THE EFFECT OF PHENYLALANINE ANALOGUES ON THE TRANSPORT OF METABOLISM OF PHENYLALANINE: WITH SPECIAL REFERENCE TO THE POSSIBLE USE IN PHENYLKETONURIA

by

DAVID ROBIN LINES

A thesis submitted to the Faculty of Medicine in the University of Adelaide for the degree of Doctor of Medicine.

Department of Paediatrics,
University of Adelaide, 1984.

I hereby certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University; and that to the best of my knowledge and belief it does not contain any material previously published or written by another except where due reference is made in the text.

The developmental work in establishing the gut sac model was undertaken in conjunction with A.J. Bedworth and this is also included in his Honours Thesis "Inhibition of Phenylalanine Transport in the Everted Gut Sac" Flinders University. The candidate supervised Mr. Bedworth in this work but all the experiments described in this thesis are separate and conducted on a different rat species.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the Degree of Doctor of Medicine.

DAVID R. LINES.

ACKNOWLEDGEMENTS

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To my wife, Denise Anne, without whom this thesis would never have been written, and to my father, Edward Wolryche Low Lines who taught me "If you do an experiment and the result is as you expected, you have learnt nothing; if the result is unexpected then you have learnt something".

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SYNOPSIS

This thesis examines the possibility that the administration of analogues of phenylalanine such as β-2-thienylalanine, cycloleucine, parachlorophenylalanine or phenylalaninol might be used as treatment agents in phenylketonuria. Elevated serum phenylalanine appears to be the mechanism whereby the virtual absence of effective phenylalanine hydroxylase in classical phenylketonuria leads to brain damage. The reduction of these elevated levels by a strict low phenylalanine diet prevents the brain damage in phenylketonuria.

Because the analogues cited above could possibly reduce the absorption of phenylalanine (from ingested food) by the small intestine and the reabsorption of phenylalanine by the proximal renal tubule (from the glomerular filtrate) they could lower serum phenylalanine and hence be potential treatment agents for phenylketonuria.

The initial study examined the effect of intravenous β-2-thienylalanine on the urinary excretion of phenylalanine in Rhesus monkeys rendered hyperphenylalaninaemic by the simultaneous infusion of phenylalanine. It showed that β-2-thienylalanine doubled urinary excretion of phenylalanine.

The next study was conducted on Rhesus monkeys using cycloleucine and parachlorophenylalanine in a similar fashion but the monkeys were also infused with inulin so that glomerular filtration could be measured. This showed that both produced substantial increases in urinary excretion of phenylalanine by hyperphenylalaninaemic monkeys, but not by inhibiting renal tubular reabsorption of phenylalanine, rather, they increased glomerular filtration.

The effect of β-2-thienylalanine, cycloleucine and parachlorophenylalanine on the intestinal absorption of phenylalanine in Rhesus monkeys was examined and each analogue reduced absorption of phenylalanine.

Cycloleucine was known to be relatively non-toxic to humans and parachlorophenylalanine quite toxic to a range of experimental animals, but little is known about the toxicity of β-2-thienylalanine, so a long term feeding of β-2-thienylalanine was undertaken to infant monkeys, both with and without phenyl-alanine. With the possible exception of predisposing to Shigella enteric infection, no toxic effects of β-2-thienylalanine was found clinically or in the haematological indices studied.

The β-2-thienylalanine appeared to be partially effective in preventing the phenylketonuria symptoms that occurred in infant monkeys when phenylalanine alone was fed to them (3 grams/kg/day).

With no ill effect from β-2-thienylalanine noted, it was believed justified to give an acute load of it to phenyl-ketonuria patients and 20mg/kg/hr. was given intravenously in saline. This increased phenylalanine excretion by 2.25 fold when compared to saline alone. An oral load of phenylalanine 20mg/kg was found to reduce the absorption of phenylalanine from a 100mg/kg load by 47%.

Because of the possibility that cycloleucine might be successful in lowering serum phenylalanine levels, and the fact that it has been used as an anticancer therapy, it was given orally over one month in daily dose of 20mg/kg. It did not lower serum phenylalanine levels compared to a placebo capsule given for 1 control month.

As phenylketonuric man is the only known animal with no effective phenylalanine hydroxylase, it was decided to see if the variable phenylalanine levels and the fact that the clinical protection achieved by β-2-thienylalanine was only partial, may have been due to inhibition of phenylalanine hydroxylase - such inhibition would have increased serum phenylalanine thereby countering its beneficial effect on lowering serum phenylalanine by intestinal and renal mechanisms. This was done in vitro with rat phenylalanine hydroxylase and β-2-thienylalanine was found to inhibit radioactive phenylalanine to tyrosine conversion, but not as powerfully as parachlorophenylalanine. Cycloleucine was not an inhibitor of phenylalanine hydroxylase at all. The ability of the analogues to inhibit phenylalanine hydroxylase in vivo was measured by their impairment of the conversion of intraperitoneally injected universally ^{14}C labelled phenylalanine to ^{14}C carbon dioxide; by this method parachlorophenylalanine was found to be an effective inhibitor of phenylalanine hydroxylase while neither cycloleucine nor β-2-thienylalanine were.

An unresolved problem was how much increased glomerular filtration rate accounted for the increased phenylalanine excretion in the face of β-2-thienylalanine and whether the mechanism whereby any stimulation of glomerular filtration rate occurred might be potentially harmful to phenylketonuria patients. As the monkey was no longer available to the candidate, he examined cardiac and renal parameters in the anaesthetized dog. It was found that glomerular filtration rate was stimulated but only over the first observation period of 30 minutes, and that this was accompanied by a rise in renal plasma flow but no change in cardiac output, cardiac work nor change in peripheral or pulmonary resistance. It was concluded that such stimulation of glomerular filtration rate would not pose a threat to the patient. No inhibition of renal reabsorption of phenylalanine was demonstrable in these studies.

Because of conflicting results of the effect of β-2-thienylalanine on renal reabsorption of phenylalanine in different species (monkey, man and dog) and under difficult experimental conditions, it was decided to examine its effect on an

isolated system.   The isolated brush border vesicles from the
proximal tubule of the rat kidney was chosen.   It was found that
β-2-thienylalanine and cycloleucine were inhibitors in this system
but a new analogue, phenylalaninol, was not.

All previous studies described in this synopsis which
had examined the effects of intestinal absorption had really
measured   *effective absorption*   of phenylalanine because they
used the whole animal and looked at rises of blood phenylalanine
level.   Thus, the effects that the absorbed analogue might have
had on renal excretion or hepatic (or other) metabolism of phenyl-
alanine were included in this measurement.   To examine more
critically the role of the small intestine, the everted gut sac
of the rat was used.   Although more physiological than isolated
brush borders (which can be prepared from the intestine), it
was a system to which Michaelis-Menton kinetics could be applied.
It was found that cycloleucine was the best inhibitor of phenyl-
alanine uptake, β-2-thienylalanine did inhibit but least while
phenylalaninol was intermediate in its ability to reduce phenyl-
alanine transport into the gut wall.

As a final check on the toxicity or otherwise of
β-2-thienylalanine, human fibroblast cultures were exposed to
it alone or in combination with phenylalanine, and the growth
of the fibroblasts compared to those exposed to phenylalanine
alone or control cells to which no amino acids were added.
β-2-thienylalanine did not appear to be toxic unless given in
extremely high doses and at those doses phenylalanine appeared
to be just as toxic.

The general discussion starts with a review of other
authors' work on the toxicity of β-2-thienylalanine and the concerns
that have caused ethical committees not to allow the candidate
to use β-2-thienylalanine in a medium term experiment in phenyl-
ketonuric patients.

The work of K.J. Brown in showing that β-2-thienylalanine
does not favour infection of the gut with Shigella or other bacterial
infection is discussed in detail.

The anomalous results of the effectiveness of cyclo-leucine in some systems but not in long term experiments are examined in detail, as are similar disparities in the results with phenylalaninol. Parachlorophenylalanine's toxicity, as demonstrated by others, is detailed.

Future approaches in the same area of non-dietary treatment of phenylketonuria are then discussed.

CHAPTER I

HISTORICAL INTRODUCTION TO THE THESIS

Phenylketonuria is a disease in which modern medicine and biochemistry has been highly successful. In the 50 years since its original description by Fölling (1), the essential nature of the disease as a total deficiency of effective phenylalanine hydroxylase inherited as a single autosomal recessive gene, has been established by Jervis. (2, 3) In addition, a method of detecting the biochemical disease before the occurrence of mental retardation, by screening newborn populations, has been devised by Guthrie (4, 5). Finally, a low phenylalanine diet has been shown to be of benefit to children suffering with phenylketonuria by Bickel, Gerrard and Hickmans. (6)

The efficacy of the low phenylalanine diet has been challenged by Birch and Tizard (7) because in treated retarded phenylketonuria, children had not made exceptional gains in Intelligence Quotient (IQ). They also claimed that sampling could account for the inverse relationship between pre-treatment IQ and age, and that the length of treatment was not related to the size of IQ increment. Bessman (8) pointed out that diseases are usually recognised in their most serious form, then more subtle manifestations are observed. Eventually mild forms of the disease are found to outnumber the serious ones. He argued that many of the apparent 'cures' by low phenylalanine diet are really the result of increased efficiency in identification of milder examples of phenylketonuria.

Despite these objections, most investigators treating phenylketonuria believe the low phenylalanine diet to be effective, e.g. Berman, Waisman and Graham (9,10); Coates (11); Dobson, Koch, Williamson, Spector, Frankenburg, O'Flynn, Warner and

Hudson (12); Hudson, Mordaunt and Leahy (13); Kang, Kennedy Jr., Gates, Burwash and McKinnon (14); Knox (15); Koch, Shaw, Acosta, Fishler, Schaeffler, Wenz and Wohlers (16); McBean and Stephenson (17); O'Flynn and Hsia (18), Sutherland, Umbarger and Berry (19); and Woolf, Griffiths and Moncrieff (20).

As to Bessman's point (8) that the treatment of many of the biochemically discovered children with phenylketonuria was unnecessary because they would have a milder form of the disease, two factors have arisen. The first is the discovery of a group of children who have elevated plasma phenylalanine levels, but these levels do not normally exceed 20 mg. per 100 millilitres on a normal diet. The cause or causes, definition and necessity for treatment of this condition are by no means agreed upon, but the subject is well reviewed by Carpenter, Auerbach and DiGeorge (21). Many investigators agree that this condition need not be treated, as mental development will probably be normal; Berman and Ford (22); Berman, Cunningham, Day, Ford and Hsia (23); Carpenter, Auerbach and DiGeorge (21); Hsia, O'Flynn and Berman (24); Levy, Shih, Karolkewicz, French, Carr, Cass, Kennedy Jr., and MacCready (25); Szeinberg, Cohen, Golan, Peled, Lavi and Crispin (26); Yu, Stuckey and O'Halloran (27). To avoid unnecessarily treating these patients with hyperphenylalaninaemia it is usual to leave the newborn child who is suspected to have phenylketonuria, on a normal diet until the plasma phenylalanine exceeds at least 20 mg. per 100 millilitres. Later in the first year of life, infants being treated for phenylketonuria with a low phenylalanine diet are returned to a diet of normal phenylalanine content to ensure that the plasma phenylalanine again exceeds this value.

The second factor which bears upon Bessman's (8) objection to dietary therapy for phenylketonuria is the discovery of patients with classical phenylketonuria (plasma phenylalanine

persistently above 20 mg. per 100 millilitres on a normal diet), who have normal intelligence. A number of such patients have been described (Hsia, O'Flynn and Berman (24); Knox (15); Perry et al, (28) and Pitt (29)). However, such cases are believed to be rare, Brown and Waisman (30).

Thus, while one cannot but agree with Birch and Tizard (7) that "...there is still need for an adequately designed controlled clinical trial to assess the effectiveness of the management of phenylketonuric children by a low-phenylalanine diet", such a controlled trial would no longer be considered ethical.

Hudson, Mordaunt and Leahy (13) summarised the view of most researchers in 1970:

> "Proof that early treatment with a low phenylalanine diet is necessary to prevent mental retardation developing in infants who show the biochemical characteristics of PKU could only be obtained by a random controlled trial. In the light of recent clinical experience, as reviewed in this article, such a trial would be unethical. Certainly, there are a few subjects, with an I.Q. of 70 or higher and the classical biochemical changes of PKU, who have never received treatment, but the number reported is very small. It is unlikely that many exist undetected in the general population, for estimations of the serum phenylalanine in 83,000 samples submitted to Massachusetts Public Health Department for a serological test for syphilis revealed only one case of PKU in an individual thought to be retarded".

As regards the difficulty of administering such a diet, there is little argument. To quote from Keleske, Solomons and Opitz (31):

> "...that the efforts to impose dietary restriction of phenylalanine intake affect the emotional climate of the total family; that the parents need constant support, orientation and counselling to enable them to maintain a realistic attitude towards their child with PKU; and that termination of the dietary restriction frequently improves the emotional climate of the family".

These findings were confirmed by Wood, Friedman and Steisel (32) who not only noted the disturbances of psycho-social climate in families with phenylketonuric children, but also commented: "Our data also suggested that learning in PKU children was severely curtailed by prohibitions against free explorations and play, and the opportunity to make meaningful discriminations."

Thus, an alternative approach to the treatment of phenylketonuria would be of value. Such an approach was suggested by the bacterial inhibition assay designed by Guthrie and Susi (4). In this, β-2-Thienylalanine prevents the growth of a bacteria bacillus subtilis except in the presence of excess phenylalanine. Although the exact basis of this impairment of bacterial growth has not been elucidated, the reversal of the inhibition by phenylalanine would suggest that β-2-Thienyl-alanine competitively inhibits bacterial transport and/or metabolism of phenylalanine.

If β-2-Thienylalanine or any other phenylalanine analogue could partially inhibit phenylalanine transport across the renal tubular cell, urinary phenylalanine excretion would be promoted, and hence a greater phenylalanine intake could be allowed to the phenylketonuric child. Likewise, if intestinal absorption of phenylalanine was blocked by one of its analogues, greater dietary phenylalanine would be permissible.

This thesis is concerned with the effect of phenyl-alanine analgoues on the transport and metabolism of phenylalanine to determine whether they might be useful in reducing the severity of dietary phenylalanine restriction in children with phenyl-ketonuria.

CHAPTER II

METHODS

Three phenylalanine analogues were chosen for study:

(1) β-2-Thienylalanine - this compound was first synthesised in 1938 by Barger and Easson (33). It is chemically designated α amino thiopheneproprionic acid and its chemical structure is shown in Figure 1.

(2) Parachlorophenylalanine - this compound was first synthesised in 1893 by Emil Erlenmeyer Jnr. (34). The chemical structure is shown in Figure 1.

(3) Cycloleucine - this compound was first synthesised in 1906 by Zelinsky and Stadnikoff (35). The chemical structure is shown in Figure 1.

The rationale for choosing these three compounds was as follows:

(1) β-2-Thienylalanine. This synthetic amino acid is known to inhibit phenylalanine metabolism and/or transport in bacteria. The inhibition is competitively reversed by excess phenylalanine. If such competition is at the cellular transport level, and is also found in man, it might effectively block renal tubular reabsorption of phenylalanine, thereby promoting urinary excretion of phenylalanine. A similar effect on intestinal transport would reduce intestinal absorption of phenylalanine. Both these effects would lower plasma phenylalanine levels.

(2) Parachlorophenylalanine. This compound was chosen because it has already been shown to have an effect on phenylalanine metabolism (36, 37, 38, 39, 40). Thus, further investigation of possible transport effects as outlined in the preceding paragraph on β-2-Thienylalanine were warranted. Toxic side effects of this compound have been reported in the rat, monkey and cat (40, 41, 42, 43, 44, 45), but it was decided to ascertain if this compound would have an effect in primates at a dose level less than that which produced toxic levels in other species.

Figure 1
Structure of Phenylalanine and Analogues

PHENYLALANINE

β - 2 - THIENYLALANINE

PARACHLOROPHENYLALANINE

CYCLOLEUCINE

(3) <u>Cycloleucine</u>. As can be seen in Figure 1, the chemical structure of this compound least resembles phenylalanine among the three analogues chosen. However, Oxender and Christensen (46) have shown that it inhibits both the '<i>leucine</i> preferring' and '<i>alanine</i> preferring' systems in the pigeon red blood cell. As phenylalanine uses both these transport systems, cycloleucine might well inhibit phenylalanine transport. Both Goyer et al (47) and Brown (48) have shown that cycloleucine causes an aminoaciduria, including phenylalanine, in rats and man. Finally, cycloleucine has been used in man as an anticancer therapy without serious side-effects, whereas the preceding two compounds had not been given to humans, before these experiments.

The study was designed to evaluate the effects on phenylalanine transport and metabolism in the following manner:

(a) Renal Studies –   (i) Rhesus monkey;
                       (ii) Dog;
                       (iii) Man.

(b) Intestinal Studies – (i) Rhesus monkey;
                         (ii) Man.

(c) Phenylalanine hydroxylase studies – rat and pig <i>in vitro</i>;
                                        rat <i>in vivo</i>.

(d) Cell Growth Studies – Human tissue.

(e) Safety Studies –   (i) Infant Rhesus monkey;
                       (ii) Acute loading studies in man;
                       (iii) Short (2 week) studies in man.

<u>ASSAY METHODS</u>

(1) <u>Amino Acids</u>. All determinations of synthetic amino acids were made by use of an amino acid analyser by the method of Spackman, Stein and Moore (49). Standard molarities of these compounds were run together with the naturally occurring amino acids each time a new batch of ninhydrin was made up.

The synthetic amino acids separate as distinct peaks by this method. A minimum of five, and usually seven, runs were used to calculate the ratio peak area to amino acid quantity. These results were quite reproducible - indeed, β-2-Thienylalanine has been suggested as an internal standard for amino acid analysis.

Preparation of serum and urine for amino acid analysis was performed by the method of Gerritsen, Reckberg and Waisman (50).

The concentration of phenylalanine in samples was measured by amino acid analysis when this was used for measuring the synthetic amino acids. At times, amino acid analysis was not available, and phenylalanine was determined by the spectrofluorometric method of McCamman and Robins (51) as modified by Wong et al. (52)

The synthetic amino acids did not interfere or contribute to this or any other assay procedure used. There was a high degree of correlation between the two methods of phenylalanine determination: the degree of variation always being less than 10% and usually less than 5%.

(2) Phenylalanine Hydroxylase. Rat or pig liver was used for studies of this enzyme. Each study was undertaken using one liver from one animal which was immediately homogenized with 0.1 Tris HCl buffer pH 7.4, 6 millilitres per 2g. of liver, using an ice water jacket.

The homogenate was centrifuged at 10,000 G for 30 minutes. The supernatant was sieved through wire gauze (0.5 mm^2) to remove lipids. The precipitate was resuspended in 2 ml. of the same buffer for each initial 2g. of liver and rehomogenized and centrifuged in the same fashion.

The pooled supernatants were then assayed in the following manner:

100 µl of supernatant

+ 100 µl of phenylalanine solution (2 µ moles/100 µl cold phenylalanine, containing 0.0014 µ moles of ^{14}C phenylalanine (universally labelled 0.25 µcuries).

+ 100 µl (pterine cofactor) 6-7 dimethyl-5,6,7,8 tetrahydro hydrochloride (concentration 0.25 µM/100 µl)

+ 100 µl dithiothreitol (1 µM/100 µl)

+ 100 µl Tris buffer pH 7.4 (100 µM/100 µl)

Thoroughly shaken and incubatd for 30 mins. at 37° and reaction stopped by 100 µl of Trichloracetic acid 30 g/100 ml.

After centrifugation at 1800 G for 10 minutes, 50 µl of the supernatant was spotted onto Whatman 3 mm. chromatography paper and allowed to dry.

The spot was then overlaid with cold tyrosine 15 µl of 60 µg/100 ml. Two samples, one containing 60 µg/100 ml. of phenylalanine and the other 60 µg/100 ml. tyrosine were applied to the paper for reference.

The paper was submitted to 16 hours of ascending chromatography using a solvent, Butanol:Acetic:water 6 : 1.5 : 2.5). After drying, the paper was sprayed with 0.2 g/100 ml. ninhydrin in acetone with 2 drops of pyridine. Colour development was obtained in an oven of 40°C for 30 minutes. Strips of paper were cut in line corresponding to the tyrosine spots and the radioactive tyrosine estimated by placing the paper in 10 mls. toluene scintillation solution (6g. of 2,5-diphenylonazole and 100 mgm. dimethyl POPOP/litre toluene). Preliminary studies showed that elution of tyrosine from paper with this solution was negligible in 24 hours, and all samples were counted within 2 hours.

The ß radiation was counted in a Packard liquid scintillation spectrometer for a period of 5 minutes, using internal standard for determination of quenching and using scintillation solution as background. The counts per minute were converted to disintegrations per minute using an efficiency curve constructed with known quench samples.

Thus, the percentage conversion of phenylalanine to tyrosine was obtained.

PHENYLALANINE METABOLISM *IN VIVO*

To determine the effect of analogues on metabolism of phenylalanine *in vivo*, rats were injected intraperitoneally with universally labelled C^{14} phenylalanine and the radioactive carbon dioxide measured. Adult, male Norwegian hooded rats weighing 260 to 350 grammes were fasted overnight but were allowed water *ad libitum*. After injection of labelled phenyl-alanine, 30 µg/kg. with or without the analogues in physiological saline, the rats were immediately placed in a flask. An air flow of 6 litres per minute drew their expired gases into sulphuric acid and thence to Driite. The dried air containing the rats' expired carbon dioxide was then sucked into a vibrating reed electrometer.

The electrical potential between the inner and outer surfaces of the sphere created by the radioactive carbon dioxide was measured over five minutes and recorded as a deflection on a continuous record. A pilot study revealed that 90% of the radioactivity injected was expired in the first hour, so this period was used to monitor the metabolism of phenylalanine. The carbon dioxide radioactivity was calculated by measuring the total deflection above baseline and comparing this with the standard curve. The sensitivity of the vibrating reed was checked daily by use of a cobalt source at a set distance from the sphere.

CELLULAR GROWTH STUDIES

Human fibroblasts from the skin of the one healthy child which had been maintained in subculture for 9 months were used in all experiments. All cultures were set up and media changes were all performed in an ultraviolet hood which was cleaned with 70% alcohol before each procedure. All pipette ends were flamed when opened and 90% of their length was flamed immediately prior to use. Before transfers or counting, the cultures were checked microscopically to ensure viability and that no bacterial contamination had occurred, and then the medium was sucked out using a new flamed pipette for each flask.

The medium used was minimal essential medium supplemented with
5% fetal calf serum, 5% neonatal calf serum, pyruvate 110mg
and NaHCO$_3$ 2.24 g.

After the medium was removed Versene solution was
added, the bottle shaken and then the flasks lain so that the
cells were bottom-most and covered with Versene. Then 7 mls.
of Trypsin solution added to subculture bottle and allowed
to interact with cells for 4 to 5 minutes at which time a micro-
scopic check was undertaken to ensure that separation of cells
had occurred. Such separation always occurred by 6 minutes.
The fluid containing the cells was then either counted or trans-
ferred to a petri dish for refeeding.

To count the cells the fluid containing them was
sucked up and transferred to a measuring tube and the volume
made up to a convenient figure, e.g. 10 ml. with phosphate
buffered saline. 1.0 ml. of this solution was then counted
in a Coulter counter Model ZF and the number of cells per plate
calculated.

To start an experiment approximately 250,000 cells
were added to 10 ml. of medium in a 60 mm. petri dish gently
swirled and placed in a 5% CO$_2$ incubator. After 48 hours,
4 plates were taken for counting and remaining plates used
for the experiment. It was assumed that the mean cell count
for the experimental plates would be the same as that of the
mean for the four that were counted. The number of experimental
plates required was calculated from the length of the intended
experiment and the number of experimental conditions required,
plus some extra plates to allow for possible growth failure.
As an example, in the first study the following petri dishes
were set up initially:

26 control plates containing 2 ml. of culture medium only.

20 plates with 2 ml. of medium plus 0.4 ml. of 500 mg%
β-2-thienylalanine in water added.

20 plates with 2 ml. of medium plus 0.2 ml. of 500 mg%
β-2-thienylalanine in water added.

20 plates with 2 ml. of medium plus 0.1 ml. of 500 mg%
β-2-thienylalanine in water added.

Later, phenylalanine or physiological saline or various com-
binations of phenylalanine and β-2-thienylalanine were added
to the medium.

The day following the experiment, after microscopic examination, 3 plates from each class were trypsinated as above and counted. Plates were then counted daily to see what effect the various additions had upon growth of the fibroblasts.   The cells were re-fed with 2.0 ml. of new medium with or without experimental additions every 48 hours.

BRUSH BORDER TRANSPORT STUDIES

These were conducted on the kidneys of 6 adult albino rats. The brush border vesicles were prepared by the method of Booth and Kenny (53) with the exception that magnesium chloride was used instead of calcium chloride. Transport of phenylalanine with and without analogues was measured using universally labelled ^{14}C phenylalanine by the method of Evers, Murer and Kinne (54).

Radioactive D-[^{3}H]-glucose was used to check that transport was not non-specifically inhibited, and both sodium and potassium runs were undertaken to ensure that the transport was active. Incubations were conducted for zero seconds to measure non-specific binding, and at 30 seconds, which a time course showed to be before the peak of the sodium facilitated transport.

Vesicles were separated by use of a Millipore filter using 0.22 μM Millipore filter paper, and the radioactivity counted in a Kontron MR300 automatic liquid scintillating system, using automatic adjustment for quenching and calculation of disintegrations per minute. The scintillation fluid used was Butyl-PPD 1% in toluene. The disintegrations per minute were adjusted to moles of phenylalanine and glucose transported. The brush border preparation had aliquots removed for protein analysis by the Lowry method (55). In pilot studies the enzymes alkaline phosphatase and ATPase were measured and showed that alkaline phosphatase was concentrated several fold in the presumed brush border component, while that of Na/K dependent ATPase was only marginally or not at all increased, indicating that partial purification and concentration of brush borders had been obtained while baso-lateral membrane had not. Vesicles were also demonstrated on electron microscopy (Figure 2). The electron microscopy was kindly performed by Dr. David Haynes of the Department of Histopathology at Flinders Medical Centre.

A typical time course of phenylalanine and glucose uptake is shown in Figure 3 . It can be seen that both phenylalanine and glucose uptake are dramatically increased by the presence of sodium facilitated transport and expressing the uptake as a function of the protein in the brush border concentrate added to the incubation mixture.

FIGURE 2(A)   PROXIMAL TUBULE BRUSH BORDER VESICLES FROM THE RAT
              KIDNEY.
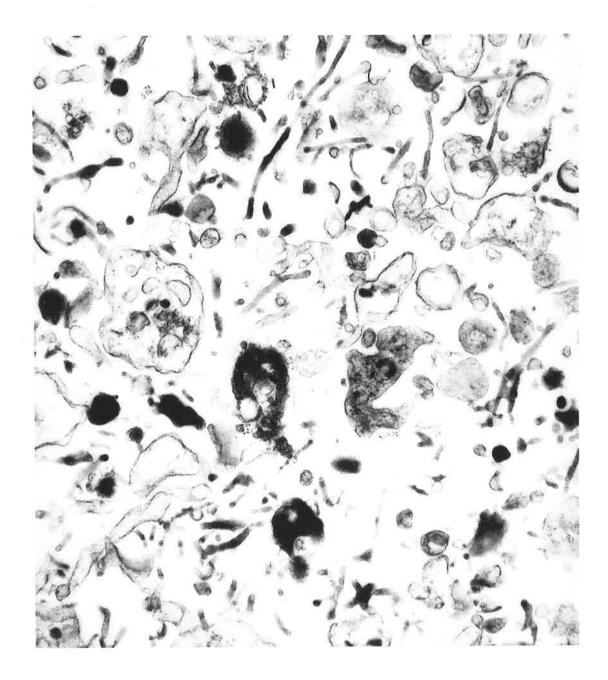
FIGURE 2(A):   ELECTRON MICROSCOPY PHOTOGRAPH REVEALS VESICLES AS
CIRCULAR HOLLOW STRUCTURES TOGETHER WITH OTHER CELLULAR DEBRIS.

FIGURE 2(B)   PROXIMAL TUBULE BRUSH BORDER VESICLES FROM THE RAT
              KIDNEY.
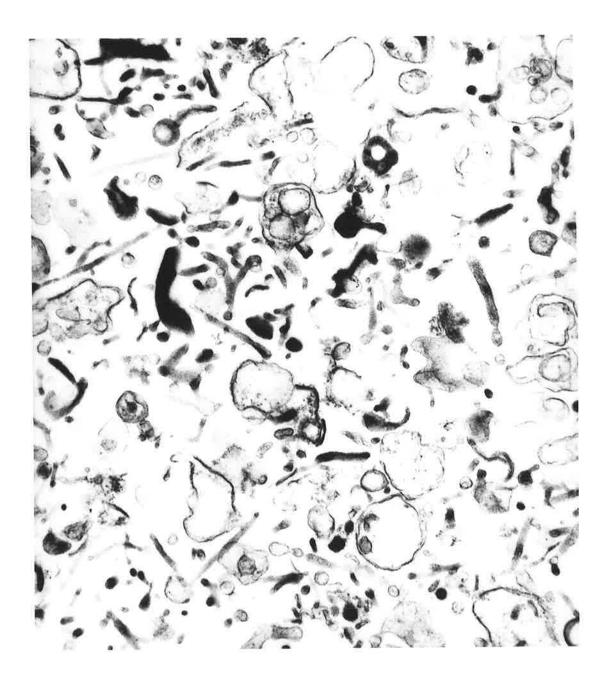
FIGURE 2(B):   ELECTRON MICROSCOPY PHOTOGRAPH REVEALS VESICLES AS
CIRCULAR HOLLOW STRUCTURES TOGETHER WITH OTHER CELLULAR DEBRIS.

FIGURE 3. TIME COURSE OF UPTAKE OF PHENYLALANINE AND GLUCOSE BY ISOLATED BRUSH BORDER VESICLES
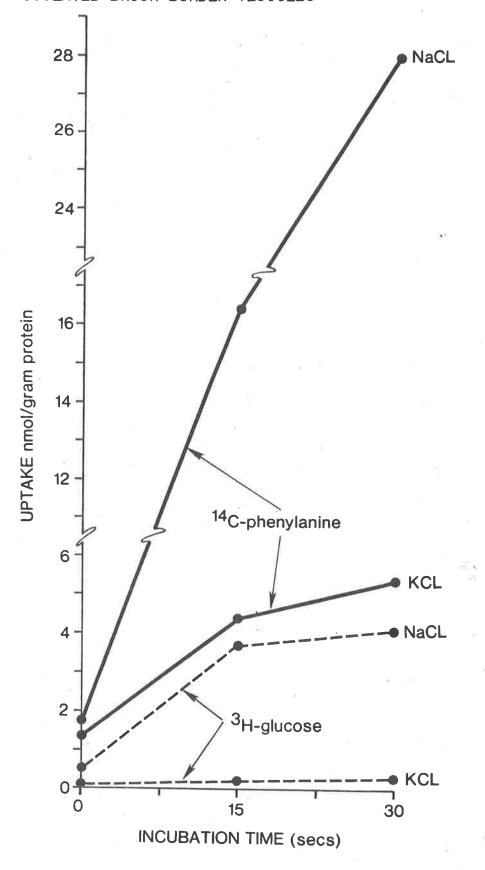
FIGURE 3: Uptake of universally labelled ^{14}C phenylalanine and ^{3}H glucose at 0,15 and 30 seconds is facilitated by the presence of sodium ion.

The concentrations of phenylalanine and its analogues used to determine inhibition are stated in Chapter X .

CHAPTER III

THE EFFECT OF β-2-THIENYLALANINE AND OF OTHER PHENYLALANINE ANALOGUES ON THE RENAL EXCRETION OF PHENYLALANINE IN THE RHESUS MONKEY (MACACA MULATTA)

This work was conducted while I was employed as a research fellow in the Joseph P. Kennedy Junior Laboratories at the University of Wisconsin, Madison, Wisconsin under the Director, Professor Harry A. Waisman. I was responsible for the concept and conduct of the experiments described, and undertook the majority of the assays described – some being performed by the technical staff of the Kennedy Laboratories.

INTRODUCTION

β-2-thienylalanine is known to inhibit phenylalanine usage by the bacteria Bacillus subtilis as shown by the absence of growth of that organism in the Guthrie test (4), where β-2-thienylalanine is included in a medium which would normally support the multiplication of Bacillus subtilis. That such inhibition by β-2-thienylalanine is reversible is also shown by the Guthrie test (4) when a blood sample containing unusually high phenylalanine, allows growth of Bacillus subtilis.

Similarly, β-2-thienylalanine affects phenylalanine metabolism in the rat, as shown by Godin and Dolan. (60) They demonstrated an increase of radioactivity in expired carbon dioxide and in urinary metabolites when DL β-2-thienylalanine was injected intravenously with DL-phenylalanine-3-^{14}C compared with the same dose of DL-phenylalanine alone.

They also showed that when DL β-2-thienylalanine-3-^{14}C was injected, negligible amounts of radioactivity appeared as carbon dioxide, while 30% to 40% of the radioactivity appeared in the urine in 12 hours.

Further, Godin and Dolan (60) demonstrated that incorportation into tissue proteins of intravenously injected β-2-thienylalanine-3-^{14}C was very low, and this compound was almost not to be found in tissue proteins after 24 hours.

These findings suggested that β-2-thienylalanine was unlikely to have a permanent untoward effect, and that if it affected phenylalanine transport, this effect might be most prominent in the renal tubule because of the high urinary excretion of β-2-thienylalanine.

To examine these suggestions and to see if the inhibition by β-2-thienylalanine existed in a higher species, the compound was tested in the Rhesus monkey (Macaca mulatta).

Effect of Infused β-2-thienylalanine on the Rhesus Monkey's Urinary Excretion of Phenylalanine while receiving Intravenous Phenylalanine

METHODS

Four, healthy, adult, male monkeys weighing 6.26kg. to 8.75kg. were lightly anaesthetised with intravenous 5-allyl-5(1-methyl butyl)-2 thiobarbiturate to allow insertion of 2.g% dextrose plus 0.4g% sodium chloride drip. While still anaesthetised, the monkeys were strapped into a restraining chair (61) and a collector attached so that urine could be collected into a plastic bottle containing a few thymol crystals and kept in ice.

When the monkey regained consciousness fully and had passed a control sample of urine, phenylalanine was given as a *statim* dose intravenously (200mg/kg). Following this, two monkeys were started on an infusion of phenylalanine in physiological saline at a concentration of 2.0g% at such a rate as to deliver 2g/kg/24 hours. After 24 hours, the intravenous fluid was changed to 2.0g% phenylalanine plus 0.5g% β-2-thienylalanine in physiological saline at the same rate.

In the other two monkeys, the reverse order was used: the combination of phenylalanine and β-2-thienylalanine was infused for the first period of 24 hours, followed by phenylalanine alone.

During these 24 hour periods, urines were collected. At the end of each infusion, suprapubic pressure was applied which caused the monkeys to void, and it was assumed the bladder emptied completely. In addition, three blood samples were drawn at 3, 18 and 24 hours. These were prepared for amino acid analysis, as described in the section on methods. The urine was also prepared for amino acid estimation by acidification and filtration. From the phenylalanine, tyrosine and β-2-thienylalanine values obtained, 24 hour urinary excretion and renal clearances were calculated.

RESULTS

The 24 hour urinary excretion of phenylalanine and tyrosine are shown in Tables I(a) and I(b).

TABLE I(a)

The excretion and clearance of Phenylalanine
added to infusion with and without β-2-thienylalanine

Monkey	Phenylalanine Excretion in mg. per 24 hours			Phenylalanine Clearance in mg/min		
	Without β-2-thienyl-alanine	With β-2-thienyl-alanine	Ratio of with thienyl-alanine to without	Without β-2-thienyl alanine	With β-2-thienyl-alanine	Ratio of with thienyl-alanine to without
I	216	521	2.42	0.290	0.890	3.07
II	70	116	1.66	0.085	0.175	2.07
III	172	375	2.17	0.208	0.771	3.71
IV	19	32	1.67	0.071	0.162	2.28

TABLE I(b)

The excretion and clearance of Tyrosine with and without
β-2-thienylalanine added to infusion

Monkey	Tyrosine Excretion in mg. per 24 hours			Tyrosine clearance in ml/min		
	Without β-2-thienyl-alanine	With β-2-thienyl-alanine	Ratio of with thienyl-alanine to without	Without β-2-thienyl alanine	With β-2-thienyl-alanine	Ratio of with thienyl-alanine to without
I	92	227	2.47	0.49	1.42	2.90
II	102	97	0.95	0.194	0.838	4.32
III	60	398	6.65	0.159	0.875	5.5
IV	17	57	3.37	0.197	0.566	2.82

Each monkey approximately doubled his excretion of phenylalanine when β-2-thienylalanine was added to the infusion (range 1.66 to 2.42). When phenylalanine clearance is examined, the increase is even more striking (range 2.07 to 3.71). There was a large individual variation of phenylalanine excretion from animal to animal which rendered a simple student t test for paired data insignificant. To allow for this individual variation (which has been noted in normal and phenylketonuric children (62)), the ratios of results with β-2-thienylalanine to without β-2-thienylalanine were expressed as a log and then compared with the expected value of zero. By this method, the phenylalanine excretion ($p < 0.005$) and clearance ($p < 0.01$) increased significantly.

Tyrosine excretion showed an increase in all but one animal but this was not significant ($p < 0.20$), and the clearance of tyrosine was significantly increased ($p < 0.005$). There was a fall of serum tyrosine level when β-2-thienylalanine was infused in monkey II. This fall in serum tyrosine might well be due to inhibition of phenylalanine hydroxylase (as shown in Uderfriend and Cooper (63) *in vitro*) with consequent reduced conversion of phenylalanine to tyrosine.

The excretion and clearance of other neutral and acidic amino acids was not consistently altered by the addition of β-2-thienylalanine to the infusate. These results are shown in Tables II(a) and II(b).

TABLE II(a)

Excretion of Neutral and Acidic Amino Acids Before (1) & After (2)
β-2-thienylalanine Added to Phenylalanine Infusion in
Rhesus Monkeys

| | MONKEYS | | | | | | | |
| | I | | II | | III | | IV | |
Amino Acid	1	2	1	2	1	2	1	2
Threonine	20.3	30.4	1.84	13.8	14.8	36.6	4.82	0.407
Serine	10.5	16.8	4.80	9.66	7.73	45.04	4.57	3.46
Glutamine	96.0	70.3	11.0	15.8	28.4	56.8	31.3	4.44
Glutamic Acid	19.3	8.59	4.10	14.12	6.35	7.20	29.2	22.6
Glycine	24.2	11.4	14.9	35.6	27.5	7.20	37.2	45.8
Alanine	6.2	2.5	1.95	0.36	3.12	4.63	15	83.5
Valine	0.0	0.0	3.93	2.34	–	3.98	6.03	1.73
Methionine	–	4.4	1.62	–	–	18.3	–	1.06
Isoleucine	2.54	4.46	6.72	–	0.859	6.94	–	1.27
Leucine	2.54	2.4	1.90	–	0.603	0.66	–	0.511

Column 1 indicates excretion in mgm/day whilst phenylalanine 2.0g/k/day infused. Column 2 indicates excretion whilst phenylalanine 2.0g/k/day and thienylalanine 0.5g/k/day infused. (−) indicates amino acid not detected in urine. No significant increases or decreases in excretion are found.

TABLE II(b)

Clearance in ml/min of Neutral and Acidic Amino Acids Before and After β-2-thienylalanine Added to Phenylalanine Infusion in Rhesus Monkeys

	MONKEYS							
	I		II		III		IV	
Amino Acid	1	2	1	2	1	2	1	2
Threonine	1.57	1.32	0.06	0.52	0.83	1.93	0.28	0.39
Serine	0.49	0.59	0.14	0.24	0.19	1.73	0.21	0.16
Glutamine	0.71	0.46	0.14	0.07	0.24	0.65	0.16	0.03
Glutamic Acid	0.40	0.30	0.38	1.31	0.30	0.72	3.65	2.55
Glycine	0.69	0.30	0.37	0.76	0.59	0.20	1.08	1.38
Alanine	0.19	0.07	0.03	0.01	0.08	0.12	0.44	2.97
Valine	0	0	0.06	0.02	–	.10	0.06	0.06
Methionine	–	0.43	0.08	–	–	3.28	–	0.20
Isoleucine	0.23	0.24	0.18	–	0.05	0.35	–	0.08
Leucine	0.13	0.10	0.06	–	0.02	0.02	–	0.02

Column 1 indicates clearance whilst phenylalanine 2.0g/k/day infused. Column 2 indicates excretion whilst phenylalanine 2.0g/k/day and thienylalanine 0.5g/k/day infused. (–) indicates amino acid not detected in urine. No significant increases or decreases in clearance are noted.

DISCUSSION AND FURTHER EXPERIMENTAL DATA

The demonstration that β-2-thienylalanine increased the urinary phenylalanine excretion in monkeys rendered hyper-phenylalaninaemic (by phenylalanine infusion) suggested that this compound might be of value in lowering blood phenylalanine levels in phenylketonuria.

The mechanism of this increased phenylalanine excretion was not shown by the above experiment. Although the fact that β-2-thienylalanine is an analogue of phenylalanine suggested competition at the level of the proximal renal tubular cell, increased glomerular filtration rate, which is a known property of ingested or infused amino acids (64,65,66) might be responsible.

To elucidate the mechanism of increased urinary excretion of phenylalanine and to test two further analogues of phenylalanine, the above experiment just described was repeated using cycloleucine or parachlorophenylalanine (3.3mg/kg/24 hours). In addition, both the phenylalanine alone and the phenylalanine plus analogue infusates contained inulin, so that glomerular filtration rate could be estimated and tubular reabsorption of phenylalanine derived.

As shown in Table III(a) both analogues increased glomerular filtration rate (p < 0.1 in each case) while Table IIIb shows that tubular reabsorption of phenylalanine was un-affected by the addition of cycloleucine or parachlorophenyl-alanine, so that the increase in excretion of phenylalanine with these analogues was the result of stimulation of glomerular filtration rate.

Although these results did not support the original concept that phenylalanine analogues might block phenylalanine reabsorption in the renal tubular cell, the increased excretion

might still be beneficial to the patient with phenylketonuria. The increased excretion of phenylalanine in the urine should allow an increased ingestion of phenylalanine, as the only other pathways of phenylalanine metabolism were either absent (phenylalanine hydroxylase) or of minor consequence as a means of removing phenylalanine from the blood (conversion of phenylalanine to phenylketones).

TABLE III(a)

Effect of Cycloleucine and Parachlorophenylalanine on inulin clearance in Hyperphenylalaninaemic Monkeys

Monkey	Inulin Clearance (ml/min)		
	Phenylalanine alone	Phenylalanine plus Cycloleucine	Phenylalanine plus Parachlorophenylaline
I	11.5	24.2	19.8
II	12.6	20.4	21.0
III	8.9	32.3	29.7
IV	17.1	15.2	15.8
	12.5	23.0	21.6

The increased glomerular filtration, as measured by inulin clearance, associated with the addition of phenylalanine analogue (cycloleucine or parachlorophenylalanine) is significant at the $p < 0.1$ in each case.

TABLE III(b)

Effect of analogues on phenylalanine reabsorption in the renal tubule

Monkey	Phenylalanine Alone			Phenylalanine plus cycloleucine			Phenylalanine plus Para-chlorophenylalanine		
	Filtered	Excreted	% Reab-sorbed	Filtered	Excreted	% Reab-sorbed	Filtered	Excreted	% Reab-sorbed
I	1435	5.38	99.7	2750	33.1	98.8	2680	3.10	99.9
II	1830	17.7	99.0	3010	12.7	99.6	3590	2.27	99.0
III	2200	41.5	98.1	5520	36.0	99.3	5600	4.80	99.9
IV	2540	11.4	99.5	2460	15.7	99.4	2700	1.35	99.9
Mean	2001	19.0	99.1	3435*	24.4	99.3	3643#	2.88	99.9

Filtered loads and excretion expressed in mg/24 hours. The filtered load of phenylalanine increased when cycloleucine (* $p < 0.10$) or parachlorophenylalanine (# $p < 0.20$) but no effect on tubular reabsorption could be detected.

CHAPTER IV

THE EFFECT OF PHENYLALANINE ANALOGUES ON INTESTINAL
PHENYLALANINE ABSORPTION IN THE RHESUS MONKEY (MACACA MULATTA)

This work was conducted under the same circumstances as described in Chapter III.

INTRODUCTION

Having demonstrated that β-2-thienylalanine, cyclo-leucine and parachlorophenylalanine promoted the renal excretion of phenylalanine when given intravenously, it was important to see if they had an effect orally, oral administration being the only practical mode of therapy for a child as injection would not be justified when dietary measures, albeit very strict ones, were effective alone.

It was therefore decided to give oral phenylalanine loads with and without the analogues, and follow the rise in serum phenylalanine.

METHODS

The same four healthy adult monkeys as described in Chapter III were used 3 months after the intravenous experiments were concluded. They were fasted for 8 hours before the experiment but allowed water in this period.

At time zero, each monkey had venous blood drawn, a nasogastric tube inserted, a urine collector attached to the external genitalia and the animal was placed in a special metabolic chair.(61) L phenylalanine 750mg/kg. dissolved in 300ml. of the tap water was given via the nasogastric tube. This dose and the timing of blood samples were chosen after 5 preliminary administrations had shown this was the maximum dose that could be given without inducing vomiting. Blood samples

were drawn at 1, 2, 3, 4 and 6 hours.

After, at least, a three day rest between experiments, the animals were given the same dose of phenylalanine with one of the analogues. The experiment was repeated after a further 3 day, or more, rest with another of the analogues until each of the four monkeys had had phenylalanine alone, phenylalanine plus β-2-thienylalanine 187.5mg/kg., phenylalanine plus cycloleucine 225mg/kg. and phenylalanine plus parachloro-phenylalanine 225mg/kg. These doses were chosen on the basis of solubility characteristics and previously described effects on phenylalanine metabolism.

Acidified, filtered urine and all blood samples were run on the amino acid analyzer for neutral and amino acids. The analogues previously had been shown not to interfere with these assays.

RESULTS

A typical experiment is shown in Figure 4. The serum phenylalanine in mg. per 100 ml. is shown at zero time and 1, 2, 3, 4 and 6 hours after the oral load. In each case, the addition of the analogue to the phenylalanine load reduces the absorption of phenylalanine. To quantitate this reduction the area under each curve above base line was calculated and *phenylalanine absorption* expressed in arbitrary units. *Phenylalanine absorption* has the italics to indicate that the graph really expresses the absorption of phenylalanine less the amount excreted in the urine and that passes from the blood into tissues, for incorporation or metabolism.

These calculated areas are shown in Table IV where the mean *phenylalanine absorption* in monkeys fed phenylalanine alone is compared with the mean *absorption* when given phenyl-

FIGURE 4. THE EFFECT OF PHENYLALANINE ANALOGUES ON THE EFFECTIVE
INTESTINAL ABSORPTION IN THE RHESUS MONKEY
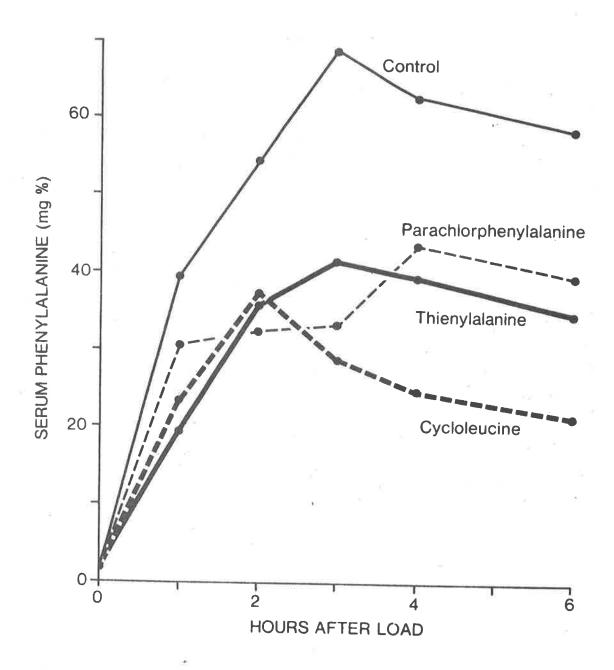
FIGURE 4: RESPONSE OF SERUM PHENYLALANINE ON MONKEY 3 TO 4 ORAL
LOADINGS. THE CONTROL LOAD OF L-PHENYLALANINE WAS 750 MG/KG,
EACH OF THE OTHER LOADS CONTAINED THE SAME QUANTITY OF
L-PHENYLALANINE (750 MG/KG) PLUS EITHER β-2-DL-THIENYLALANINE
(187.5 MG/KG), CYCLOLEUCINE 225 MG/KG OR PARACHLOROPHENYLALANINE
225 MG/KG.

alanine plus an analogue. The *phenylalanine absorption* when given phenylalanine alone, shown in Table IV, is the mean of two experiments on each animal with a 750mg/kg. L phenylalanine load.

Each analogue decreased the amount of *phenylalanine absorbed* with the exception of animal I when given β-2-thienyl-alanine. When cycloleucine is added, the mean absorption is significantly lower (at the 10% level) using a student t test for paired data. When the effect of the analogue is expressed as a ratio to allow for individual variation, as was done with the data in Chapter III, all analogues significantly reduced *phenylalanine absorption.*

Serum tyrosines were all estimated and the rise after the phenylalanine load (due to conversion of phenylalanine to tyrosine by hepatic phenylalanine hydroxylase) was less when the phenylalanine analogues were simultaneously administered. This again suggests these analogues inhibited phenylalanine hydroxylase - however, the differences were not statistically different.

TABLE IV

Phenylalanine Absorption Expressed as the Area under Curve after 750 mg/kg of Phenylalanine Nasogastric Tube with and without Analogues.

LOAD	I	II	III	IV	MEAN	
1) Phenylalanine	340	428	364	80.2	303	
2) Phenylalanine + β-2-thienylalanine	356	286	304	50.3	249	(N.S.)
3) Phenylalanine + cycloleucine	140	182	124	67.2	128	$(p < 0.:)$
4) Phenylalanine + Parachlorophenyl-alanine	178	268	143	29.9	155	$(p < 0.2)$
Ratio line 2/line 1	1.05	0.67	0.84	0.63	0.80	$(p < 0.1)$
Ratio line 3/line 1	0.41	0.43	0.34	0.84	0.51	$(p < 0.05)$
Ratio line 4/line 1	0.52	0.63	0.39	0.37	0.48	$(p \ll 0.001)$

Each monkey received 4 loads: in each he received 750 mg/kg of phenylalanine; in load 2 he also received β-2-thienylalanine 187.5 mg/kg; in load 3 cycloleucine 225 mg/kg; in load 4 parachlorophenylalanine 225 mg/kg. p values are for analogue plus phenylalanine compared to phenylalanine alone.

DISCUSSION

Although the term 'phenylalanine absorption' is not correct as the graph in Figure II reflects intestinal phenylalanine absorption, phenylalanine metabolism and urinary excretion, the fall of serum phenylalanine suggests that the analogues may be of benefit to patients with phenylketonuria.

Reduction of serum phenylalanine by dietary means appears to prevent brain damage, as discussed in Chapter I. The steps between high phenylalanine blood levels and damage to neurones is not known, although many theories have been put forward. (28 and 88). It is noteworthy that all these theories start with the elevated blood phenylalanine level as the point from which their hypothesis grows. Hence, any decrease in the blood phenylalanine level might be beneficial, no matter how it was achieved.

The alternative theory, known as the 'justification hypothesis' proposes that the mental retardation of phenylketonuria is due to tyrosine deficiency, both pre and post birth (87). However, normal cord blood tyrosine levels in phenylketonuric babies (88), the fact that patients with classical phenylketonuria have their intelligence protected by low phenylalanine diets (9-20), and the inability to detect tyrosine deficiency in treated and untreated phenylketonuric patients (89) suggest that tyrosine deficiency is not the cause of brain damage in phenylketonuria.

Thus, further investigation of phenylalanine analogues as a means of treating phenylketonuria appeared warranted.

CHAPTER V

THE SAFETY AND CLINICAL EFFICACY OF β-2-THIENYLALANINE IN INFANT MONKEYS RENDERED HYPERPHENYLALANINAEMIC BY ORAL PHENYLALANINE SUPPLEMENTATION.

The studies involving the use of β-2-thienylalanine described hereunder were entirely my own. However, the work on infant monkeys who were fed phenylalanine alone and the control monkeys fed no supplement had previously been conducted at the same laboratories (Joseph P. Kennedy Jnr. Laboratories, University of Wisconsin, Madison, Wisconsin) under the auspices of Professor H.A. Waisman.

INTRODUCTION

Having determined that the three analogues tested reduced the plasma phenylalanine increment obtained over 6 hours after an oral phenylalanine load, the feasibility of this as a practical method of treating phenylketonuria needed to be determined.

Two outstanding questions presented themselves:

(a) Would these compounds alone protect against mental retardation in animals rendered hyperphenylalaninaemic?

(b) Would these compounds be toxic to the animals in their own right?

In an endeavour to answer the questions about the protection afforded by β-2-thienylalanine and the safety of β-2-thienylalanine, four infant monkeys were fed it; two in combination with a dose of oral phenylalanine, previously found to render monkeys hyperphenylalaninaemic and mentally retarded (90). The two other infant monkeys were fed the same dose of β-2-thienylalanine alone.

Previous work by Professor H.A. Waisman and colleagues had developed an animal model of phenylketonuria, in that the animals were rendered hyperphenylalaninaemic and brain damaged by feeding them high dose phenylalanine from birth (61,90-92). The β-2-thienylalanine-fed monkeys were compared with these phenylalanine-fed and control monkeys. All 3 groups were in the same monkey laboratory under identical conditions, other than their feeds.

METHODS

All Rhesus monkeys (Macaca mulatta) were taken from their mothers immediately following birth and bottle fed in a special nursery. The control group of all monkeys born and not used for experimental purposes were fed a commercial milk formula designed for human infants (Simalac*). The second group were fed the same formula supplemented with L-phenylalanine (L-2-amino-3-phenyl-L-propanol) so as to give a target intake of supplemental phenylalanine equal to 3 grams per kilogram body weight per day.

Two β-2-thienylalanine-fed animals (designated L36 and L46) were given the same milk formula with added β-2-thienylalanine so as to give a target of 0.75 grams per kilogram per day if all milk was consumed. The final group of 2 monkeys (L80 and M11) had the same formula supplemented with both L-phenylalanine (target 3.0 grams per kilogram per day) and β-2-thienylalanine (0.75 grams per kilogram per day).

This diet was introduced by experienced handlers and intake carefully charted. The technique and the general care of monkeys was that described by Waisman and Harlow (61, 90, 91, 92).

The dietary intake and weight of each monkey was recorded daily while height and head circumference were measured weekly. After a four hour fast, blood was taken for phenylalanine

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* Ross Laboratories, Cleveland, Ohio.

tyrosine and β-2-thienylalanine estimation as described in the initial methods section. At the same time blood was sent for haematological estimations. These complete blood pictures were conducted by the Clinical Laboratory Service of the University of Wisconsin Hospital, Madison, Wisconsin. The diet and these estimations were continued for 240 days.

RESULTS

Clinical

On the final day of the special diet, one monkey (L36) who had been fed a diet supplemented with β-2-thienylalanine developed Shigella gastroenteritis. He was treated with antibiotics and intravenous fluids but died on the tenth day of this illness. Autopsy showed no other abnormality other than findings compatible with gastroenteritis.

The brain and liver were assayed for β-2-thienylalanine incorporation into protein after hydrolysis of the trichloracetic acid precipitable protein – the brain contained no β-2-thienylalanine while the liver protein had a phenylalanine: tyrosine; β-2-thienylalanine ratio of 22:7:1. Thus very little β-2-thienylalanine had been incorporated into protein.

Another monkey fed both phenylalanine and β-2-thienylalanine supplements also developed Shigella gastro-enteritis (at 200 days) but recovered completely in 7 days when given parenteral physiological saline and anti-biotics.

The quantity of phenylalanine added to the milk was increased at standard times in an effort to obtain the target of 3 grams per kilogram per day. Overall, both the animals given phenylalanine alone and the two given phenylalanine plus β-2-thienylalanine received an average of 2.5 grams of phenylalanine/kg/day after the first month of life. The target was not achieved because the phenylalanine-fed animals consumed a lesser volume of milk per day on a body weight basis.

β-2-thienylalanine appeared to be particularly distasteful to the monkeys, as all four that had it drank less than those who had phenylalanine alone or no supplement at all.

Weight gain in the monkeys fed β-2-thienylalanine was not significantly different from the control animals except for L36 and M11 when they suffered gastroenteritis. The growth rates of all monkeys fed β-2-thienylalanine whether in combination with L-phenylalanine or not was superior in velocity to that of the 4 monkeys fed phenylalanine alone. This was particularly noticeable in the period 200 to 240 days. These findings are represented graphically in Figure 5.

Figure 6 shows the volume of milk consumed by the infant monkeys, the phenylalanine intake achieved and the amount of phenylalanine added to Simalac in an endeavour to reach the target of 3.0 g/kg/day of phenylalanine in those animals fed extra phenylalanine.

The upper panel of Figure 6 shows that the animals fed Simalac alone (—+—) took in a greater volume of milk (expressed as cc/kilogram/day) than any of the animals fed supplements. Initially they took in about 400 cc/kg/day and towards the end of their first year the figure was approximately 350 mls/kg/day. The 4 monkeys fed phenylalanine supplements alone (3.0 g/kg/day) and represented by the hatched area in the Figure) take in about 325 cc/kg/day for the first 6 months but after that, mean intake falls below 300 cc/kg/day. The 4 monkeys fed β-2-thienylalanine (with or without phenylalanine) supplements all had lower intakes during the 240 days during which they received supplemented Simalac. The 2 monkeys, L46 and L80, who did not develop Shigella gastroenteritis were followed for the 120 days after all supplements were stopped. It can be seen that their intakes promptly rose and exceeded that of the phenylalanine supplemented animals. This strongly suggests that the monkeys found β-2-thienylalanine more distasteful than phenylalanine.

The middle panel of Figure 6 compares the phenylalanine intake of the 2 monkeys fed β-2-thienylalanine (0.75 g/kg/day) and phenylalanine (3.0 g/kg/day) supplements and the 4 monkeys fed phenylalanine (3.0 g/kg/day) supplements (hatched area = mean ± 1 S.D.). It can be seen that despite the lower total
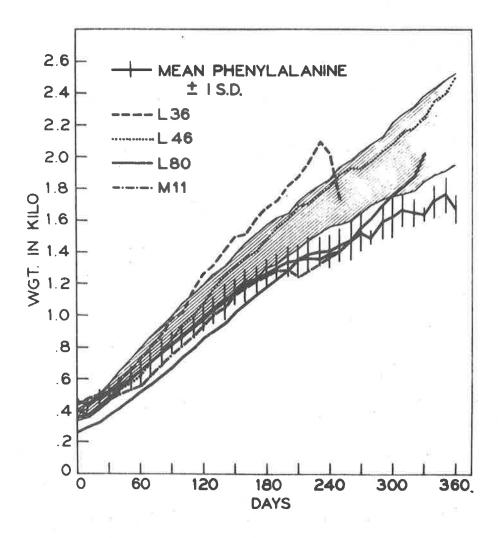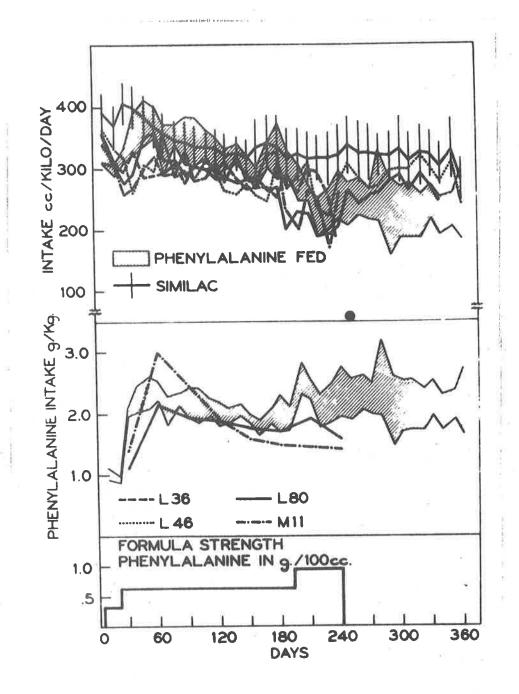
FIGURE 5 : 2 Monkeys (L36 & L46) were fed 0.75 g/kg/day -2-thienyl-
alanine for 240 days. L80 and M11 were fed -2-thienylalanine
(0.75 g/kg/day) plus L-phenylalanine (3.0 g/kg/day) for 240 days.
The hatched area represents the mean plus standard deviation of
monkeys fed no supplements —+— represents 4 monkeys fed L-phenyl-
alanine 3.0 g/kg/day mean ± S.D.

FIGURE 6   PHENYLALANINE INTAKE BY RHESUS MONKEYS ON NORMAL MILK
AND MILK SUPPLEMENTED WITH PHENYLALANINE AND/OR
B-2-THIENYLANININE

FIGURE 6   THE UPPER PANEL SHOWS THAT ANIMALS FED PHENYLALANINE
(3.0 G/KG/DAY) AS REPRESENTED BY THE HATCHED AREA HAD A LOWER INTAKE
THAN MONKEYS FED SIMILAC ALONE (+) MEAN SHOWN AS THE UPPER CONTINUOUS
LINE WITH VERTICAL BARS REPRESENTING 1 STANDARD DEVIATION. THE 4
MONKEYS L36, L46 & M11 FED B-2-THIENYLALANINE HAVE GENERALLY LOWER
INTAKES. THE MIDDLE PANEL COMPARES PHENYLALANINE INTAKE OF MONKEYS
FED 3.0 G/KG/DAY PHENYLALANINE AND 0.75 G/KG/DAY B-2-THIENYLALANINE
WITH THE MEAN ± S.D. OF 4 MONKEYS FED PHENYLALANINE ALONE. THE
LOWEST PANEL SHOWS THE CONCENTRATION OF PHENYLALANINE IN MILK FED TO
BOTH GROUPS OF ANIMALS GIVEN 3.0 G/KG/PER DAY.

intake the amount of phenylalanine consumed is roughly comparable. This is because, in these 2 monkeys, their intake was relatively high in the first 90 days. The bottom panel shows the amount of phenylalanine supplement given. This was basically an exercise in chasing one's tail in that when the phenylalanine intake fell below the targe of 3.0 g/kg/day, the concentration of phenyl-alanine in the milk was increased. To avoid differences between each group, standard times for each concentration of phenylalanine in milk were adopted. In the animals fed phenylalanine alone, milk was supplemented at 10 g/100 ml from 240 to 360 days, while in those given β-2-thienylalanine, all supplements (including phenylalanine) were ceased at 240 days.

