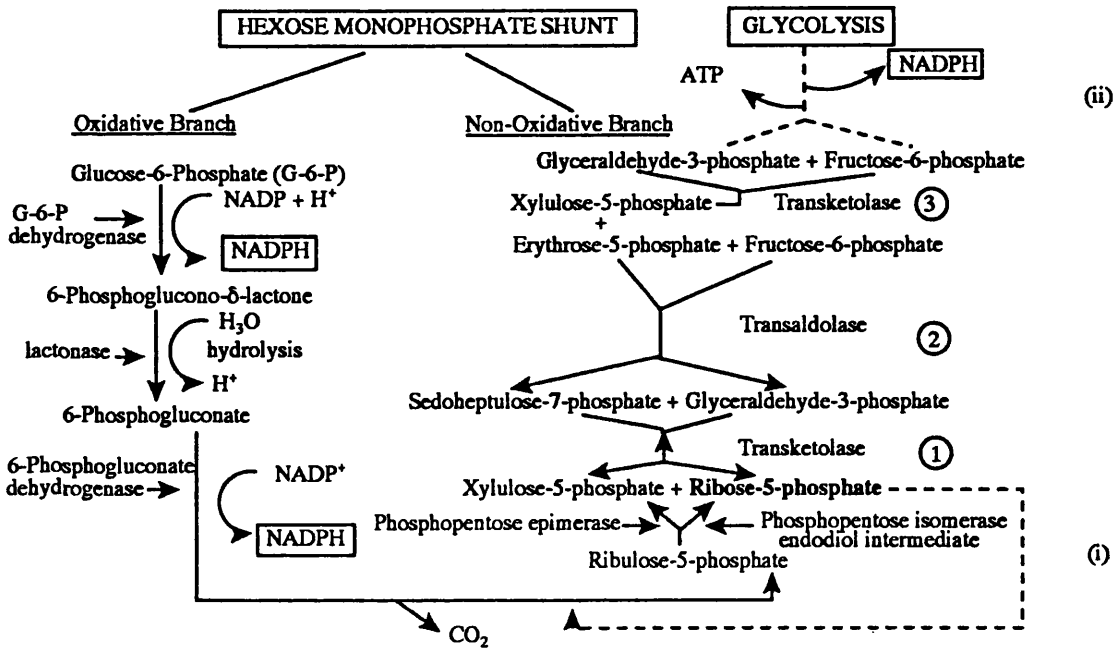


Figure 1.3: Metabolic pathway of hexose monophosphate shunt. Products required for reductive biosynthesis (NADPH) and nucleotide synthesis (ribose-5-phosphate) are illustrated. The flow of glucose-6-phoshate depends on the need of:

a) ribose-5-phosphate > NADPH then the flow of reactions 1,2, & 3 are reversed by transketolase and transaldolase.
b) ribose-5-phosphate = NADPH then flow as illustrated above
c) ribose-5-phosphate < NADPH then
   i) Recycling of ribose-5-phosphate into glucose-6-phosphate in the oxidative branch
   ii) Proceeding of glyceraldehyde-3-phosphate and fructose-6-phosphate to glycolysis.

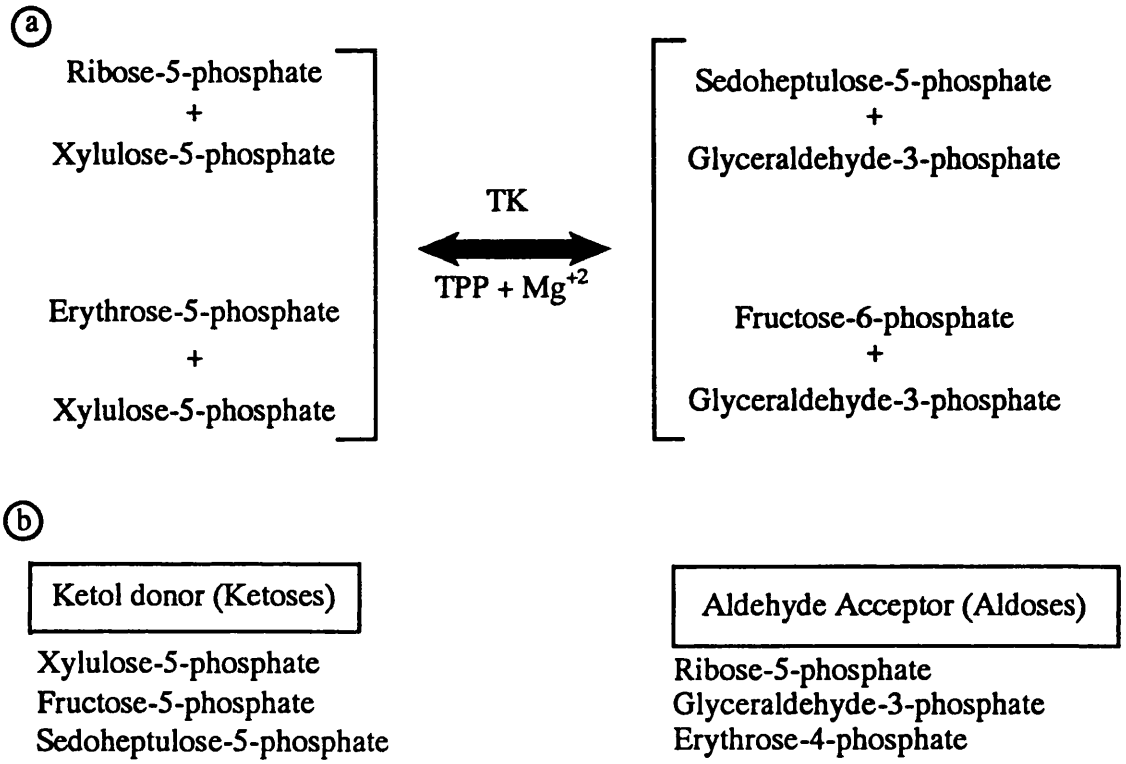


Figure 1.4: Reactions catalyzed by transketolase (TK) in the hexose monophosphate shunt.
a) specific substrates utilized by TK and the products formed
b) list of ketoses and aldoses transfered by TK in the presence of cofactors thiamine pyrophosphate (TPP) and Mg^{++}

cerebral gluthione (GSH) in the reduced state, thus a decline of NADPH will result in low GSH impairing the function of the cerebral metabolic pathway.

1.3.3 Clinical Application of Transketolase Assay

In 1958 it was discovered that TK activity could be monitored in red blood cells (RBC) and an abnormal activity may be restored by the addition of TPP, the TK activation test (Brin et al., 1960). This abnormality also reflects similar changes in the brain (Pratt et al., 1985). It was the work of Brin and associates that demonstrated the presence of TK in RBC, and that it was the only thiamine dependent enzyme in RBC. They were also the first to develop a method for the estimation of TK in red cell hemolysates (Brin et al., 1960). Two years later, Dreyfus and Moniz developed a simple and reproducible method for direct assay of TK activity from microgram quantities of tissues and microlitre amounts of whole blood using a calorimetric method. Their findings suggested that TK activity varies with levels of coenzyme present. The addition of TPP into the assay would increase enzyme activity and is related to the degree of the saturation of enzyme with cofactor. This is termed the "TPP effect" (TPPE) and is expressed as a percentage of the basal activity. TPPE was found to reflect the amount of apoenzyme not saturated with thiamine.

1.3.3.1 Measurement of Erythrocyte Transketolase Activity

The method of Dreyfus and Moniz (1962) requires two haemolyzed blood samples, both incubated with excess R-5-P substrate reagent and one with excess TPP. ETK activity is determined by the formation of sedoheptulose-7-phosphate (S-7-P) measured spectrophotometrically, and expressed in μg/ml haemolysates. The difference in enzymatic activity with and without the addition of TPP in vitro is expressed as percentage of TPP.

Despite the modification (Warnock et al., 1975; Takeuchi et al., 1984)

of the previous method (Dreyfus & Moniz, 1962) extreme care and caution were required to produce reliable results (Rosalki, 1984). Therefore, an enzymatic method for determining ETK activity has been employed widely in the last twenty years or so which provides more consistent and reproducible results. The method employed is based on the coupled reaction formed by linking TK reaction to a dehydrogenation step that can be monitored by following the change in absorbance at 340nm spectrophotometrically (section 2.1.4.1). This was first described and applied by Smeets et al. (1971) (on 10 samples) who reported mean ETK activity to be 0.368 IU/gHb at a haemoglobin (Hb) concentration in hemolysate of 45-50g/l. That is, one unit of TK activity was defined as the amount of enzyme that catalysed the formation of 1μmol of NAD per minute.

To summarize, the principle of the ETK activity test is based on four main criteria. The first criterion is that the basal activity of TK in erythrocytes (ETK) represents the endogenous enzyme (holotransketolase, HoloTK) activity depending on amount of coenzyme already present in erythrocyte. The second criterion is that the determined stimulated TK (ETKs) activity by the addition of excess TPP in vitro represents the maximum potential enzyme activity. Both ETK and ETKs activity can be expressed as per gram of Hb, per number of RBC's or in terms of erythrocyte volume (mL). The third criterion compares the values ETK and ETKs activity to indicate the degree of enzyme unsaturated with coenzyme. The final criterion regards the expression of data in terms of activity coefficient or the elevation of TPPE, expressed as a percentage. It must also be noted that the ratio of ETKs to ETK is used due to the elevation of ETK activity in inter-subject variation, and on the assumption that the apotransketolase (ApoTK) levels are not affected by TD.

1.3.3.2 Index for Thiamine Deficiency and Other Disorders

In 1958 Brin et al. demonstrated the activity of TK to be markedly depressed in the erythrocytes of TD rats and in humans with WE. The use of ETK activation by TPP as an indirect measure of thiamine content was applied since it was inversely related to thiamine concentration in erythrocytes. It was also concluded that

in TD, in vitro ETK activity falls whereas TPPE rises (Sauberlich, 1967). Following this, several workers began applying the ETK activity and TPPE test for thiamine assessment in population studies, in disorders relating to malnutrition, and in the diagnosis of WKS.

Population studies assessing nutritional status by ETK activity and TPPE tests have been carried out using colorimetric methods (Vuilleumier et al., 1983; Londsdale, 1988; Fidanza et al., 1989; Hill & Truswell, 1990) or enzymatic methods (Duffy et al., 1981). Duffy et al. (1981) measured ETK, ETKs activity and TPPE on 1920 Melanesians from Torres Strait Islands and found 21% had elevated TPPE (>15%) while 2.2% had a high TPPE (>20%). It was concluded that 10-20% of the Australian population had a daily thiamine intake below the recommended amount. In 1983 Vuilleumier et al. performed ETKA activation test on 150 blood donors in Switzerland and found that 6.7% had a moderate risk for TD. Lonsdale (1988) studied the ETK, ETKs activity and TPPE in 1011 patients from 1983 to 1986 attending a private practice specializing in nutritional correction. It was found that 24% of patients were thiamine deficient (elevated TPPE) while 28% had either/both abnormal ETK activity and TPPE, suggesting an abnormality of ApoTK or other factors. Lonsdale concluded that there is a widespread health problem in the U.S. relating to modern nutrition (Lonsdale, 1988). Hill and Truswell (1990) evaluated ETK activity and TPPE in 107 homeless men from two hostels and a clinic in Sydney, Australia to assess nutritional status. Their findings showed 36% of those men had elevated TPPE (21% had marginal and 15% had severe deficiency) and concluded that the nutritional status results not only from dietary effects but also from environment, ill-health and genetic factors. All these studies indicate the reliability of ETK activity and TPPE for thiamine assessment and its relation to nutritional status. (Duffy et al., 1981; Vuilleumrer et al., 1983; Lonsdale, 1988; Fidanza et al., 1989; Hill & Truswell, 1990). Finally TPPE has been shown to be independent of age and sex (Duffy et al., 1981; Jeyasingham, 1987; Lonsdale et al., 1988).

The TK activation test has been employed in the assessment of several other disorders. In 1977 Kjosen & Seim studied ETK activity and TPPE in patients

with diabetes mellitus, anemia, polyneuritis and malnourishment secondary to vascular disease of brain. Their findings showed ETK activity to be reduced in patients with diabetes mellitus and polyneuritis while elevated in those with pernicious anaemia. ETK activity in the malnourished alcoholic group, control alcoholic group and a normal control group did not vary significantly nor did TPPE between all groups regarding TPPE. This would suggest a variation in ApoTK levels that results from disease and not malnutrition (Kjosen & Seim, 1977). However, ETK activity and TPPE assessed in beri beri patients showed a decline in ETK activity with elevated TPPE as a result of TD (Kuriyama et al., 1980; Kawai, 1980). Gibson et al. (1988) studied ETK activity in Alzheimer's disease and found a reduction of ETK activity to 45% in the brain and peripheral tissues with other abnormalities in RBC and fibroblasts. These studies suggest that ETK activity and TPPE should be applied independently with regard to the aetiology of the disorder (Sauberlich, 1984).

ETK activity and TPPE in chronic alcohol abusers have been studied in order to establish alcohol neurotoxicity, thiamine deprivation and the development of WKS. Fennelly et al. (1967) evaluated ETK activity in malnourished (TD) alcoholics (n=17) with liver cirrhosis and signs of neuropathy and found that 10 patients had reduced levels of ETK activity. It was also found that ETK activity in TD patients without liver disease was increased significantly after in vitro addition of TPP in RBC hemolysates whilst having a negligible effect on those with liver cirrhosis. Similar results were observed after oral administration of thiamine which did not improve peripheral neuropathy nor ETK activity in patients with severe liver cirrhosis. This suggests that the abnormality of ETK activity was unrelated to the blood thiamine level (Fennelly et al., 1967). Melgaard and associates (1989) also reported no differences in ETK activity between a group of alcoholics and a normal group which suggests no relation between alcoholic neuropathy and nutritional deficiency. Thiamine status in alcoholic patients (n=20) before and after intramuscular thiamine hydrochloride supplementation (200mg) was studied by the TK activation test. It was found that ETK and ETKs activity in alcoholic patients were significantly different from normal controls (McLaren et al., 1981; Gradual et al., 1985) and that the treatment had no significant effect on ETK and ETKs activity

but TPPE was significantly decreased. Kerr and associates also studied the effect of oral treatment with thiamine on ETK activity in alcoholic WKS patients (n=16), and normal controls (n=11) maintained for several months and then stopped abruptly. These findings showed a rapid decline (completing in 4 weeks) of ETK activity in WKS while there was a gradual decline of ETK activity in normal controls. This difference between the response of the two groups to thiamine withdrawal supports the hypothesis that at least some WKS patients possess an abnormality in TK (Kerr et al., 1986). Finally, there was one report describing an abnormality of TK in a WKS patient due to magnesium deficiency which was improved by treatment with intramuscular $MgSO_4$ suggesting importance of Mg^{++} ion as a cofactor (Traviesa, 1974). All these studies (Fennelly et al., 1967; McLaren et al., 1981; Gradual et al., 1985; Kerr et al., 1986) strongly suggest that abnormality in ETK activity reflects abnormality in TK and that of TPPE reflects abnormality in thiamine status only. Morevoer, both chronic abuse and TD can result in WKS (Sauberlich, 1967; Victor et al., 1971) as a result of an abnormality in TK (Sauberlich, 1989; Victor et al., 1989).

1.3.4 Biochemical Analysis of Purified Holotranskelotase

Human tissue TK, unlike yeast and some mammalian species, has not been fully characterized with respect to its catalytic and chemical properties due to a lack of homogeneous enzyme preparation (Jung et al., 1988). Most of the known properties on TK were derived from studies on purified TK from spinach in 1971, yeast in 1961, pig liver in 1960 and from rat liver in 1953 (Paoletti, 1983; Himmo et al., 1988). The purification of rat liver TK allowed the determination of its molecular weight (MW) 139kD (made up of two subunits of 69kD) with multiple forms having pI values from 7-8 as detected by isolectric focusing (IEF). (Paoletti, 1983; Paoletti Aldinucci, 1986). The isolation of TK from the rabbit liver was reported to have a MW of 162kD near to that of Baker's yeast (158-159kD) (Masri et al., 1987).

Several reports have attempted the isolation of TK from human

erythrocytes and other tissues, in a purified homogenous form, to characterise its properties. Purification techniques involved mostly ion exchange chromatography (Warnock & Prudhomme, 1982; Greenwood et al., 1984; Takeuchi et al., 1986; Jung et al., 1988; Sheu et al., 1988), gel filtration (Pratt et al., 1985; Mocali & Paoletti, 1989), affinity chromatography (Himmo et al., 1988; Mocali & Paoletti, 1989) and/or application of sophisticated techniques such as fast protein liquid chromatography (FPLC) (Takeuchi et al., 1986; Jung et al., 1988).

Preparations of pure homogenous human TK showed that it existed in more than one form differing in MW (Warnock & Prudhomme, 1982; Pratt et al., 1985, Sheu et al., 1988), ionic strength (Greenwood et al., 1984; Takeuchi et al., 1986; Mocali & Paoletti, 1989), and TPP binding affinity (Warnock & Prudhomme, 1982; Greenwood et al., 1984; Pratt et al., 1985; Jung et al., 1988). Such preparations also provide a mean of estimating Km values for substrates utilized by TK (3.0-4.0 x 10^{-4} M for R-5-P and 1.5-2.0 x 10^{-4} M for X-5-P) with an optimum pH of 7.6-8.0 (Warnock & Prudhomme, 1982; Takeuchi et al., 1986; Himmo et al., 1988) at temperature 40-50°C (Takeuchi et al., 1986; Himmo et al., 1988). Purified TK was shown to be inhibited by phosphate ions (0.01M) (Prudhomme & Warnock, 1982; Himmo et al., 1988). This was not confirmed by Takeuchi and associates (1986) who reported a maximum velocity for TK (4.2 X 10^2 mol/min/mol of enzyme) and that 1 mol TK subunit contained 0.9 mol TPP. It was also found that HoloTK was more heat stable than ApoTK (Takeuchi et al., 1984; Sheu et al., 1988) and could be stored below 0°C for several weeks without loss of activity (Warnock & Prudhomme, 1982; Takeuchi et al., 1986; Sheu et al., 1988; Mocali & Paoletti, 1989). Finally, the homogenous preparations of TK allowed the analysis of the amino acid composition (Takeuchi et al., 1986) and generation of polyclonal antibodies in order to characterize catalytic activity of TK by immunoaffinity and physical properties of TK by immunochemical staining of western blots (Takeuchi et al., 1986; Jung et al., 1988; Sheu et al., 1988; Mocali & Paoletti et al., 1989).

Partial purification of ETK by ion-exchange chromotography eluted in two peaks which were well separated from hemoglobin (Kaczmarek & Nixon, 1983; Greenwood et al., 1984) and differed in the proportion of ApoTK present. The later

eluted peak of ETK activity required the addition of TPP in order to express activity (Greenwood et al., 1984). Similarly, gel filtration also yielded two peaks of ETK activity differing in binding affinity and MW. The low affinity enzyme (which requires TPP for activation) was shown to be half the MW of the high affinity enzyme (which is firmly bound to TPP) as determined by its position on the chromatograph (Pratt et al., 1985; Thomson et al., 1987). Other work has shown heterogeneity of TK with respect to its MW with 50-60, 26-30 and 33kD (Warnock & Prudhomme, 1982).

The detection of multiple forms of TK, differing in pI values, by IEF analysis of partially purified human ETK was first reported by Kaczmarek and Nixon (1983). This finding indicated the presence of 8 individual species of ETK (detected by TK specific activity stain) with pI values ranging from 6.6-9.2 and the presence of six different patterns of these species in 25 healthy individuals having common isoenzymes at pI 7.5, 7.8 and 8.1. Kaufmann et al. (1987) confirmed the presence of at least two ETK bands with pI 7.3 and 8.6 in all 63 healthy individuals by the same method. A somewhat similar analysis was reported by Paoletti et al. (1989) who applied IEF techniques and western blotting for separation of TK from fresh human leukocytes. Their findings also confirmed the presence of at least two TK variants differing in pI (range 7.4-8.4) and specific activity. The rare variant (1.5% of 217 healthy individuals) had 20-25% less activity from the standard as detected by immunostaining (Paoletti et al., 1989).

The above reports and others, on the purification and partial purification of TK have shown TK to be heterogenous in ionic strength, pI values (Kaczmarek & Nixon, 1983; Greenwood et al., 1984; Paoletti et al., 1989), MW (Warnock & Prudhomme, 1982; Kaczmarek & Nixon, 1983; Pratt et al., 1985; Thomson et al., 1987; Sheu et al., 1988) binding affinity and activation by TPP (Greenwood et al., 1984; Pratt et al., 1985; Thomson et al., 1987; Jeyasingham & Pratt, 1988).

1.4 Studies of Human Erythrocyte Transketolase in Wernicke-Korsakoff Syndrome

Several reports have described variation in TPPE values and its elevation (Smeets et al., 1971; Bayoumi & Rosalki, 1976; Duffy et al., 1981; Kjosen et al., 1977; Sauberlich, 1984; Lonsdale, 1988) in TD and related disorders with particular emphasis to WKS (Victor et al., 1971; Sauberlich, 1984). In a particular study, Bayoumi and Rosalki (1976) investigated the TK activation test and concluded that abnormal ETK activity and TPPE are directly related to clinical evidence of TD based on their study of normal and alcoholic subjects. Of 47 alcoholic and malnourished inpatients, 13 had abnormal ETK activity (one geriatric patient, 8 alcoholics with WKS and 4 alcoholics with no clinical TD) while those patients with no clinical evidence of TD had normal ETK activity. This report and others suggested the finding of TK variation results from the dissociation of part of the enzyme into its ApoTK (Jeyasingham et al., 1987) or from an abnormality in the binding affinity of ApoTK to coenzyme (Kjosen, 1977; Gradual et al., 1985; Kerr et al., 1986; Takeuchi et al., 1988) and that the abnormality is most prominent in WKS (Blass & Gibson, 1977; Greenwood et al., 1984; Pratt et al., 1985; Kerr et al., 1986; and Jeyasingham et al., 1987).

The classical WKS which occurs in a small number of patients, most commonly as a secondary effect of chronic alcohol abuse may be caused by thiamine depletion superimposed on a genetic abnormality of TK. This was first postulated by Blass and Gibson in 1977 who examined TK in cultured cells from four patients with WKS. They found a significant difference ($p<0.001$) between the apparent Km values for TPP in fibroblast TK of patients and normal controls. This abnormality of TK in fibroblasts continued through several passages in culture medium containing excess thiamine and no alcohol. Blass and Gibson (1977) thus concluded that the biochemical abnormality in TK from WKS fibroblasts appear to be genetic and may be a result of an apoenzyme mutation, rather than a consequence of disease (Olson & Doisy, 1979).

There are several clinical and epidemeolgical observations supporting

a genetic aetiology of WKS: (i) there is only a small minority of alcoholics, both TD and non TD, and other chronically malnourished persons who develop WKS (Victor et al., 1971; Blass & Gibson, 1979), (ii) it occurs more frequently in Europeans than non-Europeans on a similar thiamine diet whereas Asians develop "wet" cardiac beri beri (Sauberlich, 1967), (iii) the variation and overlap of ETK activity among and between normal populations (Sauberlich, 1967), (iv) the existence of patients in whom the addition of TPP in vitro and large thiamine doses in vivo does not restore normal ETK activity nor reverse symptoms (Sauberlich, 1967). These, and the fact it is rare, provide evidence of a genetic predisposition (Blass & Gibson, 1979).

Further evidence of a genetic abnormality of TK in WKS comes from a report of a pair of monozygotic twins, one of whom suffered from WKS (verified at autopsy) and one unaffected. Both twins had low basal ETK activity, similar to that described by Blass and Gibson in 1977 (Leigh et al., 1981). Mukherjee and associates (1986) studied ETK activity from fibroblasts of a diabetic patient who developed WE after treatment with tolzamide, and 3 diabetic kindred with no history of WE. It was found that the Km value for TPP of the patient and one diabetic sib were similar to the values estimated in WKS. Mukherjee and associates had also reported a TK abnormality in familial chronic alcoholic males, in their non alcoholic sons and in 3 generations of a non-alcoholic Amish family. These findings indicated that TK abnormality may exist in a non-alcoholic population and that it may be present in male and female sibs (Mukherjee et al., 1987). In 1988 Lonsdale described a TPPE abnormality in an 8 year old girl who was suffering from recurrent infection, similar to her mother and father suggesting an unknown familial component. Finally, the work performed by Nixon and associates strongly suggested genetic variation of TK. By applying IEF techniques on ETK in 42 patients with WKS and 36 normal controls, a specific isozyme pattern, (characterized by pI values ranging 6.6-9.2) was detected in 39 patients with WKS but in only 8 normal controls (Nixon et al., 1989).

Recent studies have implied that the abnormality of TK in WKS may be a result of environmental factors and not genetic. It has been reported that a severe alcoholic state is the immediate cause of damage to TK which produces an

abnormal variant more readily in thiamine deficient individuals (Pratt et al., 1985). This variant may be structural (e.g. smaller molecule), catalytic (low binding affinity to TPP) or a combination of both and is present in high proportions in patients with WKS (Greenwood et al., 1984; Pratt et al., 1985; Jeyasingham et al., 1987; Thomson et al., 1987; Nixon, 1983; Nixon et al., 1984). It had also been reported that the decline in ETK and ETKs activity may result from loss of both apoenzyme and coenzyme. This can lead to a decreased synthesis, or increased destruction of apoenzyme, to a greater degree in malnourished alcoholics and in those with WKS (Kerr et al., 1986; Takeuchi et al., 1988). The formation of a damaged or degraded TK variant in WKS, with respect to its binding affinity with TPP, may cause impaired brain metabolism that can result in brain tissue damage (Pratt et al., 1985; Thomson et al., 1987; Jeyasingham et al., 1987).

Pratt, Jeyasingham and associates (1985-1988) provided a tentative explanation on how brain damage occurs in TD and why only certain individuals were susceptible to the more severe form of WKS. Their conclusions were supported by Lishman (1990) who also suggested that TK variants could have arisen from damage to the TK system (Lishman, 1990), although a real genetic component may be present (Pratt et al., 1985). Chronic alcohol abuse may cause damage to TK which is accelerated in a thiamine deficient state and could lead to an impairment of TK activity (Pratt et al., 1985; Lishman, 1990). Thus, high energy phosphate will be reduced below the normal level and is compensated for by increasing the rate of brain glucose utilization. This will lead to excessive production and accumulation of lactate in the brain cells due to a limitation of substrate transport across the blood-brain barrier, and results in brain damage associated with WKS (Pratt et al., 1985). It has also been suggested that ageing effects and liver status may also contribute to abnormality in ETK activity and brain damage in WKS (Fennelly, 1967; Thomson et al., 1987).

1.5 AIMS OF THE STUDY

The aim of this study is to examine the role of TK in the aetiology of WKS by application of multiple methods of TK analysis.

Three complimentary approaches are used:

1. Quantitative investigation of TK by evaluation of existing (published) methods of TK assay and development and application of an optimised method to measure endogenous TK activity before and after activation with cofactor in erythrocytes from patients with WKS and normal controls.

2. Qualitative investigation of endogenous ETK and partially purified ETK using a variety of protein separation methods to search for evidence of structural variations in WKS patients.

3. Characterisation of TK by preparation of a purified enzyme suitable for analysis by amino acid composition and amino acid sequencing.

CHAPTER 2: MATERIALS & METHODS

2.1 ANALYSIS OF ERYTHROCYTE TRANSKETOLASE IN CRUDE SAMPLE

2.1.1 Sample Collection and Storage

Control blood samples (30ml) were obtained by venepuncture from a total of 25 students and members of staff at the Duncan Guthrie Institute of Medical Genetics and collected in 3 x 10ml lithium heparin tubes. Blood was also collected from 30 patients with WKS by Dr. Ian Smith. The age of each subject and date of collection was noted (Table 2.1). The median age for normal controls was 27 years and for the patients 65 years.

The samples were centrifuged at 2000 RPM for 10 minutes in an MSE MULTEX centrifuge. The plasma and buffy coat were discarded by careful aspirations using a pasture pipette. To the packed red blood cells, an equal volume of 0.9% saline was added and mixed. The tubes were spun down as beforehand. The saline (upper layer) was aspirated and discarded. This step was repeated until a clear upper layer was obtained (three washes were adequate). The upper layer was discarded and an equal volume of buffered citrate glycerol (2 parts glycerol, 3 parts citrate solution containing 0.6% K_2HPO_4 and 0.47% KH_2PO_4, pH 7.4) was added and mixed thoroughly. This acts as a cryo-protective agent and prevents red cell hemolysis on freezing. The packed red blood cells (RBC) were stored in plastic tubes (3.5ml) at -20°C.

Table 2.1 List of red blood cell samples collected from patients with Wernicke-Korsakoff syndrome. Samples were stored at -20°C in buffered citrate glycerol.

Collection & Storage Date	Sample	Sex	Age
11/1988	100194	M	70
11/1988	100195	M	64
11/1988	100196	M	63
11/1988	100197	M	78
11/1988	100198	M	64
18/9/1989	100100	F	65
3/1990	100101	M	66
3/1990	100102	M	79
3/1990	100103	M	64
3/1990	100104	M	65
3/1990	100105	M	61
3/1990	100106	F	
3/1990	100107	F	
3/1990	100108	M	62
3/1990	100109	M	72
3/1991	100110	M	69
3/1990	100111	M	71
3/1990	100112	M	62
16/5/1990	100113	M	64
16/5/1990	100114	M	68
16/5/1990	100115	M	60
16/5/1990	100116	F	66
28/5/1990	100117	F	65
28/5/1990	100118	M	78
28/5/1990	100119	M	72
28/5/1990	100120	M	71
30/5/1990	100121	M	70
30/5/1990	100122	M	69
24/7/1991	100134	M	57
24/7/1991	100135	F	57

2.1.2 Haemoglobin Estimation

Since Hb concentration is proportional to the quantity of red cells in the haemoysate, red cell enzyme activity may be expressed as a ratio of the Hb concentration. This is measured by the addition of ferricyanide-cyanide reagent (Drabkin's solution) to the haemolysate which converts Hb to cyanmethemoglobin. The optical density at 540 nm is proportional to the concentration of cyanmethemoglobin.

30μl of haemolysate was added to 1ml of Drabkin's solution (Appendix II.1) in a 1.5ml eppendorf and mixed. The reaction tubes were left at room temperature for 5 minutes to allow the complete conversion of Hb to cyanmethoglobin. The optical density of the sample was read spectrophotometically against a reagent blank at 540nm. The Hb concentration was calculated by using the following formula:

(2.1)

$$C = \frac{d \times OD \times 540 \times M \times 10^{-4}}{540 \; Hi\; CN}$$

$$d = \frac{vol.\ of\ Haem.\ (0.03ml) + vol.\ of\ reagent\ (1ml)}{vol.\ of\ haemoly.\ (0.03ml)}$$

C = Hb concentration (gm/dL)
d = dilution factor = 34.3
HiCN = millimolar extinction coefficient of cyanmethemoglobin at 540nm = 11.0
M = molecular weight of Hb (16,520).

All estimations were performed in duplicate and Hb concentration is expressed as grams/dL.

2.1.3 Protein Assay

Red cell enzyme activity may also be expressed as a ratio of total protein concentration. In order to estimate the protein concentration of a given solution, a dye-binding assay was used (Bradford, 1976). This method is based on the fact that when protein binds to a dye, (Commassie Brilliant Blue, CBB, G-250), the absorbance maximum shifts from 465nm to 595nm. A protein standard is used to allow the estimation of the unknown sample. Albumin is widely used since the extinction coefficient of the albumin-dye complex is constant over a 10-fold concentration range (Spector, 1978). A commercially available protein assay kit (BioRad) was used. The protein standard used was bovine serum albumin (BSA).

A microassay procedure was employed to avoid the use of excessive volumes of sample. Several dilutions of protein standard (BSA) were prepared containing 5-25 mg/ml. Into 1.5ml eppendorf, 20μl at protein standard or protein sample (previously diluted with water) was added to 1ml of a 1:5 dilution of dye reagent (Phosphoric acid). The solutions were mixed gently to avoid excessive foaming and left at room temperature for a least 10 minutes up to one hour. The solution was then transferred to a plastic cuvette and read against a "blank" at 595nm in PYE UNICAM PU8800 spectrophotometer. The absorbances at 595nm of the protein standard were plotted against standard concentration. The concentration of protein in the unknown sample were read from the standard curve. All samples were assayed in duplicate.

2.1.4 Quantitative Analysis of Erythrocyte Transketolase

The concentration of an enzyme in a haemolysate is proportional to its activity which can be estimated using a suitable reaction system. By adjusting the assay conditions, such as pH, temperature and concentration of substrates and intermediates of the reaction system, the enzyme analyzed is made the true rate-limiting step. The rate of reaction, which is proportional to the enzyme activity, is measured in terms of the quantities of products formed per unit of time. If those

quantities do not have light-absorption maxima and therefore present difficulties in measuring optical density, then the reaction is coupled to another enzymatic reaction which gives an easily measured optical change. This system is known as a coupled reaction. The rate of the product accumulation is directly proportional to the change in absorbance measured by a spectrophotometer. This change in optical density is linear with time under optimum conditions. It may be measured by following the rate of reaction over a period of time recorded continuously with a pen and chart plotter (Kinetic Assay). An alternative approach is an endpoint assay which reads the initial optical density of the reaction (t_0) and once again after a given period of time (t_1), thus measuring the change in optical density over a given period (t_1-t_0). To establish if the enzyme activity is altered within a patient group, it is essential to first determine a normal range of enzyme activity. This range can be defined by performing statistical analysis of results obtained from normal individuals.

2.1.4.1 Erythrocyte Transketolase Assay

Transketolase activity was assayed based on a method described by Smeets et al. (1971), modified by Bayoumi and Rosalki (1976), using the following coupled reaction:

1. Ribose-5-phosphate + Xylulose-5-phosphate $\xrightarrow{TK}$ Sedoheptulose-7-phosphate + Glyceraldehyde-3-phosphate

2. Glyceraldehyde-3-phosphate $\xrightarrow{TPI}$ Dihyroxyacetone-phosphate

3. Dihydroxyacetone phosphate + NADH $\xrightarrow{GDH}$ Glycerol-3-phosphate + NAD

In the presence of excess triosephosphate isomerase (TPI), glycerol-3-phosphate dehydrogenase (GDH) and NADH, glycerol-3-phosphate is rapidly converted to dihydroxyacetone phosphate by TPI in reaction (2) which is subsequently reduced to glycerol-3-phosphate and NAD. For each molecule of

dihyroxyacetone-phosphate converted from glycerol-3-phosphate formed in reaction (1) and reduced, one molecule of NADH is oxidized to NAD. This results in a decrease in absorbance at 340nm which is followed spectrophotometrically (340nm absorption maximum for NADH). Since TK activity is to be measured, it is made the rate-limiting component by the appropriate adjustment of the reaction system. All reagents and concentrations used are listed in Appendix II.2a.

Sample Preparation:

Red cell samples stored at -20°C in buffered citrate glycerol were retrieved and thawed at room temperature. To 1ml of packed RBC, 1.5ml of 1% Triton X-100 was added and vortex mixed. To precipitate the cell debris, the samples were spun at 12800 RPM for 10 minutes. The Hb concentration was estimated as described in section 2.1.2, and adjusted by further dilution with Triton (if necessary), to approximately 40 gm/dl. Two aliquots for each haemolysate were prepared (i) 75% haemolysate, and 25% TPP solution for assay with cofactor (ETKs activity), and (ii) 75% haemolysate, 25% Tris-HCl, for assay without cofactor (ETK activity). These along with the reaction reagents, were preincubated at 37°C for 15 minutes in a covered water bath.

Assay Protocol:

The reaction reagents were freshly prepared. To the substrate buffer (R-5-P), 0.4% GDH/TPI solution and 2% NADH solution were added. The total volume was divided into two bottles; 20% of TPP solution was added to one portion and 20% Tris-HCl pH 7.6 to the other. Each sample was assayed with and without cofactors, in duplicate.

Each reaction tube contained 1.27ml of substrate reagents and 30μl of haemolysate to provide an intial optical density (OD) reading (t_0) and final OD reading (t_1). Four tubes were set up for each haemolysate as follows:

Aliquot	1	2	3	4
Substrate Reagent	1.27ml	-	1.27ml	-
Blank Reagent	-	1.27ml	-	1.27ml
Haemolysate with TPP	0.03ml	0.03ml	-	-
Haemolysate	-	-	0.03ml	0.03ml

All aliquots were preincubated at 37°C as before for 15 minutes (t_0) and for a further 45 minutes (t_1). The tubes were then removed from water bath and placed on ice to stop any further reaction. 1ml of reaction mixture was placed in a quartz curvette and read against a reagent blank at 340nm.

Calculation of TK activity:

The change in optical density (Δ OD) was determined from the difference between OD at t_1 and OD at t_0, and ΔOD/min was determined by dividing ΔOD by the difference in incubation time between (t_0) and (t_1). ETK and ETKs activity is calculated from the following formula.

(2.2)

$$ETK\ activity = \frac{\Delta OD/\text{min.} \times V_e \times 100}{Hb\ (gm/dL) \times E \times V_H}$$

where V_e = final volume in eppendorf (ml)
Hb = haemoglobin concentration (gm/dL)
E = millimolar Extinction of NAD = 6.22
V_H = volume of haemolysate (ml)

ETK activity is expressed in International Units per gm of Hb. (IU/gmHb).

To calculate TPPE the following formula was used:

(2.3)

$$TPPE = 100 - \left(\frac{ETKs\ activity}{ETK\ activity} x\ 100\right)$$

This value is expressed as % activation and any value exceeding 25% is considered abnormal (Sauberlich, 1967).

2.1.4.2 Optimizing the Assay

The assay was further modified by varying conditions such as incubation times, addition of $MgCl_2$ etc, as suggested by Tate and Nixon (1987) and Heinrich et al. (1972) respectively to obtain complete activation of TK with cofactor and to reduce Hb interference. All reagents, sample and reaction mixtures were prepared as described in section 2.1.4.1 unless otherwise specified. The modifications and variations carried out are listed in Table 2.2.

2.1.4.3 Application of Transketolase Endpoint Assay for the Analysis of Patients and Control Samples

From the modification of the endpoint assay, optimum conditions were derived and applied to the analysis of normal control and patient samples. The reagents used and their concentrations are listed in Appendix II.2b.

Table 2.2 List of modifications and their specification performed to optimize the endpoint assay.

Modification	Stage in Assay	Purpose	Other Specification
Boiling Haemolysate	Prior to Adding TPP	↓ Hb Interference	2 min, 5 min.
Addition 10% TCA	"	"	-
Addition 20% NaOH	"	"	-
Heating Reaction Mix	Prior to Measuring OD	↑ ETK Activation	60°C, 80°C
Use Shaker Water bath	Incubation of Reaction Mix	↑ Binding of TPP to TK	-
Addition of 1.0mM DTT	Prior to incubating Haemolysate	↑ ETK Activation	-
Reducing Hb concentration	Prior to Adding TPP	↓ Hb Interference	From 40gms to 20gms/dl
Addition of 1.2mM $MgCl_2$	Prior to Incubating Reaction Mix	↑ TPP Binding + ETK Activation	-
Addition of 7.0mM X-5-P	"	↑ Complete Reaction with Substrate	-
Varying Incubation Times	$t_1 - t_0$	Best Constant Rate of Reaction	10,30,45,60,90,120 mins.

↑ increase
↓ decrease

Sample Preparation:

The samples were retrieved as before (2.1.4.1) and thawed out at room temperature. The concentration of haemolysate to be used was adjusted to give a final protein concentration of approximately 20mg/ml. Protein estimation was determined by using Bio-Rad protein assay (Section 2.1.3). After final dilution of haemolysate with 1% Triton, the samples were spun at 12800 RPM for 10 minutes. The haemolysates and reagents were preincubated for 15 minutes at 37°C in a covered water bath.

Assay Protocol:

The reagents (Appendix II.3) were preincubated at 37°C for 10 minutes. For each sample, four aliquots were prepared in a 1.5ml eppendorf containing:

Aliquot	**1**	**2**	**3**	**4**
Substrate Buffer	920μl	920μl	920μl	920μl
GDH/TPI	5μl	5μl	5μl	5μl
NADH	20μl	20μl	20μl	20μl
TPP	40μl	-	40μl	-
$MgCl_2$	5μl	5μl	5μl	5μl
Tris-HCL (pH 7.75)	-	40μl	-	40μl
Haemolysate	20μl	20μl	20μl	20μl

This gives a total of 1.1ml in each eppendorf. Two sets of reaction mixture were prepared for each sample in addition to a reagent blank. One set was incubated for 30 minutes (t_0) and the other for 60 minutes (t_1). The reaction mixture (1ml) was then placed in a quartz curvette and read against a reagent blank at 340nm. All samples were assayed in duplicate.

Calculation of TK activity:

ETK and ETKs activity were calculated using formula 2.2 with the following slight modification in formula:

(2.4)

$$ETK\ activity = \frac{\Delta OD/\min \times 1000 \times V_e}{NAD \times V_H}$$

ETK and ETKs activity are expressed in IU/L haemolysate. To obtain ETK activity in IU/mg protein, the following formula was applied:

(2.5)

$$ETK\ activity\ (IU/mg\ protein) = \frac{ETKA\ (IU/L)}{P_c}$$

where P_c = protein concentration mg/L.

2.1.4.4 Statistical Analysis

To perform statistical analysis on ETK and ETKs activity in patients with WKS and normal controls, the mean and standard deviation were calculated. Significant differences between the mean ETK and ETKs activity was calculated using the parametric student's t-test in the following formulae:

(2.6)

$$MEAN = \frac{\sum x}{n}$$

$$Standard\ deviation = \sqrt{\frac{\sum (x-\bar{x})^2}{n}}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\theta_1^2}{n_1} + \frac{\theta_2^2}{n_2}}}$$

x = ETK or ETKs activity of each sample
n = number of sample
x = mean
θ = standard deviation
t = student's t-test

2.1.5 Qualitative Analysis of Erythrocyte Transketolase

Electrophoresis achieves high resolution of protein mixtures, hence allowing characterization of proteins in terms of microheterogeneity, degradation and subunit structure. It functions as a transportation system of ions or charged molecules through a stable solution contained in a viscous medium (supporting matrix) under an electrical field. The macromolecules (mixture of proteins) migrate in the supporting matrix according to their size, shape and ion charge. Thus the rate of migration enables the distinction and measurement of the proteins separated. The mobility of proteins is influenced by choice of buffer, pH and pore size of the support gel matrix. The matrices minimize the diffusion of sample components which may occur in free solution, and reduce any convection resulting from heat generation during electrophoresis. The separation of proteins is obtained in gels by free passage of small molecules and impeded passage of larger molecules, therefore producing a molecular sieving effect. There are different types of supporting gel matrix such as polyacrylamide, agarose, cellulose acetate and starch which differ in structure and filtration properties. The choice of matrix to be used is related to the type of separation required.

2.1.5.1 Starch Gel Electrophoresis

The separation of protein mixture using this method is achieved not

only on net ionic charge but also on differences in molecular size. In an electrical field, the anions would migrate towards the positive electrode (Anode) and the cations migrate toward the negative electrode (Cathode). The strength of the electric field and the size of the net charge on an enzyme molecule influence the speed of migration. The pH of the buffer solution determines migration speed (based on the ionic strength) and the voltage setting, on the power supply, determines strength of the electrical field (through the electrodes). The method of starch gel electrophoresis was developed by Smithies, 1959. All reagents and buffers used are listed in Appendix II.3.

