SOME ASPECTS OF THE BIOCHEMISTRY OF MALABSORPTION FROM THE UPPER INTESTINAL TRACT

by

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SUMMARY

A method has been introduced which was suitable for the routine measurement of vitamin E levels in serum. It has been used to assess the vitamin E status of 220 patients with gastrointestinal diseases. These patients serum levels were found to be significantly lower than those of blood donor normals. The levels found correlated with the degree of ileal disease. Surgical intervention in any area of the gastrointestinal tract produced a lowering of serum levels. Vitamin E levels were not correlated with haemoglobin or MCV. There was a correlation with serum albumin and iron and with faecal fat excretion, all of which reflect gastrointestinal damage.

When tritiated DL-α-tocopherol was given by mouth to rats, the major site of absorption was found to be in the jejunum and proximal ileum. The method used to monitor absorption of an oral dose of vitamin E by humans was not sensitive enough to follow accurately the small changes of level involved. The same methodological limitation applied when fasting levels of vitamin E were compared in serum from hepatic or hepatic portal with that from the ante-cubital vein; there was no significant correlation between serum vitamin E and cholesterol levels in these samples.

The method used for the measurement of vitamin B_6, in the form of pyridoxal-5'-phosphate (PLP), is convenient to use but further mechanisation is necessary to improve reproducibility. 182 gastrointestinal patients and 124 normals have had their serum PLP measured. Amongst normals, women had a statistically significant lower mean serum PLP. This sex difference was abolished when the subjects had gastrointestinal disease. The presence of an active

disease process was found to have the most marked effect on the levels found. An attempt was made to correlate serum PLP level with the excretion of abnormal levels of tryptophan metabolites after an oral load of L-tryptophan.

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Abbreviations

The abbreviations used for units of mass, time, length, concentration, volume are as described in directions to authors for Clinical Science. Their system of referencing has also been used. Other abbreviations are as listed below:-

ACD	Adult coeliac disease
ACTH	Adrenocorticotrophic hormone
BA	4,7,-diphenyl-1,10,-phenanthroline
butyl PBD	2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)1,3,4,-oxadiazol
CNS	Central nervous system
C.P.M.	Counts per minute
CV	Coefficient of variation
GFD	Gluten free diet
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindolyl-3-acetic acid
IAA	Indole-3-acetic acid
IV	Intravenous
KA	Kynurenic acid
Kyn	Kynurenine
MCV	Mean corpuscular volume
n	number
NAD	Nicotinamide adenine dinucleotide
NS	Not significant
OD	Optical density
p	Probability
PLP	Pyridoxal-5'-phosphate
PUFA	polyunsaturated fatty acid
r	Correlation coefficient

RBC	Red blood cell
RE	Regional enteritis
rpm	revolutions per minute
SD	Standard deviation
TCA	Trichloracetic acid
UC	Ulcerative colitis
$\bar{x}$	Mean
XA	Xanthurenic acid

Statistical Formulae used

Mean $$\bar{x} = \frac{\sum_{1}^{n} X_i}{n}$$

Standard deviation $$SD = \sqrt{\frac{\sum_{1}^{n}(X_i - \bar{x})^2}{n - 1}}$$

Correlation coefficient $$r = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{N}}{\sqrt{\left(\Sigma X^2 - \frac{(\Sigma X)^2}{N}\right)\left(\Sigma Y^2 - \frac{(\Sigma Y)^2}{N}\right)}}$$

t for correlation coefficient $$t = \frac{r}{\sqrt{\frac{1 - r^2}{n - 2}}}$$

Students 't' test $$t = \frac{\bar{x}_1 - \bar{x}_2}{\text{SE difference}}$$

Degrees of freedom $DF = (n_1 - 1) + (n_2 - 1)$

SE difference $$= \sqrt{\frac{\left[\sum_{1}^{n} X_i^2 - \frac{(\Sigma X_i)^2}{n_1}\right] + \left[\sum_{1}^{n} X_j^2 - \frac{(\Sigma X_j)^2}{n_2}\right]}{(n_1 - 1) + (n_2 - 1)} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

Standard deviation of pairs $$SD = \sqrt{\frac{\Sigma d^2}{2N - 1}}$$ where d is the difference between the pairs and N is the number of pairs

Coefficient of variation $$CV = \frac{SD}{x} \times 100$$

Probability p from statistical tables, using the appropriate number of degrees of freedom

All calculations were performed on a Sumlock 142 statistical electronic calculator.

1. GENERAL INTRODUCTION

The task of assessing the state of nutrition of an individual is a complex one. Fig.1.1. (after Krehl, (1967)) illustrates some of the factors which intervene between food supply in the community as a whole, and the levels of any particular nutrient which may be measured in that individual. Of special interest in this study is the situation where malabsorption is likely to take place. The two conditions which are of most interest here are Adult Coeliac Disease (ACD) and Regional Enteritis (RE).