Figure 7 shows growth expressed as a function of intake of milk (i.e. grams gained per 10 litres of milk consumed). It can be seen that the Simalac-fed animals (shaded area) and phenylalanine alone supplemented monkeys (—+—) had very similar gains, while all 4 infant monkeys given β-2-thienylalanine (2 with phenylalanine and 2 given β-2-thienylalanine alone) gained better. This is a reflection of the infants being able to maintain a reasonable weight gain (Figure 5) despite poor intake (Figure 6). No significant differences were noted for height and head circumference measurements between the β-2-thienylalanine-fed and control monkeys.

Neurologically, the monkeys fed L-phenylalanine alone appeared retarded and convulsed as did one monkey (L80) fed β-2-thienylalanine in addition to L-phenylalanine. The other monkey (M11) who was fed the combination appeared to be neurologically protected as he did not convulse and was indistin-guishable in behaviour from the control animals.

Blood Data

The phenylalanine levels of the monkeys receiving the β-2-thienylalanine supplement (0.75g/kg/day) with or without L-phenylalanine (3.0g/kg/day) are compared with those who received phenylalanine (3.0g/kg/day) alone in Fig. 8. β-2-thienylalanine alone did not cause serum phenylalanine levels to vary from normal. When β-2-thienylalanine was given together with phenylalanine, the serum levels varied markedly but, generally, were lower (mean 26mg.% [S.D. = 19]) than that of the animals supplemented with phenylalanine alone (mean 44mg.% [S.D. = 11]).

FIGURE 7   WEIGHT GAIN AS A FUNCTION OF MILK INTAKE IN CONTROL-FED, PHENYLALANINE SUPPLEMENTED AND B-2-THIENYLANININE SUPPLE[42.] MENTED INFANT MONKEYS.
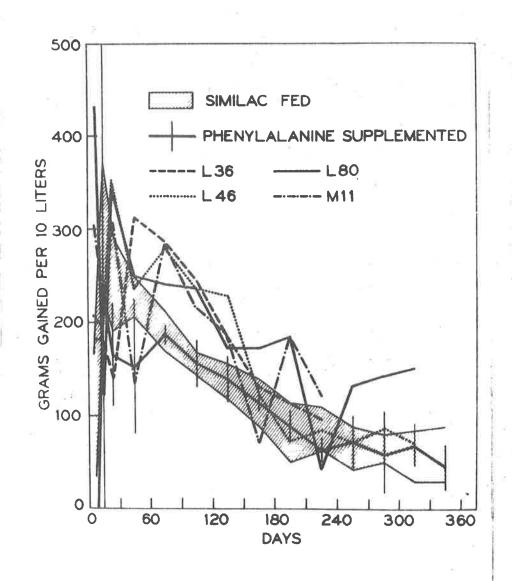
FIGURE 7 : WHEN EXPRESSED AS A FUNCTION OF MILK INTAKE THE WEIGHT GAIN OF THOSE INFANT MONKEYS FED A SUPPLEMENT OF PHENYLALANINE (+) WAS SIMILAR TO THOSE FED SIMALAC ALONE. (NOTCHED AREA). MONKEYS L36 & L46 FED B-2-THIENYLANININE (0.75 G/KG/DAY) ALONE AND L80 & M11 FED THE SAME SUPPLEMENT OF B-2-THIENYLANININE AND PHENYLALANINE (3.0 G/KG/DAY) ALL GAINED WEIGHT AT A GREATER RATE OVER THE 240 DAYS THEY WERE FED SUPPLEMENTS.

FIGURE 8   SERUM PHENYLALANINE LEVELS IN INFANT MONKEYS FED
            SUPPLEMENTED B-2-THIENYLALANINE AND/OR PHENYLALANINE
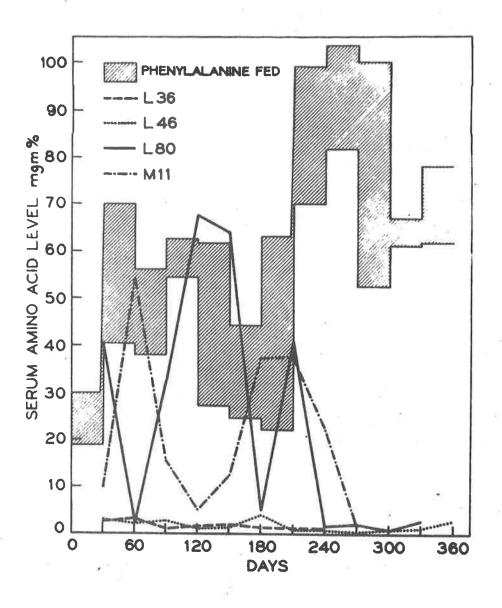
FIGURE 8 : SERUM PHENYLALANINE LEVELS (Y AXIS) FOR THE 4 MONKEYS FED
PHENYLALANINE ALONE SUPPLEMENT (HATCHED AREA) ARE EXPRESSED AS THE
MEAN MONTHLY FIGURE ± S.D.. THE TWO MONKEYS FED β-2-THIENYLALANINE
(0.75 G/KG/DAY) - L36 AND L46 - SHOW NO ELEVATION OF SERUM PHENYLAL-
ANINE. THE TWO MONKEYS FED COMBINATION OF PHENYLALANINE (3.0 G/KG/DA
AND β-2-THIENYLALANINE (0.75 G/KG/DAY) SHOW MARKED VARIATION IN
SERUM PHENYLALANINE BUT LEVELS ARE GENERALLY LOWER THAN THOSE ON
MONKEYS FED PHENYLALANINE 3.0 G/KG/DAY ALONE.

When the two monkeys receiving the combination of β-2-thienylalanine and l-phenylalanine were returned to a normal diet at 240 days, the serum phenylalanine levels rapidly fell to normal where they remained.

The mean serum β-2-thienylalanine levels of those monkeys fed the combination of it plus phenylalanine were higher than those of the monkeys fed the same amount of β-2-thienylalanine alone. However, the values were extremely variable as were the serum tyrosine values. These results are shown in Table V.

There were no haematological abnormalities in any of the groups of monkeys supplemented with amino acids compared with the control animals. (Figures 9 and 11).

DISCUSSION

These long term feeding experiments of infant monkeys were designed for two purposes:

1. To provide further evidence about the efficiency of β-2-thienylalanine in reducing serum phenylalanine levels and hence, possibly preventing brain damage.
2. To test the safety of this compound in new born monkeys fed it on a long term basis.

The studies confirmed that β-2-thienylalanine did lower serum phenylalanine levels but that the serum phenylalanine levels showed wide variation from day to day. This might be due to inhibition of the infant monkeys' phenylalanine hydroxylase by the β-2-thienylalanine as reported by Uderfriend and Cooper (63). This inhibition of the major degradative metabolic pathway of phenylalanine would tend to elevate serum phenylalanine, while the renal and intestinal effects already described would tend to reduce it. To overcome this problem one would have to use an animal totally deficient in phenylalanine hydroxylase, as is the phenylketonuric patient. An animal partially deficient in phenylalanine hydroxylase, such as the dilute *lethal* mouse, would not be suitable, as the residual phenylalanine hydroxylase

TABLE V

Serum β-2-thienylalanine (β-2-T) and Tyrosine*(Tyr.) Levels in
Infant Rhesus Monkeys fed the antagonist with and without
Phenylalanine at various ages.

Period	β-2-Thienylalanine Fed				β-2-T and Phenylalanine Fed			
Age in days	L-36		L-46		L-80		M-11	
	β-2-T	Tyr.	β-2-T	Tyr.	β-2-T	Tyr.	β-2-T	Tyr.
– 30	3.1	2.3	2.6	2.9	8.8	22.8	9.5	13.5
30 – 60	2.4	3.2	5.9	3.6	1.2	2.5	12.6	41.8
60 – 90	3.2	3.0	6.4	4.2	9.5	7.4	9.5	60.8
90 – 120	3.9	3.0	6.5	4.0	11.4	20.7	7.0	6.7
120 – 150	10.3	0.44	6.0	2.2	10.4	10.6	8.1	45.2
150 – 180	7.7	2.2	11.6	6.1	1.5	3.9	6.3	12.4
180 – 210	4.1	1.4	3.3	2.0	7.7	4.7	10.0	9.2
210 – 240	9.6	1.6	4.4	1.4	Gastroenteritis		17.2	2.1
240 – 270	2.0	1.0	9.1	0.78	5.2	3.5	0.22	1.8
270 – 300	died		–	0.97	–	–	–	2.5

* mg per 100 ml.

TABLE V: Infant monkeys fed β-2-T 0.75g/kg/day demonstrate similar
levels of β-2-T whether given Phenylalanine (3g/kg/day)
or not. Tyrosine levels were significantly higher in those
fed phenylalanine as well as β-2-T.

FIGURE 9   HAEMOGLOBIN LEVELS IN INFANT MONKEYS FED SUPPLEMENTS OF
          B-2-THIENYLALANINE ALONE OF WITH PHENYLALANINE SUPPLE-[46.]
          MENT
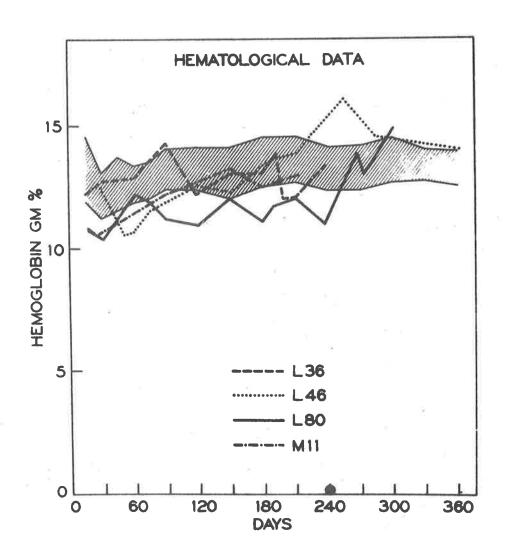
FIGURE 9 : NORMAL INFANT MONKEY LEVELS (MEAN ± ONE S.D.) ARE SHOWN
IN THE HATCHED AREA. MONKEYS L36 AND L46 (FED B-2-THIENYLALANINE
0.75 G/KG/DAY ONLY SHOWNORMAL VALUES. THOSE FED THE COMBINATION
- L80 AND M11 - HAVE LOWER LEVELS BUT STUDENT T TEST FAILS TO RE-
VEAL A SUBSTANTIAL DIFFERENCE. N.B. L80 WAS SMALL AT BIRTH SHOW-
ED HIGHER PHENYLALANINE LEVELS THAN M11 AND WAS NOT CLINICALLY PRO-
TECTED AGAINST THE EFFECTS OF PHENYLALANINE - SUPPLEMENTED FEEDING
(SEE TEXT). ALL ANIMALS HAD SUPPLEMENTS CEASED AT 240 DAYS

FIGURE 10   RED CELL COUNTS IN INFANT MONKEYS FED SUPPLEMENTS OF
B-2-THIENYLALANINE ALONE OR WITH PHENYLALANINE
SUPPLEMENT.
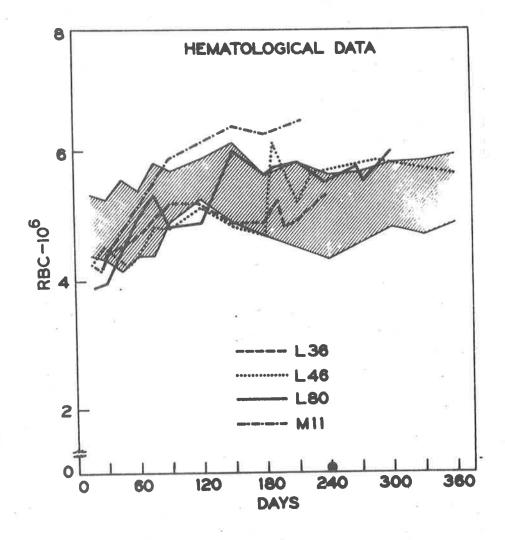
FIGURE 10 : NORMAL INFANT MONKEY LEVELS (MEAN ± ONE S.D.) ARE SHOWN
IN THE HATCHED AREA. ALL 4 ANIMALS FED B-2-THIENYLALANINE (WITH OR
WITHOUT PHENYLALANINE) DEMONSTRATE NORMAL RED BLOOD CELL COUNTS
BETWEEN 4 AND 6 MILLION/C MM.

FIGURE 11   PERCENTAGE OF RETICULOCYTES IN INFANT MONKEYS FED
SUPPLEMENTS OF B-2-THIENYLALANINE ALONE OR WITH
PHENYLALANINE SUPPLEMENT
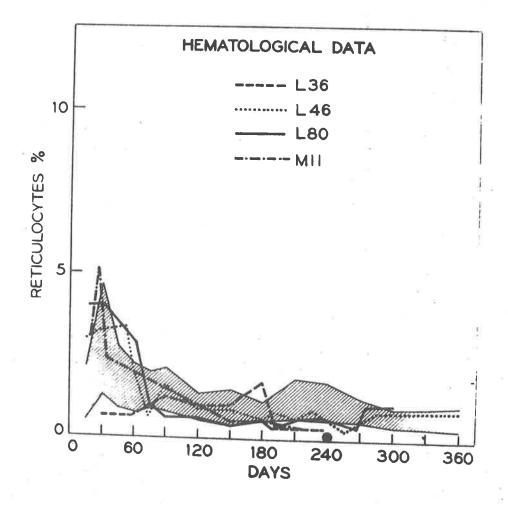
FIGURE 10 : PERCENTAGE OF RETICULOCYTES IN CONTROL INFANT MONKEYS
(MEAN ± ONE STANDARD DEVIATION) ARE SHOWN IN THE HATCHED AREA.
NO SIGNIFICANT DIFFERENCES BETWEEN MONKEYS FED B-2-THIENYLALANINE
ALONE (L36 AND L46) OR WITH PHENYLALANINE (L80 AND M11) ARE FOUND.

would be inhibited, causing the same problem. Unfortunately, the only animal totally deficient in phenylalanine hydroxylase that has been described is phenylketonuric man. Thus, to obtain a satisfactory answer, human beings with this disease would have to be used. This further emphasises the importance of the safety or otherwise of β-2-thienylalanine.

In the above study, infant monkeys fed β-2-thienylalanine grew normally in height, weight and head circumference, haematological indices were unremarkable and, in one autopsy, no abnormalities were found. Also, brain and liver taken from those studies showed little or no incorporation of β-2-thienylalanine into protein, which was comforting and confirmed work by Godin and Dolan (60) in rats.

However, a major concern was the appearance of Shigella gastroenteritis in two of the infant Rhesus monkeys fed β-2-thienylalanine. The reason for this might be –

(a) Coincidence: Shigella is endemic in most primate colonies and this laboratory was no exception. However, it was a matter of great concern that one monkey died – an event that had not occurred for many years at this particular laboratory.

(b) Impairment of the monkey's defence mechanism – β-2-thienylalanine might depress immune mechanism or damage an organ (e.g. gut mucosa) to allow a Shigella colonisation to become an active infection.

(c) Conferring an advantage to the Shigella organism, e.g. if a non-pathogenic organism such as E. coli were selectively disadvantaged by β-2-thienylalanine, thus reducing this organism's number and allowing superinfection with Shigella not so disadvantaged.

The autopsy study, clinical examination and haematological studies gave no indication of which factor was involved, or whether some other might be responsible.

As there is well known species variation in toxicity of drugs and other chemicals, and because of the problems caused

by the inhibition of phenylalanine hydroxylase by β-2-thienylalanine, it was thought that an acute load of β-2-thienylalanine given to phenylketonuric patients was justified.   This is described in the next section.

CHAPTER VI

THE EFFECT OF β-2-THIENYLALANINE ON SERUM LEVELS OF
PHENYLALANINE FOLLOWING AN ORAL LOAD OF PHENYLALANINE
IN PHENYLKETONURIA PATIENTS.

The studies were conducted with the technical assistance
of Dr. Carol Krips who was a fifth year medical student at the
time. They were performed at the Strathmont Centre for the
mentally retarded with chemical estimations in the Department
of Child Health at the University of Adelaide.

INTRODUCTION

As β-2-thienylalanine had been shown to both promote
the renal excretion of phenylalanine and also reduce the intestinal
absorption of phenylalanine in monkeys rendered hyperphenylalanin-
aemic, it was of interest to study the effect of this compound
in patients with phenylketonuria. As the compound had been
given on a chronic basis to infant monkeys without detrimental
effect (with the possible exception of predisposing the recipient
to Shigella gastroenteritis), it was considered justified to
use this compound on an acute basis.

Accordingly, two experiments were undertaken:

(a) Renal

Three female mentally retarded, untreated, phenyl-
ketonuria patients,whose parents or guardians had given permission
for the studies to be undertaken, were infused with physiological
saline alone for four hours, followed by a further four hour
period of β-2-thienylalanine in physiological saline at a rate
that delivered a dose of 20mg/kg/minute. Urine was collected
by indwelling Foley catheter into a plastic vessel containing
thymol. Blood was drawn by venepuncture at the start, mid-point
and finish of each infusion (0,2 & 4 hours).

Urinary and blood creatinine and phenylalanine levels were measured as described in the analytical section, while urinary phenylketones were determined by gas liquid chromatography. (59)

From the data glomerular filtration rate, phenylalanine excretion and renal tubular reabsorption of phenylalanine and phenylketone excretion rates were derived for both the control (saline only) and experimental (β-2-thienylalanine infusion) periods.

The results are shown in Tables VI(a) & VI(b) Glomerular filtration rate was increased appreciably by β-2-thienylalanine infusion in two of the three patients, while in the third it fell minimally. In all three patients the renal tubular reabsorption of phenylalanine was reduced by the β-2-thienylalanine and, as a consequence, phenylalanine excretion increased in all three patients. The mean increase was 2.25 fold.

The excretion of urinary phenylpyruvic acid and phenyllactic acid was increased, while the effect on phenylacetic and orthohydrophenylacetic acid was variable. Without blood levels of the phenylketones it is impossible to determine the relative importance of renal tubular absorption and glomerular filtration rate, but the fact that all three patients (including the patient whose glomerular filtration rate did not alter appreciably), increased the excretion of phenylpyruvic acid suggests an effect on renal tubular reabsorption. It is also possible that β-2-thienylalanine inhibited the metabolism of phenylpyruvic acid to other phenylketones.

Discussion

This study confirmed the increased excretion of phenylalanine when β-2-thienylalanine is infused. It further demonstrated that in humans, at least, the basis of the increased excretion was a reduced tubular reabsorption of phenylalanine, while in 2 of the 3 phenylketonuric patients there was an increase in glomerular filtration rate as when tested in the monkey.

TABLE VI(a)

Effect of β-2-thienylalanine (20mg/kg/min) on Renal
Handling of Phenylalanine in Untreated Phenylketonuric Women.

Patient	Glomerular Filtration Rate* ml/min		Phenylalanine Excretion mg in 4 hours		% Reabsorption	
	Control	Experimental +	Control	Experimental	Control	Experimental
1	60.4	77.1	142.5	266.7	97.8	85.1
2	108.6	104.2	117.1	277.9	95.0	83.9
3	44.9	53.4	90.0	225.4	95.6	91.1
Mean	71.3	78.2	116.5	256.7	96.1	86.7

* Uncorrected for surface area.

+ Experimental = period in which β-2-thienylalanine infused.

TABLE VI(b)

Effect of β-2-thienylalanine (20mg/kg/min) on Renal
Handling of Phenylketones in Untreated Phenylketonuric Women.

Patient	Phenylpyruvic Acid		Phenyllactic Acid		Phenylacetic Acid		Orthohydroxy Phenyl-acetic Acid	
	Control	Exp.+	Control	Exp.	Control	Exp.	Control	Exp.
1	118	193	83	463	3.08	8.05	17.0	20.4
2	424	791	156	166	12.6	7.57	30.9	15.6
3	78	345	37	92	6.95	13.2	9.55	23.1
	207	443	92	240	7.54	9.61	19.15	19.7

Excretion in mg/4 hours.

+ Exp = period in which β-2-thienylalanine infused.

These results suggest that β-2-thienylalanine might be the most appropriate analogue (of the three studied) to use for the treatment of phenylketonuria, as the other two did not block tubular reabsorption but merely had an effect via the increased glomerular filtration.

It should be noted that in two of the three phenyl-ketonuria females, glomerular filtration rate was increased. The mechanism of this stimulation was not clear from this study, although the patients did not appear clinically affected at the time of the study or in the 12 years since. It is conceivable, however, that should the increased rate be due to increased cardiac work leading to greater renal blood flow, this might have deleterious effects on the patients' health, such that β-2-thienylalanine might not be justified as a long term treatment of phenylketonuria. Thus, the basis of the stimulation of glomerular filtration rate was studied and is reported in a later section of this thesis.

The major result of this study was that the results found in one primate, Macaca mulatta, were confirmed in the intended recipient phenylketonuric man: namely that β-2-thienylalanine would stimulate the urinary excretion of phenylalanine. It thus had potential as a treatment of phenyl-ketonuria.

Discussion

These results confirmed those found in the Rhesus monkey: β-2-thienylalanine increased the excretion of phenylalanine in the urine by decreasing renal tubular absorption of phenylalanine and in 2 or the 3 patients, increasing glomerular filtration rate, as measured by creatinine clearance. In addition, urinary phenylpyruvic and phenyllactic acid excretion increased in each patient. The other phenylketones (phenylacetic, orthohydroxy-phenylacetic) excretion increased in 2 patients but fell in the third.

Further β-2-thienylalanine depressed the level of serum phenylalanine after an oral load of phenylalanine in phenyl-ketonuric patients. This depression lasted for 6 hours and was on average 47.5%.

Lönnerblad (93) has shown that the average time for material to reach the ileo-caecal valve, after ingestion is 178 minutes, with a shorter period for infants and children. Thus, in most cases the phenylalanine swallowed with β-2-thienylalanine would have passed the absorptive surface by 6 hours. Hence, a late rise in serum phenylalanine would not be expected.

As β-2-thienylalanine is absorbed when taken orally and is largely excreted by the kidney (as discussed in Chapter III), renal excretion of phenylalanine would be expected to increase. This, together with the mean 47.5% reduction of phenyl-alanine absorption, suggests that by taking β-2-thienylalanine 20mg/kg with meals, the phenylketonuric patient could consume twice the usual amount of phenylalanine without elevating serum phenylalanine.

This would enable a much more liberal diet and hence relieve many of the tensions presently associated with management of the disease.

(b) Intestinal

Eight mentally retarded, untreated, phenylketonuria patients were used including the three females used for the renal study – as in that study, permission for the study had been granted by parents or their guardians.

On two consecutive days they were fasted for 8 hours before the experiment. Throughout the study period they received no food but were allowed water with cordial flavouring after each blood sampling, if they so desired.

After a control blood sample (0 hours) was collected, the subject drank a load of L-phenylalanine 100 mg/kg with or without 20 mg/kg of β-2-thienylalanine. This was dissolved in an orange cordial drink at a concentration of 20 mg. of phenylalanine per ml. Repeat blood samples were drawn at 1, 1$\frac{1}{2}$, 2 4 and 6 hours after the injection of phenylalanine.

On the first day, half the patients had phenylalanine only and the other 4 the combination of β-2-thienylalanine and phenylalanine. On the next day those who had received phenylalanine only were given the combination and vice versa.

A typical study is shown in the accompanying Figure 12 where β-2-thienylalanine clearly depresses the serum phenylalanine level response to the oral load of 100 mg. of L-phenylalanine. A quantitative value for the elevation of serum phenylalanine above fasting levels during the 6 hours was obtained by calculating the area under the curve above the base line; i.e. the area in square inches on the graph paper which was the same in each instance.

These areas are shown in Table VII. It can be seen that, in each case, the addition of β-2-thienylalanine to the oral phenylalanine reduced the elevation of serum phenylalanine. Using the student "t" test for paired data, this reduction was significant (t = 5.353, p = < .001). The percentage by which the elevation of phenylalanine was reduced by simultaneous ingestion of β-2-thienylalanine was between 11.0% and 70.3% with a mean reduction of 47.5% (Standard Deviation 9.78%).

TABLE VII

Absorption of Phenylalanine After Oral Load of Phenylalanine
100 mg/kg With and Without β-2-thienylalanine

| | | Area (arbitrary units) | | | |
| | | A | B | | |
Subject	Age	Phenylalanine 100 mgm/kg	Phenylalanine 100 mgm/kg+ β-2-T 20mgm/kg	Difference A-B	Percentage Reduction in area
AA	13	606.25	537.5	66.75	11.01
AW	19	794.5	236.5	558.00	70.23
CM	17	803.5	445.75	357.75	44.5
GC	18	703.25	395.5	403.75	57.4
RA	22	350.25	225.0	125.25	35.76
WJ	66	362.5	117	245.5	67.72
FH	49	691.25	375.5	315.75	45.67
FH	39	395.5	230	165.5	41.84
Mean		588.35	320.34	279.78	47.55
S.D.		±32.82	±21.50	±57.56	

B < A p= 0.001, T = 5.353

Elevation of serum phenylalanine following an oral load of phenyl-alanine (100 mg/kg) is expressed in arbitrary units as the area under the curve above base-line phenylalanine levels as shown in Figure 1. The addition of β-2-thienylalanine (20 mgm/kg) constantly and significantly reduced this elevation.

These patients who received this β-2-thienylalanine have been followed for 12 years since receiving the first acute load of β-2-thienylalanine. None have shown any ill effects clinically. Nearly two years later, prior to another study, haemoglobin, red blood cell and white blood cell counts, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin concentration, white blood cell differential; serum electrolytes; plasma urea, glucose, total protein, albumin, globulin, creatinine, serum glutamic oxaloacetic acid transaminase, alkaline phosphatase, gamma glutamyl transpeptidase, lactic dehydrogenase (include isoenzymes), were measured. No consistent or significant deviations were found. These results are shown in Table X.

In addition, the patients showed no clinical disturbance attributable to β-2-thienylalanine in the 12 years following the acute load of this compound.

While these results are comforting, they by no means prove the safety of the compound. Further studies in experimental animals while on chronic dosage extending over several generations (with challenges by pathogenic intestinal organisms), chronic and acute studies in the healthy human volunteers, and, finally, long term exhibition of β-2-thienylalanine in infants and children with phenylketonuria are needed. Such extensive studies are beyond the scope of this thesis but are presently being undertaken by the author or students under his supervision, and are discussed in Chapter XIII.

FIGURE 12   SERUM PHENYLALANINE LEVELS AFTER AN ORAL LOAD OF
100 MG/KG PHENYLALANINE WITH (B) OR WITHOUT (A)
β-2-THIENYLALANINE 20 MG/KG.
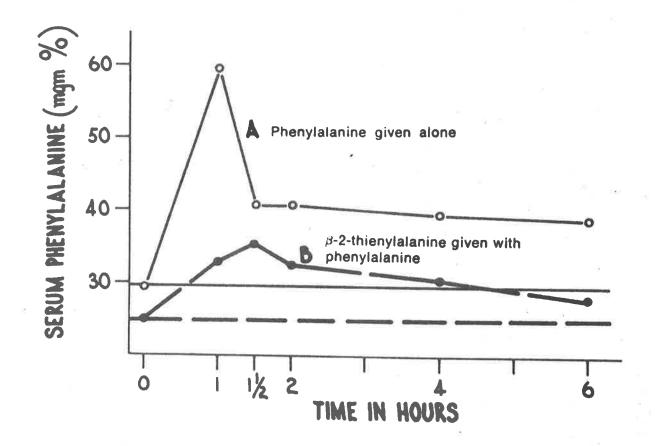
FIGURE 12: TYPICAL SERUM PHENYLALANINE RESPONSE CURVE IN A RETARDED
UNTREATED PHENYLKETONURIC ADULT GIVEN 100 MG/KG OF PHENYLALANINE
ALONE ON ONE DAY (A) AND THE SAME LOAD OF PHENYLALANINE WITH β-2-
THIENYLALANINE (20 MG/KG) ON THE NEXT (B) EFFECTIVE ABSORPTION IS
EXPRESSED AS THE AREA UNDER THE CURVE ABOVE BASELINE FROM 0 TO 6
HOURS. (ON STUDY A FASTING SERUM PHENYLALANINE AT 0 HOURS WAS 29 MG%.
IN STUDY B IT WAS 15 MG%). β-2-THIENYLALANINE SIGNIFICANTLY REDUCES
THE INTESTINAL ABSORPTION OF PHENYLALANINE.

<center>CHAPTER VII</center>

<center>THE EFFECT OF 2 WEEKS OF ORAL CYCLOLEUCINE ON SERUM PHENYLALANINE</center>

<center>LEVELS OF PHENYLKETONURIC PATIENTS</center>

While β-2-thienylalanine had been shown to be effective in reducing serum phenylalanine levels in phenylketonuric patients in an acute load, as a hitherto unused compound in humans, its possible harmful effects were yet to be fully investigated. Because of this uncertainty, it was decided to test cycloleucine, which, although it had a lesser effect on renal excretion of phenylalanine in the monkey, had two advantages: it had been shown to cause an aminoaciduria in man including phenylalanine (48) as well as rats (47), and it has been used as anti-cancer chemotherapy in humans on a long term basis with limited success but, more importantly, little toxicity (94 & 95).

METHODS

6 of the 8 untreated, retarded phenylketonuric patients previously described were entered into the study. 3 were male and 3 female and all estimations were conducted after an 8 hour fast.

For the first two weeks the patients were maintained on their normal diet. On day 1 and day 15 blood was drawn for haematological and biochemical studies (Tables IX,X), and phenylalanine measurement by the method of Wong et al (52). Pilot studies had shown that cycloleucine does not interfere with phenylalanine estimations by this method.

TABLE VIII

Serum Phenylalanine levels in Phenylketonuric Sufferers (on normal diet) on no Treatment for 2 weeks and then on Cycloleucine 20 mg/kg/day for 2 weeks.

SERUM PHENYLALANINE LEVELS MG %

Patient	Age	CONTROL PERIOD							ON CYCLOLEUCINE				
		Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 15	Day 17	Day 19	Day 22	Day 24	Day 26
A.W.	21	26.5	31.0	46.8	42.5	38.6	37.6	48.8	41.7	36.2	33.0	41.5	43.3
C.M.	19	16.3	19.5	24.5	21.0	21.6	18.5	26.2	22.4	23.7	25.7	29.0*	26.5
G.C.	20	32.8	21.3	36.8	42.8	-	-	28.8	29.6	26.5	25.5	21.0	31.7
R.A.	24	25.8	30.6	45.3	38.0	30.0	36.4	34.2	34.8	30.2	33.5	29.0	26.3*
W.J.	68	23.1	33.8	44.5	35.0	30.8	31.8	35.3	44.0	34.6	32.5	34.6	32.5
F.H.	41	25.4	28.7	37.8	30.7	22.7*	27.2	27.2	26.4	23.4	25.3	27.5	32.5
MEAN		25.0	27.5	39.3	31.5	28.7	30.3	33.4	33.1	29.1	29.2	30.4	32.2

MEAN BEFORE CYCLOLEUCINE... | 30.8   MEAN ON CYCLOLEUCINE... | 30.8

* Indicates not Fasting

– Indicates no sample obtained

TABLE VIII: The oral administration of Cycloleucine 20 mg/kg/day does not decrease serum phenylalanine levels. Samples taken on day 29 were unable to be analysed because of spectrofluorophotometer breakdown.

TABLE IX

Haematology of Phenylketonuric Patients before Cycloleucine (Days 1 and 15)
and after two weeks of Cycloleucine 20 mg/kg/day.

PATIENT	DAY	Hb	RBC	PCV	MCV	MCHC	WBC	NEUTRO-PHILS	EOSINO-PHILS	BAS	LYM	MONO
	1	13.1	4.55	41%	89	32%	6.3	50%	12%	1%	29%	8%
A.W.	15	13.0	4.45	38.5%	86	34%	7.8	53%	14%	3%	24%	6%
	29	12.5	4.21	37.5%	87	33.5%	5.9	48%	11%	0	34%	8%
	1	12.7	4.14	39%	90	32.5%	9.7	69%	1%	1%	25%	4%
C.M.	15	11.8	3.81	34%	88	34.5%	8.1	67%	3%	0	21%	9%
	29	12.4	4.28	36%	85	34.5%	11.3	77%	1%	0	17%	5%
	1	14.5	4.71	43%	90	34%	7.8	64%	3%	2%	27%	4%
G.C.	15	14.0	4.75	41.5%	87	33.5%	9.5	78%	2%	1%	16%	3%
	29	13.9	4.81	40%	82	35%	8.5	56%	5%	0	31%	8%
	1	14.0	4.88	45%	91	32.5%	8.2	66%	2%	1%	26%	5%
R.A.	15	14.0	4.76	39.5%	83	35.5%	7.3	62%	3%	4%	28%	3%
	29	13.9	4.89	41%	83	34%	8.2	73%	1%	1%	21%	4%
	1	15.1	5.40	47%	96	32%	6.7	62%	5%	0	20%	13%
W.J.	15	14.8	5.13	46%	89	32%	8.2	63%	7%	0	18%	12%
	29	14.4	5.31	45.5%	84	31.5%	8.1	62%	5%	0	22%	11%
	1	14.9	5.15	44%	84	34%	9.1	53%	21%	1%	19%	6%
F.H.	15	13.5	4.58	39%	85	34.5%	8.1	47%	29%	0	23%	1%
	29	14.1	5.20	41.5%	80	34%	9.3	50%	26%	2%	19%	3%

TABLE IV: Administration of Cycloleucine 20 mg/kg/day has no consistent effect on red or white blood cells. In each patient on all 3 samplings platelets were reported as plentiful.

TABLE X: Serum Biochemistry of Retarded Phenylketonurics on Normal Diet (Days 1–14) and on Normal Diet plus Cycloleucine (20 mg/kg/day) Days 15–29.

Patient	Day	Sodium*	Potassium*	Chloride*	Urea*	Glucose*	Total+ Protein	Albumin+	Globulin+	Creatinine*	G.O.T^u	Alkaline^u Phosphatase	G.O.T^u	L.D.H^u
A.W.	1	144	4.1	103	3.5	4.2	6.6	4.4	2.2	0.070	12	39	5	187
	15	142	4.0	107	3.7	4.1	6.6	4.7	1.9	0.075	6	37	7	239
	29	146	4.2	106	3.7	3.9	6.3	4.2	2.1	0.075	7	34	6	150
C.M.	1	142	4.6	100	4.3	3.7	7.4	4.8	2.6	0.075	12	24	9	218
	15	139	4.1	111	3.2	4.3	7.3	5.0	2.3	0.075	6	21	13	173
	29	140	4.7	99	3.9	4.0	7.3	4.7	2.6	0.085	12	23	7	198
G.C.	1	141	4.5	99	2.7	4.1	7.8	5.1	2.7	0.070	11	18	3	167
	15	137	4.6	101	2.8	4.7	7.5	5.2	2.3	0.075	6	16	2	132
	29	140	4.6	103	2.8	5.6	7.3	4.5	2.8	0.080	11	16	<1	161
R.A.	1	143	4.6	103	5.3	4.1	6.5	4.4	2.1	0.105	18	35	7	191
	15	140	4.3	104	5.3	4.4	6.6	4.6	2.0	0.105	8	31	6	173
	29	142	4.3	103	4.9	4.2	6.6	4.1	2.5	0.100	11	29	6	204
W.J.	1	142	5.0	99	3.7	3.9	7.2	4.4	2.8	0.100	20	33	8	307
	15	143	4.2	106	5.0	3.9	7.1	4.7	2.4	0.100	7	29	9	171
	29	143	4.4	104	5.9	3.8	6.9	4.5	2.4	0.090	10	30	9	210
F.H.	1	142	4.6	101	4.6	3.9	7.2	4.8	2.6	0.075	12	24	9	218
	15	141	4.5	104	3.0	4.8	6.6	4.7	2.3	0.075	6	21	13	173
	29	140	4.5	103	3.8	3.8	6.8	4.7	2.6	0.085	12	23	7	198

TABLE X: Serum biochemistry of phenylketonuric patients in Strathmont Hospital for the Mentally Retarded. Bloods drawn on Day 1 and 15 were before the administration of cycloleucine 20 mg/kg/day. Those on Day 29 were after 2 weeks of cycloleucine. No consistent variations were seen. Many of the lactic dehydrogenase (LDH) values obtained were slightly elevated but these occurred in both the control and experimental periods. Plasma albumin levels on Day 29 were always the same or less than those on Day 15 but the variation was generally less than occurred between Days 1 and 15 when no cycloleucine occurred.

* Units in mM/l.
+ Grams per 100 ml.

u = Units for glutamic-oxaloacetic transaminase (GOT) alkaline phosphatase (international units), gamma glutamyl transpeptidase (GGT) and lactic dehydrogenase (LDH).

On days 3, 5, 8, 10 and 12 blood was drawn for phenyl-
alanine and tyrosine estimation only. On day 15 cycloleucine
was started in a dose of 20mg/kg/day in 3 divided doses immediately
before meals in the form of 200mg capsules, and blood was drawn
for estimations as listed above. On days 17, 19, 22, 24, 26
blood was drawn for phenylalanine and tyrosine. On day 28 blood
was drawn for estimations as on day 1 and 15. Also on days 1,
15 and 28 the patients were weighed.

RESULTS

The phenylalanine levels are shown in Table VIII .
No results were obtained on day 28 because of a malfunction of
the spectrofluorophotometer. However, the haematological and other
biochemical estimations were conducted on that day.

It can be seen that cycloleucine did not reduce serum
phenylalanine compared with the control period. Similarly, no
significant variation between control and cycloleucine period
were noted in haematological, biochemical or patients' weight
data, (Tables IX,X,XI). No clinical abnormality attributable
to cycloleucine was detected at that time, nor in the 10 years
following the administration of cycloleucine.

DISCUSSION

The inability of cycloleucine at this dosage 20mg/day
to reduce serum phenylalanine levels is perhaps not surprising
in view of the minor degree of phenylalaninuria induced by it
in man (Brown (48) and Rhesus monkey (see Chapter III).

TABLE   XI   Body weights of Retarded Phenylketonuric
                  Patients on Day 1 and 15 (before Cycloleucine)
                  and on day 29 after 14 days of Cycloleucine
                  20 mg/kg/day.

	DAY 1	DAY 15	DAY 29
A.W.	41.0	40.7	40.4
C.M.	49.3	48.4	47.1
J.C.	54.4	54.5	55.9
R.A.	52.0	51.7	52.8
W.J.	64.1	63.0	63.3
F.H.	69.5	69.3	69.8

TABLE   XI   Body weights in kilograms show no significant variation
                  between when on cycloleucine (Day 29) when compared to
                  control weights (Days 1 and 15).

The haematological and biochemical data plus clinical observations did not show any toxicity of this compound in the phenylketonuric patients tested. Thus, this compound might be of more value in the aminoacidopathies where the amino acids elevated are markedly excreted in the urine in response to cyclo-leucine, e.g. maple syrup urine disease. From this study cyclo-leucine at the dose of 20mg/kg. would not appear to be of value in the treatment of phenylketonuria.

CHAPTER VIII

THE EFFECT OF β-2-THIENYLALANINE AND OTHER PHENYLALANINE ANALOGUES ON PHENYLALANINE HYDROXYLASE AND PHENYLALANINE METABOLISM *IN VIVO*

As mentioned previously, a difficulty in the interpretation of the effect of β-2-thienylalanine and parachlorophenylalanine on phenylalanine metabolism is the reported effect of them on phenylalanine hydroxylase (37-39, 63). Thus, while these two analogues would tend to lower serum phenylalanine by their renal and intestinal actions, their inhibition of phenylalanine hydroxylase would tend to increase it.

It was, therefore, decided to confirm the inhibition of this enzyme by β-2-thienylalanine and parachlorophenylalanine and to seek possible inhibition by cycloleucine. In addition to studying the effect of these three analogues on phenylalanine hydroxylase, an *in vivo* study of their effect on phenylalanine metabolism in the rat was conducted, the rat being chosen because of its very high phenylalanine hydroxylase content in the liver.(96,97)

METHODS

In vitro phenylalanine hydroxylase activity was measured as described in the analytical methods section. To each of the initial solutions was added either 0.1 ml of physiological saline or 0.1 ml physiological saline containing one of the analogues to give the final following concentrations 1,2,4,8,16 or 32 micromoles per ml. Each concentration of analogue and control saline was compared with an identical homogenate of the same rat liver

and spotted on the same paper. Both the experimental and control samples were counted as a batch. This removed the possibility of error due to biological variation of activity of phenylalanine hydroxylase from one rat liver to another. Such variation was quite evident so that inhibition is expressed as a percentage of control activity. Each strength of inhibition was tested on five different rat livers.

RESULTS

These are shown in Table XII .

It can be seen that cycloleucine did not inhibit phenylalanine hydroxylase except at 16.0 millimolar concentration, and even that inhibition is to a much lesser degree than with the other two analogues, both of which appear to definitely inhibit phenylalanine hydroxylase *in vitro*.

TABLE XII

In vitro inhibition of phenaylalanine hydroxylase

Strength of Inhibitor (millimolar in final solution)	1.0	2.0	4.0	8.0	16.0	32.0
β-2-thienylalanine	+7.5 *	-4.1	-24.0	-11.8	-31.8	-50.9
Parachlorophenyl-ananine	-7.2	-20.0	-28.0	-27.0	-27.0	not soluble
Cycloleucine	+7.4	+8.2	+20.3	+2.3	-11.8	+1.6

* Inhibition expressed as percentage (increase or decrease of radioactive tyrosine production) of value obtained when no inhibition added. Each value is a mean of estimations from five different rat livers.

IN VIVO

Universally labelled ^{14}C phenylalanine was injected intraperitoneally as described in the methods section. Control and experimental (where analogues were added) rats were alternated. The quantity of analogue injected was 150, 300 and 600 mg/kg for both cycloleucine and parachlorophenylalanine corresponding approximately to 1, 2 and 4 millimolar concentration, assuming an even distribution throughout body tissues.

Similarly, the 83, 187, 333 and 667mg. of β-2-thienylalanine corresponded to 1,2,4 and 8 millimolar concentration if distribution throughout tissues was even. As it is very likely that concentration would be higher in hepatic water than in other parts of the rats' tissues, the actual molarity to which phenylalalnine hydroxylase was exposed would probably have been higher.
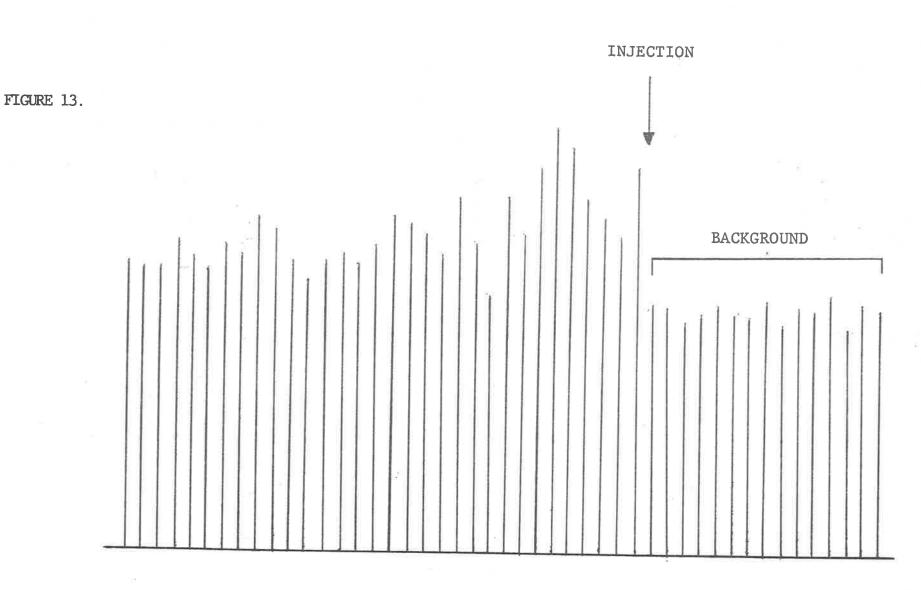
The rats were adult, male, Albino weighing 260g. to 350g. All had food removed for at least 4 hours before testing, but were allowed free access to water. Intraperitoneal injection of universally labelled ^{14}C phenylalanine, with or without the analogue was performed under light ether anaesthesia. The rats were then placed in a flask through which a flow of room air of 6 litres per minute was maintained. The radioactivity of the expired carbon dioxide was measured as described in the analytic methods section.

Five rats were used, each from 3 to 6 times. The amount of carbon dioxide excreted in one hour in arbitrary units expressed as the total excreted in the same units over 24 hours

varied markedly from 30% to 73%. The time of peak radioactive excretion varied in each rat from day to day. However, in all but two instances, the peak excretion occurred before 30 minutes had elapsed. On this data it was decided to measure the total radioactivity excreted over one hour with and without analogues.

RESULTS

These are shown in tabular form (Table XIII). The units are arbitrary and equal the number of mm. of deflection above mean background that the needle-measuring activity deflected. The graphic printout is shown in Figure 13. Because of the direction of rotation of the drum, the graph is read from right to left, i.e. the control period when no radioactivity had been injected precedes the experimental on the right. In fact, there was still some mild elevation above baseline 12 hours after injection. Thus, only one rat per day was used and the machine checked for calibration by a radioactive cobalt source on each day.

FIGURE 13.

INJECTION

BACKGROUND

EXAMPLE OF RECORD OBTAINED BEFORE AND AFTER
INJECTION OF RADIOACTIVE PHENYLALANINE

TABLE XIII

Carbon dioxide production by rats injected with universally labelled
14C phenylalanine with and without an analogue.

ANALOGUE	CYCLOLEUCINE						PARA–CHLOROPHENYLALANINE						β–2–THIENYLALANINE							
Dose in mg/kg.	150 #		300		600		150		300		600		83		167		333		667	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
	60	80	79	83	69	75	89	38*	69	46	41	21+	54	53	62	71	54	69	70	88

C = Radioactive phenylalanine alone injected.

E = Same dose of radioactive phenylalanine plus analogue in dose stated given.

10 control and 10 experimental animals used for each dosage.

* $p < .02$

+ $p < .01$

Comment: Only parachlorophenylalanine significantly reduces radioactive carbon dioxide from ^{14}C phenylalanine in rats.

It can be seen that, as in the *in vitro* experiment, parachlorophenylalanine had a profound effect on phenylalanine metabolism. β-2-thienylalanine did not appear to affect the production of radioactive carbon dioxide from phenylalanine in contrast to its inhibition of phenylalanine hydroxylase *in vitro*. Cycloleucine neither inhibits phenylalanine hydroxylase *in vitro* nor affects the carbon dioxide production from phenylalanine.

DISCUSSION

In phenylalanine there are 6 carbon atoms in the Benzene ring compared to 3 in the side chain. The Benzene ring is not split (a necessary requisite for the carbon atoms to enter the citric acid pathway) until homogenistic acid is converted to maleyl acetoacetic acid. The first step of the pathway from phenylalanine to homogenistic acid is its conversion to tyrosine from phenyalalanine. Here, any inhibition of phenylalanine hydroxylase would be expected to severely reduce the carbon dioxide production from phenylalanine.

Thus, the β-2-thienylalanine results appear anomalous. *In vitro*, this compound inhibits phenylalanine hydroxylase but, *in vivo*, it does not affect carbon dioxide production from phenylalanine. If anything, it stimulates it, but this trend is not statistically significant. However, these results are consistent with those of Uderfriend and Cooper (63) who found that β-2-thienylalanine does inhibit phenylalanine hydroxylase *in vitro*, and Godin and Dolan (60) who reported a small increase in radioactive carbon dioxide from phenylalanine labelled with ^{14}C at the one or three position. Their dose of β-2-thienylalanine

was 10mg. in 150g. rat (67mg/kg) which is smaller than the lowest dose used in our experiment, but they collected carbon dioxide for much longer periods. They suggest that the increased catabolism of phenylalanine is due to β-2-thienylalanine competing with phenylalanine for incorporation into protein. However, they did not demonstrate any consistent effect of β-2-thienylalanine on incorporation of phenylalanine into protein except pancreatic protein.

The difference between the *in vitro* and *in vivo* effects could be explained on the basis of the assay for phenylalanine hydroxylase. In the *in vitro* experiment a homogenate is used in which the hydroxylase is released from cells. If, as in the *in vivo* experiment, β-2-thienylalanine was slow to enter intact cells (a possible explanation for its low incorporation into protein), then little or no effect on phenylalanine hydroxylase might be expected.

These experiments do throw a doubt on the validity of using an experimental animal with any phenylalanine hydroxylase as a long term model of the use of the analogues in phenylketonuria. Cycloleucine, which does not appear to affect phenylalanine hydroxylase *in vitro or in vivo*, would appear to be an exception to this. However, a 2 week trial in phenylketonuric patients of this compound had shown no effect. (Chapter VII).

It therefore appears that β-2-thienylalanine or parachlorophenylalanine would have to be further tested in phenylketonuric man. The known side effects of parachlorophenylalanine

in experimental animals make it unjustified to use this compound in man. (36,38-45) Thus, β-2-thienylalanine, which had an acute effect of increasing renal excretion of phenylalanine and appeared to decrease intestinal absorption of phenylalanine, justified further examination. As this has not been used in man, safety studies would be required. Studies previously described in this thesis indicated two major areas of concern. The first is micro-biological effects, and the second, the basis of increased glomerular filtration rate.

The microbiological studies required are extensive and beyond the scope of this thesis: indeed, they are the subject of a Ph.D. thesis by Mr. K.J. Brown at Auckland University. One published paper has shown that Shigella species are inhibited by β-2-thienylalanine in $vitro$, as are most gram negative organisms, but Salmonella species are not. (Brown, Tannock, Elliott and Lines (98)). However, magnesium levels that could be expected to be found in the human intestine protected the usual micro-biological flora room from inhibition by β-2-thienylalanine.(99)

The basis of the increased glomerular filtration rate was studied and is described in the next section.

CHAPTER IX

THE BASIS OF GLOMERULAR FILTRATION RATE STIMULATION

BY β-2-THIENYLALANINE AND PHENYLALANINE

In 1944 Pitts (64) showed that both infusing glycine and increasing dietary protein elevated glomerular filtration rate. Moustgard (65) showed similar effects by infusing casein hydrolysate and oral protein feeding. Both these workers used dogs. Lindheimer et al. (66), using the same animal, showed that infused amino acids increased glomerular filtration rate in the absence of an expanded extracellular fluid. Hiatt and Hiatt (100) had also shown a stimulation of glomerular filtration by protein feeding in seals at the same time as Pitts.

The work described in Chapter III showed that parachloro-phenylalanine and cycloleucine stimulated glomerular filtration in Rhesus monkeys (Macaca Mulatta), while β-2-thienylalanine increased it in 2 of 3 female phenylketonuric patients, as described in Chapter VI.

It therefore seemed important to study further the effect of β-2-thienylalanine on glomerular filtration rate and the mechanism of the increase in rate, if this occurred. If glomerular filtration rose as a result of higher cardiac output, with increased cardia work, it might preclude the use of β-2-thienylalanine as a treatment of phenylketonuria.

The dog was used for this study because the laboratory the candidate was working in at the time was very experienced in cardiovascular studies in this animal. It was also known that the dog did increase glomerular filtration rate in response to natural amino acids. (64-66)

METHODS

In the first instance, each dog was used as its own control and four experimental groups and one control group (in which no amino acid was infused) of 5 or 6 dogs were chosen. As there were changes with time, it was decided a further experiment with 2.0g% phenylalanine infusion which had shown most effect be conducted with a further 6 dogs to compare the first 90 minutes with the first 90 minutes of the control dogs.

Infusions used in the experimental groups were 0.5g% phenylalanine, 0.5g% of β-2-thienylalanine, 2.0g% phenylalanine, 2.0g% phenylalanine plus 0.5g% β-2-thienylalanine. These solutions were given intravenously in physiological saline (0.9g% Na Cl) containing inulin and para-amino-hippurate to allow determination of glomerular filtration rate and renal blood flow as described in the methods section.

Each dog was prepared in the following fashion: they were premedicated with morphine and anaesthetised one hour later with 50% Nembutal and 50% Dial with Urethrane 1.1ml/kg. This method had been used in over 1,000 dogs in the laboratory for cardiovascular studies and did not cause any significant effect (after the immediate induction period) on cardiac parameters. The following were then inserted into the anaesthetised dog – a cuffed endotracheal tube, Foley indwelling urethral catheter, intravenous drip in the foreleg, bilateral femoral canulae, and a cardiac catheter was advanced into the pulmonary artery. Cardiac output was determined by the cardio-green method of Hetzel et al. (101).

In this experiment phenylalanine was determined by the spectrofluorometric method described in the methods section, as amino acid analysis was not available.

In the initial experiments, using the dog as its own control, three 30 minute control periods were followed by three 30 minute experimental periods. To compensate for possible time changes, a two way analysis (dog/time) was carried out by Spearman's Rank Correlation Coefficient (102). This showed a significant correlation ($p < 0.1$) with time, and thus to determine the statistical significance of the various treatments and time periods Scheffé's method of simultaneous confidence interval was performed on various contrasts (103).

This statistical analysis was conducted by Miss J. Herriman of the Medical Statistics Unit of the Auckland University Medical School.

RESULTS

Table XIV shows glomerular filtration rate in millilitres per minute while Table XV gives the same values expressed on a body weight basis. A two-way analysis showed no overall difference when 0.5g% phenylalanine or β-2-thienylalanine were infused. However, both 2.0g% phenylalanine alone and in combination with 0.5g% β-2-thienylalanine increased glomerular filtration rate (in mls/kg/min). This increase was significant at the less than one percent level.

For 2.0g% phenylalanine infusion and for 2.0g% phenyl-alanine plus 0.5g% β-2-thienylalanine infusion, Scheffés method of simultaneous confidence showed that during the 30 minutes

TABLE XIV   79.

Changes in Glomerular Filtration Rate (ml/min) during three
30 minute control periods; and three 30 minute experimental
periods following addition of Amino Acid to Infusion.

		CONTROL			EXPERIMENTAL		
			NO AMINO ACID				
S.A. m²	WT. kgs.	0-30	30-60	60-90	0-30	30-60	60-90
0.85	18.6	51.6	37.4	41.3	33.5	32.9	32.7
0.84	13.0	61.5	135.0	47.5	83.3	67.8	88.0
0.75	16.2	53.2	61.3	66.8	67.8	56.9	83.2
0.97	22.6	85.3	71.4	68.6	46.6	58.5	70.7
0.68	12.3	48.8	55.6	51.0	50.9	46.5	52.8
0.64	12.3	62.1	42.3	35.8	41.2	39.8	41.5
			0.5 grams % PHENYLALANINE STUDY				
0.78	18.1	62.2	51.3	76.1	103.8	78.3	47.6
1.10	28.1	73.2	89.7	80.3	158.2	177.9	111.7
0.97	24.1	63.2	64.8	56.8	61.2	76.4	80.8
0.64	12.0	51.3	92.1	73.3	91.8	58.1	59.2
0.72	15.9	36.1	39.4	43.2	46.3	48.1	37.1
			0.5 grams % β-2-THIENYLALANINE STUDY				
0.84	18.2	61.9	50.9	50.4	68.3	45.7	66.2
0.90	20.2	68.2	30.8	50.8	72.9	70.5	70.4
0.56	13.0	45.2	22.0	30.0	110.8	29.2	35.2
0.84	18.0	40.5	41.2	60.2	65.1	47.9	47.6
0.78	17.0	58.8	69.7	59.1	52.4	51.1	63.0
			2.0 grams % PHENYLALANINE STUDY				
0.60	14.1	36.0	31.2	20.2	64.2	40.2	43.7
0.92	20.0	52.3	76.6	56.5	78.4	82.2	77.6
0.60	14.2	41.0	39.1	41.0	52.6	39.8	25.5
1.04	24.9	43.8	52.4	65.4	87.7	73.3	72.2
0.84	19.3	28.1	24.2	29.8	57.5	39.0	33.2
1.05	23.1	65.1	71.0	68.0	87.5	75.7	80.1
			COMBINED β2 + PHENYLALANINE STUDY				
0.59	14.0	49.4	40.4	42.4	83.6	54.9	51.3
0.75	16.5	21.5	23.7	28.1	40.5	33.5	30.1
1.05	24.6	50.9	49.3	47.7	50.2	43.0	47.7
0.79	17.2	54.4	50.9	53.5	67.9	57.2	37.8
0.44	10.2	22.4	32.4	32.8	38.6	40.1	34.1
0.97	23.0	49.7	53.9	55.8	66.2	59.1	63.1

TABLE   XV

Changes in Glomerular Filtration rate expressed on a body weight
basis, comparing three 30 minute control periods with 3 similar
experimental periods when amino acid added to infusion.

GLOMERULAR FILTRATION RATE mls/kg/min.

	CONTROL			EXPERIMENTAL		
	0-30	30-60	60-90	0-30	30-60	60-90
	2.77	2.01	2.22	1.80	1.77	1.76
	3.42	7.50	2.60	4.63	3.77	4.89
	3.33	3.83	4.18	4.24	3.56	5.20
	3.77	3.16	3.04	2.06	2.59	3.13
	3.97	4.52	4.15	4.14	3.78	4.29
	5.05	3.44	2.91	3.35	3.24	3.37
MEAN	3.72	4.08	3.19	3.37	3.12	3.67
± 1 SD	0.70	1.71	0.74	1.09	0.73	1.25

0.5 grams % PHENYLALANINE STUDY

	3.43	2.83	4.20	5.73	4.33	2.62
	2.60	3.19	2.86	5.63	6.33	3.98
	2.62	2.69	2.36	2.54	3.17	3.35
	4.27	7.68	6.10	7.65	4.84	4.93
	2.27	2.48	2.72	2.91	3.03	2.33
MEAN	3.04	3.77	3.65	4.89	4.34	3.44
± 1 SD	0.72	1.97	1.38	1.91	1.21	0.94

0.5 grams % β-2-THIENYLALANINE STUDY

	3.40	2.80	2.77	3.75	2.51	3.64
	3.38	1.52	2.52	3.61	3.49	3.49
	3.48	1.69	2.31	8.52	2.25	2.71
	2.25	2.29	3.34	3.62	2.66	2.64
	3.46	4.10	3.48	3.08	3.01	3.71
MEAN	3.19	2.48	2.88	4.52	2.78	3.24
± 1 SD	0.47	0.93	0.46	2.0	0.43	0.46

2.0 grams % PHENYLALANINE STUDY

	2.55	2.21	1.43	4.55	2.85	3.10
	2.62	3.38	2.83	3.92	4.11	3.88
	2.89	2.75	2.89	3.70	2.80	1.80
	1.83	2.18	2.73	3.65	3.05	3.01
	1.46	1.25	1.54	2.98	2.02	1.72
	2.82	3.07	2.94	3.79	3.28	3.47
MEAN	2.36	2.55	2.39	3.76	3.02	2.83
± 1 SD	0.53	0.81	0.65	0.46	0.62	0.81

COMBINED β2 + PHENYLALANINE STUDY

	3.53	2.89	3.03	5.97	3.92	3.66
	1.30	1.44	1.70	2.87	2.03	1.82
	2.07	2.00	1.94	2.04	1.78	1.94
	3.16	2.96	3.11	3.95	3.33	2.20
	2.20	3.18	3.22	3.78	3.93	3.34
	2.16	2.34	2.43	2.88	2.57	2.74
MEAN	2.40	2.47	2.57	3.58	2.93	2.62
±1 SD	0.74	0.61	0.59	1.24	0.86	0.69

TABLE XVI.a

Changes in Cardiac Output (litres per minute)
three 30 minute control periods and three similar
periods after amino acid added to infusion.

CARDIAC OUTPUT L/min.

CONTROL			EXPERIMENTAL		
NO AMINO ACID					
0-30	30-60	60-90	0-30	30-60	60-90
5.48	4.16	4.63	2.40	2.02	2.08
3.47	3.44	3.28	2.67	2.45	2.15
3.70	2.26	2.89	2.47	2.55	2.21
4.41	4.13	3.70	4.13	3.77	2.70
3.19	2.33	1.69	1.46	1.40	1.47
5.75	3.81	2.78	2.63	1.57	1.75
0.5 grams % PHENYLALANINE					
4.16	4.16	1.74	1.50	1.58	1.43
2.93	3.73	3.54	2.94	2.56	2.31
5.55	2.90	3.36	2.78	2.90	3.30
2.45	1.51	1.46	1.48	1.58	2.43
4.85	3.19	2.51	1.78	1.69	2.18
0.5 grams % β-2-THIENYLALANINE STUDY					
1.78	1.54	1.38	1.72	1.74	1.57
2.73	3.02	3.63	2.88	3.08	3.44
2.03	3.03	3.68	1.47	1.74	2.07
2.63	3.17	2.75	2.32	1.96	2.36
3.80	3.32	1.68	1.35	1.17	1.06
2.0 grams % PHENYLALANINE STUDY					
2.31	2.31	1.94	1.42	1.76	1.83
6.31	4.55	3.39	2.69	3.18	3.23
2.22	2.61	2.54	2.36	1.98	1.61
5.83	4.02	2.59	2.42	2.22	2.06
4.50	2.36	2.03	2.08	1.73	2.21
3.43	4.92	2.66	2.04	1.93	2.11
COMBINED β2 + PHENYLALANINE STUDY					
2.51	2.33	1.97	1.72	1.18	1.86
2.47	1.56	1.44	0.89	0.54	0.54
4.67	4.45	4.27	3.88	2.74	2.05
3.56	3.70	2.72	1.69	2.27	1.61
3.70	5.78	2.27	2.18	1.74	1.53
3.96	2.78	2.60	2.19	1.64	1.46

TABLE **XVIb**

Changes in Cardiac Output per kg. body weight
comparing three 30 minute control periods and
three similar after amino acid added to infusion.

CARDIAC OUTPUT mls/kg/min.

| | CONTROL | | | EXPERIMENTAL | | |
| | NO AMINO ACID | | | AMINO ACID | | |
	0-30	30-60	60-90	0-30	30-60	60-90
	295	224	249	129	109	112
	193	191	182	148	136	119
	198	140	178	152	157	136
	195	183	164	183	167	120
	259	189	137	119	114	120
	467	310	226	214	128	142
MEAN	268	206	189	158	135	125
±1 SD	97	58	38	32	21	11

0.5 grams % PHENYLALANINE STUDY

	230	230	96.1	82.8	87.2	79
	104	132	125	104	91.1	82.2
	230	120	139	115	120	136
	204	126	122	123	132	203
	308	201	158	112	106	137
MEAN	215	162	128	107	107	127
±1 SD	66	45	20	13.7	17	45.3

0.5 grams % β-2-THIENYLALANINE STUDY

	97.8	84.6	103	94.5	95.6	86.2
	135	150	180	142	152	170
	156	233	283	113	133	159
	146	176	153	129	109	131
	224	195	98.8	79.4	68.8	62.3
MEAN	152	168	164	111	112	122
± 1 SD	38	45	61	23	29	41.5

2.0 grams % PHENYLALANINE STUDY

	164	164	138	101	125	130
	316	228	170	135	159	162
	156	184	179	166	139	113
	243	168	108	101	92.5	85.8
	233	164	130	113	112	113
	148	213	115	88	84	91
MEAN	210	187	140	117	119	116
± 1 SD	60	25	26	26	26	25

COMBINED β2 + PHENYLALANINE STUDY

	179	166	114	123	84.2	133
	150	94.5	87.2	53.9	32.7	32.7
	190	181	174	158	111	83.3
	207	215	158	98.2	132	93.6
	363	567	223	214	171	150
	172	121	113	95.2	71.3	63.4
MEAN	210	224	149	124	106	99
±1 SD	71	158	44	51	46	41

TABLE XVII

Cardiac Index (litres/min/M^2) comparing three 30 minute
control periods and three similar periods after amino
acids added to infusion.