Red cells stored at -20°C in Glycerol Citrate were retrieved, thawed and diluted with 1% Triton as described in section 2.1.4.1. and 200μl of haemolysate containing 0.3mM TPP was prepared. The samples were preincubated at 37°C for 15 minutes prior to loading onto gel. The hydrolysed starch gel solution (0.5L) was poured immediately into gel mould plates (15 x 22cm), avoiding formation of air bubbles, to give a 6mm depth. The gel was left at room temperature to set for 10-20 minutes and then transferred to the cold room (to solidify) for at least six hours. The upper glass plate was lifted up slowly with a spatula so not to break the gel. A wide strip of gel (2cm) was cut out and removed. Along 6cm, the gel was sliced and slid backward to provide a gap between the two portions of the gels. Sample inserts (1 x 0.5cm 3mm filter paper) were loaded with 10-20μl of sample. The inserts were blotted and inserted vertically between the two gels. The cut sections of gel was slid back together and the 2cm end strip replaced. Any remaining gaps were filled with liquid paraffin. Electrophoresis was performed on an LKB Multiphor overnight (approx. 19 hours) at 0.34 kV and constant current of 2mA with cooling (4°C using LKB Multitemp).

2.1.5.2 Cellogel Electrophoresis

Cellogel is a form of cellulose acetate which is available commercially. It is supplied in 2.5cm or 15cm wide x 20cm long strips contained in 30% (w/v) methanol packs. The gel is of porous structure and the separation of protein mixture is based on the difference in charge and size. Electrophoresis using this method is

quick and requires small volumes of sample (as little as 5μl). The reagents and buffers used are given in Appendix II.3.

The cellogel strip (15 x 20cm) was soaked in gel buffer for 30 minutes. Six samples were applied to the dull side of the strip using a special applicator that was loaded from drops of haemolysate (prepared as for starch gel electrophoresis). Two applications were made on a predetermined line (three quarters of the way up) on the Cellogel taking care to avoid smears. The cellogel strip was placed on the racks in the electrophoresis tank, so as the end nearest the sample line was toward the cathode buffer. The electrode wicks were soaked in appropriate buffers and placed on either end of the cellogel to act as electrode bridge. Electrophoresis was performed at 4°C for 30-90 minutes under constant current of 0.2-0.3mA.

2.1.5.3 Polyacrylamide Gel Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) is a useful technique for separating specific protein fractions. The gel pore-size can be accurately controlled by varying the acrylamide and bis-crosslinker concentration, hence contributing a molecular sieving effect. PAG is stable over a wide range of pH, temperature and ionic strength and is chemically inert in polymerized form. It is prepared by polymerising the monomeric form of acrylamide together with methylene bisacrylamide which provides a rigid inert cross-linked gel matrix. Tetramethyl-ethylendiamine (TEMED) and ammonium persulphate are required as catalysts to initiate the polymerisation of the gel. The ratio of bis-acrylamide to acrylamide determines the density, elasticity, mechanical strength and pore size of the gel. By varying concentration of the acryclamide bis-acrylamide solution, (3%-25%) manageable gels can be obtained. An alternative crosslinker is DHEBA (N,N^1-(1,2, dihydroxyethylene) bis-acrylamide) which possesses both a periodate-sensitive 1,2 diol structure and two amido methyl bonds that are cleavable under alkaline conditions. By using DHEBA as crosslinker, the gel structure can be solubilized after electrophoresis, and seperated protein bands can be isolated. All reagents and buffers are listed in Appendix II.4.

Preparation of gel:

For a 1.5mm thick gel (16 x 16cm), 36ml of gel solution was required consisting of two parts acrylamide-DHEBA solution, one part TEMED, four parts ammonium persulphate, and one part Tris-glycine buffer pH 8.2. The gel solution was poured into a vertical gel mould apparatus. The gel comb (10 x 1.5mm sample wells) was then inserted in the space between the two plates. Gel polymerisation required a minimum of one hour at room temperature but best results were obtained by leaving gels overnight. After assembly of the electrophoresis apparatus (BioRad Protean II Cell), the gel comb was removed and 2L of 1 in 10 dilution of Tris-glycine buffer pH 8.2 was poured into the tank and the upper chamber.

Sample Preparation and Application:

One volume of packed RBC in glycerol citrate at -20°C was added to two volumes of 1% Triton. The haemolysates were centrifuged at 12800 RPM for ten minutes and divided into two aliquots, each containing 97μl haemolysate and 3μl of 3mM TPP. The samples were then incubated for one hour at 37°C. Two volumes of sample were the mixed with one volume glycerol, to prevent any smearing of Hb and facilitate free migration of proteins during electrophoresis. 20μl of sample glycerol solution was loaded into the sample wells. Bromophenol blue (BPB) was used as a tracking dye to indicate the migration velocity. The running conditions for the electrophoresis were 0.5kV constant voltage with 300mA current and running time of approximately one hour for 16 x 16cm gel and 90 minutes for 20 x 20cm with cooling (4°C).

2.1.6 Staining

Two staining methods were used: specific enzyme staining and a general protein staining. The former is used for detection of enzyme activity in situ, and for direct demonstration of individual components of an enzyme (isoenzymes) in the electrophoretic matrix. The latter was used to estimate protein quantity by band intensity and monitoring protein purification. Commassie-Brilliant Blue R-250

(CBB R-250) and Silver Staining were used to stain proteins.

2.1.6.1 Specific Transketolase Stain

This technique detects specific proteins on the basis of enzymatic activity, thus requires the presence of specific substrates and cofactors. The technique used is an electron-transfer dye staining method which requires the coenzymes NAD or NADP and transfer dyes. Suitable dyes are tetrazolium salts (methyl thiazolyl tetrazolium, MTT; nitroblue tetrazolium, NBT) which are reduced at the end of the reaction sequence to yield an insoluble dark-purple formazan at the site of activity. This reaction is catalysed by the presence of phenazine methosulphate (PMS). It can be applied to detect enzymes which lead to the production of NADH and NADPH as well as other enzymes that can be coupled by intermediate reactions to reduce NAD and NADP to NADH and NADPH. The stain is light sensitive hence incubation must be carried out in the dark. Visualization of electrophoretic profiles can be achieved by three techniques. Firstly, the gel can be immersed directly into the staining solution containing all required reagents; secondly, by immersing a Whatman 3mm filter paper in the staining solution and placing it on gel to visualize enzyme activity in situ. Thirdly, the reagents are immobilized in an agrose gel which is used as an overlay which reduces the rate of diffusion of soluble reaction products. A specific enzyme staining method for TK was modified from that described by Kaczmarek and Nixon (1983). Figure 2.1 illustrates the Transketolase staining reactions. A coupled enzyme reaction was used to incorporate an intermediate coenzyme (NAD). The reagents used are listed in Appendix II.5.

(i) Staining Starch Gels

A 3mm Whatman filter paper was cut to the same size as that part of the gel containing Hb band and immersed in the freshly prepared stain (stable for 2-3 hours). The filter paper is laid on the cut surface of the starch gel. The gel was placed in a LEEC incubator at 37°C for two hours. The filter paper was replaced by a fresh one at least once. The filter paper was then removed and bands were visualized.

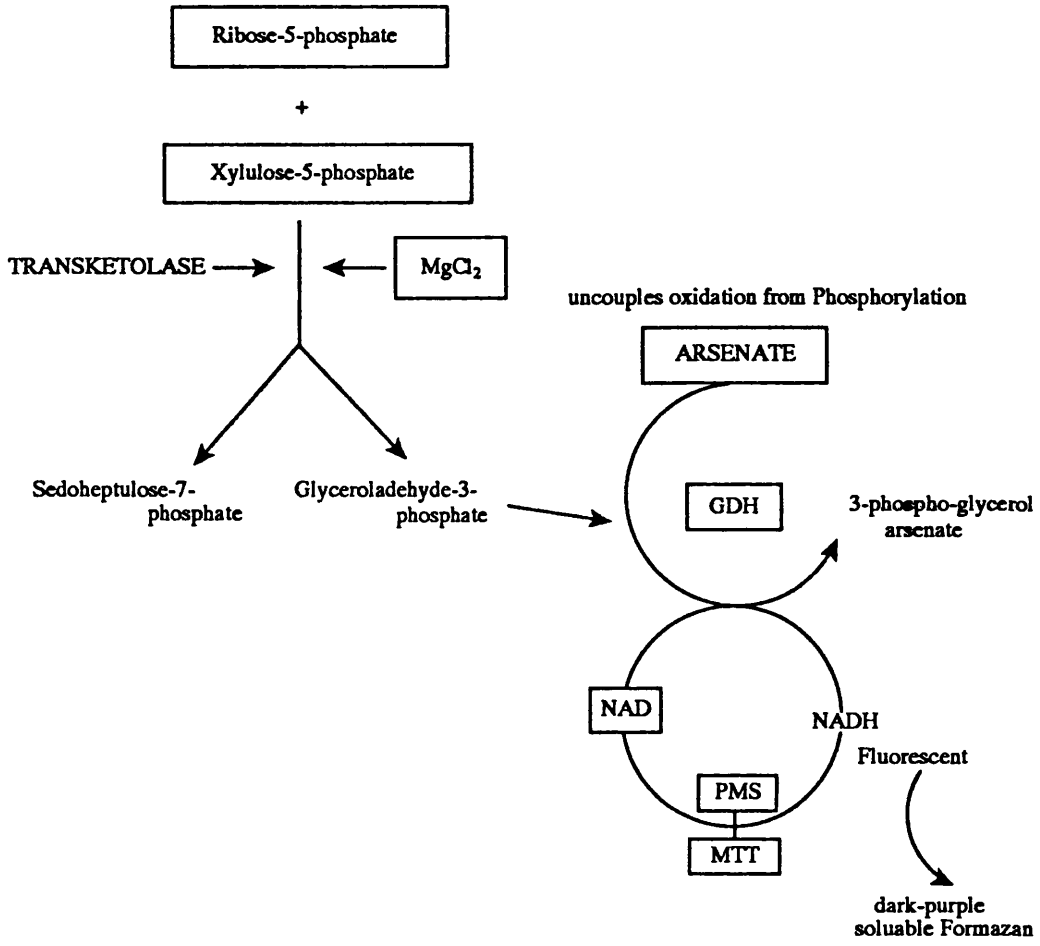


Figure 2.1: Diagrammatic representation of the specific transketolase (TK) stain. In this reaction the substrates (xylulose-5-phosphate and ribose-5-phosphate) are added in the stain with $MgCl_2$ so TK catalyzes the first reaction where two products are formed. Glycerolaldehyde, one of the products, enters a secon reaction (as a substrate with arsenate) which glycerol-3-phophate dehydrogenase (GDH) catalyzes to form 3-phosphate-glycerol. This reaction is linked to a transfer dye reaction that utilizes methyl thiazolyl tetrazolium (MTT) as a substrate and is catalyzed by phenazine methasulphate (PMS) which requires an electron. This is provided from the reduction of coenzyme NAD to NADH. The product formed at the site of activity is the fluorescent NADH in the form of formazon.
(Chemicals inside the squares are the required reagents).

(ii) Staining Cellogels

As with starch gels, a 3mm filter paper was cut to the size of the cellogel and immersed in the freshly prepared stain. The soaked filter paper was laid on top of the cellogel and incubated at 37°C (LEEC incubator) for 45 minutes. The filter paper was then removed, cellogel placed on a water moistened filter paper to avoid drying of cellogel and bands were visualised.

(iii) Staining of Polyacrylamide Gels

The preparation of the stain varies from that described earlier. The concentration of PMS was reduced to minimize heavy background staining. Since the filter paper had a tendency to adhere to the PAGE gel and tear when lifted, an agrose gel overlay was developed as an alternative. Once the electrophoretic run was completed, the gel was removed from the glass plates. The section of gel to be stained was cut and a mould built around it with plasticine. The agarose is added to 40ml Tris-HCl pH 7.6 and brought to boil on a hot plate. The agarose solution was cooled gradually to about 60°C at which time, the staining components were added. It was necessary to avoid solidification of agrose gel prior to addition of reagents. The staining solution was poured on top of the gel and allowed to cool and set in the dark prior to incubation at 37°C (LEEC incubator) for one hour. The agarose was then removed from the surface and bands of enzyme activity were visualized.

To confirm that the bands stained were TK activity, a control experiment was performed. One half of the gel was stained as described and the other stain depleted from the substrate.

2.1.6.2 General Protein Stain

(i) Coomassie Brilliant Blue R-250

Coomassie Brilliant Blue (CBB) R-250 is a non-poly sulphated triphenylamine dye which forms electrostatic bonds with ammonium groups and non-

covalent bonds with non-polar regions in the protein. This type of staining is stable, uniform and ideal for densitometry. It has an adequate sensitivity to detect as little as 0.1mg of protein.

Staining was carried out in a solution containing 0.1% (w/v) CBB R-250 in 45% distilled water, 45% methanol and 10% acetic acid. Staining time varies and depends on the thickness of the gel and polyacrylamide concentration. Usually, for a 16 x 16cm, 1.5mm thick gel, one hour was sufficient. However, it was sometimes necessary to stain the gel for a longer period (overnight) to obtain higher resolution of bands. The gel was then placed in destain solution and the time required for destaining also varied according to the thickness and polyacrylamide concentration of the gel. It can take as long as 24 hours to destain, with several changes of destain solution (45% (w/v) methanol, 10% (w/v) acetic acid) to reduce heavy background staining.

(ii) Silver Staining

The type of silver staining used was derived from the method of Merril, et. al. (1981). It is highly sensitive, 10-50 fold more than CBB R-250 and can detect up to 0.1ng/mm^2. Therefore it is ideal for detection of trace impurities during protein purification. It may be applied on most forms of PAGE gels and IEF gels. This method, however, is time consuming and expensive.

The protocol was based on that described by the manufacturer (BioRad silver stain kit). Staining time depends on the thickness of the gel and volume depends on the size of the gel and is carried out at room temperature.

The method was a modified to improve the quality of staining (Appendix II.7) by gentle agitation at each staining step. Therefore, the size of the glass container for staining gels should be of appropriate size to allow free movement of the gel while having sufficient volume to cover the gel during shaking. Gels stained with CBB R-250 prior to a silver stain did not require first fixing step but must be destained completely. These gels gave best resolution of bands.

2.2 PURIFICATION OF ERYTHROCYTE TRANSKETOLASE BY ELECTROPHORETIC TECHNIQUES

Purification was undertaken in order to characterise the protein in terms of its activity, structure and function. Upon planning a purification strategy, the following points must be considered; the amount of purified protein required, the importance of maintaining its activity, and the degree of purity. The amount of protein recovered after the purification will depend on the amount of starting material and yield. As protein tends to be lost in each purification step, the amount of starting material should be maximized and the number of purification steps minimized.

Purity is defined in terms of the percentage of the total protein which has the desired characteristics. It is essential to have some information on the chemical and physical properties of an enzyme in order to design a suitable purification method. In the purification of a protein, its catalytic activity may be used to monitor the effects of each stage in the process. Purification of TK was attempted using three different methods for preparative and analytical purposes.

2.2.1 Polyacrylamide Gel Electrophoresis

Transketolase was purified from red blood cells by PAGE. This method was developed from the technique used to isolate a CF protein by Jamieson (1983). Gels (16 x 16cm) were prepared using the BioRad vertical electrophoresis apparatus (Protean II cell) as described in section 2.1.5.3 with a DHEBA cross-linker in place of the methylene bis-acrylamide. Sample wells were formed using a 10mm wide comb.

2.2.1.1 Sample Preparation and Application

RBC from normal controls and patients were retrieved from RBC storage at -20°C in buffered citrate glycerol. The samples were prepared by adding 300μl of 1% Triton to 200μl of packed RBC and 100μl Glycerol, and vortex mixed. 20μl of tracking dye (bromo phenyl blue) was loaded into one well and 20μl of

sample into the remaining wells on each gel. Each sample was run in triplicate. Electrophoresis was performed under a constant voltage of 0.5Kv with 300mA current at 4°C for 90 minutes.

2.2.1.2 Excision of Transketolase Bands

The region of gel, from the anode end up to 10mm cathodic to the Hb band, was cut and discarded. The remainder of the gel was cut into three portions each containing 3 wells into which the same sample had been loaded. These portions were further sliced, from the cathodic end, to 4.5-5 cm wide and 2 cm long. This region of the gel contained TK activity as detected from PAGE using gel specific enzyme stain (Figure 2.2). A 1 x 2cm gel strip was sliced, from the track loaded with BPB, and used as the blank control. The three portions were each sliced in halves to yield six strips of 2 x 2.5cm.

2.2.1.3 Solubilization of gels

Each strip, incorporating sample, was placed in a universal tube containing 20ml of 0.01M Tris/HCl pH 10.0. The universal tubes were left overnight at room temperature, to dissolve the gel strips. On the following day, if the gel(s) was not dissolved, the tube(s) was placed in a 37°C water bath until it dissolved completely. The pH of each gel solution was measured and to those below pH 8.5, 1M Tris/HCl pH 10 was added to bring pH above 8.5.

2.2.1.4 Dialysis and Lyophilisation

Dialysis tubing (2-18/32) was prepared by soaking it in distilled water overnight. The solutions were then dialyzed for 24 hours, changing dialysis buffer twice, against 0.001M Tris-HCl pH 10.0 at 4°C. All solutions comprising one sample were combined and placed in a 250ml conical flask and freeze-dried (Edwards Freeze-dryer Modulyo) overnight. The freeze-dried samples were weighed and stored at -20°C in bijou tubes.

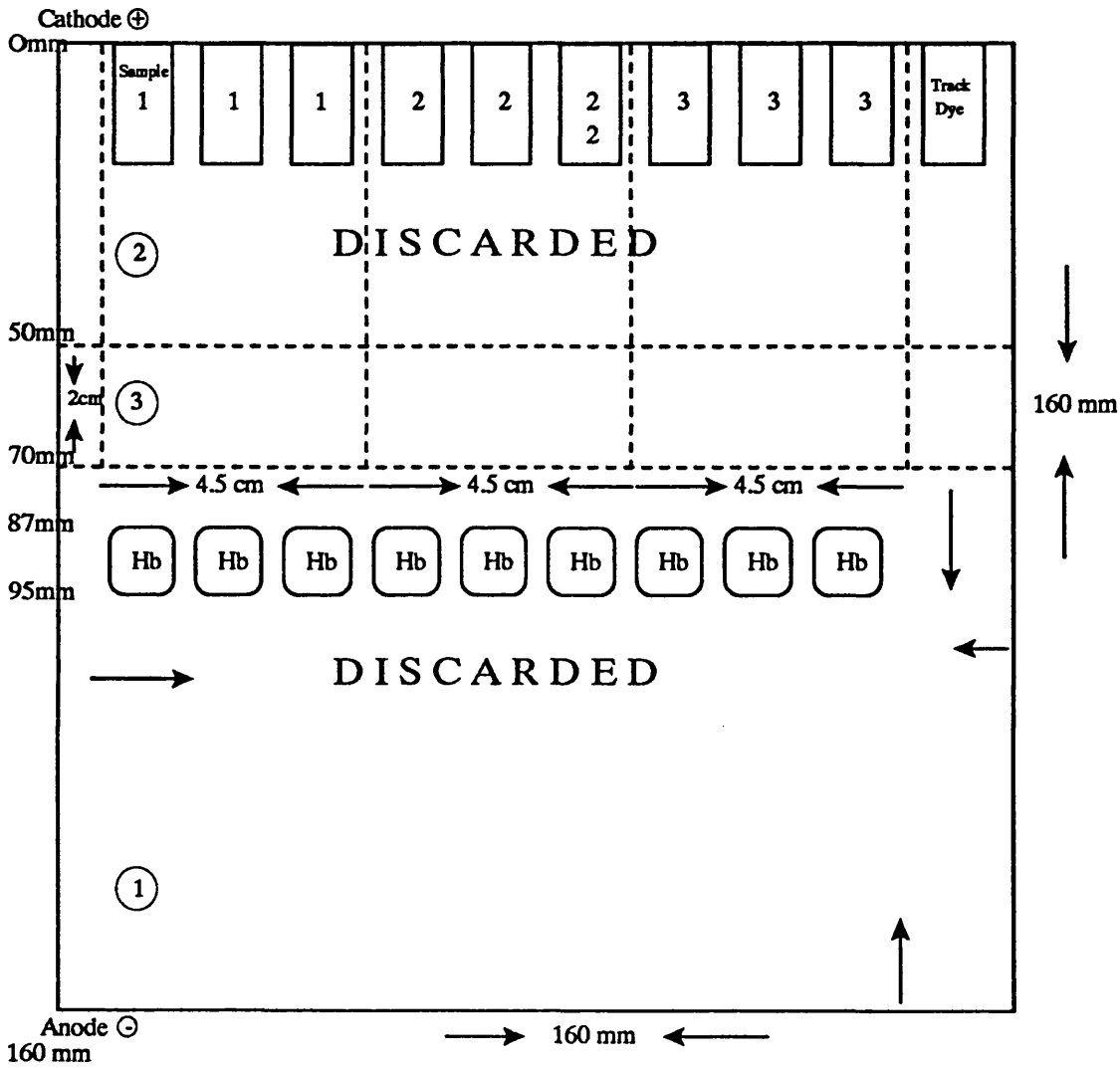


Figure 2.2: Diagrammatic representation of slicing gel strips incorporating transketolase bands. Three samples were loaded on one gel (1,2,3). Hb - Haemoglobin

2.2.2 Analysis of Purified Erythrocyte Transketolase

2.2.2.1 Quantitative Analysis

Protein estimation was performed as described in section 2.1.3 using BioRad protein assay kit. The extracted freeze-dried fractions were reconstituted in 2ml distilled water. 400μl of aliquots were assayed by adding 400μl dH_2O and 200μl Biorad protein assay reagent.

The isolated protein fractions were assayed for TK activity using the assay as described in section 2.1.4.3 with minor modifications. For each sample, four aliquots were prepared in a 1.5ml eppendorf containing:

Aliquots	1	2	3	4
Substrate solution without $MgCl_2$/TPP (in 0.1M Tris-HCl)	700μl	700μl	-	-
Substrate solution + $MgCl_2$/TPP	-	-	700μl	700μl
Sample	500μl	-	500μl	-
0.1M Tris HCl	-	500μl	-	500μl

This gave a total of 1.2ml in each eppendorf. The reaction mixtures were preincubated at 37°C for two hours. 500μl aliquots were removed and the initial optical density (t_0) measured at 340nm. The remaining reaction mixture was left incubating at 37°C in a water bath overnight. The final optical density (t_1) was measured at 340nm. A blank, using a protein free fraction, was subtracted from final change in optical density (t_1 - t_0). ETK and ETKs activity were determined by applying formula 2.4 and 2.5.

2.2.2.2 Qualitative Analysis

Electrophoretic profiles of the isolated fractions were analyzed by PAGE and IEF. Protein detection was performed initially by specific TK stain (to

confirm that the protein bands represented TK activity) and finally by silver staining using BioRad silver stain kit. The reagent concentrations and all buffers used in the following sections are given in Appendix II.10.

(i) Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were prepared as described previously in section 2.1.5.3 a (16 x 16 cm 1.5mm thick). Sample wells were made using a 10 well gel comb. Into each well, 100μl of reconstituted fraction from each sample was loaded with 20μl of BPB track dye. Electrophoresis was performed at 4°C for 60 minutes under a constant 0.4KV and 350mA current. The gel was initially stained once with TK enzyme stain to detect TK activity, as described in section 2.1.6.1. The gel was also stained with BioRad silver stain kit (section 2.1.6.3) for a 16 x 16 cm gel of 1.5cm thickness.

(ii) Isoelectric Focusing

IEF is an electrophoretic technique for the separation of a mixture of proteins with regard to net charge. It involves the separation of proteins in a continuous pH gradient that increases from the anode (acidic) to the cathode (basic). The migration of proteins continues as long as they retain a negative or a positive charge. Once the net charge on the molecule becomes zero, migration ceases. This point in the pH gradient is the Isoelectric point (pI) of the protein. IEF allows the detection of electrical charge variants of an enzyme. This technique is best performed using slab gels run on a horizontal flat-bed apparatus, with an efficient cooling platten to cope with the heating effect caused by the high electrical field strengths.

PREPARATION OF IEF GELS

The gel solution was prepared by adding 10ml of 29.1% acrylamide, 10ml of 0.9% bis-acrylaride, 7ml of 87% Glycerol and 1.5ml ampholine (Pharmacia, pH 3.5-10) to 31.5ml distilled water. The solution was degassed for 10 minutes, and

1.5ml of 1% ammonium persulphate and 50μl of TEMED were added. With a 60ml syringe, the gel solution was loaded carefully in the gel mould (avoiding formation of air bubbles). The gel was left at room temperature for one hour to polymerise and then placed at 4°C for at least one hour before use.

LOADING AND RUNNING SAMPLE

The gel plate was placed on the cooling platten of the Multiphor apparatus (LKB) and one double layer of 15 x 0.5cm electrode wick (1cm wide 3mm Whatman filter paper) was soaked in phosphoric acid and placed on the anode end while another wick was soaked with sodium hydroxide and placed on the cathode end. 40μl of reconstituted sample was applied to a double layer sample applicator (3mm filter paper) placed 1cm below the cathode wick 1.5cm apart. 20μl of pI markers (Pharmacia, pI 3.5-10) were applied on two applicators. The gel was focused for 30 minutes under a constant power of 30W, limited voltage 2kV and a starting current of 75mA at 4°C. After 30 minutes, power was cut off and a further 25μl of each sample was applied. The power supply (LKB2103) was reconnected and electrophoresis continued under the same conditions for a further 30 minutes. The previous step was repeated so that the total volume of sample applied was 100μl and electrofocusing was carried out for 90 minutes in total.

PROTEIN DETECTION OF IEF GELS

The gel was initially stained once with TK enzyme stain. The staining solution (100ml) was prepared by mixing all the staining reagents (section 2.1.6.1) in 0.1M Tris-HCl pH 7.6 in a dark container. The IEF gel was placed in the freshly prepared stain solution in a stainless steel tray and covered. The tray was incubated in a 37°C oven for approximately two hours. This method was applied to verify that any bands detected represented TK activity. IEF gels were also stained with BioRad silver stain kit in order to establish exact pI of the TK bands and to ensure no contaminating proteins were present. The IEF gel was fixed with 500ml of 12% TCA (trichlorocetic acid), 3.5% SAA (sulphosalicylic acid), 30% methanol, 54.5% dH_2O (Fix I) for one hour to precipitate the proteins and wash away the ampholines

prior to staining. This solution was decanted, and 500ml of 12% TCA, 30% Methanol, 48% dH_2O (Fix II) was added into the tray and left for one hour. The solution was decanted and a fresh fixing solution (Fix II) was added for a further hour. The gel was then placed in 400ml of 10% ethanol, 5% Acetic Acid for 30 minutes to start the silver staining. Subsequent steps performed were similar to those described in section 2.1.6.3.

(iii) SDS-Polyacrylamide Gel Electrophoresis

This technique allows the separation of a protein mixture with regard to its molecular weight only. An effective solubilizing agent such as sodium dodcyl sulphate (SDS) is employed to mask the intrinsic charge of a polypeptide chain so that the net charge is constant. Therefore electrophoresis can only be performed in a molecular sieving matrix (PAGE). Effective separation of a protein mixture, of a given range of molecular weight, is determined by the concentration of the polyacrylamide gel. That is, for proteins of MW 20-350kD, a 5% Polyacrylamide gel would be ideal whereas for a protein with a MW15-200kD a 10% polyacrylamide gel would be used.

The discontinuous buffer system devised by Lammeli (1970) and based on the system of Ornstein (1964) and Davies (1964) with the addition of 0.1% (w/v) SDS has been applied. This improves the resolution of protein since the buffer ion concentrations differ between the gel and electrode solutions. It also allows the concentration of a sample prior to its separation. Hence, a stacking gel of low ionic strength with large pores (ie. lower polyacrylamide concentration) is laid on top of the resolving gel. The sample is applied on the stack gel and upon application of an electrical field, proteins will migrate rapidly through the stack gel and concentrate into a narrow sharp zone. The ions from the discontinuous buffer migrates behind the protein and causes it to unstack once the protein enters the resolving gel, where the pH gradient increases and the pore size decreases. In the resolving gel, the protein will separate according to its molecular radius (approximate MW). All SDS-proteins have a net negative charge at pH 7.2, and migrate toward the anode (lower electrode).

In order to achieve the complete binding of SDS (1.4g SDS to 1g of protein) the polypeptide chain configuration must be unfolded and its disulphide bonds reduced. It is therefore important to pretreat the sample prior to loading on gel by boiling the sample in a solution containing SDS and a reducing agent (β-Mercaptoethanol, Dithiothreitol).

GEL MOULD PREPARATION

The resolving gel was prepared by mixing 6ml of Polyacrylamide solution with 3.3ml of gel buffer (Appendix II.9b), 1ml Glycerol and 3.7ml dH_2O and degassing the solution for approximately fifteen minutes. To that, 225μl of 10% ammonium persulphate and 22.5μl TEMED was added and mixed gently. The resolving gel was poured up to a height of about 13cm, and overlayed with isobutanol and left to polymerize for several hours in the BioRad Protean II gel mould apparatus. The isobutanol was poured off and the top of the gel was rinsed several times with the dH_2O and finally with the gel buffer. The stack gel was prepared by mixing the following: 700μl polyacrylamide buffer, 2ml gel buffer, 40μl of 10% ammonium persulphate and 7.5μl TEMED (total stacking gel solution ~9ml) and poured on top of the resolving gel. The gel comb (10 well, 1.5mm thick) was inserted at an angle into the stack gel and the gel was left overnight to polymerise.

LOADING AND RUNNING OF SAMPLE

To an eppendorf, one part reconstituted TK sample (fractions isolated by PAGE) and one part sample buffer (Appendix II.9a) were added. The aliquots were mixed, boiled for 2-3 minutes and spun at 12800 RPM for 10 minutes. The MW protein marker (Sigma, 20-215kD) were reconstituted with 1 to 1 dilution of sample buffer, and heated as described above.

The sample wells were rinsed firstly with dH_2O and last with cathode buffer. The gel was assembled in the Protean II Cell apparatus. In the upper chamber 500μl of cathode buffer was added and 2L of anode buffer was added to the lower chamber. About 150μl of sample was added to the well and 50μl of protein

marker was added to a different well. The gel was run for approximately 2 hours at 4°C with constant 0.35kV and current of 200mA.

In the denaturing conditions applied in this method, TK activity would be lost, so the specific enzyme stain was not used. The gel was immersed in 500ml of 0.1% CBB R-250 (in 45% methanol, 10% acetic acid) for several hours to visualize the bands. Destaining was carried out overnight with several changes of destain solution (45% methanol, 10% acetic acid). To obtain a better resolution, the gel was then silver stained starting at the second fixation step (section 2.1.6.3) using BioRad silver stain kit.

2.2.2.3 High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique for separating a mixture of macromolecules into single compounds by passing them through a separation matrix under high pressure. Subsequently, qualitatative and quantitative analysis can be performed on collected fractions. HPLC is a form of liquid-solid chromatography and is referred to as "High Performance" due to the level of high sensitivity and resolution that it can achieve.

There are many advantages for the use of HPLC. These include the achievement of better resolution than any other form of chromatography and reproducibility is improved. The automation and quantification provides control over the operating conditions such as column temperature, inlet pressure, flow rate and separation time. In addition, it is fast and versatile and the column may be used many times without the need of regeneration (Hamilton & Sewell, 1982). Compounds can be separated on the basis of polarity, MW, thermal instability, ionic strength, organic strength and any combination of these factors.

The operation of HPLC undergoes two main competing phases, the solid stationary phase and the liquid mobile phase which is forced through, under high pressure. The sample, mixture of components to be separated, is injected and carried through by the mobile phase (solvent, eluting agent) to the stationary phase

(column) where it is retained, and where separation of components occurs. The component of interest is eluted with the solute and passes through the detector and out into the fraction tube.

The basic apparatus of HPLC consists mainly of a solvent reservoir (mobile phase), and sample injector, high pressure pumping system, a column (stationary phase) and a sensitive detector (Figure 2.3). A programmable controller and a recorder are other useful but non-essential components. The controller is capable of delivering accurate solvent flow (0.1ml/min - 10ml/min), provide a gradient elution, produce non-pulsatile flow and store useful programmes. The chromatographic profile is deliniated on a chart recorder. The HPLC system used consists of: LKB 2210 Recorder (2-Channel), LKB 2158 Uvicord SD, LKB 2152 HPLC Controller, LKB 2150 HPLC Pump, and LKB 2133-500 DEAE-5PW Column (8.0 x 7.5 mm ID x cm)

The mode of separation performed on the extracted TK by ion-exchange chromatography in order to establish presence of ETK variants. In this type of separation, the exchange of ions (opposite charges) takes place in the column by binding a molecule with a strong negative or positive site on the matrix. Therefore, this separation depends on the strength of the interaction between the solute's ions and the exchange site. There are two types of ion-exchange chromatography, anion-exchange and cation-exchange. In the former, the components pH would be lower than that of the column thus gains a positive charge, while in the latter the opposite holds true. Depending on the ionic strength of the component, a weak or strong ion exchanger is applied to prevent very strong or very weak retention respectively. A short and broad column is ideal for this mode of separation and a gradient elution is required.

The column must be first equilibrated with filtered and degassed with solvent A (20mM Tris-HCl pH 7.5). Prior to injecting the sample, 50μl of solvent A was injected into the DEAE-5PW column and a gradient run (Table 2.3a) was applied for 10 minutes to detect any background trace (recorded on the chart). The column was allowed to re-equilibrate for 5 minutes, and 100μl of sample was

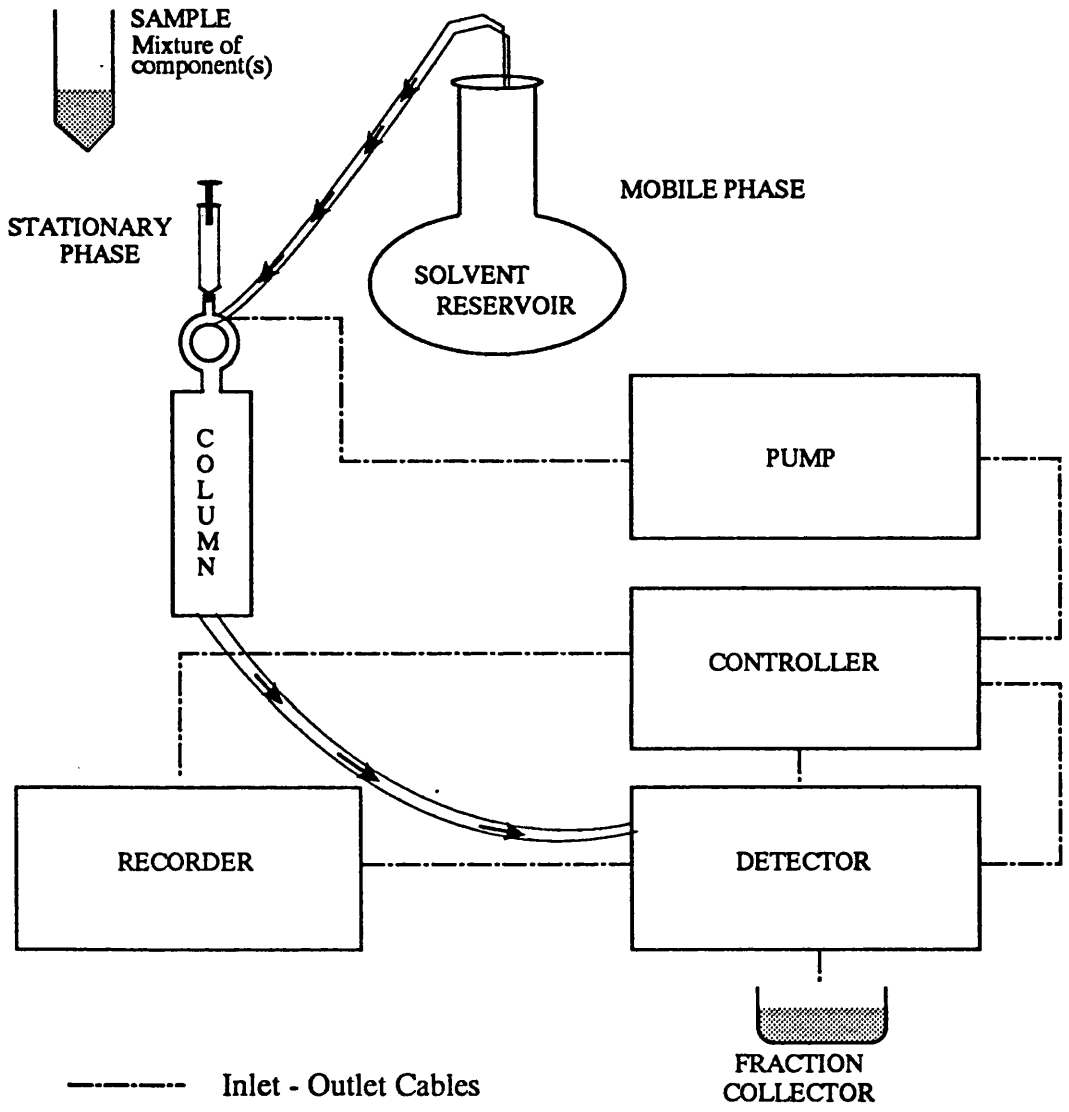


Figure 2.3: Instrumentation and operation of high performance liquid chromatography. The sample is injected to the column and the components seperated and eluted with the solvent which is forced through the pump. The solute is monitered by the detector and forced through to the fraction collector, while the elution profile recorded on a chart. All operations are controlled by the controller.

Table 2.3 Gradient elution programmes used for the seperation of transketolase by ion-exchange chromatography with high performance liquid chromotography. Solvent A contained 200μ Tris-HCl pH 7.5 and Solvent B contained 1M NaCl in solvent A. These programmes were stored in the LKB2152 controller.

Table 2.3a Gradient elution programme 1 applied for a 10 minute washing of the column before and after sample injection.

Time (min)	Flow Rate (ml/min)	% Solvent A	% Solvent B
0	.8	100	0
1	.8	100	0
4	.8	65	35
6	1.0	0	100
8	1.0	0	100
10	1.0	100	0

Table 2.3b Gradient elution programme 2 applied for the seperation of transketolase in 40 minutes.

Time (min)	Flow Rate (ml/min)	% Solvent A	% Solvent B
0	.8	100	0
5	.8	100	0
25	.8	65	35
30	1.0	0	100
35	1.0	0	100
40	1.0	100	0

injected. The gradient elution programme (Table 2.3b) was initiated from the controller and the solute was monitored at 280nm wavelength with a sensitivity setting of 0.002 absorbance units (Au). The chart recorder speed was set at 5mm/minute and the gradient run was set for forty minutes.

2.3 PURIFICATION OF TRANSKETOLASE FROM WHITE BLOOD CELLS

In 1989, Mocali and Paoletti developed a new technique to purify TK from white blood cells (WBC). The purification was performed in five steps; sample preparation, desalting and gel filteration, DEAE-cellulose adsorption, ammonium sulphate fractionation and finally affinity adsorption on CM-cellulose. Modifications of this method included sample preparation, the column dimension for gel filteration, and the omission of afinity adsorption step using CM-cellulose.

2.3.1 Quantitative Analysis of Transketolase Fractions

(Fractions collected at each step were assayed for protein and TK activity).

Protein estimation was performed using Bradford's method (1976), a modification of the method described in section 2.1.3. Briefly, the reagent was prepared by dissolving 100mg of CBB G-250 in 50ml of 95% Ethanol. To this 100ml of 85% phosphoric acid was added and then diluted to one litre with dH_2O water. The solution was stored in a dark bottle at room temperature. The protein assay was performed by adding 20μl of sample into a test tube. To it, 1ml of Bradford's reagent was added and mixed gently. The tube was left at room temperature for at least 10 minutes up to a maximum of 60 minutes. Absorbance was measured at 595nm. BSA (0-200mg/ml) was used as protein standard.

TK activity was determined using a kinetic assay (Appendix II.10).

Two aliquots were prepared for each fraction to be assayed containing the following reaction mixture:

Alliquot	1	2
0.1M Tris-HCL pH 7.6	-	920μl
0.1M Tris-HCl pH 7.6 containing substrates	920μl	-
GDH/TPI	5μl	5μl
NADH	20μl	20μl
TPP	40μl	40μl
$MgCl_2$	5μl	5μl

The reaction mixture was placed in a quartz curvette (1cm light path) at 37°C, monitored at 340nm with band width of 0.2 AU and recorded at chart speed setting of 0.5mm/min for 5 minutes on a Guildford 2600 spectrometer. Then 20μl of sample was added and mixed. The reaction was monitored on the chart at the same settings for 10 minutes. TK activity was calculated from the following formula: (2.7)

$$TK\ activity = \frac{\Delta OD/\text{min} \times 1000 \times V_c}{E \times V_c}$$

where:

ΔOD/min = calculated from the chart (ΔOD/min for TK activity - ΔOD/min for TK activity without substrates)

V_f = Total volume in cuvette (1.01ml)

E = Millimolar Extinction of NAD = 6.22

V_c = sample volume (20μl)

Total TK activity was expressed in IU/L and specific TK activity was expressed in IU/mg of protein.

2.3.2 Sample Preparation

WBC were extracted from a pool of 400ml of buffy coats obtained from several healthy donors from the Regional Blood Transfusion Service at Law Hospital. Four extractions were carried out, and once the required amount was obtained, all the WBC extracted and stored at -20°C were pooled prior to cell lysis.

2.3.2.1 Preparation of Human Leukocytes from Buffy Coats

About 10ml of buffy coat was placed in a 50ml conical tube. To it 40ml of dextran solution (1.2g/100ml isotonic solution) was added, mixed well and left at room temperature for approximately one hour. The supernatant was carefully removed into a 20ml Sterilin universal tube and centrifuged at 3700 RPM for 15 minutes. The supernatant was discarded and the pellets were suspended in 2ml saline and spun at 2000 RPM for 5 minutes. The supernatant was discarded and if the pellet was contaminated with RBC 1.5ml of ice-cold water was added, resuspended and mixed thoroughly with a pasteur pipette to lyse the RBC. The mixture was left for 90 seconds at room temperature and 0.5ml of ice-cold 3.6% NaCl was added and mixed by inverting the tube. The tubes were spun at 2000 RPM in MSE centrifuge for 60 seconds. The supernatant was discarded and the pellet was transferred to a 1.5ml eppendorf. If RBC were still present, the lysis step was repeated. The final pellet was weighed and stored in 0.5ml saline at -20°C until all extractions were complete and ready for use.

2.3.2.2 Lysis of White Blood Cells

The pellets were retrieved, thawed and pooled together. The total dry weight of white blood cells (3.0g) was resuspended in 30ml of 0.01M Tris-HCl pH 7.8 and kept on ice. The cells were disrupted by six cycles of 5 second sonication at 70W with 60 second intervals. The sonicate was left on ice for 20 minutes then spun at 16000 x g in a high speed Beckman centrifuge for 20 minutes at 4°C. A clear supernatant (30ml) was collected and assayed for protein and TK activity.

2.3.3 Gel Filtration and Desalting

The chromatographic separation, by gel filteration and desalting, is based on the molecular size of a compound. In this mode an isocratic elution of varying buffer conditions is used. The efficiency of the resolution depends on the column packing particle's pore size, distribution, density, porosity, and flow rate and on the viscosity of the elution buffer. A thin and long column is ideal for this type of separation. The column must be calibrated prior to use.

For a 4 x 30cm Amicon column, 200ml of packing media (Sephadex G-25 coarse, Pharmacia) was used. The sephadex was swollen by dissolving 100g of G-25 in 1L of dH_2O and left overnight at room temperature. The thick slurry was poured slowly in the column against a glass rod while stirring occasionally to prevent formation of air bubbles. The column was then equilibrated with 400ml of 0.01M Tris-HCl pH 7.8 at a flow rate 225ml/hr controlled by a peristaltic pump (Pharmacia P-1). The column was calibrated by loading a mixture of 4mg of dextran blue and 2mg of DNPH in 1ml of 0.01M Tris-HCl pH 7.8. The mixture was eluted with 1L of 0.01M Tris-HCl pH 7.6 at a flow rate of 225ml/hr. Fractions were collected every three minutes with LKB2112 Redirac fraction collector.