A diagnosis of ACD is made when a biopsy taken from the jejunum appears flat, when examined under a binocular microscope. Trier and Browning (1970) found that biopsies taken from the duodenal-jejunal flexure of ACD patients incorporated twice as much thymidine-^{3}H into cell nuclei as the cells in the biopsies from normal controls. The labelled cells had migrated to the surface epithelium within 24 hours in the untreated patients, whilst labelled cells did not migrate further than the lower third in the normals. Most of the evidence indicates that the villous "atrophy" results from a shortened life span of differentiated epithelial cells, which is not compensated for by an increased rate of cell replication and differentiation in the elongated crypt. The surface epithelial cells of the untreated ACD jejunal biopsy show a considerable amount of intra-cellular disorientation when examined under an electron microscope (Rubin, Ross, Sleisenger, Weser, (1966)); the micro villi were short and deranged and the terminal web was irregular and incompletely developed. Villous atrophy combined with microvillar abnormalities produce a drastic reduction in the number of functional absorptive cells. Many of the known absorptive abnormalities in untreated ACD have been attributed to known, or assumed, deficiencies

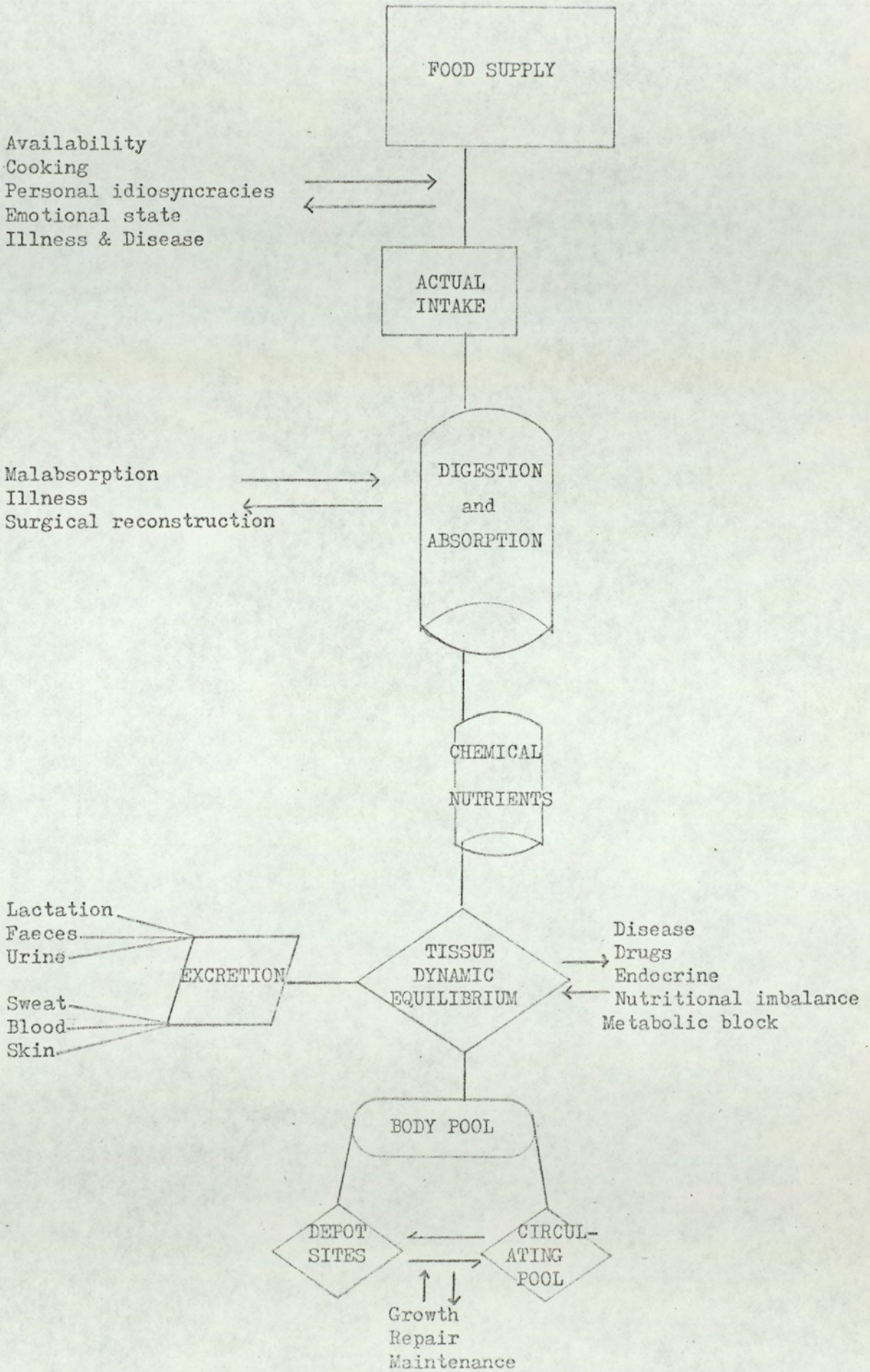


FIG. 1.1.
Factors affecting food intake, nutrient requirements, digestion, absorption and excretion.

of enzymes and carriers which are associated with the altered morphology of the surface epithelial cell.