CARDIAC INDEX

	CONTROL			EXPERIMENTAL	
		NO AMINO ACID			
0-30	30-60	60-90	0-30	30-60	60-90
6.45	4.89	5.45	2.82	2.38	2.45
4.13	4.10	3.90	3.18	2.92	2.56
4.27	3.01	3.85	3.29	3.40	2.95
4.55	4.26	3.81	4.26	3.89	2.79
4.69	3.43	2.49	2.15	2.05	2.16
8.98	5.95	4.34	4.11	2.45	2.73
MEAN 5.51	4.27	3.97	3.30	2.84	2.61
±1 SD 1.73	0.96	0.87	0.72	0.63	0.26
		0.5 grams % PHENYLALANINE STUDY			
5.33	–	2.23	1.92	2.03	1.83
2.66	3.39	3.22	2.67	2.33	2.10
5.72	2.99	3.46	2.87	2.99	3.40
3.83	2.36	2.28	2.31	2.47	3.80
6.74	4.43	3.49	2.47	2.35	3.03
MEAN 4.86	3.29	2.94	2.45	2.43	2.83
±1 SD 1.44	0.75	0.56	0.32	0.31	0.75
		0.5 grams % β-2-THIENYLALANINE STUDY			
2.12	1.83	2.24	1.05	2.07	1.87
3.00	3.40	4.00	3.20	2.42	3.82
3.63	5.41	6.57	2.63	3.11	3.70
3.13	3.77	3.27	2.76	2.33	2.81
4.87	4.26	2.15	1.73	1.50	1.36
MEAN 3.35	3.73	3.65	2.47	2.49	2.71
±1 SD 0.90	1.17	1.61	0.52	0.70	0.97
		2.0 grams % PHENYLALANINE STUDY			
3.85	3.85	3.23	2.37	2.93	3.05
6.86	4.95	3.68	2.92	3.46	3.51
3.70	4.35	4.23	3.93	3.30	2.68
5.61	3.87	2.49	2.33	2.13	1.98
5.36	2.86	2.42	2.48	2.06	2.63
3.27	4.69	2.53	1.94	1.84	2.00
MEAN 4.78	4.08	3.09	2.66	2.62	2.64
±1 SD 1.26	0.69	0.68	0.64	0.64	0.54
		COMBINED β2 + PHENYLALANINE STUDY			
4.25	3.95	3.34	2.92	2.00	3.15
3.29	2.08	1.92	1.19	0.72	0.72
4.45	4.24	4.07	3.69	2.61	1.95
4.51	4.68	3.44	2.14	2.87	2.04
8.41	13.1	5.16	4.95	3.95	3.48
4.08	2.87	2.68	2.26	1.69	1.51
MEAN 4.83	5.15	3.59	2.86	2.31	2.14
±1 SD 1.65	3.6	1.06	1.21	1.01	.94

Renal Plasma Flow in mls/min; combining three
30 minute control periods with three similar
periods after amino acid added to the infusion.

RENAL PLASMA FLOW mls/min.

CONTROL			EXPERIMENTAL		
0–30	30–60	60–90	0–30	30–60	60–90
221	168	180	168	125	155
276	444	180	238	196	213
330	308	289	260	267	299
400	369	370	230	256	199
194	208	165	150	138	139
213	188	174	202	218	156

0.5 grams % PHENYLALANINE STUDY

291	265	246	228	381	160
219	252	209	178	230	225
292	195	189	174	244	303
196	208	203	197	164	183
242	215	276	259	250	202

0.5 grams % β-2-THIENYLALANINE STUDY

190	152	160	159	126	116
255	92	149	210	140	191
110	131	217	291	153	202
150	238	264	318	222	265
197	188	201	181	152	140

2.0 grams % PHENYLALANINE STUDY

240	127	167	320	173	148
230	219	214	209	221	235
130	150	142	161	103	99
266	227	193	262	198	194
209	181	173	213	160	136
254	242	285	282	263	243

COMBINED β2 + PHENYLALANINE STUDY

186	198	242	253	211	196
143	163	218	263	189	187
241	244	252	281	196	179
254	221	232	304	194	175
142	166	170	152	135	128
291	272	261	308	242	220

Renal Plasma Flow in millilitres per kg. body weight
per minute comparing three 30 minute control periods
with three similar periods after amino acid added to
the infusion.

RENAL PLASMA FLOW mls/kg/min.

	CONTROL			EXPERIMENTAL		
	0-30	30-60	60-90	0-30	30-60	60-90
	11.9	9.0	8.6	8.0	6.0	8.3
	15.3	24.7	10.0	13.2	10.9	11.8
	20.6	19.0	17.8	16.0	16.5	18.5
	17.7	16.3	16.4	10.2	11.3	8.80
	15.8	16.9	13.4	12.2	11.2	11.3
	17.3	15.3	14.1	16.4	17.7	12.7
MEAN	16.4	16.8	13.4	12.7	12.3	11.9
±1 SD	2.9	5.1	3.6	3.0	3.9	3.3

0.5 grams % PHENYLALANINE STUDY

	16.1	14.6	13.6	12.6	21.0	8.8
	7.8	9.0	7.4	6.3	8.7	8.0
	12.1	8.1	7.8	7.2	10.1	12.6
	16.3	17.3	16.9	16.4	13.7	15.3
	15.2	13.5	17.4	16.3	15.7	12.7
MEAN	13.5	12.5	12.6	11.8	13.8	11.5
±1 SD	3.2	3.5	4.3	4.3	4.4	2.7

0.5 grams % β-2-THIENYLALANINE STUDY

	10.4	8.35	8.79	8.74	6.92	6.37
	12.6	4.6	7.4	10.4	6.93	9.46
	8.46	10.1	16.7	22.4	11.8	15.5
	8.3	13.2	14.7	17.7	12.3	14.7
	11.6	11.1	11.8	10.6	8.9	8.2
MEAN	10.3	9.48	11.9	14.0	9.4	10.8
±1 SD	1.7	2.9	3.5	5.2	2.3	3.6

2.0 grams % PHENYLALANINE STUDY

	17.0	9.0	11.8	22.7	12.3	10.5
	10.5	11.0	10.7	10.5	11.1	11.8
	9.2	10.6	10.0	11.3	7.3	7.0
	11.1	9.5	8.1	10.9	8.3	8.1
	10.8	9.4	9.0	11.0	8.3	7.0
	11.0	10.5	12.3	12.2	11.3	10.5
MEAN	11.6	10.0	10.3	13.1	9.8	9.2
±1 SD	2.5	0.73	1.5	4.3	1.9	1.9

COMBINED β2 + PHENYLALANINE STUDY

	13.3	14.1	17.3	18.1	15.1	14.0
	8.7	9.9	13.2	11.3	11.5	11.3
	9.8	9.9	10.2	11.4	8.0	7.3
	14.7	12.8	13.5	17.7	11.3	10.2
	13.9	16.3	16.7	14.9	13.2	12.5
	12.7	11.8	11.4	13.4	10.5	9.6
MEAN	12.2	12.5	13.7	14.5	11.6	10.8
±1 SD	2.2	2.3	2.6	2.2	2.2	2.1

TABLE XIX 86.

Phenylalanine reabsorption (% of filtered load) comparing three
30 minute control periods and three experimental periods where
the amino acid (1) indicated was added.

	CONTROL			EXPERIMENTAL		
	0–30	30–60	60–90	0–30	30–60	60–90
	67.0	84.8	94.7	96.9	96.3	96.1
	80.3	89.2	87.9	97.1	96.2	96.0
	90.5	91.0	92.5	90.2	90.1	92.6
	85.6	88.02	91.1	95.6	93.2	93.9
	78.74	66.5	76.2	71.0	61.3	69.6
MEAN	80.5	83.9	88.5	88.2	87.4	89.6
±1 SD	7.9	8.9	6.5	8.4	9.7	8.5

	0.5 grams % PHENYLALANINE STUDY					
	92.1	93.2	93.9	98.8	97.8	98.0
	94.3	80.0	80.1	98.1	98.8	98.0
	93.4	93.9	95.8	98.2	98.4	98.1
	97.6	98.6	98.6	99.6	99.5	99.5
	61.2	60.4	60.4	92.7	96.1	97.0
MEAN	87.7	85.0	85.8	97.4	98.1	98.1
±1 SD	13.4	14.2	14.2	2.4	1.15	0.79

	0.5 grams % β-2-THIENYLALANINE STUDY					
	88.7	91.0	91.3	92.7	92.6	93.9
		83.9	94.2	90.0	91.7	87.1
	97.8	99.6	98.3	99.3	99.3	98.7
	89.7	95.3	95.0	82.6	89.5	
	98.8	99.2	99.1	99.0	99.0	99.2
MEAN	93.8	93.8	95.6	92.7	94.4	94.7
±1 SD	4.6	5.8	2.8	6.2	4.0	4.9

	2.0 grams % PHENYLALANINE STUDY					
	81.9	90.0	80.9	94.3	96.9	97.1
	81.3	93.5	84.1	99.4	99.6	99.4
	88.4	84.1	88.7	99.2	98.6	99.3
	81.8	89.2	90.9	98.1	99.0	99.2
	84.1	78.8	80.2	96.8	97.3	97.9
MEAN	83.5	87.2	85.0	97.6	98.3	98.6
±1 SD	2.63	5.2	4.2	1.9	1.0	0.92

	COMBINED β2 + PHENYLALANINE STUDY					
	92.4	86.2	90.7	99.2	98.9	98.9
	94.3	87.8	94.4	98.8	98.1	98.7
	93.0	96.0	96.1	98.8	98.7	98.8
	96.5	97.7	96.7	99.8	99.7	99.7
	84.0	81.6	81.9	97.4	97.8	98.8
MEAN	92.0	89.9	92.0	98.8	98.6	99.0
±1 SD	4.3	6.1	5.5	0.79	0.66	0.37

TABLE XX　　　　　　　　　　　　　　　　　　　　　　　　　　87.

Stroke Volume in Millilitres comparing three 30 minute
control periods add three similar periods after amino
acids added to infusion.

STROKE VOLUME

NO AMINO ACID

	0-30	30-60	60-90	0-30	30-60	60-90
	73	55	66	34	31	30
	39	23	31	41	31	27
	46	35	48	41	39	37
	59	59	67	75	63	49
	34	39	31	26	28	27
	44	29	40	33	22	21
MEAN	49	40	47	42	36	32
±1 SD	14	14	16	16	13	9

0.5 grams % PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	32	-	32	25	18	24
	45	29	44	53	43	39
	69	45	52	43	39	35
	38	25	24	17	18	26
	54	35	28	24	21	24
MEAN	48	33	36	32	28	30
±1 SD	13	8	10	13	11	6

0.5 grams % β-2-THIENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	36	31	38	34	35	31
	68	86	81	58	62	69
	34	47	49	17	20	19
	48	53	46	39	33	34
	54	42	34	25	26	24
MEAN	48	52	50	35	35	35
±1 SD	12	19	16	14	14	18

2.0 grams % PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	26	31	26	19	22	23
	74	70	57	41	42	46
	30	37	39	34	33	29
	69	57	37	37	32	32
	38	31	29	26	22	23
	49	55	35	34	30	30
MEAN	45	47	37	32	30	31
±1 SD	17	15	10	7	7	8

COMBINED β2 + PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	39	36	33	31	20	31
	25	16	21	12	9	9
	72	63	63	60	42	41
	39	37	36	28	23	29
	31	36	24	23	18	17
	34	25	29	29	25	21
MEAN	40	36	34	31	23	25
±1 SD	15	14	14	15	10	10

TABLE XXI LEFT VENTRICULAR WORK kg-M/min
(To obtain left ventricular work in Watts divide by 6.12)

	CONTROL			EXPERIMENTAL		

NO AMINO ACID

	0-30	30-60	60-90	0-30	30-60	60-90
	10.06	7.64	8.50	4.08	3.21	3.11
	5.43	6.08	5.58	4.56	4.17	3.80
	6.31	4.61	5.90	4.20	5.03	4.21
	6.30	5.62	5.03	6.18	5.64	4.42
	3.82	2.79	1.93	1.67	1.52	1.84
	9.78	5.96	4.35	3.93	2.56	2.98
MEAN	6.95	5.45	5.22	4.1	3.69	3.39
±1 SD	2.4	1.6	2.1	1.32	1.42	0.87

0.5 grams % PHENYLALANINE STUDY

	6.51		2.84	2.55	2.58	2.24
	4.18	5.33	4.81	4.20	3.66	3.46
	9.81	5.32	6.40	4.92	5.13	5.83
	3.17	2.05	2.08	1.91	2.26	3.47
	7.59	4.56	3.75	2.90	2.87	2.73
MEAN	6.25	4.32	3.97	3.30	3.30	3.55
±1 SD	2.4	1.3	1.52	1.10	1.02	1.23

0.5 grams % β-2-THIENYLALANINE STUDY

	2.66	2.41	2.94	2.81	2.96	2.56
	3.90	4.72	6.17	4.90	5.03	5.38
	2.45	4.94	5.51	2.30	2.72	3.15
	3.76	4.32	3.55	3.09	2.18	3.21
	5.58	5.19	2.28	2.02	1.67	1.17
MEAN	3.88	4.31	4.09	3.02	2.91	3.09
±1 SD	0.99	0.99	1.5	1.01	1.15	1.36

2.0 grams % PHENYLALANINE STUDY

	3.93	3.93	3.30	2.51	3.23	3.24
	9.87	7.74	5.30	4.76	5.62	5.49
	3.47	3.90	3.80	3.69	3.37	2.63
	9.12	6.01	3.87	4.11	3.77	3.64
	8.87	4.01	3.45	4.24	3.29	3.91
	4.48	6.42	3.47	2.89	2.83	2.44
MEAN	6.62	5.33	3.86	3.70	3.68	3.72
±1 SD	2.7	1.48	0.62	0.78	0.91	0.88

COMBINED β2 + PHENYLALANINE STUDY

	3.58	3.64	3.08	2.81	1.85	3.04
	3.53	2.02	1.76	1.27	0.80	0.84
	6.35	6.66	6.39	5.80	4.29	3.21
	6.54	7.55	4.44	3.06	4.26	3.28
	5.28	9.43	3.39	3.85	2.96	2.29
	6.19	5.29	4.60	4.32	3.41	3.14
MEAN	5.25	5.77	3.94	3.52	2.93	2.63
±1 SD	1.26	2.46	1.4	1.4	1.26	0.87

TABLE XXII

RIGHT VENTRICULAR WORK kg-M/min

NO AMINO ACIDS

	CONTROL			EXPERIMENTAL		
	0-30	30-60	60-90	0-30	30-60	60-90
	1.27	0.91	0.94	0.49	0.44	0.48
	0.47	0.61	0.45	0.40	0.45	0.38
	0.65	0.40	0.47	0.37	0.28	0.33
	0.30	0.51	0.35	0.42	0.41	0.33
	0.56	0.38	0.18	0.20	0.19	0.20
	0.94	0.62	0.34	0.39	0.23	0.24
MEAN	0.70	0.57	0.46	0.38	0.33	0.34
±1 SD	0.32	0.19	0.26	0.90	0.10	0.90

0.5 grams % PHENYLALANINE STUDY

	CONTROL			EXPERIMENTAL		
	0.62		0.24	0.22	0.28	0.23
	0.28	0.46	0.34	0.32	0.28	0.28
	1.36	0.59	0.69	0.57	0.71	0.81
	0.33	0.21	0.20	0.22	0.26	0.40
	0.79	0.52	0.44	0.24	0.21	0.33
MEAN	0.68	0.45	0.38	0.31	0.35	0.41
±1 SD	0.39	0.14	0.18	0.13	0.18	0.21

0.5 grams % β-2-THIENYLALANINE STUDY

	CONTROL			EXPERIMENTAL		
	0.29	0.25	0.28	0.26	0.31	0.30
	0.41	0.53	0.59	0.58	0.63	0.70
	0.22	0.29	0.25	0.14	0.14	0.17
	0.43	0.52	0.45	0.16	0.32	0.42
	0.59	0.47	0.18	0.18	0.18	0.16
MEAN	0.39	0.41	0.35	0.26	0.32	0.35
±1 SD	0.39	0.12	0.15	0.16	0.17	0.20

2.0 grams % PHENYLALANINE STUDY

	CONTROL			EXPERIMENTAL		
	0.38	0.44	0.32	0.41	0.55	0.60
	1.11	0.87	0.60	0.55	0.69	0.75
	0.51	0.55	0.55	0.55	0.46	0.35
	0.95	0.60	0.42	0.49	0.42	0.42
	0.92	0.39	0.33	0.37	0.35	0.45
	0.51	0.54	0.25	0.19	0.25	0.29
MEAN	0.73	0.56	0.41	0.42	0.45	0.47
±1 SD	0.27	0.15	0.13	0.13	0.14	0.16

COMBINED β2 + PHENYLALANINE STUDY

	CONTROL			EXPERIMENTAL		
	0.24	0.22	0.19	0.21	0.16	0.25
	0.37	0.21	0.16	0.12	0.08	0.09
	0.70	0.54	0.55	0.53	0.41	0.36
	0.58	0.50	0.37	0.28	0.37	0.35
	0.45	0.86	0.28	0.33	0.28	0.27
MEAN	0.47	0.43	0.30	0.29	0.24	0.25
±1 SD	0.15	0.23	0.13	0.13	0.12	0.90

TABLE XXIII   90.

TOTAL PERIPHERAL RESISTANCE DYNES/kg/min.

	CONTROL NO AMINO ACIDS		EXPERIMENTAL		
0-30	30-60	60-90	0-30	30-60	60-90
1969	2594	2330	4163	4629	4227
2649	3020	3046	3742	4078	4832
3621	5304	4148	4045	4544	5063
1903	1935	2160	2129	2332	3539
2205	3018	3972	4598	4567	5002
1737	2412	3306	3343	6108	5709
MEAN 2347	3047	3160	3670	4376	4729
± 1 SD 638	1076	748	789	1110	686

2= 0.5 grams % PHENYLALANINE STUDY

4995		5511	6660	6078	6436
2864	2250	2257	2854	3277	3771
1872	3720	3330	3737	3583	3148
3099	5293	5748	5130	5311	3453
1895	2631	3502	5388	5911	4583
MEAN 2945	3474	4070	4754	4632	4278
± 1 SD 1139	1181	1345	1384	1163	1180

3= 0.5 grams % β-2--THIENYLALANINE STUDY

4939	7922	4889	5576	5742	6109
3074	3043	2752	3469	3114	2672
4921	3165	2389	6252	5282	4324
3191	2521	2761	3376	4077	3386
2313	2768	4757	6512	7172	8294
MEAN 3688	3884	3510	5037	4957	3776
± 1 SD 1058	2031	1082	1354	2027	459

4= 2.0 grams % PHENYLALANINE STUDY

4325	4325	5149	7317	6130	5677
1457	2196	2711	3862	3267	3093
4140	3368	3461	3894	5045	5957
1576	2187	3394	4128	4500	5043
2575	4233	4921	5763	6468	4701
2237	1559	2884	4074	4472	4545
MEAN 2718	2978	3753	4839	4980	4839
± 1 SD 1137	1064	946	1287	999	858

5= COMBINED β2 + PHENYLALANINE STUDY

3343	3945	4665	5576	7789	5156
3397	4867	4995	9429	16280	17020
1711	1976	2059	2266	3354	4483
3031	3240	3526	6290	4859	7440
2268	1659	3873	4766	5741	5746
2321	4025	3996	5292	7456	8649
MEAN 2679	3285	3852	5603	7580	8083
± 1 SD 621	1143	941	2123	4172	4234

TABLE XXIV

TOTAL PULMONARY RESISTANCE DYNES/kg/min.

	CONTROL	NO AMINO ACIDS		EXPERIMENTAL		
	0-30	30-60	60-90	0-30	30-60	60-90
	248	307	259	500	633	653
	230	302	244	329	440	483
	375	460	332	356	251	398
	91	174	151	145	170	265
	326	412	378	547	571	544
	176	252	259	334	560	457
MEAN	240	318	271	369	438	467
±1 SD	94	95	71	130	172	120

0.5 grams % PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	792		459	586	652	671
	191	193	158	217	250	311
	259	413	357	431	496	436
	326	529	543	594	607	395
	198	301	414	449	246	403
MEAN	353	359	387	455	486	443
±1 SD	225	125	130	137	143	121

0.5 grams β-2-THIENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	539	623	468	511	597	713
	322	344	264	416	389	348
	315	185	109	381	276	232
	365	303	349	396	489	440
	242	253	381	592	751	829
MEAN	357	342	314	459	500	512
±1 SD	99	150	122	80	164	224

2.0 grams % PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	415	484	494	118	104	105
	165	246	306	446	402	421
	612	459	503	576	686	794
	165	219	370	495	504	582
	266	406	472	500	693	542
	256	130	210	274	373	378
MEAN	313	324	393	402	460	470
±1 SD	158	132	108	157	202	211

COMBINED β2 + PHENYLALANINE STUDY

	0-30	30-60	60-90	0-30	30-60	60-90
	223	241	284	418	677	430
	356	512	444	898	1628	1776
	188	162	170	206	321	507
	269	211	294	567	432	794
	190	152	317	403	551	679
	182	201	215	292	341	493
MEAN	235	246	287	464	658	837
±1 SD	62	122	86	224	450	486

immediately after the amino acids were started, there was a significantly higher glomerular filtration rate than for the control periods as a group. This first 30 minute experiment period was also significantly higher than the immediately preceding control 30 minute period. The last two periods (1 hour) of the experimental period were not significantly different from the 3 control glomerular filtration rates.

The same method of analysis showed that the first 30 minutes of the experimental group demonstrated a significantly higher glomerular filtration rate than the last half hour or the last hour of the experimental infusion. It therefore appears that there was an initial increase in glomerular filtration rate after the amino acid was added to the infusion, but that this falls away with time.

Tables XVI to XXIV show cardiac output, cardiac index, renal plasma flow, tubular reabsorption of phenylalanine, stroke volume, left ventricular work, right ventricular work, total peripheral resistance and total pulmonary resistance, and these results will be discussed as a group.

There is a fall in cardiac output to almost half the value of the first control period; however, a similar trend is seen in those dogs which received no amino acid and statistical analysis by both two-way variance and Chi squared show no significance. Similarly, cardiac index and stroke volume did not fall at a greater rate after amino acid infusion than in dogs that did not have amino acids in the last 90 minutes.

When inspecting the renal plasma flow as shown in Tables XVIIIa & XVIIIb, it is clear that there is a slight tendency for this to fall with time in the dogs that received no amino acid, and this trend is not altered by the addition of 0.5g% of phenylalanine to the infusate. With the other three amino acid infusions (β-2-thienylalanine 0.5g%, 2.0g% phenylalanine and phenylalanine 2.0g% plus β-2-thienylalanine 0.5g%) there is a rise in renal plasma flow immediately after the amino acid is added. This corresponds to the period when glomerular filtration rate increased most. However, statistical analysis showed no significant rise at this time.

Examination of the filtration fraction in Table XXV shows that while the value tended to rise with addition of amino acid to infusate, this was of much smaller degree than the rise in glomerular filtration rate. It also tended to rise with time in the dogs into which no amino acid was infused. Statistical analysis of filtration fraction showed a significant difference over time in all infusates (including when no amino acid added) except when 0.5g% β-2-thienylalanine was added. However, no pattern in change when amino acid was added could be detected.

Thus, it can be concluded that glomerular filtration rate does increase significantly, albeit temporarily, with the addition of 2.0g% phenylalanine to the infusate with and without 0.5g% of β-2-thienylalanine. This rise is accompanied by a rise in renal plasma flow and filtration fraction which, however, is not statistically significant. This suggests the possibility

TABLE XXV

Filtration Fraction comparing three control periods of 30 minutes
with three similar periods after amino acids added to infusion.

	CONTROL			EXPERIMENTAL		
	0-30	30-60	60-90	0-30	30-60	60-90
	NO AMINO ACIDS					
	0.23	0.22	0.26	0.23	0.30	0.21
	0.22	0.30	0.26	0.35	0.35	0.41
	0.16	0.20	0.23	0.27	0.22	0.28
	0.21	0.19	0.19	0.20	0.23	0.36
	0.25	0.27	0.31	0.34	0.34	0.38
	0.29	0.22	0.21	0.20	0.18	0.27
MEAN	0.23	0.23	0.24	0.26	0.27	0.32
	0.5 grams % PHENYLALANINE STUDY					
	0.21	0.19	0.31	0.46	0.21	0.30
	0.33	0.36	0.38	0.89	0.77	0.50
	0.22	0.33	0.30	0.35	0.31	0.27
	0.26	0.44	0.36	0.47	0.35	0.32
	0.15	0.18	0.16	0.18	0.19	0.18
MEAN	0.23	0.30	0.30	0.47	0.37	0.31
	0.5 grams % β-2-THIENYLALANINE STUDY					
	0.33	0.33	0.32	0.43	0.36	0.57
	0.27	0.33	0.34	0.35	0.50	0.37
	0.41	0.17	0.14	0.38	0.19	0.17
	0.27	0.17	0.23	0.20	0.22	0.18
	0.30	0.37	0.29	0.29	0.34	0.45
MEAN	0.32	0.27	0.26	0.33	0.32	0.35
	2.0 grams % β-2-THIENYLALANINE					
	0.15	0.25	0.12	0.20	0.23	0.30
	0.23	0.35	0.26	0.38	0.37	0.33
	0.32	0.26	0.29	0.33	0.39	0.26
	0.16	0.23	0.34	0.33	0.37	0.37
	0.13	0.13	0.17	0.27	0.24	0.24
	0.26	0.29	0.24	0.31	0.29	0.33
MEAN	0.21	0.25	0.24	0.30	0.31	0.31
	COMBINED β-2-THIENYLALANINE (0.5g%) AND PHENYLALANINE (2.0g%)					
	0.27	0.20	0.18	0.33	0.25	0.26
	0.15	0.20	0.13	0.15	0.18	0.16
	0.21	0.20	0.19	0.18	0.22	0.27
	0.21	0.23	0.23	0.22	0.29	0.27
	0.16	0.20	0.19	0.25	0.30	0.27
	0.17	0.20	0.21	0.21	0.24	0.29
MEAN	0.19	0.20	0.19	0.22	0.25	0.24

of shunting of renal blood flow, as cardiac output, cardiac index and stroke volume do not show any significant difference that could not be explained by time alone.

While it is not possible to determine such changes of flow within various areas of the kidneys, it is possible to measure both peripheral and pulmonary resistance. If these altered substantially it might be reasonable to infer an alteration in renal vascular resistance. Tables XXIII and XXIV show that both these rose with time. Statistical analysis shows that there were no significant differences in those dogs infused with 2.0g% phenylalanine (with or without β-2-thienylalanine) when compared to the dogs that received no amino acid during the experimental period.

One of the purposes of this study was to see if the increased glomerular filtration rate induced by amino acids and β-2-thienylalanine in particular was associated with any deleterious effects such as increased cardiac work. It has already been seen that cardiac output did not increase, and in Tables XXI and XXII it can be appreciated that neither left nor right ventricular work increased as a result of the amino acid infusion.

Finally, the opportunity was taken to measure the effect of β-2-thienylalanine on the tubular reabsorption of phenyl-alanine.

As expected, there was a high rate of phenylalanine reabsorption which tended to increase with time in those animals

that received no amino acid in the experimental period. The addition of phenylalanine at 0.5g% had no effect on this trend, while at 2.0g% the effect was damped. β-2-thienylalanine alone, or in combination with phenylalanine, did not block the reabsorption of phenylalanine. Thus, in the dogs tested in this way β-2-thienylalanine did not block renal tubular reabsorption of phenylalanine. Because the effect of the passage of time appeared more pronounced than the addition of any amino acid, it was decided to infuse 6 dogs with 2.0g% phenylalanine from the outset, and to compare them with the 6 control dogs from the previous study, using the 3 first half hour periods. 2.0g% phenylalanine was chosen because it had caused the most marked deviations from control trends in the previous study.

Glomerular filtration rates in mls/min are shown in Table XXVI and in Table XXVII. This value is shown as a function of body weight.

No significant differences can be detected when glomerular filtration is expressed as a function of body weight which is in contrast to when the dogs were used as their own controls; in that experiment when 2.0g% of phenylalanine was added after 90 minutes, a significant but transitory rise in glomerular filtration rate was seen.

This difference is probably due to the high individual variations between the glomerular filtration rates of each dog, even when this value is expressed as a function of body weight. The dogs were not selected by breed so this is perhaps not surprising.

TABLE XXVI

Glomerular Filtration Rate in 6 Dogs infused with
2.0g% Phenylalanine in 3 thirty minute periods compared
with control animals receiving Intravenous Saline only.

GLOMERULAR FILTRATION RATE mls/minute

Exp. Wt.kgs.	Exp. 0-30	(con) mins.	Exp. 30-60	(con) mins.	Exp. 60-90	(con) mins.
12.7	39.0	(51.6)	31.5	(37.4)	36.6	(41.3)
13.7	31.5	(61.5)	76.9	(135)	59.0	(47.5)
20.0	78.5	(53.2)	52.9	(61.3)	56.2	(66.8)
37.6	61.5	(85.3)	63.7	(71.4)	63.2	(68.6)
19.3	77.2	(48.8)	116	(55.6)	97.7	(51.0)

TABLE XXVII

Glomerular Filtration Rate, expressed per kg. body
weight in 6 Dogs Infused with 2.0g% Phenylalanine
in 3 thirty minute periods compared with Control
Animals receiving Intravenous Saline only.

GLOMERULAR FILTRATION RATE mls/kg/minute

	Experimental	Control	Experimental	Control	Experimental	Control
	0-30 minutes		30-60 minutes		60-90 minutes	
	2.99	(2.77)	2.48	(2.01)	2.89	(2.22)
	2.30	(3.42)	5.61	(7.50)	4.31	(2.60)
	3.93	(3.33)	2.65	(3.83)	2.81	(4.18)
	1.64	(3.77)	1.69	(3.16)	1.68	(3.04)
	4.00	(3.97)	6.01	(4.52)	5.06	(4.15)
	2.04	(5.05)	1.88	(3.44)	1.79	(2.91)
MEAN	2.81	3.72	3.39	4.08	3.09	3.19
±1 SD	0.91	0.77	1.75	1.87	1.24	0.81

No significant differences in cardiac output, renal plasma flow, stroke volume or ventricular work were seen. (Tables XXVIII to XXXI). However, percentage phenylalanine reabsorption was markedly increased when 2.0g% phenylalanine was infused initially, compared to those animals that received saline only. (Table XXII)

DISCUSSION

The study described in this section set out to examine the basis of the increase in glomerular filtration rate caused by the infusion of phenylalanine and/or β-2-thienylalanine. In the dogs used, it was found that a significant, albeit transient, rise did occur when amino acid in the form 2.0g% phenylalanine was added to the infusate after 90 minutes. A similar result was obtained when the combination of 2.0g% phenylalanine plus 0.5g% β-2-thienylalanine was added at the same time. However, neither 0.5g% phenylalanine or β-2-thienylalanine caused a significant rise in glomerular filtration rate but a similar trend of a brief rise in glomerular filtration rate was observed.

These changes in glomerular filtration rate were accompanied by rises in renal plasma flow and filtration fraction not associated with increased cardiac output or ventricular work. This suggests that the short-lived stimulation of glomerular filtration rate was probably due to effects upon the renal vasculature. Certainly, the findings do not preclude the use of β-2-thienylalanine on the basis of undesirable cardiovascular side-effects.

TABLE XXVIIIa

Renal Plasma Flow in mls/min in 6 dogs infused with 2.0g% Phenylalanine compared to those infused receiving saline: three thirty minute periods used.

Exp. 0-30	(con)	Exp. 30-60	(con)	Exp. 60-90	(con)
151	221	155	168	113	180
214	276	105	444	172	180
259	330	287	308	221	289
345	400	219	369	256	370
735	194	1022	208	686	165
738	213	266	188	327	174

No significant differences.

TABLE XXVIIIb

Renal Plasma Flows as a function of Body Weight (mls/kg/min) in 6 dogs infused with 2.0g% Phenylalanine compared to control dogs receiving Saline. Renal Plasma Flow mls/kg/min.

	Exp. 0-30	(con)	Exp. 30-60	(con)	Exp. 60-90	(con)
	11.9	11.9	12.2	9.0	8.9	8.6
	15.6	15.3	7.7	24.7	12.6	10.0
	13.0	20.6	14.4	19.0	11.1	17.8
	9.2	17.7	5.8	16.3	6.8	16.4
	38.1	15.8	53.0	16.9	35.5	13.4
	30.4	17.3	10.9	15.3	13.5	14.1
MEAN	19.7	16.4	17.3	16.8	14.7	13.4
± 1 SD	10.7	2.9	16.2	5.1	9.6	3.6

No significant differences.

TABLE XXIXa

Cardiac Output in litres per minute in
6 dogs Infused with 2.0g% Phenylalanine
in 3 thirty minute periods compared with
Control Animals receiving Intravenous
Saline only.

CARDIAC OUTPUT litres/minute

Exp. 0-30	(con)	Exp. 30-60	(con)	Exp. 60-90	(con)
2.10	5.48	1.79	4.16	1.28	4.63
4.31	3.47	3.53	3.44	2.39	3.28
5.21	3.70	4.62	2.26	2.10	2.89
4.24	4.41	2.83	4.13	2.70	3.20
3.49	3.19	3.74	2.33	3.45	1.69
2.24	5.75	2.75	3.81	2.28	2.78

No significant differences

TABLE XXIXb

Cardiac Output as function of body weight
(mls/kg/min) in 6 dogs infused with 2.0g%
Phenylalanine compared to control dogs
receiving Saline

	Exp. 0-30	(con)	Exp. 30-60	(con)	Exp. 60-90	(con)
	151	221	155	168	113	118
	214	276	105	444	172	190
	159	330	287	308	221	189
	345	400	219	369	256	370
	735	194	1022	208	686	165
	738	213	266	188	327	174
MEAN	188	268	169	206	121	189
±1 SD	78	97	65	58	41	38

No Significant Differences

TABLE   XXIXc

Cardiac Stroke volume of 6 dogs infused with 2.0g% Phenylalanine
compared to 6 control dogs receiving Saline: 3 thirty minute
periods used.

	Exp.	(con)	Exp.	(con)	Exp.	(con)
	0-30		30-60		60-90	
	35	73	36	55	38	66
	54	39	50	23	43	31
	65	46	51	35	21	48
	65	59	47	59	49	67
	39	34	39	39	43	31
	43	44	55	29	33	40
MEAN	50	49	46	70	38	47
± 1 SD	12	14	6.7	14	9	16

No significant differences.

TABLE   XXX

Left ventricular work of 6 dogs infused with 2.0g%
Phenylalanine comparing 3 thirty minute periods with those
of 6 control dogs infused with Saline only.

	Exp.	(con)	Exp.	(con)	Exp.	(con)
	0-30		30-60		60-90	
	2.97	10.06	2.82	7.64	2.02	8.50
	2.81	5.43	2.11	6.08	1.43	5.58
	8.22	6.31	7.50	4.61	3.08	5.90
	7.15	6.30	4.80	5.62	4.70	5.03
	5.30	3.82	4.60	2.79	4.30	1.93
	3.17	9.78	3.74	5.96	2.85	4.35
MEAN	4.9	6.95	4.26	5.45	3.06	5.22
± 1 SD	1.76	2.5	1.73	1.6	1.16	2.1

TABLE XXXI

Right Ventricular work of 6 dogs infused with
2.0g% Phenylalanine comparing 3 thirty minute
periods with those of 6 control dogs infused
with Saline only.

	Exp.	(con)	Exp.	(con)	Exp.	(con)
	0–30		30–60		60–90	
	0.32	1.27	0.27	0.91	0.21	0.94
	0.41	0.47	0.34	0.61	0.16	0.45
	0.43	0.65	0.63	0.40	0.29	0.47
	0.92	0.30	0.65	0.51	0.59	0.35
	0.57	0.56	0.56	0.38	0.52	0.18
	0.24	0.30	0.30	0.62	0.22	0.34
MEAN	0.48	0.70	0.46	0.57	0.33	0.46
± 1 SD	0.22	0.32	0.16	0.19	0.16	0.26

No significant difference.

TABLE XXXII

Percentage reabsorption of phenylalanine from glomerular
filtrates of 6 dogs infused with 2.0g% phenylalanine,
comparing 3 thirty minute periods with those of 6 control
dogs infused with saline only.

PERCENTAGE OF PHENYLALANINE REABSORBED

	0-30 minutes		30-60 minutes		60-90 minutes	
DOG	Control	Experimental	Control	Experimental	Control	Experimental
1	67.6	98.9	90.5	99.8	78.7	99.3
2	84.8	99.6	91.0	99.9	66.5	99.7
3	94.7	99.7	92.5	99.9	76.2	99.7
4	80.3	99.5	85.9	96.3	No data	99.9
5	89.2	99.8	88.0	99.3	No data	99.4
6	87.9	99.9	91.1	99.5	No data	99.5
MEAN	84.1	99.6	89.8	99.1	73.8	99.6
± 1 SD	14.3	2.48	9.8	0.98	8.1	0.67

The phenylalanine reabsorption was significantly higher ($p < 0.001$)
when phenylalanine was infused.

The comparison of dogs infused from the onset with 2.0g% phenylalanine against those which had no amino acid added to the infusate showed no significant changes due to high individual variation, with one exception: phenylalanine reabsorption by the renal tubule was increased when 2.0g% phenylalanine was infused. In the author's opinion this result is probably spurious. It is noted from Tables XIX and XXXII that percentage reabsorption is always higher when serum phenylalanine was rendered higher by the addition of phenylalanine. This suggests that at low levels of phenylalanine in the urine the spectroflurometric method measured falsely high in quantitating phenylalanine. Certainly, the result is incompatible with all other studies where it was found that tubular phenylalanine reabsorption remained relatively consistent for any one animal, no matter what serum level of phenylalanine was present.

This finding does not invalidate the results where urinary phenylalanine was measured in previous chapters because, in those instances, serum and urinary levels of phenylalanine were high. In addition, the measurements done in Wisconsin were by amino acid analyser, a method known to give satisfactory results both in that laboratory and elsewhere.

The primary purpose of this study was to determine whether β-2-thienylalanine might cause undesirable cardiovascular effects. Such undesirable effects were not seen and, thus, this compound appears safe to use in phenylketonuria from the cardiovascular aspect.

CHAPTER X

INHIBITION OF PHENYLALANINE TRANSPORT IN ISOLATED BRUSH BORDER
VESICLES IN THE RAT PROXIMAL TUBULE BY β-2-THIENYLALANINE,
CYCLOLEUCINE AND L-PHENYLALANINOL

The work described in this Chapter was initially
conducted at the Department of Biochemical Genetics, McGill Uni-
versity, Montreal under the auspices of Professor Charles Scriver
and later at the Division of Human Nutrition, Commonwealth Scientific
Industrial Research, Glenthorne, South Australia while the candidate
was employed at Flinders University. (The candidate chose to
work at the Division of Human Nutrition because of the facilities
that were readily available to him there, and because there was
a long-standing cooperative research effort in other areas between
the Department of Paediatrics at Flinders University and the
Division of Human Nutrition, C.S.I.R.O.)

The candidate is grateful for the instruction in
brush border techniques given by Susan Tanenhouse in the Division
of Biochemical Genetics, Montreal. However, all the experiments
described herein are my own work, except for the enzyme assays
for which I received technical assistance from Lilliana Lattanzio
as it was impossible to conduct both the brush border experiment
and measure enzymes on freshly prepared samples.

The concept that β-2-thienylalanine can inhibit the
renal tubular reabsorption of phenylalanine has been challenged.
Previous work (Ch.III,VI,IX), has shown conflicting results with
glomerular filtration rate being increased in some species by
β-2-thienylalanine, but not others. Yet, in all, renal excretion
of phenylalanine is increased when β-2-thienylalanine is given

concurrently with phenylalanine.

Cycloleucine presents a similar picture with it being an agent that increases phenylalanine concentration in the urine of man (47,48) yet as shown in Chapter III, it did not decrease renal tubular reabsorption of phenylalanine. Unfortunately, it did not appear to reduce serum phenylalanine levels in phenyl-ketonuric patients. (Chapter VII)

Parachlorophenylalanine was in no way superior to these two agents in promoting renal excretion and, as it has shown itself to produce unacceptable side-effects in a number of species (40-45), it was no longer investigated. However, Shimomura et al. (105), have shown that phenylalaninol (Figure 14) is an agent that reduces effective intestinal absorption of phenyl-alanine in the rat, and reduces the absorption of phenylalanine by the everted gut sac of the Wistar rat. As jejunal transport and proximal renal tubular transport of amino acids show many similar characteristics, phenylalaninol was included rather than parachlorophenylalanine in these studies.

METHODS

Brush border membrane vesicles were prepared by the method of Booth and Kenny (53) and phenylalanine transport in them measured by the technique of Evers, Murer and Kinne (54). As did Booth and Kenny, we checked that brush border membranes had been obtained by enzyme analysis, but the techniques used and enzymes chosen were different.

To measure a brush border associated enzyme alkaline phosphatase was chosen. To measure an intracellular enzyme succinate-cytochrome C reductase was determined, and for the

FIGURE 14 :   PHENYLALANINOL
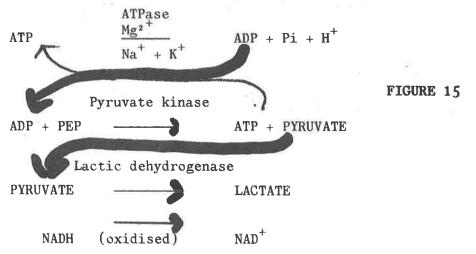
FIGURE 14:   THE STRUCTURE OF PHENYLALANINOL (L-2-AMINO-3-
PHENYL-1-PROPANOL)

basolateral (plasma) membrane F1-ATPase was chosen and, on some occasions, Na-K ATPase as well.

In all cases, the enzyme was measured in the initial homogenate and on an aliquot of the brush border preparation, which was actually used for determining the transport of phenylalanine.

Na-K ATPase was measured by doing three determinations of the amount of NAD generated by the homogenate from Tris ATP. The first determination (A) was in the absence of sodium, the second $(B)^+$ in the presence of sodium but with the inhibitor ouabain added and, finally, (C) total activity was measured with sodium present and ouabain absent. In this manner Na-K ATPase activity could be measured, as could potassium stimulated Mg ATPase. *

---

* The reaction involves the cyclic regeneration of adenosine triphosphate (ATP) via pyruvate kinase (PK) in the presence of phospho-enol-pyruvate (PEP). The assay is linked to the oxidation of NADH via lactic dehydrogenase (LDH). The end result is the conversion of ATP by ATPase with NADH oxidation (see Figure 15)

FIGURE 15

In solution (C) the total activity is measured (by decreasing absorbance as the oxidation of NADH to NAD is measured). In solution (B) ouabain is present and this is a specific inhibitor of Na-K ATPase and thus any rate obtained is due to Mg^{2+} ATPase. In solution (A) there is no sodium and the first step requires both sodium and potassium. Thus the rate obtained is due to sodium insensitive ATPase activity. *

Calculation is done by subtracting the rate obtained when ouabain is in the solution (solution (B)) from the total rate (solution (C)) as a measure of magnesium ATPase activity. This value is then subtracted from the total rate (solution (C)) and a value obtained for the ouabain sensitive ATPase which is Na-K ATPase. The sodium sensitive rate is calculated in the same fashion except rate used in solution (A) is measured instead of that in (B). This also is a measure of Na-K ATPase.

* See footnote next page.

To 500 ul of each of the soluctions (A), (B) and (C),[+] was added an appropriate quantity of homogenate (usually 20 or 40 ul), the content of the vial mixed and incubated at 38° C for 2 minutes. To this was added 14.3ul of Tris-ATP 60mM which gives a final concentration of 1.5mM.

The NAD generated was measured at 340nM in a Gilford spectrophotometer.

Alkaline phosphatase was measured by using p-nitrophenyl phosphate as substrate which is hydrolysed by alkaline phosphatase to p-nitrophenol and phosphate. While the substrate p-nitrophenyl phosphate is colourless, p-nitrophenol absorbs light strongly at 405nM. The absorbance rate is directly proportional to the amount of alkaline phosphatase in the homogenates.

450ul glycylglycine buffer made as indicated above but with pH adjusted to 8.0 had 20ul of homogenate added (if this did not give a sufficient reaction the quantity was doubled), mixed and incubated at 38° C for 2 minutes. To this was added 10ul of 2mM p-nitrophenyl phosphate and the absorbance measured immediately at 405nM using a blue Tungsten lamp in a Gilford spectrophotometer.

+ Solution (B) (total ATPase) contains (in assay tube) lactic dehydrogenase 14.3 U/ml, pyruvate kinase 23.3 U/ml, phosphenopyruvate 3.14mM, sodium chloride 80mM, ouabain 2mM, nicotinamide adenosine dinucleotide reduced form (NADH) 0.28mM in a glycylglycine buffer which gives a final concentration in the assay tube of glycylglycine 100mM magnesium sulphate $7H_2O$ 2mM, H_4 EDTA 0.2mM, potassium chloride 20mM and sucrose 250mM - this buffer is adjusted to pH7.6 with IM Tris before being used for solution (C). Solution (A) is the same as (C) with the sodium chloride being omitted and replaced with water. Solution (C) is the same as (B) with ouabain omitted and replaced with water.

Succinate cytochrome C reductase was measured following bursting open the mitochondria with potassium phosphate-potassium cyanide in the presence of the mitochondrial enzyme succinate cytochrome C reductase and succinate. It absorbs at 550nM.

To 450µL of potassium phosphate-potassium cyanide mixture [#] the homogenate was added, (initially 10µl but adjusted to obtain a sufficiently measurable response). This combination was mixed and incubated at $38^{o}C$ for 2 minutes. Then 10µl of potassium succinate 0.5M in 50mM potassium phosphate (pH 7.2) was added followed by 80µl of cytochrome C 80mg/ml in the same potassium phosphate buffer. The optical density was immediately measured in the visible light of 550nM in a Gilford spectrophotometer.

F1-ATPase was determined by measuring total conversion of NADH to NAD and by measuring the quantity that was not inhibited by oligomycin. (2 µl added to each assay of 4mg/ml dyomycin in 0.4% ethanol).

To a F1-ATPase mix containing lactic dehydrogenase, pyruvate kinase and potassium cyanide (as in succinate cytochrome C reductase assay) was added sodium ATP as substrate to obtain a final concentration of 2mM. this was incubated at $38^{o}C$ for 2 minutes following mixing. The homogenate was added in sufficient quantity to allow effective measurement; usually 20µl was sufficient.

[#] Potassium cyanide mixture is identical to solution (C) of the Na-ATPase estimation with the addition of 60 µl of potassium cyanide (3.3mg/ml) to each 12 mls of it.

The optic density was measured at 340nM using a UV filter and Tungsten Blue lamp in a Gilford spectrophotometer.

Assuming the oligomycin completely inhibited F1-ATPase the quantity of this enzyme could be determined.

The enzyme activities were converted to specific activities by expressing them as μ mole of product formed per hour per mg. of protein. Protein determinations were made on aliquots of the original homogenate and the brush border preparation. In this way, the purification of the various cellular fractions could be determined. For example, in the experiment illustrated in Figure 3, the specific activity of alkaline phosphatase increased from 19.96 in crude homogenate of 96.50, a relative enrichment of 11.6, suggesting that the brush border component had been increased by at least this amount, the succinate cytochrome C reductase specific activity fell from 6.36 in the crude homogenate to 0.41, suggesting that nearly all mitochondria had been removed, while the F1- ATPase altered little - 8.19 in homogenate and 9.57 in brush border preparation - this enzyme is associated with the baso-lateral membrane which presumably was little altered by the purification step.

The Na-K ATPase was not measured in this particular experiment but in others it paralleled changes in F1-ATPase.

The mean changes in these enzymes throughout all these experiments expressed on enrichment factor (enzyme activity in brush border preparation/enzyme activity in crude homogenate are shown in Table XXXIII.

The above studies strongly suggested that our preparations of renal tubular cells were purified usually five fold

TABLE XXXIII

Enrichment of Enzymes Associated with Subcellular Functions during
Purification of Brush Borders from Rat Kidney

Enzyme Specific Activity

	Alkaline Phos-phatase	F_1ATPase	Succinate Cytochrome C Reductase	Na/K AtPase
Crude Homogenate	14.2 (2.98–31.0)	17.3(11.4–37.1)	15.8(4.04–48.0)	18.7(2.11––33.1)
Brush Border Preparation	42.4 (19.3–75.2)	14.4(3.54–45.9)	0.556(0.05–1.21)	26.8(3.87–72.3)
Ratio of Brush Border Preparation: Crude Homogenate	5.2 (1.17–10.3)	0.922(0.43–1.9)	0.049 (.003–.145)	1.41(0.21–2.18)

Table XXXII: Figures expressed are units of enzyme activity/mg of protein. Figures in brackets ( ) represent the range. For Alkaline Phosphatase and F_1 ATPase 8 experiments are represented, for Succinate Cytochrome C Reductase 7, and for Na/K ATPase 3. In every case the ratio of Alkaline phosphatase brush border to crude homogenate rats was greater than 1, indicating enrichment by the brush border. Succinate Cytochrome C Reductase was always markedly reduced, indicating clearance of mitochondria. F_1ATPase showed inconsistent purification/removal.

in respect of brush border membranes and, therefore, any effect of the analogues detected would be a reflection of their action at the brush border of the renal tubule. It was therefore decided to proceed with an investigation of the effect of β-2-thienylalanine, cycloleucine and phenylalaninol on phenylalanine transport.

Brush Border Transport Studies

Initially a number of incubation times, phenylalanine and β-2-thienylalanine concentrations were used, and the results are shown in Table XXXIV. For all cycloleucine and phenylalaninol studies, the concentration of phenylalanine doses was 1mM which was not only the concentration at which Evers, Murer and Kinne did their studies (54) but corresponds to a level below which many clinicians feel phenylalanine levels should be kept to protect children with phenylketonuria from brain damage.

Two concentrations of these inhibitors were finally investigated, equimolar, e.g. 1mM, and half that amount 0.5mM. The total volume of incubation solution was kept constant by reducing the volume of the buffer by the same amount as the amount of inhibitor added. Four control experiments with no inhibitors were conducted and the uptake of phenylalanine and glucose in the presence of sodium, or in its absence (replaced by potassium) are shown in Figure 3. Clearly, the transport of both phenylalanine and glucose are sodium dependent. The final active transport of phenylalanine was calculated by subtracting the amount of phenylalanine transported in its presence at 30 seconds. A similar Na-K phenylalanine transported at 0 seconds was measured and this was subtracted from the 30 second value to allow for non-

TABLE XXXIV

The effect of β-2-thienylalanine on Phenylalanine Uptake by the Isolated Brush Border of the Renal Proximal Tubule of the Rat.

Experiment Number	Incubation time in seconds	Control Phenylalanine only	Experimental Phenylalanine & β-2-thienylalanine	Concentration of Phenylalanine	Concentration of β-2-thienylalanine
1	15	158($\pm$71)[+]	119($\pm$97)	0.025mM	0.1mM
	120	321($\pm$124)	207($\pm$108)	"	"
	90 x 60	132($\pm$27)	123($\pm$47)	"	"
2	15	170($\pm$118)	80($\pm$37)	0.05mM	0.1mM
	30	285($\pm$132)	226($\pm$29)	"	"
	60	1092($\pm$112)	860($\pm$88)	"	"
	90	300($\pm$79)	294($\pm$78)	"	"
	120	164($\pm$118)	91($\pm$49)	"	"
3	15	402($\pm$289)	254($\pm$132)	"	"
	15 + EDTA	288($\pm$78)	230($\pm$62)	"	"
4	15	2643($\pm$719)	1676($\pm$253)	0.5mM	0.1mM
	15 + EDTA	2185($\pm$991)	1744($\pm$492)	"	"
5	30	19746($\pm$3702)	15960($\pm$3934)	1.0mM	0.5mM
6	30	24537($\pm$6642)	22480($\pm$3868)	1.0mM	1.0mM

Transport of phenylalanine into brush border vesicles is expressed as picamoles of phenylalanine/g of protein in brush border solution in the time shown. EDTA was added to remove magnesium used in preparing brush borders because it has been reported to counteract the inhibition of bacterial growth caused by β-2-thienylalanine but it did not increase inhibition in this system. Because of wide individual variation reflected in high standard deviation ( ), student T test did not show a decrease in phenylalanine uptake by β-2-thienylalanine. However, Sign test is strongly significant $p < .001$ as each time β-2-thienylalanine reduces uptake in each case, albeit only slightly.

specific binding of phenylalanine to the vesicles. This transport was then expressed in picamoles of phenylalanine transported per mg. of protein (as measured by the Lowry method (55) in the brush border solution). Glucose transport was determined in the same way. The action of the three analogues are illustrated in Figs. 16, 17 & 18. Tables XXXVa, b, and c sho the effect of each concentration of analogue compared to control values in their absence on the same day, and on the same brush border preparation.

FIGURE 16   The effect of β-2-Thienylalanine on Phenylalanine
and Glucose uptake by the Isolated Brush Border
Vesicles from the Rat Proximal Kidney Tubule
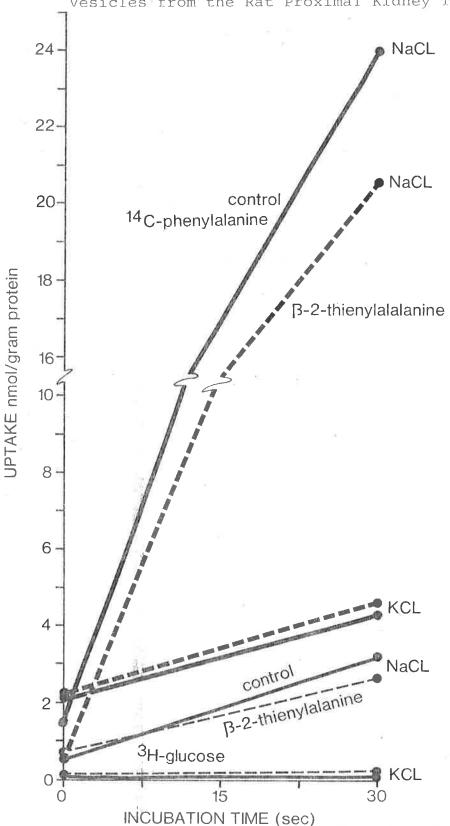
FIGURE 16:   Active transport at 30 seconds calculated as
outlined in Figure 17 and described in Chapter 2 shows that
β-2-Thienylalanine mildly reduces uptake of Phenylalanine
but has no effect on the active transport of glucose.

FIGURE 17   The Effect of Cycloleucine on Phenylalanine and
            Glucose uptake by the Isolated Brush Border
            Vesicles from the Rat Proximal Kidney Tubule
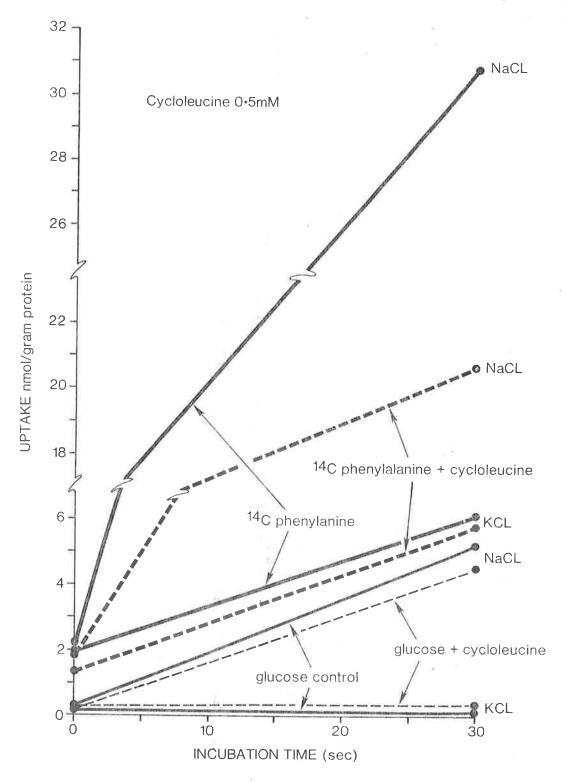
FIGURE 17:   The active transport of Phenylalanine with or
without Cycloleucine is calculated by subtracting the uptake
at 30 seconds in K Cl from the uptake in NA Cl. The zero
second value (which represents non-specific binding) is
subtracted from the 30 second value. Active glucose
transport is calculated in the same way. Cycloleucine reduces
Phenylalanine uptake while having little effect on glucose
transport.

FIGURE 18   The Effect of Phenylalaninol on Phenylalanine and
Glucose uptake by the Isolated Brush Border
Vesicles from the Rat Proximal Kidney Tubule
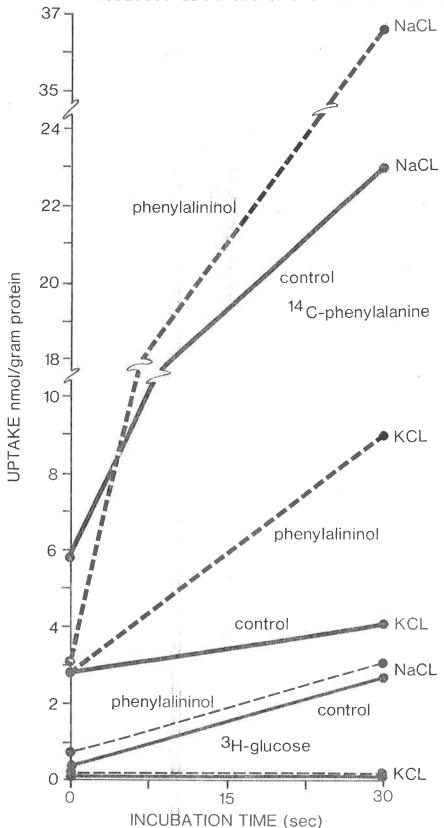
FIGURE 18:   The active transport of Phenylalanine (calculated
as outlined in Figure 17 and described in Chapter 2) appears
to be unaffected or stimulated by Phenylalaninol. Glucose
transport is unaffected by Phenylalaninol.

TABLE XXXVa

Percentage Change of phenylalanine and glucose transport by β-2-thienylalanine

Experiment	Time in seconds	Phenylalanine Transport Change	Glucose Transport Change	Concentration of Phenylalanine	Concentration of β-2-thienylalanine
1	15	−24.7%	−5.7%	0.025mM	0.1mM
	120	−35.5%	+9.6%	"	"
	90 x 60	−6.8%	+18.8%	"	"
2	15	−52.9%	−2.4%	0.05nM	0.1mM
	30	−20.7%	+7.8%	"	"
	60	−21.1%	−2.9%	"	"
	90	−2.0%	+9.6%	"	"
	120	−44.5%	−0.7%	"	"
3	15	−36.1%	+7.3%	0.05nM	0.1mM
	15 + EDTA	−20.1%	+17.9%	"	"
4	15	−36.6%	+9.2%	0.5mM	0.1nM
	15 + EDTA	−20.2%	−1.7%	"	"
5	30	−19.2%	−30.5%	1.0mM	0.5mM
6	30	−8.4%	+5.4%	1.0mM	1.0nM

Transport of phenylalanine and glucose into brush border vesicles expressed as percentage change when β-2-thienylalanine is added to incubation mixture as shown. Phenylalanine data is the same as in Table **XXXIV** but results have been expressed as percentages and the reduction of phenylalanine is significant $p < .001$ by Sign test. Glucose transport does not alter significantly in the presence of β-2-thienylalanine.

TABLE XXXVb

Effect of 0.5mM or 1.0mM (β -2-thienylalanine), cycloleucine and phenylalaninol on phenylalanine transport into isolated rat proximal tubular brush border vesicles.

Ratio of inhibitor to phenylalanine	PHENYLALANINE TRANSPORT (PICAMOL/GRAM PROTEIN)					
	CYCLOLEUCINE		PHENYLALANINOL		β-2-THIENYLALANINE	
	Absent	Present	Absent	Present	Absent	Present
0.5	9,070	3,820	18,700	27,500	19,700	16,000
1.0	7,530	2,594	30,600	42,500	30,600	28,000

TABLE XXXVc

Effect of 0.5mM or 1.0nM cycloleucine, phenylalaninol and β-2-thienylalanine on phenylalanine and glucose transport into isolated rat proximal tubular brush border vesicles expressed as percentage change

Ratio of inhibitor to phenylalanine	% Change in Phenylalanine transport			% Change in glucose transport		
	Cyclo-leucine	Phenyl-alaninol	β-2-thienyl-alanine	Cyclo-leucine	Phenyl-alaninol	β-2-thienyl alanine
0.5	-57.9%	+47.1%	-19.2%	-7.8%	+6.2%	-30.5%
1.0	-54.4%	+38.9%	-8.4%	+4.1%	-17%	+5.4%

DISCUSSION

These results show that β-2-thienylalanine is an inhibitor of phenylalanine transport, but that the inhibition of uptake into the rat renal brush border isolated vesicle is only of minor degree. This inhibition is achieved without any consistent effect on glucose transport. Cycloleucine also appears to be a potent inhibitor of phenylalanine uptake by the isolated brush border vesicles in the rat renal proximal tubule but, surprisingly, phenylalaninol, if anything, appeared to promote the uptake of phenylalanine. This last result is surprising in view of the fact that Shimomura et al. (105) had shown that phenylalaninol inhibited intestinal uptake of phenylalanine both in the everted gut sac and in the whole rat. Generally, amino acid transport in the small intestine and the renal rat proximal tubule is very similar in mammalian species. However, there are differences as has been described by Rosenberg et al. (106) in the heterozygous state of cystinuria.

Cycloleucine appeared to be the most potent inhibitor of uptake of phenylalanine and this, of course, fits with the work of Brown et al. (48) and Goyer et al. (47) in showing an amino acid caused by this agent in both man and rats. In Chapter VII the candidate was unable to obtain any significant reduction in phenylalanine levels in phenylketonuric patients given a supplement of cycloleucine 20mg/kg. per day. In the preceding Chapters the candidate has outlined the fact that although β-2-thienylalanine consistently increased the phenylalanine excretion by various mammalian species, this appeared to be, in some cases, due to inhibition of phenylalanine reabsorption by the proximal tubule and in others, by increased glomerular filtration rate,

or a combination of these two factors. The promotion of excretion in the Rhesus monkey is outlined in Chapter III and is quite significant: an increase of phenylalanine excretion of at least two-fold. There was a small but significant increase of phenylalanine excretion in three phenylketonuric female patients given β-2-thienylalanine (Chapter VII). Because studies in human phenylketonuric patients have produced a 47% reduction of phenylalanine absorption when a β-2-thienylalanine load was given along with phenylalanine, and because of the finding in this Chapter of no inhibition of phenylalanine transport by phenylalaninol, it was decided to look at these three agents – cycloleucine, phenylalanine and β-2-thienylalanine in a model of intestinal transport, and this work is outlined in Chapter XI.