The crude extract (30ml) was loaded onto the column at a flow rate of 3.7ml/min and the components were eluted with 600ml of 0.01M Tris-HCl pH 7.8. Fractions were collected every 3 minutes. Protein estimation in column effluents was obtained by measuring absorbance in alternate fractions at 280nm. Fractions containing high protein concentrations were assayed for TK activity, pooled (66ml) and stored at 4°C.

2.3.4 DEAE-Cellulose Adsorption

DEAE-cellulose was prepared by dissolving 50g of DE-52 in 500ml of 50mM Tris-HCl pH 7.8, centrifuged for 10 minutes, and the supernatant then decanted. The cellulose was equilibrated with 10mM Tris-HCl pH 7.8 for 20 minutes

while shaking occasionally. It was centrifuged for 10 minutes and the supernatant was decanted. The wash was repeated and the DEAE-cellulose was stored at 4°C.

TK activity in void fractions (66ml) was mixed with 4.5g wet DEAE-cellulose by gentle stirring for 15 minutes. A supernatant was collected under a mild vaccum and was assayed for TK activity and protein concentration.

2.3.5 Ammonium Sulphate Fractionation (45-90%)

To 71ml protein solution 19.738g of ammonium sulphate was added slowly and gradually while stirring on ice for 15 minutes to obtain a 45% saturation. The solution was spun down at 16000 x g for 20 minutes at 10°C and the supernatant collected was assayed for TK activity and protein concentration. To a final supernatant (80ml) 26.07g of ammonium sulphate was added while stirring on ice to yield a 90% final saturation. The fraction was centrifuged at 16000 x g at 10°C for one hour. The supernatant was collected and the precipitate (45-90% ammonium sulphate) was dissolved in 3ml 0.01M Tris-HCl pH 7.8. The supernatant and suspended pellet were assayed for TK activity and protein concentration and then stored at 4°C.

2.4 PURIFICATION OF ERYTHROCYTE TRANSKETOLASE BY CHROMATOGRAPHIC TECHNIQUES

The following method used was modified from that of Takeuchi et. al. (1986). The complete isolation of TK was achieved in three steps: sample preparation, ion-exchange chromatography on DEAE-sepharose, and fast protein liquid chromatography (FPLC). Fractions were collected at the end of each step and assayed for TK activity at 340nm using the Kinetic assay, for 10 minutes at 37°C with and without the substrates described in section 2.3.1. Total protein estimation, from pooled fractions from each step, was determined by a modification of Bradford's method described previously in section 2.3.1.

2.4.1 Sample Preparation

Blood (60ml) was collected from a normal volunteer and placed in 6 heparin tubes, each containing 10ml. Packed, washed red cells were prepared as described in section 2.1.1, and the cells from all six tubes were pooled in 500ml conical flask giving a total volume of 36ml. The packed RBC were shock frozen with liquid nitrogen (-196°C) for at least five minutes and thawed in a 37°C water bath. Three freeze/thaw cycles were carried out and 36ml of deionized water was added and mixed. The final solution was dialyzed (dialysis tubing 2-18132) against 4L of 0.01M Tris-HCl pH 8.3 for 36 hours at 4°C with a single change of dialysis buffer after 24 hours. The dialyzed heamolysate was spun at 17,000 x g for one hour at 4°C. From that, 200μl was taken and diluted with 800μl 0.1M Tris-HCl pH 7.6 to assay for TK activity and protein concentration.

2.4.2 Ion Exchange Chromatography on DEAE-Sephorose

The 500ml preswollen DEAE-Sepharose Fast Flow (Pharmacia) was poured into an Amicon column (10 x 10cm) at 4°C. The column was equilibrated with several volumes of Buffer A (0.01M Tris-HCL pH 8.3) until the pH of the elutant was 8.3.

The hemolysate (70ml) was loaded on the column at a flow rate 90ml/hr. The column was washed with 500ml Buffer A. Fractions were collected at 12 minute intervals in a 20ml test tube. A linear gradient of equal volumes (2L in total) of Buffer A and Buffer B (1M NaCl in Buffer A) was eluted at a flow rate of 1.5ml/min. Absorbance of alternate fractions was measured at 280nm to give a rough estimate of protein concentration. TK activity was assayed in every four fractions and conductivity was measured in every fifth fraction.

Subsequently fractions containing TK activity were pooled into one fraction. Two fractions of pooled TK activity (A & B) were collected and assayed for protein concentration. The two fractions (A & B) were diaylised (Medical

International Dialysis Tubing, 1-8/32) against 0.01M Tris-HCl pH 8.3 for 36 hours with a single change of dialysis buffer after 18 hours. The dialyzed fractions were assayed for TK activity and protein concentration.

2.4.3 Fast Protein Liquid Chromatography

FPLC is a specific modular HPLC system supplied by pharmacia-LKB. Separation of a protein mixture can be achieved in a short time with high resolution. It has the same characteristics as HPLC and is used widely for the purification and analysis of proteins. FPLC® Pharmacia-LKB comprises a liquid chromatography controller (LCC-500), a FPLC Pump (R-500), a single path monitor control unit (UK1), a chart recorder, a fraction collector (FRAC-100), and a 100ml sample superloop. The mode of separation applied on the FPLC in the third step of purification was ion-exchange chromatography on Mono Q column.

The FPLC system was washed with Buffer A and Buffer B prior to connecting the Mono Q column. The column was equilibrated with Buffer A for 5 minutes, then 10 minutes with Buffer C (0.2M NaCl in Buffer A) and finally 15 minutes with Buffer A at a flow rate 1.0ml/min. The sample superloop was also washed with Buffer A prior to use. The chart recorder was set at 0.5cm/min, absorbance band width was set at 0.5 Au and the UV monitor was set at 280nm. A baseline for background absorbance was recorded on the chart prior to loading sample.

Fraction A (50ml) was injected in the superloop at a flow rate 0.5ml/min. The sample was then eluted in the column with Buffer A at a flow rate 1ml/min. No fractions were collected at this point. The gradient elution (0-100% Buffer C) programme (Table 2.4) was initiated and 1.3ml fractions were collected. The absorbance at 280nm was monitored in each fraction and recorded on the chart. The column was washed as described previously with Buffer A and C prior to loading Fraction B. Fraction B (63ml) was injected in the superloop and onto the column at a flow rate 0.75ml/min and eluated in the same way as Fraction A. Fractions containing high protein absorbance (peaks on the chart) were assayed for

Table 2.4: Gradient elution programme used for the separation of transketolase by fast protein liquid chromatography. This programme was used to elute dialyzied fractions A and B from ion exchange chromatography on DEAE-Sepharose. Buffer A contained 0.01M Tris-HCl pH 8.3 and Buffer C contained 0.2M NaCl in Buffer A.

Time (min)	Flow rate (ml/min	% Solvent A	% Solvent C
0	1	100	0
25	1	0	100
30	1	0	100
35	1	100	0

TK activity and protein concentration.

2.4.4 SDS-Polyacrylamide Gel Electrophoresis

The four fractions collected (desalted and lyophilized) were analyzed for electrophoretic profile and molecular weight estimation using SDS-PAGE method. The buffer system and polyacrylamide concentration used varies slightly from the previous method described in section 2.2.2.2.

2.4.4.1 Sample Preparation

The sample fractions were diluted with 0.5% ammonium bicarbonate (elution buffer) to give a total volume of 2.5ml per fraction. The sample fraction was desalted on a pre-equilibrated PD-10 Sephadex G-25 column and the proteincomponents were eluted with 3.5ml elution buffer. The diluted fractions (3.5ml per fraction) were frozen on dry-ice ethanol bath for several minutes and lyophilized (Flexi-dry Lynphilizer Fissystem Jar) overnight. The freeze-dried fractions were reconstituted with SDS-PAGE sample buffer (Appendix II.9a) and boiled for 3 minutes.

2.4.4.2 Estimation of Molecular Weight

ETK purified fractions were subjected to SDS-PAGE for estimation of MW. The gel mould was prepared and set up using BioRad Protean II Cell Electrophoresis. The 10% acrylamide resolving gel (30ml) was prepared by mixing the following: 10ml polyacrylamide solution, 11.3ml 1M Tris pH 8.8, 150μl 20% SDS, and 8.7ml dH_2O (Appendix II.9c). The solution was degassed for 10 minutes prior to the addition of 100μl 10% ammonium persulphate and 20μl TEMED. The resolving gel was poured in the gel mould, overlayed with isobutanol, as described in SDS-PAGE (Method 1, section 2.2.2.2), and left to polymerise for 2 hours at room temperature. The 4% acrylamide stacking gel (20ml) was prepared by mixing: 2.66ml polyacrylamide solution, 2.5ml 1M Tris pH 6.8, 100μl 20% SDS, 100μl 10% ammonium persulphate and 20μl TEMED. The isobutanol was decanted, and the gel

mould washed several times with dH_2O followed by one wash with 1M Tris pH 6.8, and was dried prior to pouring the stacking gel. The gel comb (10 wells x 1.00cm) was inserted and the stacking gel was left to polymerise for one hour.

The gel comb was removed and the wells were washed and filled with running buffer (Appendix II.9b). 100μl of each fraction, 20μl of haemolysate (1 in 5 dilution) pretreated with sample buffer and 20μl of pretreated BSA were loaded in sample well. 500ml of running buffer was poured in the upper chamber and 2.0 L was poured in the lower chamber of BioRad protean II cell tank. Electrophoresis was performed at 4°C under constant voltage of 0.4kV and a current of 350-500mA.

The gel was immersed in 500ml of CBB R-250 staining solution for at least 6 hours to visualize bands and destained for 24 hours with several changes of destain solution (50% Methanol, 10% Acetic Acid). The gel was then silver stained with BioRad silver stain kit starting with the second fixature step.

2.5 AMINO ACID ANALYSIS

In order to characterize a protein in terms of its amino acid constituent, number, type and sequence and determining its primary structure, a protein must be obtained in a purified form. Sufficient amount of pure protein required for such analysis may be as little as 10 pmol (ie. 0.5-5mg for a 50kD protein), however it is always better to have more starting material. A rough estimate of protein concentration and its molecular weight are required.

The protein analyzed must be in solution or solid support that does not interfere with subsequent analysis and which can be obtained either by chromatography (HPLC) or by electrophoresis (SDS-PAGE). Direct analysis can be performed on proteins isolated by HPLC. However, protein analyzed on SDS-PAGE must be extracted by electroelution or electroblotting. The protein band must be first visualized with a stain dye, cut and eluted into a solution in the former or electroblotted, visualized, cut and directly analyzed, in the latter.

2.5.1 Western Blotting

This technique, also referred to as Transblotting, involves the transfer of a protein from a gel onto the surface of a thin matrix. The protein(s) would be immobilized on the surface and is accessible whenever required. An advanced technique of transferring a protein is that performed under an electric field perpendicular to the plane of the gel. This is referred to as electoblotting and was developed by Towbin et al. in 1979. Electroblotting apparatus may either be horizontal (semi dry with graphite plate electrodes) or vertical (having large buffer reservoir with platinum electrodes). Transblotting conditions vary depending on the thickness of gel, molecular weight of protein, buffer ions and concentration, type of matrix and purpose of transfer. Proteins electroblotted for amino acid analysis must be seperated with electrophoretic buffers (such as Tricine in place of Glycine) that do not interfere with the analysis. Electroblotting must also be performed on a matrix that would not be damaged by reagents and solvents used in automated analytical instruments. Such membrane used is Immobilon P supplied by Millipore and manufactured from Teflon-like material polyvinylidene diflouride (PVDF) which possess high mechanical strength, retention and sensitivity. Gels are best blotted in CAPS (3-{ Cyclohexylamino}-1- propanesulfonic acid) to reduce levels of Tris and Glycine contamination.

The electroblotting buffer was prepared by mixing 400ml of 10X stock 100mm CAPS buffer (pH 11.0 with 2M NaOH, 400ml methanol and 3200ml Milli Q H_2O). The PVDF membrane (Millipore 16 x 16cm) sheet was cut to the dimension of the resolving gel, immersed in 100% methanol (Analytical Grade) for few seconds, transferred to 50% Methanol for few more seconds and placed in a plastic container containing blotting buffer. Two sheets of 3mm filter paper (dimension as resolving gel) were also soaked in blotting buffer.

Purified TK fraction (2-3μg/100ml) prepared from a normal control by PAGE (section 2.2) was subjected to SDS-PAGE (section 2.2.2.2). After electrophoresis had completed, the gel was preequilibrated with 2 changes of 400ml blotting buffer. A fibre pad sponge (presaturated with blotting buffer) placed on the

cathode panel was built up with one sheet of presoaked 3mm Whatman filter paper followed by the gel on top (laid down carefully to avoid trapping air bubbles in between). The sandwich was completed by carefully laying the PVDF membrane on top of the gel (rubbing side to side to expel any air bubbles) and then on top of that was the Whatman paper, saturated sponge and anode panel. The sandwich was secured by closing the gel holder. This was placed in the half filled buffer reservoir (BioRad 170-3910 Trans-Blot-Electrophoretic Transfer Cell). The cooling plastic coil connected to a circulator was immersed and the reservoir was filled (to cover the sandwich) with blotting buffer. Electroblotting was performed under a constant current (Sartorius Blotting Power Supply) of 125A, 25V for 90 minutes under a cool temperature of circulating ice water.

The membrane was removed, rinsed with Milli-Q H_2O for 5 minutes and dipped in 100% methanol for few seconds prior to staining. Bands were visualized by staining PVDF membrane in 0.1% CBB R-250 for 5 minutes and destaining in several changes of 50% methanol until bands were visualized and the background reduced. The PVDF membrane was handled with sterile forceps at all times and was placed in a plastic bag, sealed and stored at -20°C.

2.5.2 Amino Acid Composition

This analysis involves the cleaving of the peptide chain at random and identifying the nature and quantity of the amino acid residues. This is important prior to sequencing as it provides information on the quantitation of a protein/peptide which may be N-terminally blocked. It can also provide the approach to cleaving a protein if an internal sequence was required.

Complete analysis and quantification of amino acid analysis may be routinely performed in less than an hour with as little as 1nmol of polypeptide chain using automated analyzer. This analysis provides the ratio of amino acid residues per polypepeptide chain. The molar ratio of each amino acid residue can be calculated from MW and amount of sample analyzed. This analysis can be performed, by either ion-exchange chromatography on a cation exchange resin or by reverse phase

chromatography based on polarity at of an amino acid, on HPLC.

Amino acid composition analysis was performed on two TK bands from the PVDF membrane, by the Molecular Paleontology Laboratory in the Department of Geology & Applied Geology, using Applied Biosystems 420-H amino acid analyzer with automatic hydrolysis head. Separation of amino acids was performed by ion-exchange HPLC. The method used was obtained from Aitken et al., 1989.

2.5.2.1 Sample Preparation - Hydrolysis

Polypeptides can be broken down by either proteolytic enzymes or most commonly by acid hydrolysis.

Two TK bands (0.1-10μg) were cut and collected in a precleaned (by heating in 6M HCl) small hard glass tubes. Hydrolysis was performed in a hydrolysis vessel containing 0.5-1ml 6M HCL and 0.5% phenol (v/v). The vessel was flushed with 99.998% argon for two minutes, and closed with a teflon disc bonded to silicon rubber and a heat-resistant screw cap. The reaction vessel was heated in an oven at 165°C for one hour. Once the vessel is cool, it was placed in vacuo in a desiccator containing sodium hydroxide pellets to remove excess acid.

2.5.2.2 Derivatization with Phenylisothiocynate

The following procedure was carried out initially on amino acid standard. To the vial containing dried amino acid samples, 100μl of coupling buffer (0.7ml acetonitrile, 0.2ml triethylamine, 0.1ml water) was added, vortex mixed and removed by vacuum centrifugation. A further 100μl of coupling buffer with 5μl PITC (phenyisothiocynate) was added, vortex mixed and incubated at room temperature for 20 minutes. The sample was loaded onto the column of the Amino Acid Analyzer and PTC (phenylthiocarbamyl) yields were monitored at 254nm. Amino acid composition of TK was determined against amino acid standard and quantitation was analyzed by Applied Biosystem 920A Data Analysis Module, based

on given MW. Analysis report was provided by the Molecular Paleontology Laboratory.

2.5.2.3 Statistical Analysis

In order to verify the protein bands detected and analysed for amino acid composition (molar ratio %) are of the same enzyme (TK), non-parametric statistical analysis was performed by employing Kendall's rank correlation coefficient (K) and Wilcoxon signed rank test. The former allows the calculation of the correlation coefficient between two sets of data and does not require any assumptions about the data distribution. The data from the amino acid composition (% molar ratio) of one protein band are arranged from the lowest to the highest order, while placing the corresponding values of the other protein band. Then K is calculated by the following formula:

(2.7)

$$K = \frac{2S}{n(n-1)}$$

where S = P - Q where
- P = number of values higher than the top value of the first protein band.
- Q = number of values lower than the top value of the second protein band.
- n = number of values compared.

Since "K" measures the "purity" of correlation then:

k = +ve	positive correlation
k = 0	no correlation
k = -ve	negative correlation

The significance of "K" is determined by calculating Z, the Standardized normal deviate in the following formula:

(2.8)

$$Z = \frac{S\sqrt{18}}{\sqrt{[N(N-1)(2N+5)]}}$$

where S = P - Q
N = number of values compared

Then if		
	$Z < 1.96$	correlation not signficant ($p > 0.05$)
	$Z \geq 1.96$	correlation signficant ($p \leq 0.05$)
	$Z > 2.58$	correlation highly signficant ($p \leq 0.01$)

The Wilcoxon signed rank test is a non parametric sign test for paired data which measures the magnitude of differences contributing to the tested statistics. The null hypothesis for this test is : the distribution from two sets of observations, which are sampled, are identical. This test was performed by using the software SPSS/PC+.

In addition, the molar ratio (%) of amino acid composition from each protein band were compared by plotting the values on a histogram and on a scatter plot.

An upward trend	positive correlation
A downward trend	negative correlation
No straight line	no correlation

2.5.3 Amino Acid Sequencing

The primary structure of a protein can be characterized from deriving sequentially the order of its amino acid residues. The amino acid sequence of a protein would distinguish it from other proteins. It can also characterize the chemical and physical properties of a protein as well as define its higher levels of structure. Determining the sequence of amino acid residues is performed by the sequential liberation and identification of one amino acid at a time from either end of the polypeptide chain under controlled conditions. There are chemical and enzymatic

methods for determining the amino acids at either the amino terminus (N) or carboxyl (C) terminus.

Enzymatic methods involves the use of exopeptidase which attacks only peptide bonds at the end of the polypeptide chain. For cleavage of the C-terminal, carboxypeptidases are used and for the N-terminal, aminopeptidases are applied. Sequencing can only be determined by close and continuous monitoring of the kinetics of the amino acid release as rapid digestion of the polypeptide chain can occur. Amino acid sequencing by chemical methods is most commonly used. This method involves the chemical labelling of the amino acid at either end, peptide hydrolysis and identification of the labelled amino acid. A chemical method for identification of C-terminal residues is hydrozinolysis. The chemical hydrazine reacts with the α-carboxyl group of each peptide bond and cleaves it. Each amino acid is then converted and released as a hydrazide derivative, except for the C-terminal residue which remains unmodified. Following chromatographic separation, the unmodified amino acid C-terminal residue can be identified.

The majority of protein sequences are obtained from the N-terminal. The first devised method was described by Sanger in 1953 who used 2,4-dinitro-fluorobenzene (DNFB), which reacts with the five unprotonated α-amino group, to form a yellow 2,4-dinitrophenyl derivates that is cleaved by acid hydrolysis. The amino acid residue is identified by chromatographic separation. A more sensitive method is the labelling of α-amino group with a fluorescent dansyl chloride and the derivative is detected by fluorometric methods. The most important and commonly used procedure is based on the Edman degradation method. It involves a three step cycle for the sequential identification of each amino acid residue starting at the N-terminal (Figure 2.4). The first step of each cycle is to couple the α-amino group by reacting it with phenylisothiocyanate (PITC) in an alkaline medium. This results in a phenylthiocarbamyl (PTC) derivative. The second step would be to cleave the PTC-peptide by treatment with anhydrous acid such as trifluoracetic acid (TFA). This yields an anilinothioxolinone ATZ-derivative and the remaining intact polypeptide chain. The ATZ-derivative is then converted to phenylthiohydantain (PTH) amino acid in the third step. This derivative can be identified by

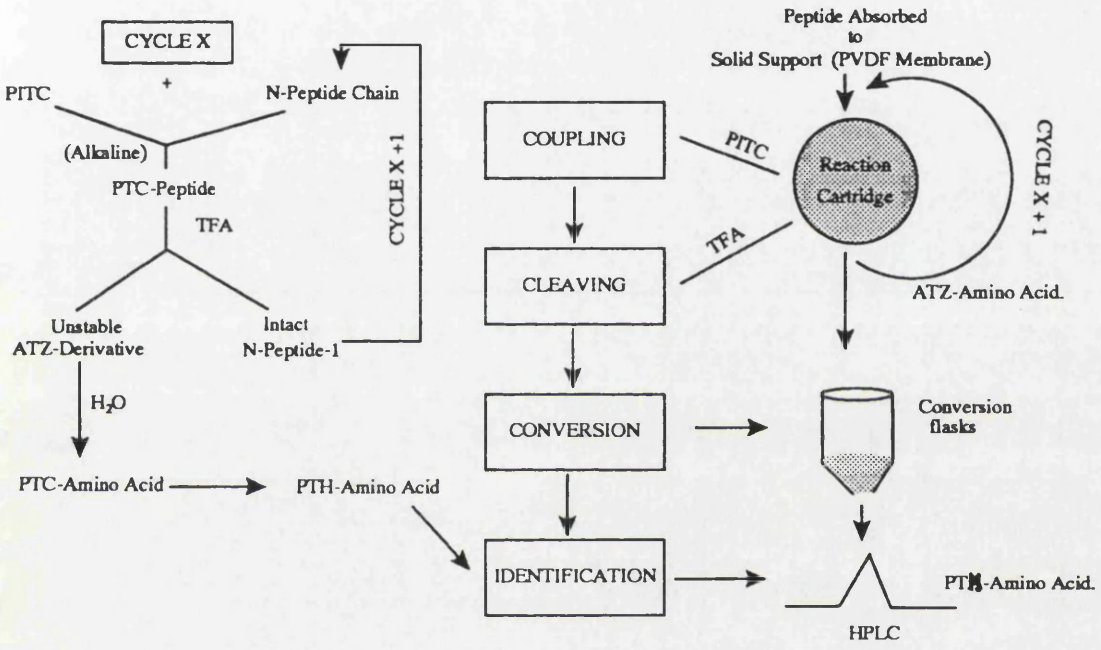


Figure 2.4: Chemical reaction of the Edman degradation method in an automated sequencing instrument. In the coupling step, the PITC modifies the N-terminal residue of a peptide followed by the cleaving step in which the N-terminal residue is cleaved by TFA resulting in an unstable ATZ-Derivative and an intact shortened peptide with a reactive N-terminus to enter another cycle (cycle x + 1). These two steps are performed on a protein solid support (PVDF - Immobilin membrane) which takes place in a reaction cartridge. The next step is the conversion of unstable ATZ-Derivative (in a flask) to a stable PTH amino acid that can be identified by the elution time of a peak on HPLC. (After Matsudaira, 1989)

chromatographic separation. The shortened polypeptide is then subjected to another cycle and so on until the last remaining amino acid residue is modified. This method was first automated by Edman and Begg in 1967. The development of the gas-liquid or pulsed-liquid chromatography improved the sensitivity, speed and the ease of sequencing operation. The employment of microbore columns for PTH amino acid detection in computerized HPLC allows longer sequencing (40-60 cycles) of peptides from as little as 10 pmol.

TK amino acid sequence analysis (15 cycles) was performed from eight bands on the PVDF membrane by the Molecular Paleontology Laboratory at Glasgow University. Automated sequencing of the Edman degradation method, by Pulse-liquid Chromatography on Applied Biosystems (477A) Protein Sequencer with gradient on-line (120A) HPLC, was used.

Prior to sequencing TK, four precycles of 100pmol standard amino acid sequence, with 1.5mg polybrene (identify sequence by-products), was carried out by commencing with the coupling step. The background of PTH-amino acids must fall prior to loading TK bands. The eight bands excised from PVDF were loaded in a glass scintillation vial and inserted in the cartridge. To the cartridge, 5% (v/v) PITC was delivered in a volume enough to wet the filter(s). The PTC-peptide was washed, with 12.5% (v/v) trimethylamine and 0.001% DDT, and cleaved by the addition of 25% anhydrous TFA, and 0.001% DTT. Reagents were delivered automatically from these reservoirs via a teflon tube. The unstable ATZ-derivative is passed to the conversion flasks and where excess PITC, exposed to water, forms N,N^1 diphenylthiourea (DPTU). Unreacted PITC is dissolved out to waste from the cartridge and conversion flasks by delivering n-heptane solvent. Excess DPTU, and other by-products N, N^1-diphenylurea (DPU), were dissolved out to waste by delivering a second solvent (ethyl acetate). Delivery of solvent (n-chlorobutane), allowed the precipitation of potentially extractable hydrophobic samples, was made directly into 25% TFA to initiate immediate conversion.

Once the ATZ-derivative was converted to the PTH-amino acid, the next cycle was initiated. The PTH-amino acid was dried and reconstituted with 20%

(v/v) acetonitrile solvent and transferred to the HPLC. PTH-amino acids were identified by gradient elution on a reverse-phase column and HPLC elution profile at 269nm was recorded for each cycle. Amino acids were identified by their characteristic retention time. The sequence was determined by the appearance of a PTH-amino acid in cycle 1, and the simultaneous disappearance of that amino acid with the appearance of the next (if different) PTH-amino acid in cycle 2. The amino acid detected in each cycle and the by-products elution were recorded on a chromatograph. The derivation of the amino acid analysis is illustrated in Figure 2.5. The amino acid sequence was analyzed for homologies in the Gen Bank Database.

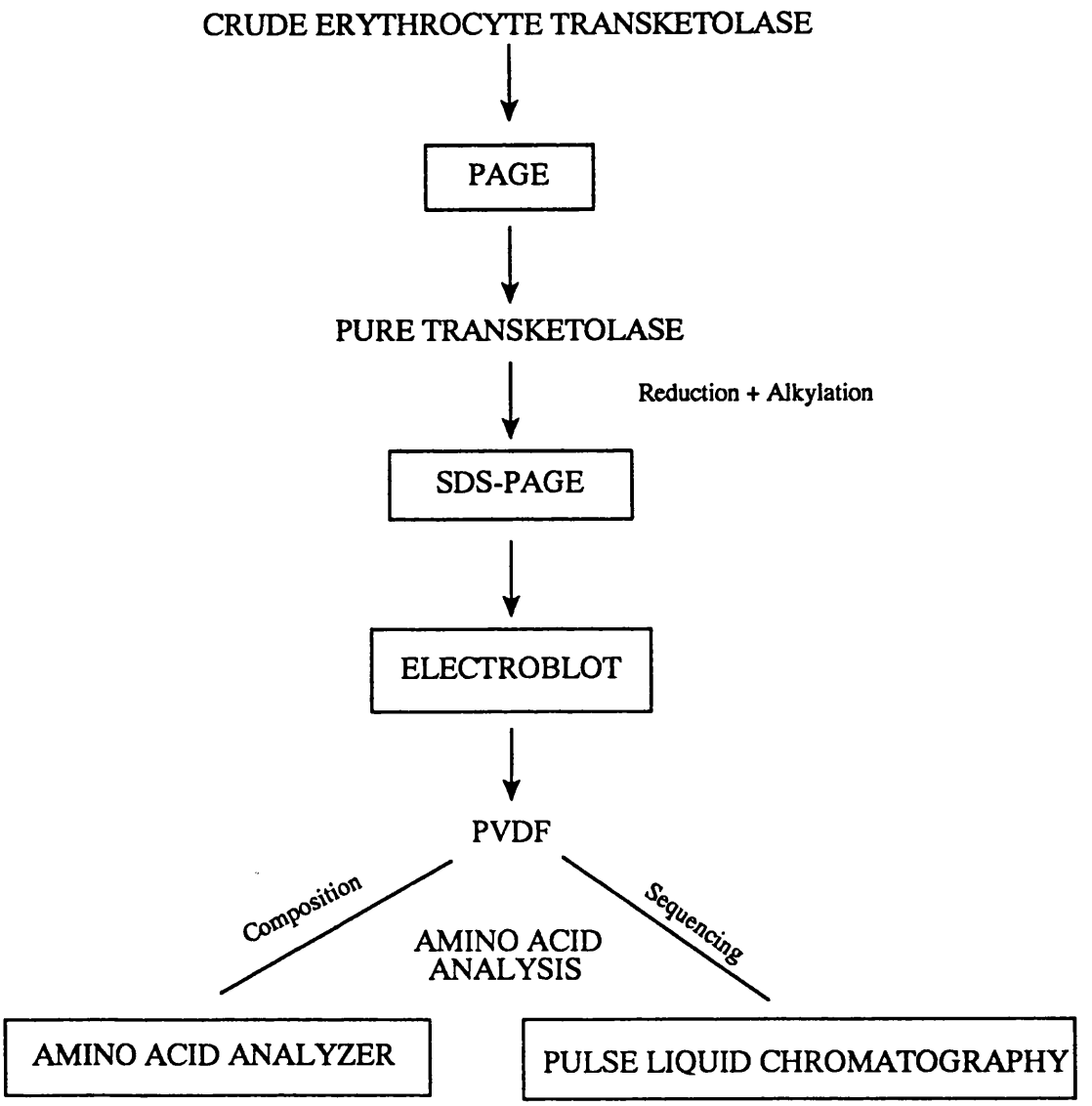


Figure 2.5: Summary of obtaining amino acid analysis from purified erythrocyte transketolase. The first step is the isolation of homogenous pure erythrocyte transketolase from a normal individual by polyacrylamide gel electrophoresis (PAGE). The purified transketolase is then subjected to SDS-PAGE and the protein band were electroblotted on an Immobilon (PVDF) membrane. The bands were excised from PVDF; treated and subjected to amino acid analysis.

CHAPTER 3: RESULTS

3.1 ANALYSIS OF ERYTHROCYTE TRANSKETOLASE IN CRUDE SAMPLE

3.1.1 Quantitative Analysis of Erythrocyte Transketolase

3.1.1.1 Transketolase Endpoint Assay

The initial endpoint assay, developed to measure the activity of TK in RBC, was performed on 7 normal controls. ETK and ETKs activity were measured using formula 2.2 and the results are listed in Table 3.1. The mean ETK activity was 0.51 ± 0.11 IU/g Hb and the addition of TPP did not significantly ($p > 0.1$) increase the ETK activity (ETKs) which was 0.47 ± 0.12 IU/g Hb with a mean Hb concentration of 37.19 ± 2.52 g/dL.

The endpoint assay was modified by varying several conditions and measuring ETK and ETKs activity on the same samples (7 normal controls). The summary of these modifications and their result are listed in Table 3.2. It was apparent that heating the sample and precipitating Hb by the addition of TCA or NaOH resulted in a loss of TK activity. Other variations (boiling haemolysate and incubating in a shaker waterbath) resulted in negative TPPE due to the low values of ETKs obtained from the inhibition of TPP binding to TK.

However, reducing the Hb from 40g/dL to 20g/dL improved the assay slightly (Table 3.3). With this modification, TK endpoint assay was performed on 14 normal controls and 6 patients (results are listed in Table 3.3). Performing the Student t-test on mean ETK activity from normal controls (0.41 ± 0.14 IU/g Hb) and patients (0.54 ± 0.16 IU/g Hb) gave a value of $t = 0.679$ which was not statistically signficant ($p>0.1$). The mean ETKs activity for normal controls (0.45 ± 0.18 IU/g Hb) and patients (0.56 ± 0.17 U/g/dL Hb) gave a t-test value of $t=0.542$ which was also not statistically significant ($p>0.1$) with mean Hb concentration for normal controls 20.38 ± 1.34 g/dL and patients 21.53 ± 1.36 g/dL.

Table 3.1 Erythrocyte transketolase (ETK) activity and stimulated erythrocyte transketolase (ETKs) acitivity in 7 normal controls estimated by the developed endpoint assay. Result are averages of duplicates.

Sample	Haemoglobin g/dL	ETK activity IU/g Hb	ETKs activity IU/g Hb	TPP Effect (%Activation)
100180	35.7	0.46	0.30	-34.78
100181	37.5	0.45	0.32	-28.89
100182	41.9	0.63	0.46	-26.98
100183	37.2	0.68	0.57	-16.18
100184	38.2	0.36	0.47	30.56
100185	35.9	0.50	0.52	4.00
100186	33.9	0.50	0.62	24.00
MEAN	37.19	0.51	0.47	-6.89
S.D (±)	2.52	0.11	0.12	26.54

Table 3.2 Summary of the modifications performed on the end point assay and their results. The mean transketolase activity with (ETKs) and without TPP (ETK) are listed. The result of each variation is expressed as a negative effect (-ve) or a positive effect (+ve). All samples applied were normal controls and the number of samples (n) varied.

Modification	**Mean ETK activity IU/g Hb**	**Mean ETKs activity IU/g Hb**	**Effect**
Heating Haemolysate (n=7)	0.35(±.08)	-	-ve
Boiling Reaction Mix (n=6)	0.47(±.10)	0.30±.05)	-ve
Incubating in Shaker Bath (n=5)	0.50(±.20)	0.27(±.12)	-ve
Addition of 10% TCA (n=3)	0.02(±.01)	0.005(±.002)	-ve
Addition of 20% NaOH (n=5)	0.28(±.09)	-	-ve
Addition of DTT (n=6)	0.61(±.21)	0.59(±.22)	-ve
Addition of DTT + $MgCl_2$ (n=5)	0.65(±.11)	0.61(±.08)	-ve
Increasing Incubation Δt	Table 3.5	Table 3.5	+ve
Reducing Hb concentration	Table 3.4	Table 3.4	+ve
Addition $MgCl_2$ (n=7)	0.50(±.12)	0.54(±.16)	+ve
Addition xylulose-5-phosphate	Table 3.5	Table 3.5	+ve

3.1.1.2 Application of Transketolase Assay in Wernicke-Korsakoff syndrome

The modified endpoint assay (reducing Hb concentration, increasing incubation time, adding X-5-P and $MgCl_2$) gave a more reliable, reproducible and sensitive method for analysis of ETK in normal controls and patients with WKS. Specificity and accuracy of ETK and ETKs activity were achieved by expressing these values in IU/mg protein. The modified endpoint assay was performed on 20 patients and 10 normal controls to establish any difference between ETK and ETKs activity with cofactor and coenzyme between the two groups. The t value ($t = 0.073$) obtained by comparing the mean and standard deviation (with the Student t-test) between the mean ETK activity from normal controls (0.418 ± 0.193 IU/mg protein) and patients (0.406 ± 0.155 I.U/mg protein) was not statistically significant ($p>0.1$). The mean ETKs activity between normal controls (0.738 ± 0.208 IU/mg protein) and patients (0.680 ± 0.19 IU/mg protein), was also not statistically significant ($t = 0.33$, $p>0.1$). The mean protein concentrations in the normal controls was 18.72 ± 0.07 mg/ml and in patients 18.43 ± 0.06mg/ml.

3.1.2 Qualitative Analysis of Erythrocyte Transketolase

3.1.2.1 Starch Gel Electrophoresis

The electrophoretic mobility of ETK was initially investigated by starch gel electrophoresis. Samples from one normal control and one patient were run in duplicate after preincubation with the cofactor TPP. The gel was sliced horizontally and stained with specific TK stain. A faint blue band was detected which represents the site of ETK activity (Figure 3.1) which was cathodic to the Hb band. The separation of ETK from Hb was achieved using this method.

3.1.2.2 Cellogel Electrophoresis

Cellogel electrophoresis was performed from RBC of 13 normal controls and 7 patients with WKS. All samples were preincubated with the cofactor TPP prior to electrophoresis. Specific TK stain allowed the detection of two ETK

Table 3.3a Haemoglobin concentration and erythrocyte transketolase activity with (ETKs) and without (ETK) coenzyme TPP (ETK) in 14 normal controls. Transketolase endpoint assay performed after the reduction of haemoglobin (Hb) concentration from 40g/dL to 20g/dL. The values listed are the average of duplicates.

Sample	Haemoglobin g/dL	ETK activity IU/g Hb	ETKs activity IU/g Hb	TPP Effect (%Activation)
100180	20.8	0.31	0.40	29.03
100181	19.6	0.62	0.72	16.12
100182	21.5	0.40	0.28	-30.00
100183	20.2	0.41	0.23	-43.90
100184	17.8	0.26	0.27	3.85
100185	17.6	0.13	0.31	138.46
100186	20.8	0.32	0.52	59.38
100187	20.8	0.50	0.58	16.00
100188	21.3	0.62	0.64	3.23
100189	20.4	0.52	0.77	48.08
100190	21.6	0.45	0.48	6.67
100191	21.8	0.46	0.24	-47.83
100192	19.5	0.32	0.38	18.75
100193	21.6	0.39	0.43	16.26
MEAN	20.38	0.41	0.45	16.29
S.D (±)	1.34	0.14	0.18	46.65

Table 3.3b Haemoglobin (Hb) concentration and erythrocyte transketolase activity with (ETKs) and without (ETK) coenzyme TPP for the 6 patients with Wernicke-Korsakoff syndrome.

Sample	Haemoglobin g/dL	ETK activity IU/g Hb	ETKs activity IU/g Hb	TPP Effect (%Activation)
100100	22.8	0.48	0.54	12.5
100194	22.9	0.60	0.62	3.33
100195	19.2	0.65	0.59	-9.23
100196	21.2	0.24	0.28	16.67
100197	21.2	0.57	0.81	42.10
100198	21.9	0.68	0.53	22.06
MEAN	21.53	0.54	0.56	7.22
S.D (±)	1.36	0.16	0.17	22.26

Table 3.4: Mean erythrocyte transketolase (ETK) activity and stimulated erythrocyte transketolase (ETKs) activity in 30 samples analyzed by the modified endpoint assay. ETKs was obtained by the addition of thiamine pyrophosphate and $MgCl_2$. The sample included 10 normal controls and 20 patients with Wernicke-Korsakoff syndrome.

Sample	Protein concentration mg/ml	ETK activity (IU/mg protein)	ETKs activity (IU/mg protein)
Normal Mean ± S.D	18.72 0.07	0.418 0.193	0.738 0.208
Patient Mean ± S.D	18.43 0.06	0.406 0.155	0.680 0.190

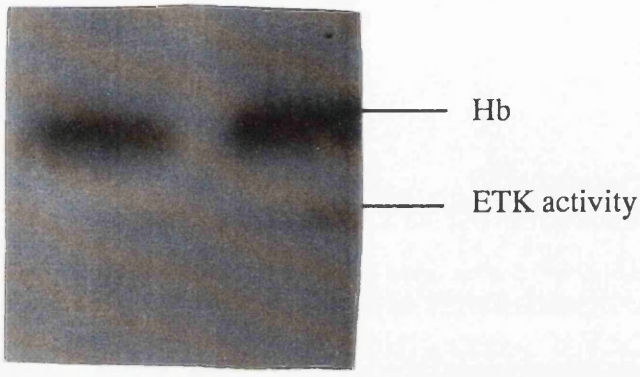


Figure 3.1: Illustration of Erythrocyte transketolase (ETK) activity band on starch gel detected with specific transketolase stain. It is apparent that the ETK band was well separated from the haemoglobin (Hb).

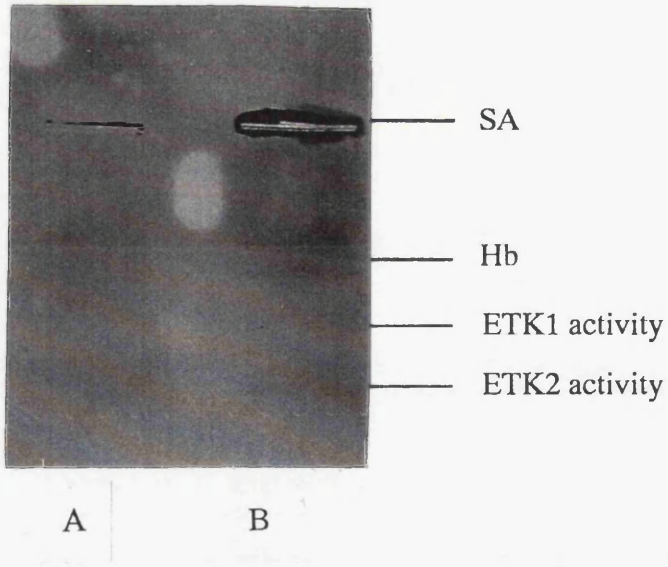


Figure 3.2: Illustration of erythrocyte transketolase (ETK) activity bands on cellogel detected with specific transketolase. Transketolase was resolved into two bands (ETK 1 and ETK 2) that varied in stain intensity and which were separated from haemoglobin (Hb). (SA = sample application).

A. Electrophoretic profile representing a normal control
B. Electrophoretic profile representing a patient with Wernicke-Korsakoff syndrome.

bands cathodic to the Hb band for all samples (Figure 3.2). There was some variation in band intensity between different samples, but all showed a characteristic pattern of two ETK bands, one (ETK 2) which stained more densely than the other (ETK 1). In some gels, it was difficult to detect the second ETK 1 band due to the faint staining. Although the separation of ETK from Hb was improved (from the previous method) there was no difference observed between WKS patients and normal controls.

3.1.2.3 Polyacrylamide Gel Electrophoresis

Prior to analyzing samples from normal controls and patients with WKS using PAGE, the specific TK stain was modified in an attempt to reduce the initial heavy background staining. The first modification involved the replacement of NBT with MTT which resulted in better band intensity and reduced background staining (Figure 3.3). To confirm that the stained bands represented ETK activity one half of the gel ("gel 1") was stained with specific TK stain and the other half ("gel 2") with both substrates (R-5-P and X-5-P) omitted (Figure 3.4A,B). These were then stained with CBB R-250 to confirm the bands detected in gel 1 and undetected in gel 2 do exist and are protein in nature (Figure 3.4C). Two protein bands were visualized in "gel 2" after staining with CBB R-250. To obtain an accurate localization of the ETK bands in relation to the Hb position, the gel was stained with silver stained (Figure 3.5D).

A total of 15 normal control samples and 11 patient samples were run in duplicate. All samples revealed two bands of ETK activity (varying in band intensity) approximately 10-15mm cathodic to the Hb band (Figure 3.5). The two ETK bands were qualitatively similar in both the normal control (n=15) and patient samples (n=11) analyzed.