CHAPTER XI

THE EFFECT OF β-2-THIENYLALANINE, CYCLOLEUCINE AND PHENYLALANINOL ON PHENYLALANINE TRANSPORT IN THE EVERTED GUT SAC FROM THE RAT.

In Chapter VI it was shown that β-2-thienylalanine reduced the absorption of phenylalanine by phenylketonuric patients to a significant degree. It has also been shown in three female phenylketonuric patients (Chapter VI) that β-2-thienylalanine increased the excretion of phenylalanine in the urine, but this effect was minor. Thus, it seems that the major effect of β-2-thienylalanine is at the gut level, and these studies were undertaken to see if β-2-thienylalanine inhibited intestinal transport of phenylalanine in an isolated gut system, and also to compare this inhibition with that of cycloleucine and phenylalaninol.

It was also decided to demonstrate whether the inhibition was competitive or non-competitive by the use of Michaelis-Menton kinetics. To use this kinetic model the rate of uptake, V, must vary with the phenylalanine concentration in the manner as shown in Figure 19. That phenylalanine uptake obeys Michaelis-Menton kinetics has been established by Shimomura et al. (105) Evers et al. (54) and Wapnir and Moak (107). The Michaelis-Menton equation describing the kinetics of this transport is -

$$V = Vmax \frac{(PHE)}{(PHE)+KM}$$

where Vmax is the maximum rate of phenylalanine uptake and KM the Michaelis constant and V is equal to the phenylalanine concentration at which the uptake rate is half the maximum value (see Figure 20). Measurement of the rates of uptake at different concentrations of phenylalanine and the analogue will distinguish between competitive and non-competitive inhibition. In competitive

124.

FIGURE 20 : THEORETICAL PLOT OF VELOCITY OF PHENYLALANINE UPTAKE BY EVERTED GUT SAC IF SUCH UPTAKE FOLLOWS MICHAELIS-MENTON KINETICS.
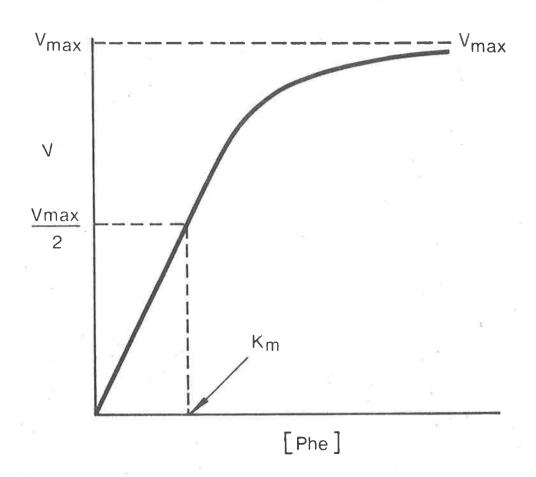
FIGURE 20: IF UPTAKE OF PHENYLALANINE BY THE EVERTED GUT SAC FOLLOWS MICHAELIS-MENTON KINETICS, VELOCITY OF UPTAKE AS A FUNCTION OF PHENYLALANINE CONCENTRATION WILL DESCRIBE A CURVE OF THE SHAPE ILLUSTRATED. Km (MICHAELIS CONSTANT) IS THE CONCENTRATION OF PHENYLALANINE AT WHICH THE VELOCITY OF UPTAKE IS HALF MAXIMAL (VMAX).
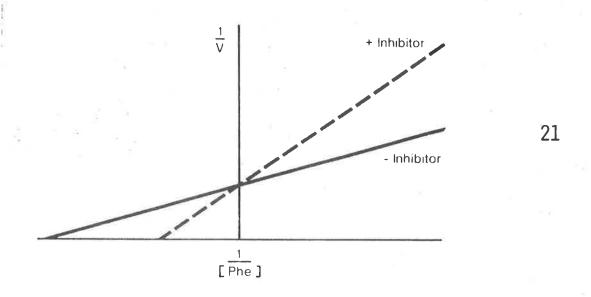
inhibition, the intercept of the double reciprocal plot of the uptake curve, as shown in Figure 21, is the same in the presence and absence of inhibitors, although the slope is different. This reflects the fact that Vmax is not altered by a competitive inhibitor. The hallmark of competitive inhibition is that the inhibition can be overcome by sufficiently high concentration of the substance inhibited. In non-competitive inhibition Vmax is decreased since the inhibition cannot be overcome by increasing the substrate and this is illustrated in Figure 22.

The double reciprocal plot of phenylalanine uptake can be used to determine Vmax as shown in Figure 23. This plot also yields an apparent KM value of phenylalanine uptake in the presence of inhibitor. The true KM value and also the inhibitor constant, (Ki), can be determined by drawing the secondary plot the slope of the double reciprocal lines shown in Figure 24. The decision to obtain kinetic data on the small intestinal absorption of phenylalanine suitable to apply to the Michaelis – Menton model influenced the choice of intestinal transport model used. The whole animal is not much use because the number of potential fates of a given dose of phenylalanine, and Michaelis-Menton kinetics can only be applied to the transport phenylalanine across one surface at a time.

Data obtained from a cellular homogenate as in the renal studies in the previous Chapter will be of use in terms of a potential treatment for phenylketonuria only if it could be established that such data also held for more physiological systems. The everted gut sac appears to occupy an intermediate position between an isolated system and the whole animal, having the advantage of being more physiological than the isolated brush border and yet measuring transport across only one surface and therefore allowing the application of Michaelis-Menton kinetics.

The rat was chosen as the experimental animal because Munck (108) states that at the intestinal absorption level there is little species difference in amino acid uptake. Such amino acid uptake has been measured in the small intestines

FIGURES 21 & 22 : THEORETICAL DOUBLE RECIPROCAL PLOTS OF PHENYLALANINE
UPTAKE WITH COMPETITIVE (21) INHIBITORS AND NON-
COMPETITIVE (22) INHIBITORS OF PHENYLALANINE 126.
TRANSPORT.
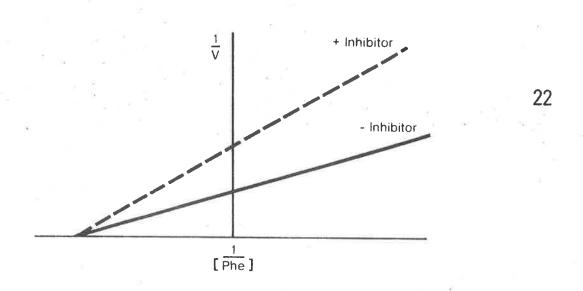
FIGURE 21: IF THE INHIBITOR OF PHENYLALANINE UPTAKE IS COMPETITIVE,
THE VELOCITY OF PHENYLALANINE TRANSPORT EXPRESSED AS A FUNCTION OF
PHENYLALANINE CONCENTRATION IN A DOUBLE RECIPROCAL FASHION WILL
INTERCEPT THE Y AXIS AT THE SAME POINT WHETHER THE INHIBITOR IS PRESENT
OR NOT.

FIGURE 22: IF THE INHIBITOR IS NON-COMPETITIVE THE 2 LINES INTERCEPT
THE Y AXIS AT DIFFERENT POINTS BUT TOUCH THE X AXIS AT THE SAME POINT.

FIGURE 23 : DOUBLE RECIPROCAL PLOT OF PHENYLALANINE UPTAKE
BY THE GUT SAC IN THE PRESENCE OF THEORETICAL
COMPETITIVE INHIBITOR.
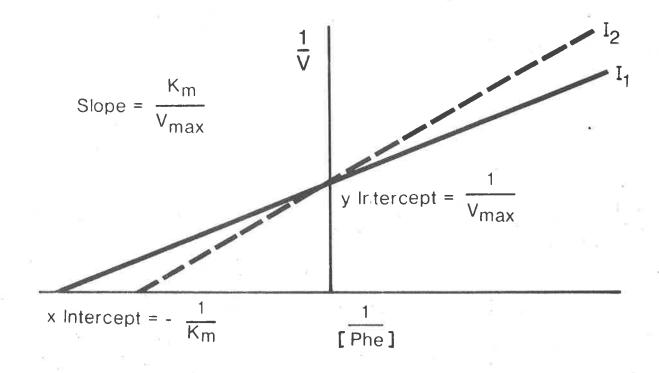
FIGURE 23: DOUBLE RECIPROCAL PLOT CAN BE USED TO DETERMINE V_{MAX} (SLOPE = $\frac{K_M}{V_{MAX}}$). THE APPARENT K_M FOR PHENYLALANINE UPTAKE VARIES WITH INHIBITOR CONCENTRATION.

FIGURE 24 : SECONDARY PLOT OF DOUBLE RECIPROCAL SLOPES IN THE
PRESENCE OF INHIBITOR AGAINST INHIBITOR CONCENTRATION.
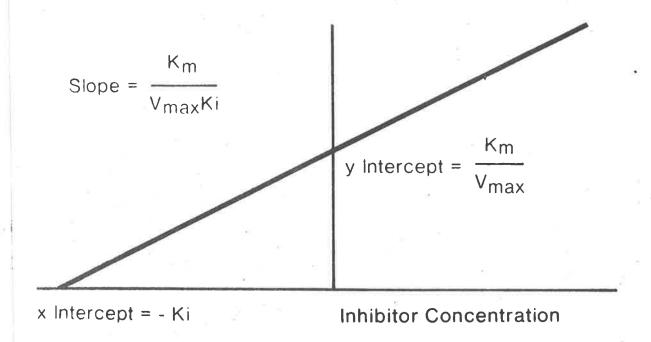
FIGURE 24: ILLUSTRATED IS A THEORETICAL LINE PLOTTING SLOPES
OF LINES OBTAINED BY DOUBLE RECIPROCAL PLOTS OF PHENYLALANINE
UPTAKE (AS A FUNCTION OF PHENYLALANINE CONCENTRATION) AT VARIOUS
INHIBITOR CONCENTRATIONS, AGAINST THE INHIBITOR CONCENTRATIONS
USED. THE INTERCEPTS OF THE X AND Y AXES CAN BE USED TO CALCULATE
TRUE K_m AND K_i VALUES.

of rats, pigs, rabbits, mice, guinea pigs and even man, and the results from these systems have been comparable within experimental systems (108). Thus, species differences can be considered negligible and the rat is a readily available cheap animal and was therefore used in these studies. The rats used were adult male Norwegian hooded. They were kept in cages in groups of varying size but never more than six. Unless otherwise stated, they were fed commercial rat pellets and given water freely. They were sacrificed as required throughout the experiment by stunning and transection of the cervical cord. The jejunum and ileum were removed and the duodenal-jejunal junction was chosen as the upper point because it is readily identifiable where the gut is closely bound to the abdominal wall. The entire length of small intestine was then removed and washed in Ringer solution and everted by tying one end to a glass rod of approximately $1\frac{1}{2}$mm. diameter and pushing this through the lumen of the gut. The everted intestine was then washed in Ringer again to remove the intestinal contents and cut into lengths of about 5cm. These were then tied off at one end with a cotton ligature and a loose ligature applied to the other end, loose enough to allow the insertion of a blunt needle which was used to inject $\frac{1}{2}$ - 1ml. of serosal medium in a solution along with a small bottle of air. The needle was withdrawn and the ligature pulled tight to prevent further fluid escaping.

EXPERIMENTAL METHOD

The filled everted gut sacs were preincubated in Ringer solution at a temperature of $38^{o}C$ and an atmosphere of 5% CO_2/95% O_2 for 30 minutes. They were then blotted and weighed. They were incubated for an appropriate time (usually 5,15,20,30,60 and 120 seconds) in the same medium plus phenylalanine and, in inhibition experiments, the analogue also added was the radioactive glucose to ensure that the inhibition obtained was not general.

After the incubation, the gut sacs were taken from the medium, washed rapidly three times in 4°C 0.3M mannitol solution 100mls. to remove medium and stop further transport. The sacs were emptied and an aliquot of the solution which blotted the serosal surface taken. They were again washed in mannitol, blotted and reweighed empty. Each sac was then placed in 0.1M nitric acid for 2 hours to obtain the intracellular fluid from the mucosal cells. An aliquot of this was then taken, as was one from the incubation medium after removal of the sac. Each of these aliquots was placed in a vial to which was added 10mls. of ACSII scintillation fluid and counted in an ear (model 6892). The original medium before incubation was also counted in the same way.

The amount of glucose or phenylalanine transported was expressed as:

$$\frac{\text{counts per minute/sac}}{\text{standard counts per minute}} \div \text{sac weight in grams}$$

and it is this figure or its reciprocal that is plotted on the graphs in this Chapter.

The active transport of glucose and phenylalanine in the jejunum and ileum is sodium dependent as it is in the renal tubule. Thus that transport that occurs in the absence of sodium (i.e. potassium Ringer) represents non-specific transport, e.g. diffusion. To obtain this figure in each experiment, potassium Ringer was used on sacs from the same three rats for all washing, preincubation and incubation procedures. The composition of both sodium-Ringer and potassium Ringer is shown in Table XXXVI.

To choose the most physiological range of phenylalanine at which to estimate transport of phenylalanine, three rats were sacrificed after three different feeding regimes –

(a) free access to food for 24 hours
(b) fasted for 24 hours but allowed water *ad libitum*
(c) fasted for 23 hours and then allowed free access to food and allowed free access to water throughout.

TABLE XXXVI

Ringer Solutions used for Incubation in
Gut Sac Transport Experiments

	Na^+-Ringer	K^+-Ringer
NaCl	140mM	–
KCl	–	140mM
$KHCO_3$	10mM	10mM
K_2HPO_4	1.2mM	1.2mM
KH_2PO_4	0.2mM	0.2mM
$CaCl_2$	1.2mM	1.2mM
$MgCl_2$	1.2mM	1.2mM
Glucose	1.0mM	1.0mM

Composition of the Na^+-Ringer and the K^+-Ringer (i.e. sodium-free Ringer) for phenylalanine uptake experiments. Glucose was omitted from the Ringers used in the washing and pre-incubation procedures.

All three rats were kept in darkness for the last hour to encourage eating for rats (a) and (c). They were then sacrificed as above, their small intestines removed and cut into lengths of approximately 5cms. (as with gut sacs). The order of the segments was recorded and their contents milked a test cylinder to measure volume. This chyme was suspended in 1ml. of water, vigorously shaken and then centrifuged for 10 minutes at 6,000 r.p.m. The supernatant was decanted and an equal volume of 6% sulphosalicylic acid containing norleucine as an internal standard was added. After mixing, the solution was spun at 10,000 r.p.m. The, now clear, supernatant was subjected to aminoacid analysis as in Chapter II . In this way a phenylalanine concentration in the sequential segments of small intestine was obtained for each of the rats. These results are in Table XXXVII and show that no constant pattern down the small intestine was obtained and, surprisingly, the completely fasted rat had the highest mean level of phenylalanine. The overall mean phenylalanine level was 19mM/l so that studies were conducted on rats that were allowed free access to phenylalanine and the phenylalanine concentrations used to determine Michaelis-Menton kinetics were 0-20mM/1.

CONTROL STUDIES

Field, Schultz and Curran (109) have shown that a steady state isotope flux across the gut serosa is not established until 25-45 minutes and Davey, Rapmore and Smyth (110) have shown that periods as short as 5 seconds give useful results for methionine uptake by everted gut sacs. Thus, a maximum period of two minutes was the initial time course chosen.

To demonstrate that the sacs were not leaking, ^{125}I albumin was placed in the Ringer inside sacs in a pilot study. Because albumin cannot permeate through the gut wall, its presence in the incubating medium must be due to leakage and/or contamination of the mucosal surface on insertion. The three sacs were incubated for 5,15,20,30,60 or 120 seconds and the incubation medium counted for ^{125}I. The results were expressed as a percentage of the amount put inside the sacs. The results of each sac are shown

TABLE XXXVII

Phenylalanine Content of Small Intestine of the
Norwegian Hooded Rat under conditions of (1) Feeding *ad libitum*
(2) Fasting except for Water (3) Fasted and then Allowed Food
for 1 hour

Position of Intestine relative to Dueodenal-Jejunal Junction	(1) Free Access to Food	(2) Fasted except for water	(3) Fasted and then fed for 1 hour
(1)	8.43mM	No data	30.3mM
(2)	3.40mM	7.77mM	14.4mM
(3)	2.28mM	32.0mM	24.3mM
(4)	17.6mM	12.4mM	12.5mM
(5)	10.1mM	30.3mM	13.4mM
(6)	30.3mM	50.0mM	18.0mM
Mean	12.0mM	26.5mM	18.8mM

TABLE Segment 1 in each rat was the first 5cms. below the duodenal-jejunal junction; segment 2 the next 5cms. etc. In rat 2, segment 1, no internal standard was added to the chyme for analysis. No consistent pattern of phenylalanine content was found, the overall mean concentration was 19.1mM.

in Table XXXVIII and indicate that in each case, less than 1% of the inserted radioactivity was found in the incubation medium - a negligible amount and consistent with contamination on insertion of the ^{125}I albumin.

To further check that serosal transport was negligible over the 2 minute period in which mucosal transport was to be assessed, non-everted gut sacs were incubated in medium containing ^{14}C phenylalanine at the same time as everted gut sacs from the same rat. The results obtained are shown in Figure 25, and demonstrate that uptake by the serosal surface showed a similar curve to that of uptake by the mucosal surface. However, the degree of uptake of the serosal surface was one-third to one-half of that of the mucosal surface.

While this might indicate that the serosal surface was capable of actively transporting phenylalanine, it could merely be a reflection of diffusion and non-specific binding. If the latter was the case, the serosal uptake should (a) plateau with longer incubation, and (b) be equal in potassium Ringer solution as in sodium Ringer.

Thus a 10 minute time course was undertaken with everted and non-everted gut sacs and incubation in potassium Ringer was compared with sodium Ringer for both everted and non-everted gut sacs.

Figure 26 shows that in the 10 minute time course phenylalanine uptake plateaued at about 5 minutes in the non-everted gut sacs, but continued to rise throughout the 10 minutes in the everted gut sacs whose mucosa was exposed to the phenyl-alanine.

Figure 27 shows that the everted gut sacs in potassium Ringer (i.e. no sodium) had a markedly reduced uptake of phenyl-alanine compared to when sodium was present. The absence of sodium had no effect on phenylalanine transport in the non-everted sacs. Both in the presence and absence of sodium the non-everted sacs accumulated phenylalanine more rapidly than did the everted gut sacs in the absence of sodium. This suggests that phenylalanine

TABLE XXXVIII

Use of ^{125}I-albumin as a Check on
Gut Sac Leakage

Incubation Period	Volume of Leakage/Contamination as % of amount inside Sac		Mean Value for 3 Sacs
			%
5 seconds	Sac 1	0.03%	
	Sac 2	0.13%	0.08
	Sac 3	0.05%	
15 seconds	Sac 4	0.03	
	Sac 5	0.03	0.03
	Sac 6	0.03	
20 seconds	Sac 7	0.03	
	Sac 8	0.04	0.03
	Sac 9	0.03	
30 seconds	Sac 10	0.11	
	Sac 11	0.16	0.10
	Sac 12	0.02	
60 seconds	Sac 13	0.09	
	Sac 14	0.09	0.67
	Sac 15	0.02	
	Sac 16	0.17	
	Sac 17	0.06	0.11
	Sac 18	0.10	
Mean contamination			0.34

TABLE   Mean contamination of mucosal fluid of 0.34% indicates leakage has not occurred.

FIGURE 25 : PHENYLALANINE UPTAKE BY EVERTED AND NON-EVERTED RAT
GUT SACS.
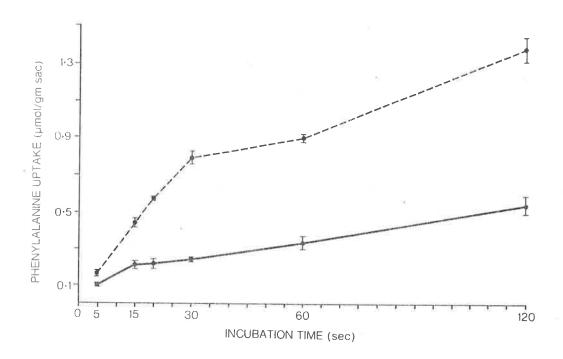
FIGURE 25: PHENYLALANINE UPTAKE BY NON-EVERTED GUT SACS ●——● SHOW
A SIMILAR CURVE TO THE EVERTED GUT SACS ●– – –▲ (BUT THE DEGREE IS
ONLY ABOUT 1/3 THAT OF THE EVERTED GUT SACS) OVER A 2 MINUTE
INCUBATION.

FIGURE 26 : PHENYLALANINE UPTAKE BY EVERTED AND NON-EVERTED
RAT GUT SACS OVER TEN MINUTES.
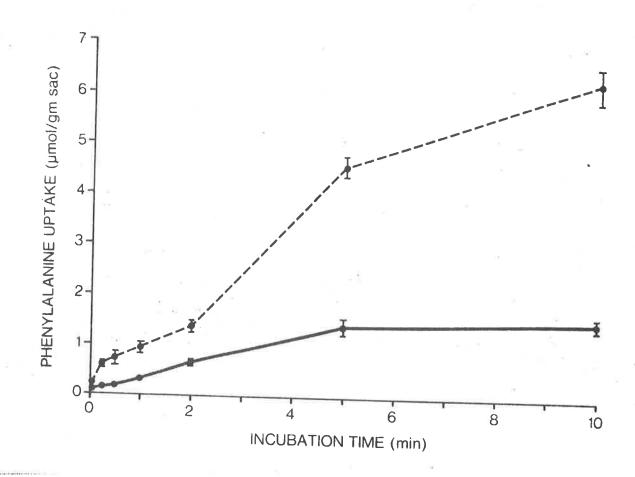
FIGURE 26: PHENYLALANINE UPTAKE BY NON EVERTED GUT SACS ●━━● SHOWS
NO INCREASE AFTER 5 MINUTES WHILE EVERTED GUT SACS ●━ ━● CONTINUE
TO SHOW INCREASING UPTAKE OVER THE FULL TEN MINUTES.

FIGURE 27 : PHENYLALANINE UPTAKE BY EVERTED AND NON-EVERTED
GUT SACS IN THE PRESENCE AND ABSENCE OF SODIUM.
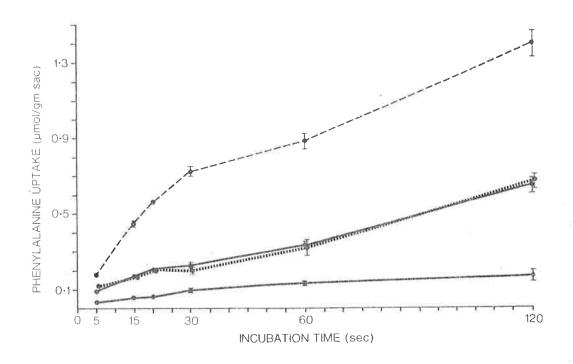
FIGURE 27: PHENYLALANINE UPTAKE BY EVERTED GUT SACS IN THE
PRESENCE (•━ ━ ━•) OF SODIUM IS MARKEDLY GREATER THAN IN ITS
ABSENCE (BOTTOM LINE). IN THE NON-EVERTED GUT SAC THE ABSENCE
OF SODIUM (• ||| •) DOES NOT ALTER THE AMOUNT OF PHENYLALANINE
TRANSPORTED AS THE MIDDLE TWO LINES INDICATE NON-EVERTED GUT SAC
TRANSPORT OF PHENYLALANINE WITH OR WITHOUT SODIUM AND ARE ALMOST
IDENTICAL.

diffuses more rapidly across (or is more readily non-specifically bound to) the serosal surface of the gut sac. However, the transport is not sodium dependent and because of the failure to continue beyond 5 minutes, not active. Thus, any effect of phenylalanine analogues in this system can be attributed to their effect on the mucosal transport of phenylalanine.

Active transport of phenylalanine and glucose in everted gut sacs was calculated by subtracting values obtained in potassium Ringer from those obtained in sodium Ringer. A time course of phenylalanine and glucose transport calculated in this fashion is shown in Figure 28 . It can be seen that this is most linear over 20 seconds and, therefore, this period was taken for calculation of Michaelis-Menton kinetics and evaluation of the action of the three analogues effect on phenylalanine transport.

EXPERIMENTAL STUDIES

Two concentrations of each inhibitor were used, 5mM and 20mM. The latter was approximately equimolar with the mean intraluminal intestinal concentration of phenylalanine in the rat, and the former one-quarter of the concentration - approximately the ratio in the acute loading experiment in phenylketonuric patients given an acute load of phenylalanine with or without β-2-thienylalanine (Chapter VI). The phenylalanine uptake by the everted gut sac was compared in the presence or absence of each inhibitor at these two concentrations.

Figure 29 shows that β-2-thienylalanine 5mM reduced phenylalanine uptake by 17%, while 20mM β-2-thienylalanine reduced uptake by 25%. Figure 30 demonstrates that cycloleucine reduced uptake by 17% at 5mM concentration but 40% at 20mM. Figure 31 indicates that phenylalaninol reduced uptake at 5mM and 20mM by 24% and 35% respectively. The inhibitors had no effect on glucose uptake as shown in Table XXXIX.

By plotting uptake of phenylalanine and concentration both as reciprocals (double reciprocal plot) it was shown that

FIGURE 28 : ACTIVE TRANSPORT OF PHENYLALANINE AND GLUCOSE INTO
EVERTED GUT SACS FROM RATS.
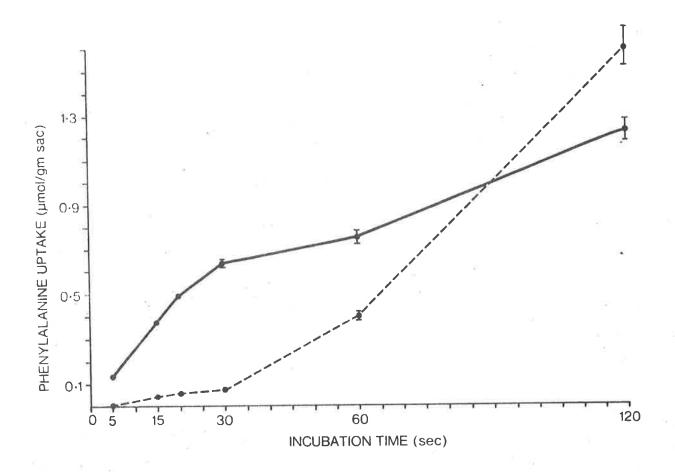
FIGURE 28: SODIUM-DEPENDENT (ACTIVE) UPTAKE OF PHENYLALANINE
(●——●) AND GLUCOSE (●— —●) INTO EVERTED GUT SAC FROM AN INCUBATION
SOLUTION CONTAINING IMM GLUCOSE AND IMM PHENYLALANINE. AS UPTAKE
IS MOST LINEAR OVER FIRST 20 SECONDS, THIS PERIOD WAS USED FOR
ALL SUBSEQUENT GUT SAC EXPERIMENTS.

FIGURE 29   PHENYLALANINE UPTAKE BY THE EVERTED GUT-SAC IN THE PRESENCE AND ABSENCE OF β-2-THIENYLALANINE.
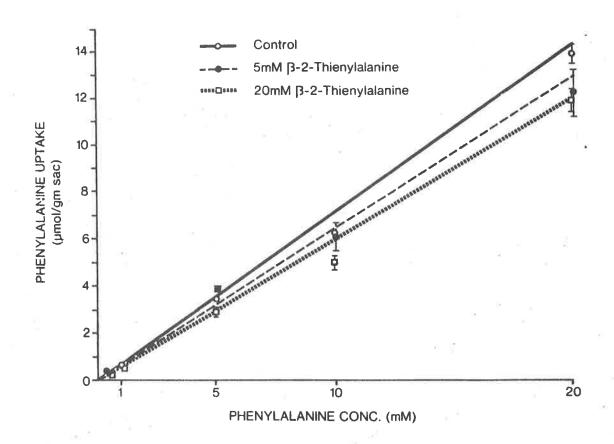
FIGURE 29:   BOTH 5 MM AND 20 MM β-2-THIENYLALANINE REDUCE THE TRANSPORT OF PHENYLALANINE INTO THE GUT-SAC WALL AT THE CONCENTRATIONS OF PHENYLALANINE SHOWN.

FIGURE 30   PHENYLALANINE UPTAKE BY THE EVERTED GUT-SAC IN THE
PRESENCE AND ABSENCE OF CYCLOLEUCINE.
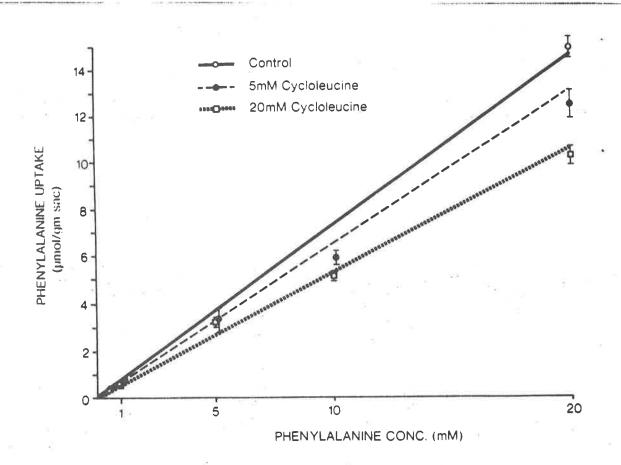
FIGURE 30: BOTH 5 MM AND 20 MM CYCLOLEUCINE REDUCE THE TRANSPORT
OF PHENYLALANINE INTO THE GUT-SAC WALL AT THE CONCENTRATIONS OF
PHENYLALANINE SHOWN.

FIGURE 31   Phenylalanine Uptake by the Everted Gut-Sac in
the Presence and Absence of Phenylalinol.
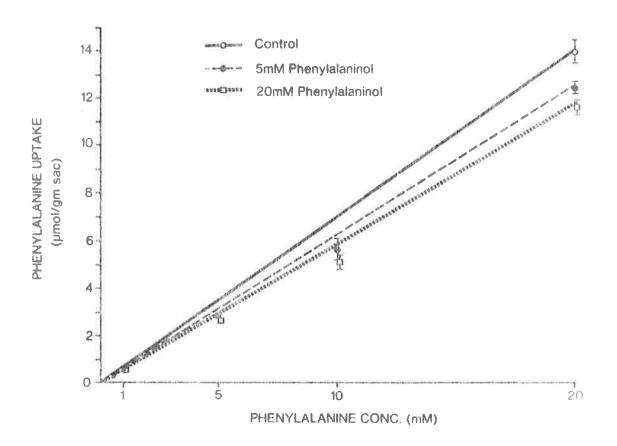
FIGURE 31:   Both 5mM and 20 mM Phenylalaninol reduce the
transport of Phenylalanine into the Gut-Sac wall at the
concentrations of Phenylalanine shown.

all lines passed through the same point on the uptake axis which indicates that the inhibition is competitive (Figures 32, 33 & 34). A secondary plot of the slopes can then be obtained as shown in Figure 35 which then enables one to calculate the Km for phenylalanine (1.857 x 10   M), phenylalaninol (7.364 x 10   M) and cycloleucine (2.928 x 10   M). Thus, although β-2-thienylalanine inhibits intestinal transport of phenylalanine into the mucosal cell of the rat's small intestine, it is inferior in this respect to phenylalaninol and cycloleucine. Table XXXIX shows that neither varying the concentration of phenylalanine nor the presence of analogue affected the transport of glucose.

DISCUSSION

Both this study and the renal brush border study (Chapter X), show that both β-2-thienylalanine and cycloleucine inhibit phenylalanine transport with β-2-thienylalanine being the less effective phenylalaninol inhibited in the gut sac but not the renal brush border. In phenylketonuric man and Rhesus monkeys β-2-thienylalanine appeared to reduce the level of serum phenylalanine levels after oral loading with phenylalanine. Despite the fact that cycloleucine is a significantly better inhibitor of phenylalanine transport *in vitro*, in a chronic administration study at 20mg/kg., no reduction of phenylalanine levels would be obtained.

This failure of cycloleucine to reduce phenylalanine levels could reflect -

(a) The patient did not receive the drug. The investigation was dependent on the nursing staff to give the cycloleucine but, as all subjects were mentally retarded, administration may have been difficult. Also, staff may have been reluctant to give an experimental drug. Unfortunately, amino acid analysis was not available to the investigator and, thus, cycloleucine levels could not be measured, nor did the investigator have the prescience to include a marker such as riboflavin.

(b) Failure of capsules to dissolve in time for the cycloleucine to be able to affect phenylalanine uptake. To ease adminis-

FIGURE 32   DOUBLE RECIPROCAL PLOT OF PHENYLALANINE UPTAKE INTO
THE EVERTED GUT-SAC IN THE PRESENCE AND ABSENCE OF
β-2-THIENYLALANINE.
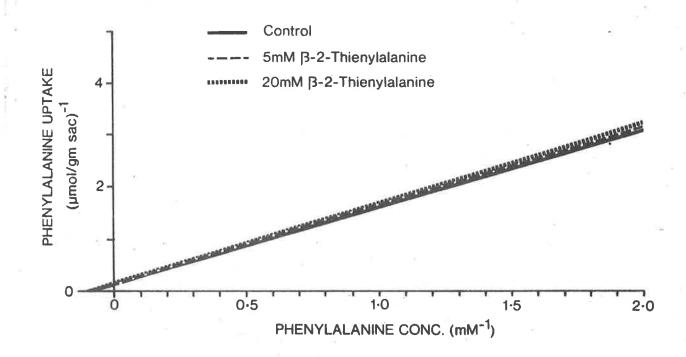
FIGURE 32: THE CONTROL AND BOTH β-2-THIENYLALANINE LINES PASS
THROUGH THE SAME POSITION ON THE Y AXIS INDICATING THAT β-2-
THIENYLALANINE IS A COMPETITIVE INHIBITION OF PHENYLALANINE UPTAKE
INTO THE GUT-SAC WALL OF THE RAT.

FIGURE 33   DOUBLE RECIPROCAL PLOT OF PHENYLALANINE UPTAKE INTO
THE EVERTED GUT-SAC IN THE PRESENCE AND ABSENCE OF
CYCLOLEUCINE.
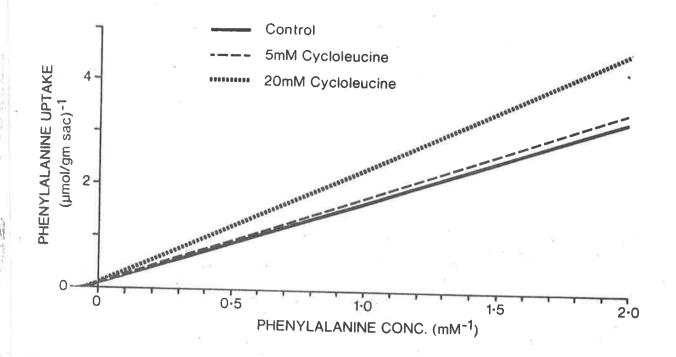
FIGURE 33:   THE CONTROL AND BOTH CYCLOLEUCINE LINES PASS THROUGH
THE SAME POSITION ON THE Y AXIS INDICATING THAT CYCLOLEUCINE IS A
COMPETITIVE INHIBITION OF PHENYLALANINE UPTAKE INTO THE GUT-SAC
WALL OF THE RAT.

FIGURE 34   DOUBLE RECIPROCAL PLOT OF PHENYLALANINE UPTAKE INTO
THE EVERTED GUT-SAC IN THE PRESENCE AND ABSENCE OF
PHENYLALANINOL.
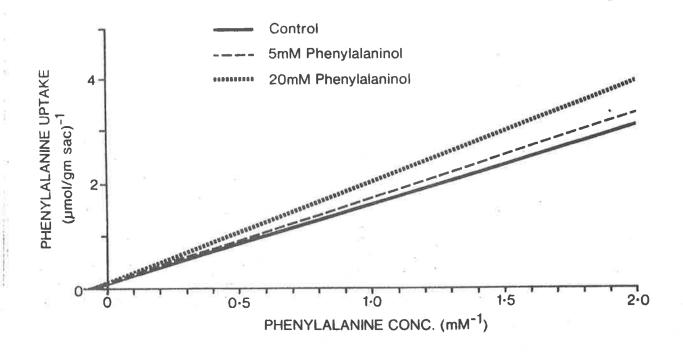
FIGURE 34:   THE CONTROL AND BOTH PHENYLALANINOL LINES PASS THROUGH
THE SAME POSITION ON THE Y AXIS INDICATING THAT PHENYLALANINOL IS A
COMPETITIVE INHIBITOR OF PHENYLALANINE UPTAKE INTO THE GUT-SAC WALL
OF THE RAT.

FIGURE 35   SECONDARY PLOT DERIVED FROM THE SLOPE OF THE DOUBLE
RECIPROCAL PLOT OF EFFECT OF β-2-THIENYLALANINE,
CYCLOLEUCINE AND PHENYLALANINOL ON PHENYLALANINE
UPTAKE BY THE EVERTED GUT-SAC OF THE RAT.
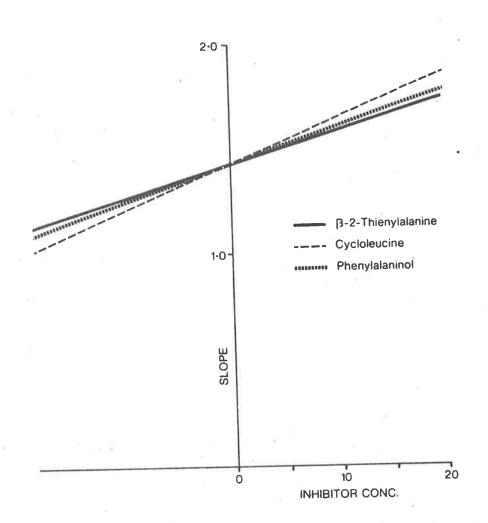
FIGURE 35: THE SLOPES OF THE AFFECT OF THE ANALOGUES ON
PHENYLALANINE UPTAKE AS SHOWN IN FIGURES 32, 33 & 34 ARE PLOTTED
AGAINST THE CONCENTRATION OF INHIBITOR USED. THIS ENABLES THE
CALCULATION OF K_M FOR PHENYLALANINE AND THE K_I VALUE FOR EACH
INHIBITOR.

TABLE XXXIX

Glucose uptake in the presence and absence of inhibitors of phenylalanine uptake by everted gut sacs. Standard deviations (from three sacs) are shown in brackets.

Phenylalanine Concentration (mM)	GLUCOSE UPTAKE (μmol glucose/gm sac)			
	Phenylalanine only	20mM Cycloleucine	20mM Phenylalaninol	20mM β-2-thienylalanine
0.5	0.075 (0.008)	0.077 (0.013)	0.058 (0.016)	0.085 (0.009)
1	0.062 (0.010)	0.072 (0.005)	0.090 (0.005)	0.064 (0.009)
5	0.088 (0.014)	0.091 (0.010)	0.063 (0.017)	0.084 (0.011)
10	0.092 (0.011)	0.069 (0.006)	0.067 (0.018)	0.071 (0.006)
20	0.073 (0.009)	0.097 (0.012)	0.071 (0.008)	0.082 (0.009)

TABLE XXXIX

No consistent effect of phenylalanine concentration or presence of analogue on glucose transport is seen.

CHAPTER XII

THE EFFECT OF β-2-THIENYLALANINE AND/OR HIGH LEVELS OF PHENYLALANINE ON FIBROBLAST CELL CULTURES.

This work was performed in the Department of Biochemical Genetics, McGill University, Montreal. The candidate is grateful to Gerry Lancaster for instruction in the techniques and the supply of the original fibroblasts. All the work described is the candidate's own but he was assisted in the counting of cells by Denise Major.

BACKGROUND

The previously described work in this thesis has shown that β-2-thienylalanine was effective in reducing the effective absorption of phenylalanine in phenylketonuric patients by 47% (Chapter VI), but β-2-thienylalanine had been associated with Shigella gastroenteritis in primates (Chapter V). The possible causes of this might be –

(a) β-2-thienylalanine might damage normal flora of the gut while not affecting Shigella growth. The studies of Brown, Tannock, Elliott and Lines (98), Brown and Lines (99), Brown, Vesey, Tannock, Bell, Lines and Elliott (112), Brown, Tannock, Eyres, Elliott and Lines (113), Brown and Lines (114,115), and Brown, Elliott, Tannock and Lines (116) have shown that this is not likely. Growth of Shigella was inhibited by β-2-thienylalanine in both agar and liquid media. While Escherichia strain were inhibited by β-2-thienylalanine, this inhibition was reversed by the presence of magnesium (0.1g/1 of Mg SO_4o $7H_2O$) which is equivalent to 0.41mM/1 of magnesium. As the normal range of magnesium in children's serum is 0.75 – 1.00mM/1 (Flinder's Medical Centre, Department of Clinical Biochemistry, Hadbook 1981), presumably all bacteria would be protected against inhibition of β-2-thienylalanine. Of 127 anaerobes isolated from the faecal samples of three diet-managed phenylketonuric patients only 9 were inhibited by β-2-thienylalanine, and in all

cases this inhibition was abolished by the simultaneous presence of phenylalanine (13mg. per 100ml.) in Columbia agar - a level that would be expected in phenylketonuric syndrome.

Finally, Brown et al. (113) fed mice β-2-thienylalanine by oesophagial tube (350mg. per mg. per day) and found they were no more susceptible to gastrointestinal tract colonization with Salmonella typhimurium or Shigella flexneri III than control mice fed water.

(2) Cellular damage. β-2-thienylalanine is only minimally incorporated into protein in monkeys fed it in infancy (Chapter V) and in rats as shown by Godin and Dolan.(60) Nevertheless, it may be responsible for the development of *false proteins* which may predispose the recipient to immune deficiency leading to predisposition to infection or other significant cellular damage.

To further investigate the possibility that β-2-thienylalanine might damage human tissue it was decided to expose growing fibroblast cultures from a normal child to β-2-thienylalanine, high level phenylalanine as might be found in a phenylketonuric child and a combination of phenylalanine and β-2-thienylalanine as might be found in a phenylketonuric child treated with β-2-thienylalanine and to see if these additions to the culture medium affected growth of fibroblasts as measured by cell numbers over successive days. The details of cell culture and counting techniques are in Chapter II . Two controls were used: (a) in which only medium was used, and (b) medium with physiological saline (0.9g%) was added, same volume as the phenylalanine, β-2-thienylalanine or phenylalanine + β-2-thienylalanine additions. Initially, approximately 250,000 cells were put in the petri dishes and left for 24 hours at which time counting was undertaken. Refeeding with the same medium ± additions of the uncounted plates was undertaken every third day. From thereon counting was done every day and refeeding on alternate days. The concentration and amount of β-2-thienylalanine, phenyl-

alanine or saline added is shown in Table XL .

TABLE XL

Effect of β-2-thienylalanine and/or phenylalanine on cell numbers in fibroblast cultures

	FIRST EXPERIMENT	SECOND EXPERIMENT
Solution added	500mg. % β-2-thienylalanine	500mg % β-2-thienylalanine
Volumes	100 μl 200 μl 400 μl	100 μl 200 μl 400 μl
Solution added		1.0g% Phenylalanine
Volume		400 μl
Solution added		500mg.% β-2-thienylalanine + 1.0g% phenylalanine
Volumes		100 μl 200 μl 400 μl

	THIRD EXPERIMENT	FOURTH EXPERIMENT
Solution added	500mg. % β-2-thienylalanine	500mg.% β-2-thienylalanine + 1.0g% phenylalanine
Volumes	50 μl 100 μl 200 μl	50μl 100μl 200μl
Solution added	1.0g% phenylalanine	0.9g% sodium chloride
Volumes	50 μl 100 μl 200 μl	50μl 100μl 200μl

The solutions were added in 2.0mls of medium every third day. In addition to the physiological (0.9g%) saline controls, cells were grown in medium with no additions in all 4 experiments.

RESULTS

These are shown graphically in Figures 36, 37, 38, 39. In each case the number of cells per plate is a mean of at least 3 plates. In the fourth experiment to allow comparison more readily, the cell growth is expressed as a straight line using the method of least squares. (Figure 39).

In experiment one (Figure 36), where β-2-thienylalanine was compared to no additions to the normal growth medium, all 3 solutions of β-2-thienylalanine appeared to mildly decrease fibroblast growth. The 100μl volume of 500mg.% β-2-thienylalanine is equivalent to a final concentration of 1.46mM in the medium, 200μl ≡ 2.92mM and 300μl ≡ 5.84mM. These are all concentrations well above that which would be expected to be achieved in the serum of phenylketonuric patients given 20mg/kg. of β-2-thienylalanine.

In experiment two (Figure 37), the combination of β-2-thienylalanine and phenylalanine also slightly depresses cell growth - if anything, slightly more than β-2-thienylalanine alone. When 400 μl of 1.0g% phenylalanine alone (equivalent to 13mM phenylalanine in final solution), growth was similarly depressed compared to cells grown in medium with no additions. This suggested that perhaps the slight dilution occasioned by the 0.1ml. addition to 2.0mls. might account for the decreased cell growth, and this point was checked in experiment three.

Experiment three (Figure 38) shows that no consistent effect from physiological saline could be seen, nor was there any consistent dose response seen with β-2-thienylalanine or β-2-thienylalanine plus phenylalanine. However, with phenylalanine alone, there was an apparent relationship between phenylalanine dose and inhibition of cell growth. There were no viable cells available to count on day 7 in the phenylalanine exposed cells. It should be noted that the 50 μl addition of phenylalanine gives a final concentration of 1.5mM, a level that would be expected to be exceeded in untreated phenylketonuric children.

FIGURE 36   EFFECT OF β-2-THIENYLALANINE ON GROWTH OF HUMAN
             FIBROBLASTS IN CELL CULTURE
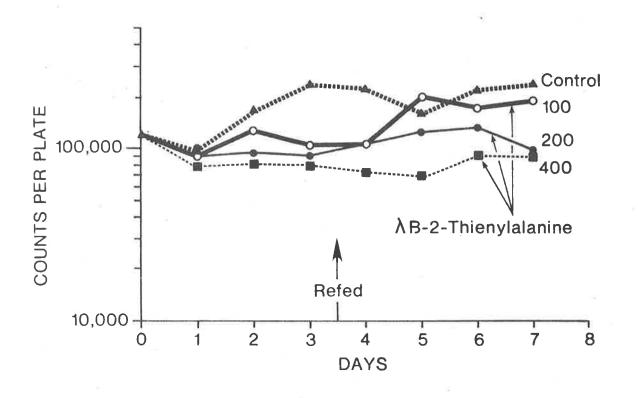
FIGURE 36: β-2-THIENYLALANINE 100 µL, 200 µL, AND 400 µL OF
-0.5 G% SOLUTION ADDED ON FIRST AND THIRD DAYS SLIGHTLY DECREASES
CULTURED FIBROBLAST NUMBERS.

FIGURE 37 EFFECT OF β-2-THIENYLALANINE AND/OR PHENYLALANINE 156. ON HUMAN FIBROBLASTS IN CELL CULTURE
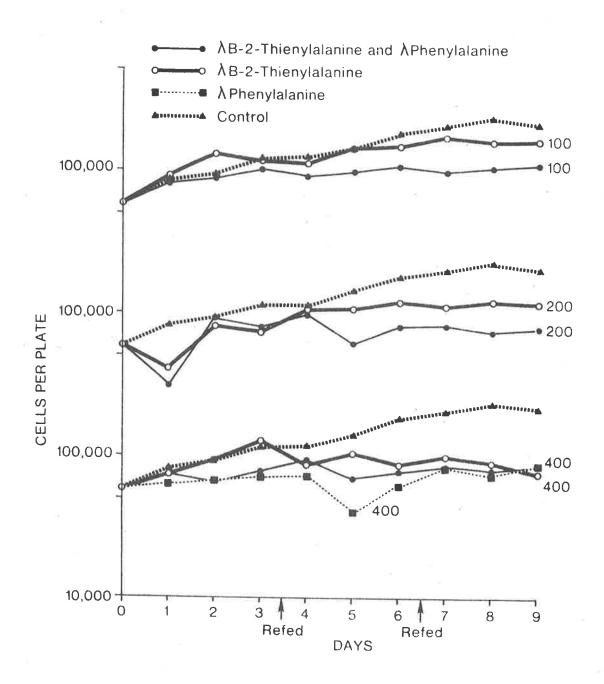
FIGURE 37: THE COMBINATION OF β-2-THIENYLALANINE (0.5%) AND PHENYLALANINE (1.0 G%) APPEARS TO REDUCE FIBROBLAST MULTIPLICATION MORE THAN β-2-THIENYLALANINE (0.5 G%) ALONE WHEN 100 OR 200 λ (μL) ADDED. THE 200 λ ADDITIONS OF β-2-THIENYLALANINE (0.5 G%) ALONE OR WITH PHENYLALANINE (1.0 G%) DEFINITELY AFFECT FIBROBLAST NUMBERS BUT NOT MORE THAN PHENYLALANINE (1.0 G%) ALONE.

FIGURE 38   EFFECT OF β-2-THIENYLALANINE AND/OR PHENYLALANINE ON[157.] HUMAN FIBROBLASTS IN CELL CULTURE
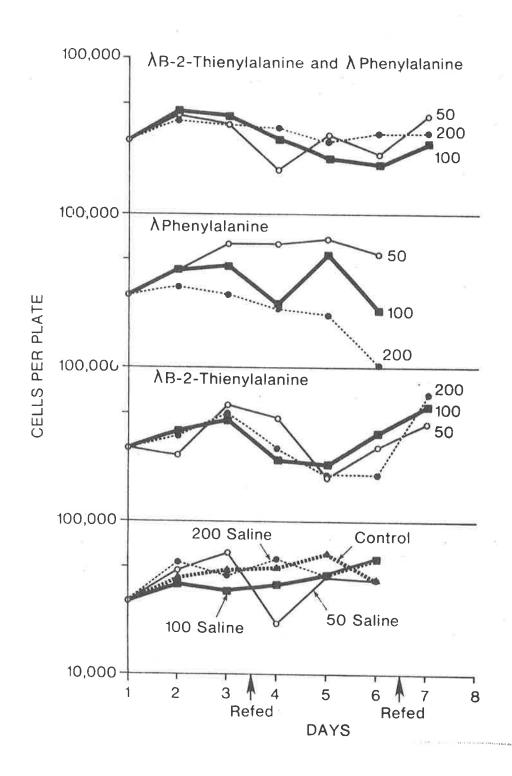
FIGURE 38:   SEE FIGURE 37 FOR CONCENTRATIONS OF β-2-THIENYLALANINE AND PHENYLALANINE, SALINE WAS PHYSIOLOGICAL (0.9 G%). β-2-THIENYLA-LANINE ALONE OR IN COMBINATION DOES NOT SHOW A CLEAR DOSE/TOXICITY RELATIONSHIP WHILE PHENYLALANINE CLEARLY SHOWS A DOSE/TOXICITY RELATIONSHIP. THE CONTROL IN THE BOTTOM PANEL APPLIES FOR ALL UPPER PANELS AND THE SALINE ADDITIONS DO NOT REDUCE CELL GROWTH INDICATING THAT THE EFFECTS IN THE UPPER PANELS ARE NOT DUE TO DILUTION.

Experiment four (Fig. 39) was identical to experiment three but the growth curves have been expressed as straight lines by the method of least squares (103). This allows easier comparison of the effect of β-2-thienylalanine alone in combination with phenylalanine, phenylalanine alone and physiological saline. It can be seen that all additions cause growth patterns scattered around the control growth pattern. Three of the experimental results show increased growth rates compared to control - 50μl β-2-thienylalanine (0.73mM) 100 μl of phenylalanine (3mM) and 100μl of 0.9g% sodium chloride.

The other 8 additions reduced fibroblast growth with the 200 μl additions having the most effect. No consistent pattern can be seen and no particular addition appears more deleterious than another, nor is it statistically significant.

DISCUSSION

No significant or consistent effect on fibroblast growth by β-2-thienylalanine could be demonstrated, and at concentrations of 0.73mM β-2-thienylalanine the growth of fibroblasts was, if anything, superior to control samples. Assuming an even distribution of β-2-thienylalanine in body water, a dose of 20mg/kg (a dose effective in reducing effective absorption of phenylalanine in man), and 100% absorption, and 75% body weight is water, the average concentration of β-2-thienylalanine would be .15mM. Thus, little evidence of cellular damage by β-2-thienylalanine would be expected.

It may be that continuous administration of β-2-thienylalanine might lead to β-2-thienylalanine accumulation but more than 90% of a given dose of β-2-thienylalanine infused over 24 hours is excreted in the urine (Chapter III). In addition, long-term feeding experiments in monkeys could show no β-2-thienylalanine in brain protein and very little in liver protein, nor could any clinical evidence of cellular damage be detected in the two monkeys fed β-2-thienylalanine alone (Chapter V).

The safety of β-2-thienylalanine as found by other workers will be discussed in Chapter XIV.

FIGURE 39   EFFECT OF β-2-THIENYLALANINE AND/OR PHENYLALANINE ON HUMAN FIBROBLASTS IN CELL CULTURE
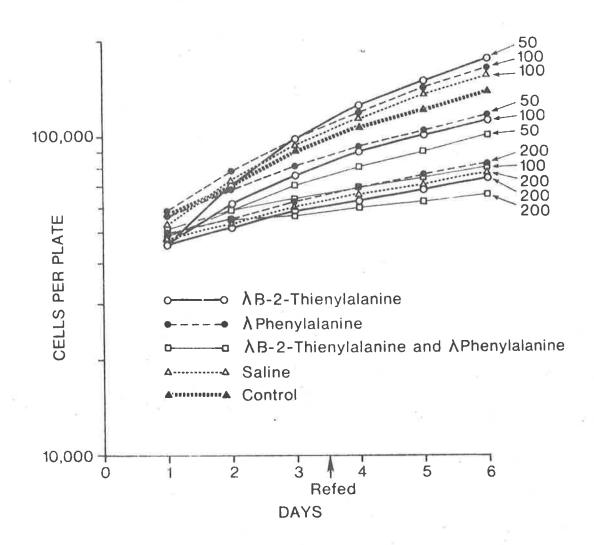
FIGURE 39: CONDITIONS ARE IDENTICAL TO THOSE OUTLINED IN FIGURE 38 BUT THE LINES HAVE BEEN REPRESENTED AS STRAIGHT BY THE METHOD OF LEAST SQUARES. FOR DISCUSSION SEE TEXT.

CHAPTER XIII

STUDIES TO EXAMINE THE EFFECT OF LOW CONCENTRATIONS OF β-2-THIENYLALANINE, CYCLOLEUCINE AND PHENYLALANINOL ON TRANSPORT OF PHENYLALANINE IN THE EVERTED GUT SAC OF THE RAT

In Chapter XII it was shown that phenylalaninol was an effective inhibitor of phenylalanine transport into the mucosa of the everted gut sac from the rat. Both 5mM and 20mM conentrations of phenylalaninol inhibited uptake of phenylalanine from a 20mM phenylalanine solution in Ringer's solution. However, in Chapter X phenylalaninol at 0.5mM or 1.0mM did not inhibit phenylalanine uptake, from a 1mM solution, by isolated brush border vesicles prepared from the proximal tubules of rat kidneys. These results appeared anomalous as it is generally accepted that amino acid transport in the proximal renal tubule and the small intestine are very similar, as is indicated by nature's experiments such as cystinuria and Hartnup's disease - conditions which appear to be due to an antosomal recessive genetic disease and in which both intestinal and renal transport of certain amino acids are affected. In addition, the results in the gut and renal preparations appeared congruent for β-2-thienylalanine and cycloleucine.

The reason for this anomaly was suggested by the work of Izbicka-Dmitrijevic, Mastalerz and Kochman (117), who found that their phenylalanine analogues and phenylalanine itself stimulated pyruvate kinase at low concentrations 1-5mM, while inhibiting at higher concentrations. Although they were looking at an energy enzyme, it could be that either the synport enzyme responsible for transport behaved in the same way, or that the energy supply necessary for active transport was stimulated.

The work described in this Chapter was designed to look at the effect of lower doses of phenylalaninol on phenylalanine transport. In addition, β-2-thienylalanine and cycloleucine at low dose were tested.

in Table XLII.

It can be seen that low (0.5mM or 1.0mM) concentrations of these analogues (β-2-thienylalanine or cycloleucine) do not inhibit or stimulate the uptake of phenylalanine to any significant degree.

DISCUSSION

The finding that phenylalaninol at low concentration (0.5mM) stimulates the uptake of phenylalanine by the everted gut sac confirms the work of Izbicka-Dimitrijevic et al. (117) who showed that other analogues of phenylalanine and phenylalanine itself stimulated pyruvate kinase. The mechanism of the stimulation of phenylalanine uptake was not explored but the results are compatible with the renal brush border studies described in Chapter X when phenylalaninol at 0.5mM or 1.0mM far from reducing uptake by the isolated vesicles, appeared to stimulate such uptake.

Similarly, the lack of stimulation by β-2-thienylalanine on low level (0.5mM or 1.0mM) cycloleucine in the everted gut sac is congruent with the fact that these compounds inhibited phenylalanine uptake by the isolated vesicles derived from the proximal brush borders of the rat kidney as described in Chapter X.

Shimomura et al. (105) showed that phenylalaninol 40mg/kg. appeared to be more potent than cycloleucine 100mg/kg. in inhibiting effective absorption of phenylalanine (300mg/kg. *stat* dose) in the intact Wistar rat.

As found by the candidate in Chapter XI, they found cycloleucine to be a more potent inhibitor than phenylalaninol in the everted gut sac using 10mM concentrations of inhibitor. They measured transport by determining the appearance of phenyl-alanine inside the sac (i.e. in serosal fluid) after 1 hour of incubation - thus, they were examining transport at both the mucosal and serosal surfaces. However, their results are largely in agreement with those of the candidate that above 5mM concentration phenylalaninol is an inhibitor of phenylalanine transport by the intestine.

METHODS

These were identical to those described in Chapter XI but the concentrations of phenylalaninol tested were 0.5mM, 1.0mM, 2mM and 4mM respectively. β-2-thienylalanine and cyclo-leucine were tested at 0.5mM and 1.0mM only.

RESULTS

The effects of 1.0mM to 4.0mM phenylalaninol have been combined with the results from Chapter XI where phenylalaninol was used and are consistent with a typical dose response effect for 2mM to 20mM phenylalaninol, but 1mM phenylalaninol gives a line slightly above the control line. The value for both the 1mM and 2mM concentrations fall within the mean $\pm$ one standard deviation of the control line (Fig. 40). These results are expressed as the double reciprocal plot in Figure 41 and show that the inhibition is competitive with the various lines including that of 1mM intersecting the y axis at the same point. Figure 42 plots the slopes of these lines against the concentration of inhibitor and, from this, a kM for phenylalanine of 1.49×10^{-2} M and Ki for phenylalaninol of 6.77×10^{-2} M were derived – these are very similar figures to those derived in Chapter XI (Km = 1.86×10^{-2} and Ki 7.65×10^{-2} respectively).

When 0.5mM phenylalaninol was used a distinct stimu-latory effect on gut uptake of phenylalanine was observed (Figure 43). The slope of the lines when phenylalanine uptake is plotted against concentration in the presence of various concentrations of phenylalaninol is expressed in Table XLI as are slopes of the lines when the double reciprocal is used. Thus, the non-inhibition, in fact, stimulation, of phenylalanine uptake by isolated brush borders of proximal renal tubule when 0.5mM or 1.0mM phenylalaninol is used, is congruent with the studies described in this Chapter.

β-2-thienylalanine and Cycloleucine in Low Concentrations

When the stimulatory effect of low concentration was found, it was decided to check the effect of 0.5mM and 1.0mM β-2-thienylalanine and cycloleucine on phenylalanine uptake by the everted gut sac. 0.5mM concentrations of these analogues showed no significant differences from control values as shown

FIGURE 40 : EFFECT OF PHENYLALANINOL (1-20 mM) ON PHENYLALANINE UPTAKE IN THE EVERTED GUT SAC OF THE RAT.
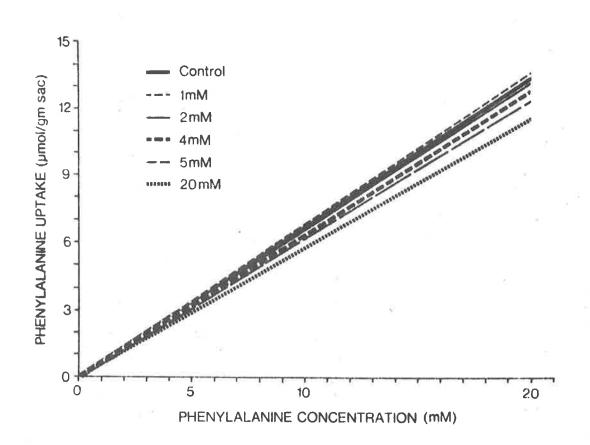
FIGURE 40: PHENYLALANINOL 4, 5 AND 20 MM REDUCES PHENYLALANINE UPTAKE. 1 AND 2 MM CONCENTRATIONS HAVE NO EFFECT (WITHIN 2 STANDARD DEVIATIONS OF CONTROL VALUE).

FIGURE 41 : DOUBLE RECIPROCAL PLOT OF PHENYLALANINE UPTAKE BY
THE EVERTED GUT SAC AND THE EFFECT OF PHENYLALANINOL
(1-20 mM).
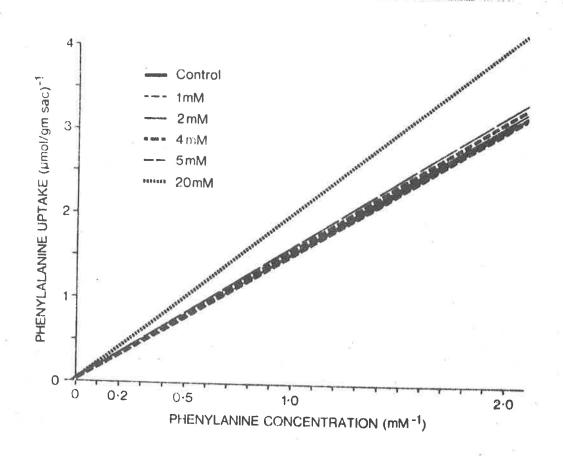
FIGURE 41: DOUBLE RECIPROCAL PLOT OF THE EFFECT OF PHENYLALANINOL
ON PHENYLALANINE UPTAKE SHOWS THAT EFFECT OF PHENYLALANINOL IS
COMPETITIVE IN THAT LINES CROSS Y AXIS AT APPROXIMATELY SAME POINT
(0.0105 - 0.013).

FIGURE 42 : SLOPES OF LINES OF PHENYLALANINE UPTAKE AS A
FUNCTION OF PHENYLALANINE CONCENTRATION PLOTTED
AGAINST CONCENTRATION OF PHENYLALANINOL (1-20 mM)
IN INCUBATION MEDIUM OF EVERTED GUT SAC.
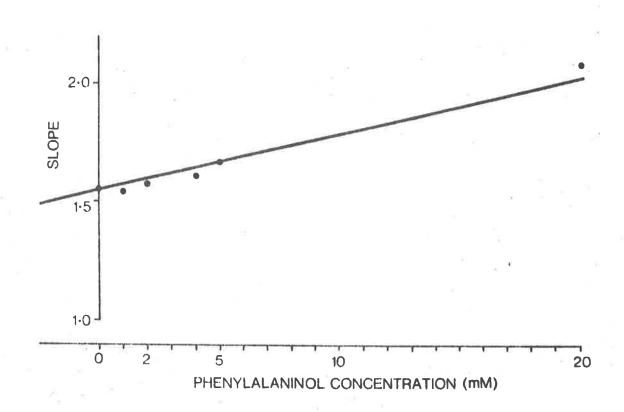
FIGURE 42: SLOPES OF LINES DERIVED FROM DOUBLE RECIPROCAL PLOT
OF PHENYLALANINE UPTAKE IN THE PRESENCE OF PHENYLALANINOL
(1-20 mM) PLOTTED AGAINST THE CONCENTRATIONS OF PHENYLALANINOL
USED. FROM THIS FIGURE THE K_I OF PHENYLALANINOL IS DERIVED AND
IS 6.77×10^{-2}M.

FIGURE 43 : EFFECT OF 0.5 mM PHENYLALANINOL ON PHENYLALANINE UPTAKE BY EVERTED GUT SACS.
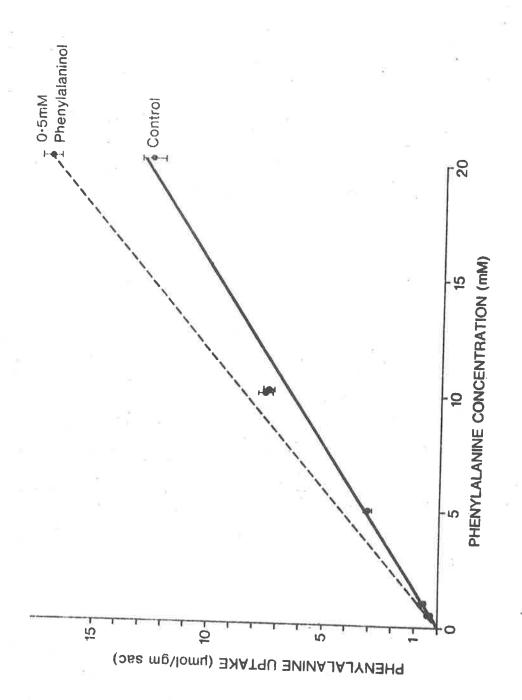
FIGURE 42: EACH POINT (PLUS STANDARD DEVIATION FOR 5, 10 AND 20 mM) IS SHOWN. 0.5 mM PHENYLALANINOL STIMULATES PHENYLALANINE UPTAKE.

TABLE XLI   Slope of the Line expressing Phenylalanine Uptake by the Everted Gut Sac
as a Function of Phenylalanine Concentration at Various Levels of Phenylalaninol

Phenylalanine Uptake as Function of Phenylalanine Concentration				Double Reciprocal Plot			
Concentration of Phenylalaninol	Slope of line	Intercept on y axis	Correl-ation (R)	Concentration of Phenylalaninol	Slope of line	Intercept	Correl-ation (R)
Zero (control)	0.696	0	0.99	Zero	1.551	.0105	0.99
0.5mM	0.858	0	0.98	0.5mM	1.262	.0107	0.97
1.0mM	0.680	0	0.98	1.0mM	1.33	.0105	0.98
2.0mM	0.661	0	0.98	2.0mM	1.45	.0106	0.99
4.0mM	0.635	0	0.96	4.0mM	1.62	.0120	0.97
5.0mM	0.622	0	0.97	5.0mM	1.66	0.130	0.99
20.0mM	0.575	0	0.99	20.0mM	1.98	.0104	0.98

Table XLI: Phenylalanine Uptakes are expressed graphically in Figures 40 and 41.   The slopes
are expressed numerically in this Table where uptake is expressed as a function of
phenylalanine concentration.   In the left-hand Table, the lower the figure in the
slope column, the lower the uptake for each concentration of phenylalaninol.   In the
double reciprocal plot the higher the figure for slope, the less the uptake.   It can
be seen that 0.5mM phenylalaninol clearly stimulates phenylalanine uptake, whereas
1.0mM and 2mM have little effect and 4, 5 and 20mM phenylalaninol inhibit phenylalanine
uptake.

TABLE XLII Slope of the Line expressing Phenylalanine Uptake by the Everted Gut Sac as a Function of Phenylalanine concentration and the effect of 0.5 and 1.0mM β-2-thienylalanine or Cycloleucine

Phenylalanine Uptake as a function of Phenylalanine concentration				Double Reciprocal Plot			
Concentration of Inhibitor	Slope of Line	Intercept of y axis	Correlation coefficient (R)	Concentration of inhibitor	Slope of line	Intercept on y axis	Correlation coefficient (R)
Zero (control)	0.649	0	0.99	Zero (control)	1.590	0.0109	0.99
0.5mM β-2-thienyl-alanine	0.651	0	0.99	0.5mM	1.623	0.0011	0.99
1.0mM β-2-thienyl-alanine	0.565	0	0.96	1.0mM	1.730	0.012	0.99
Zero (control)	0.676	0	0.98	0	1.452	0.011	0.99
Zero (control)	0.653	0	0.96	Zero (control)	1.5616	0.012	0.98
0.5mM Cycloleucine	0.648	0	0.98	0.5mM	1.511	0.009	0.96
1.0mM Cycloleucine	0.542	0	0.97	1.0mM	1.801	0.0140	0.96
Zero (control)	0.662	0	0.99	0	1.497	0.0133	0.97

Table XLII : Phenylalanine uptake in the presence of β-2-thienylalanine (0.5mM or 1.0mM) is not significantly different from control values nor does Cycloleucine have any effect on the slope of the line representing phenylalanine uptake as a function of phenylalanine concentration.

The Shimomura et al. (105) study also indicates indirectly that phenylalaninol is absorbed by the intestine. They gave phenylalaninol 200mg/kg. orally alone or with 800mg/kg. of phenylalanine. The urinary sodium level after phenylalaninol alone was significantly higher than in control rats given water, as was the sodium to potassium ratio. The combination of phenylalaninol plus phenylalanine gave similar results. However, the urinary potassium level in the group given phenylalanine and phenylalaninol was significantly reduced compared to the group given phenylalanine 800mg/kg. alone. While it would be preferable to have measured blood levels of phenylalaninol directly, the fact that phenylalaninol appears to affect the urinary levels of electrolytes when given orally is suggestive evidence that it is absorbed. It would be possible to argue that the effects are secondary to changes in phenylalanine or tyrosine levels in blood induced by phenylalaninol's effect on their absorption, but they found phenylalaninol 300mg/kg. alone had no significant effect on phenylalanine or tyrosine levels in blood of their rats.

If phenylalaninol is absorbed it would be important to determine the level in blood and, hence, the likely level in glomerular filtrate. If these levels were to be in the 0.5mM – 1.0mM range then phenylalaninol would stimulate reabsorption of phenylalanine by the proximal tubule which would be undesirable. If the levels of phenylalaninol in blood were to be similar to the levels of β-2-thienylalanine in infant monkeys when fed β-2-thienylalanine 0.75mg/kg/day (1.2 – 11.4mg/100ml.) then predicted levels of phenylalaninol would be 0.08mM to 0.75mM (M.W. of phenylalaninol = 151), i.e. in the stimulatory range.

This Chapter explains the apparently anomalous results of Chapters X and XI. It casts doubt on using phenylalaninol as a treatment agent and suggests that given orally β-2-thienylalanine would have little effect on the renal handling of phenylalanine if given at 0.75g/kg/day - a dose that is well above the apparently effective dose of 20mg/kg/day. If cycloleucine shows similar absorption characteristics to β-2-thienylalanine its predominant effect would be on intestinal absorption - not renal - too.

Because cycloleucine in low does 20mg/kg/day was ineffective, and because it would cause a general aminoaciduria, it would appear less desirable than β-2-thienylalanine as a treatment agent. Thus, β-2-thienylalanine would seem to be the theoretically best agent out of it, phenylalaninol and cycloleucine. Because of this, further studies on its potential toxicity were warranted, and these were undertaken as described in the previous Chapter and by K.J. Brown, as discussed in Chapter XIV.

CHAPTER XIV

GENERAL DISCUSSION AND CONCLUSIONS OF THE APPROACH TREATING
PHENYLKETONURIA BY THE ADMINISTRATION OF PHENYLALANINE ANALOGUES

This thesis set out to examine the possibility of whether β-2-thienylalanine or other phenylalanine analogues could inhibit phenylalanine transfer across either the intestine or increase the urinary excretion of phenylalanine, hence allowing greater intake of phenylalanine to be consumed by the phenylketonuric child. This, in turn, would allow liberalisation of the diet taken by the child and therefore probably lead to improved social function of the child.

In Chapter III it was shown that β-2-thienylalanine doubled the excretion of β-2-thienylalanine in Rhesus monkeys who were rendered hyperphenylalananaemic by phenylalanine infusion. Unfortunately, the mechanism of this excretion was not examined but when parachlorophenylalanine and cycloleucine were infused in the same monkeys, also rendered hyperphenylalananaemic by phenylalanine infusion, it was found that the excretion of phenylalanine increased mainly due to an increase of glomerular filtration rate. Regrettably, these monkeys were not available to the candidate for his work in Australia and New Zealand, and he had to use a different species for looking at the mechanism of the increased excretion of phenylalanine that was induced by β-2-thienylalanine. Using the isolated brush border of the proximal renal tubule of the rat, it could be shown that β-2-thienylalanine did reduce uptake by the vesicles in this model, which would suggest that β-2-thienylalanine was inhibiting renal tubular transport of phenylalanine from the glomerular filtrate. However, in dogs under general anaesthesia, no such inhibition of phenylalanine transport could be seen. Indeed, β-2-thienylalanine appeared to increase the renal tubular reabsorption of phenylalanine, although this result is considered to be fallacious because whenever the filtered load of phenylalanine was increased, the renal tubular reabsorption of phenylalanine increased, and this probably reflects the inability of the spectrofluorometric method used to measure phenylalanine levels in urine

accurately when such levels are low. In these dogs a statistically significant increase in glomerular filtration rate was detected after the infusion of either phenylalanine, β-2-thienylalanine or the combination. However, this increase was only evident in the 30 minute period under study.

Because of the species difference, the results of most interest are those that were attained in phenylketonuric humans, and in Chapter VI it was shown in 3 phenylketonuric women who were untreated by low phenylalanine diet, that β-2-thienylalanine did, indeed, increase the excretion of phenylalanine, and that this was associated with reduced renal tubular reabsorption of phenylalanine in all 3, plus an increase in glomerular filtration in two of the three women.

The oral feeding of β-2-thienylalanine produced more consistent and hopeful results. In Rhesus monkeys given β-2-thienylalanine along with phenylalanine, the *effective absorption* of phenylalanine was reduced. In infant monkeys fed from birth on β-2-thienylalanine along with oral phenylalanine supplements that would give them blood levels in the phenylketonuric range, β-2-thienylalanine did reduce the serum phenylalanine levels and appeared to be at least partially protective in preventing the consequences of high serum phenylalanine, that is, mental retardation and fits. This work was confirmed in acute oral load of β-2-thienylalanine to phenylketonuric patients wherein it was shown that when a patient was given 100mg/per kg. of oral phenylalanine, a 20mg/per kg. concurrent load of β-2-thienylalanine reduced the *effective absorption* of phenylalanine by 47% on average. Thus, β-2-thienylalanine appeared to have a potential for treatment agent in phenylketonuria.

The second agent looked at was cycloleucine which is known to produce aminoaciduria in man which includes phenylalanine in man. Cycloleucine increased the urinary excretion of phenylalanine mainly by increasing the glomerular filtration rate. It also reduced the intestinal absorption of phenylalanine in the Rhesus monkey, and these effects were confirmed both in the isolated brush border of the rat proximal

renal tubule and in the everted gut sac of the rat. Indeed, cycloleucine in these systems would appear to have an advantage over the β-2-thienylalanine. However, a chronic feeding study of cycloleucine 20mg/per kg. per day to phenylketonuric patients did not demonstrate any lowering of serum phenylalanine levels in these untreated patients.

Initially, parachlorophenylalanine was tested to see whether it was a much more potent inhibitor of transport of phenylalanine from the other two agents. This certainly was not the case where the urinary studies of phenylalanine were concerned, nor did it appear to be particularly more effective than β-2-thienylalanine and cycloleucine when given orally to monkeys. Parachlorophenylalanine is known to inhibit the serotonin pathway and to produce quite obvious clinical effects such as increase in libido and insomnia in experimental animals. Because of these known side effects and the fact that it did not seem to confer greater advantage in treatment of phenylketonuria, it was not tested in later experiments. Instead, phenylalaninol was substituted because of the work of Shimomura et al. ( ) which was published after the candidate had conducted his initial primate studies. Phenylalaninol, according to Shimomura et al., significantly reduces intestinal absorption of phenylalanine, and this work was confirmed by us in Chapter 11 on the everted gut sac of the rat. However, we were not able to find any effect of phenylalaninol on phenylalanine uptake by the isolated brush border of the rat renal proximal tubule. Thus, it appeared that β-2-thienylalanine, cycloleucine and possibly phenylalaninol have potential as treatment agents in phenylketonuria. Cycloleucine has been used in a short-term chronic study and not found to be of value. Objections to human testing of β-2-thienylalanine and phenylalaninol are largely ethical.

The candidate has chosen to concentrate on β-2-thienylalanine because of his long-term monkey experiments which indicated that it might be safe, and the potential of phenylalaninol was not evident until after he left the United States of America where he had access to monkeys. Ethical committees in New Zealand have indicated that they were unwilling

to allow studies on phenylketonuric patients with β-2-thienylalanine because of some toxic effects mentioned by other authors which will be reviewed below. The only toxic effect that the author had been able to elicit in monkeys was a possible predilection to Shigella infection, and this is considered to be coincidence as the work of K.J. Brown showed in his Ph.D. thesis entitled "The Effects of β-2-thienylalanine a possible treatment agent of phenylketonuria, on bacterial growth". The published works by Dr. Brown and the candidate have been reviewed in Chapter XII and will also be discussed in detail below.

To quote from the general conclusions of K.J. Brown's thesis:

"The completed studies suggest that β-2-thienylalanine would have little effect on indigenous bacteria populations in treated PKU patients. Few genera of bacteria which were tested in pure culture in minimal media were inhibited by β-2-thienylalanine and the inhibition of pure cultures was reversed in the presence of phenylalanine and tyrosine. Under solid media conditions, magnesium was also effective in abolishing β-2-thienylalanine inhibition of cultures. Bacteria from phenylketonuric patients seemed to be similar in reaction to β-2-thienylalanine as cultures of the same genera as non-phenylketonuric sources. Only 7% of culturable strict anaerobic bacteria were inhibited in pure culture. Even for these inhibited analogues phenylalanine seemed to be protective against β-2-thienylalanine inhibition. The mouse studies were also promising in that animals fed β-2-thienylalanine were no more susceptible to infection with intestinal pathogens than were controls. Indigenous bacteria appeared to be present in equal numbers in β-2-thienylalanine and water treated animals, and no histologically detectable changes were noted in examined tissues from β-2-thienylalanine-fed animals.

The general conclusion that one can make from these studies is that β-2-thienylalanine is of low toxicity both for bacteria and mammalian tissues."

The other objection to using β-2-thienylalanine in phenylketonuric patients is that β-2-thienylalanine might cause *false proteins* to be made and lead to cellular damage. As outlined in Chapter V, monkeys fed β-2-thienylalanine from birth during infancy showed no haematological abnormalities,

no clinical abnormalities other than the possible Shigella gastro-enteritis, and when one underwent autopsy studies, no β-2-thienylalanine could be found in brain protein and the amount found in liver protein was very small indeed, with a ratio of phenylalanine to β-2-thienylalanine being 22:1.   Chapter XII of this thesis looked at the effect of β-2-thienylalanine on fibroblast growth in culture and found no inhibition of growth occurred at levels that were considerably above those which could be expected in patients treated with β-2-thienylalanine 20mg./per kg. per day.   It could be argued from the data in Chapter XII,   that some non-specific addition was causing the inhibition of fibroblast growth found when 200 microlitres of the additions were added to the culture medium because β-2-thienylalanine, phenylalanine, combination of phenylalanine and β-2-thienylalanine all have much the same effect.   At lower doses of these agents very little, if any, inhibition was observed. Certainly, no statistically significant inhibition could be found in these studies.   The literature relevant to β-2-thienylalanine causing cellular damage is discussed in the next section of this Chapter.

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Literature concerning β-2-thienylalanine and Cellular Damage

Hermann in 1953 used explanted chick embryos after approximately 24 hours of incubation (late headfold to 6-somite) which were kept on a mixture of agar and egg extract for 18 hours during which time primitive central nervous system, optic vesicle and otocyst, a beating heart and a regular string of somites can be observed. 2.5mg/ml. (approximately 14mM) of β-2-thienylalanine reduced growth (normally 4.0cm length was reduced to 2.9cm) which was partly reversed by adding equimolar amounts of phenylalanine (3.4cm length achieved). In addition, zigzag neural tube, irregular brain and somite formation occurred and this was not reversible by the addition of phenylalanine. He noted that β-2-thienylalanine produced malformations only in concentrations 10 times higher than those found to produce malformations with leucine analogues. (118)

Munro and Clark (119) using 400mg. tissue slices from rat livers in Krebs-Ringer solution, found that 7mM β-2-thienylalanine inhibited uptake of glycine into protein (63%) and orthophosphate occurred after 1 hour. When equimolar phenylalanine was added with β-2-thienylalanine, partial reversal of the inhibition occurred. However, when it was added after 1 hour, there was no reversal of inhibition. They suggested that phenylalanine acted by preventing penetration of β-2-thienylalanine into the cell and that the inhibitory effects of intracellular β-2-thienylalanine are rather general and non-specific. The latter point was sustained by the finding that intracellular β-2-thienylalanine also inhibited para-aminohippuric acid formation from para-amino-benzoic acid and glycine. Again, the concentration of β-2-thienylalanine used was very high, and if it were used for treatment of phenylketonuric children, all cells would be simultaneously exposed to phenylalanine which they showed, at least partially, reverses the effects of β-2-thienylalanine.

Caveness (120) showed that β-2-thienylalanine is toxic to the rabbit macrophage and can cause morphological changes and cell death. Despite this toxicity, he demonstrated little

inhibition of protein synthesis (using radioactive valine incorporation) and no inhibition of protein breakdown. The toxicity of β-2-thienylalanine (2mM) was largely, if not totally, prevented by the concurrent inclusion of phenylalanine in the Eagle's medium. If no phenylalanine was present, β-2-thienylalanine was incorporated into the macrophage at about 40% rate of normal for phenylalanine.

In a further study using the same concentration of β-2-thienylalanine, Caveness (121) examined incorporation of exogenous adenine and hypoxanthine into ribonucleic acid (RNA). He found that β-2-thienylalanine had no discernible effect on adenine incorporation into the adenine fraction of RNA over the first 6 hours, but slightly decreased it in the following 6. However, β-2-thienylalanine increased the uptake of both adenine and hypoxanthine into the guanine fraction of the macrophage's RNA.

He then labelled RNA with ^{14}C-8-adenine and examined the effect of 2mM β-2-thienylalanine on the loss of adenine from RNA over 8 hours. β-2-thienylalanine had no effect on the loss rates from the adenine fraction, but increased the loss by 40% from the guanine fraction. He concluded that β-2-thienylalanine did not significantly alter the rate of RNA synthesis, or break down in the cell.

Again, these studies used levels of β-2-thienylalanine much higher than would be expected if 20mg/kg. of β-2-thienylalanine was used in patients, but the levels are much less than those used by Munro and Clark (119), suggesting that toxicity is unlikely if 20mg/kg. of β-2-thienylalanine were used. His finding of complete or near complete protection by equimolar phenylalanine is encouraging because, in the candidate's proposed treatment, the level of phenylalanine would always be higher than that of β-2-thienylalanine.

Jacquez, Barclay and Stock (122) were the first to look at the effect of β-2-thienylalanine on cell cultures. They were predominantly interested in its possible efficacy as an anti-tumour agent. They selected cultures of heart and of sarcoma T-241 from C57 black mice. They looked for cytotoxic

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effects such as rounding up of cells, increased granularity
of the cytoplasm and disintegration of cells and graded such
0 to 4+.

It was found that 2.92 μM/ml. of β-2-thienylalanine
produced dubious toxic effects on the heart but 1+ toxicity
on sarcoma cells. Doubling concentrations of β-2-thienylalanine
produced increased toxicity and at 1.68 μM/ml. 4+ toxicity was
found in both heart and sarcoma cultures. They noted that macro-
phages which sometimes migrated out of the sarcoma implants appeared
more resistant to toxicity induced by β-2-thienylalanine. They
found that the toxicity could be blocked by l-phenylalanine
but not by d-phenylalanine. Describing inhibition ratios as
that required for half-nullification of 4+ toxicity, they found
that over their range of concentrations (2.92 - 11.68mM) both
in heart and sarcoma the ratio was 1 mole of l-phenylalanine
to 12 to 24 moles of β-2-thienylalanine. The ratio expected
to be found in phenylketonuric patients treated with β-2-thienylalanine
would be the reverse, probably of the order of 3 moles of phenyl-
alanine to 1 mole of β-2-thienylalanine. Thus, on the data
of Jacquez et al. (122) phenylalanine would be very protective.
Unlike Dittmer, Ellis, McKennis and du Vigneaud (123) and Brown,
as described in the previous section of this Chapter, Jacquez
et al. (122) could not find any protection from tyrosine; nor
could tryptophan protect in this system.

Rabinovitz, Olson and Greenburg (124) using the Ehrlich
ascites carcinoma *in vitro* examined the incorporation of phenyl-
alanine, valine, leucine and lysine into protein in the presence
and absence of β-2-thienylalanine (and o-flurophenyl-DL-alanine).
In order to estimate the extent of incorporation of amino acids
by mechanisms other than the usual metabolic pathways, a comparison
was made between incorporation into the ascites cell protein
of labelled carbon from leucine and norleucine and from valine
and norvaline. Leucine carbon was incorporated at a rate 300
times that of norleucine and valine carbon 100 times that of
norvaline, indicating a high order of specificity for the incor-
porative process.

They found that, by Michaelis-Menton kinetics using the Lineweaver and Burke plot, inhibition of phenylalanine incorporation (into protein) by β-2-thienylalanine was strictly competitive. They found the inhibition ratio (molar ratio of antagonist to amino acid for 50% inhibition) was 100:1 for β-2-thienylalanine: phenylalanine (and 20:1 for ο-fluorophenylalanine).

Rabinovitz et al. also found that β-2-thienylalanine (and ο-fluorophenylalanine) competitively inhibited the incorporation of the other amino acids tested but, unlike phenylalanine, the two antagonists had equal inhibitory potency. In addition, phenylalanine itself inhibited the incorporation of the other amino acids in the same manner as did its analogues - a finding compatible with the candidate's findings on the effect of β-2-thienylalanine and phenylalanine on fibroblast cultures (Chapter XII). Phenylalanine and its analogues inhibited valine incorporation more than they did that of leucine or lysine. At 0.05M concentration they required a 100:1 molar ratio to inhibit valine incorporation 50% while this degree of inhibition was not achieved for leucine or lysine at 1000:1 ratio.

These findings fit with the hypothesis of Christensen, Riggs, Fischer and Palatine (125) that the effect of phenylalanine and its analogues upon other amino acids is due to an imbalance with decreased absorption of one amino acid at low concentration when the cell is presented with a high concentration of another. The very high β-2-thienylalanine:phenylalanine ratio and the fact that the inhibition was strictly competitive strongly suggests that in the phenylketonuric patient, where the β-2-thienylalanine: phenylalanine ratio would be less than one, that inhibition of phenylalanine incorporation would be negligible.

Rabinovitz et al. (124) also showed that the inhibition of phenylalanine incorporation into protein was in part by preventing its absorption by the cell and in part, by inhibiting the incorporation of intracellular phenylalanine into protein, but as regards the latter inhibition, it was nowhere near as effective as ο-fluorophenylalanine. Nor did β-2-thienylalanine inhibit the incorporation of intracellular valine, leucine or lysine into protein.

Godin and Dolan (60) showed that negligible amounts of β-2-thienylalanine itself were possibly incorporated in protein of rats. They injected DL-β-2-thienylalanine (the same as used in all experiments described in this thesis), labelled with ^{14}C at the 3 position into the left jugular vein and measured radioactive carbon incorporation into protein at 2, 6 and 24 hours later. Table is derived from their results.

TABLE XLIII

Incorporation of ^{14}C in Tissue Proteins after Injection of Phenylalanine and DL-β-2-thienylalanine-3-^{14}C

Time after Injection	Liver	Kidney	Pancreas	Brain	Plasma
2 hours	2.8×10^{-4}	8.7×10^{-4}	3.6×10^{-3}	1.6×10^{-4}	1.0×10^{-3}
6 hours	3.0×10^{-4}	8.9×10^{-4}	1.3×10^{-3}	1.9×10^{-4}	5.3×10^{-4}
24 hours	1.3×10^{-4}	5.7×10^{-4}	3.1×10^{-4}	1.1×10^{-4}	1.4×10^{-4}

Table XLIII: The radioactivity contained in the tissue proteins is expressed as a percentage of the amount injected intravenously. The highest uptake is 0.0036% into pancreas protein 2 hours after injection, and this declines ten-fold by 24 hours.

It could be argued that ^{14}C incorporation does not represent β-2-thienylalanine incorporated into protein. However, Godin and Dolan (60) found that only 0.45% of the radioactivity injected as β-2-thienylalanine was recovered as carbon dioxide 2 hours after injection, and that this quantity did not increase over the following 10 hours.

30% of the radioactivity injected as β-2-thienylalanine was found in the urine 6 hours after injection and 37% by 12 hours. These results suggest that very little, if any, of β-2-thienylalanine was extensively metabolised. Radioacetography of the urine demonstrated that this compound produced a large number of urinary metabolites and unchanged β-2-thienylalanine. In contrast, the candidate found 90% of β-2-thienylalanine was excreted unchanged in monkeys infused with it. This may represent a species difference or differences in measurement technique as the candidate used amino acid analysis not radioacetography) or the fact that the labelling of β-2-thienylalanine with ^{14}C at the 3 position may have lead to increased metabolism of the β-2-thienylalanine.

However, the findings of Godin and Dolan (60) agree with the candidate's finding (Chapter V) that incorporation of β-2-thienylalanine into protein is negligible, and Godin and Dolan have shown that such proteins are metabolisable and, hence, would not accumulate.

All the studies in this Section suggest that β-2-thienylalanine will not, in the quantities sufficient to reduce intestinal absorption of phenylalanine, produce a reduction of phenylalanine incorporation into protein, nor will it be significantly incorporated into protein itself. Thus, this concern should not prevent a short, e.g. two week trial, as outlined for cycloleucine in Chapter VII, of β-2-thienylalanine being undertaken in phenylketonuric patients.

Could β-2-thienylalanine replace phenylalanine as the brain damaging agent?

When the candidate has presented aspects of this work at scientific meetings, it has been suggested that the circulating β-2-thienylalanine would act on the brain in a similar way to elevated phenylalanine and, thus, the lowering of serum phenylalanine would be offset by the benefit of β-2-thienylalanine in the serum.

In Chapter V it was shown that the levels of β-2-thienylalanine that occurred on long-term oral feeding of it were significant, although incorporation into protein was negligible. However, the two monkeys who were not fed supplemental phenylalanine but were given β-2-thienylalanine had similar levels of β-2-thienylalanine to those fed the combination, and did not develop clinical evidence of mental retardation. This does not address the problem of whether the β-2-thienylalanine levels plus moderately elevated phenylalanine levels might be just as damaging as high phenylalanine levels alone - the evidence cited in Chapter V was that one monkey fed the combination was clinically brain damaged, while the other was not. However, the experimental animal has phenylalanine hydroxylase which is probably inhibited by β-2-thienylalanine as shown by Uderfriend and Cooper (63), and by the candidate in Chapter VIII. Thus,

the reduction of serum phenylalanine in the experimental animal is less than would be achieved in phenylketonuric man.

To further explore the possibility of β-2-thienylalanine causing brain damage in combination with moderately elevated phenylalanine levels, this section looks at the various theories of mechanism of brain damage and how the added presence of β-2-thienylalanine could be expected to affect same.

Theory of serotonin deficiency

In 1957 Pare, Sandler and Stacey (67) found that in 10 phenylketonuric children, the serum 5-hydroxytryptamine levels and urinary 5-hydroxyindoleacetic acid levels (expressed as a function of urinary creatinine excretion) were markedly reduced, compared to 12 normal children, 15 hospitalised children awaiting tonsillectomy and 9 mentally defective children without phenylketonuria living in the same institution as the phenyl-ketonuric children. They postulated that a reduction in serum phenylalanine level would favour hydroxylation of tryptophan and result in an elevated serum 5-hydroxytryptamine (serotonin), and that this is important for brain function. In 1959 the same workers (67) compared 49 patients with phenylketonuria and 32 mental defectives and confirmed their earlier work that the phenylketonuric patients had lower urinary 5-hydroxyindole acetic acid levels (2.2mg/g. creatinine) than their mentally defective non-phenylketonuria group (7.2mg/g. creatinine). In addition, the phenylketonuria group again had much lower serum 5-hydroxytryptamine levels (71.2ng/ml. compared to 283ng/ml. in non-phenylketonuric controls).

They then examined the relationship between 5-hydroxy-indoleacetic acid and intelligence and found no significant correlation. They postulated that high phenylalanine levels might affect other neurotransmitter pathways. Knox (68) has made similar observations including finding low serotonin levels in cerebrospinal fluid. McKean (127) has shown low serotonin levels in brain tissue on autopsy samples from untreated institutional patients with phenylketonuria. He also found low catechol-amine, tryptophan and tyrosine levels. McKean with Schanberg and Giarmann showed that the uptake of the serotonin precursor

5-hydroxytryptophan into the brain was reduced by elevated serum phenylalanine levels, while Huang and Hsia (129) showed that 5-hydroxytryptophan decarboxylase was inhibited by phenylalanine and phenylketones.

However, Davison and Sandler (130) found that the phenylketones, particularly phenylacetic acid and phenylpyruvic acid, were much more powerful inhibitors of 5-hydroxytryptophan decarboxylase than phenylalanine itself. Using 3.3mM concentrations with phenylacetic acid, they obtained an 84% inhibition of 5-hydroxytryptophan decarboxylase and phenylpyruvic acid inhibited by 76%, phenyllactic acid by 52%, while phenylalanine alone only inhibited this enzyme 13%.

If it is the metabolites of phenylalanine that inhibit rather than phenylalanine itself, then β-2-thienylalanine would not be toxic to the brain.

Wooley and van der Hoeven (131) used a combination of both d and l phenylalanine plus l tyrosine in mice and showed that there was a dubious reduction in the ability of the mice to escape from a bright light. They (131) then showed that if 5-hydroxytryptophan or another serotonin congener, melatonin, was given with the phenylalanine and tyrosine, the learning impairment was prevented. However, using a different phenyl-ketonuria model of parachlorophenylalanine plus phenylalanine feeding to rats, Butcher, Vorhees and Berry (133) showed learning deficits not found in rats fed the same dose of parachlorophenyl-alanine. Yet, both groups of rats showed an identical reduction in serotonin. The same group (134) injected parachlorophenyl-alanine plus phenylalanine into neonatal rats and compared them to another group which received the same combination plus melatonin: no difference was found but both groups had reduced learning ability compared to uninjected controls.

Finally, Schaeffer, Barrett, Sanders, Bush and Vorhees (135) showed that p-chloroamphetamine, a specific inhibitor of cerebral tryptophan hydroxylase, did not inhibit learning; rather, it improved learning.

It seems unlikely that serotonin deficiency is the likely cause of the mental retardation in phenylketonuria.

If it were, enzyme studies on the effect of β-2-thienylalanine on the tryptophan to serotonin pathway would be warranted, as would investigation into the effect of β-2-thienylalanine on the uptake of 5-hydroxytryptophan into the central nervous system.

Other neurotransmitter substances have been implicated in the pathogenesis of phenylketonuria; as mentioned above, Pare, Sandler and Stacey (67) theorised that such may be the case, while McKean showed low levels of catecholamines in phenylketonuric post-mortem brain – the catecholamines reduced were dopamine and especially norepinephrine. Nadler and Hsia (136) found reduced catecholamines including norepinephrine in blood and urine of phenylketonuria sufferers. The mechanism of this reduction appears to be via the presence of phenylethylamine in the brain (137); as discussed below, this is unlikely to be increased by the presence of β-2-thienylalanine in blood.

Lasala and Coscia (138) found a tetrahydroisoquinoline derivative in phenylketonuric urine and in the brains of rats with experimental PKU which inhibits dopamine-β-hydroxylase. Again, the presence of β-2-thienylalanine in blood is not likely to increase the formation of this substance.

Phenylethylamine

The role of phenylethylamine and associated Vitamin B6 (pyridoxine) metabolism disturbance is a very interesting one in the various hypotheses of the pathogenesis of brain damage in phenylketonuria. It has been pursued predominantly by Loo and Mack (139). They showed that the phenylethylamine-pyridoxal complex is neurotoxic in mice (140) and that phenylethylamine increases in rat brain with increasing serum phenylalanine (141). They showed that the pyridoxamine formed prevented the accumulation of acidic phenylalanine metabolites, probably by binding with the first phenylketone derived from phenylalanine, i.e. phenyl-pyruvic acid, and transferring it out of the brain cells. Phenyl-ethylamine also depletes B6 by combining with it and could lead to an effective Vitamin B6 deficiency which, in turn, could lead to both impaired myelin synthesis (140) and neurotransmitter abnormalities. Certainly, at post-mortem, phenylketonuric brains appear to have been improperly myelinated *(vide infra)*.

They behaviourally supported their biochemical hypo-
thesis (140) by showing that weanling rats fed phenylalanine
plus pyridoxine had normal learning.   They could find no effect
of a pyridoxine free diet but an antimetabolite of Vitamin B6
did (and the complex of phenylethylamine and pyridoxine has
anti-vitamin B activity).   Thus, in the absence of pyridoxine,
the phenylalanine metabolites which are acidic would not be
transported out of the brain and could cause damage in their
own right, the presence of an anti-vitamin B6 substance would
be formed, Vitamin B6 would also be consumed.   All of these
actions could damage the brain.   Lastly, Loo and Mack (141) found
that giving B6 along with its anti-metabolite prevented learning
deficits.

Phenylethylamine formation in phenylketonuric patients
is   a   direct   result   of   elevated   serum   phenylalanine.
β-2-thienylalanine would not increase the formation of phenyl-
ethylamine.   Thus, if the phenylethylamine hypothesis of brain
damage pathogenesis is correct,   β-2-thienylalanine would not
merely replace phenylalanine as the first step towards brain
damage.

Brain Polyribosome Disaggregation

As stated in Chapter I , this theory has been advanced
by Aoki and Siegel (73) and has been supported by other workers.
(143-146). Taub and Johnson pointed out that the 'disaggregation'
does not fit with other findings of altered amino acid concen-
trations in the brain (discussed below), as a rate limiting
supply of amino acids has been shown to apparently stabilise
cell polyribosomes and decrease the transition to monomeric
ribosomes (145).   Tryptophan deficiency is the only amino acid
deficiency according to Hori, Fisher and Rabinovitz (147) that
led to 'disaggregation' of polyribosomes in rabbit reticulocytes.

Zomzely, Roberts, Brown and Provost (148) reported
mammalian brain polyribosomes to be more unstable than those
of other tissues.   While Aoki and Siegel (73) noted that in
response to high levels of phenylalanine it was the brain tissue,
particularly in neonatal animals, that was most susceptible.
These findings are compatible with human phenylketonuria where

tissue damage other than to brain is negligible (or explainable by tyrosine deficiency or phenylketone production), and where it is known that neonatal brain is more susceptible to damage than are adult brains.

Taub and Johnson's (145) paper was directed towards the "elucidation of the mechanism by which phenylalanine disrupts brain polyribosomes and the effects of aging on polyribosome mobility in the presence of high concentrations of amino acid". They injected mice with either 1 or 2 mg/kg. phenylalanine and examined sedimentation profiles of brain ribosomes 30 minutes later. They used saline injected mice as controls. From their studies they considered 'disaggregation' a misnomer and showed that the increase in monomeric ribosomes (28%-42%, depending on whether free or bound polyribosomes were examined) could possibly be explained by an increase in robosomal sub-units. They also showed that the increase in monomeric ribosomes was greater in the cerebral cortex (68%) than in the cerebellum (33%). They subjected their ribosomes to potassium disassociation which did not appreciably alter the number of active monoribosomes, but did increase the number of ribosomal sub-units. This, they argued, was evidence that the increase in monomeric ribosomes was not mediated by RNA-use since enzymic hydrolysis of mRNA+ would result in monoribosomes within mRNA and peptidyl-t-RNA attached and these particles would have been resistant to potassium dissociation. They confirmed that the brains of 60 day old mice were relatively resistant to high phenylalanine exposure, while those of mice aged between 1 and 16 days were equally sensitive.

Unfortunately, none of these studies indicate why high phenylalanine increases the ratio of monomeric ribosomes to polyribosomes. Taub and Johnson (145) hypothesise that as high phenylalanine levels, through amino acid imbalance (discussed below) may alter the availability of anylated-t-RNA, this may influence protein initiation as shown in other systems by Vaughan and Hansen (149). This, Taub and Johnson (145) argue, would reduce

the percentage of ribosomes that are bound as polyribosomal complexes. Thus, the only effect that β-2-thienylalanine could have, would be through adding to the amino acid imbalance. This is discussed in the next paragraph.

Inhibition of amino acid transport into the brain

High phenylalanine levels have been shown to decrease transport of other amino acids into the brain (150) and to reduce protein synthesis in brain (151, 152, 153, 154)

Mitoma and Le Valley (155) indicated that the incorporation of amino acids into brain protein could be influenced by transport of those amino acids into the cell or by the events of synthesis. Hughes and Johnson (156) pointed up the difficulty in distinguishing alterations at the translational level from inhibited amino acid transport. High phenylalanine level does reduce the production of methionyl-t-RNA but the acylation of t-RNA for other amino acids is unaffected (157) This suggests a highly specific role for phenylalanine and, therefore, β-2-thienylalanine would be unlikely to mimic the action of phenylalanine.

The much lower levels of β-2-thienylalanine achieved during treatment of infant monkeys (Chapter V) would be unlikely to cause inhibition of transport of other neutral amino acids. The levels achieved in those monkeys were usually less than 10mg%, whereas most clinicians believe that levels of phenylalanine up to 15mg% (0.9mM) are quite safe. It is difficult to conceive of the elevated phenylalanine levels plus β-2-thienylalanine being summated, because the addition of one of the branched chain amino acids in experimental phenylketonuria appears to be protective (158,159)

Abnormal or Reduced Myelin

Chase and O'Brien (160) on the basis that young patients with phenylketonuria have impaired formation of myelin (161), as do patients dying of homocystinuria (162), and the reported decreased total brain lipids, especially cerebroside and sulfatide in branched-chain ketoaciduria (163),hypothesised that the condition common to these three aminoacid-opothics was impairment of myelin formation, in particular, cerebroside sulfate (sulfatide).

They injected newborn rats from 12 hours of birth thrice daily until 18 days of age, giving 5g/kg/day subcutaneously either one of 8 amino acids, phenylpyruvic acid or ammonium carbonate (only 2.5g/kg/day). Control animals were injected with physiological saline. To obtain growth reduced rats, they fed 16 pups from one mother rat while all others were 3 or 4 animals to one dam.

In initial experiments the *in vivo* formation of sulfatide in myelin from intraperitoneal ^{35}S-sulfate was determined at different periods after birth. Later, to examine the reversibility of the effects of excess phenylalanine, the injections were discontinued at intervals and the animals nursed normally until 18 days when *in vivo* sulfatide formation was measured.

Determination of *in vitro* sulfatide synthesis was measured in three ways –

1) ^{35}S-sulfate served as the radioactive precursor;

2) ^{35}S-adenosine 3'-phosphate 5'-phosphosulfate (PAPS) served as the sulfatide precursor and exogenous cerebroside was added;

3) was the same as 2) but whole homogenate of brain was the cerebroside source as it was in method 1).

They found that sulfatide synthesis started at 6 days old and peaked at 18 days reaching a low stable rate at 23 days. The rats injected with phenylalanine from day 1 began synthesis of sulfatide on the same day, but at a lesser rate (at day 18 synthetic rate was only half that of control saline injected animals). Body weights were not significantly less (although that may have been a Type II statistical error here as 24 controls weighed 46.2 ± 4.9g. and 11 phenylalanine injected animals 40.0 ± 4.5g) but brain weights and total brain lipids cholesterol, cerebroside and sulfatide were significantly reduced by the phenylalanine injections. Only sulfatide levels were significantly reduced if these lipids were expressed as mg. per gm. wet weight of brain. Galactolipid sulfotransferase activity *in vitro* using ^{35}S-sulfate as the precursor was lower in phenylalanine brains, but the activity was not decreased

if ^{35}S-PAPS was used. This suggests that the mechanism of decreased sulfatide formation was due to reduced PAPS. They also found decreased DNA and protein content in both the cerebrum and cerebellum.

In the later experiments looking for reversibility they found that 4 days of phenylalanine was sufficient to result in significantly reduced brain weight, total brain lipid weight and *in vivo* formation of sulfatide.

When they compared amino acids they found methionine (5g/kg/day) most inhibited sulfatide formation, tyrosine and argenine had a similar effect to phenylalanine; histidine, leucine and valine had lesser but detectable effects, but isoleucine did not inhibit sulfatide formation. Clearly, the effect of the amino acids were not equal and thus no accurate statement of the effect of β-2-thienylalanine can be predicted. It is of interest that ammonium carbonate (used to see if ammonia toxicity was a common pathway) inhibited sulfatide formation by 37%, and significantly reduced total brain weight and brain lipid weight. Arginine, although it reduced sulfatide synthesis, did not reduce total brain weight, nor did isoleucine or phenyl-pyruvate. The poorly nourished 16 pups showed similar reductions in brain weight, total brain lipid and sulfatide formation. Thus, it is hard to postulate ammonia as the common factor. As Chase and O'Brien (160) comment, the decreased DNA and protein contents "suggest that the toxicity of phenylalanine is broad and is not limited only to a reduced formation of myelin lipids."

The findings of Chase and O'Brien have been supported by Prensky, Fishman and Daftari (164) and it has been shown that incorporation of labelled acetate (165) or labelled glucose (166 ,167) is reduced in the presence of high phenylalanine level. Unfortunately, unlike Chase and O'Brien (160) they did not compare phenylalanine with other amino acids. Therefore, the effect of β-2-thienylalanine on these models can only be speculative.

Impaired Energy Metabolism

Gimenez, Valdivieso and Mayor (167) have shown that phenylalanine inhibits pyruvate kinase and hexokinase, but Weber (168) showed pyruvic acid had the same effect. If phenylalanine

exerts its effect by conversion to phenylpyruvic acid then β-2-thienylalanine would not cause brain damage. Other studies in this field supporting a disturbance of glycolysis as a mechanism for the pathogenesis of phenylketonuric brain damage do not give data such as the article of Chase and O'Brien (160) supplied, which enable one to ascertain if β-2-thienylalanine is likely to have a similar effect as phenylalanine because other amino acids are not used, but a common theme of phenylpyruvic acid affecting pyruvate decarboxylation is present (168,169). Similarly, phenylacetate has been shown to reduce synaptic boutons in rat brain by Robain, Wisniewski, Loo and Wen (171). And others have supported the role of phenylacetate in reduction of synapses and/or dendrites (172,173). Any assignment of the pathogenetic role for brain damage in phenylketonuria to the phenylketones would be evidence against β-2-thienylalanine also causing such brain damage.

Disturbed Gultamate Metabolism

Perry et al. (174) proposed a role for decreased glutamic acid being the pathogenetic mechanism for brain damage in phenylketonuria, but this view has been challenged (*vida supra*). Taishan (175) examined the possibility that 1-keto and 1-hydroxy acids of valine and leucine and the aromatic acid derivatives of phenylalanine (phenylketones) acted via their ability to inhibit glutamic acid decarboxylase which would lead to reduced γ-aminobutyric acid and that, in turn, would lead to brain damage. He found o-hydroxylphenylacetic acid to be the most potent inhibitor of glutamic acid decarboxylase obtained from rat brain, among the phenylketones, but that 1-ketoisovaleric acid and 1-keto-isocaproic acid (keto derivatives of valine and leucine) to be even more powerful inhibitors. Regrettably, he did not test phenylalanine itself, nor leucine or valine. Again, if it is the phenylketones themselves which are the responsible agents for the brain damage in phenylketonuria through the glutamic acid to γ-aminobutyric acid pathway, it is reasonable to assume that β-2-thienylalanine would not cause similar damage.

In conclusion, regarding the pathogenesis of the

brain damage in phenylketonuria, Scriver and Clow (176) have summed it up well:

> "The brain phenotype in PKU is specific; therefore, its mechanism is of great interest. Unfortunately, a biochemical basis for the irreversible mental retardation and the more reversible behavioural disorders remains unclear, or at least untidy. No particular metabolite of phenylalanine can be considered the prime culprit; no single aspect of cellular metabolism is implicated. Phenylalanine and several of its derivatives, acting in different ways on various structural and functional components of the brain, probably account for the irreversible and reversible outcomes of prolonged severe postulated hyperphenylalaninemia ...The possibilities include perturbations of energy metabolism, amino acid transport, cellular amino acid pools and synthesis of neuro-transmitters and macromolecules pertinent to brain structure and function."

In this state of uncertainty of the mechanism of brain damage in phenylketonuria, it is impossible to predict the likely effect of β-2-thienylalanine plus modest phenylalanine elevation against higher phenylalanine levels alone.

In looking at all the mechanisms, none suggest that β-2-thienylalanine would be more toxic to brain than phenylalanine. Where amino acids have been compared, definite variations in toxicity have been noted. In many cases, the phenylketones or other phenylalanine metabolites appear more damaging than phenylalanine itself. The only direct work in this field has been the candidate's, as described in Chapter V, and there it was found that β-2-thienylalanine alone appeared non-toxic and in one of two monkeys, the combination of β-2-thienylalanine and modest elevation of phenylalanine was less toxic than higher levels of phenylalanine. Unfortunately, the lowering of phenylalanine levels in the monkey experiment was less than one would expect in phenylketonuric man. It is the candidate's opinion that a short-term feeding experiment with β-2-thienylalanine would be justified if a more suitable analogue of phenylalanine cannot be found.

As discussed above and in Chapters V and XIII, a concern over using β-2-thienylalanine as a treatment agent for phenylketonuria was the fact that it might predispose the patients receiving it to Shigella enteric infection. Two possible mech-

anisms for such an effect can be postulated –

(1) that cells could be damaged by β-2-thienylalanine and thereby their ability to resist non-pathogenic doses of Shigella impaired;

or

(2) that the normal bacterial flora of the gut may be reduced by the ingestion of β-2-thienylalanine and organisms such as Shigella which might be relatively or absolutely resistant to the effect of β-2-thienylalanine would then be at an advantage in colonizing the gut.

These concerns were among the reasons why ethical committees were reluctant to allow any chronic administration of β-2-thienylalanine to phenylketonuric patients. Accordingly, these postulates, particularly the latter, were examined by Dr. K.J. Brown in his Ph.D thesis which was supervised by the candidate in conjunction with Dr. G.W. Tannock of the Department of Microbiology, University of Otago, Dunedin, N.Z. The candidate was co-author of many of the published papers that arose from that work. These will now be discussed.

Initially, it was decided to see if bacterial genera could be differentiated by their inhibition or otherwise by β-2-thienylalanine (114).

The bacteria were cultured on Demain's (177) medium with the following modifications: manganese sulphate, ferrous sulphate and calcium chloride were omitted. It was found by colony counting that bacterial growth in this medium was comparable to that on the complex medium Columbia agar. Using this, it was found that all 28 Shigella cultures tested were inhibited by β-2-thienylalanine as were 30 of 32 strains of E. coli tested. It was found that the 8 Salmonella typhi strains tested were not inhibited, nor were the 45 Salmonella typhimurium tested.

The following organisms were not inhibited by β-2-thienylalanine – Proteus mirabilis (6 strains tested), Proteus vulgaris (2), Proteus morgani (3), Proteus rettgeri (1), Pseudomonas aeruginosa (2), Acinetobacter species (4), Enterobacter species (3), Citrobacter species (1). Of the 5 strains of Klebsiella species tested, 2 were inhibited and 3 were not inhibited

by β-2-thienylalanine which is possibly due to taxonomic non-uniformity. Thus, Shigella would not have an advantage in patients fed β-2-thienylalanine but possibly Salmonella would.

During the studies on differentiating bacteria on the basis of their reaction to β-2-thienylalanine, it became evident that magnesium played a critical role and this was further examined by Dr. K.J. Brown and the candidate (99), and it was found in the Guthrie tests (5) that if magnesium sulphate was in the medium at 0.05g/l the test became unreliable and at 0.1g/l Bacillus subtilis overgrew the medium and phenylalanine levels could not be determined. Similarly, E. coli and Shigella species which were inhibited when magnesium levels in the medium were low became non-inhibited at 0.1g/l Mg. $SO_4.7H_2O$ in the medium. This is 0.41mM magnesium and the normal range of the serum magnesium is 0.75 to 1.0mM. Thus, in the human intestine no inhibition of bacterial growth pathogen or non-pathogen would be expected.

The above two studies were conducted on solid agar. The next study by Brown, Elliott, Tannock and Lines (98) was conducted in 3 different liquid, chemically defined media where it was found that β-2-thienylalanine inhibited all three genera, and in these media neither magnesium nor zinc abolished inhibition but phenylalanine and tyrosine did.

To determine the mechanism of these differences between solid and liquid media, Brown, Tannock, Elliott and Lines (98) placed β-2-thienylalanine in an agar plug and allowed it to diffuse from there into the liquid medium. It was found that Salmonella was again protected from the effects of β-2-thienylalanine. The differences occurring in β-2-thienylalanine inhibition between liquid and plate assay conditions were not due to composition of the culture plates, time of challenge of cultures with β-2-thienylalanine, availability of oxygen, differences in ratio of volume to available surface area, selection of mutants or extractable substances in the agar. It was concluded that the delay in diffusion of β-2-thienylalanine to the Salmonella allowed those species to activate a mechanism that protected them. It is difficult to conceptualize such a delay in the

intestine and, hence, Salmonella should not have an advantage in patients fed β-2-thienylalanine.

As the predominant bacteria in the large bowel are anaerobes, it was important to ensure that these would not be inhibited by β-2-thienylalanine. A study by Brown, Vesey, Tannock, Bell, Lines and Elliott (112) showed that of the 127 anaerobes isolated from the gut of 3 treated phenylketonuric children, only 9 were inhibited by β-2-thienylalanine and, in all cases, this inhibition was reversed by a phenylalanine level (13mg%) that would be expected in the serum of phenylketonuric children and was exceeded in the chyme of both fasted and fed rats (Chapter XI.

Finally, mice were challenged with Salmonella typhimurium or Shigella flexneri III when they were fed β-2-thienylalanine and when they were not (Brown, Tannock, Eyres, Elliott and Lines(113)), 350mg/kg/day was given for 7 days. 12 mice were given $10^3 - 10^4$ bacteria on the penultimate day of β-2-thienylalanine feeding. Another group of 12 mice received water by gavage and were challenged with the same dose of bacteria. Half the β-2-thienylalanine group and half the control group were killed 24 hours after bacterial challenge and the other half 48 hours later. A segment of spleen (and liver) was taken for histological examination, the rest of the spleen, the small intestine and the large intestine were cultured for bacteria.

Larger doses of bacteria were given in a similar fashion but there were only 4 mice in the β-2-thienylalanine group and 4 in the control group for Salmonella cultures with the challenge inoculation being 10^5, 10^6, 10^7 and 10^8 bacteria in each group. In addition, 4 mice were given 50mg. of Streptomycin in addition to β-2-thienylalanine, and 4 control animals were given Streptomycin in their water. These animals were challenged with 10^3 Salmonella on the penultimate day.

For Shigella 12 mice were used for bacterial challenges of 10^4, 10^5 and 10^7, 8 mice for 10^8 challenge and 4 mice for a 10^4 challenge when Streptomycin was given with β-2-thienylalanine or water.

The results are shown in Table XLIVa & b which is taken from the article by Brown et al. (113) and clearly show that β-2-thienylalanine does not increase the colonization rate with either Salmonella or Shigella.

The most common other bacterial isolates were Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa and Klebsiella species. No obvious differences were noted in either the types of bacteria recovered or their numbers on plates from water-fed or β-2-thienylalanine-fed mice unchallenged with pathogenic bacteria or given low inoculi. With increasing size of pathogenic inoculum there were more lactose-fermenting bacteria from both water and β-2-thienylalanine-fed animals compared to those given smaller inoculums and there was a narrower range of bacterial types. No obvious histological changes were noted in spleen or liver samples taken from mice given β-2-thienylalanine or water, but not challenged with pathogens.

Thus, the paper of Brown and his co-workers cited above show that there are good theoretical reasons why β-2-thienylalanine would not favour colonization of the intestine with pathogens and, in practice, β-2-thienylalanine did not make mice more susceptible to Salmonella or Shigella enteric infection.

Would Cycloleucine or Phenylalanine be an Effective Treatment Agent in Phenylketonuria?

In Chapter III it was shown that cycloleucine increased the urinary excretion of phenylalanine in hyperphenylalaninemic monkeys predominantly by increasing glomerular filtration rate. In Chapter IV it reduced intestinal absorption of phenylalanine in the same monkeys when given together with a phenylalanine load.

These results were confirmed in Chapter X where cycloleucine inhibited the uptake of phenylalanine by isolated brush border vesicles from the rat kidney proximal tubule, and in Chapters XI - XIII where cycloleucine was found to inhibit phenylalanine uptake by the everted gut sac of the rat - it was a more powerful inhibitor than β-2-thienylalanine or phenyl-alaninol. In addition, cycloleucine has been used in humans suffering cancer with negligible side-effects (94,95).

TABLE XLIV:　　　　Bacterial Colonization of Mice fed β-2-Thienylalanine from Article
by K. Brown et al.

(a) Positive colonization samples with *Salmonella typhimurium*.

Samples	Number of bacteria in challenge dose *					
	No streptomycin treatment					50 mg Streptomycin
β-2-Thienylalanine fed	10 –10 * (12 mice)	10 (4 mice)	10 (4 mice)	10 (4 mice)	10 (8 mice)	10
Small intestine	0	0	0	0	4/25%	0
Small intestine T	0	0	0	1/19%	5/44%	2/31%
Large intestine	0	0	0	1/13%	6/53%	1/19%
Large intestine T	0	1/6%	1/6%	1/9%	8/100%	0
Spleen	0	0	0	0	0	0
Spleen T	0	0	0	1/25%	3/38%	2/25%
Water fed	(12 mice)	(4 mice)	(4 mice)	(4 mice)	(4 mice)	(4 mice)
Small intestine	0	0	0	0	7/50%	2/25%
Small intestine T	0	0	0	0	5/63%	1/25%
Large intestine	0	0	1/6%	1/6%	8/81%	1/25%
Large intestine T	0	1/6%	0	0	8/97%	3/56%
Spleen	0	0	0	0	3/28%	0
Spleen T	0	0	1/3%	0	4/47%	1/13%

T = tetrathionate broth enriched cultures.
a/b: a = number of animals positive for *Salmonella*,　b = % of plates that were positive (duplicate plates of four media = 32 plates for four mice or 64 plates for eight mice).
4 of 4 β-2-T-fed animals and 3 of 4 water-fed animals were positive in at least one sample.

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(b) Positive colonization samples with *Shigella flexneri* III.

Samples	Number of bacteria in challenge dose		
	No streptomycin		50 mg streptomycin
β-2-thienylalanine fed	10 , 10 , 10 (12 mice)	10 (8 mice)	10 (4 mice)
Small intestine	0	1/4%	1/17%
Large intestine	0	5/46%	2/33%
Spleen	0	0	0
Water fed	(12 mice)	(8 mice)	(4 mice)
Small intestine	0	2/13%	1/25%
Large intestine	0	4/30%	3/42%
Spleen	0	1/8%	0

a/b: a = number of animals positive for *Shigella*, b = % of positive plates (duplicate plates of three. Media = 24 plates for four mice and 48 plates for eight mice).

However, in Chapter VII oral cycloleucine 20mg/kg/day did not reduce phenylalanine levels in 6 untreated phenylketonuric patients when given over 2 weeks. Although this is much less than is given as anticancer therapy (300mg/kg/day), it is equivalent to the 20mg/kg/dose of β-2-thienylalanine given to the same patients which reduced absorption of phenylalanine from a 100mg/kg. load by 47%. A higher dose of oral cycloleucine would appear justified to see if it could achieve the desired lowering of serum phenylalanine levels.

In considering this approach, the candidate was aware of the work of Oxender and Christensen (46) who showed that cycloleucine inhibited the transport of many amino acids - not just phenylalanine and tyrosine as β-2-thienylalanine appears to do.

Goyer et al. (47), using rats, found that the amino-aciduria following cycloleucine was predominantly dibasic, but cystinuria did not occur unless the rats' diets were supplemented with 2% cystine. They could not find a reduction in plasma levels of phenylalanine after 3 days of oral cycloleucine 300mg/kg/day - indeed, levels went up from 6.46 µM/100ml. (range 5.51-720) to 8.17 µM (7.72-8.66) despite "increases in renal clearances of urine glycine, alanine, tyrosine, phenylalanine and histidine". From the figure in their article, it appears that phenylalanine clearance increased from approximately 2.5ml/minute x 10^{-3} to about 12.5ml/minute x 10^{-3}. They did not measure glomerular filtration rate, nor did they weigh their rats to see if catobolism accounted for the elevation of serum amino acids.

Brown (48) found a marked dibasic aminoaciduria and cystinuria in advanced cancer patients given intravenous cycloleucine 300mg/kg/day. Again, phenylalanine excretion is only expressed graphically - it is clearly increased, and again, plasma levels, if anything, are increased. One would imagine that the patients would have been in a catabolic state as they were suffering advanced cancer. However, Brown states that plasma "levels were essentially unchanged by drug treatment".

Oxender and Christensen (46) and Akedo and Christensen have shown that cycloleucine has an inhibitory pattern in respect

to neutral amino acids similar to that of methionine in that it inhibits both the 'leucine preferring' and 'alanine preferring' transport systems. This supports the renal work of Brown (48) and Reynolds (47) suggesting that cycloleucine affects the transport of a wide range of amino acids. From this, it could be inferred that although 300mg/kg/day of cycloleucine might inhibit phenylalanine intake, it would also reduce other neutral amino acid uptake causing a pellagra-like state or kwashiorkor. If the intestinal transport of dibasic amino acids and cystine was affected to the same degree as renal transport, cycloleucine would cause a marked inhibition of these amino acids, while only reducing phenylalanine absorption modestly with consequent malnutrition.

Phenylalaninol showed anomalous results. As shown in Chapter XI, it was intermediate inhibition-power (i.e. more powerful than β-2-thienylalanine but less than cycloleucine) as regards phenylalanine uptake by the everted gut sac. However, it showed no inhibition of phenylalanine uptake by brush border vesicles derived from the proximal renal tubule of the rat (Chapter X). Indeed, it appeared to stimulate phenylalanine transport in this mode. The explanation of this would be a dual effect initially described by Izbicka-Dimitrijevic, Mastalerz and Kochman (117) when they examined the effect of phenylalanine analogues on rabbit-muscle pyruvate kinase activity. The analogues they examined were 1-phenylalanyl methyl ester, 1-1-amino-2-phenylethyl phosphoric acid, 1-alanine and 1-1-aminoethyl phosphoric acid, in addition to phenylalanine itself. At appropriate pH and substrate concentrations all the analogues and phenylalanine exhibited activation of the pyruvate kinase at low concentrations (1mM to 5mM). The concentration of phenylalaninol used in the brush border experiments described in Chapter X was in their low range 0.5mM to 1.0mM, while in the intestinal studies, the amount used was 5mM or 20mM and in the studies of Izbicka-Dimitrijevic et al. (117), they found all their analogues inhibited at 5mM or greater.

In Chapter XIII it is clear that phenylalaninol does have a dual effect — at 0.5mM concentration it stimulates phenylalanine uptake, at 4mM concentration and above it inhibits phenylalanine uptake by the everted gut sac. No such duality could be found with β-2-thienylalanine or cycloleucine.

This does not suggest that phenylalaninol would prove a useful treatment agent in treating phenylketonuria for, while one might be able to ensure high levels of phenylalaninol in the intestine of phenylketonuric patients that would inhibit uptake of injected phenylalanine, the levels at the proximal renal tubule would be low, thus stimulating reabsorption of phenylalanine from the glomerular filtrate. This potential disadvantage combined with the absence of even preliminary safety studies makes phenylalaninol a less attractive proposition for further investigation than β-2-thienylalanine.

CONCLUSION

β-2-thienylalanine does have potential as a treatment agent for phenylketonuria. It clearly does reduce phenylalanine absorption from the intestine of phenylketonuric patients and promotes renal excretion of phenylalanine by the same patients.

Its incorporation into protein is minimal and in the safety studies that have been conducted, has not caused concern other than possible presdisposition of the recipient to Shigella infection. This potential disadvantage has been extensively investigated by K.J. Brown (reviewed above) and no such disadvantage appears to exist.

Parachlorophenylalanine, cycloleucine and phenylalaninol have either proven or theoretical disadvantages when compared to β-2-thienylalanine as a potential treatment agent. While it is possible that a superior analogue to β-2-thienylalanine may be found and should be sought, a further investigation of β-2-thienylalanine (e.g. a medium term [weeks] feeding of it to untreated phenylketonuric patients) appears warranted.

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PUBLICATIONS RELATED TO THE THESIS RESEARCH

Renal Amino Acid Reabsorption in Hyperphenylalaninemic Monkeys Infused with β-2-Thienylalanine[1] (34944)

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β-2-Thienylalanine (α amino-thiophenepro-prionic acid), first synthesized by Barger and Easson in 1938 (1), is a potent inhibitor of phenylalanine metabolism in bacteria as shown by the bacterial-inhibition test developed by Guthrie (2) and employed in the screening of human neonates for the detection of phenylketonuria. It has been shown by Godin and Dolan (3) to affect phenylalanine metabolism in rats. When administered to mammals, β-2-thienylalanine is largely excreted by the kidney. The present study examines the effect of β-2-thienylalanine on renal excretion of phenylalanine and other amino acids in the monkey.

Methods. Four healthy, adult, male rhesus monkeys (*Macaca mulatta*) which weighed 6.26-8.75 kg were used. The animals were usually fed Purina Monkey Chow, fruits, and vitamins but were fasted for 8 hr before and throughout the experiment. They were lightly anesthetized with intravenous 5-allyl-5(1-methyl butyl)-2 thiobarbiturate (Surital, Parke-Davis, Co.) 30 mg/kg and an intravenous drip of 2.5% dextrose plus 0.45% sodium chloride was inserted. A collector was attached by tube to the external genitalia so that urine could be collected in a plastic bottle kept in ice. A few thymol crystals were placed in the plastic bottle to prevent bacterial growth. A control blood sample was drawn.