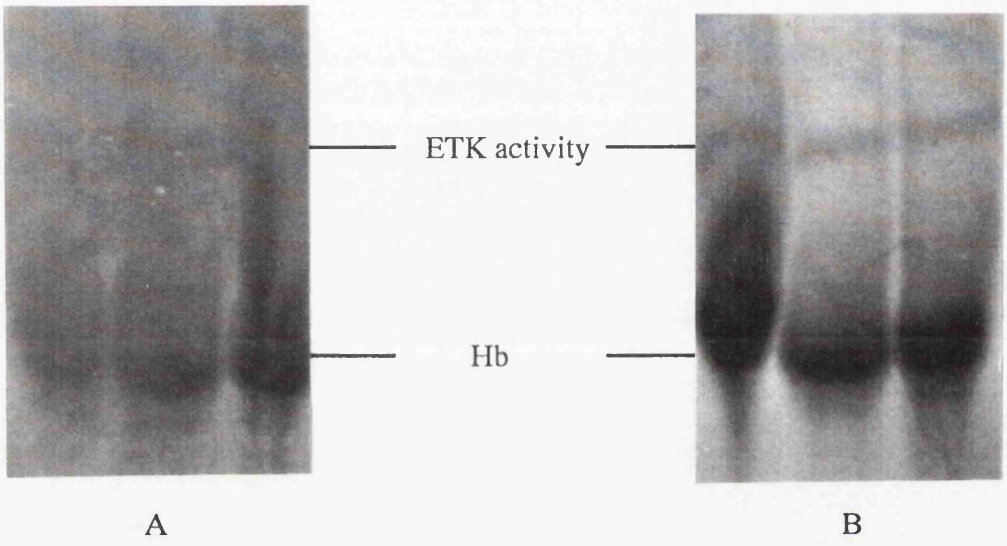


Figure 3.3: Illustration of the improved specific transketolase stain. The gel was sliced in two halves after polyacrylamide gel electrophoresis.

A. The one half of the gel subjected to the unmodified specific transketolase stain*. Erythrocyte transketolase (ETK) activity is faintly visualized but well separated from haemoglobin (Hb)

B. The other half of the gel subjected to the modified specific transketolase stain**. The resolved ETK is clearly visualized and background staining reduced

* with nitroblue tetrazolium

** with methyl thiazolyl tetrazolium

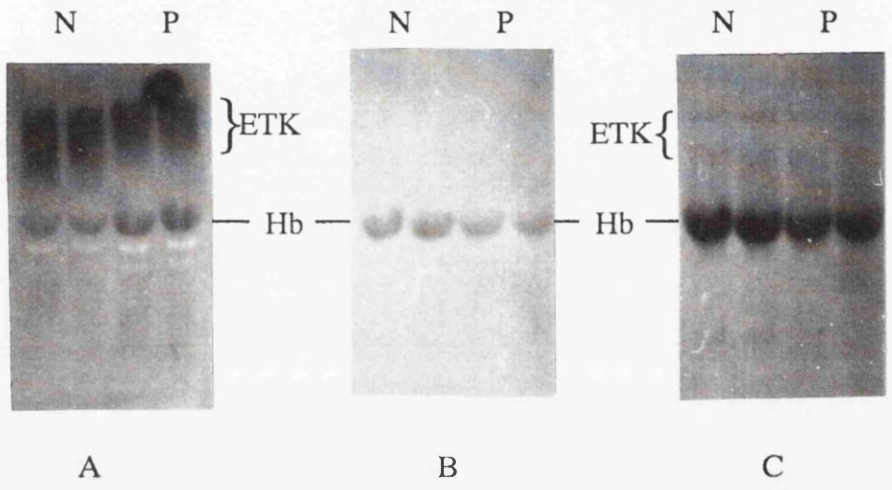


Figure 3.4: Illustration on the sensitivity of the specific transketolase (TK) stain. A normal control (N) and a patient (P) sample with Wernicke-Korsakoff syndrome were run in quadreplicate on a polyacrylamide gel (PAG). The PAG was sliced in two halves and subjected to specific TK stain. The site of erythrocyte transketolase (ETK) activity separated from haemoglobin (Hb) was analyzed to confirm stain sensitivity.

A. The one half of the gel subjected to specific TK stain with the detected ETK bands

B. The other half of the gel subjected to specific TK stain but depleted substrates (xylulose-5-phosphate and ribose-5-phosphate), no bands except for Hb detected

C. Restaining of B with coomassie brilliant blue R-250, ETK band was resolved into 2 distinct bands on both halves of the gel

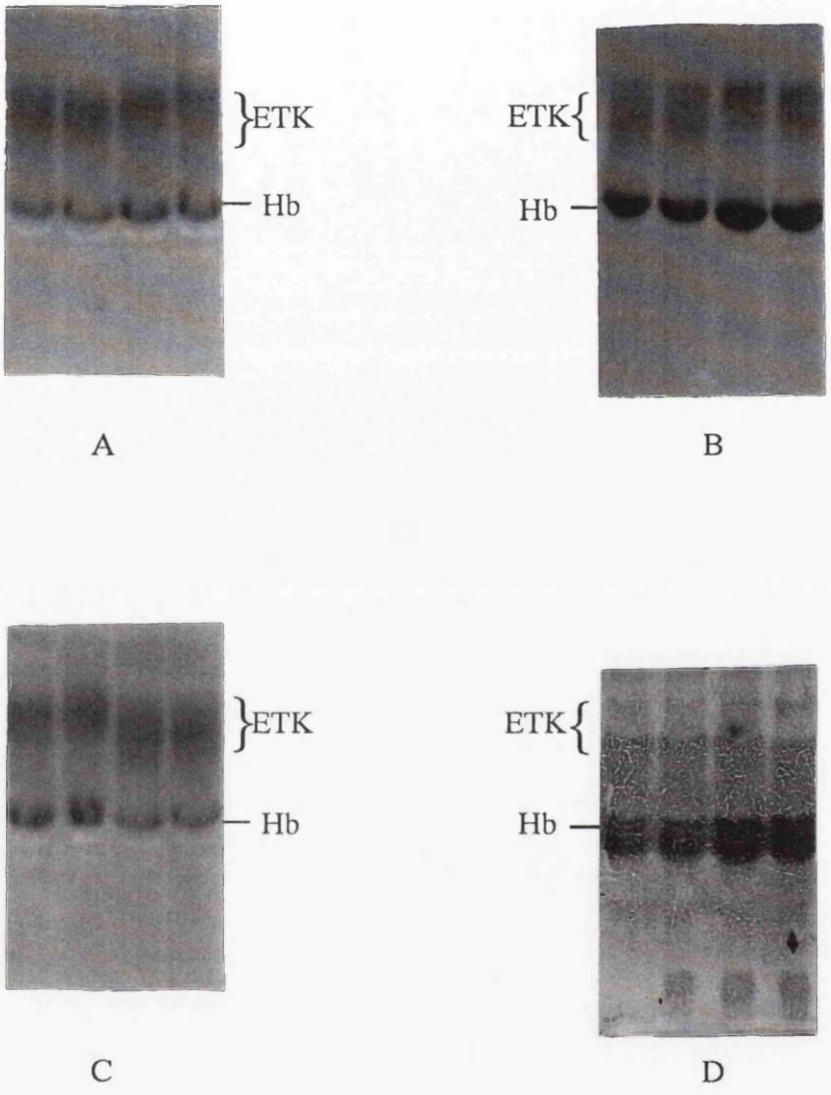


Figure 3.5: Electrophoretic profile of erythrocyte transketolase (ETK) on polyacrylamide gel (PAG). The samples analyzed were normal controls and patients with Wernicke-Korsakoff syndrome.

A. PAG subjected to specific TK stain revealing the site of ETK activity and separation from haemoglobin (Hb)

B. Restaining of A with coomassie brilliant blue R-250 allowed further resolution of ETK

C. PAG stained with specific TK stain

D. Restaining of C with BioRad's silver stain allowing the resolution of ETK into two quantitatively distinct ETK bands

3.1.3 Analysis of Transketolase in Wernicke-Korsakoff syndrome

Preliminary work on the quantitative analysis of ETK by the development of the TK assay gave indication of difficulties that may arise from Hb interference and insufficient binding of cofactor to enzyme. Although these were overcome by several modifications negative values for TPPE and wide variation in values within and between samples persisted (Table 3.3). ETK and ETKs activity was expressed in IU/mg protein rather than IU/g Hb to obtain a more specific and accurate value for the analysis in WKS. Quantitative analysis of ETK and ETKs activity using the modified TK assay in 10 normal controls and 20 patients with WKS showed no systematic statistically significant differences ($p>0.1$) between the two groups (Table 3.4).

Preliminary work using electrophoretic techniques allowed the analysis of ETK mobility by specific TK stain and its separation from Hb on a gel matrix (starch gel). This was examined further by cellogel electrophoresis. Although this method was rapid, reliable and inexpensive the ETK bands were difficult to visualize due to the smearing of Hb and difficulties with specific TK stain. By employing PAGE it was possible to improve the sensitivity of the stain for qualitative analysis and to apply silver staining for quantitative analysis of ETK electrophoretic profile. Qualitative analysis of ETK in 15 normal controls and 11 patients with WKS revealed the presence of two ETK bands with varying intensities as detected by specific TK stain (Figures 3.5A). ETK bands were shown to vary quantitatively within each sample tested by the silver stain method (Figure 3.5D).

Although ETK was shown to be heterogenous by electrophoretic techniques, quantitative and qualitative analysis did not reveal any differences between TK in crude samples of normal controls and patients with WKS.

3.2 PURIFICATION OF ERYTHROCYTE TRANSKETOLASE BY CHROMATOGRAPHIC TECHNIQUES

3.2.1 Qualitative Analysis of Purified Erythrocyte Transketolase

3.2.1.1 Polyacrylamide Gel Electrophoresis

All purified and reconstituted samples were run on PAGE and protein detection was performed by silver staining following specific TK stain to verify any protein bands separated represent ETK activity*. Partially purified fractions of ETK from 11 normal controls and 25 patients with WKS were subjected to PAGE. Silver stained gels resolved two ETK bands in all samples analyzed. The electrophoretic profile of ETK on PAGE is illustrated in Figure 3.6.

3.2.1.2 Isoelectric Focusing

The isolated fractions from both normal control and patient samples were concentrated and focused on IEF gels. One gel containing fractions from one normal control and one patient sample was subjected to specific TK stain. Two ETK bands of very low intensity were detected on the IEF gel*. The gel was then subjected to silver staining to verify the bands detected by specific TK stain were true proteins and to test for the presence of contaminating proteins (ETK). Partially purified fractions of ETK (n=36), pI protein markers and a polyacrylamide control blank were subjected to IEF followed by silver staining. ETK was resolved into two bands with no contaminating bands present in the polyacrylamide control blank thus confirming the purity of the ETK fraction. All normal controls (n=11) and patient samples (n=25) were resolved into two ETK bands with pI 7.35 and 6.55 (estimated from protein markers) as illustrated in Figure 3.7.

* Due to the heavy background staining and low intensity of the bands, electrophoretic profile of ETK detected by specific TK stain is not illustrated

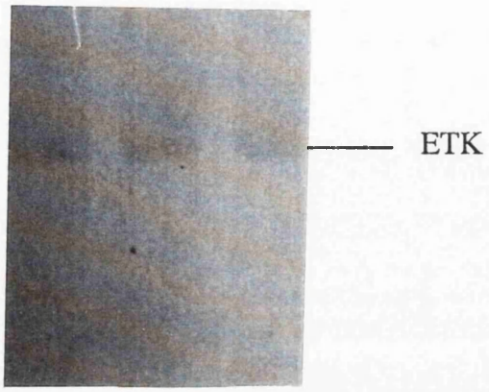


Figure 3.6: Electrophoretic profile of purified transketolase fractions on a polyacrylamide gel. Purified erythrocyte transketolase (ETK) from a normal control was resolved into two distinct bands detected by BioRad's silver stain.

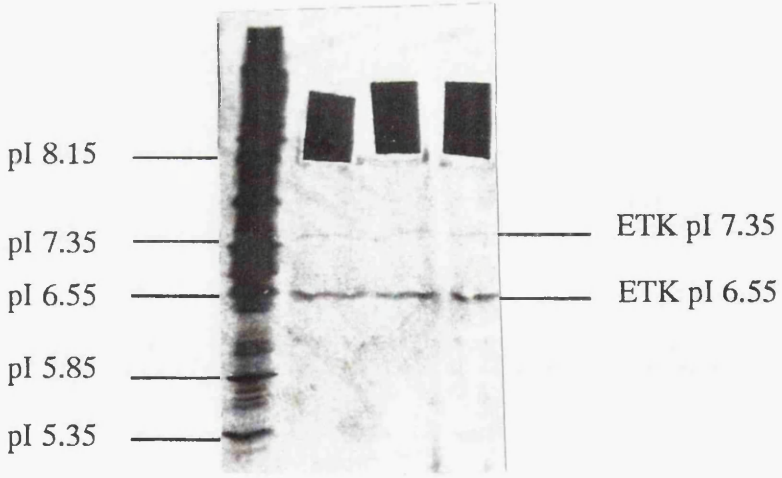


Figure 3.7: Electrophoretic profile of purified transketolase by isoelectric focusing. The gel was stained with BioRad's silver stain and the isoelectric points (pI) of the two resolved erythrocyte transketolase (ETK) bands in the normal sample were estimated from protein markers (pI 5.35-8.15).

3.2.1.3 High Performance Liquid Chromatography

Fractions isolated by PAGE from one normal control and one patient with WKS were subjected to ion-exchange HPLC. Two peaks were recorded on the chromatograph in each case (Figure 3.8, 3.9). In both samples the first peak eluted at 19 minutes under an increasing gradient (0-35%) of 1M NaCl in 20mM Tris-HCl pH 7.5. The second peak eluted at 31 minutes under an increasing gradient (35-100%) of 1M NaCl in 20mm Tris-HCl pH 7.5. These two peaks were consistent with the ETK bands detected in IEF which also contained TK activity. The elution profile shows that the purified fractions were free from contaminating proteins since no other peaks were eluted. It also demonstrates the heterogeneity of TK with respect to ionic strength (thus retention time).

3.2.2 Analysis of Partially Purified Transketolase in Wernicke-Korsakoff Syndrome

The extraction of TK from RBC using the PAGE purification method was performed on 36 samples (11 normal controls and 25 patients). The isolated fractions from all samples were subjected to quantitative analysis and qualitative analysis.

Paritally purified fractions of TK from RBC of 36 samples were assayed for ETK activity by the modified endpoint assay. It was found that partially purified ETK requires both cofactors TPP and $MgCl_2$ for activity. The mean ETK activity estimated in 11 normal controls was 6.79 ± 3.61 IU/mg protein (6.41 mg/L) and 5.89 ± 2.02 IU/mg protein (6.60mg/L) in 25 patients with WKS. These values were not statistically significant ($t = 1.41$, $p>0.1$).

Although qualitative analysis of purified fractions of ETK illustrated its heterogeneity with respect to pI value and ionic strength no differences between normal controls (n=11) and patients (n=25) with WKS were observed.

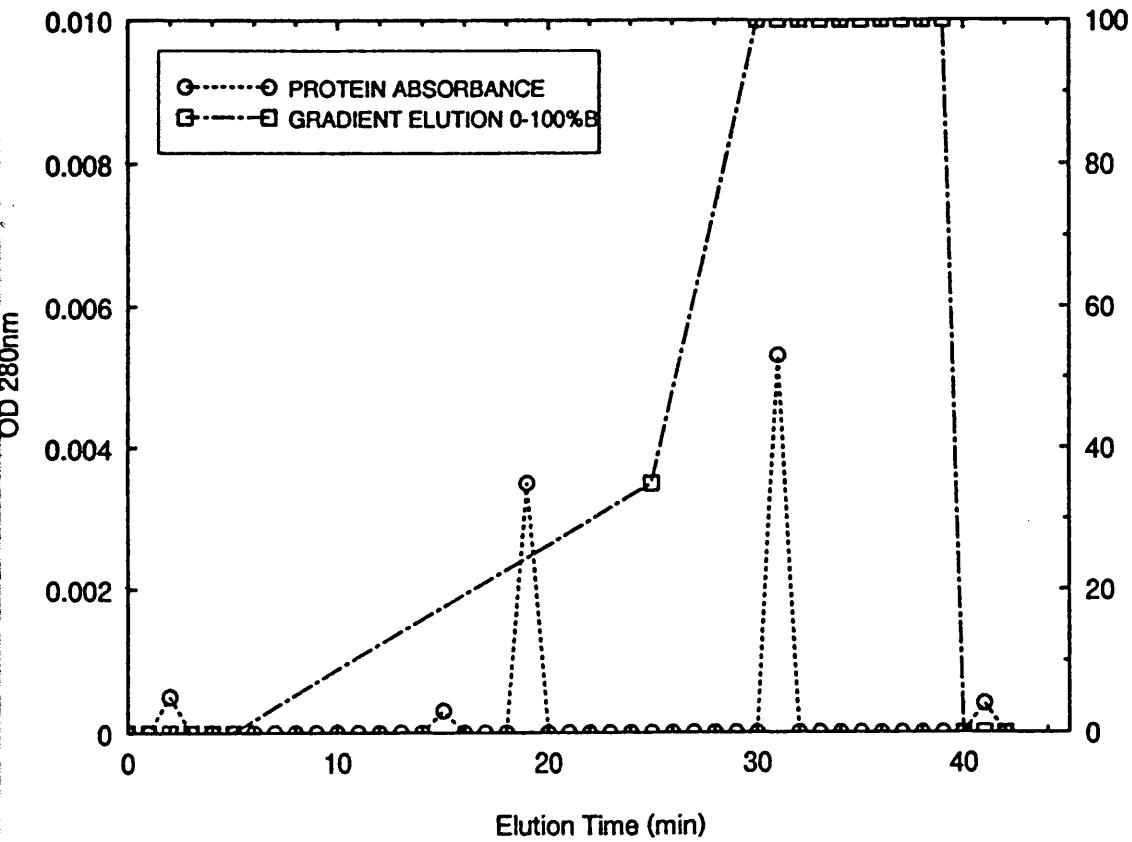


Figure 3.8: Elution profile of purified erythrocyte transketolase (ETK) from a normal control (100133) by high performance liquid chromatography (HPLC). The sample (2.5mg/100ml) was loaded on an ion-exchange column and eluted with 1M NaCl in 20mM Tris-HCL pH 7.6 gradient for 40 minutes. The first ETK peak was eluted at 19 minutes under an increasing gradient 0-35%; the second peak eluted at 31 minutes under 35-100% gradient.

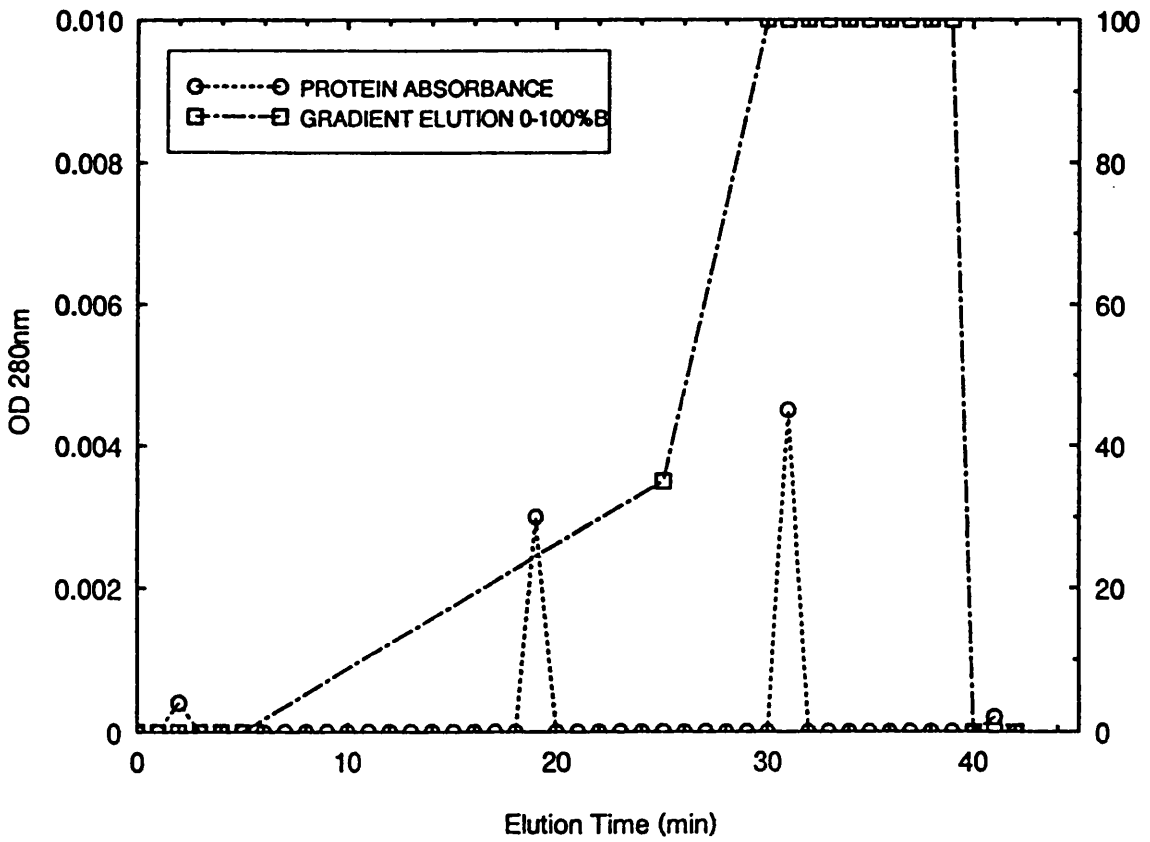


Figure 3.9: Elution profile of purified erythrocyte transketolase (ETK) from a patient with Wernicke-Korsakoff syndrome (100100) by high performance liquid chromatography (HPLC). The sample (1.5mg/100ml) was loaded on an ion-exchange column and eluted with 1M NaCl in 20mM Tris-HCl pH 7.6 gradient for 40 minutes. The first ETK peak eluted at 19 minutes under an increasing gradient of 0-35%; the second peak eluted at 31 minutes under an increasing gradient of 35-100%.

3.3 PURIFICATION OF TRANSKETOLASE FROM WHITE BLOOD CELLS

The crude extract of WBC (30ml) was loaded onto the Sephadex G-25 column. The protein mixture was equilibrated and eluted with 600ml of 10mM Tris-HCl pH 7.8. Fractions (11ml) were collected every 3 minutes and every second fraction was monitored at 280nm for protein absorbance. The results were plotted on the graph (Figure 3.10) and peaks containing high protein absorbance (fractions 12-20) were assayed for TK activity by a kinetic assay (section 2.3.1). TK activity was recovered in a total void volume of 66ml and those in fractions 12-19 were plotted on the same graph (Figure 3.10).

The progress of the purification was monitored by assaying protein concentration and TK activity at every step of the purification (Table 3.5). Enzyme activity after DEAE-cellulose adsorption was only 0.0102 U/mg. Following ammonium sulphate fractionation the final recovery declined to 0.01140 U/mg. Enzyme recovery became poorer as the purification steps progressed, thus no further purification steps were carried out.

3.4 PURIFICATION OF ERYTHOCYTE TRANSKETOLASE BY CHROMATOGRAPHIC TECHNIQUES

3.4.1 Ion Exchange Chromatography on DEAE-Sepharose

Dialyzed haemolysate (72ml) was loaded onto the pre-equilibrated DEAE-Sepharose. Elution was performed with a linear gradient of equal volumes of 1L 10mM Tris-HCl and 1L 0.1M NaCl in 10mM Tris-HCl pH 8.3. Fractions (18ml) were collected every 12 minutes and every second fraction was monitored at 280nm for protein absorbance. TK activity was assayed in every fourth fraction starting from the 20th fraction (last fraction collected prior to the gradient run) up to the 100th fraction. These results were plotted on a graph alongside the protein

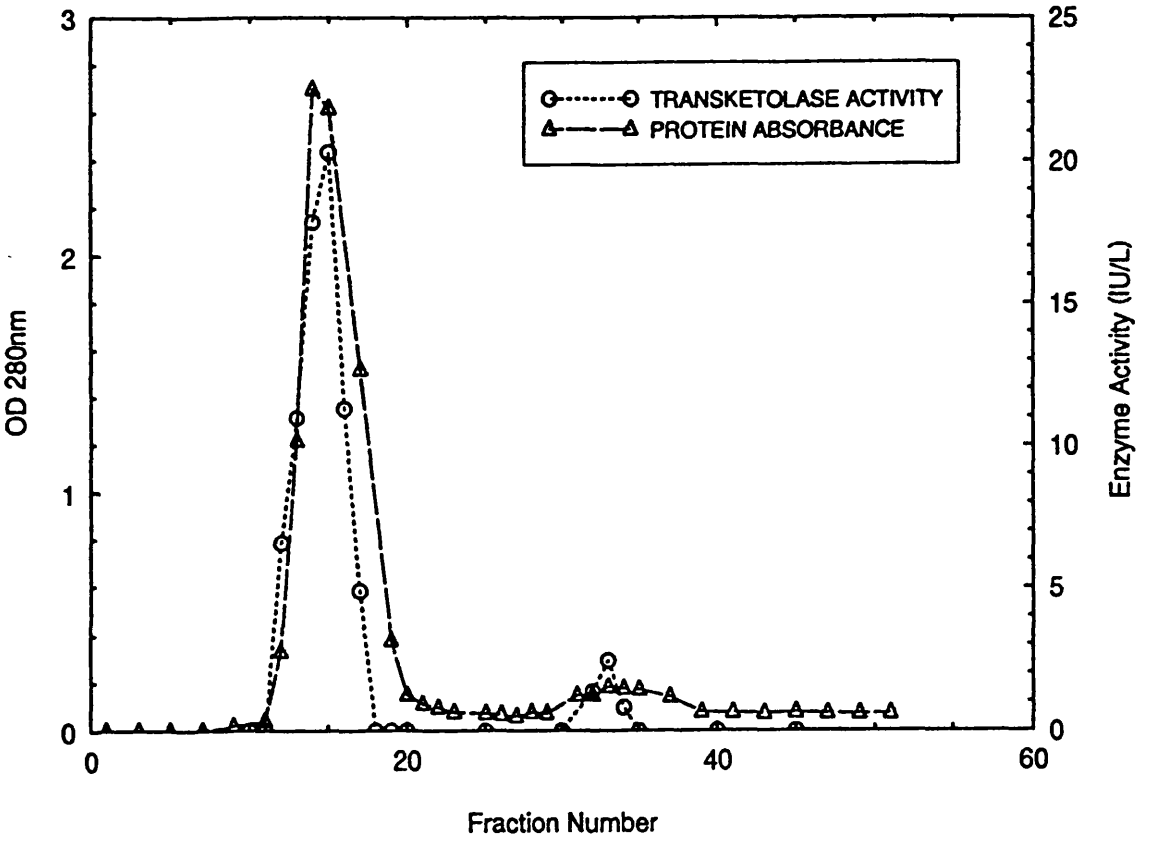


Figure 3.10: Elution profile of transketolase (TK) from white blood cells (WBC) by gel filtration. Protein mixture (75mg/30ml) from WBC was loaded on a Sephadex G-25 column. TK activity was eluted in fractions 12-19 with 10mM Tris-HCl, pH 7.8.

Table 3.5 Purification summary of transketolase from white blood cells (3g). Enzyme activity was obtained from 20μl of sample and protein estimation was performed using Bradford's method.

STEP	Volume (ml)	Activity (IU/L)	Protein (mg)	Specific Activity (IU/mg)	Purification (-fold)
Crude extract	30	9.4	75	0.00376	1
DEAE-Sephadex	72	7.6	54	0.0103	2.7
45% Amm. Sulph.	80	4.9	40	0.0098	2.6
45-90% Amm. Sulph.	91	4.3	34	0.0114	3

absorbance where TK activity was recovered in two peaks (Figure 3.11). Peak A was eluted from fractions 28-32 in a void volume of 80ml and peak B was eluted from fractions 53-58 in a void volume of 100ml. Fractions from Peak A were pooled and fractions from peak B were pooled and assayed for ETK activity (using the kinetic assay in section 2.3.1) and protein concentration. Hb eluted in fractions 64-100 where very low ETK activity (1.4nmol/min/ml) was also detected in fraction 82 (peak C). Further analysis was not performed on peak C while peaks A and B were treated and analyzed separately. Conductivity was also measured in fractions 30-145 and plotted on the same graph (Figure 3.11).

3.4.2 Fast Protein Liquid Chromatography

After dialyzing peaks A and B, 68ml of peak A was loaded onto a Mono Q column and eluted with 0.2M NaCl in 10mM Tris-HCl pH 8.3 with a linear gradient intiated 25 minutes after the start of elution. Fractions (1.2ml) were collected and monitored continuously at 280nm; two peaks were resolved and assayed for TK activity. The results are presented in Figure 3.12. Peak A1 eluted in fraction 48 (1.1ml) and peak A2 eluted in fraction 52 (1.1ml). Peak B (78ml) also resolved into two peaks (B1 and B2) after a gradient elution was initiated. Peak B1 was eluted in fraction 21 (1.2ml) and B2 in fraction 24 (1.2ml). The results were also plotted on a graph (Figure 3.13). All four fractions were dialyzed and stored at 4°C.

3.4.3 Analysis of Purified Erythrocyte Transketolase

Protein concentration and ETK activity was performed at each step of the purification (Table 3.6). Enzyme was recovered at all steps, however, most of it was lost (peak B: 30.6mg to 0.1mg, peak A: 18.3mg to 0.09mg) due to either complete binding to the Mono Q column or its unbinding and immediate elution (retention time=elution time). Although sufficient material was not recovered for amino acid analysis this purification method allowed some characterisation of TK.

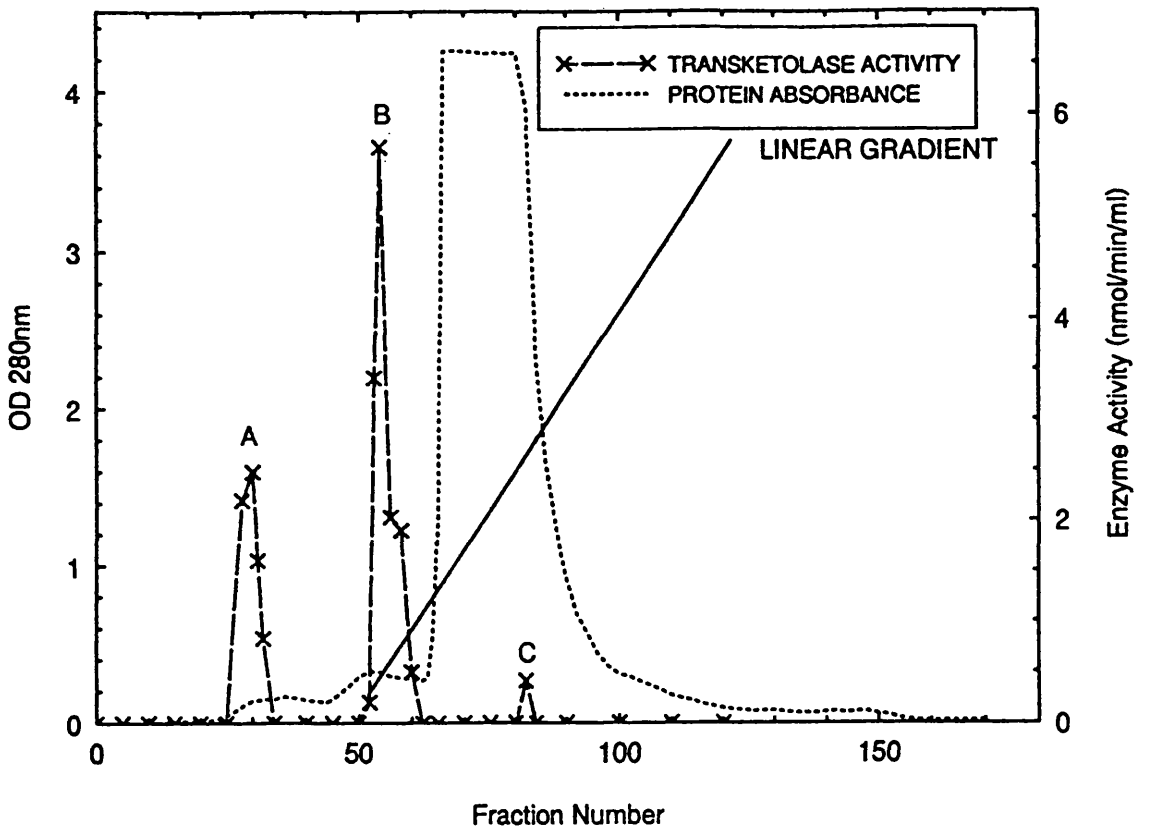


Figure 3.11: Elution profile of erythrocyte transketolase (ETK) and haemoglobin (Hb) by ion-exchange chromatography. The dialyzed haemolysate (72ml) was loaded on DEAE-Sepharose and eluted with a linear gradient of equal volumes (1L 10mM Tris-HCl and 1L 0.1M NaCl in 10mM Tris-HCl pH 8.3). Transketolase activity was eluted in fractions 28-32 (Peak A) and in fractions 53-58 (Peak B). Peak C contained very low ETK activity.

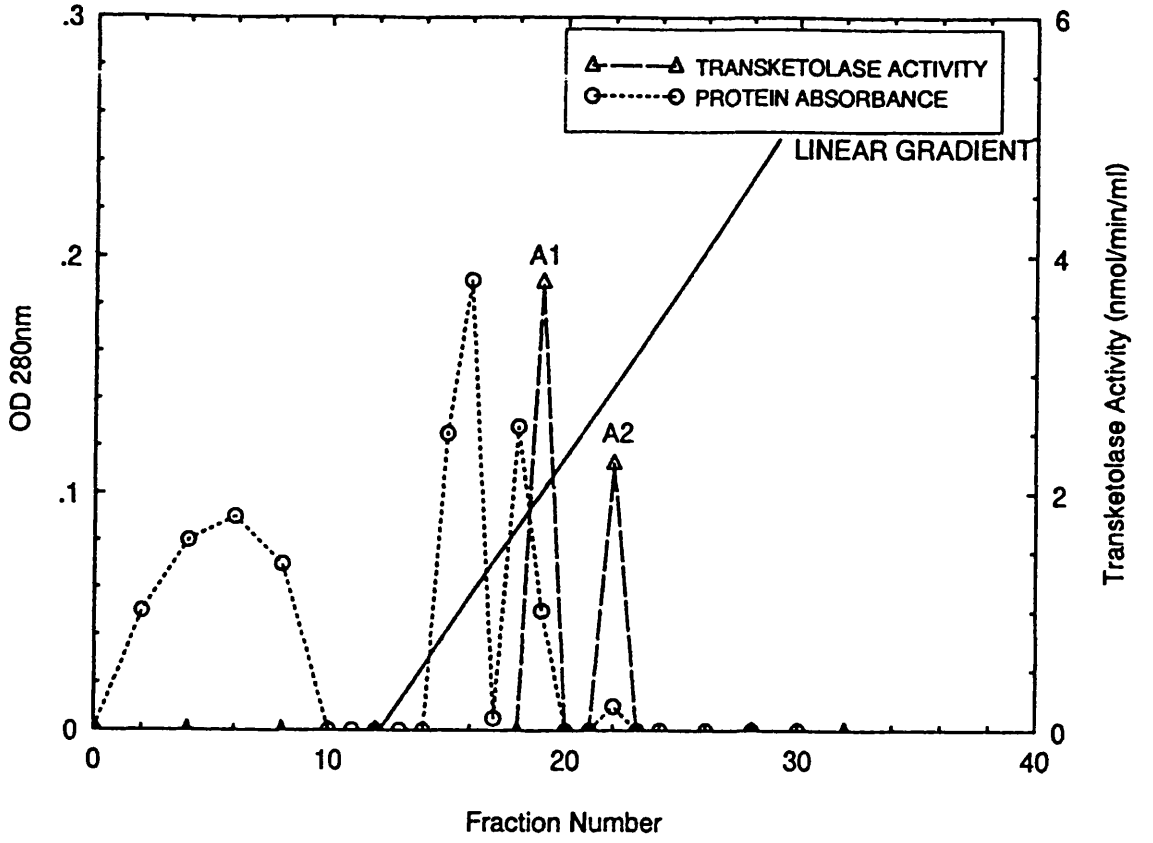


Figure 3.12: Elution profile of purified erythrocyte transketolase (ETK) fraction (peak A) by ion-exchange on fast protein liquid chromotography (FPLC). Peak A (68ml) was loaded onto a Mono Q column and ETK activity was eluted with 0.2M NaCl in 10mM Tris-HCl, pH 8.3 linear gradient. Peak A was resolved further into two peaks (A1 & A2). Peak A1 eluted in fraction 48 and peak A2 eluted in fraction 52.

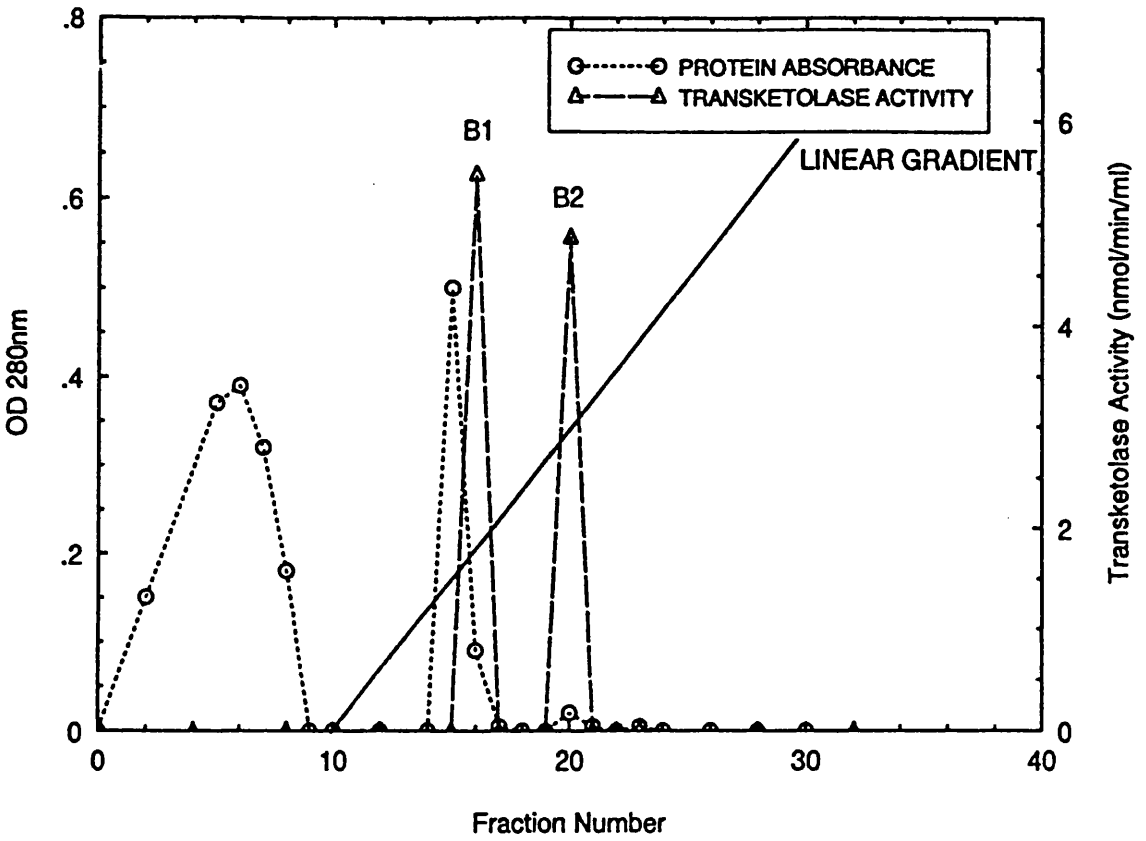


Figure 3.13: Elution profile of purified erythrocyte transketolase (ETK) fraction (peak B) by ion-exchange on fast protein liquid chromotography (FPLC). Peak B (78ml) was loaded onto a Mono Q column and ETK activity was eluted with 0.2M NaCl in 10mM Tris-HCl, pH 8.3 linear gradient. Peak B was resolved further into two peaks (B1 & B2). Peak B1 eluted in fraction 21 and peak B2 eluted in fraction 24.

Table 3.6: Purification summary of erythrocyte transketolase (ETK) by chromatographic techniques. ETK activity was estimated from 20μl of sample using kinetic TK assay and protein estimation was performed using Bradford's method.

Purification Step	Total Volume (ml)	Total Protein (mg)	Total Activity (mol/ min)	Specific Activity (nmol/ min/mg)	Yield (%)	Purifi-cation (Fold)
60ml whole blood 36ml Packed red blood cells	72	20,563	2376	0.115	100	1
DEAE-Sepharose:						
Peak (A)	80	18.36	156	8.5	6.6	74
Peak (B)	100	30.60	427	13.95	18	121
Dialysis:						
Peak (A)	68	10.40	112.88	11.25	4.76	98
Peak (B)	78	18.21	274.47	15.07	11.55	131
FPLC MONO Q:						
Peak (A1)	1.2	0.0612	5.034	82.25	0.21	715
Peak (A2)	1.1	0.0281	3.575	127.45	0.15	1108
Peak (B1)	1.2	0.0459	6.577	143.29	0.28	1246
Peak (B2)	1.2	0.0612	5.844	95.49	0.25	830

The heterogeneity of ETK was confirmed with regard to its ionic strength as analyzed by ion-exchange chromatography (Figure 3.11), FPLC (Figures 3.12 and 3.13) and quantitatively in relation to the proportion of ETK activity eluted in each peak. Peak A had ETK activity of 8.5 nmol/min/mg protein while peak B contained 13.95nmol/min/mg protein.

Following dialysis and freeze-drying, fractions A1, A2, B1 and B2 were analyzed for MW estimation. Protein markers (20μl), original haemolysate (20μl) and fractions A1, A2, B1 and B2 (100μl each) were loaded on a 10% SDS-PAGE. The purified TK fractions were resolved into one band (68kD) detected only with silver staining after CBB R-250 (Figure 3.14). Since these fractions were undetectable with CBB R-250, they were not suitable for amino acid sequencing.

3.5 AMINO ACID ANALYSIS OF TRANSKETOLASE

3.5.1 Isolation of Homogenous Erythrocyte Transketolase and Western Blotting

Homogenous ETK was isolated using the PAGE purification method (section 3.2) since it has achieved the best enzyme recovery, high purity and sufficient material (3μg/160ml) for amino acid analysis. The purification steps and enzyme recovery are summarized in Table 3.7. The isolated fraction (250μg) was subjected to qualitiative analysis to confirm that it was TK (Figures 3.6, 3.7, 3.8).

Molecular weight estimation was determined by SDS-PAGE. Two species were detected with apparent MW of 51kD for the dense band and 35kD for the faint band as determined by the MW markers and detected by CBB R-250 and silver staining (Figure 3.15). Following SDS-PAGE the isolated fraction was loaded onto 10 wells in SDS-PAGE and transfered onto a PVDF membrane by western blotting. The membrane was stained with CBB R-250 and two ETK bands were detected (Figure 3.15). Amino acid analysis was performed on all the ETK tracks in PVDF.

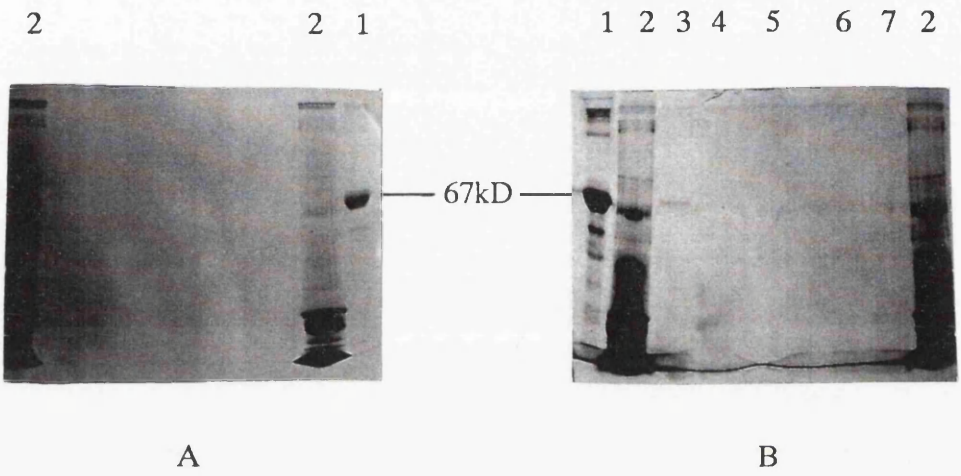


Figure 3.14: Electrophoretic profile of purified erythrocyte transketolase (ETK) by chromatographic techniques. SDS-polyacrylamide gel electrophoresis (SDS-PAGE method 2) was used for the molecular weight estimation of ETK (68kD).