Each monkey was strapped into a special metabolic chair and when full consciousness was regained a control urine specimen was obtained. The animal was infused with 200 mg/kg phenylalanine[2] intravenously as a stat dose. Two monkeys (B56 and E27) were started on an infusion of phenylalanine in physiological saline at concentration of 2.0 g/100 ml and at a rate to deliver 2 g/kg/day. After 24 hr the infusion was changed to 2.0 g/100 ml phenylalanine plus 5 g/100 ml β-2-thienylalanine[3] in saline at the same rate. In the other two monkeys (D43, E65) the reverse order was used. The combination of phenylalanine and β-2-thienylalanine was infused for the first 24 hr followed by phenylalanine alone.

At the end of each 24-hr urine collection suprapubic pressure was applied to empty the bladder completely. During each 24-hr period three blood samples were drawn; at 3, 18, and 24 hr after the infusion was started. Acidified and filtered urine and sera samples (4) were analyzed on a Beckman-Spinco amino acid analyzer by the method of Spackman, Stein, and Moore (5) for neutral and acidic amino acids. β-2-Thienylalanine is clearly separated from phenylalanine by this method, appearing as a peak before tyrosine. Twenty-four-hour excretion and standard clearances were calculated.

Results. The 24-hr excretion and clearance of tyrosine, phenylalanine, and β-2-thienyl-alanine are shown in Table I. All monkeys increased their excretion of both phenylalanine and tyrosine when receiving β-2-thienyl-alanine. There was a marked variation in excretion of these amino acids from monkey to monkey but the increase in excretion of phenylalalanine and tyrosine whilst being infused with β-2-thienylalanine as well as

TABLE I. *The Excretion and Clearance of Phenylalanine and Tyrosine Before and After Thienylalanine Added to Infusion.*[a]

Amino acid	Monkey no. B56			D48			E27			E65		
	1	2	3	1	2	3	1	2	3	1	2	3
Excretion in mg/24 hr												
Tyrosine	93.7		2.47	102.0	97.4	0.95		59.9	6.65		56.7	3.37
Phenylalanine	216.0	524.0	2.42	69.0	116	1.66	172	375	2.17	16.8	31.9	1.67
Thienylalanine	—	955	—	95.6	95.6	—	568	568	—	—	1480	—
Clearance in ml/min												
Tyrosine	0.49	1.42	2.90	0.194	0.838	1.32	0.150	0.875	5.5	0.197	0.566	2.82
Phenylalanine	0.29	0.89	3.07	0.0847	0.175	2.07	0.208	0.771	2.71	0.071	0.162	2.28
Thienylalanine	—	4.82	—	—	0.618	—	—	4.65	—	—	20.0	—

[a] Column 1 indicates values while phenylalanine 2.0 g/kg/day infused; column 2, values while phenylalanine 2.0 g/kg/day and thienylalanine 0.5 g/kg/day infused. Column 3 is the ratio of column 2 to column 1. The ratio is significant for phenylalanine excretion ($p < .005$) but not for tyrosine excretion. The clearance ratios are significant for both phenylalanine ($p < .010$) and tyrosine ($p < .005$).

phenylalanine was approximately double that while on phenylalanine alone. The excretion of phenylalanine is expressed as a ratio derived by dividing excretion while on both phenylalanine and β-2-thienylalanine by the excretion while on phenylalanine alone. The ratio was significant for phenylalanine ($p < .005$) but not for tyrosine ($p < .20$). The clearance values for phenylalanine and tyrosine show an even greater ratio which was statistically significant (phenylalanine $p < .010$, tyrosine $p < .005$).

The excretion and clearance of other neutral and acidic amino acids show no consistent alterations when β-2-thienylalanine was added to the infusion. Detailed clearance and excretion values of neutral and acidic amino acids before and after β-2-thienylalanine are shown in Tables III and IV.

Control serum and urine amino acids are shown in Table II. The urinary amino acids were determined from a single untimed sample of urine, rather than stressing the animal by further restriction and fasting. A specific aminoaciduria was not found in the monkeys prior to the experiment, and it is unlikely that a mild generalized renal aminoaciduria would have affected the results. The normal blood levels exclude an overflow aminoaciduria.

Discussion. In the human phenylketonuric patient blood levels of phenylalanine are usually elevated at least 20-fold. Even at levels of this magnitude renal tubular reabsorption of phenylalanine is almost complete (6). Berry and her co-workers (7) were able to show an increase in excretion of phenylalanine in phenylketonurics, using paper chromatography. There was overlap with excretion levels of normal children. No clearance studies were performed, however. It is unlikely that a significant percentage of the filtered load of phenylalanine was not reabsorbed in the phenylketonuric patients.

Competition for renal tubular reabsorption between amino acids is well established (8-10) but the only study on the competition between β-2-thienylalanine and phenylalanine in mammalian kidney by Godin and Dolan (3) showed an increase of ^{14}C derived

TABLE II. Control Urinary and Serum Amino Acid Levels.[a]

| | Monkey no. | | | | | | | |
| | B56 | | D43 | | E27 | | E65 | |
Amino acid	Urine	Serum	Urine	Serum	Urine	Serum	Urine	Serum
Threonine	1.53	2.24	0.76	1.45	0.42	2.23	0.21	1.28
Serine	0.87	2.21	0.49	3.28	0.27	4.16	0.15	0.81
Glutamine	0.79	7.83	1.61	17.3	2.22	16.4	1.70	17.1
Glutamic acid	0.42	2.59	0.63	1.33	1.41	0.89	0.16	1.79
Glycine	0.11	4.14	1.68	4.15	2.89	4.39	1.84	4.04
Alanine	0.25	4.20	0.39	2.71	0.36	4.00	0.36	2.73
Valine	—	3.06	Trace	1.42	—	2.39	Trace	2.83
Methionine	—	1.40	0.35	0.77	0.10	0.65	0.89	0.59
Isoleucine	—	1.52	0.10	0.73	0.18	1.19	0.45	0.87
Leucine	—	2.94	0.11	1.75	0.12	2.30	—	1.71
Thienylalanine	—	—	—	—	—	—	—	—
Tyrosine	Trace	1.37	Trace	2.02	Trace	1.06	Trace	1.33
Phenylalanine	0.73	2.48	Trace	1.52	0.09	1.51	Trace	1.30

[a] Serum and urine concentration of amino acids in milligrams per 100 ml while on iv saline and dextrose. Trace indicates present but not measurable; (—) indicates not detected.

from phenylalanine in the urine of rats with simultaneous injection of β-2-thienylalanine. Most of this ^{11}C was found as phenylalanine metabolites, only 29% being phenylalanine itself.

In the present study, three monkeys showed significant quantities of phenylalanine and tyrosine in their urine while being infused with phenylalanine alone. The excretion and clearance of phenylalanine and tyrosine doubled in all four monkeys when β-2-thienylalanine was infused. This suggests a marked competition between β-2-thienylalanine and phenylalanine for tubular reabsorption. The degree of competition could be more accurately assessed had concurrent

TABLE III. Excretion of Neutral and Acidic Amino Acids Before and After Thienylalanine Added to Infusion in mg/24 hr.[a]

| | Monkey no. | | | | | | | | | | | |
| | B56 | | | D43 | | | E27 | | | E65 | | |
Amino acid	1	2	3	1	2	3	1	2	3	1	2	3
Threonine	20.3	30.4	1.50	1.84	13.8	7.50	14.8	36.6	2.47	4.82	0.407	0.19
Serine	10.5	16.8	1.59	4.80	9.66	2.01	7.73	45.04	5.83	4.57	3.46	0.76
Glutamine	96.0	70.3	0.73	11.0	15.8	1.43	28.4	56.8	2.00	31.3	4.44	0.14
Glutamic acid	19.3	8.59	0.44	4.10	14.12	3.44	6.35	7.20	1.13	29.2	22.6	0.77
Glycine	24.2	11.4	0.47	14.9	35.6	2.39	27.5	7.20	0.26	37.2	45.8	1.23
Alanine	6.2	2.5	0.40	1.95	0.36	0.18	3.12	4.63	1.48	15	83.5	5.57
Valine	0.0	0.0	—	3.93	2.34	0.59	—	3.98	—	6.03	1.73	0.29
Methionine	—	4.4	—	1.62	—	—	—	18.3	—	—	1.06	—
Isoleucine	2.54	4.46	1.75	6.72	—	—	0.859	6.94	8.08	—	1.27	—
Leucine	2.54	2.4	0.94	1.90	—	—	0.603	0.66	1.09	—	0.511	—

[a] Column 1 indicates excretion while phenylalanine 2.0 g/kg/day infused; column 2, excretion while phenylalanine 2.0 g/kg/day and β-2-thienylalanine 0.5 g/kg/day infused. Column 3 is the ratio of column 2 to column 1; — indicates amino acid not detected in urine. There is no statistically significant difference in the excretion of these amino acids when β-2-thienylalanine is added to the infusion.

TABLE IV. Clearance in Milliliters per Minute of Neutral and Acidic Amino Acids Before and After Thienylalanine Added to Infusion.[a]

Amino acid	Monkey no.											
	B56			D43			E27			E65		
	1	2	3	1	2	3	1	2	3	1	2	3
Threonine	1.57	1.32	0.84	0.06	0.52	8.67	0.83	1.93	2.32	0.28	0.39	1.39
Serine	0.49	0.59	1.20	0.14	0.24	1.71	0.19	1.73	9.10	0.21	0.16	0.76
Glutamine	0.71	0.46	0.65	0.14	0.07	0.50	0.24	0.65	2.71	0.16	0.03	0.19
Glutamic acid	0.40	0.30	0.75	0.38	1.31	3.45	0.30	0.72	2.4	3.65	2.55	0.70
Glycine	0.69	0.30	0.43	0.37	0.76	2.05	0.59	0.20	0.34	1.08	1.38	1.28
Alanine	0.19	0.07	0.37	0.03	0.01	0.33	0.08	0.12	1.50	0.44	2.97	6.75
Valine	0	0	—	0.06	0.02	0.33	—	.10	—	0.06	0.06	1.00
Methionine	—	0.43	—	0.08	—	—	—	3.28	—	—	0.20	—
Isoleucine	0.23	0.24	1.04	0.18	—	—	0.05	0.35	7.00	—	0.08	—
Leucine	0.13	0.10	0.77	0.06	—	—	0.02	0.02	1.00	—	0.02	—

[a] Column 1 indicates clearance while phenylalanine 2.0 g/kg/day infused; column 2, excretion while phenylalanine 2.0 g/kg/day and β-2-thienylalanine 0.5 g/kg/day infused. Column 3 is the ratio of column 2 to column 1; — indicates amino acid not detected in urine. There is not a statistically significant difference in the excretion of these amino acids when β-2-thienylalanine is added to the infusion.

inulin or creatinine clearance studies been performed. However, the values obtained and the statistical analyses of the results clearly indicate significant competition between β-2-thienylalanine on the one hand and phenylalanine and tyrosine on the other.

Other amino acids were not consistently affected. Thus, there appears to be a specific mechanism for phenylalanine and tyrosine reabsorption in the monkey kidney which is inhibited by β-2-thienylalanine.

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The Inhibition of Intestinal Absorption of Phenylalanine in the Rhesus Monkey (35159)

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Phenylketonuria is the commonest of the genetic aminoacidopathies and is an important disease in which mental retardation is the most prominent feature. The administration of a diet low in phenylalanine is generally accepted as useful (1–3) although its efficacy has been questioned (4, 5). This diet is very restrictive and may be a major factor in the emotional climate of the home (6) and any method that could make the dietary intake more liberal and varied without unduly increasing the phenylalanine content would be useful. One approach that might allow this would be to feed synthetic amino acids which compete with phenylalanine for active transport in the ileum.

We have previously reported that β-2-thienylalanine inhibits renal tubular reabsorption of phenylalanine (7) and because ileal and renal absorptive mechanisms for amino acids share many common characteristics, it was decided to try the effect of this phenylalanine antagonist upon the absorptive phenomenon. In addition, parachlorophenylalanine, a well known inhibitor of phenylalanine metabolism (8) and cycloleucine, a substance known to produce a generalized aminoaciduria in man (9) were used. Cycloleucine is currently under trial (Phase II of Clinical Drug Evaluation Program of Cancer Chemotherapy National Cancer Institute) in patients with leiomyosarcoma in whom side-effects are mild and reversible.

Materials and Methods. Four healthy, adult male rhesus monkeys (*Macaca mulatta*), which weighed 6.25 to 8.75 kg, were used. They were usually fed Purina monkey chow, fruits and vitamins, but were fasted for 8 hr before the experiment during which they were offered water *ad libitum*.

Each monkey had a control blood sample drawn, nasogastric tube passed, and urine collector attached to the external genitalia, and the animal was placed in a special metabolic chair (10). The dose of phenylalanine administered orally to each monkey with or without the antagonist was 750 mg/kg of body weight. This dose, and the timing of blood samples was chosen after 5 preliminary administrations were shown to produce maximum effect and no emesis. Blood samples were drawn at 1, 2, 3, 4, and 6 hr.

The dose of the inhibitors was as follows: β-2-thienylalanine, 187.5 mg/kg; cycloleucine, 225 mg/kg; parachlorophenylalanine, 225 mg/kg. These doses were chosen on the basis of solubility characteristics and previous known effects on phenylalanine metabolism. Both the phenylalanine and antagonists were dissolved in 300 ml of tap water.

Urine was collected in a plastic bottle containing a few crystals of thymol and kept in ice. At the end of the 6-hr period suprapubic pressure was applied to empty the bladder.

Acidified urine and all sera samples (11) were analyzed on a Beckman-Spinco amino acid analyzer by the method of Spackman *et al.* (12) for neutral and acidic amino acids. β-2-thienylalanine and parachlorophenylalanine are clearly separated by this method, each appearing as a peak before tyrosine.

Results. Phenylalanine absorption. A typical experiment is shown in Fig. 1: the blood level of phenylalanine (mg/100 ml) is shown at zero time, 1, 2, 3, 4, and 6 hr. In each case the addition of the inhibitor to the 750 mg/kg of phenylalanine administered reduces the absorption of phenylalanine. To quantitate this reduction the area under each curve above base line was calculated and phenylalanine absorbed expressed in arbi-

TABLE I. Total Phenylalanine Absorption Expressed as Area Under Curve After 750 mg/? of Phenylalanine Load by Naso-Gastric Tube With and Without Synthetic Amino Acids[a]

Load	B56	E27	D43	B65	Mean
1. Phenylalanine	339.8	428.0	363.65	80.25	302.9
2. Phenylalanine + β-2-th.	356.0	286.0	303.9	50.3	249.0 (NS)
3. Phenylalanine + cycloleucine	139.7	182.4	124.4	67.2	128.4 ($p < .100$)
4. Phenylalanine + Parachloro	178.1	268.0	142.8	29.9	154.7 ($p < .200$)
Ratio					
5. line 2/line 1	1.05	0.67	0.84	0.63	0.80 ($p < .10$)
6. line 3/line 1	0.41	0.43	0.34	0.84	0.51 ($p < .05$)
7. line 4/line 1	0.52	0.63	0.39	0.37	0.48 ($p < .001$)

[a] Each monkey received 4 loads: in each he received 750 mg of phenylalanine/kg; in load he also received β-2-thienylalanine, 187.5 mg/kg; in load 3 cycloleucine, 225 mg/kg; in load parachlorophenylalanine, 225 mg/kg. Each analog decreased the amount of phenylalanine absorbed when given the analog (with the exception of B56 given β-2-thienylalanine). When results expressed as a ratio for each individual monkey (thereby reducing individual variance) each analog significantly reduced absorption.

trary units.

These calculated areas are shown in Table I where the mean absorption of phenylalanine in monkeys fed phenylalanine is compared with the mean absorption when given phenylalanine plus each synthetic amino acid. The phenylalanine absorption in Table I is the mean of two experiments on each animal with a 750 mg/kg dose. All three phenylalanine antagonists reduce phenylalanine absorption. When cycloleucine is added, the mean absorption is significantly lower and when parachlorophenylalanine is added it only approaches significance ($p < 0.2$). The variation in intestinal absorption of phenylalanine by individual monkeys is shown by line 1. The absorption of phenylalanine when both phenylalanine and inhibitor are given is compared in lines 5, 6, and 7 for each individual monkey. In each case, except monkey B56 given β-2-thienylalanine, the value of this ratio is less than unity, indicating that the amino acid analogue reduces intestinal absorption. Expressed this way the reduction of absorption is significant and especially so in the case of parachlorophenylalanine ($p < .001$).

Serum tryptophan. As previously reported (13) the phenylalanine load lowered serum tryptophan levels. After 4 hr, the tryptophan levels started to rise again as shown in Fig. 2. When the synthetic amino acid was administered in addition, the tryptophan fell again

with the same recovery after 4 hr. In T II the fall of serum tryptophan (expresse the area between the curve and base for the first 4 hr is shown. β-2-Thienylala failed to produce a significantly different in serum tryptophan compared with ph alanine alone but both cycloleucine parachlorophenylalanine did. When pressed as a ratio to allow for indivi variation, parachlorophenylalanine prod
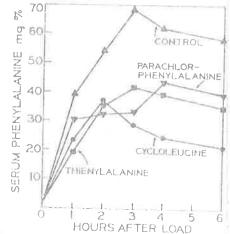
FIG. 1. Rise in serum phenylalanine in mo E27 following four oral loads of phenylalanine antagonist. Control load was phenylalanine, mg/kilo. Each of the 3 other loads contained phe alanine, 750 mg/kg plus either β-thienylalan 187.5 mg/kg; cycloleucine, 225 mg/kg; or p chlorophenylalanine, 225 mg/kg.
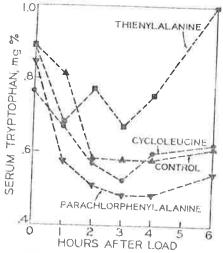
FIG. 2. Fall in serum tryptophan in monkey E27 following four oral loads of phenylalanine ± antagonist as described under Fig. 1.

a significantly greater fall in tryptophan than phenylalanine alone. Also in the three monkeys not shown in Fig. 1 the plasma level of tryptophan failed to rise by the usual 6 hr when parachlorophenylalanine was administered.

Serum tyrosine. This was measured either by the method of Wong *et al.* (14) or by amino acid analyzer. The rise in serum tyrosine over 6 hr was small but consistent. Although the synthetic amino acids produced a trend for slower rise in tyrosine this was not significant with any analogue. These data are

not presented but are available from the authors.

Urinary amino acids. The urinary excretion of phenylalanine during the 6 hr of oral load is shown in Table III. In all cases, the excretion is less when the synthetic amino acid is concurrently administered with one exception: β-2-thienylalanine given to Monkey B56 promotes greater excretion but this was the only instance in which intestinal absorption of phenylalanine was greater when synthetic amino acid was given (see Table I). This confirms the impression that the reason for the lower urinary excretion of phenylalanine in the presence of analogues is the lower absorption from the intestine.

Discussion. The results shown indicate that both cycloleucine and parachlorophenylalanine are potent inhibitors of intestinal absorption of phenylalanine, as is β-2-thienylalanine but to a lesser extent. The finding that this compound also inhibits the kidney transport system again demonstrates the similarity of transport systems in these two tissues.

Parachlorophenylalanine caused a marked rise in plasma phenylalanine in two out of five patients who received it on a chronic basis (15). There was an initial fall in plasma phenylalanine in two of the five patients, but no acute studies on intestinal absorption were performed. Presumably the elevation of plasma phenylalanine after chronic dosage

TABLE II. Fall in Serum Tryptophan Level Expressed as Area Above Curve After 750 mg/kg of L-Phenylalanine by Naso-Gastric Tube With and Without Synthetic Amino Acids.

Load	B56	E27	D43	E65	Mean and significance
1. Phenylalanine only	0.571	0.595	0.755	0.415	0.588
2. Phenylalanine + β-2-thienylalanine	0.581	1.100	0.730	0.730	0.820 ($p < .200$)
3. Phenylalanine + cycloleucine	1.16	1.18	0.85	0.920	1.04 ($p < .05$)
4. Phenylalanine + parachlorophenylalanine	1.45	2.28	1.84	1.37	1.235 ($p < .05$)
Ratio					
line 2/line 1	1.018	1.849	0.967	1.759	1.148 (NS)
line 3/line 1	2.035	1.983	1.126	2.217	1.840 ($p < .05$)
line 4/line 1	2.541	3.832	2.437	3.301	3.028 ($p << <.001$)

[a] Experiment as described under Table 1; β-2-thienylalanine failed to produce a significantly greater fall in serum tryptophan than phenylalanine alone. When cycloleucine or parachlorophenylalanine was added to the oral load the fall in serum tryptophan was significantly greater than when the same dose of phenylalanine was administered alone.

TABLE III. Urinary Excretion of Phenylalanine for 6 hr After 750 mg/kg Load of Ph[...] alanine by Naso-Gastric Tube With and Without Synthetic Amino Acids.[a]

Load	B56	D43	E27	E65	Mea[...]
Phenylalanine only	13.4	148.5	26.1	73.3	65.[...]
Phenylalanine					
+ β-2-thienylalanine	22.9	4.6	4.9	1.2	8.4
+ parachlorophenylalanine	2.1	0.8	5.8	0.8	2.[...]
+ cycloleucine	2.5	19.2	6.4	0.5	7.[...]

[a] Each monkey excretes less phenylalanine when the synthetic amino acid is concurrent[...] ministered with the exception of B56 when given β-2-thienylalanine.

was due to inhibition of phenylalanine hydroxylase. Parachlorophenylalanine has been found to be a potent inhibitor of rat phenylalanine hydroxylase *in vivo* (8, 14) although a poor inhibitor *in vitro* (16).

Weitzman *et al.* (17) also administered parachlorophenylalanine intraperitoneally to monkeys (*Macaca mulatta*) but only measured sleep patterns and brain serotonin. Mouret *et al.* (18) performed a similar study in the rat. Unfortunately neither paper cites data from which absorption characteristics can be derived.

Cycloleucine has been shown to be an inhibitor of amino transport into the Ehrlich cell by Oxender and Christensen (19) affecting both their postulated systems for neutral amino acid transport. Brown (9) showed that cycloleucine administration markedly increased human urinary excretion of cysteine, ornithine, lysine, arginine, taurine, threonine, serine, valine, tyrosine, and histidine. Brown did not specifically mention phenylalanine but the figure in the text indicates that its excretion is increased. Goyer (20) found a similar aminoaciduria in rats injected with cycloleucine intraperitoneally in which urinary phenylalanine excretion was definitely elevated. Thus there is additional evidence that cycloleucine inhibits transport of phenylalanine in both intestinal and renal tissues.

Elevated plasma phenylalanine is known to be associated with lowered plasma tryptophan levels (13). Phenylalanine inhibits transport of tryptophan in the intestine (21) and at the blood brain barrier (13). The more severe and prolonged depression of plasma tryptophan by parachlorophenylalanine is of interest as this compound inhibits

tryptophan hydroxylase (8, 22, 25) action in the brain has been conside[...] the cause of the lowered brain seroto[...] 23–26). These low levels of tryptoph[...] real concern when considering the us[...] synthetic amino acids as a mode [...] ment in phenylketonuria. Although t[...] shows that the analogues decrease[...] sorption of phenylalanine, Aoki an[...] (27), have shown that cerebral try[...] deficiency may be the mechanism [...] brain damage occurs with elevate[...] phenylalanine. Further reduction of [...] tryptophan by the administratiion [...] synthetic amino acids may even fur[...] plete the brain of tryptophan. Such [...] ing of cerebral tryptophan seems [...] nonspecific phenomenon in that a nu[...] amino acids when elevated in plasm[...] the brain free tryptophan (28). Such [...] ing by branched chain amino acids [...] can occur without lowering of plasn[...] (13). Thus the possible detrimental e[...] these synthetic amino acids can only [...] sessed by their prolonged oral admin[...] —this is currently being undertaker [...] laboratory.

Summary. β-2-Thienylalanine, [...] cine, and parachlorophenylalanine ha[...] shown to acutely reduce intestinal ab[...] of an oral phenylalanine load. C[...] with phenylalanine, they reduce plasm[...] tophan levels more than phenylalanin[...]

Whether their oral administrati[...] be of benefit in allowing a more libera[...] phenylketonuric children requires n[...] vestigation.

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PHENYLKETONURIA: REDUCTION OF SERUM LEVELS OF PHENYLALANINE FOLLOWING ORAL ADMINISTRATION OF B-2 THIENYLALANINE

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PHENYLKETONURIA: REDUCTION OF SERUM LEVELS OF PHENYLALANINE FOLLOWING ORAL ADMINISTRATION OF B-2 THIENYLALANINE

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SYNOPSIS

8 mentally retarded but otherwise healthy phenylketonuric individuals were given two oral loads of phenylalanine (100 mgm/Kg). One of these loads was accompanied by β-2-thienylalanine (20 mgm/Kg). Blood levels of phenylalanine in the 6 hours after the two phenylalanine loads were compared. β-2-thienylalanine reduced the levels of phenylalanine in every case.

This suggests that if the phenylketonuric child were to take an appropriate amount of β-2-thienylalanine with meals, he might be able to eat more phenylalanine, and this would allow a considerably more liberal diet.

As diet has been shown to be a dominant factor in the emotional climate of families of phenylketonuric children, this stress would be considerably reduced. Thus, the administration of β-2-thienylalanine might lead to a more normal social development of the patient.

Treatment of phenylketonuria by restricting the amount of phenylalanine in the diet is generally considered to be successful in preventing or arresting brain damage (Berman *et al.*, 1966, 1967; Hudson *et al.*, 1970; Koch *et al.*, 1970). However, such a diet is very restrictive and a dominant factor in the emotional climate of the patient's family (Keleske *et al.*, 1967; Wood *et al.*, 1967). Thus, an alternative mode of treatment would be desirable.

Such an alternative mode of therapy was suggested by the discovery that the synthetic amino acid β-2-thienylalanine (β-2-t) promoted the renal excretion and inhibited the intestinal absorption of phenylalanine in Rhesus monkeys loaded with phenylalanine (Lines and Waisman, 1970a, 1970b).

The Rhesus monkey loaded with phenylalanine is different from the phenylketanuric patient in that the monkey still has phenylalanine hydroxylase, and β-2-t has been reported to inhibit this enzyme (Udenfriend and Cooper, 1952). Thus this model is not entirely suitable for assessing the value of this compound in phenylketonuria, nor is there any experimental animal suffering from a total lack of effective phenylalanine hydroxylase. For these reasons it was decided to assess the effect of β-2-t on the intestinal absorption of phenylalanine in phenylketonuric patients who were not on diet.

MATERIALS AND METHODS

The 8 subjects were all mentally retarded phenylketonuric patients who were otherwise healthy and on an unrestricted diet.

On 2 consecutive days they were fasted for 8 hours before the experiment. Throughout the experiment they received no food but were allowed water with cordial flavouring as required.

After a control blood sample was collected, the subject was given a load of L-phenylalanine 100mg/kg, with or without 20mg/Kg of β-2-t. This was dissolved in an orange cordial drink as a solution of 20 mg of phenylalanine per ml. and taken orally. Further blood samples were taken at 1, 1½, 2, 4 and 6 hours after ingestion of the load.

On the first day, 4 subjects received phenylalanine only, and 4 received phenylalanine and β-2-t. On the following day, those who received phenylalanine only were given the combination of phenylalanine plus β-2-t and vice versa.

Serum phenylalanine levels were assayed using a fluorometric method (McCaman and Robins, 1962). This method was modified by increasing the pH of the succinate buffer from 5.80 to 5.88. To avoid precipitation of the copper reagent, copper sulphate was added to potassium sodium tartrate and sodium carbonate immediately before use. The phenylalanine standards were made up in 7.5% bovine serum albumin.

β-2-t was shown not to interfere with the measurement of phenylalanine by this assay. Because of lack of the patient's cooperation in obtaining accurately timed urine samples, no attempt was made to estimate the excretion of phenylalanine by the kidneys. Similarly, this lack of cooperation plus the predictable metabolism of phenylalanine by intestinal bacteria, precluded phenylalanine measurement in the faeces.

A quantitative value for the elevation of phenylalanine above fasting levels was obtained
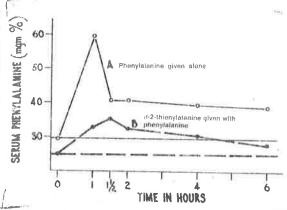
Figure 1. Serum phenylalanine levels following oral load of phenylalanine (100 mg/kg) to phenylketonuric patients.

by calculating in arbitrary units the area of curve above base line, i.e. the area in square inches on the graph paper, which was the same scale in each instance (Table I). In each case, the addition of β-2-t to the loading dose of phenylalanine reduced the elevation of serum phenylalanine and the reduction was significant ($t = 5.353$, $p = .001$). The percentage by which this elevation of phenylalanine was reduced by simultaneous ingestion of β-2-t was

TABLE I

Absorption of Phenylalanine After Oral Load of Phenylalanine 100 mg/kg With and Without β-2-Thienylalanine

Subject	Age	Area (arbitrary units)		Difference A - B	Percentage Reduction in area
		A Phenylalanine 100 mgm/kg	B Phenylalanine 100 mgm/kg + β-2-T 20 mgm/kg		
AA	13	606.25	537.5	66.75	11.01
AW	19	794.5	236.5	558.00	70.23
CM	17	803.5	445.75	357.75	44.5
GC	18	703.25	395.5	403.75	57.4
RA	22	350.25	225.0	125.25	35.76
WJ	66	362.5	117	245.5	67.72
FH	49	691.25	375.5	315.75	45.67
AW	39	395.5	230	165.5	41.84
Mean		588.35	320.34	279.78	47.55
S.D.		±32.82	±21.50	±57.56	

B < A   p = 0.001,   t = 5.353

Elevation of serum phenylalanine following an oral load of phenylalanine (100 mg/kg) is expressed in arbitrary units as the area under the curve above base-line phenylalanine levels as shown in Figure 1. The addition of β-2-thienylalanine (20 mgm/kg) constantly and significantly reduced this elevation

variable, with a range of 11.0% to 70.3% and a mean reduction of 47.5% (S.D. 9.78%).

DISCUSSION

The results show that for 6 hours at least, β-2-thienylalanine (β-2-t) depresses the level of serum phenylalanine after an oral load in phenylketonuric patients, the average depression being 47.5%.

Lönnerblad (1951) has shown that the average time for material to reach the ileocaecal valve, after ingestion, is 178 minutes, with a shorter period for infants and children. This suggests that in most cases the phenylalanine taken with β-2-t would have passed the absorptive surface by 6 hours and be unavailable for absorption. Hence one would not expect a late rise in serum phenylalanine if β-2-t was merely delaying phenylalanine absorption from the small intestine. Likewise, if β-2-t reduced the serum phenylalanine level by promoting the renal excretion of phenylalanine, a delayed rise of serum phenylalanine would not occur. It might therefore be possible for a phenylketonuric patient to ingest twice the usual amount of phenylalanine accompanied by β-2-t without elevating the level of plasma phenylalanine. This would enable a much more liberal diet and hence relieve many of the tensions currently associated with the management of this disease.

β-2-t is absorbed when taken orally (Lines and Waisman, 1970b) which has potential advantages and disadvantages. The advantage is that it is largely excreted by the kidney where it promotes the excretion of phenylalanine (Lines and Waisman, 1970a), helping to reduce serum phenylalanine. The disadvantage associated with the absorption of β-2-t is the possible effect on tryptophan metabolism. It has been suggested that the elevated plasma phenylalanine in phenylketonuria inhibits the passage of tryptophan into the brain cells thereby causing damage to those cells (Aoki and Siegel, 1970), and β-2-t in the plasma could possibly have a similar effect. In acute experiments, the oral administration of β-2-t does not lower plasma tryptophan in monkeys (Lines and Waisman,

1970b). However, the effect of β-2-t on intracellular tryptophan is not known. The role of a deficiency of intracellular tryptophan in causing brain damage in phenylketonuria is speculative, but it is generally accepted that a reduction in the level of plasma phenylalanine prevents brain damage. Thus this mode of treatment of phenylketonuria, which lowers plasma phenylalanine, warrants further investigation.

SUMMARY

β-2-thienylalanine administered simultaneously with phenylalanine to phenylketonuric individuals substantially reduced the usual rise in serum phenylalanine. Thus this compound might be taken with meals of a more nearly normal phenylalanine content without the plasma phenylalanine reaching dangerous levels in patients suffering phenylketonuria.

ACKNOWLEDGEMENTS

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Brief Communication:

EFFECT OF CYCLOLEUCINE AND PARACHLOROPHENYLALANINE ON PHENYLALANINE REABSORPTION IN THE RENAL TUBULE OF THE RHESUS MONKEY

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Summary. As cycloleucine and parachlorophenylalanine are known to affect phenylalanine metabolism, their effect on renal tubular handling of a phenylalanine load was studied. The addition of either substance to an intravenous infusion of phenylalanine did not decrease renal tubular reabsorption of phenylalanine in the Rhesus monkey. However, both cycloleucine and parachlorophenylalanine increased glomerular filtration rate as measured by inulin clearance.

INTRODUCTION.

Cycloleucine has been shown to inhibit the transport of many amino acids into the Ehrlich tumour cell (Christensen. Feldman and Hastings, 1963). In the intact animal it has caused an aminoaciduria as demonstrated in man (Brown. 1967), and in rats (Goyer, Reynolds and Elston, 1969). We have recently shown that this substance inhibits the intestinal uptake of phenylalanine in the monkey. We also demonstrated inhibition of intestinal absorption of phenylalanine by parachlorophenylalanine. a compound more potent than cycloleucine (Lines and Waisman. 1970b). Thus, it was of interest to establish whether both inhibitors caused a loss of phenylalanine in the urine in monkeys.

MATERIALS AND METHODS.

Four adult male monkeys who were normally fed Purina monkey chow. vitamins and fruit, were fasted overnight. They were lightly anaesthetized with intravenous 5-allyl-5(1-methyl butyl)-2 thiobarbiturate. 30 mg per kg, and an intravenous drip inserted. A collector was attached to the external genitalia so that urine could be collected in a plastic bottle kept in ice. The bottle contained a few thymol crystals to prevent bacterial growth. Each monkey received a load of sodium chloride (0·15M). inulin and phenylalanine, 0·1 g%. On 2 separate days cycloleucine or parachlorophenylalanine 30 mg% was added to this solution. The load size was 10 ml per kg (i.e. 10 mg/kg of phenylalanine with or without 3·3 mg/kg of inhibitor). After a 3 day rest the experiment was repeated with one of the remaining solutions. Following another rest of at least 3 days. the third solution was used. The order in which the solutions were used was completely random. No parachlorophenylalanine or cycloleucine could be detected in the control serum or urine 3 days after they had been infused.

After the initial load the intravenous drip was maintained with the same solution at a rate of 4·16 ml per kg per h. Thus. the rate of infusion of phenylalanine was 4·16 mg per

kg per h. When present, the rate of infusion of the inhibitor was 1·38 mg per kg per h. After 1 h the suprapubic region was pressed to empty the bladder and the urine discarded, a new plastic bottle being used for the 6 h collection. A venous blood sample was drawn at this time, and at 4 and 7 h. At 7 h the experiment was concluded. Throughout the collection period the monkey was strapped in a special metabolic chair (Waisman, 1963).

All sera and acidified filtered urine were analyzed in a Beckman-Spinco amino acid analyzer by the method of Spackman, Stein and Moore (1958). Inulin was measured by the method of Roe, Epstein and Goldstein (1949). From these data, filtered loads and tubular reabsorption of phenylalanine were calculated by standard methods. Because of the individual variation from monkey to monkey, the various parameters were calculated as a ratio for each animal (i.e. phenylalanine plus inhibitor:phenylalanine alone). These ratios were then expressed as a logarithm and subjected to the Student "t" test for statistical significance.

RESULTS.

The most striking result is the increase in glomerular filtration rate, as shown by inulin clearance in Table 1. The filtered load, excretion and tubular reabsorption of phenylalanine data are shown in Table 2. The percentage reabsorption by the renal tubule is unaffected by the addition of cycloleucine or parachlorophenylalanine, as shown in Table 2.

TABLE 1.

Effect on inulin clearance of the addition of cycloleucine and parachlorophenylalanine to a phenylalanine infusion.

Monkey	Inulin clearances (ml/min)		
	Phenylalanine alone	Phenylalanine plus cycloleucine	Phenylalanine plus parachlorophenylalanine
E65	11·5	24·2	19·8
E27	12·6	20·4	21·0
D43	8·9	32·2	29·7
B56	17·1	15·2	15·8
Mean	12·5	23·0	21·6

The addition of cycloleucine or parachlorophenylalanine increases glomerular filtration rate significantly (p < 0·1 in both cases).

DISCUSSION.

The increased glomerular filtration rate caused by the addition of the analogues cyclo-leucine and parachlorophenylalanine is of interest. Such a rise has previously been noted in dogs. Pitts (1944) found that infused glycine increased glomerular filtration, while Lindheimer, Lalone and Levinsky (1967) showed that feeding 200 g of casein (free of electrolytes) consistently elevated inulin or creatinine clearance, as did Moustgard (1948). Moustgard also infused a casein hydrolysate into dogs and found a 50-60% increase in glomerular filtration.

It is tempting to suggest that the increased excretion of amino acid found previously with cycloleucine (Brown, 1967; Goyer et al., 1969) or other synthetic amino acids (Lines and Waisman, 1970a) is due to the increased filtered load of amino acid surpassing the maximal tubular reabsorptive capacity, as shown in Table 2. However, it can be seen that despite the increased filtered load, excretion is not significantly increased and in fact is reduced with parachlorophenylalanine. Thus, exceeding maximum tubular reabsorption does not appear to be the mechanism involved for the aminoaciduria previously described with cycloleucine.

Does the aminoaciduria previously ascribed to cycloleucine also apply to phenylalanine? Oxender and Christensen (1963) found that cycloleucine inhibits transport in the Ehrlich

TABLE 2.

Effect of analogues on phenylalanine reabsorption in the renal tubule.

Monkey	Phenylalanine alone			Phenylalanine plus cycloleucine			Phenylalanine plus parachlorophenylalanine		
	Filtered load	Excreted	% Reabsorbed	Filtered load	Excreted	% Reabsorbed	Filtered load	Excreted	% Reabsorbed
E65	1435	5·38	99·7	2750	33·1	98·8	2680	3·10	99·9
E27	1830	17·7	99·0	3010	12·7	99·6	3590	2·27	99·9
D43	2200	41·5	98·1	5520	36·0	99·3	5600	4·80	99·9
B56	2540	11·4	99·5	2460	15·7	99·4	2700	1·35	99·9
Mean	2001	19·0	99·1	3435*	24·4	99·3	3643†	2·88	99·9

* < 0·20.
† p < 0·10.

The addition of each inhibitor to the intravenous infusion of phenylalanine (300 mg of analogue for every gram of phenylalanine) produces an increase in filtered load of phenylalanine, which approaches significance in the case of cycloleucine (p < 0.20) and is significant for para-chlorophenylalanine (p < 0·10). Renal tubular reabsorption of phenylalanine is not affected by the addition of either cycloleucine or para-chlorophenylalanine.

tumour cell affecting both "leucine-preferring" and "alanine-preferring" systems for neutral amino acids. Since phenylalanine uses these systems, one would expect phenylalanine transport to be inhibited by cycloleucine. Brown (1967) did not note a large increase in phenylalanine excretion in man, but the figure in the text indicates some increase when cycloleucine was administered. Goyer et al. (1969) found a definite increase in phenylalanine excretion when cycloleucine was given to rats. Neither of these investigators measured glomerular filtration rate, and hence the cause of the aminoaciduria was not determined.

It is possible that cycloleucine's failure to inhibit renal tubular reabsorption is a species difference peculiar to the monkey, since man and rat excrete more phenylalanine. The higher mean excretion of phenylalanine with cycloleucine shown in Table 1 suggests that a higher dosage of cycloleucine may have produced an inhibition of phenylalanine reabsorption by the renal tubule, but we used the 300 mg/kg dosage suggested for cancer chemotherapy and which Brown (1967) found caused an aminoaciduria. It is also possible that the amino-aciduria that he and Goyer et al. (1969) found was due to stimulation of glomerular filtration rate and not renal tubular transport inhibition as they surmised.

The failure of parachlorophenylalanine to block renal tubular reabsorption of phenyl-alanine is disappointing in view of its well known ability to inhibit phenylalanine metabolism in vivo (Koe and Weissman, 1966) and our demonstration that it inhibits intestinal absorption of phenylalanine (Lines and Waisman, 1970b) in the Rhesus monkey. Again, a variety of dosage schedules would be necessary to demonstrate that parachlorophenylalanine does not affect renal tubular reabsorption of phenylalanine. However, the extremely low phenylalanine excretion over the 6 h that this compound was infused suggests that it does not affect tubular transport. Thus, neither cycloleucine or parachlorophenylalanine had any demonstrable effect on the transport mechanisms in the renal tubule. This is not due to poor glomerular filtration of these compounds, as over 90% of the administered dose was excreted in the urine during the 6 h of the experiment. However, the fact that they diminished intestinal uptake of phenylalanine (Lines and Waisman, 1970b) suggests they may still have a potential for decreasing plasma phenylalanine levels.

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The Effect of Feeding β-2-Thienylalanine on Phenylalanine Metabolism in the Rhesus Monkey*

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Summary: β-2-thienylalanine, which is known to inhibit phenylalanine absorption in the intestine and to promote its excretion by the kidney, was fed to two infant Rhesus monkeys as a supplement to milk, for 240 days in a dosage of 0.75 g per kg. Two other infant monkeys received the same dose of β-2-thienylalanine plus phenylalanine 3 g per kg. These monkeys were compared with four infant animals that received phenylalanine 3 g per kg as a supplement, and a group that was fed milk alone. No adverse effect on growth or development by the β-2-thienylalanine feeding was noted. One of the monkeys which received β-2-thienylalanine with phenylalanine was not protected against the high blood phenylalanine and suffered neurological damage. The other monkey fed this combination did not appear to suffer any brain damage. However, brain damage was found in all four monkeys fed phenylalanine alone.

It is concluded that β-2-thienylalanine is worthy of further study in the treatment of phenylketonuria.

The treatment of phenylketonuria by low phenylalanine diet is usually beneficial[1, 2, 3, 4] in effectively lowering the plasma phenylalanine and in allowing normal brain development, although some had earlier questioned its efficacy[5, 6]. There is no doubt that the dietary management of the phenylketonuric child requires careful monitoring for several years, is time consuming for the parent, and that management of such a child is a major facet in the family's emotional climate[7].

Thus, any treatment that would obviate or liberalize the diet would be useful. One therapeutic approach, short of being able to supply the deficient enzyme, would be the administration of an agent that would block either intestinal absorption or promote renal excretion of phenylalanine. We have already shown that β-2-thienylalanine is able to accomplish both phenomena[8, 9], and it was decided to try this agent (on a long term basis) in young monkeys fed excess phenylalanine to establish whether this phenylalanine antagonist could effectively lower the plasma phenylalanine level in these hyperphenylalaninemic animals.

Methods and Materials

Four groups of monkeys (*Macaca mulatta*) were taken at birth from their mothers and fed several dietary regimens for various periods. The first group contained four monkeys which were fed a commercially modified milk diet (Similac)* supplemented with L-phenylalanine, approximately 3g/kg/day, as has been described elsewhere in greater detail[10]. The second group of two animals was supplemented with β-2-thienylalanine 0.75 g/kg/day. The third group consisted of two monkeys fed
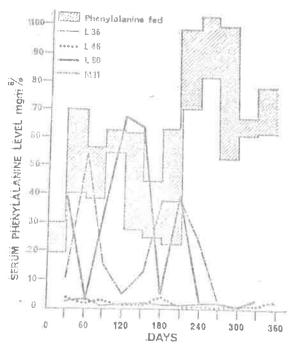
FIGURE 1: Plasma phenylalanine levels in *Macaca Mulatta* after feeding various diets. The shaded area is the mean ± SD (standard deviation) of levels in four phenylalanine supplemented animals for each 30-day period. L-36 and L-46 were fed 0.75 g/kg/day of β-2-thienylalanine for 240 days. L-80 and M-11 were fed 0.75 g/kg/day of β-2-thienylalanine plus 3 g/kg/day of L-phenylalanine for 240 days.

both L-phenylalanine 3 g/kg/day and β-2-thienylalanine 0.75 g/kg/day in their regular milk. The fourth group included all the control monkeys fed Similac from birth in this laboratory. The method of introducing this diet and the hand feeding and care of the infant monkeys has been described in detail elsewhere[10, 11, 12, 13].

The monkeys' dietary intake was recorded daily as was their weight. Height and head circumference were measured at weekly intervals. Blood for phenylalanine, tyrosine, and β-2-thienylalanine was drawn, after a four hour fast, at four day intervals in the first month of life and at two or four week intervals thereon. To estimate phenylalanine, tyrosine, and β-2-thienylalanine, the serum was deproteinized as previously described[14], and analyzed by the method of Spackman, Stein and Moore[15] for neutral and acidic amino acids. β-2-thienylalanine clearly separates as a distinct peak just before the elution of tyrosine. Blood samples for hematological investigation were drawn concurrently with those for amino acid determination.

After 240 days the β-2-thienylalanine supplemented monkeys were returned to a normal diet. One monkey, L-36, who had been fed β-2-thienylalanine contracted shigella gastroenteritis on the final day of the diet. The animal died after a ten day illness, during which he received antibiotics and parenteral fluids. Autopsy revealed findings compatible with gastroenteritis; brain and liver were removed and assayed for β-2-thienylalanine, tyrosine, and phenylalanine after hydrolysis of the trichloracetic acid precipitable protein. We have previous-

ly demonstrated that β-2-thienylalanine is recovered completely after hydrolysis.

M-11, fed the combination of phenylalanine and β-2-thienylalanine, also developed shigella gastroenteritis at 200 days, but this animal on cessation of all oral feedings, and intravenous fluids and antibiotics administration, recovered completely within seven days.

Results

Serum amino acids

The phenylalanine levels of the monkeys fed β-2-thienylalanine either with or without phenylalanine are compared in Figure 1, with those who received phenylalanine alone. β-2-thienylalanine alone at the dosage of 0.75g/kg/day did not cause serum phenylalanine level to vary from normal. When β-2-thienylalanine was combined with phenylalanine the serum phenylalanine levels varied considerably, but generally were lower [mean = 26 mg% (S.D. = 19)] than the animals fed phenylalanine alone [mean = 44 mg% (S.D. = 11)]. Both groups of β-2-thienylalanine supplemented monkeys were returned to a normal diet at 240 days and it can be seen that serum phenylalanine rapidly fell to normal levels and remained there.

The serum β-2-thienylalanine and tyrosine levels are shown in Table 1. The tyrosine levels are extremely variable in the two monkeys also fed phenylalanine. This variation was also seen in the phenylalanine only fed monkeys. The animals supplemented with β-2-thienylalanine and phenylalanine tended to have higher β-2-thienylalanine levels in their serum than those fed the same dose of β-2-thienylalanine alone.

Dietary intake and growth

The formula strength was increased at standard times in an effort to maintain phenylalanine intake near 3g/kg in the appropriate groups but this was rarely achieved. However, both the animals fed phenylalanine alone and those given phenylalanine plus β-2-thienylalanine received an average of 2.5g of phenylalanine per kilogram after the thirtieth day of life. This lower intake of phenylalanine is a reflection of the reduced volume of milk consumed per k/day. All four monkeys who received β-2-thienylalanine, either with or without phenylalanine, drank less than the animals who had phenylalanine or no supplement. This suggests that β-2-thienylalanine was particularly distasteful to the infant monkeys.
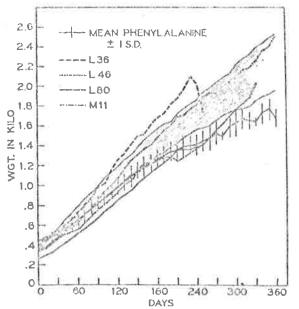
FIGURE 2 : Weight gains of *Macaca mulatta*. The shaded area shows normal weights of monkeys (means ± SD) maintained on Similac in this laboratory. The weights of the four phenylalanine supplemented monkeys are shown as a mean ± SD for each ten-day period. L-80 and M-11 were fed 0.75 g/kg/day of β-2-thienylalanine plus 3 g/kg/day of L-phenylalanine for 240 days. L-36 and L-46 were given the β-2-thienylalanine supplement for 240 days.

There was no remarkable disturbance weight gain in any of the monkeys fed β-thienylalanine as shown in Figure 2. L-80 was a small monkey at birth but grew normal despite being fed both phenylalanine and β-thienylalanine. Monkey M-11 grew at the lo range of normal until he contracted gastr enteritis at 200 days. In the monkeys fed phenyl lanine at high concentrations, slowness in weig gain occurs from about 200 days. This is n evident in the two monkeys fed the same do of phenylalanine plus β-2-thienylalanine, b these monkeys were returned to normal diet 240 days.

Figure 3 shows growth as a function of intal The weight gained per 10 l of intake is equal the normal and phenylalanine fed groups, b growth is superior in the β-2-thienylalani supplemented groups. Similar data for heig and head circumference showed no deterio effect from β-2-thienylalanine.

Other findings

Red cell indices showed no significant dev tion among the animals with variously supp

TABLE 1.

Serum β-2-Thienylalanine (β-2-T) and Tyrosine (Tyr.) Levels* in Infant Rhesus Monkeys fed the antagonist with a without Phenylalanine at various ages.

Period	β-2-Thienylalanine Fed				β-2-Thienylalanine and Phenylalanine Fed			
Age in days	L–36		L–46		L–80		M–11	
	β-2-T	Tyr.	β-2-T	Tyr.	β-2-T	Tyr.	β-2-T	Tyr.
0 – 30	3.1	2.3	2.6	2.9	8.8	22.8	9.5	13.5
30 – 60	2.4	3.2	5.9	3.6	1.2	2.5	12.6	41.8
60 – 90	3.2	3.0	6.4	4.2	9.5	7.4	9.5	60.8
90 – 120	3.9	3.0	6.5	4.0	11.4	20.7	7.0	6.7
120 – 150	10.3	0.44	6.0	2.2	10.4	10.6	8.1	45.2
150 – 180	7.7	2.2	11.6	6.1	1.5	3.9	6.3	12.4
180 – 210	4.1	1.4	3.3	2.0	7.7	4.7	10.0	9.2
210 – 240	9.6	1.6	4.4	1.4	Gastroenteritis		17.2	2.1
240 – 270	2.0	1.0	9.1	0.78	5.2	3.5	0.22	1.8
270 – 300	died		–	0.97	2.3	1.6	–	2.5

*mg per 100 ml.
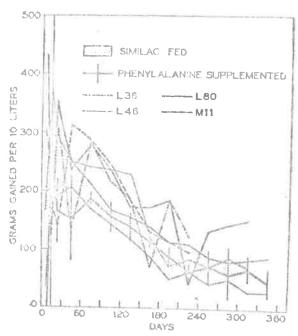
FIGURE 3: Growth of *Macaca Mulatta* as a function of intake. The shaded area shows weight gain (mean ± SD) per ten litres of formula for all Similac fed animals in this laboratory. The weights of the four phenylalanine supplemented animals are also shown as a mean ± SD. At 15 days of age there was such a great variation in the intakes that the line indicating the mean ± SD should have extended somewhat below the base line of the chart. L-36 and L-46 were fed 0.75 g/kg/day of β-2-thienylalanine for 240 days. L-80 and M-80 were fed 0.75 g/kg/day of β-2 thienylalanine plus 3.0 g/kg/day of L-phenylalanine for 240 days.

mented groups, nor were other haematological abnormalities detected. (These data are available from the author on request).

Clinically no abnormality resulting from administration of the β-2-thienylalanine was evident except in L-80 who convulsed and behaved in a manner identical to the phenylalanine fed monkeys[19]. This monkey had a higher maximal level of phenylalanine – 68 mg% compared with the other animal fed the combination of phenylalanine and β-2-thienylalanine (54 mg%), although both had an identical average serum phenylalanine level of 26 mg%. Also, L-80's low birth weight might be associated with prematurity or some other factor which rendered his brain more susceptible to damage by the elevated blood phenylalanine levels.

The post-mortem on L-36 after death from gastroenteritis revealed findings compatible with only that diagnosis. The brain protein contained

no detectable β-2-thienylalanine while liver protein had a phenylalanine:tyrosine:β-2-thienylalanine ratio of 22:7:1. Thus incorporation of β-2-thienylalanine into protein was negligible.

Discussion

The results suggest that β-2-thienylalanine added to the infant monkeys' milk formula decreased the intake but did not impair growth. Interestingly, β-2-thienylalanine fed animals grow better than phenylalanine fed animals or the control group, when weight gain is expressed as a function of intake. This probably reflects the monkey's ability to maintain growth at a slightly lowered intake.

It can be seen that the blood phenylalanine levels were generally reduced in the two monkeys fed the combination of β-2-thienylalanine and phenylalanine compared to those fed phenylalanine alone.

The trend toward higher serum β-2-thienylalanine levels in the monkeys fed phenylalanine compared to those fed β-2-thienylalanine alone suggests that phenylalanine inhibits β-2-thienylalanine metabolism in the monkey. The metabolic pathway involved is not known, but Godin and Dolan[16] have shown that rats cannot break down β-2-thienylalanine to CO_2 and, like us, found little of the analogue incorporated into protein. We have found that a large percentage of the administered dosage is recovered in the monkey's urine and therefore β-2-thienylalanine probably competes with phenylalanine for reabsorption in the renal tubule[8]. If phenylalanine were to increase β-2-thienylalanine excretion one would expect the serum level of β-2-thienylalanine to be lower. Therefore, β-2-thienylalanine appears to be metabolized by some other pathway in the monkey. Certainly β-2-thienylalanine did appear to protect the monkey against the neurologic consequences of supplemental phenylalanine feeding in one animal, but L-80 developed convulsions and signs of mental retardation. It is interesting that this monkey, L-80, had higher maximal levels of phenylalanine (67.6 and 63.9) than the other animal (M-11) fed this combination (61.9 and 37.3), which appears to be mentally normal.

As elevation of serum phenylalanine is the most direct result of the absence of phenylalanine

hydroxylase one might presume correctly that to decrease the amino acid would be beneficial as has been found in the human patient[1, 2, 3, 4]. Thus, β-2-thienylalanine may decrease the blood level of phenylalanine by inhibiting absorption.

The incorporation of β-2-thienylalanine into protein is of some concern as it may give rise to ineffective enzymes or other proteins. The fact that the red cell indices and white cell counts remained normal throughout the feeding of β-2-thienylalanine is encouraging as these cells arise from tissues of known high protein turnover. More disturbing is the fact that two out of four monkeys developed shigella gastroenteritis soon after or during β-2-thienylalanine feeding. Shigella is endemic in subhuman primates but the occurrence of this enteritis suggests a careful examination of immunological status when this antagonist is used.

These results indicate that further investigations in phenylketonuric patients are warranted. β-2-thienylalanine did not cause any abnormality of growth or development patterns when fed to infant monkeys from birth in a dose of 0.75 g/kg and did appear to be partially effective against the damaging effects of high phenylalanine feeding.

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DIFFERENTIATION OF PATHOGENIC, GRAM-NEGATIVE,

BACTERIAL GENERA IN THE PRESENCE OF β - 2 -

THIENYLALANINE

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ABSTRACT

Described is a fast screening assay for aid in the differentiation of bacterial genera. _Salmonella_ is separated from _Escherichia_ and _Shigella_, as a generic group on the basis of an inhibition assay incorporating β-2-thienylalanine, and using a defined medium. The assay is also usable for confirmation of taxonomic differences between genera.

The inhibition reaction is readable as an "all-or-none" phenomenon, follows a dose-response relationship for cultures affected by β-2-thienylalanine, and is unaffected by the growth phase of the culture.

INTRODUCTION

The phenylalanine analogue, β-2-thienylalanine (β-2-t), has been used in microbial screening assays for detection of elevated blood phenylalanine levels in newborn infants (6)

β-2-thienylalanine has also been employed in animal models, and has been shown to increase renal excretion of phenylalanine in rhesus monkeys (8). The analogue has also been shown to inhibit intestinal absorption of the amino acid in rats (5, 11), adult monkeys (9), infant monkeys (10), and in short-term phenylalanine loading experiments in man (7). Thus it shows promise as an adjunct to the normal dietary therapy used for treatment of phenylketonuria.

Among the safety studies conducted to test the effect of the analogue upon intestinal bacteria; cultures of gram-negative, pathogenic genera were grown on a defined plating medium, in the presence of β-2-thienylalanine, to determine if the various genera could be differentiated under these conditions.

MATERIALS AND METHODS

STRAINS

Cultures of Escherichia coli, Shigella flexneri II, Sh. flexneri III, Sh. sonnei, Salmonella typhi, S. typhimurium, Proteus mirabilis, P. vulgaris, P. morgani, Pseudomonas aeruginosa, Streptococcus pyogenes, Klebsiella sp., Acinetobacter sp., Enterobacter sp., were obtained as isolates from non-infant clinical specimens, from the Wallace Laboratory, Auckland Hospital. The Salmonella and Shigella strains were determined by serotyping, while the identification of the other cultures was made by comparison of biochemical reactions.

Serotyped cultures of E. coli O26/B6, O55/B5, O86/B7, O112/K66, O114/K90, O119/B16, O125/B15, O127/B8, and O128/B12, Shigella flexneri I, II, and III, Sh. boydii, Sh. sonnei, Salmonella typhi, S. typhimurium, S.typhimurium var. Copenhagen, S. newington, and S. panama, were obtained from Princess Mary Hospital for Children, Auckland, having been isolated from infant pathological specimens.

Two of the serotyped strains from each of the three genera in this group were used for determining the optimal β-2-t concentration in the test system.

MAINTENANCE OF THE STRAINS

Cultures were maintained for long term use on slants of Dorset Egg Medium (2), as prepared by the Media Department, Auckland Hospital, under the following conditions.

Sixteen large chicken eggs were rinsed in alcohol, broken under sterile conditions, and shaken with sterile glass beads for 10 minutes. To the blended eggs (approximately 800 ml) was added 200 ml of 0.9% (w/v) NaCl. Five ml aliquots were dispensed into bottles. Bubbling of the medium was reduced by placing the bottles in the autoclave, and immediately closing the exhaust to exclude air. The pressure was maintained at 15 psi for 20 minutes, by bleeding through the exhaust valve. The medium was then autoclaved normally at 121 C and 15 psi for 10 minutes, followed by slow exhaust of the steam.

For experimental use, cultures were streaked for isolation from this medium onto Columbia agar plates (BBL, Cockeysville, Md.). After overnight incubation, colonies were picked and transferred to peptone water, consisting of 2% (w/v) tryptone (Oxoid) with 0.5% (w/v) NaCl, and grown for 3 - 5 hrs at 37 C to attain a logarithmic growth phase (Absorbance of 0.1 at 450 nm as measured with a Bausch and Lomb Spectronic 20 spectrophotometer). An absorbance of 0.1 under these conditions was equal to 2×10^8 colonies/ml. Cultures not immediately tested were stored at 5 C for as long as 14 days.

MEDIA FOR INHIBITION ASSAYS

Vegetative Minimal Medium (VMM) (1) was prepared from 100 ml of a mineral salts concentrate (per litre, 30g K_2HPO_4; 10g KH_2PO_4; 5g NH_4Cl; 1g NH_4NO_3; 1g Na_2SO_4; 0.1g $MgSO_4 \cdot 7H_2O$; 0.01g $MnSO_4 \cdot 4H_2O$; 0.01g $FeSO_4 \cdot 7H_2O$;

and 0.005g $CaCl_2$), to which was added 1.47g L-glutamic
acid, 0.04g disodium ethylene diamine tetraacetate·
$2H_2O$, 13.5g agar, and distilled water to a volume of
900 ml. The medium was adjusted to pH 6.8-7.0,
and autoclaved at 121 C for 15 minutes, to reduce the
possibility of charring, which occurred at conditions
of higher temperature and pressure. After cooling,
10g of glucose in the form of a separately autoclaved
10% (w/v) solution was added aseptically, to bring
the final volume to one litre.

Modified VMM was prepared as above except that
$MnSO_4$, $FeSO_4$, and $CaCl_2$ were not added.

DETERMINATION OF COLONY COUNTS

Colony counts were performed to compare growth
of the selected intestinal pathogens in the inhibition
medium with that in a complex plating medium, as well
as for determination of the size of the inoculum for
assays. Serial dilutions, 10^0 to 10^{-8}, were done in
duplicate in 1:10 mineral salts concentrate, using
serotyped strains of the genera, Escherichia, Shigella,
and Salmonella. From each dilution, 0.05 ml was
added in duplicate to 15 ml of agar medium, and pour
plates made in sterile, disposable plastic, 85 mm
diameter Petri dishes (Tasman Vaccine Laboratories Ltd.,
Upper Hutt, New Zealand). Plates were inverted and
incubated at 37 C for 18 - 24 hours. Colonies were
counted on plates having 20 - 200 colonies.

INHIBITOR

A 0.01 M aqueous solution of β-2-thienylalanine (β-2-thienyl-DL-alanine, Sigma Chemical Co.) was sterilized by autoclaving, dispensed as 10 ml aliquots, and maintained at -20 C. The dose-response relationship of the β-2-t solution to the Escherichia, Shigella, and Salmonella test strains was determined for use in the inhibition assay (TABLE 1).

INHIBITION ASSAY

Bacteria were screened for inhibition of growth by parallel streaking of strains, 8 or fewer per plate, on Vegetative Minimal Medium or modified VMM in duplicate, followed by the placing of a sterile strip of Whatman 3MM filter paper, 6 mm x 75 mm, perpendicular to the streaks, and applying 100 µl of 0.01 M β-2-t solution onto the strip with a sterile syringe. Filter paper strips, impregnated only with distilled water, were used in control plates. Petri plates were inverted and incubated at 37 C, and were examined after 18 - 24 hours. Inhibition was defined as lack of visible growth within 1 cm of either side of the impregnated strip. Non-inhibited strains grew to the strip without a visible no-growth area. Reactions of strains showing a visible no-growth area which were not distinct enough to qualify as "inhibition", were termed, "borderline". (Figure 1).

EFFECT OF GROWTH PHASE UPON INHIBITION REACTION

The test strains were grown for 4 hr. (logarithmic phase), 18 and 36 hrs. (stationary phase) at 37 C in peptone water, and streaked into plates for measurement of inhibition responses in the presence of β-2-thienylalanine.

EFFECT OF GROWTH PHASE UPON INHIBITION REACTION

The test strains were grown for 4 hr. (logarithmic phase), 18 and 36 hrs. (stationary phase) at 37 C in

Colony counts showed that growth in the Vegetative Minimal Medium used for the inhibition assay (Figure 2) was comparable to that expected in a complex medium, such as Columbia agar (Figure 3), for the test strains used. The lines of best fit were determined by the method of least squares.

The dose-response relationship of the β-2-thienylalanine solution to growth of the test organisms showed no inhibition of the Salmonella strains at any tested concentration. Shigella and Escherichia strains displayed borderline reactions at even the lowest concentrations tested, as compared to the filter paper and water-impregnated filter paper controls. At 60 μl of inhibitor solution, both Shigella and Escherichia test strains were inhibited. No further change in reactions was noted with increasing β-2-t concentration. (Table 1). The dose selected for the inhibition assay was 100 μl of the 0.01 M solution, being safely above the minimum inhibition level of 60 μl.

Using the selected test dosage of β-2-t, the reactions of the test organisms were compared at different phases of growth. No difference was noted in the reactions of the cultures at different growth phases (Table 2). In addition, to study if the reaction changed with time, cultures were tested that had been maintained upon

Dorset Egg Medium for as long as one year after
initial testing, with no change in the inhibition reaction
noted (data available from the authors on request).

Isolates from non-infant pathological specimens of
the genera Escherichia, Shigella, and Salmonella were
compared in the assay using VMM, as growth on this
medium was satisfactory for the test organisms of the
same genera. The growth of isolates of other gram-
negative genera in the presence of β-2-t was compared
in the same assay, and results are shown in Table 3.
Known serotyped cultures derived from infant pathological
specimens were also tested in the assay, and results
are shown in Table 4. Of the 32 total E. coli cultures
tested (Tables 3 and 4), 30 were inhibited by β-2-t.
The two non-inhibited cultures could not be serotyped
with available agglutinating antisera for known patho-
genic strains. All available known pathogenic E. coli
strains tested were inhibited, as were all tested
Shigella strains (28 cultures). All of the tested
Salmonella cultures, however, were not inhibited (15
cultures). Other organisms tested which would grow
upon the medium reacted identically within a genus,
with the exception of the genus, Klebsiella.

Of the 103 cultures tested under these conditions,
no difficulty was encountered in deciding upon whether
inhibition had or had not occurred, and no borderline
reactions were noted (Tables 3 and 4).

DISCUSSION

A defined minimal medium, Vegetative Minimal Medium
described by Demain (1), is suitable for growth of the
genera Escherichia, Shigella, and Salmonella, as well as
for a number of other known gram-negative genera.

The medium may be simplified by removal of the
manganese, iron, and calcium salts with no apparent effect
upon growth or the inhibition reaction. This simplifi-
cation of the medium also lessens the formation of un-
dissolved complexes noted when the original medium is
used without the presence of agar. Proteus sp. which
grow poorly on VMM, produce enough growth upon modified
VMM to be assayed.

The assay described in this report is quick to set
up, and fast to read, as individual assays are readable
as "all-or-none" reactions, with no questionable or
borderline ones in our series. The similarity of response
of the genera Escherichia and Shigella may be a confirm-
ation of their taxonomic closeness, as compared to the
genus Salmonella, from which the former genera are almost
totally differentiated (3). The assay also confirms
other taxonomic studies between genera. For example,
the genus Citrobacter has been placed by Edwards and
Ewing (4) taxonomically closer to the genus Salmonella
than to other Enterobacteriaceae, a suggestion confirmed
by the inhibition reaction noted. The genus Klebsiella
reacts heterogeneously in the assay, suggesting taxonomic
non-uniformity.

The inhibition reaction of bacterial cultures in the presence of β-2-t provides a way of testing the effects of the inhibitor under defined medium conditions, preparatory to examination of the effects under more complex conditions such as occur within the mammalian intestinal tract. Results show that some pathogenic intestinal bacteria are affected by β-2-thienylalanine under the specific defined conditions of the assay.

Other studies are continuing on the nature of the inhibition phenomenon and will be reported separately.

ACKNOWLEDGMENTS

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TABLE 1

EFFECT OF DOSAGE OF β-2-THIENYLALANINE UPON INHIBITION REACTION

0.01 M β-2-t (μl)	E.coli O55/B5	E.coli O86/B7	Sh.boydii	Sh.sonnei	S.typhi	S. typhi-murium
0 (a)	O	O	O	O	O	O
0 (b)	O	O	O	O	O	O
5	±	±	±	±	O	O
10	±	±	±	±	O	O
20	+	±	±	+	O	O
40	+	±	±	+	O	O
60	+	+	+	+	O	O
80	+	+	+	+	O	O
100	+	+	+	+	O	O
120	+	+	+	+	O	O
140	+	+	+	+	O	O
160	+	+	+	+	O	O
180	+	+	+	+	O	O
200	+	+	+	+	O	O
400	+	+	+	+	O	O
600	+	+	+	+	O	O

(a) .. filter paper alone, no β-2-thienylalanine

(b) .. filter paper impregnated with distilled water, no β-2-t.

(+) .. culture inhibited

(O) .. culture not inhibited

(±) .. borderline reaction

TABLE 2

EFFECT OF PHASE OF GROWTH OF BACTERIAL CULTURES UPON
INHIBITION REACTION DUE TO 100µl of 0.01M ß-2-THIENYLALANINE

Phase of Growth	E.coli 055/B5	E.coli 086/B7	Sh. boydii	Sh. sonnei	S. typhi	S. typhi-murium
Logarithmic phase 4 hrs after inoculation	+	+	+	+	O	O
Stationary phase 18 hrs after inoculation	+	+	+	+	O	O
36 hrs after inoculation	+	+	+	+	O	O

(+) .. culture inhibited

(O) .. culture not inhibited

TABLE 3.

INHIBITION OF NON-INFANT PATHOLOGICAL ISOLATES BY 0.01M β-2-THIENYLALANINE

	Number of cultures tested	Cultures inhibited	Cultures not inhibited
Escherichia coli	23	21	2
Shigella flexneri II	2	2	O
Shigella flexneri III	10	10	O
Shigella sonnei	11	11	O
Salmonella typhi	7	O	7
Salmonella typhimurium	4	O	4
Other gram-negative organisms			
Proteus mirabilis (a)	6	O	6
Proteus vulgaris (a)	2	O	2
Proteus morgani (a)	3	O	3
Proteus rettgeri (a)	1	O	1
Pseudomonas aeruginosa	2	O	2
Klebsiella sp.	5	2	3
Acinetobacter sp.	4	O	4
Enterobacter sp.	3	O	3
Citrobacter sp.	1	O	1

(a) .. Culture grew too poorly on the original test medium to assay, but growth was sufficient on modified Vegetative Minimal Medium to assay the reaction

TABLE 4:

INHIBITION REACTIONS OF SEROTYPED CULTURES OBTAINED FROM INFANT PATHOLOGICAL SPECIMENS

Organism

Escherichia coli

O26/B6	+
O55/B5	+
O86/B7	+
O112/K66	+
O114/K90	+
O119/B16	+
O125/B15	+
O127/B8	+
O128/B12	+
Shigella flexneri I	+
Shigella flexneri II	+
Shigella flexneri III	+
Shigella boydii	+
Shigella sonnei	+
Salmonella typhi	O
Salmonella typhimurium	O
S. typhimurium var. Copenhagen	O
Salmonella newington	O
Salmonella panama	O

(+).. strain inhibited

(O).. strain not inhibited

CAPTIONS FOR FIGURES

Figure 1   Inhibition reactions of test strains

(a)   no β-2-thienylalanine (sterile distilled
water impregnated test strip)

(b)   100 μl 0.01 M β-2-t

Test organisms are 1. E.coli O55/B5,
2. E.coli O86/B7,   3. Sh.boydii,
4. Sh.sonnei   5. S.typhi, and
6. S. typhimurium

Figure 2   Relationship of absorbance values for gram-negative
test strains (E.coli O55/B5, E.coli O86/B7,
Sh.boydii, Sh.sonnei, S.typhi and S.typhimurium)
grown in peptone water and plated on modified
Vegetative Minimal Medium.
Line of best fit determined by the method of least
squares.
Correlation coefficient equals 0.8537 ($p < 0.001$).
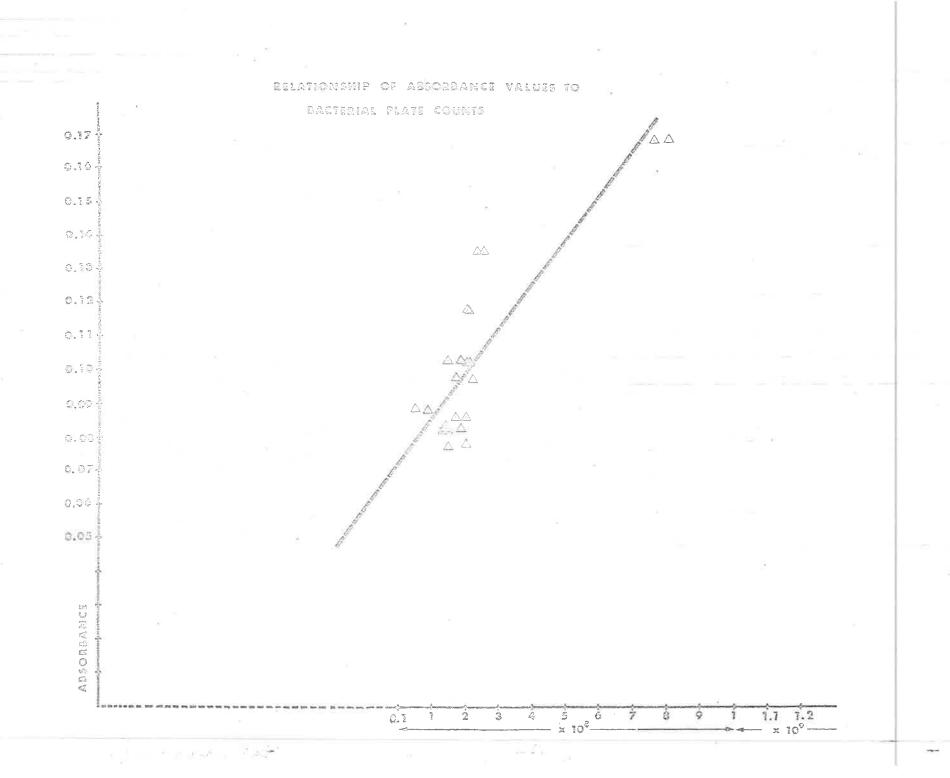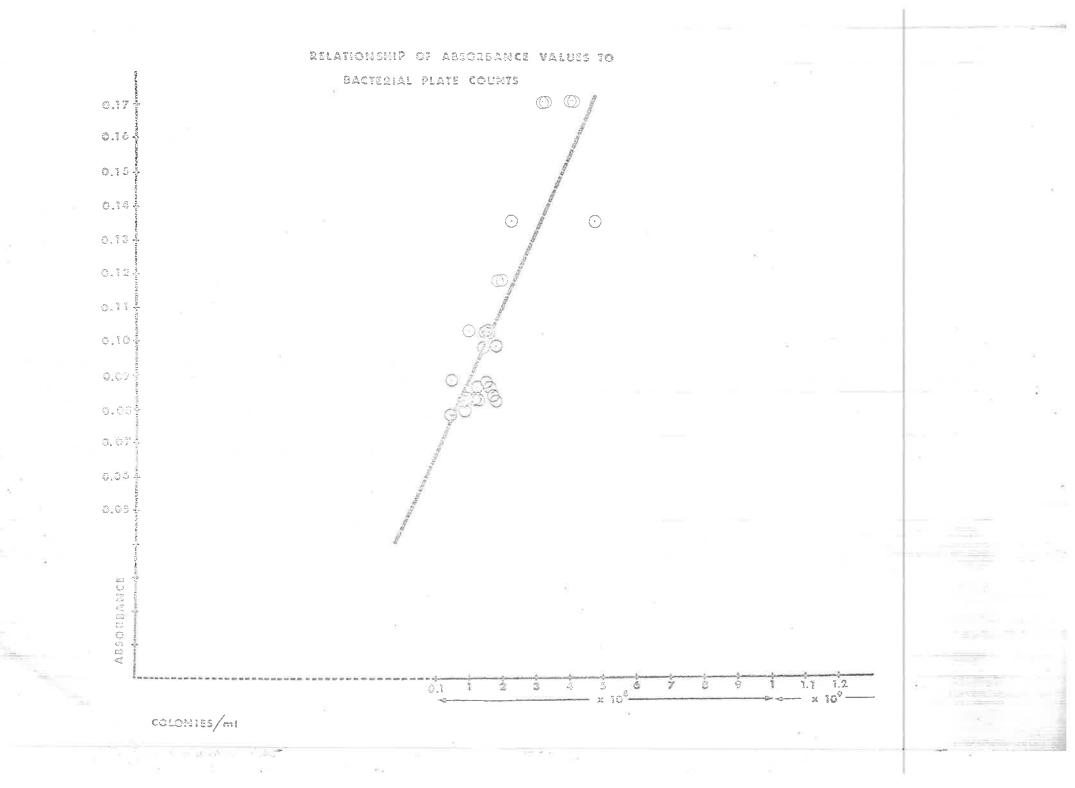
Figure 3   Relationship of absorbance values for gram-negative
test strains grown in peptone water to bacterial
plate counts on Columbia agar.
Correlation coefficient equals 0.8623 ($p < 0.001$).

RELATIONSHIP OF ABSORBANCE VALUES TO
BACTERIAL PLATE COUNTS

RELATIONSHIP OF ABSORBANCE VALUES TO
BACTERIAL PLATE COUNTS

ABSORBANCE

COLONIES/ml

PAEDIATRIC RESEARCH SOCIETY OF AUSTRALIA

Abnormal appearance of the brush border on transmission electron microscopy was associated with lowering of all disaccharidases level (p < 0.001).

Significant lowering of all enzyme levels was seen in patients with coeliac disease (p < 0.05), both active (n = 50), and in partial remission (n = 8), and with giardiasis (n = 32). In those with the post-gastroenteritis syndrome (n = 15) lowering of the lactase and sucrase levels only was significant. Enzyme levels in cows' milk protein intolerance (n = 8), and coeliac disease in complete remission (n = 7), were similar to normal patients (n = 226). Eight patients were considered to have primary disaccharidase deficiency.

Disaccharidase levels were found to behave predictably for histological damage and underlying disease. The marked effect of biopsy site on enzyme levels may partly explain the wide variation in disaccharidase values in the present study.

Effect of B-2-thienylalanine on bacterial growth — Guthrie test revisited
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The Guthrie test for the detection of elevated blood phenylalanine levels suggestive of phenylkeonuria, is based on the inhibition of growth of Bacillus subtilis by B-2-thienylalanine. This inhibition is reversed by the presence of excess phenylalanine in the blood spot from the patient. Although empirically a very successful test the mechanism of it has not been fully studied.

Our tests on a variety of bacteria have shown that Salmonella, Proteus, Pseudomonas, Acinetobacter, Enterobacter and Citrobacter species were not inhibited while Shigella species and E. coli were.

During investigation of the mechanism of this inhibition it was found that Magnesium levels are critical. If the level of this ion was increased the inhibition of E. coli and Shigella species by B-2-thienylalanine was no longer evident. Because of this the effect of varying the concentration of Mg. ion was tested in the Guthrie test. Blood spots from 112 infants were tested. None was positive at magnesium levels of 0, 0.01 or 0.02 G/L of Mg SO₄ in the medium. However, at 0.05 G/L (level in the original Guthrie medium) 4 samples gave positive reactions (equivalent to more than 4 mg% phenylalanine).

The action of B-2-thienylalanine on Salmonella (uninhibited species) and E. coli (inhibited) was further investigated. B-2-thienylalanine increased the concentration of tyrosine in the growth medium and in the intracellular non-protein fluid, suggesting that it acts at the level of tyrosine metabolism rather than phenylalanine.

Effect of bilirubin on the fungicidal capacity of human neutrophils
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Neutrophils possess several mechanisms for the killing of ingested micro-organisms. Although the relative importance of these antimicrobial systems have not been elucidated, Lehrer has provided evidence that the killing of bacteria and fungi is mediated by different mechanisms. We have previously shown that bilirubin impairs neutrophil bactericidal function. In the present studies, we have examined the effect of unconjugated bilirubin fungicidal capacity.

Fungicidal capacity of neutrophils was investigated by a colony count technique. Briefly, 1×10^6 Torulopsis glabrata was mixed with 1×10^6 neutrophils in a final volume of 0.5 ml medium 199 containing 1.0% pooled human serum. The tubes were rotated end-over-end at 37°C. At 0 and 4 hours, aliquots of 0.1 ml volumes were removed and diluted in distilled water to disrupt the neutrophils. Serial dilutions were plated on Sabouraud's agar for the quantitation of viable colonies.

Phagocytosis experiments were performed by a radiometric technique. This takes advantage of the inability of neutrophils and fungal cells to incorporate ¹⁴C-uridine. In brief, 5×10^5 T.glabrata and 5×10^5 neutrophils were mixed in a final volume of 0.5 ml medium. The tubes were rotated end-over-end for 30 min at 37°C, after which 0.1 ml aliquots were transferred to microlitre wells, 0.05 ml of ¹⁴C-uridine (10 μG/ml) added, and the plates incubated for a further 60 min at 37°C. A Skatron harvester was used to aspirate the cells onto glass fibre filter paper for the quantitation of radioactivity.

The results showed pronounced impairment of fungal killing in tubes containing unconjugated bilirubin. Percent T. glabrata killed in the presence of 1×10^{-4} M bilirubin was 7 ± 2.5 and 40.9 ± 7.2 (mean ± S.E. of 5 experiments) respectively, compared to 81.4 ± 4.1 in controls. This was

Brief Communication:

EFFECTS OF CHANGES IN Mg++ ION CONCENTRATION UPON BACTERIAL INHIBITION BY β-2-THIENYLALANINE IN DEFINED MEDIA

by KURT J. BROWN AND DAVID R. LINES[*]

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Summary. Variation in the inhibition of growth of *Escherichia coli* and *Shigella sp.* in various media containing β-2-thienylalanine was attributed to differences in concentrations of Mg++ ions.

Random blood samples from 112 infants were tested for elevated phenylalanine in the phenylketonuria (PKU) screening assay. Magnesium ion levels also affected the results of this assay. At $0 \cdot 05$ g/l MgSO$_4$, the concentration present in commercial PKU test agar, four false positives were detected, while no readings could be made due to overgrowth of the *Bacillus subtilis* test strain when the concentration was increased to $0 \cdot 1$ g/l.

INTRODUCTION.

The phenylalanine analogue, β-2-thienylalanine (β-2-t), is used in a microbial inhibition assay described by Guthrie and Susi (1963) to screen infant blood samples for PKU. This substance promotes renal excretion of phenylalanine, and inhibits intestinal absorption of phenylalanine in rats (Godin and Dolan, 1967; Wapnir and Lifshitz, 1974), monkeys (Lines and Waisman, 1970a and b, 1973) and man (Krips and Lines, 1972). It is being considered for use in therapy for PKU.

Our investigations have shown that members of the genera *Escherichia* and *Shigella* can be inhibited by β-2-t using a defined medium (Demain, 1957) while *Salmonella* and other genera, under the same conditions, are unaffected (Brown and Lines, 1976). The present study details the effects of different concentrations of components of defined media in assays dependent upon β-2-t inhibition of bacteria.

MATERIALS AND METHODS.

Procedure for Inhibition Assays

Organisms

Serotyped cultures of *Escherichia coli* 055/B5, 086/B7, 0114/K90, 0127/B8, *Shigella boydii*, *S. flexneri* III, *S. sonnei*, *Salmonella typhimurium*, *S. typhi*, and *S. panama*, isolated from infant pathological specimens, were obtained from Princess Mary Hospital for Children, Auckland.

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TABLE 1.
Medium Components.

	Vegetative* Minimal Medium (Demain, 1957) (g/l)	Medium VI	Minimal Defined Medium (Davis and Mingioli, 1950)	Spore Medium (Guthrie and Susi, 1963)	Spore Medium (Demain, 1957)
Glucose†	10·0000	10·0000	2·0000	5·0000	10·0000
K₂HPO₄	3·0000	3·0000	7·0000	15·0000	3·0000
KH₂PO₄	1·0000	1·0000	3·0000	5·0000	1·0000
NH₄Cl	0·5000	—	—		—
NH₄NO₃	0·1000	—	—	2·5000	0·5000
(NH₄)₂SO₄	—	2·0000	2·0000	0·5000	0·1000
Na₂SO₄	0·1000			0·5000	0·1000
Na citrate	—	0·5000	0·5000	—	—
L-glutamic acid	1·4700	—	—	0·5000	1·4700
L-asparagine	—	—	—	0·5000	1·3200
L-alanine	—	—	—	0·2500	0·8900
MgSO₄·7 H₂O	0·0100	0·0100	0·1000	0·0500	0·0100
MnSO₄·4 H₂O	0·0010	—	—	(MnCl₂) 0·0050	0·0010
FeCl₃·6 H₂O	—	—	—	(FeCl₃) 0·0050	—
FeSO₄·7 H₂O	0·0010	—	—	—	0·0010
CaCl₂	0·0005	—	—	0·0025	0·0005
EDTA·2 H₂O	0·0400	—	—	—	—
Agar	12·5	13·5	13·5	15·0	12·5

* All media pH 6·8–7·0.

† Autoclaved separately and added to the cooled medium.

‡ "Salts solution".

Maintenance of strains

Cultures were maintained on Dorset Egg Medium slants (Dorset, 1902). Organisms were streaked for isolation on to Columbia agar (BBL), then transferred to peptone water containing 2% (w/v) tryptone (Oxoid) and 0·9% (w/v) NaCl/litre for short term holding. For assays, strains were transferred to a defined diluent, a 1/10 dilution of a mineral salts concentrate (per litre of concentrate, 30 g K_2HPO_4; 10 g KH_2PO_4; 5 g NH_4Cl; 1 g NH_4NO_3; 1 g Na_2SO_4; 100 mg $MgSO_4 \cdot 7H_2O$; 10 mg $MnSO_4 \cdot 4H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$; 5 mg $CaCl_2$-Demain, 1957).

Inhibitor

An 0·01 M aqueous solution of β-2-t (β-2-thienyl-DL-alanine, Sigma) was sterilized by autoclaving.

Assay media

Media used were variations of the Vegetative Minimal Medium (VMM-Demain, 1957) and modified Minimal Defined Medium (MDM-Davis and Mingioli, 1950) and are shown in Table 1. The media were suspended in distilled water without heating, and autoclaved at 115° for 15 min to reduce possible charring. Glucose solutions were sterilised separately and added to the cooled media.

Inhibition assay

Eight or fewer streaks of different strains were made in parallel on duplicate agar plates. Sterile 6 mm × 75 mm strips of Whatman 3MM filter paper were placed at right angles across the streaks and impregnated by a syringe with 100 µl 0·01 M β-2-t (Brown and Lines, 1976). Strips impregnated with distilled water were used in controls. Plates were examined after incubation at 37° for 18 to 24 h. "Inhibition" was defined as lack of visible growth within 1 cm of either side of the strip. Non-inhibited strains grew to the strip while reactions with a less prominent no-growth area were termed "borderline".

Procedure for PKU Screening Assays

Random blood-impregnated specimen cards from 112 infants, and 12 standard phenylalanine control samples were tested using the Guthrie and Susi (1963) method.

For this assay, 0·3 cm diameter punched discs from the cards were placed on to the surface of 100 ml of solidified medium (modified Guthrie medium without "salts solution") in a plastic tray. Before pouring, 0·08 ml of a 1 ml spore : 5 ml water dilution of a standard B. subtilis spore suspension in 0·85% saline (ATCC 6633-BBL.), and 0·4 ml of a 0·001 M β-2-t solution were added to the medium. β-2-t inhibited spore germination, but inhibition was overcome if phenylalanine was present in the blood sample. The trays were covered, incubated at 37° for 16-18 h and read by transmitted light. For a positive sample, growth was equal to or greater than that surrounding a control disc impregnated with blood containing 4 mg/100 ml phenylalanine.

Five identical trays were prepared using the same specimen cards. The control tray contained no $MgSO_4$. $MgSO_4$ solution was added to the 4 remaining trays at concentrations of 0·01, 0·02, 0·05, or 0·1 g/l.

RESULTS

Inhibition assays with β-2-t were done using VMM and modified MDM (Table 1). *Escherichia* and *Shigella* were inhibited in VMM, while the *Salmonella* strains were not (Brown and Lines, 1976). In modified MDM, none of the organisms tested was inhibited (Table 2).

The two media were compared to determine if inhibition reactions were affected by the concentration of common components or by the different components present. By varying the concentrations of tested components, while keeping other components at a constant con-

centration, it was found that changes in medium components other than Mg$^+$ ion did not abolish β-2-t inhibition. Mg$^+$ ion effects are shown in Table 2.

In the Guthrie assay (Guthrie and Susi, 1963), none of the 112 specimens was positive for elevated phenylalanine in trays containing 0, 0·01, or 0·02 g/l MgSO$_4$. At a concentration of 0·05 g/l MgSO$_4$, the level contained in Guthrie's medium, 4 samples gave positive reactions. At 0·1 g/l MgSO$_4$ B. subtilis overgrew the medium and phenylalanine determinations could not be made.

TABLE 2.

Inhibition reactions to β-2-thienylalanine with increasing Mg^{++} ion concentration.

Organism	Media (g/l MgSO$_4$·7H$_2$O)*							
	VI 0·0100	XI 0·0200	XII 0·0400	XIII 0·0800	XIV 0·1000	XV 0·2000	VMM	Modified MDM
Escherichia coli								
055/B5	+	±	+	±	−	−	+	−
086/B7	+	±	+	±	−	−	+	−
0114/K90	+	±	±	+	−	−	±	−
0127/B8	+	±	+	±	−	−	+	−
Shigella boydii	+	±	+	+	−	−	+	−
Shigella flexneri III	+	±	±	±	−	−	+	−
Shigella sonnei	+	±	+	±	−	−	+	−
Salmonella typhimurium	−	−	−	−	−	−	−	−
Salmonella typhosa (typhi)	−	−	−	−	−	−	−	−
Salmonella panama	−	−	−	−	−	−	−	−

+ Inhibition of organism.

± Reaction of organism—borderline.

− Non-inhibition of organism.

* Constant medium components: glucose, 10·0000 g; K$_2$HPO$_4$, 3·0000 g; KH$_2$PO$_4$, 1·0000 g; (NH$_4$)$_2$SO$_4$, 2·0000 g; sodium citrate, 0·5000 g; agar, 13·5 g.

DISCUSSION.

Inhibition of growth of *Escherichia* and *Shigella* organisms by β-2-t in defined media is increasingly affected when the concentration of MgSO$_4$·7H$_2$O exceeds 0·0100 g/l. A ten-fold increase in this concentration abolishes the inhibiting effects of β-2-t. Weighing of components to 0·0001 g is necessary, as a gradation of reaction from + (inhibition) to ± (borderline) is found at MgSO$_4$·7H$_2$O concentrations between 0·0100-0·0200 g/l (Brown and Lines, unpublished data). The effect of SO$_4$ ion present in the salt can be discounted, since experiments using similar concentrations of (NH$_4$)$_2$SO$_4$ did not abolish inhibition.

A medium similar to VMM for germinating *B. subtilis* spores (Demain, 1957) has been adapted by Guthrie and Susi (1963) and is distributed commercially as "PKU Test Agar" (BBL). In Guthrie and Susi's (1963) medium, quantities of components have been greatly altered, as compared to Demain's (1957) original formulation (Table 1). A similar medium to Guthrie's is used by the Community Health Department, Auckland Medical School, except that a "salts solution" (including Mg$^+$ ions) is deleted. Their assay is accurate despite increases in concentration of ionic species other than Mg$^+$ ion, as compared to Demain's spore medium. The present observations, that changes in concentration of other ions do not abolish β-2-t inhibition, support their findings. Increasing Mg$^+$ ion concentration to that in the Guthrie medium (0·05 g/l MgSO$_4$) could increase false positives which, if not discounted by repeat testing or by amino acid analysis, may result in initiation of treatment for PKU,

It is suggested that effects of increasing Mg++ ion on *in vitro* bacterial cultures may reflect *in vivo* physiological states. Monitoring of Mg++ ion levels should therefore be considered during β-2-t administration to experimental animals or in clinical trials.

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Effects of increasing Mg^{++} ion concentration on the PKU monitoring assay

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Summary. Inaccuracies in a commonly used bacterial inhibition assay for blood phenylalanine levels arise when the Mg^{++} ion concentration in the assay medium is increased. This has practical implications in the diagnosis and management of phenylketonuria.

Gram-negative bacterial genera have been differentiated according to inhibition reactions to β-2-thienylalanine (β-2-t) in a plate assay[2]. When the defined medium[3] used in the inhibition assay was compared with the medium of Davis and Mingioli[4], it was found that inhibition was abolished for all genera in the latter medium due to increased Mg^{++} ion concentration[5].

The effects of increasing Mg^{++} ion concentration were then compared in the Guthrie phenylketonuria (PKU) screening assay[6], a test also based upon bacterial inhibition by β-2-t. Random blood samples from 112 infants were tested. 5 duplicate plates, with concentrations of 0, 0.01, 0.02, 0.05 and 0.1 g/l $MgSO_4$, respectively, were used to test identical series of specimens. In plates containing no magnesium, all samples were negative for increased blood phenylalanine levels (≤ 4 mg/100 ml). In the test plate containing 0.05 g/l $MgSO_4$, 4 of the samples were positive (> 4 mg/100 ml)[5]. Commercial 'PKU test agar' (BBL) contains 0.05 g/l $MgSO_4$. For a sample to be read as positive, growth of the *Bacillus subtilis* test strain must be equal to or greater than that surrounding a control disk impregnated with blood containing 4 mg/100 ml phenylalanine.

The observations of the effects of increasing Mg^{++} ion have now been extended to the PKU monitoring assay. The effectiveness of dietary therapy for reducing blood phenylalanine levels in PKU patients is monitored through periodic blood samples, which are measured in the Guthrie assay[6]. Although normal human levels of phenylalanine are 0.8–1.2 mg/100 ml[7], PKU blood samples are considered acceptable if in the range of 6–12 mg/100 ml in our clinic.

55 monitoring samples from PKU patients were tested in 5 duplicate Guthrie assay plates to which 0, 0.01, 0.02, 0.05, and 0.1 g/l $MgSO_4$ were added, respectively. Of 28 samples which read 6 mg/100 ml phenylalanine or less in the control plate (0 g/l $MgSO_4$), 3 samples gave readings of greater than 4 mg/100 ml on plates containing 0.05 and 0.1 g/l $MgSO_4$, while giving readings of less than 4 mg/100 ml on the control plate. Increasing of $MgSO_4$ to concentrations of 0.05 and 0.1 g/l resulted in decreasing differences between adjacent control samples in a series containing 0, 2, 4, 6, 8, 10, 12 and 20 mg/100 ml phenylalanine. As test sample phenylalanine concentrations were determined by comparison with the control series, determinations were made more difficult under conditions of high Mg^{++} ion, than in the plates containing 0–0.02 g/l $MgSO_4$ (figure).

Inaccurate readings in the Guthrie assay can lead to incorrect treatment regimens. In the screening assay, positive samples should be checked by an additional method[6]. False positives, if not discounted by determination of phenylalanine levels by amino acid analysis or by retesting, could result in initiation of treatment for PKU[5]. In the monitoring assay, PKU patients with near-maximum acceptable levels (10–12 mg/100 ml phenylalanine) could be

Plate A

Plate B
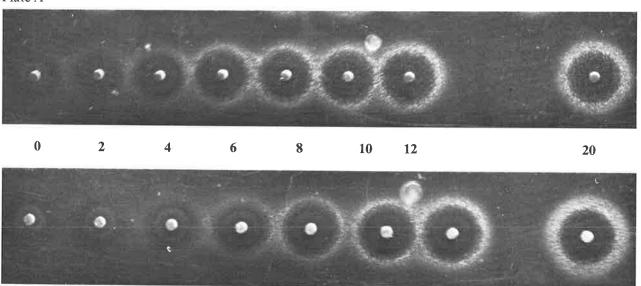
Guthrie assay[6] for PKU testing. *Bacillus subtilis* spores and β-2-thienylalanine are included in the medium. The control series of blood-impregnated filter paper disks contains increasing phenylalanine concentrations (from left, 0, 2, 4, 6, 8, 10, 12, and 20 mg/100 ml). Phenylalanine reverses the inhibition of spore germination by β-2-t. Plate A contains 0.05 g/l $MgSO_4$, the amount present in commercial PKU test agar, while plate B contains 0 g/l $MgSO_4$. Adjacent samples are more difficult to differentiate in plate A than in plate B, and the increased 'background' growth makes comparisons of test samples with the control series more difficult to read. Plates containing 0.01 g/l $MgSO_4$ read similarly to plate B.

unnecessarily restricted in protein intake, if inaccurate high readings are obtained. Blood specimens from PKU patients containing less than 4 mg/100 ml phenylalanine, which are incorrectly assessed as greater than 4 mg/100 ml, could result in dangerous overtreatment, which could lead to decreased intelligence through phenylalanine and protein deficiency[8].

It is suggested that the PKU screening and monitoring assays be modified by reducing the concentration of $MgSO_4$ in the medium to 0.01 g/l or less.

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Colonization by *Salmonella typhimurium* and *Shigella flexneri* III of the gastrointestinal tract of mice treated with β-2-thienylalanine and streptomycin

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BROWN, K. J., TANNOCK, G. W., EYRES, R. A., ELLIOTT, R. B., and LINES, D. R. 1979. Colonization by *Salmonella typhimurium* and *Shigella flexneri* III of the gastrointestinal tract of mice treated with β-2-thienylalanine and streptomycin. Antonie van Leeuwenhoek **45**: 531–546.

Mice fed β-2-thienylalanine (β-2-T) by oesophageal tube were no more susceptible to gastrointestinal tract colonization by *Salmonella typhimurium* or *Shigella flexneri* III than control mice fed water. In both β-2-T-fed and water-fed groups, the increasing dosage of *S. typhimurium*, in logarithmic increments to groups of mice, resulted in increasing numbers of these bacteria detectable on dilution plates from organ homogenates. Colonization by *S. flexneri* III only occurred at a dosage of 10⁸ bacteria for both groups. Pretreatment with 50 mg streptomycin allowed 10³ *Salmonella* or 10⁴ *Shigella* to colonize both β-2-T- and water-fed groups.

Coliforms, inhibited by β-2-T under certain conditions *in vitro*, were found in equal numbers in both groups. No obvious differences were noted in either types of other bacteria detected or numbers recovered from the two groups. No gross behavioural changes were noted in mice fed β-2-T and not challenged with pathogenic bacteria, and no pathological changes were noted in hepatic or splenic tissues. With increasing *Salmonella* dosage, collections of polymorphonuclear leucocytes, which were almost focal, and increased numbers of giant cells were noted in splenic red pulp areas, in both groups.

INTRODUCTION

The phenylalanine (Phe) analogue, β-2-thienylalanine (β-2-T), increases urinary excretion of Phe in rats without being metabolized to CO_2 or being incorporated to a great extent into proteins (Godin and Dolan, 1967). There is increased renal excretion of Phe when β-2-T is infused in adult monkeys (Lines and Waisman, 1970a). β-2-T reduces intestinal absorption of Phe in monkeys with much less fall in serum tryptophan levels than that produced by cycloleucine or p-chlorophenylalanine (Lines and Waisman, 1970b). No long-term effect on growth or development of β-2-T-treated infant monkeys has been noted (Lines and Waisman, 1973), and β-2-T depresses the serum Phe level after an oral Phe dose in phenylketonuric (PKU) patients (Krips and Lines, 1972). It interacts with Phe at the intestinal mucosa in the rat by inhibiting the specific Phe transporter, while not inhibiting transport of tyrosine (Tyr) (Wapnir and Lifshitz, 1974). The interaction appears to be a cell surface phenomenon, rather than an intracellular competition (Munro and Clark, 1958). β-2-T is therefore being considered as a possible treatment agent for phenylketonuria.

As part of a study of possible analogue-induced side effects, β-2-T-inhibition of bacterial growth has been studied. An assay has been developed which differentiates gram-negative genera according to whether the organisms are inhibited by β-2-T or not. Escherichia and Shigella strains are inhibited by β-2-T, while Salmonella, Enterobacter, Proteus, Citrobacter, and Pseudomonas strains are not inhibited in the assay (Brown and Lines, 1976). By increasing the Mg^{++} concentration in the assay medium, inhibition can be abolished for all gram-negative genera (Brown and Lines, 1978), and can be modified for Bacillus subtilis in the Guthrie and Susi (1963) PKU assay (Brown and Lines, 1978; Brown, Elliott and Lines, 1979). Phe also reverses bacterial inhibition by β-2-T in vitro (Beerstecher and Shive, 1946; Dittmer et al., 1946; Drea, 1948; Dunn and Dittmer, 1951). Only 7% of anaerobic isolates from faeces of PKU patients are inhibited by β-2-T in vitro, and the addition of Phe abolishes growth inhibition for these bacteria (Brown, Vesey, Tannock, Bell, Elliott and Lines, in preparation). Indications are then that β-2-T, while competing for intestinal absorption of Phe and reducing blood levels of Phe, may have little effect on the indigenous bacteria during in vivo treatment with β-2-T. However, infant monkeys treated long-term with this agent may be predisposed to Shigella gastroenteritis (Lines and Waisman, 1973).

In order to assess such in vivo effects, mice have been treated with β-2-T. If β-2-T were to seriously affect growth of host indigenous bacteria, then pathogenic bacteria might be expected to colonize such experimentally treated animals more easily than water-fed control animals of the same litter. Since Escherichia are inhibited by β-2-T in vitro (Brown and Lines, 1976), there may be a reduction in recovery of coliforms from β-2-T-fed animals as compared to water-fed controls. Streptomycin is known to reduce gram-negative bacterial populations, allowing Salmonella typhimurium to colonize mice at much lower dosages than in un-

treated mice (Miller and Bohnhoff, 1963). β-2-T treatment might be expected to cause similar increased colonization effects. Finally, an *in vivo* β-2-T treatment regimen would indicate not only whether indigenous bacterial flora are affected, but whether there is any resulting tissue damage allowing a pathogen to be more invasive.

Such studies then would be useful in confirming or denying indications that β-2-T should have little effect on host intestinal bacterial populations *in vivo*.

MATERIALS AND METHODS

Animals

18–25 g specific pathogen free (SPF), CBA (brown) and CD (albino) male mice were obtained from the breeding colonies of the Animal Laboratories, Auckland Medical School, and maintained on Diet 86 (N.Z. Stock Foods Co., Otahuhu, N.Z.) (estimated Phe – 0.82 % of dry content), and water, *ad libitum*.

Inhibitor

A 20 mg/ml (0.12 м) β-2-T solution (β-2-thienyl-DL-alanine, Sigma Chemical Co., St. Louis) was prepared by suspending the powder in distilled water, then solubilizing and sterilizing by autoclaving at 121 °C for 15 min. Solutions were maintained at 4 °C, and warmed to room temperature before use.

Dosage of the analogue

β-2-T solution was fed intraoesophageally using a 7 cm length of 16 gauge tubing attached to a syringe fitting (Intracath, Deseret Pharmaceutical Co., Sandy, Utah). The tubing was fitted to a sterile 1 ml tuberculin syringe (Monoject, Sherwood Medical Industries, Deland, Florida). Half a ml of the solution (350 mg per kg and per day) was fed to each of four animals in a challenge series, daily for 7 days. The other four animals of each challenge series were fed the same quantity of sterile distilled water.

Reported dosages of β-2-T used in other species include daily regimens of 187.5 and 750 mg/kg in monkey studies (Lines and Waisman, 1970b; 1973) and 20 mg/kg in human Phe loading experiments (Krips and Lines, 1972).

Bacterial strains

Serotyped cultures of *Salmonella typhimurum* and *Shigella flexneri* III were obtained from the Princess Mary Hospital for Children, Auckland, from infant pathological specimens. Strains were maintained on Dorset Egg Medium (Dorset, 1902), and transferred to peptone water [2 % (w/v) tryptone (Oxoid, London); and 0.9 % (w/v) NaCl/litre] for use in experiments.

Salmonella typhimurium challenge series

Salmonella typhimurium were fed to groups of eight animals treated with β-2-T or distilled water. Groups of CBA mice received 10^3, 10^4, 10^5, 10^6, 10^7 or 10^8 cells suspended in 0.25 ml peptone water, by tube, on the next-to-last day of the β-2-T-feeding period. Differences in colonization between pathogen dosage levels, were confirmed using groups of CD mice. The number of bacteria present in the inoculum was determined using a standard growth curve derived turbidimetrically, at an absorbance of 450 nm, using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, New York) (Brown and Lines, 1976).

Half of each experimental group (two β-2-T-fed and two water-fed animals) was killed by cervical dislocation 24 h after bacterial challenge, and the other half (two from each group) was killed at 48 h. A 1 mm cube of splenic material was taken for histological examination. The remainder of the spleen, the small intestine, and the large intestine (including the caecum) was removed, cut into segments, and each organ placed in separate McCartney bottles containing 8 ml of sterile, phosphate-buffered saline, pH 7.2. The bottles were immediately placed in an ice bath. The organs were homogenized, with the bottles placed in an ice bath, with an "Ultra Turrax" homogenizer (Janke and Kunkel, Staufen i. Breisgau, West Germany) at the top speed setting.

The homogenates were diluted 10^{-4} in sterile buffered saline, and 0.1 ml of the dilutions was spread on MacConkey (MAC) (BBL, Cockeysville, Md), Brilliant Green (BG) (Oxoid, London), Salmonella-Shigella (S-S) (BBL), and Bismuth Sulphite (BS) (BBL) agar plates, in duplicate, and incubated for 48 h at 37°C.

One tenth of a ml of each of the 10^{-4} dilutions was also used for inoculating 8 ml of tetrathionate enrichment broth (BBL), which was incubated at 37°C for 24 h. The tetrathionate broths were then diluted 10^{-2} in buffered saline, and spread-plated in duplicate on the same media as the non-enriched samples. The dilutions were made so that isolated colonies could be both quantitated and picked for serotyping. Comparable numbers of positive plates were detected with this method, and with the usual non-quantitative method of streaking tetrathionate broth samples for isolation, on the same media. The *Salmonella typhimurium* challenge strain was plated on the same media for comparison of colonial morphology. Suspected *Salmonella* isolates were confirmed by serotyping with *Salmonella* O agglutinating sera (Group B, factor 4, Wellcome Laboratories, Beckingham, U.K.).

Shigella flexneri III challenge series

Mice treated with β-2-T or water were challenged with *Shigella flexneri* III using the same regimen and procedure as for *Salmonella typhimurium*, with the following exceptions. Homogenates were diluted 10^{-2} instead of 10^{-4}, before spread-plating, and the media used were MAC, S-S, and Deoxycholate Citrate (DC) (BBL) agars. Suspected *Shigella* colonies were serotyped with *Shigella flexneri* antiserum [Wellcome, Flexneri Polyvalent (1–6, X & Y)].

Streptomycin

Fifty mg streptomycin sulphate BP (1 g streptomycin base, Glaxo Laboratories, Greenford, U.K.) in 0.5 ml distilled water were fed in some experiments to groups of mice on water or β-2-T regimens, followed by challenge with 10^3-10^4 *Salmonella* or *Shigella* bacteria 24 h later. Mice were given the daily dose of water or β-2-T one hour after dosing with streptomycin.

Spleen histology

A 1 mm cube of splenic material from animals killed 48 h after bacterial challenge was fixed and prepared histologically. Sections were stained in haematoxylin (Harris, 1900), and examined by light microscopy for possible alterations in histological appearance with increasing dosage of pathogens. Comparisons for changes in histological appearance were also made between tissues from water-fed and β-2-T-fed animals.

RESULTS

Bacterial colonization

Colonization by Salmonella typhimurium. No *Salmonella* bacteria were detected in uninoculated random animals from the mouse colonies. The increasing dosage of *Salmonella typhimurium* in logarithmic increments to groups of mice resulted in increasing numbers of these bacteria detected on dilution plates from organ homogenates (Table 1).

No *S. typhimurium* were detected in animals challenged with 10^3 or 10^4 bacteria. At a dosage of 10^5 bacteria, one animal from each group gave a single positive sample in the tetrathionate broth-enriched large intestinal homogenate. Both animals had been killed 24 h after challenge. At a dosage of 10^6 *Salmonella* bacteria, one "24 h" β-2-T-fed mouse, and two water-fed mice (one "24 h" and one "48 h") were positive in a single tetrathionate broth-enriched large intestine sample, and non-enriched large intestine and enriched spleen samples, respectively. At a dosage of 10^7 bacteria, samples from one water-fed animal and one β-2-T-fed animal were positive. At this level, a number of organ samples were positive as compared to single positive samples for mice challenged with fewer bacteria. At 10^8 bacteria in the challenge, all animals gave positive samples. All enriched large intestinal samples were positive at this dosage, with decreasing positive samples noted in non-enriched large intestinal homogenates and enriched and non-enriched small intestinal samples. Only one positive sample was detected in the enriched and non-enriched spleen samples.

In the total series, no obvious difference was noted between colonization of water-fed or β-2-T-fed animals by *S. typhimurium*. In a matching series, no notable difference was found between the two mouse strains used as regards *Salmonella* colonization.

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Table 1. Positive colonization samples with *Salmonella typhimurium*

Samples	Number of bacteria in challenge dose*					
	No streptomycin treatment					Streptomycin[3] 50 mg
β-*2-Thienylalanine* fed	10^3–10^4* (12 mice)	10^5 (4 mice)	10^6 (4 mice)	10^7 (4 mice)	10^8 (8 mice)	10^3 (4 mice)
Small intestine	0	0	0	0	4/25%	0
Small intestine T[1]	0	0	0	1/19%	5/44%	2/31%
Large intestine	0	0	0	1/13%	6/53%	1/19%
Large intestine T	0	1/6%[2]	1/6%	1/9%	8/100%	0
Spleen	0	0	0	0	0	0
Spleen T	0	0	0	1/25%	3/38%	2/25%
Water fed	(12 mice)	(4 mice)	(4 mice)	(4 mice)	(8 mice)	(4 mice)
Small intestine	0	0	0	0	7/50%	2/25%
Small intestine T	0	0	0	0	5/63%	1/25%
Large intestine	0	0	1/6%	1/6%	8/81%	1/25%
Large intestine T	0	1/6%	0	0	8/97%	3/56%
Spleen	0	0	0	0	3/28%	0
Spleen T	0	0	1/3%	0	4/47%	1/13%

[1] T = tetrathionate broth enriched cultures.
[2] a/b; a = number of animals positive for *Salmonella*, b = % of plates that were positive (duplicate plates of four media = 32 plates for four mice or 64 plates for eight mice).
[3] 4 of 4 β-2-T-fed animals and 3 of 4 water-fed animals were positive in at least one sample.

Table 2. Positive colonization samples with *Shigella flexneri* III

Samples	Number of bacteria in challenge dose*		
	No streptomycin treatment		50 mg streptomycin
β-*2-thienylalanine fed*	10^4, 10^5, 10^7* (12 mice)	10^8 (8 mice)	10^4 (4 mice)
Small intestine	0	1/4%[1]	1/17%
Large intestine	0	5/46%	2/33%
Spleen	0	0	
Water fed	(12 mice)	(8 mice)	4 mice)
Small intestine	0	2/13%	1/25%
Large intestine	0	4/30%	3/42%
Spleen	0	1/8%	0

[1] a/b; a = number of animals positive for *Shigella*, b = % of positive plates (duplicate plates of three media = 24 plates for four mice and 48 plates for eight mice).

Colonization by Shigella flexneri III. *Shigella* only colonized the gastrointestinal tract of the tested mouse strains at a dosage of 10^8 bacteria (Table 2). As with the salmonella-challenged animals, there was no notable difference in numbers of β-2-T-treated animals *versus* water-fed animals colonized. The bacteria were detected first in the large intestine, then in the small intestine, and finally, in the spleen.

Effect of streptomycin treatment. Pretreatment with 50 mg streptomycin, followed by challenge with *Salmonella* or *Shigella*, increased the ability of the bacteria to colonize the mouse gastrointestinal tract. A dosage of 10^3 *Salmonella* in streptomycin treated mice resulted in numbers of positive plates intermediate between those of non-streptomycin treated mice challenged with 10^7 and 10^8 bacteria (Table 1).
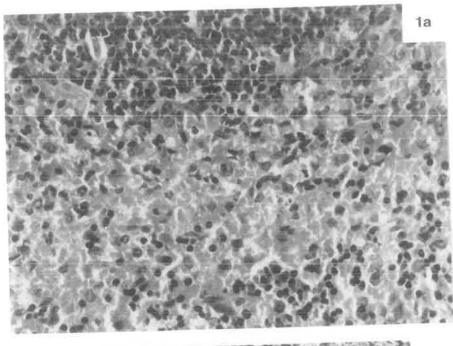
In streptomycin treated animals, a dosage of 10^4 *Shigella flexneri* III was comparable to a dosage of 10^8 *Shigella* in non-streptomycin treated animals (Table 2). For both *Salmonella* and *Shigella*, there was a marked difference between streptomycin and non-streptomycin treated animals as regards bacterial colonization.
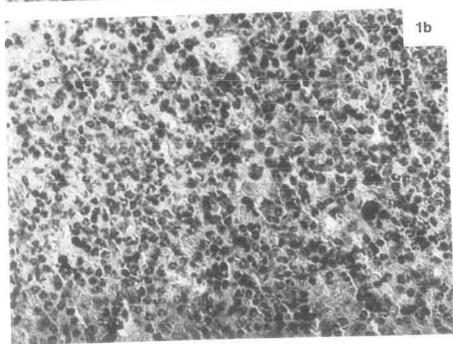
Other bacterial isolates. Most common of other bacterial isolates were *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella sp*. No obvious differences were noted in either the types of bacteria recovered or numbers of particular organisms detected on plates from water-fed or β-2-T-fed mice unchallenged with pathogens or challenged with low-pathogen inoculi.

With increasing dosage of pathogenic bacteria, there were more lactose-fermenting bacteria noted on plates from both control and experimental animals ($> 3 \times 10^7$ colonies/ml, as compared to 10^5–10^6 colonies/ml at low pathogen doses). Also, at high pathogen dosage, there were tendencies towards recovery of fewer types of bacteria, towards increased numbers of plates without bacterial growth (neither pathogens nor other bacteria) especially from spleen samples, and towards plates with pure cultures of the pathogen used for challenge.

Gross changes. As compared to non-treated controls, which tended to steadily increase in weight (mean increase $= +1.0$ g; s.d. $= 0.707$; n $= 2$), animals tube-fed daily with either water or β-2-T showed weight fluctuations when weighed on alternate days, in some experiments. There was no significant difference noted (Mann-Whitney "U" test) between β-2-T (mean $= +0.55$ g; s.d. $= 0.545$; n $= 4$), and water-fed (mean $= +0.28$ g; s.d. $= 0.512$; n $= 4$) animals, and most animals showed an overall weight gain over the total experimental period. In common with water-fed controls, β-2-T-fed animals showed no obvious behavioural changes.

At a dosage of *Salmonella* bacteria of 10^7 and greater, animals showed obvious signs of illness, had rough coats, and tended to huddle. Two water-fed animals challenged with 10^8 *S. typhimurium* without streptomycin, and 10^3 *Salmonella* after streptomycin treatment, respectively, died prior to the end of the 48 h holding period. One water-fed animal challenged with 10^8 *Shigella* also died.
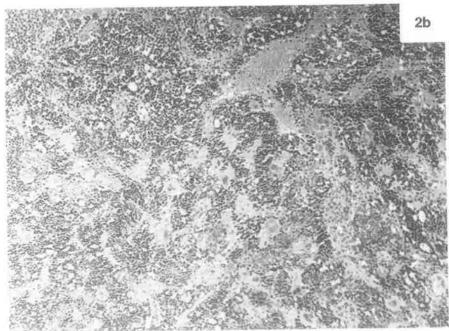
1a

1b
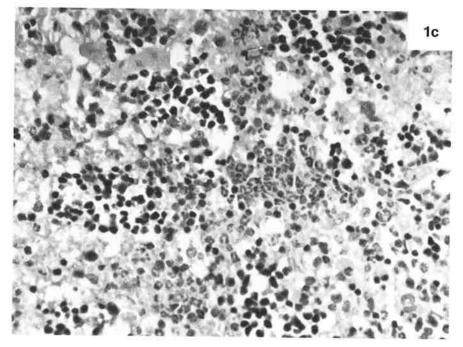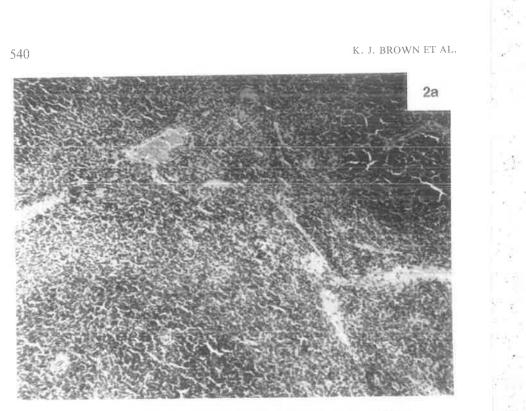
Fig. 1.

a. Mouse spleen, 48 h after inoculation with 10^3 *Salmonella typhimurium*, few polymorphonuclear leucocytes in red pulp areas. Haematoxylin and eosin, ×384.

b. 10^6 *S. typhimurium*, focal or almost focal collection of polymorphonuclear leucocytes in red pulp. HE, ×96.

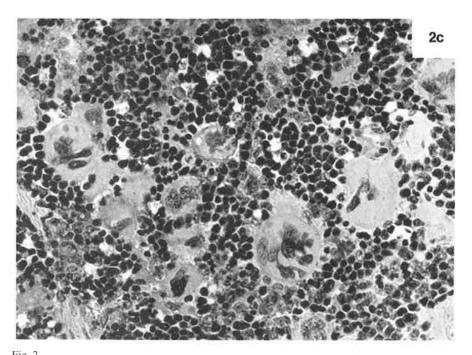c. 10^8 *S. typhimurium*, focus of polymorphonuclear leucocytes. HE, ×384.

Streptomycin treated animals showed watery stools and enlarged caeca. Signs of illness were not as extreme in the 10^3 *Salmonella* plus streptomycin animals as in end-point (10^8 bacteria) experiments.

Statistical analysis. Data were analyzed for 2 × 2 tables using the log-likelihood ratio test (G-test) for independence, and the Yates correction was employed (Sokal and Rohlf, 1969). Analyzed were 1) the numbers of animals which a pathogen colonized of the total number of animals tested, and 2) the number of plates positive for that pathogen for a particular combination of factors.

When the numbers of animals challenged with pathogens at various dosage levels were analyzed level by level, and also when total numbers of mice from all pathogen dosage levels were pooled, there was no significant difference between the water- and β-2-T-fed groups regarding ability of either *Salmonella* or *Shigella*

Fig. 2.

a. Mouse spleen, 48 h after inoculation with 10^8 *Shigella flexneri* III showing few giant cells in red pulp areas. Haematoxylin and eosin, ×96.

b. 10^8 *Salmonella typhimurium*, large numbers of giant cells in red pulp, HE, ×96.

c. 10^8 *S. typhimurium*, large numbers of giant cells, HE, ×384.

to colonize the gastrointestinal tract (i.e. $G = 0.12$; $\alpha > 0.05$, for pooled *Shigella* experiments).

Differences between numbers of positive plates noted for water- and β-2-T-fed animals challenged with pathogen were not significant at levels of 10^3, 10^4, 10^5, 10^6, or 10^8 *Salmonella* bacteria. The difference in numbers of positive plates was significant for a dosage of 10^7 *Salmonella* ($G = 19.295$; $\alpha < 0.01$). No significant differences were noted for numbers of positive plates when all *Salmonella* dosage levels were pooled ($G = 0.041$; $\alpha > 0.05$). No significant difference was noted between water- and β-2-T-fed groups for either individual *Shigella* dosage levels or for when all *Shigella* dosage levels were pooled ($\alpha > 0.05$).

Pretreatment of mice with streptomycin significantly increased colonization of both *Salmonella* and *Shigella* bacteria, both for the number of animals colonized at a particular dosage of pathogen (10^4 *Shigella*, $G = 5.17$; $\alpha < 0.05$; and 10^3 *Salmonella*, $G = 10.47$; $\alpha < 0.05$), and for the numbers of positive plates detected (for both *Shigella* and *Salmonella*, $\alpha < 0.01$).

Histological effects

No obvious histological changes were noted in spleen or liver specimens taken from animals fed β-2-T or water, but not challenged with pathogens.

With increasing *Salmonella* challenge, there were collections of polymorpho-nuclear leucocytes in the "red pulp" areas of the spleen (Fig. 1). No necrotic lesions or obvious bacterial concentrations were noted. There also was a tendency towards an increase in numbers of giant cells, possibly megakaryocytes, noted in the red pulp areas (Fig. 2). In some experiments, increased peri-arterial sheaths and slightly less prominent germinal centres were noted, but differences were not consistent throughout the series. There were no differences noted between spleens of animals fed water or β-2-T.

No consistent splenic changes were noted with increasing dosage of *Shigella* bacteria.

DISCUSSION

Mice treated with β-2-thienylalanine appear to be no more susceptible to intestinal colonization by *Salmonella typhimurium* or *Shigella flexneri* III than controls fed water. Pretreatment with streptomycin greatly increases the ability of the two pathogens to colonize whether or not β-2-T is present. β-2-T does not therefore appear to mimic streptomycin in seriously affecting the indigenous gram-negative intestinal bacterial populations.

A post-mortem on a monkey which died of *Shigella* gastroenteritis in the study of Lines and Waisman (1973) "revealed findings compatible with only that diagnosis", and "incorporation of β-2-T into protein was negligible". The present studies also suggest that any appearance of *Shigella* gastroenteritis in experimental animal populations would not be due to therapy with β-2-T.

Diet 86 contains sufficient Phe for normal rodent growth. No attempt has been made to remove Phe from the diet because β-2-T has been shown to be unable to replace Phe or methionine in rodent diets (du Vigneaud et al., 1945; Ferger and du Vigneaud, 1949), and because a diet for PKU patients which does not contain sufficient Phe for growth requirements can lead to impaired cerebral development (Berman, 1971; McKean, 1971; Ampola, 1973).

The present *in vivo* results suggest that β-2-T does not aid in increased colonization of the mouse gut by either "inhibited" *(Shigella)* or "non-inhibited" *(Salmonella)* bacteria. These results also indicate that the effects of β-2-T *in vivo* are more closely related to the *in vitro* concept of transport of β-2-T across bacterial membranes being slowed by diffusion in solid media, activating protective factors in the cell which reduce growth inhibition; than to a concept of rapid passage of β-2-T across bacterial membranes, such as occurs in liquid media (Brown, Tannock, Elliott, and Lines, in preparation).

In the present studies, no difference has been noted in the numbers or types of

bacteria isolated between β-2-T-fed and water-fed animals. Coliforms, which are inhibited by β-2-T in defined media without Phe or increased Mg^{++} present, are still detectable in treated animals in the same numbers as in control animals. In preliminary experiments in guinea pigs, when *Escherichia coli* 026/B6 has been used to measure colonization, no differences have been noted between water-fed and β-2-T-fed animals (unpublished results).

Bismuth sulphite plates from enriched samples have proved the easiest to assay and show the greatest numbers of *Salmonella* recovered in murine experiments with overwhelming infections. In animals with lesser infections, neither enrichment nor any single medium contributes to detection of larger numbers of salmonella-positive plates. The use of sixteen plates per homogenate, representing duplicate plates of four media from both enriched and non-enriched samples, has proved the greater safeguard in detecting small numbers of *Salmonella*.

Weight loss due to reduced food intake, has been reported in rats fed solid diets containing β-2-T (du Vigneaud et al., 1945; Ferger and du Vigneaud, 1949). Kaufman, Klavins and Kinney (1961) have reported weight loss in rats fed β-2-T, but have not detailed daily quantities of food consumed. Many of their histological findings in animals treated with β-2-T are noted by them to be similar to the effects of phenylalanine deficiency (in testis, bone, thymus, pituitary, thyroid, seminal vesicles and epididymis). Among the effects noted that are not similar to those of Phe-deficiency, are changes in the liver (vesicular nuclei and prominent nucleoli) and the spleen (decrease in size of Malpighian corpuscles, decrease in mature lymphocytes, and prominent reticular cells). In our series, in which mice have been fed β-2-T by oesophageal tube, there has been no consistent weight loss, and no difference in weight gain noted as compared to water-fed controls. We have observed no pathological changes in liver or splenic tissues. These differences, as compared to the results of Kaufman, may have been due to the difference in animal species used (rats *vs.* mice), or due to a shorter feeding period in our study. However, many of the histological changes reported may have been due to amino acid deficiencies resulting from greatly lowered food intake. Lines and Waisman (1973) have suggested that β-2-T is distasteful to infant monkeys. We also have found that mice show a distaste to β-2-T when they are allowed to voluntarily drink the solution. Animals seem to tolerate the dosage better when the β-2-T is completely dissolved by autoclaving as compared with when it is fed as a particulate suspension. β-2-T-treatment in the form of coated tablets or in gelatine capsules may be indicated in future human trials, to reduce taste problems.

Freter (1956) has fed mice and guinea pigs both streptomycin-resistant *Shigella* bacteria and streptomycin solution at the same time, and has been able to recover bacteria from faecal pellets. However, he has neither been able to recover *Shigella* bacteria in faecal pellets from orally infected mice not treated with streptomycin, nor has he noted any gross symptoms of disease in such mice. In our series, by assaying homogenates of the large and small intestine and spleen,

rather than faecal pellets, *Shigella* can be recovered from half of the animals fed 10^8 bacteria with no streptomycin therapy, as well as from the streptomycin-treated mice challenged with 10^3 or 10^4 bacteria. Also, one animal has died of the infection in our series. Our series, in which streptomycin is given on the day prior to *Shigella* infection, shows similar results to one of Freter's experiments involving challenge of mice with both streptomycin-resistant *Shigella* and resistant *E. coli*. We also have found that our non-resistant *Shigella* can be recovered in larger numbers from 24 h animals, as compared to animals killed 48 h after infection (both water- and β-2-T-fed groups). The reverse situation occurs with *Salmonella*, as increased numbers of bacteria are found in 48 h samples. Like Freter, we have found increasing numbers of coliforms with increasing time after infection, and some "sterile" samples. Freter has indicated that coliforms are strongly antagonistic to *Shigella* infection *in vivo*. Similarly, our findings indicate that indigenous intestinal bacteria are still protective against infection by pathogens in the presence of β-2-T.

Several studies have shown possible teratogenic effects related to β-2-T treatment of organ cultures (Fabiny, 1959; Kollar, 1968; Kollar and Baird, 1968; Baird and Verrusio, 1972). In all of these studies, any toxic effects have been reversed by equimolar or greater concentrations of Phe. In both the study of Lines and Waisman (1973), involving *in vivo* treatment of infant monkeys under conditions of elevated blood Phe; and in the present study, in which dietary levels of Phe have not been increased, animals fed β-2-T have shown normal growth as compared to control animals, and no histological changes have been found in examined tissues. Damage to host tissues may therefore prove negligible during β-2-T treatment of phenylketonuric patients.