A. SDS-PAG stained with coomassie brilliant blue R-250 (CBB R-250)

B. SDS-PAG destained from CBB R-250 and silver stained using BioRad's silver stain kit

lane 1: 20μl bovine serum albumin as protein marker (67kD).
lane 2: 20μl starting haemolysate
lane 3: 100μl Fraction A1
lane 4: 100μl Fraction A2
lane 5: 100μl Fraction B1
lane 6: 100μl Fraction B2
lane 7: 100μl track dye

Table 3.7: Quantitative analysis of isolated erythrocyte transketolase (ETK) by polyacrylamide gel electrophoresis. ETK was extracted from the haemolysate of a normal control (100133).

Variables	Crude Sample	Purified Sample
Total Volume (ml)	0.16	10
Total Activity (IU x 10^{-3})	3.248	1.45
Total Protein (mg)	3.04	0.250
Specific Enzyme Activity (IU/x 10^{-3})	1.068	5.8
Purification Field	1	5.4

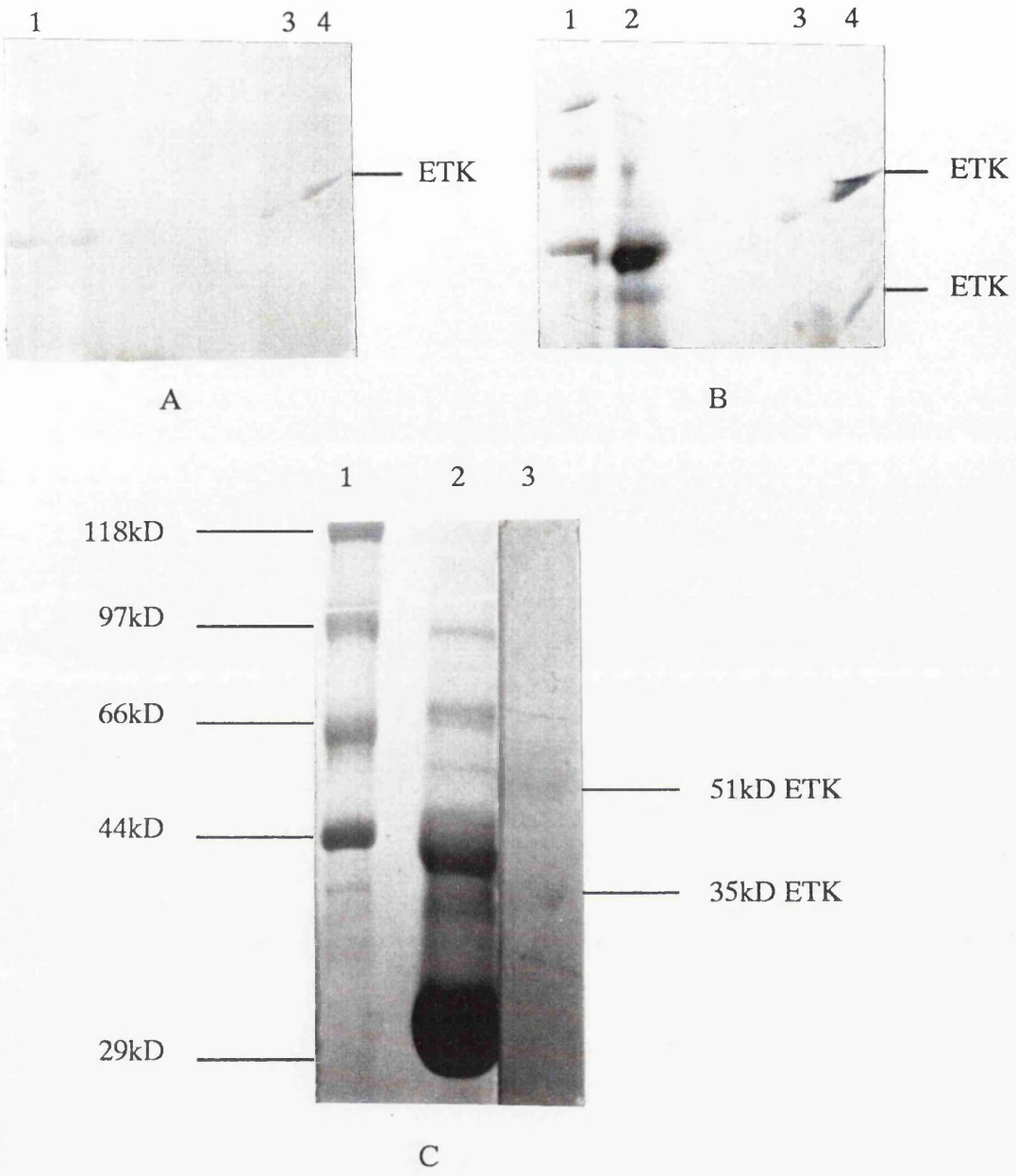


Figure 3.15: Electrophoretic profile of isolated homogenous erythrocyte transketolase (ETK). ETK was purified by the polyacrylamide gel electrophoresis (PAGE) method. ETK migrated as two bands of molecular weight (MW) 51kD and 35kD as estimated by MW markers.

A. SDS-PAG stained with coomassie brilliant blue (CBB) R-250
B. SDS-PAG stained with BioRad's silver stain.
C. Transblot membrane stained with CBB R-250.

lane 1: MW markers (20-215kD, Sigma)
lane 2: Haemolysate from the normal control sample (100133)
lane 3: Isolated ETK fraction
lane 4: Isolated ETK fraction

3.5.2 Amino Acid Composition

From two tracks of the PVDF membrane the 51kD band and 35kD band were hydrolyzed and analyzed for amino acid content. The chromatographic profiles of amino acid content by height in each bands are shown in Figures 3.16 and 3.17.

For the 51kD ETK band: 19 peaks were detected, 16 of which were matched to the standard amino acids (Appendix IV). Amino acid composition by MW and molar ratio % are listed in Table 3.8. The total weight recovered from two bands of 51kD ETK was 0.36 mg. As for the 35kD ETK band: 18 peaks were detected, 15 of which were matched to the standard amino acids. Amino acid composition by MW and molar ratio % are listed in Table 3.9 The total mass recovered from two bands of 35kD ETK was 0.18mg.

All data listed in (Tables 3.8 and 3.9) and chromatographic profiles (Figures 3.16 and 3.17) were obtained from the Applied Biosystems 920A Data Analysis report (Appendix V and VI).

To verify that the 51kD and 35kD bands are two species of the same protein on the basis of amino acid composition, statistical analysis was performed using Kendall's test (non-parametric). This gave a highly significant ($Z = 3.21$, $p \leq 1$) positive correlation ($K = 0.6$) between the two amino acid compositions. The Wilcoxon test suggests that the amino acid composition (molar ratio %) from the two protein bands are not significantly different (thus null hypothesis holds true and $P=1$, 2-tailed). In addition, the amino acid composition (molar ratio %) from both protein bands were also verified to be identical by plotting the values on a scatter plot which also showed positive correlation by an upward trend (Figure 3.18). Moreover, the histogram confirmed the close resemblance between the molar ratio (%) of amino acids from the two TK bands (Figure 3.19). Statistical analysis thus suggests that the two protein bands represent similar proteins with similar functions.

Table 3.8: Amino acid composition of 51kD transketolase (TK). Amino acid composition was determined from 0.36mg of two TK bands hydrolyzed from the transblot membrane.
(shortcode for each amino acid is shown in brackets).

Amino Acid No.	Amino Acid	Retention Time (min)	Absorbance Unit (μAu)	Composition by MW	Molar Ratio (%)
1	Aspartic Acid (D)	2.20	31903	57.0	11.95
2	Glutamic Acid (E)	2.57	20965	35.5	7.43
3	Serine (S)	3.35	22784	56.5	11.85
4	Glycine (G)	3.79	14192	31.5	6.62
5	Histidine (H)	4.25	6869	18.5	3.90
6	Arginine (R)	4.90	5984	9.5	2.00
7	Threonine (T)	5.14	11135	25.0	5.20
8	Alanine (A)	5.54	25110	44.0	9.29
9	Proline (P)	5.82	18226	34.0	7.18
10	Tyrosine (Y)	8.49	10317	16.0	3.32
11	Valine (V)	9.49	19634	34.0	7.12
12	Methionine (M)	9.78	1975	3.5	.73
13	Isoleucine (I)	11.78	9976	21.0	4.45
14	Leucine (L)	11.99	26518	46.0	9.58
15	Phenylanine (F)	12.62	13655	19.0	3.99
16	Lysine (K)	13.65	31097	25.5	5.39

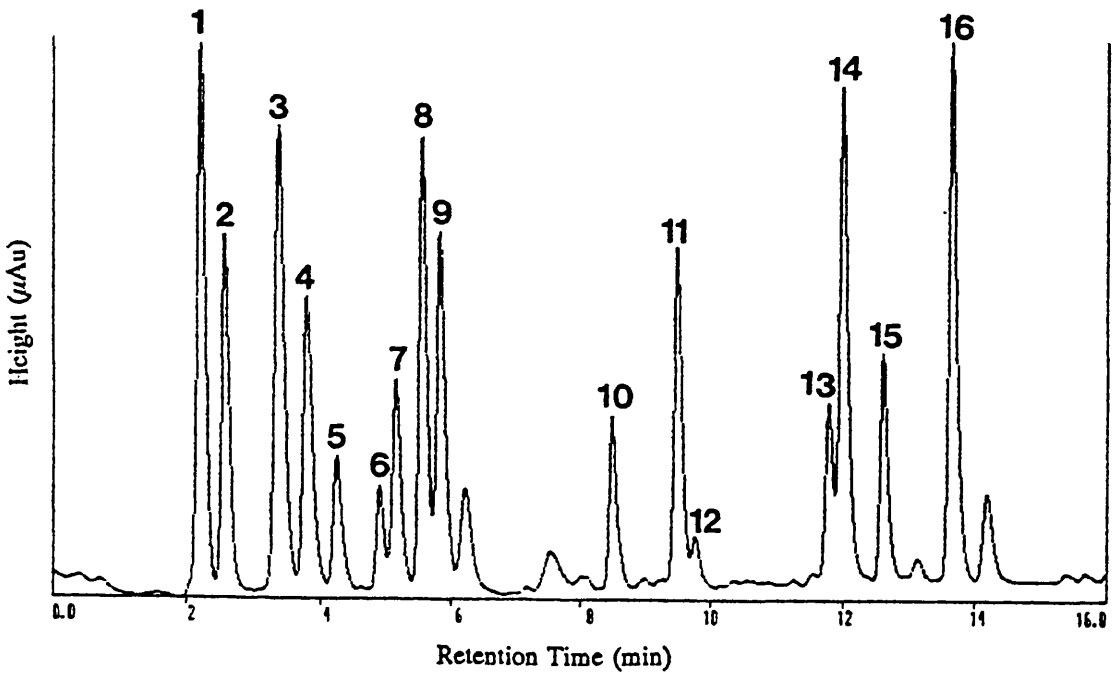


Figure 3.16: Chromatographic profile of the amino acid composition of the 51kD transketolase band. 19 peaks were detected, 16 of which were matched to the standard amino acids. Numbers 1-16 are the amino acids identified and listed in Table 3.8. (Scale = 0.032 Au).

Table 3.9: Amino acid composition of 35kD transketolase (TK). Amino acid composition was determined from 0.18μg of two TK bands hydrolzed from the transblot membrane.
(Shortcode for each amino acid is shown in brackets).

Amino Acid No.	Amino Acid	Retention Time (min)	Absorbance Unit (μAu)	Composition by MW	Molar Ratio (%)
1	Aspartic Acid (D)	2.17	10266	24.5	7.39
2	Glutamic Acid (E)	2.56	9729	22.0	6.62
3	Serine (S)	3.33	6307	21.0	6.31
4	Glycine (G)	3.78	9879	29.5	8.85
5	Histidine (H)	4.23	4796	17.5	5.24
6	Arginine (R)	4.89	3539	7.5	2.27
7	Threonine (T)	5.13	6756	20.0	6.06
8	Alanine (A)	5.53	1977	45.5	13.56
9	Proline (P)	5.82	7589	19.0	5.75
10	Tyrosine (Y)	8.49	3512	7.0	2.17
11	Valine (V)	9.49	16635	39.0	11.60
12	Methionine (M)	9.77	1032	2.5	.73
13	Leucine (L)	11.99	20017	46.5	13.90
14	Phenylanine (F)	12.62	8857	16.5	4.98
15	Lysine (K)	13.65	13774	15.5	4.58

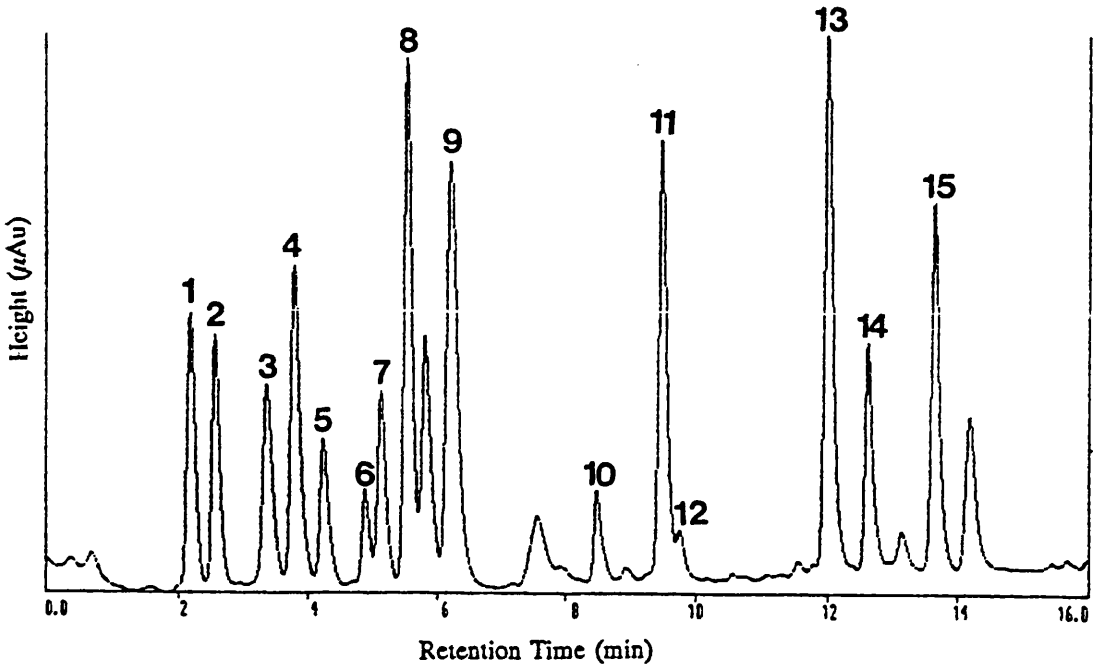


Figure 3.17: Chromatographic profile of the amino acid composition of 35kD transketolase. 18 peaks were detected, 15 of which were matched to the standard amino acids. Numbers 1-15 are the amino acids identified and listed in Table 3.9. (Scale = 0.021Au)

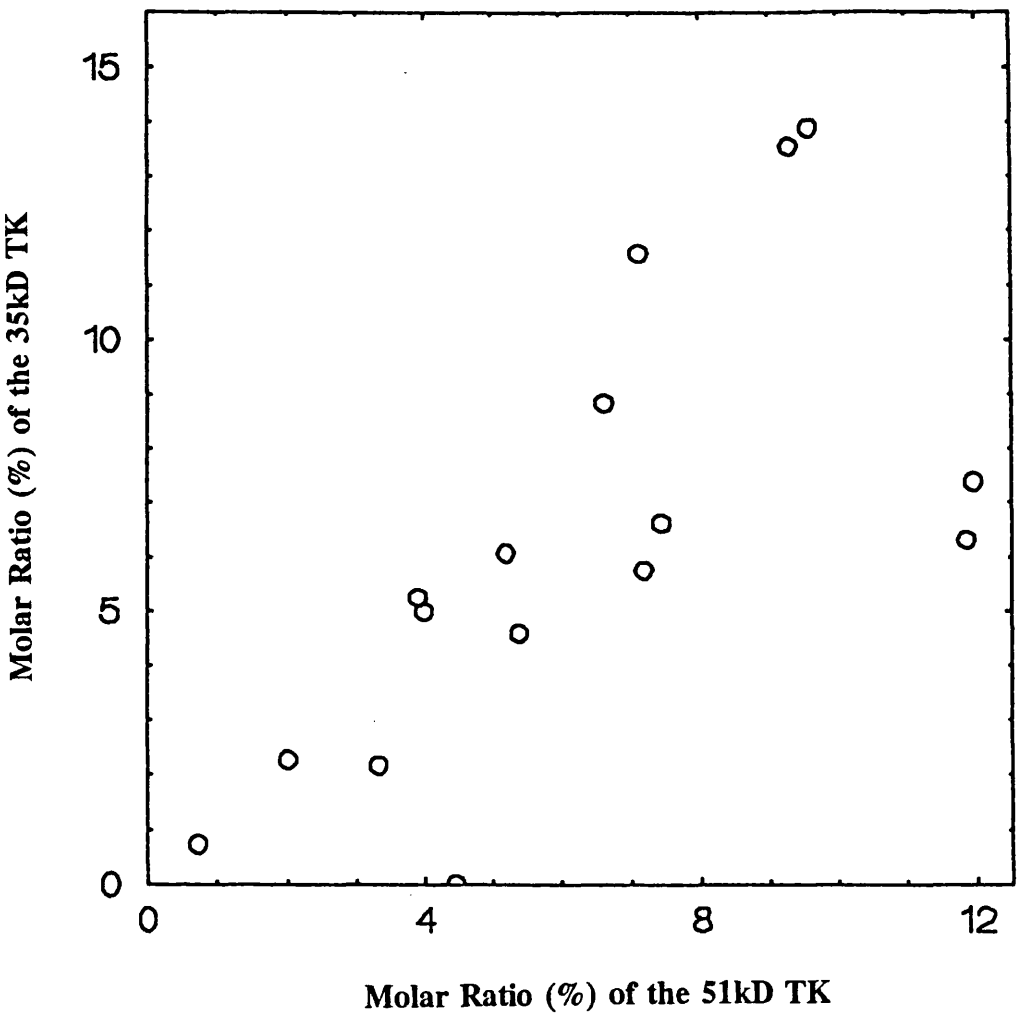


Figure 3.18: Scatter plot of amino acid composition (molar ratio %) of the 51 vs. the 35kD transketolase (TK) bands.

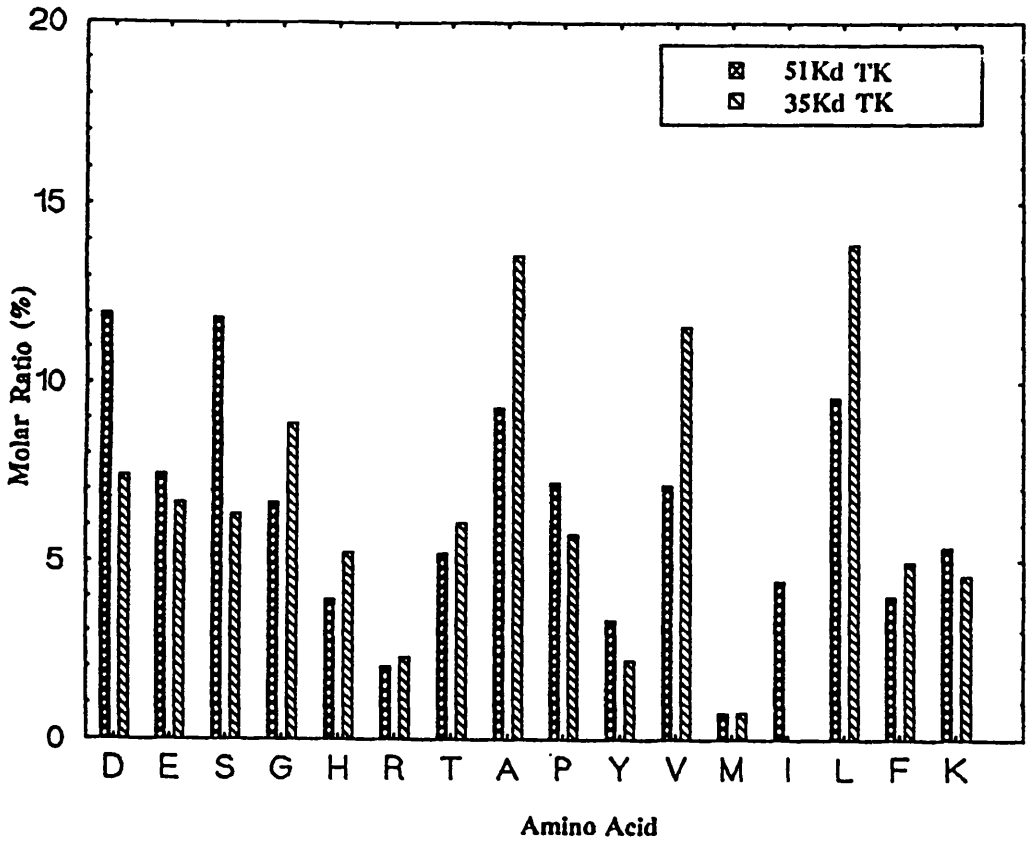


Figure 3.19: Histogram of amino acid composition (molar ratio %) comparing the 51 and 35kD transketolase (TK) bands.

3.5.3 Amino Acid Sequencing

Eight bands from the PVDF membrane were excised to perform amino acid sequencing on the 51kD (yield ~ 1.5mg) and 35kD (yield ~0.75mg) bands.

Only 15 cycles of amino acid sequence were obtained from the N-terminal of the 35kD TK band. Data on the amino acid sequence obtained is listed in Table 3.10. The average and the combined repetitive yields (percent of material that can be sequenced after each cycle) of amino acids were 105.01% and 89.07% respectively. The theoretical initial yield (percent of material that can be sequenced from the amount of sample loaded after each cycle) was 142.82 pmol. The original report on the sequence data was obtained by the Applied Biosystems 477A protein sequencer (Appendix VII). The sequence obtained represents roughly 4% of the total amino acid sequence (~350 residues).

The eluted peak, representing the amino acid in each cycle, is identified by comparing the shift in height of one cycle from the previous and subsequent cycles. That is, the peak which is not present (or has a low height) in the previous cycle begins to appear (or increase in height) in the present cycle and then disappears (or declines in height) in the next cycle. Then the peak identified is matched to the amino acid standard by its retention time (Appendix IV). The sequence profile of the first 15 cycles obtained for the 35kD TK band is provided in Figure 3.20. The amino acid sequence was analyzed for homologies in the Gen Bank Database which verified its uniqueness.

As for the 51kD TK band, no amino acid sequence was obtained due to N-terminal blockage. Although there was sufficient material for sequencing, the initial theoretical yield was only 3.78 pmol.

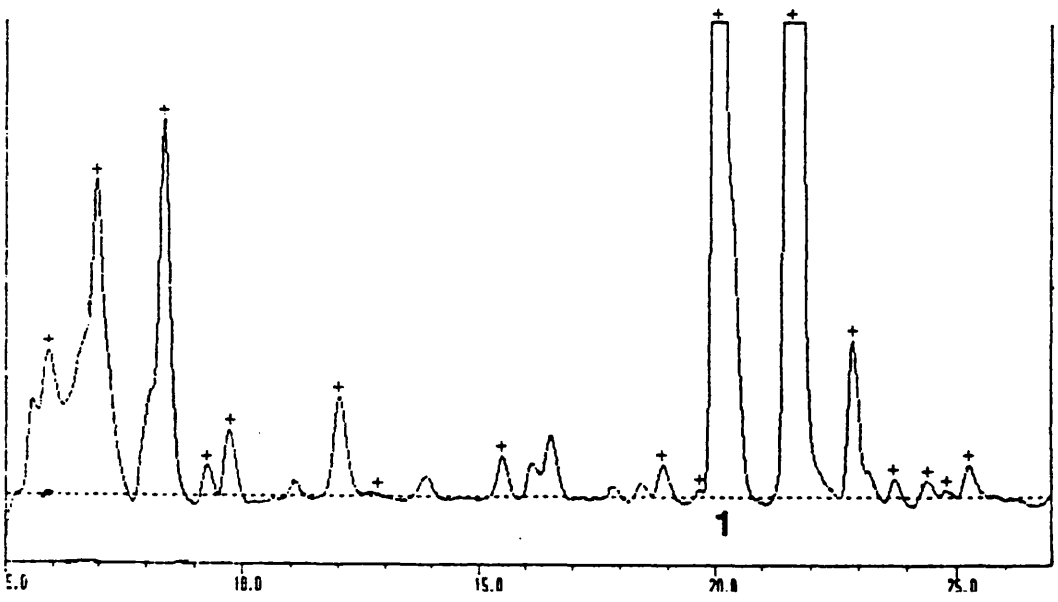
Table 3.10: Amino acid sequence data for the 15 cycles obtained from the 35kD transketolase band.

Cycle No.	Height (μAu)	Retention Time (min)	Amino Acid Identification	Ratio (pmol)	Nucleotide Codon Redundancy
1	82764	20.03	Valine (V)	195.02	4
2	28686	25.27	Leucine (L)	144.61	6
3	28212	25.25	Leucine (L)	130.86	6
4	17604	18.85	Proline (P)	73.15	4
5	20985	12.03	Alanine (A)	-	4
6	12240	9.22	Glutamine (E)	47.67	2
7	19551	9.22	Glutamine (E	75.74	2
8	8388	7.95	Threonine (T)	46.91	4
9	21081	5.95	Asparagine (N)	21.97	2
10	19293	20.00	Valine (V)	40.24	4
11	8262	24.78	Lysine (K)	-	2
12	4695	7.95	Threonine (T)	21.92	4
13	26907	12.03	Alanine (A)	33.20	4
14	14493	25.22	Leucine (L)	53.31	6
15	12213	8.35	Glycine (G)	7.15	4

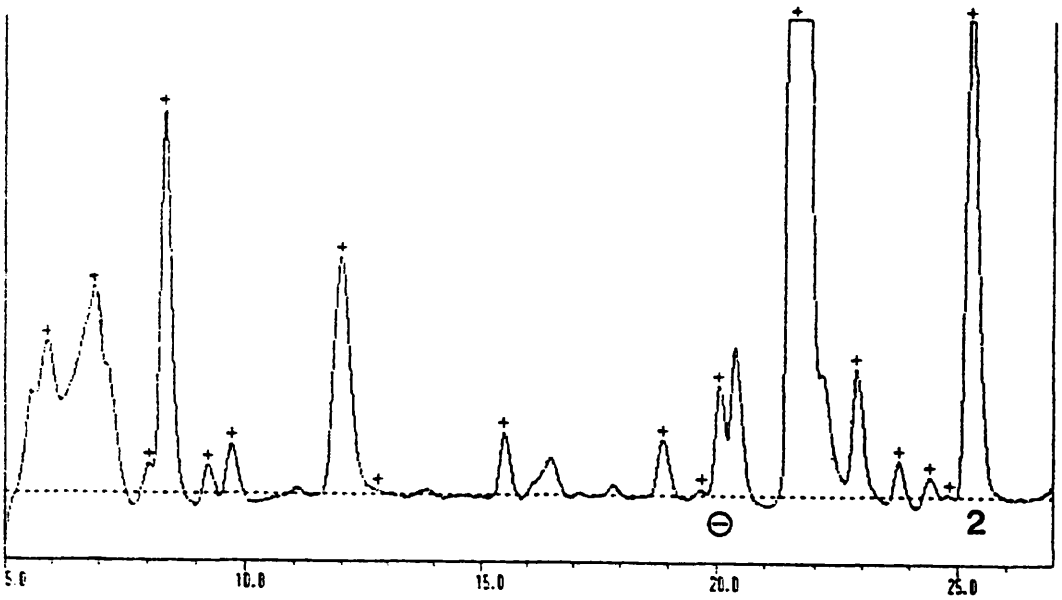
Figure 3.20: Chromatographic profile of the amino acid sequence for all 15 cycles from the 35kD transketolase band. The eluted peak in each cycle representing the identified amino acid is designated with a number (1-15). The number indicates the sequence of each amino acid residue.

+ indicates all matched amino acids with respect to retention time
⊕ indicates the appearance or increase in height of an identified peak
⊖ indicates the disappearance or decrease in height of an identified peak
x-axis: Retention Time (min)
y-axis: Height (μAu)

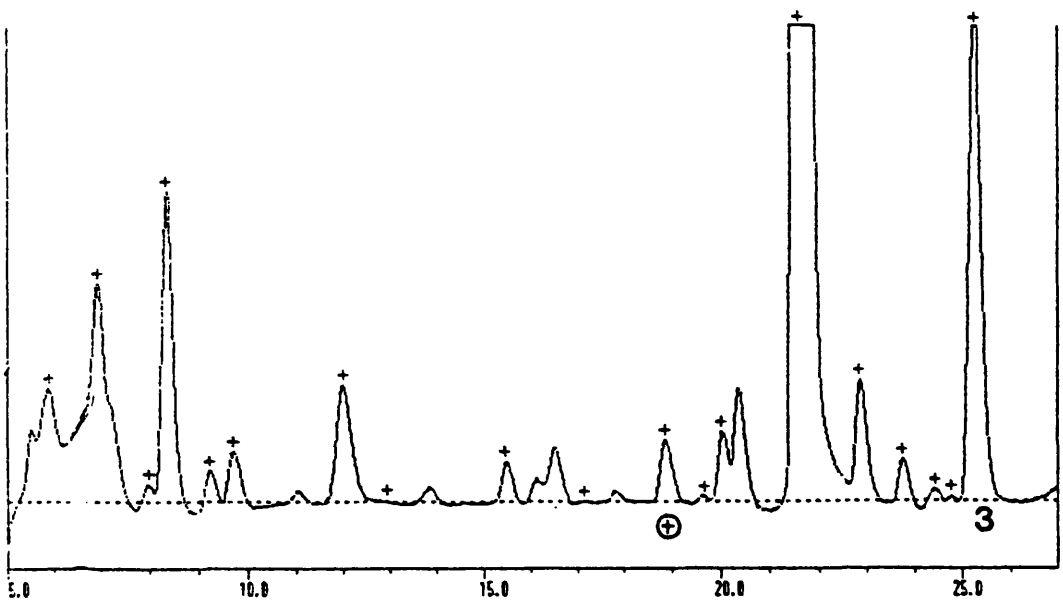
Cycle # 1:



Cycle # 2:



Cycle # 3:



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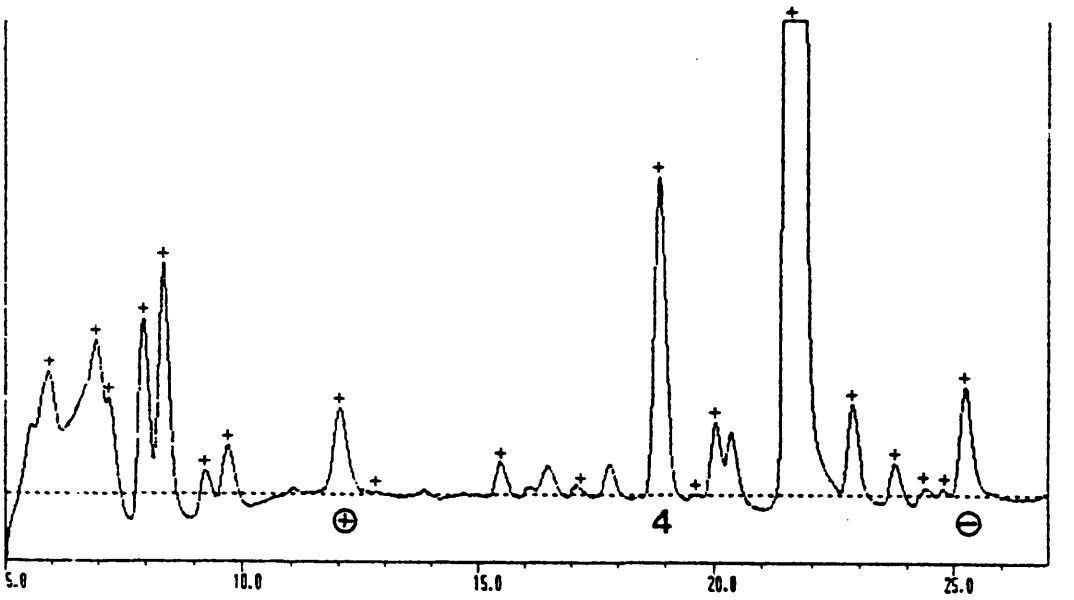

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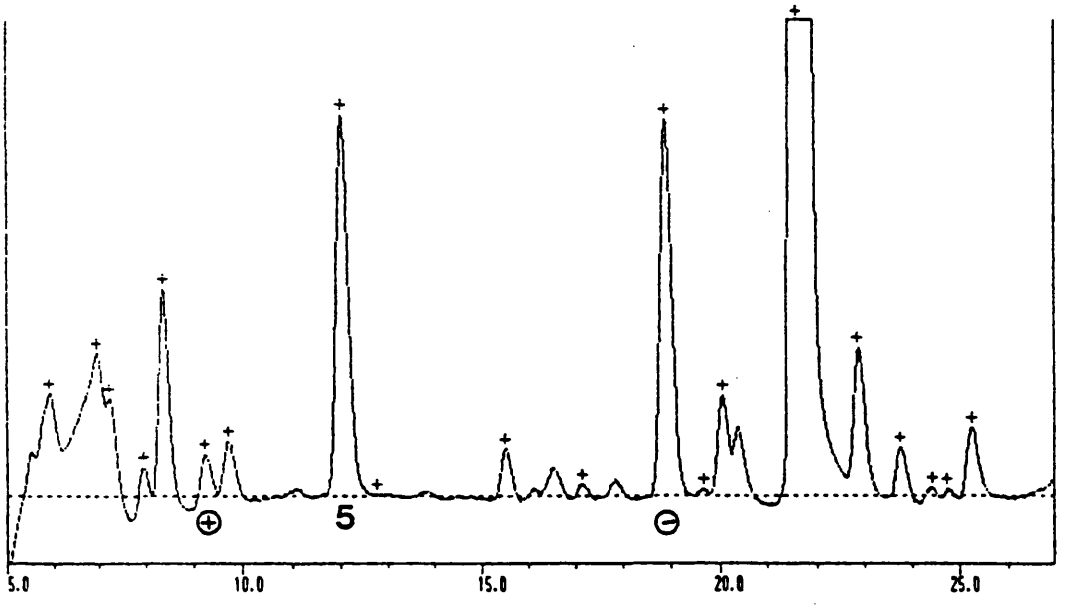
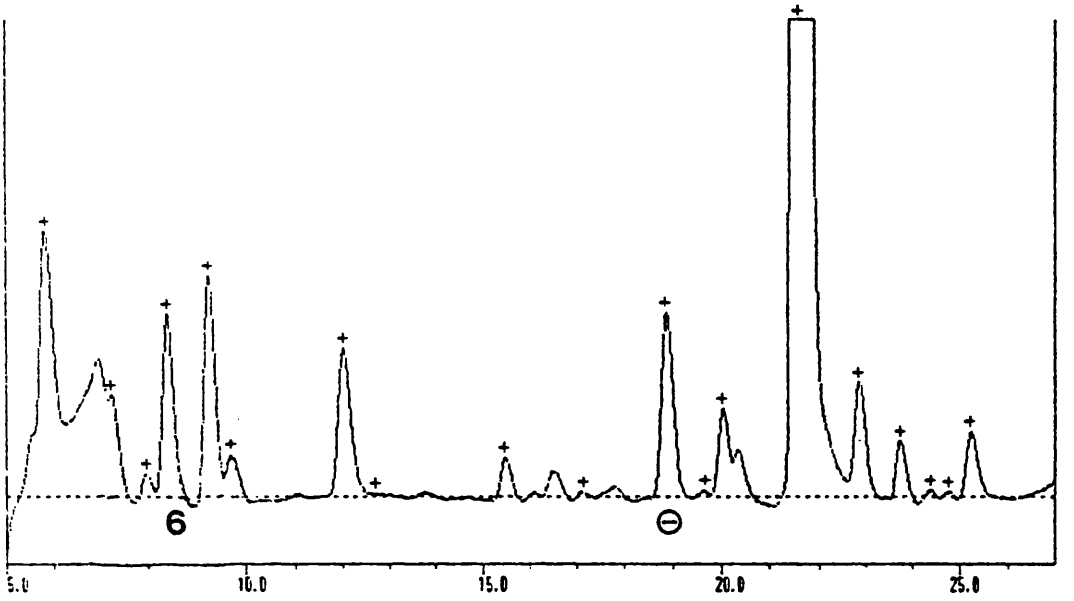
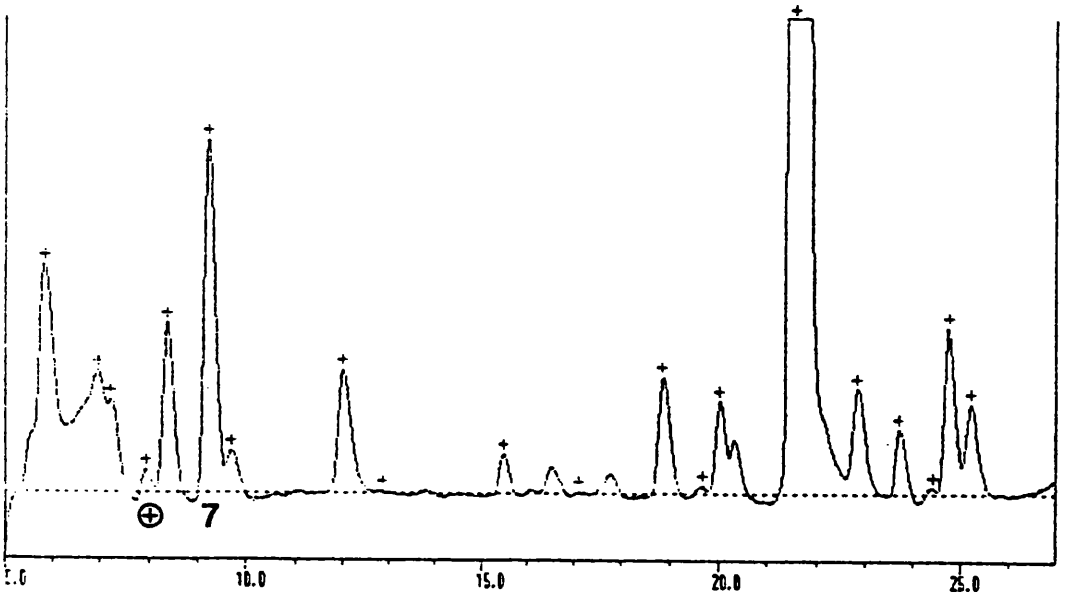


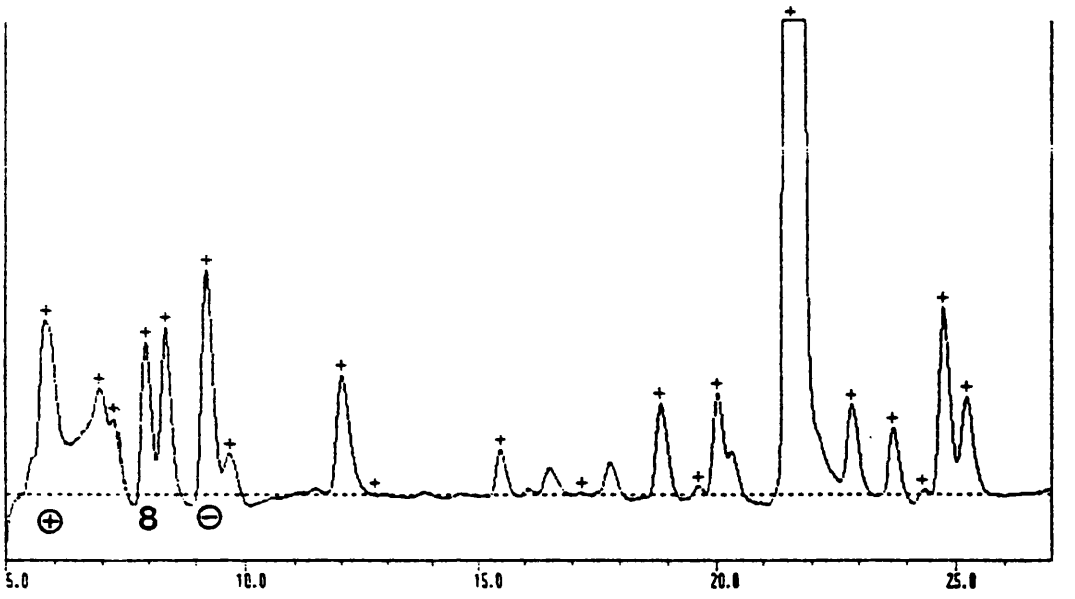
Cycle # 6:



Cycle # 7:



Cycle # 8:



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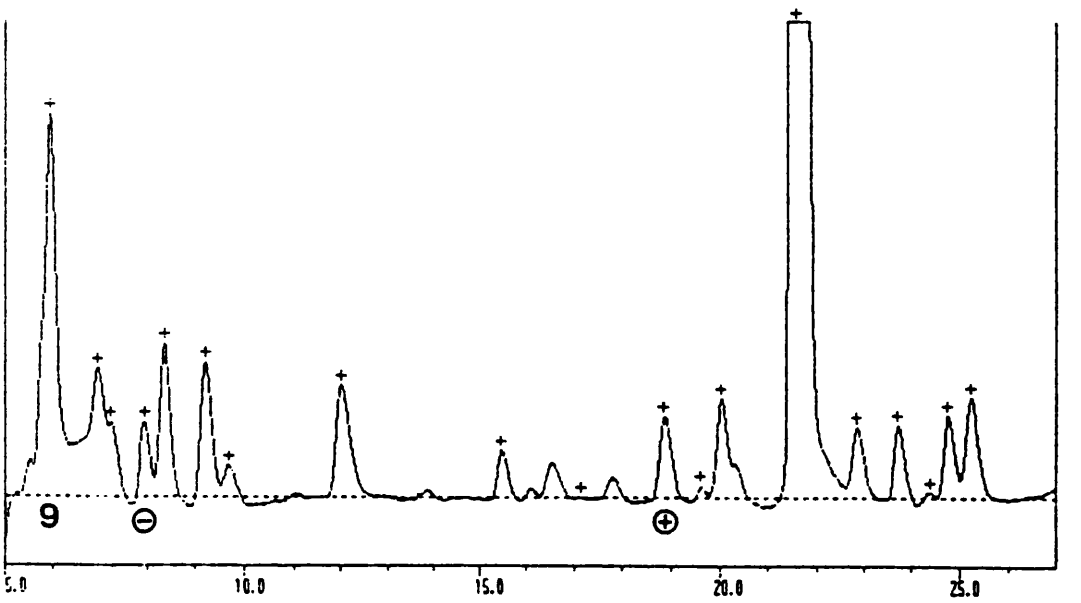