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β-2-THIENYLALANINE EFFECTS ON GROWTH OF *ESCHERICHIA*, *SHIGELLA*, AND *SALMONELLA* CULTURES IN LIQUID MEDIA CONTAINING PHENYLALANINE, TYROSINE, MAGNESIUM OR ZINC

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1. Introduction

β-2-Thienylalanine (β-2-t), a phenylalanine (Phe) analogue which competes with Phe for uptake in mammalian systems [1—5], is being considered as a treatment agent in the management of phenylketonuria (PKU). As part of the safety testing, effects of β-2-t on indigenous and pathogenic intestinal bacteria are being investigated in vitro. β-2-T displays antimicrobial activity in plate assay against pathogenic *Escherichia* and *Shigella* [6], and also *Bacillus* [7], caused by false feedback inhibition of Phe biosynthesis [8]. These genera can be made insensitive to β-2-t in plate assays when additional Mg^{2+} is added [9,10]. However, antimicrobial activity can vary when measured in agar-solidified media, as compared to liquid media assays [11,12].

In this paper, growth of *Salmonella* (not inhibited by β-2-t in a plate assay [6,9]) is compared with growth of *Escherichia* and *Shigella* (inhibited in the plate assay), in three chemically defined, liquid media. Zn^{2+} levels in prescribed PKU diets are lower than those in diets of non-PKU children (Lines, D.R. and Brown, K.J., unpublished results). Tyrosine (Tyr) is not synthesized from Phe in PKU patients because of a deficiency of phenylalanine hydroxylase, and must be supplied in the diet [13,14]. Therefore, in addition to Mg^{2+} and Phe, the effects of Zn^{2+} and Tyr on β-2-t-bacterial growth interactions are evaluated.

2. Materials and Methods

2.1. Strains

Escherichia coli O86/B6, *Shigella boydii*, and *Salmonella typhi* were obtained from Princess Mary Hospital for Children, Auckland as serotyped cultures isolated from infant pathological specimens.

2.2. Culture maintenance

Cultures were maintained on Dorset Egg Medium [15], transferred to peptone water [2% (w/v) tryptone (Oxoid, London) and 0.9% (w/v) NaCl/l], and incubated at 37°C until growth reached 0.1 A_{450} ($2 \cdot 10^8$ cells/ml) [6], measured with a "Spectronic 20" spectrophotometer (Bausch and Lomb, Rochester, NY). 0.5 ml of logarithmic phase cultures was then transferred to a defined diluent [6], grown to 0.1 A_{450} and transferred to defined media.

2.3. Test reagents

Aliquots of aqueous sterile solutions of 0.01 M β-2-t (β-2-thienyl-DL-alanine, Sigma, St. Louis, Mo.); Mg^{2+} as 10 g MgSO4/l; Zn^{2+} as 5 g ZnSO4/l; 5 g Phe/l; and 1 g Tyr/l, were used in assays.

8

2.4. Liquid culture experimental procedure

Cultures were grown at 37°C without shaking in three chemically defined media not containing agar — Vegetative Minimal Medium (VMM) [16], Medium VI [9], and Minimal Defined Medium (MDM) [17] (Table 1). A complex medium, Trypticase Soy Broth (BBL, Cockeysville, MD), containing about 0.76 g Phe/l and 0.45 g Tyr/l [18], was used for comparison studies. 5 ml of sterile medium with test reagents added, were inoculated in duplicate or triplicate with logarithmic phase cultures, and turbidity was measured directly in the sterile test tubes [19]. Measurements were made at 3 or 4 hourly intervals, for 48 to 60 h. Different species of the same genus reacted similarly in plate assays [6], and grew similarly in liquid media (unpublished results). Therefore, representative

cultures of the genera *Escherichia*, *Shigella*, and *Salmonella* were used in the present studies.

3. Results

3.1. Normal growth in three liquid defined media

In plate assays, all three genera grown on MDM (0.1 g MgSO$_4$/l), are not inhibited by β-2-t, but *Escherichia* and *Shigella* grown on VMM or Medium VI (both 0.01 g MgSO$_4$/l) are inhibited [9].

With liquid media cultures, higher maximum growth was attained by all three genera in MDM, than in VMM. Growth was intermediate in Medium VI (Fig. 1). Medium VI [9], derived from MDM [17] and VMM [16], contains the same components as
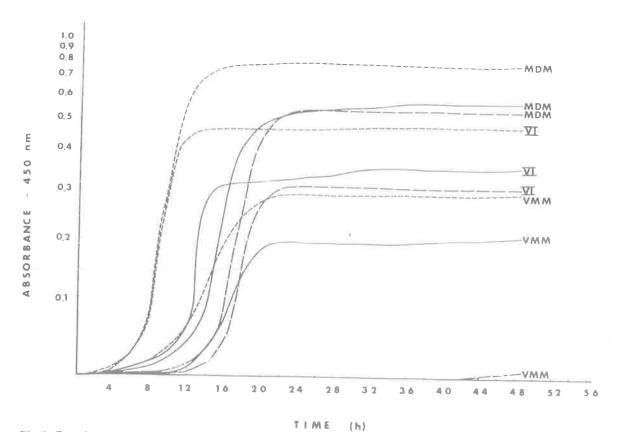
Fig. 1. Growth of cultures in MDM (0.1 g MgSO$_4$/l), Medium VI (0.01 g MgSO$_4$/l), and VMM (0.01 g MgSO$_4$/l) with no β-2-t. In all figures, lines represent the mean of two or three identically inoculated cultures. Readings were made at 3 or 4 hourly intervals, for 48 to 60 h. In all figures, *E. coli* O86/B7 (- - - - -); *S. typhi* (———); *S. boydii* (— — — —).

TABLE 1

Medium components

	Vegetative Minimal Medium [a] [16] (g/l)	Medium VI [9] (g/l)	Minimal Defined Medium [17] (g/l)
Glucose [b]	10.0000	10.0000	2.0000
K_2HPO_4	3.0000	3.0000	7.0000
KH_2PO_4	1.0000	1.0000	3.0000
NH_4Cl	0.5000	–	–
NH_4NO_3	0.1000	–	–
$(NH_4)_2SO_4$	–	2.0000	2.0000
Na_2SO_4	0.1000	–	–
Na citrate	–	0.5000	0.5000
L-Glutamic acid	1.4700	–	–
$MgSO_4 \cdot 7 H_2O$	0.0100	0.0100	0.1000
$MnSO_4 \cdot 4 H_2O$	0.0010	–	–
$FeSO_4 \cdot 7 H_2O$ [c]	0.0010	–	–
$CaCl_2$	0.0005	–	–
$EDTA \cdot 2 H_2O$	0.0400	–	–
Agar	12.5 [d]	13.5	13.5

[a] All media pH 6.8–7.0.

[b] Autoclaved separately and added to the cooled medium.

[c] "Salts solution".

[d] Modification for the present studies. Twice this concentration of agar (25 g/l) was used by Demain [16].

MDM, but at the same concentrations as in VMM (Table 1). Medium VI, like VMM, but unlike MDM, allowed β-2-t inhibition of growth of *Escherichia* and *Shigella* (but not *Salmonella*) under both solid [9] and liquid growth conditions, and allowed for better growth of bacteria in liquid cultures than did VMM. Medium VI was therefore employed in a further series of experiments.

3.2. Mg^{2+} effects

It has been found that in agar-solidified Medium VI, which contains 0.01 g $MgSO_4$/l, *Escherichia* are inhibited by ≥50 µl 0.01 M β-2-t, while *Salmonella* are not inhibited even at high β-2-t concentrations [6]. Neither genus is inhibited when the $MgSO_4$ concentration is increased to 0.1 g/l [9].

In liquid Medium VI, in contrast, growth was similar at the two concentrations of Mg^{2+} with no β-2-t

present (Fig. 2), while both *Escherichia* and *Salmonella* were inhibited by β-2-t (delay in onset of logarithmic phase; tendency of the curves to become linear) (Fig. 3). As the β-2-t concentration was increased (Fig. 4), inhibition of both *Salmonella* and *Escherichia* increased, unlike the plate assay, in spite of the higher Mg^{2+} concentration (Fig. 3). However, there was a difference between inhibition by β-2-t in media containing 0.1 g $MgSO_4$/l (less inhibition), than in those with 0.01 g $MgSO_4$/l (more inhibition) (Fig. 3), for *Salmonella* and *Escherichia* cultures.

3.3. Effects of Phe, Tyr, and Zn^{2+} in liquid media

Concentrations of Phe (0 to 20 mg/100 ml), typical of blood samples from PKU patients, were studied for effects on β-2-t inhibition. Growth of *Escherichia* was similar in media containing 0, 1, 5, 10, 15 or 20 mg Phe/100 ml medium. Growth was also similar within each series of *Shigella* or *Salmonella* cultures (Fig. 5). Growth inhibition was abolished for all three genera at ≥1 mg Phe/100 ml, and growth was comparable to that of cultures with no β-2-t (Fig. 5). Delaying the introduction of Phe in β-2-t treated cultures by ≥3.5 h, resulted in curves similar in shape to normal growth curves (no β-2-t), but displaced to the right along the x axis (not shown). Tyrosine (10 mg/100 ml) was also able to abolish inhibition (Fig. 6a). Although serum zinc levels in PKU patients are within the "normal" range, many patients on low Phe diets receive less than the recommended daily requirement of this essential mineral (Lines, D.R. and Brown, K.J., unpublished results). Zn^{2+} (10 mg $ZnSO_4$/100 ml), however, had no effect on inhibition (Fig. 6b).

3.4. Complex medium effects

By substituting trypticase soy broth, a complex medium containing both Phe and Tyr, no inhibition was noted on assay plates, and no inhibition was observed in liquid media, even at high β-2-t concentrations (not shown).

4. Discussion

In the present studies, using liquid media, both Phe (Fig. 5) and Tyr (Fig. 6a) were protected for bac-
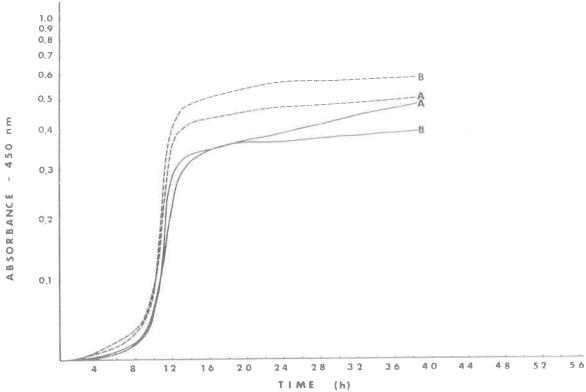
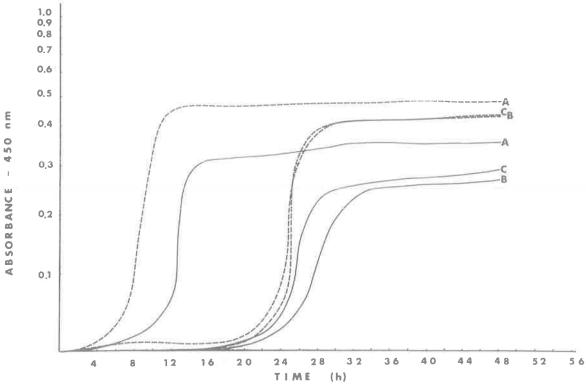Fig. 2. Effects of Mg^{2+} in liquid Medium VI on growth with no β-2-t present. A, 0.01 g MgSO$_4$/l; B, 0.1 g MgSO$_4$/l.

Fig. 3. Growth of cultures in Medium VI with 50 μl 0.01 M β-2-t, and with increasing Mg^{2+}. A, 0.01 g MgSO$_4$/l (control with no β-2-t); B, 0.01 g MgSO$_4$/l; C = 0.1 g MgSO$_4$/l. Curves for 0.5 g MgSO$_4$/l and 1.0 g MgSO$_4$/l were similar to C. Similar results were noted in MDM and VMM.
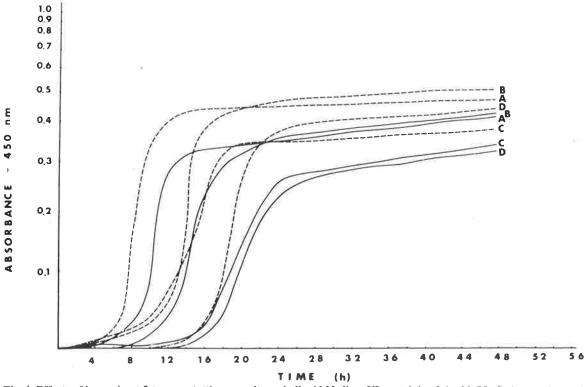
Fig. 4. Effects of increasing β-2-t concentration on cultures in liquid Medium VI containing 0.1 g $MgSO_4$/l. A, no β-2-t; B, 1 μl 0.01 M β-2-t; C, 10 μl β-2-t; D, 50 μl β-2-t. *Shigella* reacted similarly to *E. coli*.
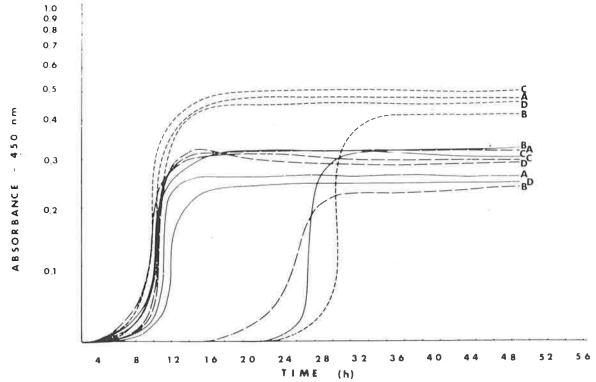
Fig. 5. Effect of increasing Phe concentration on culture growth in Medium VI with 50 μl 0.01 M β-2-t. A, 0 mg Phe/100 ml (control with no β-2-t); B, 0 mg Phe/100 ml; C, 1 mg Phe/100 ml; D, 5 mg Phe/100 ml. Curves for 10 mg; 15 mg; and 20 mg Phe/100 ml were similar to A, C, and D.
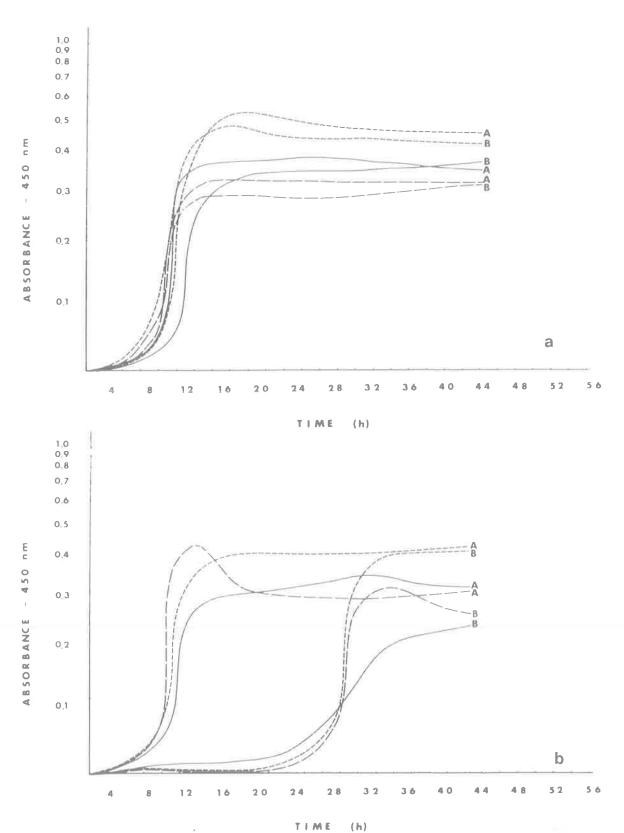
Fig. 6. Effects of Tyr (Fig. 6a) and Zn^{2+} (Fig. 6b) on bacterial inhibition by β-2-t in Medium VI. A, 10 mg Tyr/100 ml (or Zn^{2+}), no β-2-t; B, 10 mg Tyr/100 ml (or Zn^{2+}), 50 μl 0.01 M β-2-t.

terial genera both "inhibited" (e.g. *Escherichia, Shigella*) and "non-inhibited" (e.g. *Salmonella*) in the Brown and Lines [6] plate assay. Our recent studies, in vivo, have shown that mice treated with β-2-t are no more susceptible to colonization by *Salmonella* or *Shigella* than are water-fed paired control mice [20]. It is suggested from the present studies that Phe and Tyr would contribute to protection from growth inhibition by stabilizing the gastrointestinal bacterial populations in mice, and presumably in PKU patients, treated with β-2-t. As Zn^{2+} had no effect on β-2-t inhibition of bacterial growth in the present studies (Fig. 6d), lowered zinc levels in PKU diets would not appear to be harmful in regard to β-2-t effects on bacterial growth. By increasing Mg^{2+}, β-2-t inhibition in plate assays is able to be abolished [9]. In the liquid media studied, increasing the Mg^{2+} did not prevent inhibition of growth. However, for both *Escherichia* and *Salmonella,* delay in onset of logarithmic growth phase was somewhat reduced at higher Mg^{2+} concentrations (Fig. 3). Controlling the Mg^{2+} levels in PKU patients could play a more subtle, yet appreciable role, as compared to Phe and Tyr concentrations, in preventing inhibition of gastrointestinal bacterial growth by β-2-t.

Acknowledgements

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Comparison of Liquid and Agar-Solidified Defined Media Regarding the Physiological Mechanism by which β-2-Thienylalanine Inhibits Growth of *Escherichia, Shigella,* and *Salmonella* Cultures

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Abstract Growth comparisons were made, using *Shigella, Escherichia,* and *Salmonella* cultures, in liquid and agar-solidified defined media containing β-2-thienylalanine (β-2-t). The comparisons were performed to determine the nature of growth inhibition by β-2-t under different physical growth conditions.

In a plate assay, with increasing β-2-t mixed into the agar, inhibition of *Escherichia* and *Shigella* increased. However, *Salmonella* cultures were not inhibited even at the highest β-2-t concentrations used. With β-2-t added to liquid cultures, however, dose-response growth relationships were exhibited by all three genera.

The differences occurring in β-2-t inhibition between liquid and plate assay conditions were not due to composition of culture plates, time of challenge of cultures with β-2-t, availability of oxygen and associated differences in ratios of volume of media to available surface area, selection of mutants in the plate assay, or to extractable substances from the agar.

However, when β-2-t diffusion into the liquid medium was delayed by using agar plug diffusion cultures, a physiological mechanism was demonstrable which largely protected *Salmonella* cultures, but not *Escherichia* and *Shigella* cultures, from growth inhibition.

The phenylalanine (Phe) analog, β-2-thienylalanine (β-2-t), can compete for uptake with Phe in the simian kidney (29) and intestine (30), and in the rat intestine (17, 36). It has been used in the treatment of experimental hyperphenylalaninemia in neonate simians (31), and in short-term Phe loading experiments in human phenylketonurics (27). β-2-t is therefore being considered as a treatment agent in the management of phenylketonuria (PKU).

As part of the safety testing of the analog, we are investigating the effects of

agar- Noble (Difco) and Davis agar (Davis Gelatine (NZ) Ltd., Christchurch, N.Z.) were added to VMM and Medium VI for use in the Brown and Lines (2) inhibition assay. In addition, 13.5 g of the agars were suspended in 300 ml of distilled water, autoclaved, allowed to solidify, cut into $1 \times 1 \times 3$ cm blocks, suspended in 1 liter of distilled water, and washed at a flow rate of 15 ml/min for 24 hr in an Amicon Model 2000 Diafiltration cell (Amicon Corp., Lexington, Mass.) fitted with an Amicon PM-10 ultrafiltration membrane. The cell was connected to a 12 liter capacity pressure ball filled with distilled water. The cell contents were stirred at the slowest attainable rate during the washing procedure. The washed agar blocks were weighed, and one-fifth of the total added to Medium VI or VMM salts for 200 ml of medium, brought to a volume of 200 ml with distilled water, and autoclaved at 121 C for 10 min. The remainder of the agar blocks was suspended in 1 liter acetone, and stirred with a magnetic spin-bar for 2 hr. The acetone was decanted and replaced in turn with 100% ethanol, and six changes of distilled water. The solvent-washed agars were then added to fresh media, autoclaved, and used in the plate inhibition assay.

Agar Plug Diffusion Cultures

Agar plug diffusion cultures (1) were prepared to study delay of contact of inhibitory substances with, and of uptake by, bacterial suspensions in the liquid medium. For agar plug diffusion cultures, 0.5 ml of 1.5% (w/v) agar medium, with or without β-2-t added, was pipetted into the bottom of 12×100 mm culture tubes, and then overlaid with 4.5 ml of inoculated liquid culture medium above the solidified plug. In some experiments, the agar plug was first overlaid with an interface layer consisting of 0.2 ml of concentrated agar [4.5% (w/v) agar in Medium VI] before introducing the inoculated liquid medium (4.3 ml). A tube with an agar plug and with uninoculated liquid medium was used for calibration. A plug consisting of 0.7 ml of agar medium, in the bottom of the tubes, did not interfere with spectrophotometric readings.

The rate of diffusion of β-2-t from agar plugs into the liquid medium was measured for agar plug diffusion cultures, with and without overlaid concentrated agar interface layers. Liquid media tubes were used as controls. ^{14}C-labelled β-2-t [β-(thienyl-2)-DL-alanine-3-C-14; 50 μCi; Sp. Act. 25.5 mCi/mM-Calatomic, Los Angeles, California] was suspended in 1 ml of distilled water. One half milliliter of ^{14}C-β-2-t solution was mixed with 1.5 ml 0.1 M β-2-t solution (non-radioactive). Then, 0.1 ml of the mixed solution was pipetted into the bottom of culture tubes and mixed with agar medium. The agar plug diffusion cultures were prepared as usual except that the liquid medium overlaid above the solidified plug was not inoculated with bacteria. Tubes were prepared in triplicate of 1) liquid media; 2) agar plug diffusion "cultures"; and 3) agar plug cultures with concentrated agar overlays; and incubated at 37 C. Samples (0.1 ml) were taken at intervals for 48 hr (Fig. 5), placed in scintillation vials, diluted with 0.6 ml distilled water, and mixed in the vial with 8 ml of scintillation fluid [30% Triton X-100 (Rohm and Haas (NZ) Ltd., Otahuhu, N.Z.) and 70% scintillation solution]. The scintillation solution consisted of 100 mg of

1,4-bis-[2-(5-phenyloxyzolyl)]-benzene (POPOP, Sigma) and 4 mg of 2,5-diphenyloxazole (PPO, Sigma) per liter of toluene. The radioactivity in the vials was counted in a Model 2425 Automatic Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Company, Inc., Downers Grove, Illinois).

RESULTS

Development of Liquid Media Assays

To assay liquid cultures and agar plug diffusion cultures, a technique was employed for reading culture growth in standard glass test tubes directly in the spectrophotometer (1). The turbidimetric readings were previously shown to be directly proportional to the number of colonies formed (2). In the identically inoculated replicate tubes, growth curves were reproducible with respect to a) similarity of shape of the curve; b) onset of logarithmic growth phase (as measured from the first reading after the initial lag phase in which all portions of the curve between further readings gave a positive slope, until a stationary phase was reached); and c) maximum growth, the asymptotic portion of the curve (stationary phase). In preliminary experiments, different species of the same genus, and multiple isolates of particular serotypes, both fresh isolates and maintained cultures; gave the same reaction in the plate assay (2), and gave similarly shaped curves in liquid Medium VI (unpublished results). Therefore, representative *Escherichia, Shigella,* and *Salmonella* cultures were used in the following studies.

β-2-Thienylalanine Concentration Effects

Dose-response relationships between β-2-t concentration and culture growth were assayed using Medium VI under three different physical growth conditions — with β-2-t a) applied by pipette to a filter paper strip which was placed on the agar; b) mixed into the agar; and c) added to liquid media.

In the filter paper strip plate assay (2), 5–40 μl of a 0.01 M β-2-t solution in the strip caused borderline inhibition (±) of *Shigella* and *Escherichia*. Concentrations of greater than 60 μl per strip resulted in inhibition (+) (2). When β-2-t solution was mixed with 15 ml of agar medium at 46 C before pouring plates, and cultures were

Table 1. Effect of dosage of β-2-thienylalanine when mixed into
VMM* upon growth of streaked test cultures

0.01 M β-2-t (μl)	E. coli O55/B5	E. coli O86/B7	Sh. boydii	Sh. sonnei	S. typhi	S. typhimurium
0	0	0	0	0	0	0
50	0	0	0	0	0	0
100	+	±	±	+	0	0
200	+	+	+	+	0	0
400	+	+	+	+	0	0
800	+	+	+	+	0	0
1,200	+	+	+	+	0	0

* Vegetative Minimal Medium [Demain (12)].
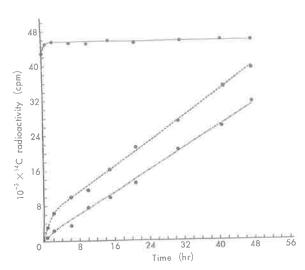(+) culture inhibited, (0) culture not inhibited, (±) borderline reaction.

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Fig. 5. Rate of diffusion of ^{14}C-β-2-t from an agar plug (⋯⋯), or from an agar plug overlaid with concentrated agar (------), and into liquid Medium VI. Control tubes (——) contain ^{14}C-β-2-t in liquid Medium VI (no agar plug).

medium conditions. The difference in reactions when cultures are grown in petri dishes and in culture tubes cannot be explained by the difference in vessel composition (glass *vs.* plastic), time of challenge with β-2-t, availability of oxygen and associated differences in surface area to volume of media ratios, or selection of mutants in the plate assay.

Agar preparations have been shown to affect bacterial-drug interactions. Sands and Bennett (33) have demonstrated that some phenolic compounds are less inhibitory to *Staphylococcus aureus* in unwashed agar, and that inhibition can be restored by washing agar cubes in distilled water before adding the agar to growth media. Similar agar effects have been shown for fatty amines (23), antibiotics (15, 24, 38), and infectivity of Dengue virus (34). Greenberg (19) has studied removal of agar factors with water, methyl alcohol, ethyl alcohol, acetone, and butanol.

After washing of agars in water and/or organic solvents, no differences have been found in inhibition reactions of bacteria in the plate assay. In all plate assays, both "inhibited" and "non-inhibited" controls are employed. Since *Shigella* and *Escherichia* cultures can still be inhibited on a dose-response basis in the plate assay, β-2-t must still be active in the agar-solidified plate media.

The observation that in the plate assay, bacteria react similarly as a genus to β-2-t (2), suggests a major metabolic difference between inhibited genera and non-inhibited genera. In preliminary experiments, we have explored the possibility that *Salmonella typhi* is protected, as compared to *Escherichia coli* O86/B7, in that β-2-t might (a) not be recognized by the aromatic amino acid transport system of the bacterial membrane, or that (b) the transporter may be more specific for Phe (in comparison

to β-2-t) than that of *E. coli,* or that (c) β-2-t may be less likely to be incorporated into *Salmonella* proteins. We have found no difference between these bacteria in ability of β-2-t either to be transported, or to be incorporated into proteins, as measured by amino acid analyses of intracellular fractions (Lines and Brown, unpublished results). D'Ambrosio et al (11) have also shown that β-2-t mimics L-phenylalanine as a transport substrate (in *B. subtilis*).

The results presented in this communication are consistent, however, with the following model. In liquid media, synthesis of 3-deoxy-D-arabinoheptulosonic acid 7-PO$_4$ (DAHP) synthetase (or prephenate dehydratase, in some bacteria) is halted almost immediately by β-2-t (14), but, due to a diffusion effect in the gel matrix (Fig. 5) resulting in fewer β-2-t molecules being available for transport across bacterial membranes at any time, inhibition is slowed in the agar system. During this period, the induction of a protective mechanism (possibly an enzyme system) past the blockage point allows for growth to proceed. Inhibited genera, not possessing the protective mechanism, recover only after a considerable delay in onset of logarithmic growth phase.

Protection of "inhibited" bacteria, and possibly most bacteria, by increased Mg^{2+} concentration in agar-solidified media, may be due to - (a) increased utilization of available shikimic acid by the Mg^{2+}-dependent shikimate kinase (16); (b) reduced loss of the ion from cell wall structures (5); or (c) increased Mg^{2+}-dependent activation to form the phenylalanyl-transfer RNA complex (8), allowing greater utilization of available Phe for protein synthesis.

In order to study the model, the solid media conditions needed to be applied to liquid cultures. We tested the model by sequentially introducing increasing delays in diffusion of β-2-t from an agar plug, through an overlay of concentrated agar, and into contact with liquid bacterial cultures (Figs. 3a, 3b, 4a, 4b). Under these conditions, protection of *Salmonella,* which are not inhibited by β-2-t in the plate assay, could be demonstrated in the agar plug cultures. The genera inhibited in the plate assays (*Escherichia* and *Shigella*) were still inhibited in the agar plug cultures, with no activation of a protective mechanism detectable.

Of the metabolites of the phenylalanine biosynthetic pathway, only phenylpyruvate and phenylalanine are past the prephenate branch-point enzyme which can be feedback-inhibited in some bacteria (6, 7). Since Conway et al (9) have shown that only 10 to 15% of the available intracellular pool of Phe is necessary for protein synthesis to proceed, as much as 85% of the Phe pool remains for other metabolic needs, including possible use as a substrate in an inducible enzyme pathway such as that suggested by the model. Guroff et al have demonstrated hydroxylation of Phe to Tyr by a pseudomonad (20, 21, 28). *Pseudomonas* is one of the genera that shows no inhibition by β-2-t in the plate assay (2), while *Escherichia,* which have been shown to be unable to convert Phe to Tyr (32), are inhibited in the assay. The plate assay (2) may then be differentiating among genera in which a phenylalanine hydroxylase pathway is inducible when blockage of the phenylalanine biosynthetic pathway by β-2-t is delayed, and genera which have no such protective alternative pathway available, even when β-2-t diffusion into the medium is retarded.

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THE ASSAY ON A DEFINED MEDIUM OF THE EFFECTS

OF β-2-THIENYLALANINE ON THE GROWTH OF

INDIGENOUS ANAEROBIC BACTERIAL ISOLATES FROM

PHENYLKETONURIC PATIENTS

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20 March 1979

SUBJECT CATEGORY

"PHYSIOLOGY AND GROWTH" OR "MEDICAL MICROBIOLOGY"

RUNNING TITLE

β-2-THIENYLALANINE AND PKU ANAEROBES

INDEX OF SUBJECTS

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   nine on
   (Brown, Vesey, Tannock, Bell, Elliott & Lines).

2. Assay of anaerobic bacterial growth, inhibition reactions
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   on anaerobic bacterial isolates from (Brown, Vesey,
   Tannock, Bell, Elliott & Lines).

Faecal samples were taken from three diet-managed phenylketonuric children in order to determine the effects of β-2-thienylalanine (β-2-t) on indigenous bacterial isolates. From the sample swabs, 127 anaerobic isolates were identified and tested for inhibition to β-2-t on a Phe-free defined medium, Anaerobe Inhibition Test (AIT) agar, devised for this study. Of the isolates, 77.9% grew sufficiently to assay inhibition reactions on at least 25% of AIT assay plates. Using Columbia agar, a complex Phe-containing medium, 86.5% of the strains could be assayed. None of 28 Bacteroides cultures was inhibited by β-2-t on AIT. Of the genera, Bifidobacterium, Eubacterium, Lactobacillus, Peptostreptococcus, and Propionibacterium, no isolates which would grow on the defined medium were inhibited. At least one isolate of each of the genera Peptococcus, Fusobacterium, and Clostridium was inhibited in the assay. Of the 127 total isolates, only 9 were inhibited by β-2-t on AIT, and inhibition was abolished for these isolates on the Phe-containing medium.

Thirty-nine aerobic isolates were taken from the same patients. Genera tested followed the same inhibition patterns as did previously tested strains of the same genera from non-PKU sources. Anaerobically isolated Escherichia coli were inhibited by β-2-t, while Streptococcus faecalis cultures were not, confirming the assays of previously

tested, aerobically-isolated cultures of the same species from non-PKU patients.

These studies are apparently the first dealing with the effects of β-2-t on anaerobic bacteria, and give a further indication that indigenous intestinal bacterial populations are unlikely to be greatly altered during in vivo treatment with β-2-t.

INTRODUCTION

β-2-thienylalanine (β-2-t) is a phenylalanine (Phe) analogue which increases the urinary excretion of Phe in monkeys (Lines & Waisman,1970a) inhibits absorbance of Phe in the monkey intestine (Lines & Waisman, 1970b), and competes with Phe for absorbance in the rat intestine (Godin & Dolan, 1967; Wapnir & Lifshitz, 1974). It has proved effective in treatment of experimental hyperphenyl-alaninemia in infant monkeys (Lines & Waisman, 1973), and in short-term Phe loading studies in man (Krips & Lines, 1972). β-2-t is therefore being considered as an adjunct therapeutic agent in the management of phenylketonuria (PKU).

In the study of Lines & Waisman (1973), a Shigella gastroenteritis was noted in some β-2-t-treated monkeys. In order to study the possible relationship between gastro-enteritis and β-2-t administration, the effects of β-2-t on the growth of gram-negative genera were determined using a plate inhibition assay (Brown & Lines, 1976), employ-ing Vegetative Minimal Medium (VMM) (Demain, 1958). Shigel-la, as well as Escherichia bacteria, were inhibited by β-2-t in the assay, while the genera Salmonella, Proteus, Pseudo-monas, Citrobacter, and Enterobacter were not inhibited (Brown & Lines, 1976).

Before further clinical testing with β-2-t is under-taken, it is necessary to determine the in vitro effects of

the analogue on growth of aerobic and anaerobic intestinal bacteria from PKU patients. Since Phe competes with β-2-t, and is able to abolish inhibition under various conditions (Beerstecher & Shive, 1946; Dittmer et al., 1946; Drea, 1948; Dunn & Dittmer, 1951; Brown et al., 1979b) it is important to use a defined medium not containing Phe for testing β-2-t effects. By using another Phe-free, defined medium in the plate assay (Medium VI-Brown & Lines, 1978), the gram-negative bacteria grow better than in VMM under both solid and liquid medium conditions (Brown et al., 1979b). We therefore used Medium VI in the present study to determine if the in vitro reactions to β-2-t of indigenous aerobic isolates from PKU patients on dietary therapy are similar to those of the gram-negative cultures from non-PKU sources, previously tested.

In order to test anaerobic bacteria from PKU patients, a new Phe-free defined medium had to be developed for both growing the isolates and assaying reactions to β-2-t.

MATERIALS AND METHODS

Bacterial strains - The following standard lyophilized strains, isolated from non-PKU sources, were obtained from the National Health Institute Type Culture Collection, Wellington, New Zealand (NHITCC): Bacteroides fragilis (NHITCC 1098; NCTC 8560), Bifidobacterium eriksonii (NHITCC 903; CDC 14370), Clostridium oedematiens (NHITCC 484; NCTC 277 Type A), C. perfringens (NHITCC 20; NCTC 8237 Type A), C. sporogenes (NHITCC 1097; NCTC 532), Eubacterium lentum (NHITCC 905; CDC 14071), E. limosum (NHITCC 906; CDC 13116), Fusobacterium (Sphaerophorus) necrophorum(s) (NHITCC 1109; NCTC 10575 Type A), F. nucleatum (NHITCC 75/1225), Lactobacillus casei ss. rhamnosus (NHITCC 299; ATCC 7469, L. plantarum (NHITCC 1100; ATCC 8014), Peptostreptococcus intermedius (NHITCC 75/1309), Propionibacterium granulosum (NHITCC 907; CDC 9055), and Streptococcus faecalis (NHITCC 1106; NCTC 775). Serotyped cultures of Escherichia coli O86/B7 and Salmonella typhi were obtained from the Princess Mary Hospital for Children, Auckland, New Zealand, as cultures on Columbia agar (BBL, Cockeysville, Md.) plates streaked for isolation from parent Dorset Egg Medium (Dorset, 1902) cultures.

Patient background - Patient P.C., aged 8 4/12 at the time of faeces collection, was diagnosed as phenylketonuric and placed on a low Phe diet at the age of one month. His dietary control, growth, and intellectual development had

all been excellent.   At the time of faeces collection,
his regimen was 60 g Aminogran (Allen and Hanbury,
Bethnal Green, London), plus a mineral supplement – Ketovite
(Paines and Byrnes, Pabyrn Labs., Middlesex) one tablet –
three times daily, and 5 ml Ketovite mixture each morning.
His Phe intake was 25 mg/kg/day, and his serum Phe level
had been 11 mg/100 ml on two successive occasions, 1) one
month before collection and 2) just prior to the faeces
collection.   When tested on the Wechsler Intelligence
Scale for Children (Wechsler, 1974), he scored in the
"superior" range.

Patient S.C., the brother of P.C., was almost 7 years
of age when faeces were collected.   He had been diagnosed
as having PKU and was started on a low Phe diet from the
age of one week.   His dietary control, growth, and
intellectual development had caused no concern.   At the
time of faeces collection, his dietary regimen was identical
to that of his brother.   Serum Phe levels immediately
before collection had been 12 mg/100 ml serum.   His intelli-
gence score on the Wechsler Scale was placed at the border
of "Bright Normal" and "Gifted".

Patient D.M., aged 1 5/12 at the time of collection,
was diagnosed as a phenylketonuric at the age of one week.
He was fed a low Phe diet beginning in the 9th day of life,
and good control had been maintained.   He was receiving
Lofenelac (Mead Johnson, Evansville, Indiana), plus cow's
milk and vegetables, supplying a total of 55 mg Phe/kg/day.

His serum Phe levels had been 12 mg/100 ml on two
successive occasions, 1) two weeks prior to collection, and
2) immediately prior to collection.   His developmental
status one month previously was assessed by the Cattell
Infant Intelligence Scale (Cattell, 1947), which placed him
at a developmental age of 15 months, one month behind his
chronological age.   However, using the Vineland Social
Maturity Scale (Doll, 1965), his development was equivalent
to "18 months".   Thus, overall development was well within
normal limits.

Anaerobic Bacteria

Isolation of anaerobes — Faecal samples from the patients
were mixed with cotton-tipped applicator sticks and trans-
ferred to isolation media within 15 minutes of collection.
The faecal swabs were streaked for isolation on to 55 mm
petri plates of Columbia agar medium, to which 5% sterile,
defibrinated horse blood was added after the medium was
cooled to $45^{0}C$.   Brain Heart Infusion (BHI) (BBL) blood
agar plates were also inoculated.   Both media were pre-
pared with and without the addition of 100 μg/ml neomycin
[Mycifradin sulphate (Upjohn), 350,000 I.U./500 mg].   Ten
ml/l of vitamin K - hemin solution (Holdeman & Moore, 1975)
were added to all of the blood agar plates.   Duplicate
plates of these media and duplicate cooked meat media
(prepared from chopped beef hearts), BHI broth, and glucose
thioglycollate broth [Thioglycollate Medium, fluid, without
dextrose or EH indicator (BBL) plus 10% glucose]

were inoculated from the swabs. Cooked meat media and thioglycollate broth were incubated at 37^0C. BHI broth and the non-inverted blood agar plates were incubated at 37^0C in an anaerobic jar with freshly charged palladium pellets (BBL), and with BBL methylene blue anaerobiosis indicators. Anaerobiosis was achieved by evacuating the jar three times with a vacuum pump, and replacing the air with hydrogen gas containing 10% CO_2. Plates were examined after 2 - 5 days for growth, and colonies picked and transferred to fresh Columbia blood agar plates. Loopsful of bacteria from the liquid media were streaked for isolation on to the series of blood agar plates after 24 h, and plates were examined as before.

Maintenance of cultures - Isolate cultures were maintained by twice-weekly transfer on to Columbia blood agar plates, and by inoculation into cooked meat medium. For long term holding, cultures were lyophilized.

A foetal bovine serum plus 5% inositol solution was sterilized by filtration through a 0.45 µ Millipore filter (Millipore Corp., Bedford, Massachusetts). A bacterial suspension was prepared by syringing 0.4 ml of the solution on to the surface of a 24 h blood plate of the isolate and sucking the suspension back into the syringe. After mixing by end-to-end rotation of the syringe, 0.1 ml aliquots of solution containing bacteria were dispensed into sterile, 6 x 100 mm glass tubes with a flame-sealed end, containing a 5 x 30 mm strip of Whatman 3MM (W. and R. Balston, Ltd., Maidstone, Kent) to absorb the sample. Using a sterile needle, the sterile cotton plug was pushed into the tube

until 1 cm above the paper strip. The contents were frozen in a dry ice and alcohol bath, and lyophilized for 3 h in an apparatus employing a lyophilizer unit (Quickfit and Quartz Ltd., Staffordshire). Tubes were sealed under vacuum with a propane torch, and stored at room temperature. For use, the vials were opened by scoring with a file at the level of the middle of the cotton plug, and cracked with a red-hot solid glass rod, following the procedure suggested by the NHITCC. The dried cultures were reconstituted in glucose thioglycollate broth.

In an alternative lyophilization procedure, some of the anaerobic isolates were also picked from blood agar plates and suspended directly into 0.1 ml defibrinated horse blood in a vial without filter paper present, before lyophilization.

Gas-liquid chromatography (GLC) - A 5 μl aliquot was taken from the supernatant solution of centrifuged glucose thioglycollate broth cultures, or directly from broth cultures, and was analysed by GLC. A standard solution, made up of a mixture of acetic, propionic, isobutyric, n-butyric, iso-valeric, n-valeric and iso-caproic acids, was used for comparison of retention times of the peaks in the sample solutions. The apparatus used was a Shimadzu GC-4APTF GLC (Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with a 3 mm internal diameter, 0.5 m glass column packed with

Chromosorb 101, 100-120 mesh (Johns-Manville, Denver, Colorado). Oxygen-free nitrogen was the carrier gas at a flow rate of about 30 ml/min. The flame ionization detector hydrogen flow rate was 30 ml/min, and the air flow rate was 300 ml/min. The injection port temperature was 200°C, and samples were run isothermally at 160°C. The chart speed was 2.5 mm/min, and samples were run for 15 min, or until there was a return to baseline.

Identification of cultures - all of the isolates were compared by colonial morphology and presence of haemolysis on blood agar, growth characteristics in glucose thioglycollate broth and cooked meat medium (including production of gas), gram reaction, cell morphology by light microscopy, spore production, and production of propionic and butyric acids in GLC determinations. Determination of acetic acid production was masked by its presence in the medium. Two-thirds of the isolates were tested for motility. The isolates from Patient D.M. were tested by CDC laboratory methods (Dowell & Hawkins, 1974), for fermentation of the following sugars - glucose, lactose, sucrose, maltose, mannitol, arabinose, xylose, trehalose, rhamnose, and fructose. These isolates were also tested for indole production and nitrate reduction, and production of catalase. Reactions and morphological

characteristics were compared with the VPI Anaerobe Labor-
atory Manual (Holdeman & Moore, 1975), Bergey's Manual
(Breed, Murray, & Smith, 1972), and the CDC Laboratory Man-
ual (Dowell & Hawkins, 1974).

The schema for isolation and identification of cultures
is detailed in Fig. 1.

Inhibitor - A 0.01 M aqueous solution of β-2-t (β-2-thienyl-
DL-alanine, Sigma) was sterilized by autoclaving.

Plate inhibition assay - Cultures were assayed for inhi-
bition by β-2-t on 88 mm plastic petri plates containing
Medium VI (Brown & Lines, 1978) or on Anaerobe Inhibition
Test (AIT) medium (Table 1).   AIT was based on Medium VI,
with the addition of specific growth factors including
succinate (Lev et al., 1971); $CaCl_2$ (Herbeck & Bryant, 1974);
volatile fatty acid mixture and cysteine sulfide solution
(Caldwell & Bryant, 1966); pyruvate, vitamin K (menadione)
plus hemin solution, Tween 80 (Holdeman & Moore, 1975)
(also, Varel et al., 1971; Macy et al., 1975 -hemin); sodium
carbonate (Varel & Bryant, 1974); carbohydrates (Labbe &
Duncan, 1975); cyanocobalamin (Caldwell et al., 1974;
Caldwell & Arcand, 1974); S^{2-} (Varel et al., 1974); NH_4^+
(Bryant & Robinson, 1962; Morishita et al., 1974; Herbeck
& Bryant, 1974); p-aminobenzoic acid (Lee et al., 1974);
and inorganic ions (Na^+, PO_4^{3-}, K^+, Mg^{2+}) (Caldwell et al.,
1973; Caldwell & Arcand, 1974; Caldwell & Hudson, 1974).

Of these inorganic ions, only elevated Mg^{2+} concen-
tration (> 0.01 g/l) affects reactions of aerobes to β-2-t
in that inhibition is abolished (Brown & Lines, 1978;

Brown et al., 1979a). Therefore, Mg^{2+} was added at a concentration of 0.01 g/l in AIT medium.

Cultures were also grown on Columbia agar plates (without blood added), to compare inhibition reactions on a Phe-containing medium. Strains were streaked in parallel, eight or fewer per plate. A filter paper strip was placed perpendicularly to the streaks and impregnated with 100 μl 0.01 M β-2-t solution (Brown & Lines, 1976; 1978). Plates were incubated for 2 to 4 days at $37^{0}C$ using the anaerobic jar procedure previously elaborated (Brown & Brown, 1978). The culture reactions were read [inhibition (+), borderline (±), or non-inhibition (0)] as previously defined (Brown & Lines, 1976; 1978).

In some experiments, "moats" were made between adjacent streaked organisms, by removing a sliver of the adjacent agar, to determine the effect of any undefined growth factors from other bacteria on inhibition reactions.

Aerobic Bacteria

Isolation of aerobes - In addition to the anaerobic isolation procedures, the faecal swabs were also streaked for isolation on to aerobically incubated MacConkey agar (BBL) and Columbia agar plates. Colonies were picked, restreaked on to Columbia agar, then transferred to Dorset Egg Medium slants for long-term maintenance, prior to testing. Cultures were identified by the Microbiology Department, Wallace Laboratory, Auckland Hospital.

Plate inhibition assay - Cultures were assayed for reactions
to β-2-t on Medium VI, using the method of Brown & Lines
(1976; 1978). *Escherichia coli* O86/B7 [inhibited (+)], and
Salmonella typhi [non-inhibited (O)] cultures were used as
assay controls.

Additional Procedure

Phenylalanine determination - 0.3 ml samples of freshly
autoclaved Columbia agar medium were hydrolyzed with 1.7 ml
of 6N HCl at 110^0C for 20 - 24 h.   The samples were then
assayed with an amino acid analyzer by Dr Ian Lyon, Depart-
ment of Community Health, University of Auckland School of
Medicine.   The medium contained 0.13 mg/ml Phe and 0.06 mg/ml
Tyr.

RESULTS

Faecal swabs were taken from three phenylketonuric children in whom the metabolic disorder was well managed by standard dietary therapy. From the swabs, 127 anaerobic isolates, and 39 aerobically isolated bacterial cultures were identified and tested for reaction to β-2-t. Additional cultures which were isolated anaerobically and grew aerobically, proved to be coliforms or Streptococcus faecalis.

Of the 39 aerobic cultures tested on Medium VI of Brown & Lines (1978), all 22 Escherichia coli cultures (both positive and negative for ornithine decarboxylation) were inhibited, and all 11 enterococci (Streptococcus faecalis) cultures were not inhibited by β-2-t. Staphylococcus sp. (3 isolates) and Micrococcus sp. (one isolate) would not grow on the defined medium used, and hence were not assayed. The results for Escherichia and Enterobacter (one isolate) from the PKU children were the same as the reactions previously noted for other non-PKU isolates of these genera (Brown & Lines, 1976). The single Acinetobacter culture isolated differed from previous findings in that it was inhibited by β-2-t.

Anaerobe Inhibition Test (AIT) agar (Table 1), a protein-free defined medium, was devised, based on Medium VI (Brown & Lines, 1978), but with the addition of a number of components known to aid growth of specific anaerobic intestinal or rumen bacteria.

Of the anaerobic standard control cultures, none of those that would grow on AIT, or on the Phe-containing Columbia agar, were inhibited by β-2-t (Table 2).

All anaerobically isolated Escherichia coli cultures, from the three PKU patients, assayed on anaerobic AIT agar plates were inhibited, while anaerobically isolated Streptococcus faecalis cultures were not inhibited by β-2-t. These results are consistent with previous results on similar non-PKU isolates incubated aerobically on Medium VI.

The 127 strictly anaerobic cultures isolated from patients D.M., P.C., and S.C., and the reactions of the cultures to β-2-t on AIT medium are shown in Table 3. On AIT, 77.9% of the isolates grew sufficiently to assay the response to β-2-t (growth on at least 25% of the assay plates). On Columbia agar (without blood), a Phe-containing complex medium, 86.5% of the isolates could be assayed. Although growth on plates was better for some strains when placed next to other bacteria, the use of "moats" in some experiments, formed by removal of slivers of media from between adjacent cultures on a single plate, showed that the basic inhibition reaction of a strain was not dependent upon diffusion of growth factors from adjacent bacteria.

Of the genera represented in the group of isolates (Table 3), none of the 28 Bacteroides isolates was inhibited by β-2-t on AIT medium. Streptococcus faecalis cultures were also not inhibited on AIT. Of the genera Bifidobacterium (10 isolates, 1 control), Eubacterium (17, 2), Lactobacillus (4, 2), Peptostreptococcus (11, 1), and Propionibacterium

(6, 1), no inhibited cultures were found, but some cultures of each genus were isolated which could not be assayed due to lack of growth on the test medium.

At least one isolate of the genera Peptococcus, Fusobacterium, and Clostridium was inhibited on AIT medium by β-2-t. The two Fusobacterium mortiferum isolates (Table 3) were inhibited, while isolates and control strains of other species of Fusobacterium were not inhibited (Table 3).

Of the 127 anaerobic isolates, only 9 (7%) were inhibited by β-2-t on AIT, and inhibition was abolished for these isolates when tested on the Phe-containing Columbia agar.

DISCUSSION

Aerobically isolated bacterial cultures and anaero-
bically isolated coliforms from diet-treated phenyl-
ketonuric patients react similarly to previously tested
intestinal bacteria from non-PKU patients, regarding in-
hibition reactions to β-2-t in defined media. Only 7% of
the strictly anaerobic intestinal bacterial isolates from
these PKU patients, assayed using Anaerobe Inhibition Test
medium, are inhibited by β-2-t in the plate assay, and this
inhibition is abolished in a Phe-containing medium.

We have previously suggested that genera such as
Salmonella, Pseudomonas, and Proteus, which are not in-
hibited in the assay, may be homogeneous in respect to
production of a factor, such as an inducible enzyme,
which is protective against inhibition of the phenylalanine
biosynthetic pathway by β-2-t (Brown et al., 1979b).
As in the case of previously studied gram-negative aerobic
genera (Brown & Lines, 1976), anaerobic genera have been
found, namely Bacteroides, Bifidobacterium, Eubacterium,
Lactobacillus, Peptostreptococcus, and Propionibacterium,
in which no strains that can be assayed are inhibited by
β-2-t. In some cases, however, strains grow poorly or
not at all on the test medium. If it can be shown that
the non-growing strains are not inhibited, by supplying
additional defined growth factors to the medium, then
members of these genera, like those of Salmonella, Pseudo-

monas, and Proteus, may also produce such a protective factor.

Other genera, Peptococcus, Fusobacterium, and Clostridium are heterogeneous in respect to response to β-2-t inhibition. The heterogeneity may reflect a lack of genetic uniformity between inhibited and non-inhibited strains. No anaerobic genera have been found in which all strains are inhibited in the absence of Phe, in contrast to the situation previously found in Escherichia and Shigella (Brown & Lines, 1976).

We have found no previous reference to the effects of β-2-t on anaerobic bacteria from either PKU patients or from other sources.

The strains recovered from treated PKU children (Table 3) prove useful in comparisons with other human populations. As the special diet for these PKU children includes small quantities of "adult" foods, it might be expected that the indigenous bacteria isolated should be similar to those recovered from adult patients.

Mata and Urrutia (1971), in a study of breast-fed Guatemalan children, found that a simplified flora consisting almost totally of Bifidobacterium occurred during the period of exclusive breast feeding. With progressive solid food supplementation, Bacteroides gradually became the most numerous bacteria. Clostridia were relatively rare in these Guatemalan children. In our study 22% of the isolates were Bacteroides, less than 8% were Bifidobacterium, and 31% were Clostridium. Attebery et al. (1972) found that

human volunteers fed a partially chemically-defined diet retained gram-positive bacteria, whereas Winitz et al. (1970) in a similar study reported no such retention. Eubacterium and Clostridium isolates were prominent in our study. Ellis-Pegler et al. (1975) also noted a persistence of Bacteroides and Clostridium bacteria in children fed on artificial formula feeds from birth. Qualitatively, therefore, the bacterial flora of these PKU patients appears to be similar neither to that of breast-fed infants nor to weaned children, but reflects instead the presence of large quantities of artificial formulae in the diet.

Finally, in the present study, only 7% of the anaerobic cultures are inhibited by β-2-t on AIT medium. Since inhibition is abolished for those cultures on a Phe-containing medium, these findings, in combination with recently completed studies in mice which will be reported separately, give a further indication that indigenous intestinal bacterial populations of PKU patients are unlikely to be greatly altered during treatment with β-2-t.

ACKNOWLEDGEMENTS

FIGURE LEGENDS

Figure 1 — Schema for isolation, identification and testing of cultures.

Portions of boxes appearing within the bounds of the anaerobic jar represent cultures incubated in the jar.

AIT = Anaerobe Inhibition Test agar

β-2-t = β-2-thienylalanine

BHI = brain heart infusion medium

BLD = horse blood and vitamin K - hemin solution added

CMM = cooked meat medium

COL = Columbia agar

GLC = gas - liquid chromatography

GLU = glucose added

LV = lyophilization vial

MAC = MacConkey agar

Medium VI (Brown & Lines, 1978)

NEO = Neomycin added

THIO = thioglycollate broth

KEY

"AEROBES" →

STRICT ANAEROBES ⇒

FAECAL SWAB

COL BLD
MAC

COL BLD NEG   COL BLD
BHI BLD NEG   BHI BLD

ANAEROBIC JAR

"AEROBIC" ISOLATES

→ MAINTENANCE
→ IDENTIFICATION

GLU THIO   CMM   BHI

ISOLATES

MAINTENANCE

COL BLD

CMM

IDENTIFICATION

GLU THIO

GLC

B-24 ASSAY   MEDIUM VI

ANAEROBICALLY ISOLATED "AEROBES"

B-24 ASSAY

COL   AIT

BIOCHEMICAL TESTING

TABLE 1

MEDIUM COMPONENTS
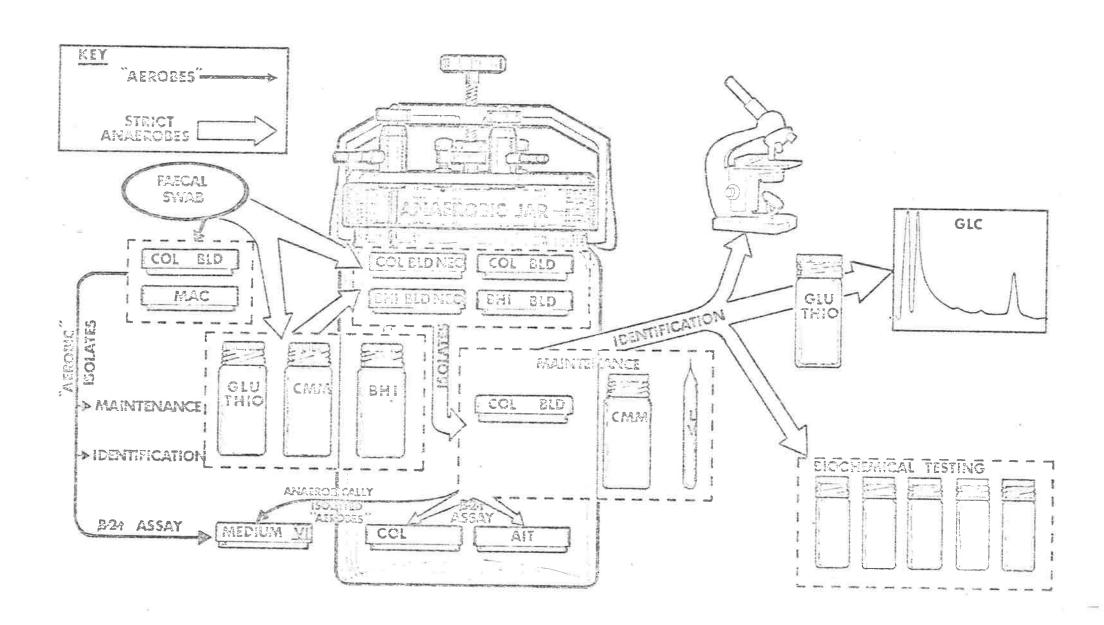
Component	Medium VI (Brown & Lines, 1978) (g/l)	Anaerobe Inhibition Test Medium (g/l)
Added before autoclaving		
K_2HPO_4	3.00	3.00
KH_2PO_4	1.00	1.00
$(NH_4)_2SO_4$	2.00	2.00
Na citrate	0.50	0.50
$MgSO_4 \cdot 7H_2O$	0.01	0.01
agar	13.5	13.5
NaCl	—	0.45
Na succinate	—	0.30
$CaCl_2$	—	0.05
pyruvic acid	—	1.0 ml/l
p-aminobenzoic acid	—	10.0 ml/l
Tween 80	—	1.0 ml/l
volatile fatty acids (*)	—	3.0 ml/l
Added after autoclaving (**)		
10% glucose solution	100.0 ml/l	100.0 ml/l
10% arabinose "	—	10.0 ml/l
10% fructose "	—	10.0 ml/l
10% maltose "	—	10.0 ml/l
8% Na_2CO_3 "	—	50.0 ml/l
Vitamin K- hemin " (***)	—	10.0 ml/l
$Na_2S \cdot 9H_2O$ - cysteine HCl·H_2O (*)	—	2.0 ml/l
0.01% Vitamin B12 - cyanocobalamin (****)	—	1.0 ml/l

(*)   — Holdeman & Moore (1975)   p. 125, as adapted from
              Caldwell & Bryant (1966).
(**)   — solutions sterilized by filtration.
(***) — Holdeman & Moore (1975), p. 127
(****) — Caldwell et al. (1973)

TABLE 2

INHIBITION REACTIONS OF ANAEROBIC CONTROL CULTURES

NHITCC† Strain Numbers		AIT	Columbia agar
1098	*Bacteroides fragilis*	O*	O
903	*Bifidobacterium eriksonii*	O	O
484	*Clostridium oedematiens*	NG	NG
20	*Clostridium perfringens*	NG	O
1097	*Clostridium sporogenes*	O	O
905	*Eubacterium lentum*	O	O
906	*Eubacterium limosum*	O	O
1109	*Fusobacterium (Sphaerophorus) necrophorum(s)*	O	O
75/1225	*Fusobacterium nucleatum*	O	O
299	*Lactobacillus casei* ss. rhamnosus	O	O
1100	*Lactobacillus plantarum*	O	O
75/1309	*Peptostreptococcus intermedius*	O	O
907	*Propionibacterium granulosum*	NG	O
1106	*Streptococcus faecalis*	O	O

†National Health Institute Type Culture Collection, Wellington, N.Z.

*= No growth on Vegetative Minimal Medium (Demain, 1958)

TABLE 2 (cont'd)

AIT = Anaerobe Inhibition Test agar

Inhibition
Reactions   Reactions readable on $\geq$ 25% of plates

O = Not inhibited

+ = Inhibited

_____

NG = Reactions readable on < 25% of plates or
     no growth on medium

| RANGE OF REACTIONS IN ASSAY | | GENERA (number of isolates) | SPECIES | PATIENTS - isolate numbers | | | CONTROLS |
AITM	Columbia Agar			D. M.	P. C.	S. C.	WHITOO
		Bacteroides (28)					
0	0		fragilis		32,34		
0	0		fragilis ss. other	15			
0	0		fragilis				1098
0	0(3),NG(3)†		vulgatus	4,11,29,30,31,32			
0	0		melaninogenicus	27,28			
0	0		putredinis	23			
0	0(2),NG(1)		sp. (+ propionic)	1	22,	59	
0	0(12),NG(1)		sp. (- propionic)		4,12,20,23,55,63,64	16,33,35,42,50,58	
		Bifidobacterium(10)					
NG	0		bifidum		23		
0	0		infantis ss.infantis	2,3			
O(5),NG(2)	O(5),NG(2)		sp.		9,17,34,60,65	38,48	
0	0		eriksonii				903
		Clostridium (40)					
0	0		cadaveris		14		
0	0		innocuum	6			
O(2),NG(1)	0		inulinum		30,52	13	
O(2),NG(1)	O(2),NG(1)		irregularis		36,53	40	
0	0		limosum		33		
0	0		novyi B		56		
NG	NG		oedimatiens				484
0	0		paraputrificum		66		
+(6),O(3)NG(2)	0		perfringens		27,39,40,50,51,57,58	35,44,45	20
0, NG	0, NG		ramosum		46	39	
0	0		sardiniensis		21		
0	0		scatologenes			9,10,14	
0	0		septicum		1		
0	0		sordellii; bifermentans			56	
0	0		sp.		38,59,62		
O(3),NG(2)	0		sporogenes, ghoni	19,26		49,51	1097
NG	0		tertium		11,12,13,15		

RANGE OF REACTIONS IN ASSAY		GENERA (number of isolates)	SPECIES	P A T I E N T S - Isolate numbers			CONTROLS
AIT®	Columbia Agar			D.M.	P.C.	S.C.	NCTC
		Eubacterium (17)					
O	O,NG		aerofaciens	24,33			
NG	O		alactolyticum		16		
O(2),NG(1)	O(2),NG(1)		contortum	13	18,19		
O	O		lentum			30	905
O	O		limosum			48	908
O	O		sp. (+ butyric)		24		
O(1),NG(2)	O		sp. (++butyric)		25,44,45		
O	O		sp. (-butyric)		51	23,25,43,47	
		Fusobacterium (3)					
+	O		mortiferum	9,14			
O	O		nucleatum				75/1225
O	O		necrophorum (s)				1109
O	O		sp.		10		
		Lactobacillus (4)					
O	O		casei				299
NG	O		catenaforme	10			
O	O		lactis		8		
O	O		plantarum				1100
O,NG	O		sp.			24,54	
		Peptococcus (6)					
NG	NG		asaccharolyticus	22			
+(1),O(1),NG(3)	O(3),NG(1)		magnus	8,31		25,27	
O	O		prevotii			52	
O,NG	O,NG		sp.		35	41	
		Peptostreptococcus (11)					
O(3),NG(1)	O(3),NG(1)		intermedius	12		26,31	75/1809
O	O		productus			5,8	
O(3),NG(3)	O(4),NG(2)		sp.		46,49	5,9,29,37	
		Propionibacterium (3)					
NG	O		granulosum				907
			sp.		28,31,37	4,7,53	
		Streptococcus (10) (facultatively anaerobic)					
O	O		faecalis				1111

TABLE 3 (cont'd)

*AIT = Anaerobe Inhibition Test agar

NHITCC = National Health Institute Type Culture Collection, Wellington, N.Z.
† = Number of cultures giving that reaction

Inhibition Reactions

Reactions readable on $\geq$ 25% of plates

 0 = not inhibited

 + = inhibited

NG = Reactions readable on < 25% of plates, or no growth on medium

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