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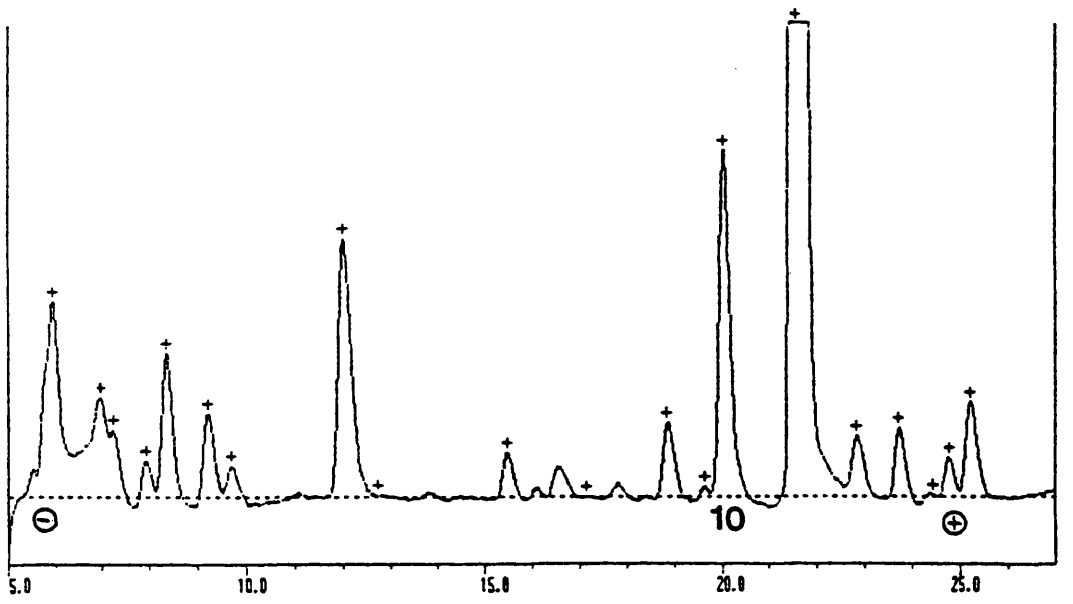

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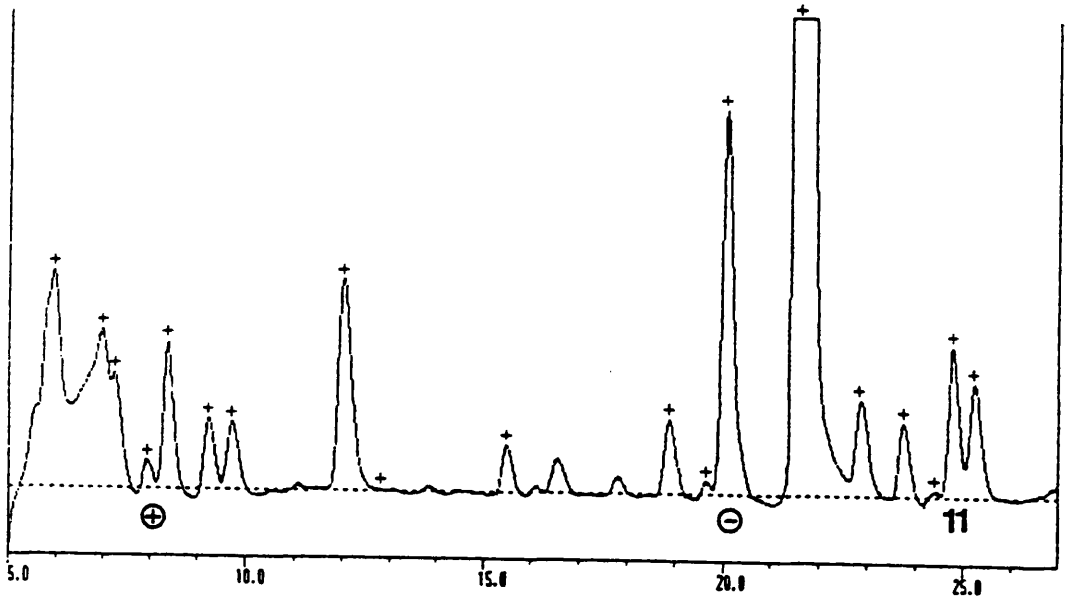

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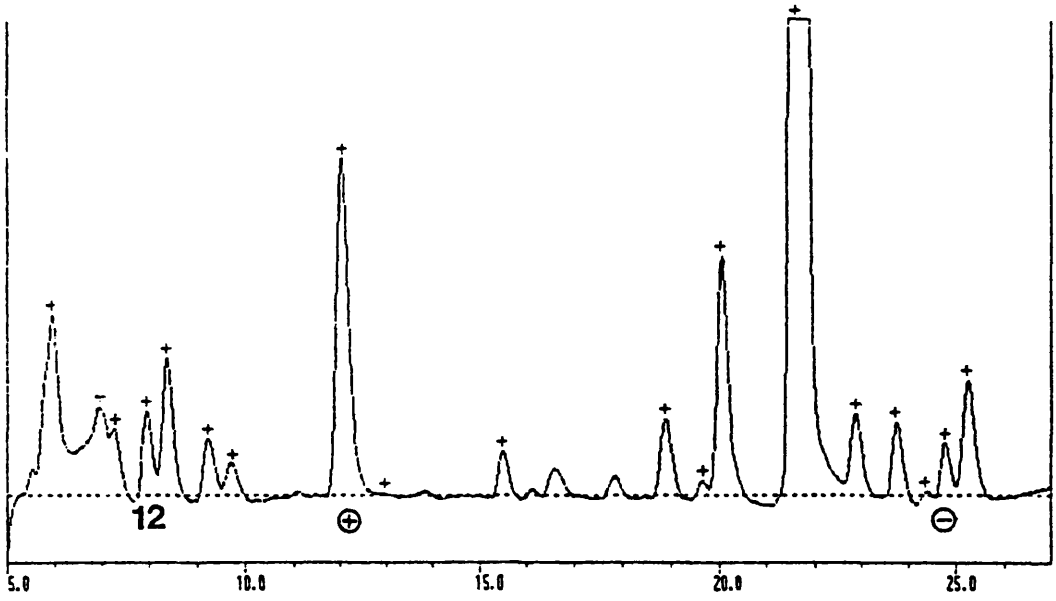

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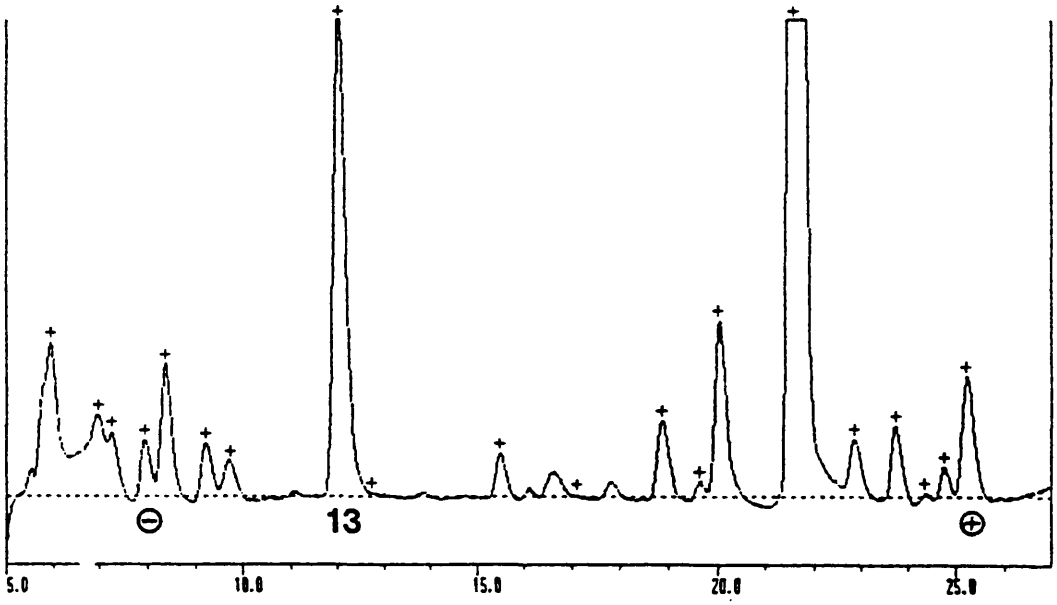

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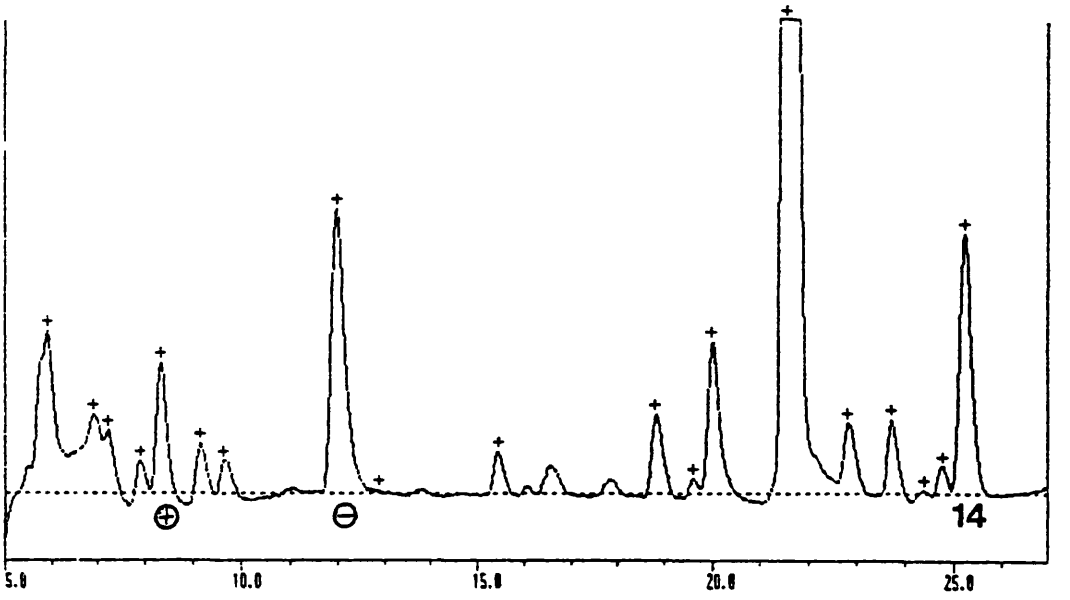

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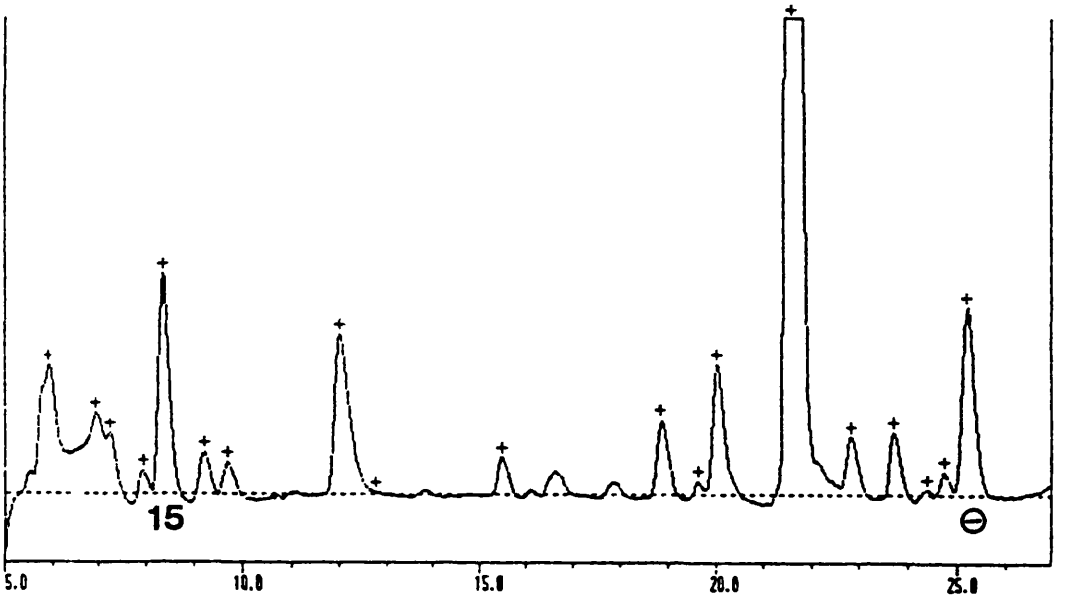


CHAPTER 4: DISCUSSION

4.1 QUANTITATIVE ANALYSIS OF TRANSKETOLASE IN WERNICKE-KORSAKOFF SYNDROME

The determination of ETK and ETKs activity and the calculation of TPPE has been used for more than twenty years as an aid to the diagnosis of WKS (Sauberlich, 1984; Victor et al., 1989). However, the calculation of TPPE in a subset of normal controls (n=14) and patients (n=6) with WKS in the present study using the endpoint assay (section 2.1.4.1) were inconsistent with wide variation (mean of normals: 16.29% ± 46.65; patient: 7.22% ± 22.26) and negative values were also observed. It has been noted by others that the calculation of TPPE for clinical diagnosis of WKS and TD can be misleading (Sauberlich, 1967; Fennelly, 1967; Wood et al., 1987; Grudzinski et al., 1989; Nixon et al., 1990) and adds little to the single measurement of ETK activity (Nixon et al., 1990). The initial value of mean ETK activity and TPPE in normal samples (n=14) are listed along with other values reported in the literature to illustrate the wide variation of mean ETK activity and TPPE and their ranges (Table 4.1). It is clear that there is no uniformity in these values nor in the ranges quoted for ETK activity and TPPE which has also been observed by Bayoumi and Rosalki (1976), Gradual et al. (1985) and Anderson and Nicol (1986). The diversity in laboratory techniques could be a contributing factor.

In this study no signficant difference ($p>0.1$) was found between the values for ETK and ETKs activity as determined by the optimized endpoint assay (section 2.1.4.3) in 10 normal controls (ETK activity: 0.418 ± 0.193IU/mg protein, ETKs activity: 0.738 ± 0.208IU/mg protein) and in 20 patients with WKS (ETK activity: 0.406 ± 0.155IU/mg protein, ETKs activity: 0.680 ± 0.190IU/mg protein). Tate and Nixon (1987) also could not demonstrate a systematic difference between WKS patients and normal controls. In 1965 Leevy and colleagues reported only 50% of patients with WKS had an abnormally low ETK activity.

The application of the TK activation test as an aid to the diagnosis of WKS has been based on the following assumption: if a patient had low stores of thiamine in the form of TPP bound to TK then upon addition of exogenous vitamin there will be a large increase in activity. The patients sampled in the present study

Table 4.1: Mean values for erythrocyte transketolase (ETK) activity and thiamine pyrophosphate effect (TPPE) reported in literature in healthy individuals. (Values in brackets are ranges reported)

Report	ETK (IU/g Hb) (mean ± SD)	TPPE (%) (mean ± SD)	Sample Size (n)
Smeets et al. (1971)	0.77 (0.59-1.06)	11 ± 4.5	10
Williams et al., (1976)	0.82 ± 0.40	(0-25)	20
Bayoumi & Rosalki (1976)	-	23 ± 12	15
Buttery et al. (1980)	0.93 (0.53-1.21)	12 (2-25)	30
Thomson et al. (1980)	0.897 ± 0.015	-	-
Duffy et al. (1981)	-	6.3 ± 3.0	120
Waring et al. (1982)	0.615 ± 0.059	11 (2-19)	360
Gradual et al. (1985)	(0.718-1.98)	(5-23)	21
Puxty et al. (1985)	0.711	9.9	11
Anderson & Nicol (1986)	0.698 ± 0.065	10.3 (2.7-15.3%)	200
Jeyasingham et al. (1987)	0.503 ± 0.221	-	40
Melgaard et al. (1989)	0.67 ± 0.42 (0.11-0.96)	12.9 ± 11 (0-34)	15
Present study	0.41 ± 0.14 (0.13-0.62)	16.29 ± 46.5	14

were hospitalized and receiving thiamine supplementation. They would be expected to have adequate stores of thiamine thus might not show signficant increase in ETK activity upon addition of TPP in vitro. This is in agreement with Gradual et al. (1985) who evaluated ETKs activity and TPPE as indices of nutritional status based on a study of alcoholic (AL, n=23) and non-alcoholic (NAL, n=21) groups. They found normal ETK activity after oral thimaine adminstration (100mg). Another study illustrating restoration of ETK activity and TPPE to normal after treatment with thiamine in WKS was reported by Leigh et al. (1981). However most other reports on the evaluation of ETK and ETKs activity in patients with WKS have only been determined prior to treatment with thiamine (Sauberlich, 1984; Bayoumi & Rosalki, 1976; Gradual et al., 1985; Kerr et al., 1986; Jeyasingham et al, 1987; Melgaard et al., 1989; Hill & Truswell, 1990) and therefore showed elevated values of ETK activity. It would appear that the apoenzyme (ApoTK) must be measured prior to thiamine supplementation in patients to assess the true value of ETK activity and TD.

It has been assumed that variation of ETK and ETKs activity and TPPE results from the dissociation of part of the enzyme into apoform (Jeyasingham et al., 1987) or from an abnormality in the binding affinity of coenzyme to ApoTK (Kjosen, 1977, Gradual et al., 1985; Kerr et al., 1986; Takeuchi et al., 1988). Such abnormalities would be expected to be prominent in WKS (Blass & Gibson, 1977; Greenwood et al., 1989; Pratt et al, 1985; Kerr et al., 1986; Jeyasingham et al., 1987). The properties, kinetics and reactivation of ApoTK have been described (Greenwood et al., 1984; Pratt et al., 1985; Tate & Nixon, 1987; Jung et al., 1988). Upon acidic treatment (e.g. ammonium sulphate, pH < 4.0) human TK can be seperated into its apoenzyme (ApoTK) and coenzyme (TPP) (Warnock & Prudhomme, 1982; Greenwood et al., 1984; Tate & Nixon, 1984; Jung et al., 1988). The resolved ApoTK, prepared from native or purified holoenzyme, has been shown to be stable at 0°C with optimum pH 7.6 and temperature 37°C and requires TPP or $MgCl_2$ for reactivation (Warnock & Prudhomme, 1982; Greenwood et al., 1984; Pratt et al., 1985; Jeyasingham et al., 1986; Tate & Nixon, 1987; Jung et al., 1988).

In order to establish the existence of ApoTK variants in the binding affinity for TPP, as first suggested by Blass and Gibson (1977), kinetic analysis (ie.

determination of Michaelis-Menten constant) on TPP and its binding to ApoTK had been performed by several workers (Greenwood et al., 1984; Pratt et al., 1985; Tate & Nixon, 1987; Jeyansingham et al., 1987). Jung et al. (1988) have found that TPP bound to enzyme has a direct influence on catalytic activity and that it was characterized by a single active site. A more recent report (Nixon et al., 1990) suggested that the finding of variation in ApoTK level reflects changes in the concentration of total TK protein (e.g. synthesis or catabolism) and are dependent upon coenzyme concentration. Although ApoTK can be estimated from the difference between ETK and ETKs activity (Nixon et al., 1990) a more reliable measurement of TK protein would be achieved by immunoassay (Takeuchi et al., 1988; Paoletti et al., 1989) or by qualitative analysis of ETK (Greenwood et al., 1984, Nixon et al., 1984; Pratt et al., 1985). This would also allow the characterization of variant ApoTK (Nixon et al., 1990) whether arising from low affinity for TK (Blass & Gibson, 1977; Mukherjee et al., 1987), altered turnover or by decreased stablity of ApoTK (Mukherjee et al., 1987; Jeyasingham & Pratt, 1988).

4.2 QUALITATIVE ANALYSIS OF TRANS-KETOLASE IN WERNICKE-KORSAKOFF SYNDROME

The reported findings of isoenzyme variants (Kaczmarek & Nixon, 1983; Nixon et al., 1984; Kaufmann et al., 1987) associated with ETK in WKS was investigated by electrophoretic methods using crude haemolysates and partially purified ETK from normal contols and patients with WKS.

Qualitative analysis by electrophoresis allowed the resolution and separation of TK variants, as well as the elimination of Hb interference. This was only achieved after improving the specific enzyme stain which had caused background staining and detection of false bands as also observed by Kaufmann et al. (1987). The application of PAGE yielded the best electrophoretic profile and allowed the detection of two TK bands in all normal controls and patients with WKS. These findings are in agreement with those of Paoletti et al. (1989) who performed IEF techniques and western blotting of TK from leukocytes and detected TK by

specific antibodies. The results obtained in the present study with regard to the electrophoretic profile of TK from crude haemolysates do not support the original reports of Kaczmarek and Nixon (1983) and Nixon et al. (1984). The former described the detection of 8 individual species of TK with pI ranging from 6.6 to 9.2 on IEF gels following partial purification of TK from stored human erythrocytes by ion-exchange chromatography and gel filtration (Kaczmarek & Nixon, 1983). Nixon and associates (1984) reported a variation in the distribution of TK isoenzymes between normal controls, alcoholic controls and patients with WKS using the method of Kaczmarek and Nixon (1983). This technique was re-examined by Kaufmann and associates (1987) who despite detecting 7 TK bands with pI values from 7.4-8.4 concluded that methodological influences may induce artifacts which is evident from the four additional marginal bands that were not always reproducible.

In an attempt to resolve this discrepency as well as to further investigate TK qualitatively in normal controls and patients with WKS, partial purification of TK was performed by PAGE. The TK preparations were subjected to various qualitiative analysis using PAGE, IEF and HPLC. All analyses supported the presence of two TK bands in all samples tested differing in MW, pI and ionic strength. Similar results have been reported by others (Greenwood et al., 1984; Pratt et al., 1985; Jeyasingham, 1987; Thomson et al, 1987; Sheu et al., 1988; Jeyasingham & Pratt, 1988) (Table 4.2). It has been suggested (Puxty et al., 1985; Kaufmann et al., 1987; Jeyasingham & Pratt, 1988) that prolonged storage of haemolysates can give rise to altered TK thus inducing artifacts. In this study, samples analyzed qualitatively from stored haemolysates in glycerol citrate buffer (at -20°C) revealed no variation in TK in all normal controls and in patients with WKS. Several reports have suggested that any variation in TK which may occur can arise from haemolysate preparation methods rather than any storage effect (Anderson & Nicol, 1986).

Smeets et al. (1971) suggested that haemolysates could be stored at −20°C for four weeks without any loss of activity. This was contradicted by Bayoumi and Rosalki (1976) and Leunis et al. (1982) who observed a 10% loss in activity after 1-2 weeks of storage. Puxty et al. (1985) also reported a signficant decline ($p<0.05$) in ETK activity after prolonged storage which allowed misleading interpretation of

results as observed by Kaufmann et al. (1987). Although such an effect may exist it was not observed in this study suggesting that it is the method of storage (i.e. storage in detergent) that gives altered froms of TK rather than prolonged storage. The storage of samples in this study were intact cells in buffer citrate glycerol thus preserving ETK activity. This is in agreement with Anderson and Nicol (1986) and Mount et al. (1987). Moreover, the detection of various isoenzymes (Kaczmarek & Nixon, 1985; Nixon et al., 1984; Kuafmann et al., 1987) may have arisen from formation of complexes between TK, TA and G-3-P as a result of storage of samples (Takeuchi et al., 1986).

Although qualitative analysis on TK from haemolysates of normal controls and patients with WKS strongly support TK heterogeneity, both quantitative and qualitative analysis revealed no systematic differences between the samples tested. This prevented the investigation of possible genetic factors by enzymatic analysis. However, quantitative analysis of TK electrophoretic profile (i.e. PAGE, IEF, HPLC) by silver staining hinted on the existence of variation between the two bands with regard to the proportion of each band present. These findings had also been reported by Greenwood et al. (1984), Pratt et al. (1985), Jeyasingham and Pratt (1988), and Takeuchi et al. (1988) who performed detailed studies on the kinetics and reactivation of TK from partially purified preparations and provided a tentative explanation on the role of TK in WKS.

Kinetic studies on TK variants were performed by Greenwood et al. (1984) using the Michaelis-Menten constant (Km) which is defined as the coenzyme or substrate concentration at which the initial reaction is half the maximal; that is the affinity of apoenzyme for TPP has a concentration at which 50% of TK is activated. Thus a high affinity apoenzyme would have a low Km for TPP. The two fractions of TK obtained by ion-exchange chromatography were separated from Hb and were each shown to contain two further variants of TK differing in their affinity for TPP (Km) (Greenwood et al., 1989). Further work on binding affinities for TPP was performed in five different groups of patients. A variant with reduced affinity for TPP (high Km) was found in the demented and alcoholic patients. Increased concentration of TPP (beyond 3μM) was shown to exhibit an inhibition effect on the high affinity

Table 4.2: Qualititave analysis of partially purified transketolase (TK) variants reported in literature. These include variants with regard to pI value, electrophoretic and chromatographic profile

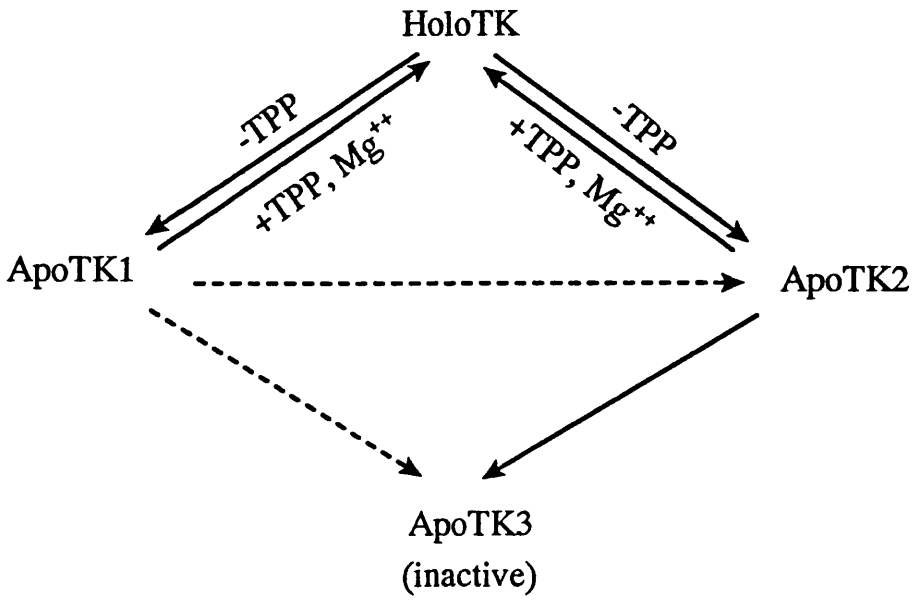
Report	Analysis	Finding	Characteristic
Kaczmarek & Nixon (1983)	IEF on 25 normals IEC & GFC	8 species of TK isoenzymes 2 peaks	pI range: 6.6 - 9.2 -
Nixon et al. (1984)	IEF on 42 WKS and 36 normals	different distribution of isoenzyme pattern	-
Greenwood et al. (1984)	IEC	at least 2 variants	differ in binding affinity to TPP concentration
Pratt et al. (1985)	GFC on normal controls	at least 2 variants varying in size and binding affinity	larger molecule had bound TPP; smaller component inactive without TPP
Thomson et al. (1987)	GFC on patients with WKS	2 variants differing in molecular weight	patients with WKS had high proportion of low molecular weight TK
Kaufmann et al. (1987)	IEF	7 TK bands and 4 marginal artifacts	pI range: 7.4-8.4
Paoletti et al. (1988)	IEF from leukocytes	at least 2 variants	pI 7.3 & 8.6
Mocali & Paoletti (1989)	IEF of isolated TK from leuckocytes	6-8 bands	pI range: 6.5-8.5
Present Study	PAGE IEF HPLC	at least 2 variants 2 variants 2 peaks as eluted by ionic strength	differ in molecular weight pI: 6.55 & 7.35 vary in proportion same as PAGE & IEF

IEC - ion-exchange chromatography
HPLC - high performance liquid chromatography
PAGE - polyacrylamide gel electrophoresis
GFC - gel filtration chromatography
IEF - isoelectric focusing

ApoTK while a low TPP concentration (under 0.3μM) would mask the low affinity ApoTK (Jeyasingham et al., 1987). Takeuchi et al. (1988) described the analysis of TK concentration and activity in human haemolysates and suggested that a heavy drinker would have a low concentration of TK thus confirming the findings of Greenwood et al. (1984).

Although RBC haemolysates provide a convenient access to enzymatic changes, it is the brain enzyme which is particularly relevant to the gene of ApoTK (Jeyasingham et al., 1986). A study on rat brain ApoTK was performed by Jeyasingham and Pratt in 1988 demonstrating existence of ApoTK in more than one form differing in binding affinities with TPP. Kinetic studies performed on rat brain ApoTK and its activation by in vitro addition of TPP suggested that one part of the apoenzyme reacts rapidly with TPP while the other appears to react slowly. Their findings also suggested that HoloTK may be converted to a third form as a result of storage changes and conditions. These findings indicate that dissociation of rat brain TK can give rise to three forms of ApoTK. The first apoenzyme (ApoTK1) can be reactivated by rapid combination with TPP and can be measured directly in the presence of $MgCl_2$ (Greenwood et al., 1984; Pratt et al., 1985). The second (ApoTK2) can be reactivated by prolonged incubation with TPP (Waring et al., 1982) and measured directly in the presence of $MgCl_2$ (Greenwood et al., 1984; Pratt et al., 1985; Jeyasingham et al., 1987). The last (ApoTK3) cannot be reactivated but can be estimated indirectly by firstly measuring HoloTK activity (ApoTK1 + ApoTK2) and then subtracting it from total TK activity (Jeyasingham & Pratt, 1988). The breakdown of HoloTK and formation of Apo1, Apo2 and Apo3 is illustrated in Figure 4.4.

The likely occurrence of such changes in vivo during thiamine deficiency producing TK variants (apoenzymes) also supports the possible role of TK in the etiology of WKS (Jeyasingham & Pratt, 1988).



HoloTK → ApoTK1 (rapidly binds with thiamine pyrophosphate (TPP) in presence of $MgCl_2$) → HoloTK
HoloTK → ApoTK1 (slowly binds with TPP in presence of $MgCl_2$) → low HoloTK
HoloTK → ApoTK1 (pH dependent and storage duration) → ApoTK2 → ApoTK3

Figure 4.1: Dissociation of holotransketolase (HoloTK) to apotransketolase (ApoTK) as a result of damage in system secondary to chronic alcoholic abuse and thiamine deficiency.

HoloTK:	Stimulated erythrocyte transketolase (ETKs) activity - erythrocyte transketolase (ETK) activity
ApoTK1:	ETK (low concentration of TPP)
ApoTK2:	ETKs (high concentration of TPP)
ApoTK3:	HoloTK - (ApoTK1 + ApoTK2)

4.3 ISOLATION AND CHARACTERIZATION OF TRANSKETOLASE

Despite the finding of TK variants and their possible role in WKS it remains to be resolved whether such variants are genetically determined (Blass & Gibson, 1977; 1979; Nixon et al., 1989; Victor et al., 1989) or are products of a damaged system (e.g. post-translational modification) (Pratt et all, 1985; Jeyasingham and Pratt, 1988; Lishman, 1990).

There are several clinical and epidemiological observations which support a genetic aetiology for WKS. Only a small majority of alcoholics (both TD and non TD) and other chronically malnourished persons develop WKS (Victor et al., 1971; Blass & Gibson, 1979), it occurs more frequently in Europeans than non-Europeans on a similar thimaine deficient diet whereas Asians develop "wet" cardiac beri beri (Sauberlich, 1967) and some WKS patients have failure of restoration of ETK activity despite addition of TPP in vitro (Sauberlich, 1967; Blass & Gibson, 1979).

In an attempt to investigate the presence of a genetic component, homogenous TK from a normal control was isolated and characterized with regard to pI value, MW and amino acid composition. Three purification methods were tested prior to the final isolation of TK.

The purification of TK from WBC was attempted using the method of Mocali & Paoletti (1989). They described three conventional steps employing desalting by gel filtration, ammonium sulphate fractionation (45-90%) and affinity chromatography. Their sample was prepared from fresh leukocytes obtained from two healthy blood donors. In this study packed leukocytes were prepared as described by Mocali and Paoletti (1989) and desalted by gel filtration. By plotting the protein absorbance against elution time, only one peak containing TK activity was eluted. This was subjected to protein fractionation after which TK activity became insufficient for further purification. The outcome of this purification method was not as successful as described by Mocali and Paoletti possibly due to any or all of the

following reasons. The starting material in this study was one third the volume of the described method (Molcali & Paoletti, 1989) and the isolation of TK was not obtained from fresh leukocyte preparation. The packed leukocytes were subjected to several freeze/thaw cycles which could have also resulted in loss of activity. Moreover, the ammonium sulphate fractionation may have resulted in further loss of TK activity. This method was also shown to be expensive, time-consuming, and to require a source of TK (leukocytes) which is inconvenient to obtain in large quantities. The results in the present study employing this method was compared to the findings of Mocali & Paoletti (1989) in Table 4.3.

Since erythrocytes are conveniently available in the required quantities (i.e. 50ml), the isolation of TK was attempted employing ion-exchange chromatography and advanced chromatographic methods (FPLC) as described by Takeuchi et al. (1986). In the present study, several of the steps described (Takeuchi et al., 1986) were modified. Different column materials were used but similar elution profiles of ETK activity and similar yields were obtained from the first ion-exchange step. Takeuchi and associates employed DEAE-Sephadex for the first purification step; however, in the present study fast flow DEAE-Sepharose (Pharmacia) was used because it had a higher tolerance for strong ionic buffers and better resolution for proteins than DEAE-Sephadex. In the present study two well-resolved ETK activity peaks were eluted with a trace of a third ETK activity peak whereas Takeuchi et al. (1986) reported the elution of a well resolved third ETK activity peak. This discrepancy could result from their use of outdated RBC or from the reaction of oxyhaemoglobin present in the third peak with NADH and R-5-P in TK assay (Grudzinski et al., 1989). The latter would be a more likely explanation for the production of an artifact since Hb was also eluted in that third peak. The third peak was not resolved in this study possibly because fresh RBCs were used but more likely because a more specific enzymatic assay was used for estimating ETK activity whereas a colormetric method was used by Takeuchi et al. (1986) Takeuchi et al. (1986) reported further resolution from the fraction in peak B into three ETK peaks on Mono P which they claimed to represent the same elution profile. In the present

Table 4.3: Comparison of results obtained by the purification of transketolase from leuckocytes using the method of Mocali and Paoletti (1989) (M & P). In the present study (PS) the dry weight of leukocytes was 3.0g and in Mocali & Paoletti 10-12g.

STEP	Total Volume (ml)		Total Protein (mg)		Specific Activity (IU/mg)	
	PS	M & P	PS	M & P	PS	M & P
Crude extract	30	105	75	665	0.0037	0.031
DEAE Column	72	212	54	155.6	0.0103	0.154
Ammonium Sulphate Fractionation (45-90%)	91	15	34	67.9	0.014	0.249

Table 4.4: Comparison of results obtained by the purification of transketolase from packed red blood cells (RBC's) using the method by Takeuchi et al. (1986). This table summarizes the four purification steps performed in this study and compares them to those in the Takeuchi et al. (1986) report. In the second purification step, desalting was performed with dialysis tubing whereas in Takeuchi's method desalting was performed on a gel filteration column. These results are those obtained from peak B only. 36ml of packed RBC was used whereas Takeuchi et al. (1986) used 100ml RBC.

STEP	Total Protein (mg)		Total Activity (nmol/min)		Yield (%)	
	Present Study	Takeuchi et al. (1986)	Present Study	Takeuchi et al. (1986)	Present Study	Takeuchi et al. (1986)
Haemolysate	20,563	83,000	2376	142,000	100%	100%
IEC	30.6	500	427	26,700	18%	18.8%
Desalting	18.2	5.6	274.47	9,560	11.55%	6.7%
FPLC (sum of all peaks eluted)	0.11	2.2	238.78	5,970	0.54%	4.2%

IEC - Ion Exchange Chromatography FPLC - Fast Protein Liquid Chromatography

study, following dialysis, the fractions from peaks A and B were subjected to FPLC on Mono Q instead of Mono P and the gel filtration step was omitted. Both fractions in peaks A and B were resolved into two further peaks by FPLC on Mono Q. This resulted in appreciable loss of TK allowing only sufficient material for SDS-PAGE analysis. This loss of TK may have resulted from the retention of TK on Mono Q. The comparison of findings between the present study and to those of Takeuchi et al. (1986) are summarized in Table 4.4. A sample from fraction B was analyzed on SDS-PAGE to estimate the MW of TK. TK migrated as a single band with MW about 68kD as reported by Takeuchi et al. (1986). Despite achieving high purity of TK using this method it was too laborious, time-consuming and expensive and resulted in only 4.2% yield.

Therefore a third purification method for the isolation of TK from erythrocytes was attempted employing electrophoretic techniques (Jamieson, 1983) used previously for the partial purification of ETK from normal controls and patients with WKS. This is the first method described for the purification of TK that employs rapid, cheap and convenient electrophoretic methods. It also provides sufficient (i.e. 250mg) protein for the qualitiative analysis (i.e. MW, IEF, HPLC) and amino acid analysis of TK. The isolation of TK from fresh haemolysate of a normal control was achieved in four steps involving PAGE, dialysis, SDS-PAGE and western blotting.

The isolated TK was shown to separate into two bands both in SDS-PAGE and IEF with estimated MW of 51 and 35kD and pI value of 7.35 and 6.55 and into two peaks eluted by ion exchange HPLC varying in ionic strength. Similar MW estimations were reported by Warnock and Prudhomme (1982) and similar pI values were also reported by Kaczmarek and Nixon (1983), Kaufmann et al. (1987) and Paoletti et al. (1988) (Table 4.2). Others have reported different MW estimation for TK by various purification method (Table 4.5). In 1971 Henrich and Wiss reported the MW of whole TK molecule to be 136kD. There is a lack of agreement on the exact MW of TK. However, all reports on the purification of TK (with the exception of Takeuchi et al., 1986) have described TK to be heterogenous comprising of at least two variants (Warnock & Prudhomme, 1982; Greenwood et al., 1984; Pratt et al., 1985; Jeyasingham & Pratt, 1988; Sheu et al., 1988; Mocali & Paloetti, 1989).

Table 4.5: Summary of the various purification methods reported. Results from the purification method by electrophoretic techniques in this study were compared to the reports given.

Report	Purification Techniques	Source	Purification (-FOLD)	Method of MW estimation	MW of TK (kD)
Warnocke & Prudhomme (1982)	IEC	RBC	400	SDS-PAGE	3 variants: 50-60, 26-30 & 33
Takeuchi et al. (1986)	IEC & FPLC	RBC	16,000	GFC SDS-PAGE	104 2 subunits (70)
Himmo et al. (1988)	Affinity Adsorption	RBC	6,200	PAGE	140
Sheu et al. (1988)	Immunoaffinity	brain	-	SDS-PAGE	2 variants: 72 & 70
Mocali & Paoletti (1989)	GFC & Affinity Adsorption	WBC	327	SDS-PAGE GFC	2 subunits (66) 135 ± 4
Present Study	PAGE & western blotting	RBC	5.4	SDS-PAGE	2 variants: 51 & 35
	IEC & FPLC	RBC	2076	SDS-PAGE	68

MW - molecular weight in kiloDaltons
IEC - Ion exchange chromatography
RBC - Red blood cells
SDS-PAGE - SDS-polyacrylamide gel electrophoresis
FPLC - Fast performance liquid chromatography
GFC - Gel filteration chromatography
WBC - white blood cells

The findings in the present study have also confirmed this heterogeneity. The disagreement on the heterogeniety of TK by Takeuchi et al (1986) may have arisen from racial differences. This may be evident because different susceptibility to WKS exists between the Caucasian and Asian races.

The discrepancy in the MW of TK among the various reports can result from changes caused by the different purification methods applied. Acylation and glycosylation of a protein can change its MW and so can other chemical and enzymatic events such as proteolysis. This was evident in this study since the purification of TK by chromatographic techniques (Takeuchi et al., 1986) showed the MW of ETK to be 68kD while by electrophoretic techniques the MW of ETK was estimated to be 51 and 35kD. This difference in TK migration on SDS-PAGE may have also arisen from the different buffer systems used (i.e. a continuous buffer was used in the former and a discontinuous buffer in the latter).

The amino acid composition of both TK bands was analysed using the material isolated from fresh erythrocytes of a normal control using electrophoretic technqiues. There has been only one report on the amino acid composition of TK (Takeuchi et al., 1986). These findings were compared to the findings in this study (Table 4.6). Statistical analysis employing Kendall's test showed the reported amino acid composition (Takeuchi et al., 1986) to have a highly significant ($Z = 2.97$, $p \leq 0.01$) positive correlation with the 51kD TK and a signficant ($Z = 2.57$, $p \leq 0.05$) with the 35kD TK in the present study. Other reports on the amino acid composition of TK included that of Kuimov et al. (1988) who described chemical modification methods which allowed the detection of specific groups essential for the activation of TK. These comprise carboxyl group, tryptophan, histidine and arginine residues which have also been detected in the study (Tables 3.8 and 3.9). Kuimov et al. (1988) also suggested that HoloTK was inhibited more slowly than ApoTK due to the presence of a tyrosine residue in the enzyme's active site. Moreover, the dependence of TK inhibition rate on the concentration of H^+ ions suggests the inhibition of a tyrosine residue.

Table 4.6: Amino acid composition (molar ratio %) of the isolated erythrocyte transketolase (TK) in the present study and in the Takeuchi et al. (1986) study. Takeuchi et al. performed amino acid composition on the TK peaks eluted from Mono P by fast performance liquid chromatography.

Amino Acid	Takeuchi et al. (1986) composition (%)		Present Study composition (%)		Occurrence in Proteins (%)*
	Peak I	Peak II	51kD	35kD	
Aspartic Acid (D)	9.7	9.9	11.95	7.39	5.5
Glutamic Acid (E)	10.9	11.2	7.43	6.62	6.2
Serine (S)	7.1	6.9	11.85	6.31	7.1
Glycine (G)	8.0	7.9	6.62	8.85	7.5
Histidine (H)	3.0	3.0	3.90	5.24	2.1
Arginine (R)	4.8	4.8	2.00	2.27	4.7
Threonine (T)	5.5	5.6	5.20	6.06	6.0
Alonine (A)	10.8	11.2	9.29	13.56	9.0
Proline (P)	2.5	2.4	7.18	5.75	4.6
Tyrosine (Y)	2.7	2.5	3.32	2.17	3.5
Valine (V)	6.2	6.4	7.12	11.60	6.9
Methionine (M)	nc	nc	0.73	0.73	1.7
Isoleucine (I)	7.7	7.5	4.45	-	4.6
Leucine (L)	7.8	8.2	9.58	13.90	7.5
Phemylamine (F)	5.3	5.1	3.98	4.98	3.5
Lysine (K)	7.6	7.5	5.39	4.58	7.0

nc = not counted although had been detected

* Frequency of occurrence of each amino acid residue in the primary structures of 207 unrelated proteins of known sequence values were obtained from M.H. Klapper, Biochemistry Biophysics Research Communications, 78: 1018-1024, 1977 (Creighton, 1983).

In the present study, amino acid sequence of 15 residues was obtained for the 35kD peptide. This sequence was compared in the Gen Bank Database for exisiting homologies which had verified the obtained sequence to be unique. The sequence of the smaller TK peptide indicates the N-terminal of TK to be hydrophobic. No sequence was obtained for the 51kD TK peptide due to N-terminal blockage which can occur from the chemical modifications of the N-terminus by either the buffers used in SDS-PAGE and western blotting or by post-translational modification. The buffers used in this study were not the cause of the blockage since all precautions were taken to avoid such an event, and since one peptide (35kD) was sequenced from the same SDS-PAGE - PVDF membrane. Therefore it would appear that the blockage may have arisen by post-translational modification. It has been suggested that greater than 75% of all intracellular proteins of eukaryotic cells have blocked N-termini and are resistant to the Edman degradation method as a result of post-translational modifications (i.e. enzymatic processes following the biosynthesis of proteins). Such modifications may occur due to the clipping off of amino acids at the N or C termini by peptidases, acylation of the amino terminus, glycosylation, phosphorylation or sulphation of certain amino acids. The report by Kuimov et al. (1988) has illustrated the inhibition of TK during acetylation by N-acetylimidazole rendering TK susceptible to acetylation (Baines & Davies, 1988; Lishman, 1990). This may also imply that the N-terminus of the 51kD peptide is the active site of TK (binding region with coenzyme TPP) since kinetic data (Kuimov et al., 1988) has shown the acetylation of at least one amino acid residue (tyrosine) would be sufficient for TK inactivation (Kuimov et al., 1988). There have been no reported TK sequences except that of Hawkins et al. (1989) who described a common structural motif in TPP binding enzymes such as transketolase formaldehyde in E.coli.

4.4 CONCLUSION AND FUTURE PROSPECTS

Preliminary work on the quantitative and qualitative analysis of TK has not resolved the role of this enzyme in the WKS as postulated by others (Sauberlich, 1984; Victor et al., 1989). Qualitative analysis and the successful isolation and characterization (in particular, amino acid composition) of TK in this study has confirmed the heterogeneity of TK (Kaczmarek & Nixon, 1983; Pratt et al, 1985; Kaufmann et al., 1987; Paoletti et al., 1989) and the existence of at least two variants differing in MW, pI value, ionic strength and amino acid composition. The role of these variants in WKS remains to be resolved. On close examination, the electrophoretic profile of silver stained TK showed the two variants may be present in different quantities. It had been suggested (Blass & Gibson, 1977; Greenwood et al., 1989; Pratt et al., 1985; Thomson et al., 1987; Jeyasingham et al., 1987) that the smaller molecule of TK represented the ApoTK which would be present in larger quantities in patients with WKS and that the larger molecule (HoloTK) would be in larger quantities in normal individuals.

Results obtained from MW estimation and amino acid analysis of TK have confirmed its susceptibility to post-translational modifications. This had also been observed by others (Pratt et al., 1985; Takeuchi et al., 1986; Mocali & Paoletti, 1989). To establish whether such modifications are genetically determined (Blass & Gibson, 1977; Kaczmarek & Nixon, 1983; Nixon, 1984; Paoletti et al., 1989; Victor et al., 1990) or are products of a damaged system induced by thiamine depletion and ethanol intoxication (Freund, 1973; Pratt et al., 1985; Jeyasingham & Pratt, 1988; Lishman et al., 1990) molecular genetic techniques must be employed for the cloning and characterization of the TK gene. This would also allow the study of the interaction between the environment and heredity in the pathogenesis of WKS and its mode of inheritance. It would also provide means for population screening of those who are susceptible to TD and/or WKS, thus assisting in the reduction of incidence of such disorders.

In 1990 Singleton et al. reported the cloning and characterizations of the human TK gene. They described the isolation of two cDNA clones specific to TK

as detected by TK polyclonal antibodies. The nucleotide and amino acid sequence of 60% of the gene had been predicted (Singleton et al., 1990) although this was not published. The amino acid sequence described in the present study may be used as a template for the synthesis of oligonucleotides and for the cloning of the 35kD gene. However, due to the number of degenerate codons in the 15 amino acid residues sequenced (Table 3.10) it would be essential to obtain more sequence data for the 35kD protein and to sequence the 51kD protein which is N-terminally blocked. As an alternative approach, the peptide can be fragmented into smaller peptides by cleaving agents specific for amino acid pairs. The number of fragments obtained must be controlled by employing site-specific proteases or chemicals for digestion of specific residues when molar ratios are known. These ratios can be obtained from amino acid composition analysis. For example, in this study, methionine was estimated to have a molar ratio of 0.73%, therefore cynanogen bromide (CNBr) can be used for cleavage at methionine-serine and methionine-threonine bonds (Matsudaira, 1989). These would result in the cleavage of the 51kD TK peptide into a few smaller peptides in which internal sequences can be obtained thus avoiding N-terminal blockage in such circumstances.

To synthesize a highly specific TK clone it would be best to obtain oligonucleotides that are low in degenerate codons and from various points on the peptide sequence. Another approach would be the synthesis of monoclonal antibodies using purified TK prepared by the PAGE purification method described in this study. This would then enable the cloning of a cDNA library specific to TK. Finally, the gene for TK can be determined by localizing and sequencing the nucleotides of the TPP binding site from the common motif in gene clones of PDH and αKGDH and then screening for the TK gene in erythrocytes (since TK is the only enzyme in RBC which utilizes TPP) by the elimination of the known PDH and αKGDH genes.

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APPENDICES

APPENDIX I: List of Samples from Normal Controls

Red blood samples collected from normal controls at different times and stored at -20°C in buffered citrate glycerol.

Collection & Storage Date	Label No.	Sex	Age
25/4/1989	100180	M	40
25/4/1989	100181	F	38
25/4/1989	100182	M	34
25/4/1989	100183	F	37
25/4/1989	100184	F	22
25/4/1989	100185	M	27
25/4/1989	100186	M	29
6/7/1989	100187	M	22
6/7/1989	100188	M	26
6/7/1989	100189	M	25
6/7/1989	100190	F	22
6/7/1989	100191	F	21
22/6/1989	100192	M	30
20/7/1989	100193	M	30
25/4/1989	100123	F	37
25/4/1989	100124	M	31
25/4/1989	100125	F	22
6/7/1989	100126	M	25
6/7/1989	100127	M	26
30/10/1989	100128	F	30
30/10/1989	100129	F	21
20/12/1989	100130	F	22
20/12/1989	100131	M	31
20/12/1989	100132	F	39
20/12/1989	100133	M	41

APPENDIX II: Materials and Reagent Concentrations

(All reagents were purchased from Sigma and Merck unless stated otherwise)

II.1 Drabkin's Solution for Haemoglobin Estimation
(stable for several months at 4°C in dark bottle)

200mg K Fe_3 CN_6	Potassium Ferricyanide
50mg KCN	Potassium cyanide
140mg KH_2PO_4	Potassium dihydrogen orthophosphate

II.2 Reagents Used in Transketalase Assay

II.2a Materials for section 2.1.4.1 and 2.1.4.2

1% Titron X-100
0.1 M Tris-HCl pH7.6 - stable for 1 month at 4°C.
14mM Ribose-5-phosphate in 0.1M tris-HCl pH 7.6
4.16% GDH + 6.25% TPI + 89.58% Tris HCl pH 7.6
10mM NADH in 0.1M Tris-HCl pH 7.6
10mM TPP in 0.1M Tris-HCl pH 7.6

II.2b Materials for section 2.1.4.3

same materials as above (II.2a)
1.2mM $MgCl_2$
7.0mM Xylulose-5-Phosphate

II.3 Reagents for Starch Gel and Cellogel Electrophoresis

(All buffers are stable for several weeks at 4°C)

10 X Anode Buffer, pH 9.2:
- 2.52% (w/v) Trizma-Base
- 0.25% (w/v) EDTA
- 0.9% (w/v) Boric Acid

10 X Cathode Buffer, pH 8.6:
- 0.515% (w/v) Sodium Barbitone
- 0.092% (w/v) Barbitone

Gel Buffer:
- 50% Cathode Buffer
- 50% Anode Buffer

II.4 Reagents for Polyacrylamide gel electrophoresis (Section 2.1.5.3, 2.2.1)

(All solutions stored at 4°C unless stated otherwise)

Glycerol
Bromophenol Blue
0.1% Triton

Tank Buffer:
Tris-Glycine Buffer pH 8.2 (x 10)
0.6% Trizma Base
2.9% Glycine

Gel Solution (stable for 1 week):
29.1% Acrylamide + 0.9% DHEBA

0.14% Ammonium Persulphate
0.28% TEMED (made up fresh)

II.5 Reagents for Transketolase Staining Method

II.5a Starch Gels and Cellogel Stain

0.1m Tris-HCl pH 7.6 (store at 4°C, stable 1 month)
5.2mM Ribose-5-phosphate
1.1mM Xylulose-5-phosphate
1.0mM NAD
500U GDH
0.05mM Phenazine Methasulphate
0.5mM NitroBlue Tetrazolium
0.5mM $MgCl_2$
2.0mM Sodium Arsenate

II.5b Polyacrylamide Gel Stain

0.1m Tris-HCl pH 7.6 (store at 4°C, stable 1 month)
5.2mM Ribose-5-phosphate
1.1mM Xylulose-5-phosphate
1.0mM NAD
500U GDH
0.05mM Phenazine Methasulphate
0.5mM Methyl Thiazolyl Tetrazolium
0.5mM $MgCl_2$
2.0mM Sodium Arsenate

II.6 Reagents for Coomassie Brilliant Blue R-250 Stain

45% (w/v) Methanol
10% (w/v) Acetic Acid
0.1% (w/v) Coomassie Brilliant Blue R-250 (SIGMA)

II.7 Reagents for Silver Stain

(Bio Rad's Silver Stain Kit)

10% Oxidising Reagent (Potassium dichromate and Nitric Acid)
(made up fresh)
10% Silver Concentrate Reagent (Silver Nitrate) - made up fresh
3.2% Developer (Sodium Carbonate and Foraformaldehyde) - can be stored for one month at room temperature

II.8 Reagents for Isoelectric Focusing

Gel solution:
- 29.1% Acrylamide - stored at 4°C
- 0.9% Bis-Acrylamide - stored at 4°C

1% Ammonium Persulphate
87% Glycerol - stored at 4°C

Electrode Buffer:
- IM Sodium Hydroxide
- 1M Ortho-phosphoric Acid

Ampholines (PI 3-10 PHARMACIA -LKB)
pI markers (3.5-10 PHARMACIA -LKB)

II.9 Reagents for SDS-Polyacrylamide Gel Electrophoresis

II.9a Sample Buffer for SDS-PAGE

6% 1M Tris pH 6.8
10% of 20% SDS
20% Glycerol - Store at 4°C 1 week
10% β-Mercaptoethanol
53% DH_2O
1% of 0.1% (w/v) Bromophenol Blue

II.9b Reagents for SDS-PAGE in section 2.2.2.2, 2.5.1

Anode Buffer: 0.2M Tris-Buffer, pH 8.9
Cathode Buffer, pH 8.25 (Adjust with Tricine):
- 0.1M Tris-Buffer
- 0.1M Tricine
- 0.1% SDS

Gel Buffer, pH 8.45 (Store at 4°C 1 week):
- 3M Tris Buffer
- 0.3% SDS

Polyacrylamide gel solution:
- 48% acrylamide
- 1.5% Bis-acrylamide

SDS-PAGE molecular weight marker 20-215kD

II.9c Reagents used for SDS-PAGE in section 2.4.4.2

20% SDS (store at room temperature for 1 week)
1M Tris pH 6.8 (store at room temperature for 1 week)
1M Tris pH 8.8 (store at room temperature for 1 week)
10% Ammonium Persulphate (make up fresh)
TEMED
Polyacrylamide gel solution (store at 4°C for 1 week):
- 30% Acrylamide
- 0.8% Bis-Acrylamide
- Iso Butanol

Running Buffer, pH 8.3 (store 4°C for 1 week):
- 0.3% (w/v) Trizma-Base
- 1.44% (w/v) Glycine
- 0.5% (w/v) of 20% SDS

Bovine Albumin Serum (MW marker 67kD)

II.10 Reagents for Kinetic Assay of TK (section 2.3.1)

0.1M Tris-HCl pH 7.6
15mM Ribose-5-phosphate
70mM Xylulose-5-phosphate
10mM NADH in 0.1M Tris-HCl pH 7.6
40ml GDH/TPI in 0.1M Tris-HCl pH 7.6
10mM $MgCl_2$ in 0.1M Tris-HCl pH 7.6
10mM TPP in 0.1M Tris-HCl pH 7.6

APPENDIX III: Endpoint Assay Raw Data

Table III.1: Optimizing the endpoint assay. All samples tested were normal controls (Appendix I). Results were summarized in Table 3.2 and are averages of duplicates and replicates.

Table III.1a: Various modifications of erythrocyte transketolase (ETK) assay to obtain full activation and reduce haemoglobin interference.

Sample	Hb conc. (g/dL)	Boiling Sample (IU/gHb)		Incubation in Shaker Bath (IU/gHb)	
		ETK	ETKs activity	ETK	ETKs activity
100180	39.3	0.51	0.27	0.70	0.35
100181	35.9	0.56	0.29	0.24	0.10
100182	30.7	0.54	0.28	0.67	0.37
100183	32.0	-	-	0.35	0.18
100184	35.1	0.32	0.30	0.56	0.35
100185	34.5	0.52	0.27	-	-
100186	35.1	0.39	0.40	-	-

Table III.1b: Various modifications of ETK activity assay.

Sample	Hb conc. (g/dL)	Heating Sample (IU/gHb)	Addition of 20% NaOH (IU/gHb
100180	42.8	0.48	0.25
100181	40.1	0.35	0.27
100182	40.7	0.20	0.33
100183	39.4	0.24	0.26
100184	110.1	0.40	0.15
100185	39.1	0.34	0.41
100186	37.3	0.35	-

Table III.1c: Various modications of ETK activity assay and its activation (ETKs) to enhance the binding of apoenzyme to coenzyme.

Sample	Hb conc. (gm/dL)	+ $MgCl_2$ (IU/gmHb)		+ DTT (IU/gmHb)		+ $MgCl_2$ + DTT (IU/gmHb)	
		ETK	ETKs	ETK	ETKs	ETK	ETKs
100180	35.7	0.53	0.74	-	-	-	-
100181	37.5	0.47	0.45	0.56	0.63	0.72	0.67
100182	41.9	0.63	0.61	0.92	0.54	0.66	0.61
100183	37.2	0.66	0.55	0.52	0.46	0.70	0.65
100184	38.2	0.31	0.73	0.60	0.41	-	-
100185	35.9	0.43	0.39	0.76	1.01	0.73	0.63
100186	35.9	0.52	0.34	0.20	-	0.46	0.47

Table III.2: Application of erythrocyte transketolase (ETK) activity assay and its stimulation (ETKs) expressed in IU /L of haemolysate and IU /mg of protein on 20 patients and 10 normal controls. All data shown are averages of duplicates and were summarized in Table 3.4.

Sample	Total Protein (mg)	ΔOD (Au)	Time (min)	ETK (ΔOD/ min)	ΔOD (Au)	Time (min)	ETKs (ΔOD /min)	ETK activity (IU)		ETKs activity (IU)		TPPE %
								(/L)	(/mg)	(/L)	(/mg)	
100100	1.11	-	-	-	-	-	-	-	-	-	-	-
100101	1.09	0.05	70	0.0007	0.086	70	0.0017	5.68	0.310	10.55	0.578	86.45
100102	1.05	0.67	70	0.0010	0.119	70	0.0017	8.12	0.464	13.80	0.789	70.04
100103	1.12	0.033	70	0.0005	0.076	70	0.0011	4.06	0.220	8.93	0.476	116.36
100104	1.16	0.021	70	0.0003	0.061	70	0.0009	2.44	0.126	7.31	0.377	199.21
100105	1.06	0.049	70	0.0007	0.078	70	0.0011	5.68	0.320	8.93	0.503	57.19
100106	1.12	0.070	70	0.0010	0.090	70	0.0013	8.12	0.433	10.55	0.563	30.02
100107	1.11	0.052	70	0.0007	0.078	70	0.0011	5.68	0.307	8.93	0.483	57.33
100108	1.18	-	-	-	-	-	-	-	-	-	-	-
100109	1.17	0.075	70	0.0011	0.092	70	0.0013	8.93	0.458	10.55	0.541	18.12
100110	1.18	0.063	65	0.0011	0.154	65	0.0024	8.93	0.452	19.99	0.987	118.36
100111	1.17	0.071	65	0.0011	0.103	65	0.0016	8.93	0.458	12.99	0.666	45.41
100112	1.18	0.032	65	0.0004	0.088	65	0.0014	3.25	0.165	11.37	0.576	249.09
100113	1.15	0.041	65	0.0006	0.100	65	0.0015	4.87	0.253	12.18	0.633	150.20
100114	1.12	0.060	65	0.0009	0.089	65	0.0014	7.31	0.390	11.37	0.606	55.38
100115	1.03	0.099	65	0.0015	0.151	65	0.0023	12.18	0.706	18.67	1.08	52.97
100116	1.01	0.090	65	0.0014	0.122	65	0.0019	11.37	0.679	15.43	0.921	35.64
100117	1.11	0.061	65	0.0009	0.097	65	0.0015	7.31	0.395	12.18	0.658	66.58
100118	1.16	0.100	65	0.0015	0.144	65	0.0022	12.18	0.633	17.86	0.928	46.60
100119	1.04	0.059	65	0.0009	0.129	65	0.0020	7.31	0.424	16.24	0.941	121.93
100120	1.00	-	-	-	-	-	-	-	-	-	-	-
100121	1.13	0.051	65	0.0008	0.088	65	0.0014	6.50	0.347	11.37	0.606	74.64
100122	1.01	0.076	65	0.0012	0.093	65	0.0014	9.74	0.581	11.37	0.679	16.87
100123	1.16	0.020	65	0.0003	0.078	65	0.0012	2.44	0.127	9.74	0.506	298.43
100124	1.05	0.048	65	0.0007	0.064	65	0.0010	5.68	0.325	8.12	0.464	42.77
100125	1.13	-	-	-	-	-	-	-	-	-	-	-
100126	1.10	0.045	65	0.0007	0.087	65	0.0013	5.68	0.311	10.55	0.578	85.85
100127	0.99	0.042	65	0.0006	0.071	65	0.0011	4.87	0.295	8.93	0.541	83.39
100128	1.16	0.021	65	0.0003	0.090	65	0.0015	2.44	0.127	12.18	0.633	398.9
100129	1.07	0.083	65	0.0013	0.144	65	0.0022	10.55	0.594	17.86	1.006	69.36
100130	1.20	0.104	65	0.0016	0.144	65	0.0022	12.99	0.650	17.86	0.893	37.38
100131	1.20	0.097	65	0.0015	0.127	65	0.0020	12.18	0.609	16.24	0.812	33.33
100132	1.17	0.096	65	0.0014	0.135	65	0.0021	11.37	0.583	17.05	0.874	49.91
100133	1.14	0.082	65	0.0013	0.164	65	0.0025	10.55	0.555	20.30	1.068	92.43
100134	1.02	-	-	-	0.104	65	0.0016	-	-	12.99	0.764	-
100135	1.17	-	-	-	0.111	65	0.0017	-	-	13.80	0.708	-

APPENDIX IV: Elution Profile of the Standard Amino Acids

= Applied Biosystems 477A Protein Sequencer Chromatogram Report =

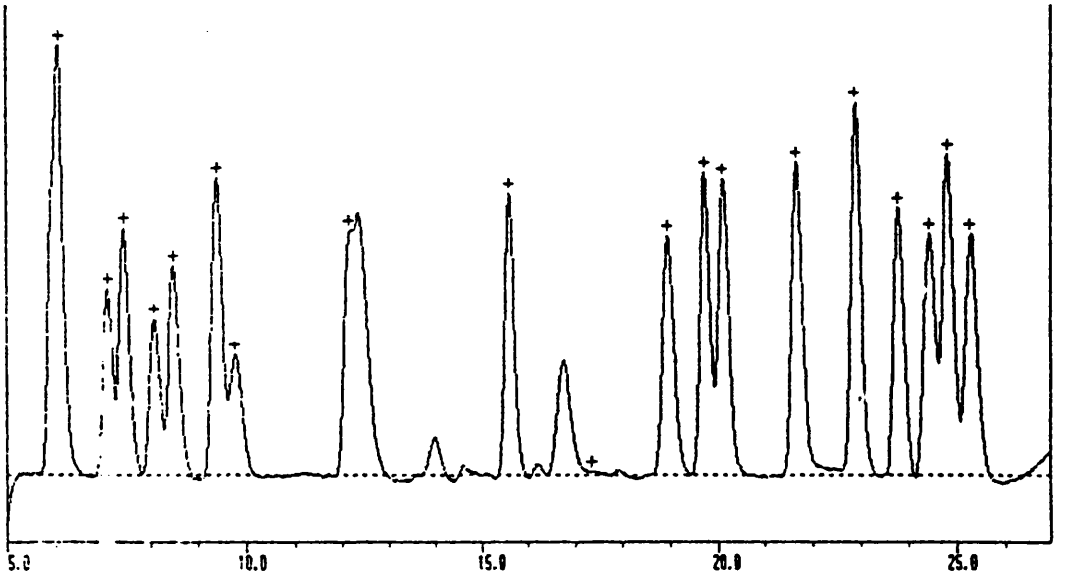
SAMPLE . 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BEGIN	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: BEGIN	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

CALIBRATION # 1 [15 Jan 1992 10:38am] 0.0100 AU

Baseline Corrected Data



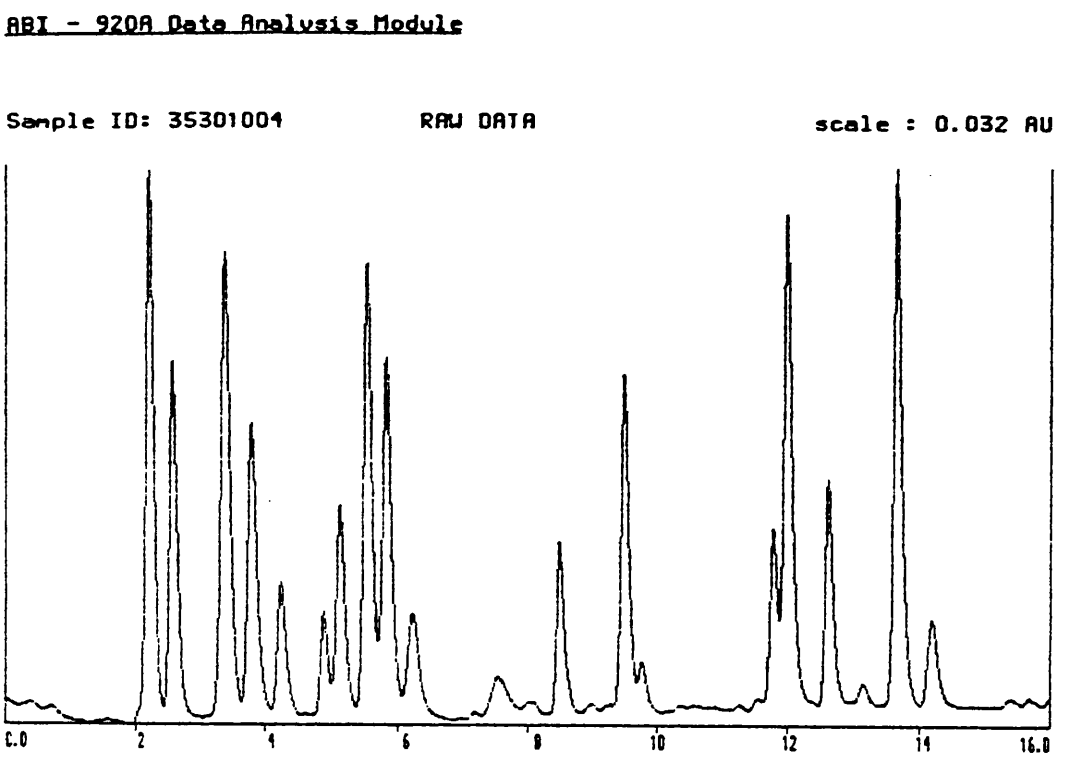
Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASN	6.07	6.00	23985	79.84	PRO	18.93	18.75	13371	58.03
SER	7.10	7.02	10470	68.27	MET	19.68	19.52	16932	63.63
GLN	7.45	7.35	13815	64.29	VAL	20.07	19.90	16551	61.40
THR	8.08	7.98	8772	62.41	OPT	21.65	21.50	17466	64.45
GLY	8.49	8.38	11793	64.44		22.42		363	
GLU	9.37	9.25	16680	60.73		22.52		363	
DMP	9.77	9.67	6765	75.54	TRP	22.90	22.73	20775	58.99
ALA	12.18	12.02	13680	69.08	PHE	23.78	23.63	14910	57.66
	12.37		14652		ILE	24.43	24.27	13521	59.29
	13.38		2100		LYS	24.80	24.65	17955	59.55
	14.32		531		LEU	25.27	25.12	13530	57.47
TYR	15.60	15.40	15705	63.56					
	16.25		573						
	16.75		6414						
ARG	17.37	17.13	237	2.48					

Tabulation threshold : 300 uAU

APPENDIX V: Amino Acid Compostion of the 51kD Transketolase Band



Pmol By Height Report

Sample ID: 35301004 (initiated 12/19/91 1:39pm) BASELINE CORRECTED

Turntable Position: 2 A
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD (initiated 12/19/91 3:0ipm)
Reference Time : 0.00 min (No ISTD Peak Specified)
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min

Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	CAL. TIME min	PEAK HEIGHT uAU	PMOL BY HEIGHT	PMOL correc. INT STD
Aspartic Acid	2.20	2.17	31903	401.70	0.00
Glutamic Acid	2.57	2.54	20965	249.56	0.00
Serine	3.35	3.32	22784	398.35	0.00
Glycine	3.79	3.75	14192	222.28	0.00
Histidine	4.25	4.21	6869	131.17	0.00
Arginine	4.90	4.86	5984	67.16	0.00
Threonine	5.14	5.10	11135	174.74	0.00
Alanine	5.54	5.49	25110	312.05	0.00
Proline	5.82	5.78	18226	241.29	0.00
	6.22	-----	3618	----------	----------
Tyrosine	8.49	8.45	10317	111.45	0.00
Valine	9.49	9.45	19634	239.39	0.00
Methionine	9.78	9.73	1975	24.49	0.00
Isoleucine	11.78	11.76	9976	149.39	0.00
Leucine	11.99	11.97	26518	322.01	0.00
Phenylalanine	12.62	12.60	13655	134.21	0.00
	13.15	-----	1033	----------	----------
Lysine	13.65	13.63	31097	180.96	0.00
	14.19	-----	3796	----------	----------

Minimum Peak Threshold: 999 uAU (29 peaks below threshold)
(19 peaks found)
(16 peaks matched)

Mol Percent Report

Sample ID: 35301004 (initiated 12/19/91 1:39pm) BASELINE CORRECTED

Turntable Position: 2 A
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD
Reference Time : 0.00 min
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min

(initiated 12/19/91 3:01pm)
(No ISTD Peak Specified)

Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	PMOL BY HEIGHT	PMOL correc. INT STD	MOL %
Aspartic Acid	2.20	401.70	0.00	11.95
Glutamic Acid	2.57	249.55	0.00	7.43
Serine	3.35	398.35	0.00	11.85
Glycine	3.79	222.28	0.00	6.62
Histidine	4.25	131.17	0.00	3.90
Arginine	4.90	67.16	0.00	2.00
Threonine	5.14	174.74	0.00	5.20
Alanine	5.54	312.05	0.00	9.29
Proline	5.82	241.29	0.00	7.18
Tyrosine	8.49	111.45	0.00	3.32
Valine	9.49	239.39	0.00	7.12
Methionine	9.78	24.49	0.00	0.73
Isoleucine	11.78	149.39	0.00	4.45
Leucine	11.99	322.01	0.00	9.58
Phenylalanine	12.62	134.21	0.00	3.99
Lysine	13.65	180.96	0.00	5.39

TOTAL PMOLS RECOVERED 3360.19

Minimum Peak Threshold: 999 uAU (29 peaks below threshold)
(19 peaks found)
(16 peaks matched)

Composition Report

Sample ID: 35301004 (initiated 12/19/91 1:39pm) BASELINE CORRECTED

Turntable Position: 2 A
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD (initiated 12/19/91 3:01pm)
Reference Time : 0.00 min (No ISTD Peak Specified)
Reference Offset 1: 0.00 min Based on Mol Wt : 51000.00
Reference Offset 2: 0.00 min

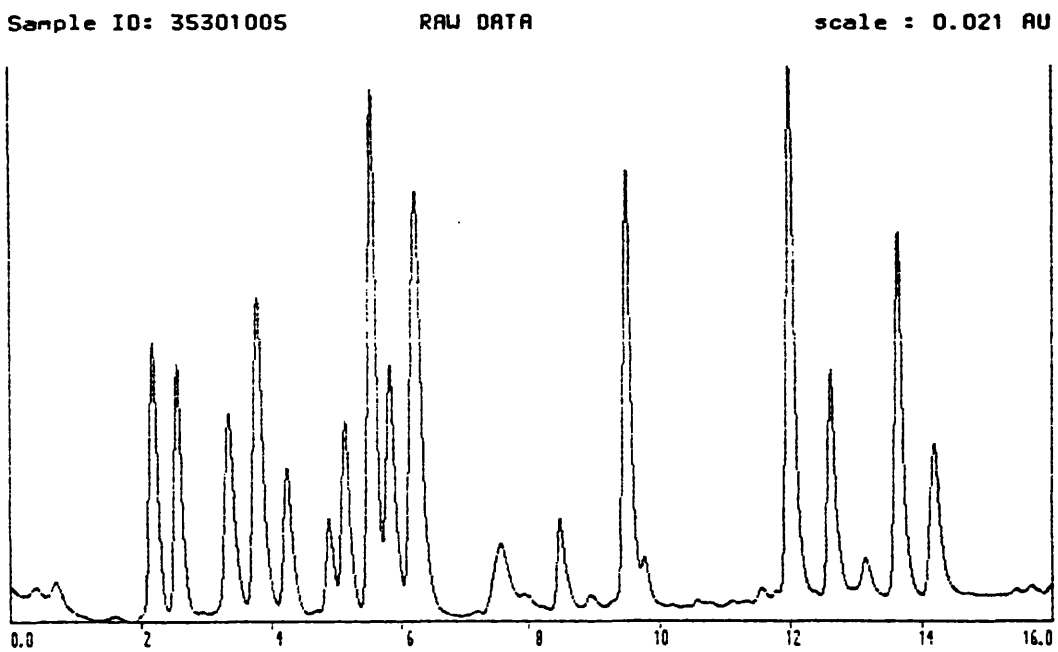
Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	PMOL BY HEIGHT	PMOL correc. INT STD	APPLIED ngms	COMP BY MW
Aspartic Acid	2.20	401.70	0.00	46.24	57.058
Glutamic Acid	2.57	249.56	0.00	32.22	35.448
Serine	3.35	398.35	0.00	34.70	56.582
Glycine	3.79	222.28	0.00	12.69	31.572
Histidine	4.25	131.17	0.00	18.00	18.631
Arginine	4.90	67.16	0.00	10.49	9.539
Threonine	5.14	174.74	0.00	17.67	24.820
Alanine	5.54	312.05	0.00	22.19	44.324
Proline	5.82	241.29	0.00	23.43	34.273
Tyrosine	8.49	111.45	0.00	18.19	15.830
Valine	9.49	239.39	0.00	23.72	34.003
Methionine	9.78	24.49	0.00	3.21	3.479
Isoleucine	11.78	149.39	0.00	16.91	21.219
Leucine	11.99	322.01	0.00	36.45	45.738
Phenylalanine	12.62	134.21	0.00	19.75	19.063
Lysine	13.65	180.96	0.00	23.20	25.704

TOTAL MASS RECOVERED 0.35 ugms
CALCULATED MW OF SAMPLE 51018.00

Minimum Peak Threshold: 999 uAU (29 peaks below threshold)
(19 peaks found)
(16 peaks matched)

APPENDIX VI: Amino Acid Composition of the 35kD Transketolase Band



Pmol By Height Report

Sample ID: 35301005 (initiated 12/19/91 2:28pm) BASELINE CORRECTED

Turntable Position: 2 B
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD
Reference Time : 0.00 min
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min

(initiated 12/19/91 3:03pm)
(No ISTD Peak Specified)

Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	CAL. TIME min	PEAK HEIGHT uAU	PMOL BY HEIGHT	PMOL correc. INT STD
Aspartic Acid	2.17	2.17	10266	129.25	0.00
Glutamic Acid	2.56	2.54	9729	115.81	0.00
Serine	3.33	3.32	6307	110.27	0.00
Glycine	3.78	3.75	9879	154.73	0.00
Histidine	4.23	4.21	4796	91.58	0.00
Arginine	4.89	4.86	3539	39.72	0.00
Threonine	5.13	5.10	6756	106.02	0.00
Alanine	5.53	5.49	19077	237.08	0.00
Proline	5.82	5.78	7589	100.47	0.00
	6.21	-----	10108	----------	----------
Tyrosine	8.49	8.45	3512	37.94	0.00
Valine	9.49	9.45	15635	202.83	0.00
Methionine	9.77	9.73	1032	12.30	0.00
Leucine	11.99	11.97	20017	243.07	0.00
Phenylalanine	12.62	12.60	8857	87.05	0.00
	13.15	-----	1068	----------	----------
Lysine	13.65	13.63	13774	80.16	0.00
	14.18	-----	4358	----------	----------

Minimum Peak Threshold: 999 uAU (27 peaks below threshold)
(18 peaks found)
(15 peaks matched)

Mol Percent Report

Sample ID: 35301005 (initiated 12/19/91 2:28pm) BASELINE CORRECTED

Turntable Position: 2 B
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU

Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD (initiated 12/19/91 3:03pm)
Reference Time : 0.00 min (No ISTD Peak Specified)
Reference Offset 1: 0.00 min
Reference Offset 2: 0.00 min

Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	PMOL BY HEIGHT	PMOL correc. INT STD	MOL %
Aspartic Acid	2.17	129.26	0.00	7.39
Glutamic Acid	2.56	115.81	0.00	6.62
Serine	3.33	110.27	0.00	6.31
Glycine	3.78	154.73	0.00	8.85
Histidine	4.23	91.58	0.00	5.24
Arginine	4.89	39.72	0.00	2.27
Threonine	5.13	106.02	0.00	6.06
Alanine	5.53	237.08	0.00	13.56
Proline	5.82	100.47	0.00	5.75
Tyrosine	8.49	37.94	0.00	2.17
Valine	9.49	202.83	0.00	11.60
Methionine	9.77	12.80	0.00	0.73
Leucine	11.99	243.07	0.00	13.90
Phenylalanine	12.52	87.05	0.00	4.98
Lysine	13.65	80.15	0.00	4.58

TOTAL PMOLS RECOVERED 1748.77

Minimum Peak Threshold: 999 uAU (27 peaks below threshold)
(18 peaks found)
(15 peaks matched)

Composition Report

Sample ID: 35301005 (initiated 12/19/91 2:28pm) BASELINE CORRECTED

Turntable Position: 2 B
Data Start : 4.00 min
Data Duration : 16.00 min
Peak Ht Threshold : 999 uAU
Sampling Interval: 0.5 sec
Samples In Run : 19
Operator ID : DEREK
Int. Std. Amt : 0 pmol

Calibration File : 35201HYD (initiated 12/19/91 3:03pm)
Reference Time : 0.00 min (No ISTD Peak Specified)
Reference Offset 1: 0.00 min Based on Mol Wt : 35000.00
Reference Offset 2: 0.00 min

Integration Interval: 0.0 to 16.0 min

PEAK ID	RET. TIME min	PMOL BY HEIGHT	PMOL correc. INT STD	APPLIED ngms	COMP BY MW
Aspartic Acid	2.17	129.26	0.00	14.88	24.730
Glutamic Acid	2.56	115.61	0.00	14.95	22.156
Serine	3.33	110.27	0.00	9.60	21.096
Glycine	3.78	154.73	0.00	8.63	29.602
Histidine	4.23	91.58	0.00	12.57	17.521
Arginine	4.89	39.72	0.00	6.20	7.599
Threonine	5.13	106.02	0.00	10.72	20.283
Alanine	5.53	237.08	0.00	18.86	45.356
Proline	5.82	100.47	0.00	9.75	19.221
Tyrosine	8.49	37.94	0.00	6.19	7.258
Valine	9.49	202.83	0.00	20.10	38.804
Methionine	9.77	12.80	0.00	1.69	2.448
Leucine	11.99	243.07	0.00	27.52	46.502
Phenylalanine	12.62	87.05	0.00	12.81	16.654
Lysine	13.65	80.16	0.00	10.28	15.335

TOTAL MASS RECOVERED 0.18 ugms
CALCULATED MW OF SAMPLE 35018.00

Minimum Peak Threshold: 999 uAU (27 peaks below threshold)
(18 peaks found)
(15 peaks matched)

APPENDIX VII: Amino Acid Sequence of the 35kD Transketolase Band

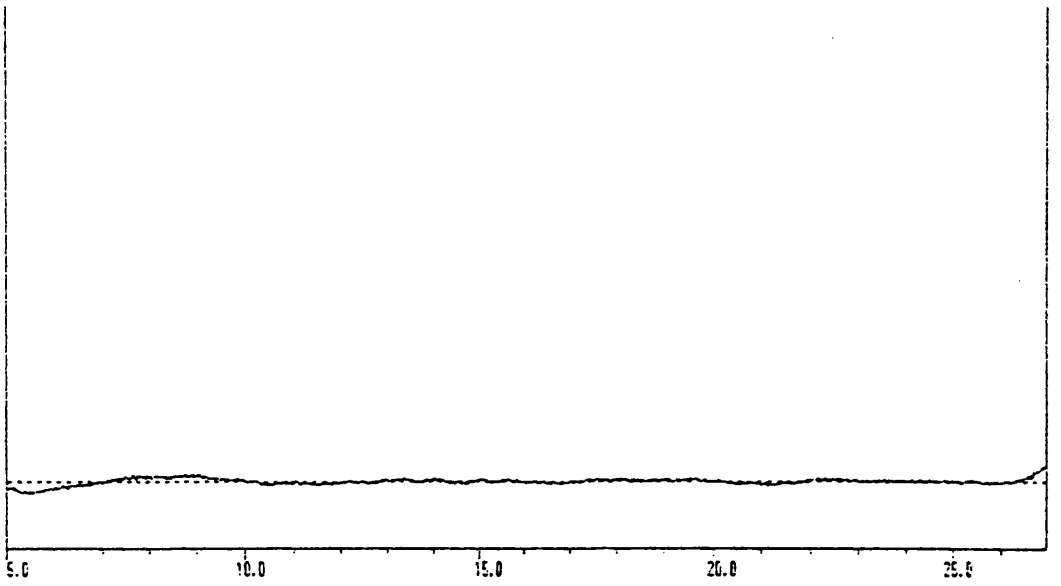
- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	:	Data collect time	: 0.0 to 29.0 min
Conversion cycle	:	Data interval	: 1.0 sec
Gradient	:	Inject volume	: 50 of 150 uL

BLANK [15 Jan 1992 9:45am] 0.0100 AU
Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	7.73		351	
	7.87		338	
	8.03		330	
	8.17		342	
	8.42		315	
	8.65		372	
	8.75		459	
	9.00		387	
	9.07		369	
	9.20		335	
	25.63		381	
	25.90		952	

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

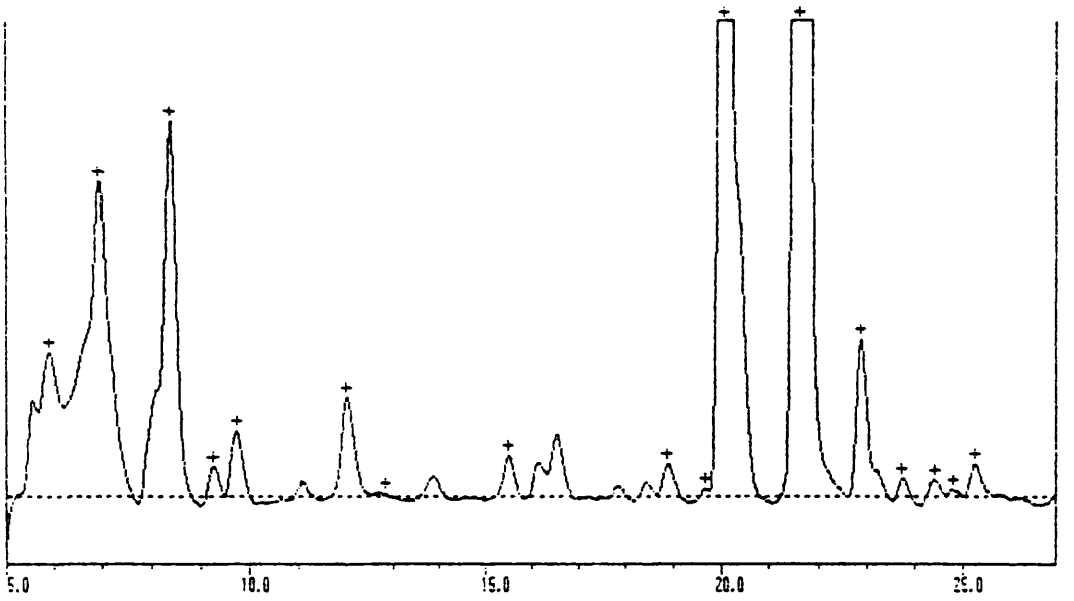
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 1 [15 Jan 1992 11:24am] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.57		5337		PRO	18.88	18.75	1612	7.55
ASN	5.90	6.00	7944	26.44	MET	19.65	19.52	462	1.74
SER	6.95	7.02	17385	113.36	VAL	20.03	19.90	82754	307.03
GLY	8.37	8.38	20757	113.43	DPT	21.62	21.50	169962	627.17
GLU	9.23	9.25	1850	6.01	TRP	22.88	22.73	8670	24.92
DMP	9.72	9.67	3636	40.60		23.20		1467	
	11.12		786		PHE	23.77	23.63	1035	4.00
ALA	12.03	12.02	5484	27.89	ILE	24.43	24.27	960	4.21
HIS	12.85	12.73	177	1.05	LYS	24.82	24.65	432	1.43
	13.90		1149		LEU	25.27	25.12	1794	7.61
TYR	15.50	15.40	2274	9.20					
	16.17		1851						
	16.53		3477						
	17.87		573						
	18.42		786						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

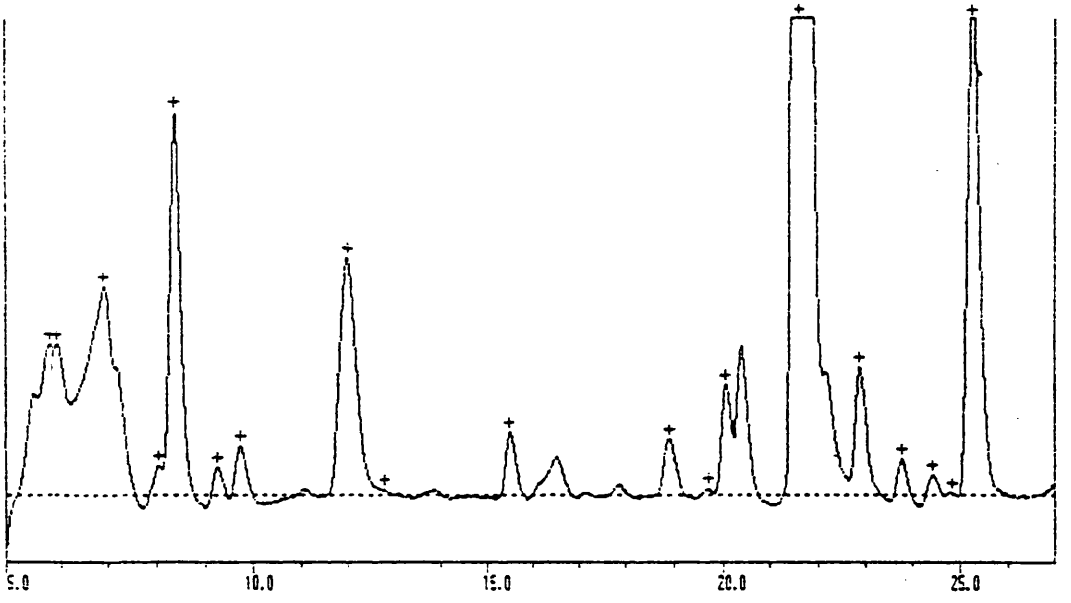
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 2 [15 Jan 1992 12:10pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.58		5586		MET	19.68	19.52	357	1.34
ASN	5.92	6.00	8373	27.87	VAL	20.05	19.90	6093	22.50
SER	6.92	7.02	11445	74.63		20.38		8206	
THR	8.03	7.88	1641	11.57	DPT	21.62	21.50	390219	1439.92
GLY	8.37	8.38	21050	115.09		22.17		6783	
GLU	9.33	9.25	1586	5.70	TRP	22.98	22.73	7038	19.99
DMP	9.73	9.67	2736	30.55	PHE	23.78	23.63	1983	7.67
	11.08		345		ILE	24.45	24.27	1083	4.75
	11.15		318		LYS	24.87	24.65	159	0.53
ALA	12.02	12.02	13083	55.05	LEU	25.27	25.12	29686	121.84
HIS	12.82	12.73	282	1.67					
TYR	15.52	15.40	3441	13.93					
	15.52		2070						
	17.85		609						
PRO	18.90	18.75	3141	13.63					

Tabulation threshold : 300 uAU

-- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

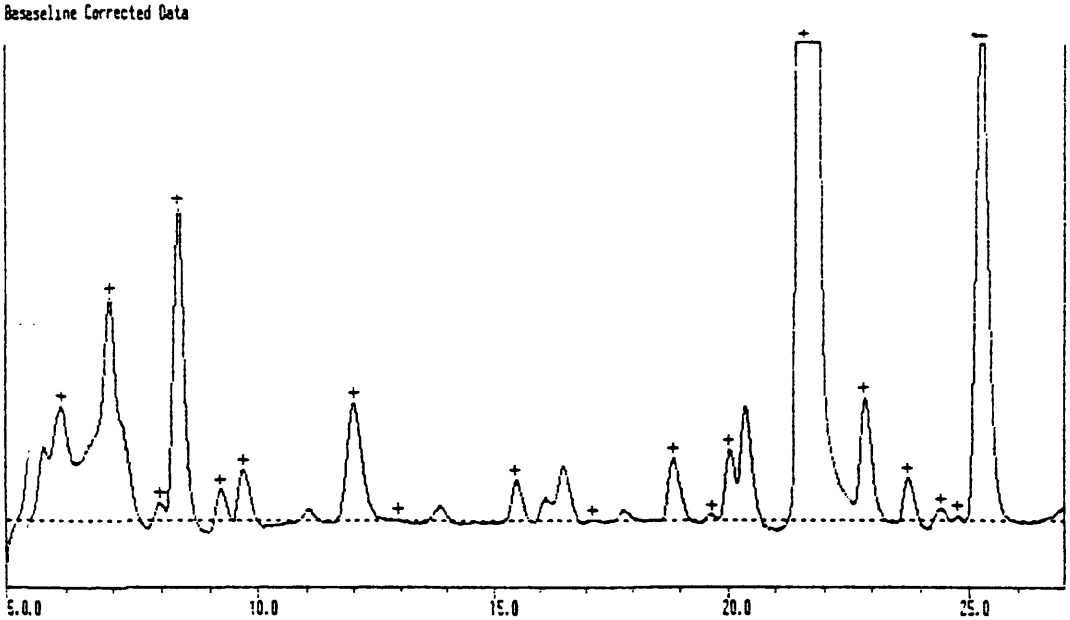
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMMINO ACID # 3 [15 Jan 1992 12:56pm] 0.0100 AU

Baseseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.53		4092		ARG	17.15	17.13	51	0.53
ASSN	5.90	6.00	6312	21.01		17.82		552	
	6.20		3240		PRO	18.87	18.75	3429	14.88
SSER	6.93	7.02	12135	79.13	MET	19.67	19.52	330	1.24
THHR	7.98	7.98	1026	7.30	VAL	20.02	19.90	3930	14.59
GULY	8.33	8.38	17214	94.07		20.37		6300	
GULU	9.22	9.25	1758	6.40	OPT	21.60	21.50	383931	1416.72
OMMP	9.70	9.67	2820	31.49	TRP	22.87	22.73	6786	19.27
	11.07		618		PHE	23.75	23.63	2391	9.25
ALLA	12.02	12.02	6531	32.98	ILE	24.43	24.27	675	2.99
HBIS	12.95	12.73	96	0.57	LYS	24.78	24.65	261	0.87
	13.88		804		LEU	25.25	25.12	28212	119.83
TYYR	15.48	15.40	2258	9.16					
	16.13		1248						
	16.52		2988						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

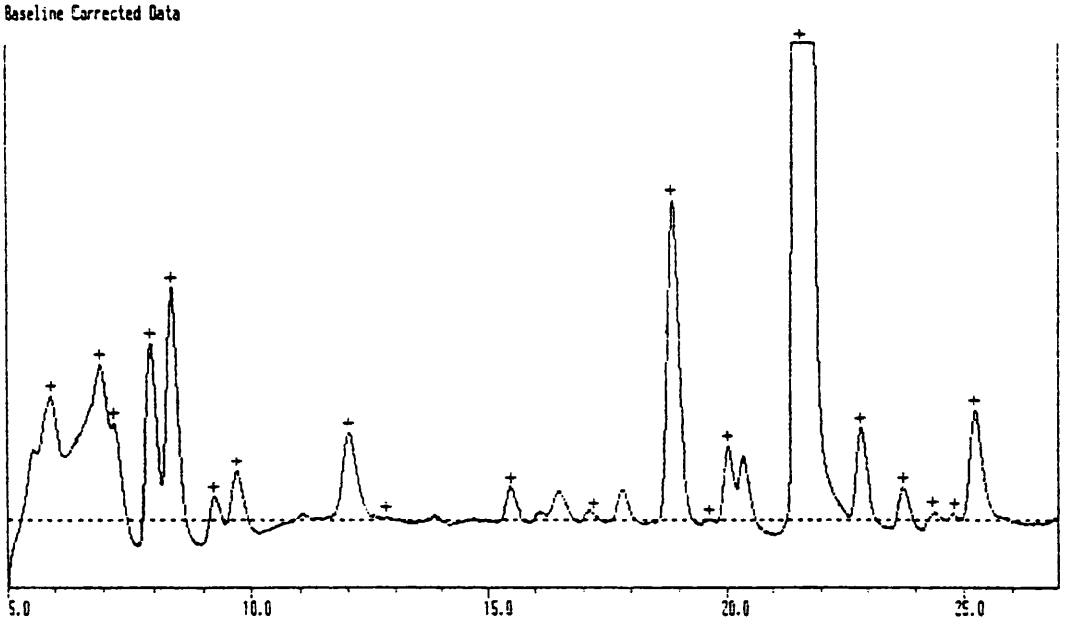
SAMPLE : 150192SA8.LQ
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 4 [15 Jan 1992 1:41pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.57		3906			17.13		558	
ASN	5.93	6.00	6888	22.93	ARG	17.22	17.13	420	4.40
SER	6.95	7.02	8513	58.16		17.83		1689	
GLN	7.22	7.35	5318	24.74	PRO	18.65	18.75	17604	76.41
THR	7.95	7.98	9790	69.58	MET	19.62	19.52	30	0.11
GLY	8.37	8.38	12985	70.41	VAL	20.02	19.90	4083	15.15
GLU	9.22	9.25	1322	4.82		20.35		3522	
DMP	9.70	9.67	2712	30.28	DPT	21.58	21.50	308829	1139.59
	11.08		389		TRP	22.87	22.73	5091	14.48
ALA	12.03	12.02	4636	24.42	PHE	23.75	23.63	1824	7.05
HIS	12.82	12.73	204	1.21	ILE	24.38	24.27	453	1.99
	13.67		306		LYS	24.80	24.65	393	1.30
TYR	15.50	15.40	1933	7.42	LEU	25.23	25.12	6096	25.89
	16.12		459						
	16.50		1635						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

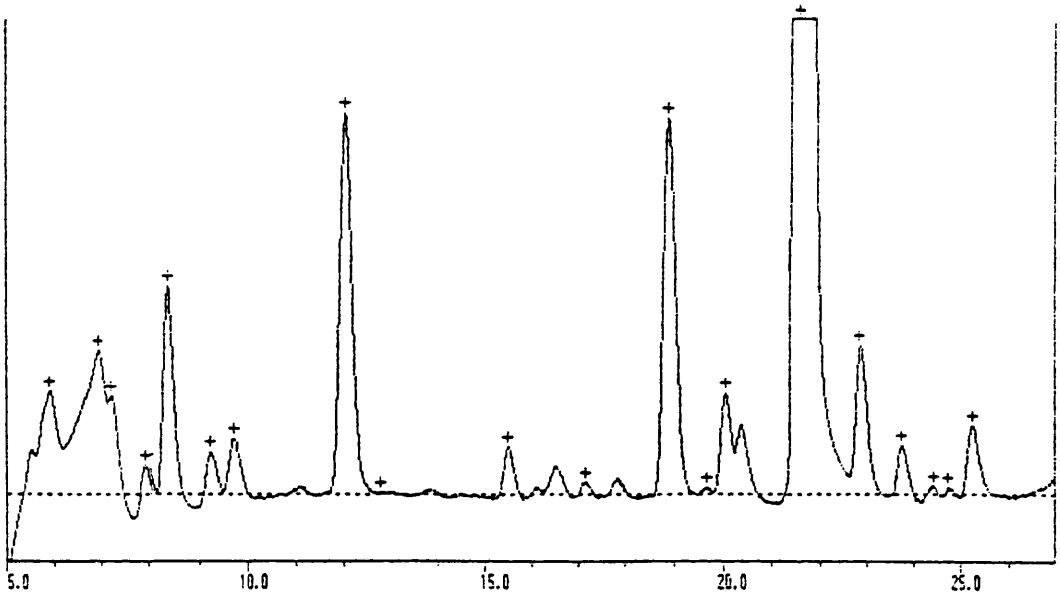
SAMPLE : 150192SAB.LD
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 5 [15 Jan 1992 2:27pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.55		2475		PRO	18.87	18.75	20992	89.77
ASN	5.92	6.00	5763	19.18	MET	19.65	19.52	351	1.32
SER	6.95	7.02	7995	52.13	VAL	20.03	19.90	5613	20.82
GLN	7.22	7.35	5442	25.33		20.35		3816	
THR	7.95	7.98	1647	11.72	DPT	21.60	21.50	461283	1702.15
GLY	8.35	8.38	11460	52.62	TRP	22.87	22.73	8241	23.40
GLU	9.22	9.25	2334	8.50	PHE	23.75	23.63	2745	10.61
DMP	9.68	9.67	3060	34.17	ILE	24.43	24.27	444	1.95
ALA	12.03	12.02	20985	105.96	LYS	24.75	24.65	275	1.24
HIS	12.80	12.73	156	0.92	LEU	25.25	25.12	3810	16.18
TYR	15.52	15.40	2567	10.79					
	15.08		381						
	16.52		1560						
ARG	17.15	17.13	660	6.92					
	17.83		915						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

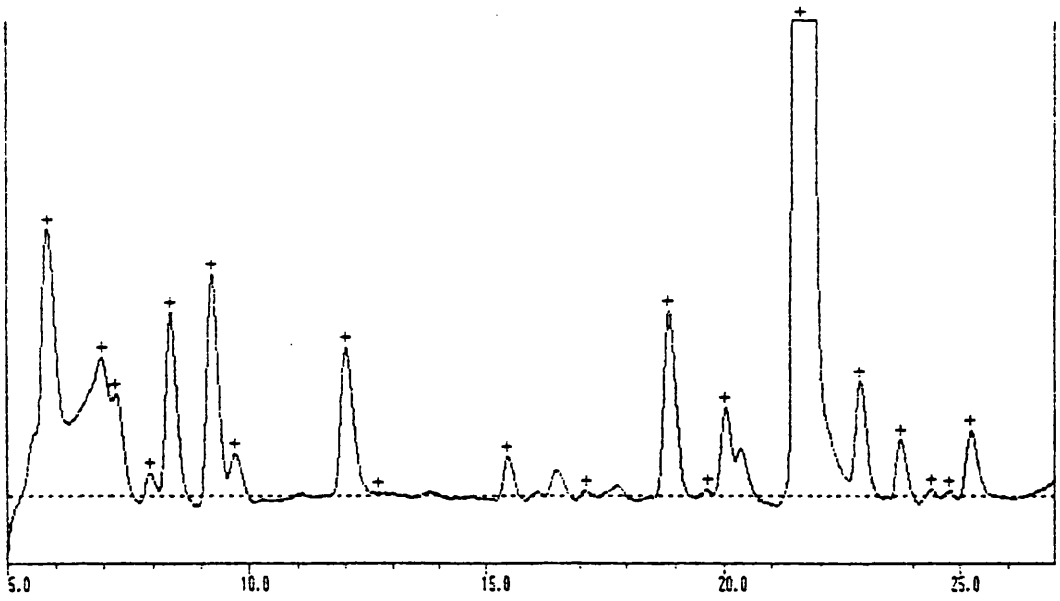
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 6 [15 Jan 1992 3:13pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASN	5.83	6.00	14685	48.88	MET	19.65	19.52	390	1.47
SER	6.97	7.02	7630	50.08	VAL	20.02	19.90	4897	18.13
GLN	7.23	7.35	5658	25.33		20.33		2629	
THR	7.95	7.98	1305	9.28	DPT	21.60	21.50	366618	1352.83
GLY	8.35	8.38	10143	55.43	TRP	22.97	22.73	5381	19.13
GLU	9.22	9.25	12240	44.57	PHE	23.75	23.63	3141	12.15
DMP	9.70	9.67	2358	26.33	ILE	24.42	24.27	429	1.89
ALA	12.03	12.02	8253	41.67	LYS	24.78	24.65	330	1.09
HIS	12.73	12.73	180	1.06	LEU	25.23	25.12	3639	15.46
TYR	15.48	15.40	2160	9.74		26.72		438	
	16.52		1419						
	17.08		309						
ARG	17.15	17.13	282	2.96					
	17.82		609						
PRO	18.85	18.75	10227	44.39					

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

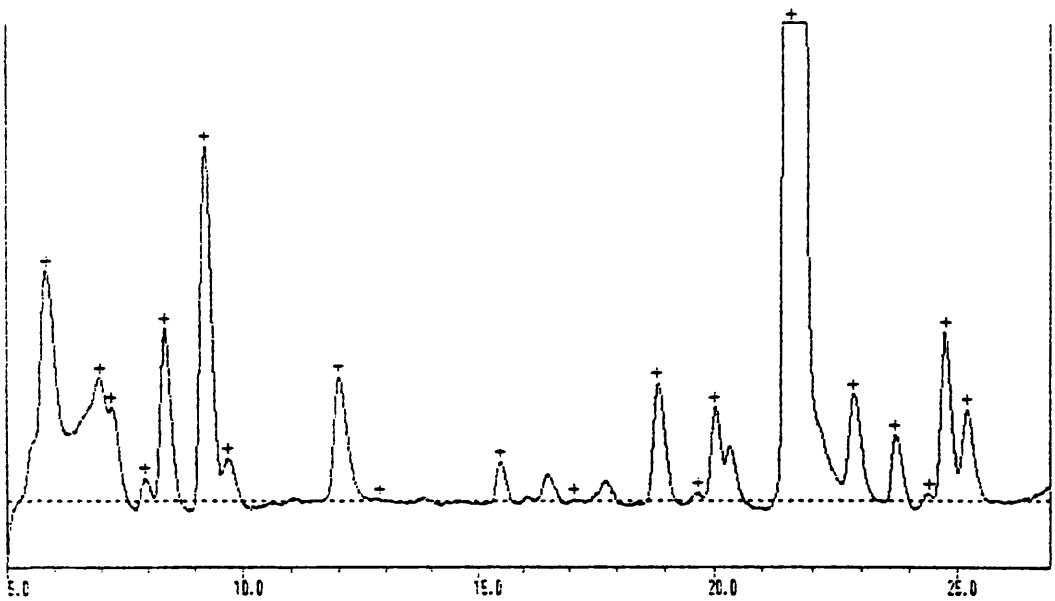
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 7 [15 Jan 1992 3:58pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASN	5.83	5.00	12569	42.17	VAL	20.02	19.90	5184	19.23
SER	6.97	7.02	6837	44.58		20.32		3033	
GLN	7.23	7.35	5220	24.29	DPT	21.58	21.50	313536	1156.96
THR	7.95	7.98	1351	8.90	TRP	22.87	22.73	5934	18.85
GLY	8.37	8.38	9510	51.97	PHE	23.73	23.63	3675	14.21
GLU	9.22	9.25	19551	71.16	ILE	24.43	24.27	390	1.71
DMP	9.68	9.67	2409	26.90	LYS	24.77	24.65	9372	31.08
ALA	12.03	12.02	6992	34.75	LEU	25.22	25.12	5073	21.55
HIS	12.88	12.73	162	0.96		26.63		363	
TYR	15.52	15.40	2154	8.72					
	16.50		1476						
ARG	17.10	17.13	111	1.15					
	17.77		1128						
PRO	18.85	18.75	6492	28.18					
MET	19.67	19.52	516	1.94					

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

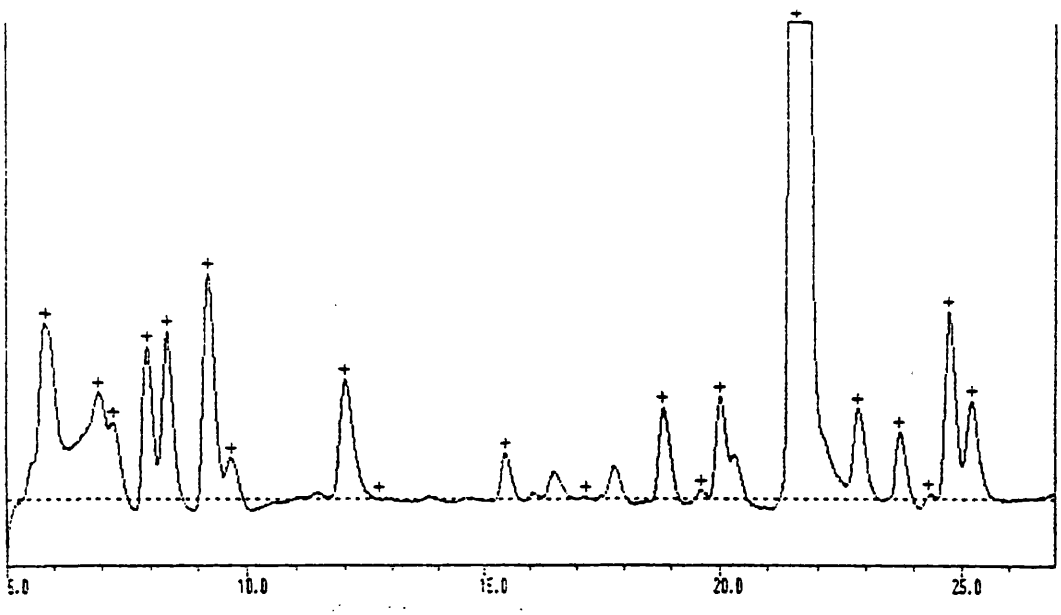
SAMPLE : 150192SAB.LQ
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 8 [15 Jan 1992 4:44pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASN	5.83	6.00	9570	31.86	PRO	18.85	18.75	5064	21.98
SER	6.93	7.02	5886	38.38	MET	19.62	19.52	483	1.81
GLN	7.25	7.35	4281	19.92	VAL	20.02	19.90	5640	20.92
THR	7.95	7.98	8399	59.66		20.32		2466	
GLY	8.35	8.38	9207	50.31	DPT	21.58	21.50	262452	968.46
GLU	9.22	9.25	12369	45.04	TRP	22.95	22.73	5034	14.29
DMP	9.68	9.67	2295	25.63	PHE	23.73	23.63	3726	14.41
	11.47		387		ILE	24.35	24.27	288	1.25
ALA	12.03	12.02	6585	33.25	LYS	24.75	24.65	10311	34.20
HIS	12.75	12.73	105	0.62	LEU	25.22	25.12	5430	23.06
TYR	15.50	15.40	2517	10.19		26.90		312	
	16.08		354			26.97		369	
	16.57		1464						
ARG	17.23	17.13	99	1.04					
	17.80		1809						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

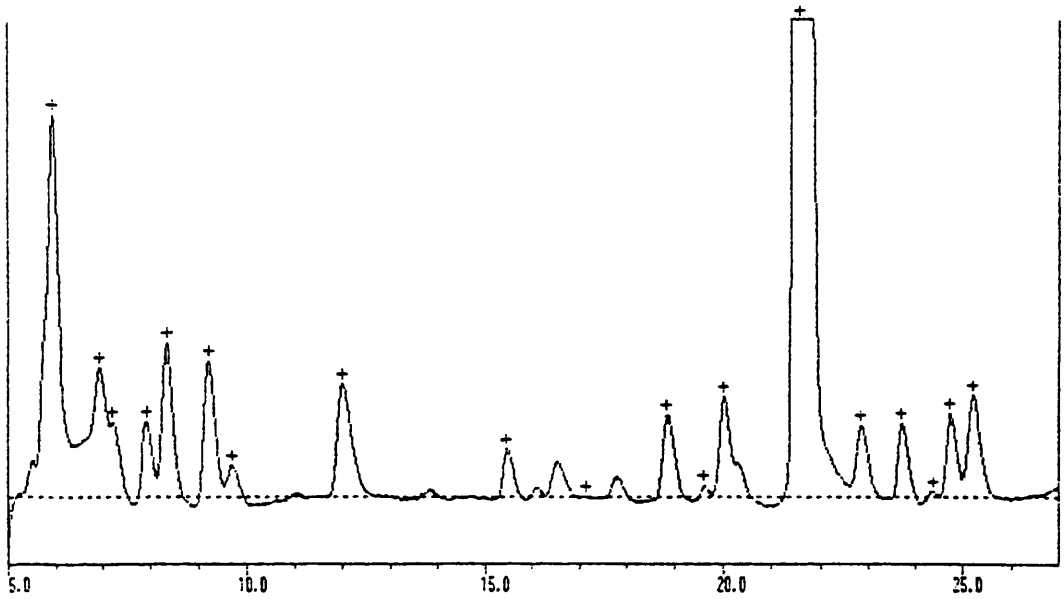
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 9 [15 Jan 1992 5:30pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.52		2037			16.55		1941	
ASN	5.95	6.00	21081	70.18	ARG	17.17	17.13	57	0.60
	6.43		2898			17.82		1104	
	6.50		2973		PRO	18.85	18.75	4566	19.82
SER	6.95	7.02	7191	46.69	MET	19.60	19.52	663	2.49
GLN	7.23	7.35	4167	19.39	VAL	20.02	19.90	5517	20.47
THR	7.95	7.98	4176	29.71		20.30		1993	
GLY	8.35	8.38	8493	46.41	DPT	21.58	21.50	202656	747.81
GLU	9.20	9.25	7536	27.44		22.58		735	
DMP	9.70	9.67	1728	19.29	TRP	22.67	22.73	3969	11.27
ALA	12.03	12.02	6276	31.69	PHE	23.73	23.63	4107	15.88
	13.88		390		ILE	24.40	24.27	334	1.42
TYR	15.48	15.40	2652	10.73	LYS	24.75	24.65	4620	15.32
	16.08		495		LEU	25.22	25.12	5843	23.97
	16.15		492						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

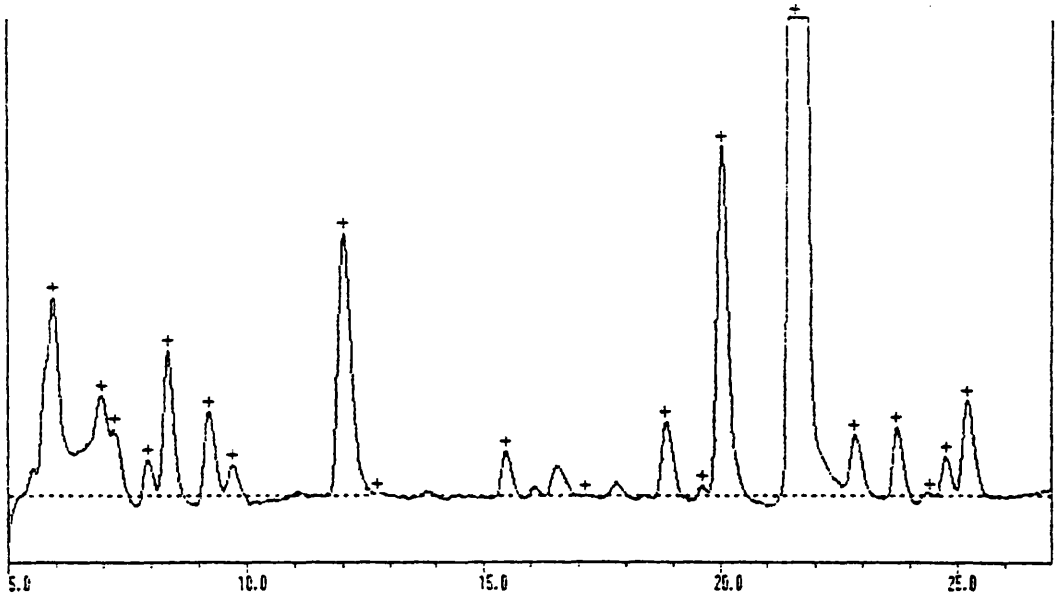
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 8:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 10 [15 Jan 1992 6:16pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.52		1539		PRO	18.85	18.75	4077	17.70
ASN	5.95	6.00	10974	35.53	MET	19.63	19.52	579	2.18
SER	6.97	7.02	5502	35.88	VAL	20.00	19.90	19293	71.57
GLN	7.35	7.35	3741	17.41	DPT	21.58	21.50	173850	641.51
THR	7.95	7.98	1980	14.05	TRP	22.87	22.73	3335	9.47
GLY	8.35	8.38	8094	44.23	PHE	23.73	23.63	3801	14.70
GLU	9.20	9.25	4644	15.91	ILE	24.45	24.27	150	0.66
DMP	9.68	9.67	1713	19.13	LYS	24.77	24.65	2205	7.31
ALA	12.03	12.02	14481	73.12	LEU	25.22	25.12	5289	22.46
HIS	12.77	12.73	150	0.89		26.85		303	
TYR	15.50	15.40	2484	10.05					
	16.07		455						
	16.58		1641						
ARG	17.15	17.13	54	0.57					
	17.82		783						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

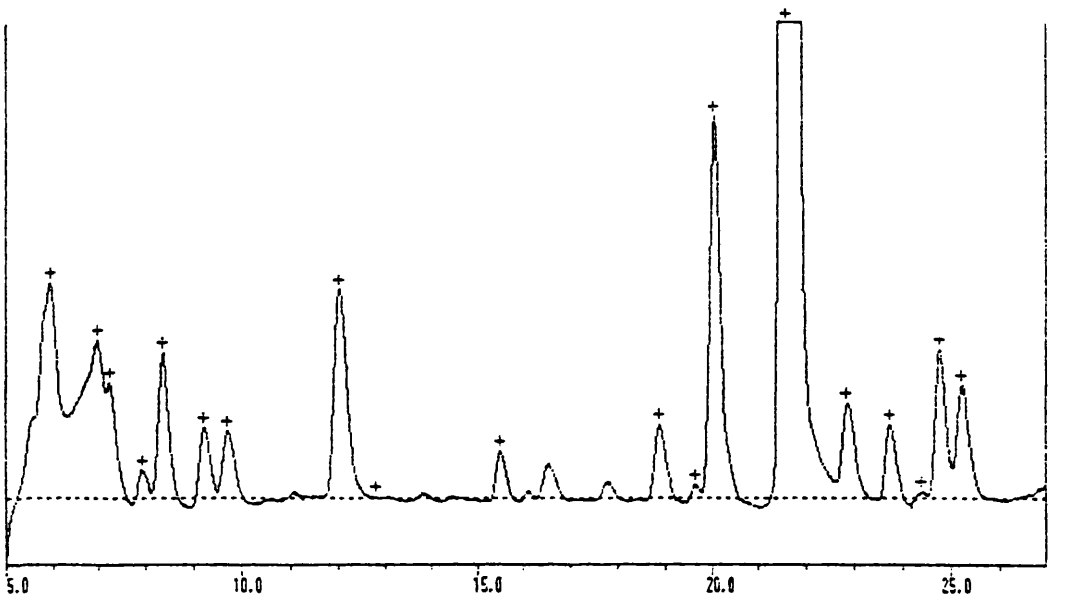
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 11 [15 Jan 1992 7:01pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.58		4494		PRO	18.87	18.75	4071	17.67
ASN	5.93	6.00	11880	39.55	MET	19.63	19.52	717	2.69
SER	6.97	7.02	8676	56.57	VAL	20.02	19.90	21057	78.12
GLN	7.25	7.35	6345	29.53	DPT	21.60	21.50	317979	1173.35
THR	7.95	7.98	1587	11.29	TRP	22.67	22.73	5271	14.97
GLY	8.37	8.36	8052	44.00	PHE	23.75	23.63	4123	15.94
GLU	9.22	9.25	3927	14.30	ILE	24.42	24.27	360	1.58
DMP	9.70	9.67	3771	42.11	LYS	24.78	24.65	8262	27.40
ALA	12.03	12.02	11523	58.19	LEU	25.23	25.12	8308	26.79
HIS	12.82	12.73	114	0.67					
TYR	15.50	15.40	2667	10.79					
	16.13		402						
	16.55		1881						
	17.78		921						
	17.85		861						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

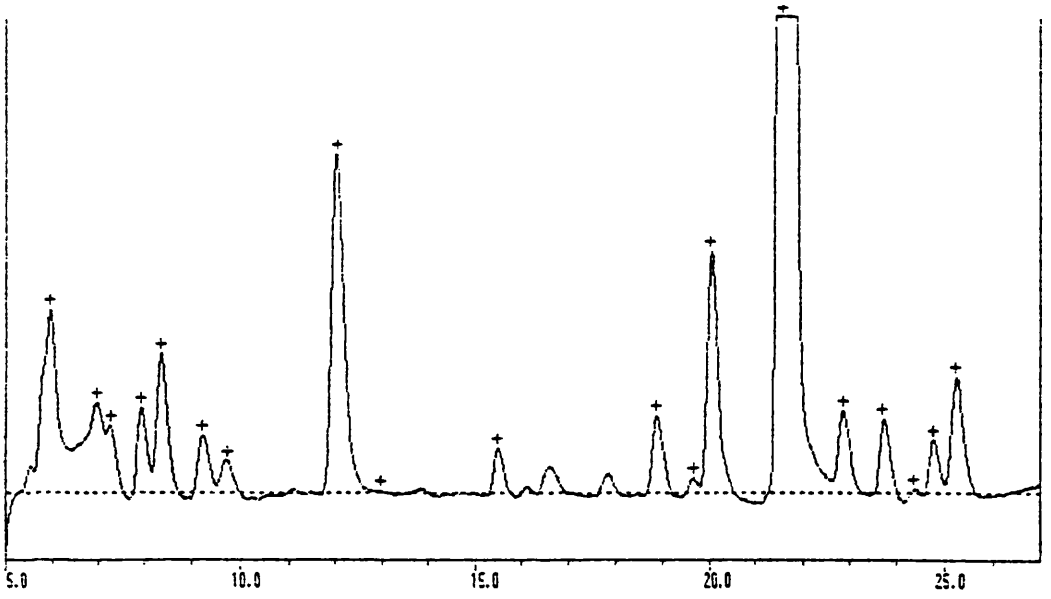
SAMPLE : 1S0192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 12 [15 Jan 1992 7:47pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.55		1479		MET	19.65	19.52	837	3.15
ASN	5.95	6.00	10182	33.89	VAL	20.02	19.90	13434	49.84
SER	6.98	7.02	4971	32.41	DPT	21.60	21.50	233520	861.70
GLN	7.28	7.35	3747	17.44	TRP	22.58	22.73	4542	12.90
THR	7.95	7.98	4695	33.40	PHE	23.73	23.63	4050	15.66
GLY	8.37	8.38	7746	42.33	ILE	24.38	24.27	204	0.89
GLU	9.22	9.25	3231	11.76	LYS	24.78	24.65	2913	9.66
DMP	9.73	9.67	1785	19.93	LEU	25.23	25.12	6420	27.27
ALA	12.05	12.02	16732	94.59		26.82		387	
HIS	13.00	12.73	99	0.59		27.00		606	
TYR	15.50	15.40	2508	10.15					
	16.13		387						
	16.58		1485						
	17.85		1074						
PRO	18.87	18.75	4266	18.52					

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

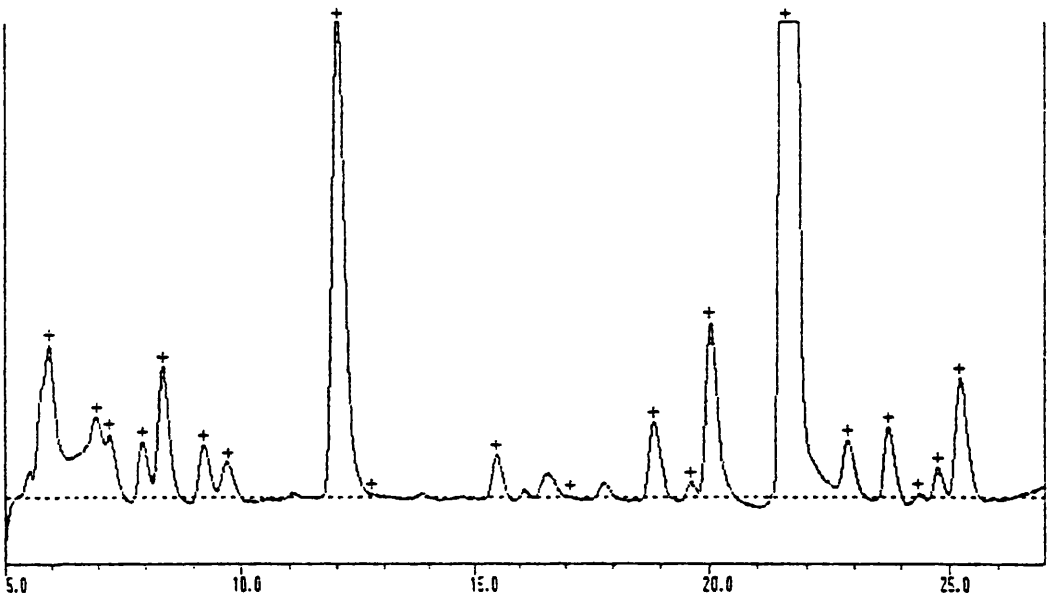
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 13 [15 Jan 1992 8:33pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.55		1464			17.82		816	
ASN	5.95	6.00	8406	27.98	PRO	18.87	18.75	4179	18.14
	6.52		2382		MET	19.63	19.52	819	3.08
SER	6.97	7.02	4467	29.13	VAL	20.02	19.90	9651	35.80
GLN	7.25	7.35	3537	16.45	DPT	21.60	21.50	157296	580.43
THR	7.93	7.98	3129	22.26	TRP	22.68	22.73	3162	8.98
GLY	8.35	8.38	7284	39.80	PHE	23.75	23.63	3924	15.17
GLU	9.22	9.25	2892	10.53	ILE	24.37	24.27	234	1.03
DMP	9.72	9.67	1908	21.30	LYS	24.78	24.65	1659	5.50
ALA	12.03	12.02	26907	135.87	LEU	25.23	25.12	6648	28.24
HIS	12.77	12.73	189	1.12					
TYR	15.50	15.40	2379	9.63					
	16.12		453						
	16.63		1341						
ARG	17.13	17.13	135	1.42					

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

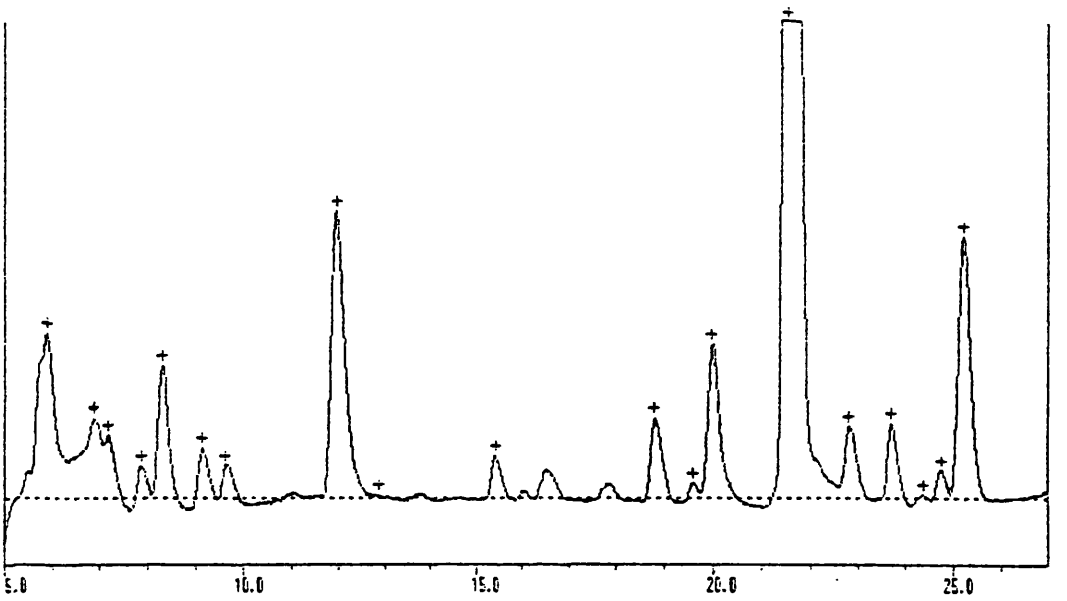
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 14 [15 Jan 1992 9:18pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.55		1491		PRO	18.82	18.75	4410	19.14
ASN	5.90	6.00	9021	30.03	MET	19.60	19.52	840	3.16
SER	6.92	7.02	4428	28.87	VAL	19.98	19.90	8532	31.85
GLN	7.23	7.35	3570	16.61	DPT	21.57	21.50	153876	567.81
THR	7.90	7.98	1800	12.61	TRP	22.83	22.73	3996	11.35
GLY	8.32	8.38	7338	40.10	PHE	23.72	23.63	4140	15.01
GLU	9.15	9.25	2805	10.21	ILE	24.40	24.27	246	1.08
DMP	9.63	9.67	1860	20.77	LYS	24.78	24.65	1602	5.31
	11.10		333		LEU	25.22	25.12	14493	61.56
ALA	11.98	12.02	15882	80.20		26.87		375	
HIS	12.90	12.73	168	0.99		27.00		432	
TYR	15.45	15.40	2379	9.63					
	16.07		372						
	16.58		1515						
	17.87		795						

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

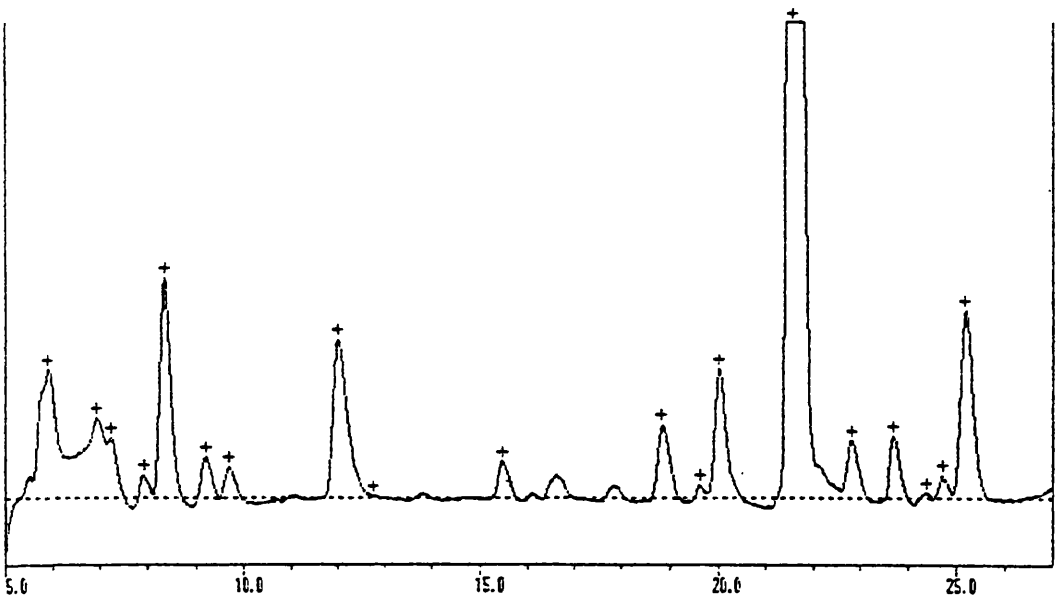
SAMPLE : 150192SAB.LO
[Initiated 15 Jan 1992 9:33am]

CYCLE SUMMARY :

Reaction cycle	: BLOTT	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 15 [15 Jan 1992 10:04pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 140192

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
	5.53		1245		MET	19.62	19.52	732	2.75
ASN	5.92	6.00	7101	23.64	VAL	20.00	19.90	7170	26.60
	6.40		2316		DPT	21.58	21.50	128439	473.94
SER	6.95	7.02	4419	28.61		22.45		804	
GLN	7.25	7.35	3411	15.87	TRP	22.87	22.73	3171	9.00
THR	7.93	7.98	1266	9.01	PHE	23.72	23.63	3450	13.34
GLY	8.35	8.38	12213	66.74	ILE	24.40	24.27	306	1.34
GLU	9.22	9.25	2295	8.36	LYS	24.73	24.65	1272	4.22
DMP	9.70	9.67	1728	19.29	LEU	25.20	25.12	10380	44.09
ALA	12.02	12.02	8769	44.28					
HIS	12.78	12.73	156	0.92					
TYR	15.50	15.40	2028	8.21					
	16.63		1272						
	17.90		630						
PRO	18.85	18.75	4071	17.67					

Tabulation threshold : 300 uAU

- Applied Biosystems 477A Protein Sequence Report -

SAMPLE : 150192SAB.LO
[initiated 15 Jan 1992 9:33am]

Sample Amount: 100 pmol

AAciid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
11	V	20.03	19.90	307.03	294.38	303.12	195.02	VAL
22	L	25.27	25.12	121.84	112.29	118.99	144.61	LEU
33	L	25.25	25.12	119.83	108.31	107.67	130.86	LEU
44	P	18.85	18.75	76.41	62.16	66.89	73.15	PRO
55	A	18.87	18.75	89.77	74.27	74.65	81.65	ALA
66	E	9.22	9.25	44.57	34.31	40.07	47.67	GLU
77	E	9.22	9.25	71.19	59.75	63.66	75.74	GLU
88	T	7.95	7.98	59.68	47.57	59.87	46.91	THR
99	N	5.95	6.00	70.18	38.60	44.60	21.97	ASN
100	V	20.00	19.90	71.57	48.22	62.55	40.24	VAL ALA
111	K	20.02	19.90	78.12	53.59	53.69	34.53	LYS
122	T	7.95	7.98	33.40	20.94	27.98	21.52	THR
133	A	12.03	12.02	135.87	95.04	105.83	33.20	ALA
144	L	25.22	25.12	61.56	31.38	43.86	53.31	LEU
155	G	8.35	8.38	66.74	19.48		7.15	GLY

REPEETITIVE YIELD ANALYSIS:

		Rep.Yield	Variance	
V:	1, 10, 11	83.32 %	0.980	:VAL
L:	2, 3, 14	89.68 %	0.997	:LEU
P:	4, 5	119.49 %	1.000	:PRO
E:	6, 7	174.15 %	1.000	:GLU
T:	8, 12	81.46 %	1.000	:THR

Average AA Repetitive Yield: 103.01 %

Combbined AA Repetitive Yield: 89.07 % 0.544

Theoretical Initial Yield: 142.82 pmol (142.82 %)