The treatment of ACD is withdrawal of gluten (a protein found in flour from wheat and certain other cereals) from the diet. This gluten-free diet has been described by Fletcher and McCririck, (1958). In the majority of cases there is an improvement in absorption and concomitant improvement in cell morphology within a few days of institution of treatment. Regeneration of villi usually requires weeks or months and is seldom complete (Yardley, Bayless, Norton and Hendrix, (1962)), (MacDonald, Brandborg, Flich, Trier and Rubin, (1964)). The means by which gluten becomes toxic and exerts its toxic effect on these patients is not fully understood. A gluten substituent, gliadin or its toxic peptides, has been shown to bind directly with coeliac jejunal mucosal cells in vitro (Rubin, Fauci, Sleisenger and Weser, (1965)). Progress is being made towards the identification of the toxic peptide(s) in gluten (Bronstein, Haeffner and Kowlessar, (1966)) despite the lack of evidence for a specific peptidase deficiency. Whatever the underlying pathogenic mechanism is, the epithelial cells are rapidly damaged as they migrate onto the surface of the coeliac mucosa, (Rubin et al, 1966)).

Regional enteritis is a chronic inflammatory disease which may affect any part of the intestinal tract, from oesophagus to anus, the inflammation usually being granulomatous in character. There is usually segmental thickening and stiffness of the bowel wall, often with single or multiple strictures. The mucosa has a cobble-stone-like appearance, resulting from longitudinal ulcerations, with the intervening rugae intersected by transverse linear ulcers. These discontinuous lesions can be found anywhere in the small and large bowel. In the active stage there is usually vascular engorgement and a fibrinous peritoneal exudate. Malabsorption may result from strictures or fistulae with secondary stasis and bacterial overgrowth, from diffuse involvement of the

small intestinal mucosa, or from resection of segments of the intestine. In the acute stage patients may suffer from a fulminant malabsorption syndrome, with fluid and electrolyte depletion and severe enteric plasma protein leakage.

A number of these ACD and RE and other patients attending the Gastrointestinal Unit of the General Hospital, Birmingham, have been found to be suffering from a persistent anaemia, for which no satisfactory cause has been found. These anaemias were not attributable to acute or chronic blood loss through the gastrointestinal tract, nor were the patients found to be deficient in iron, vitamin B_{12} or Folic Acid, as measured by serum levels. Reports in the literature indicated that deficiency in vitamin E and/or vitamin B_6 may provide an explanation of these anaemias. Since vitamin E is fat soluble and vitamin B_6 is water soluble, it was felt that this would present an opportunity to study two different modes of absorption in a variety of gastrointestinal disorders.

The primary objective was to introduce methods for measuring serum levels of vitamin E and B_6, which are suitable for use in a clinical chemistry laboratory. Having done this, the levels found in a normal population (blood donors) were to be compared with a similar number of patients attending the Unit, mainly as out-patients. An attempt will be made to correlate the vitamin levels with concurrent haematological findings and an investigation of any other related biochemical abnormality will be undertaken. It may be possible to extrapolate a site of maximum absorption from those patients who have had an area of intestine resected or a well-defined area of disease involvement.

2. VITAMIN E

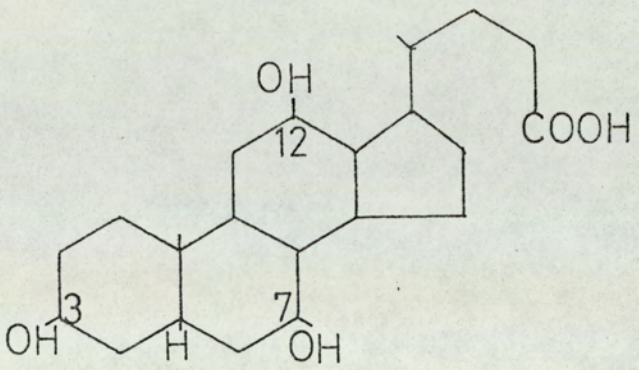
2.1.1. Introduction

Vitamin E is a fat soluble vitamin. In common with other vitamins classified in this sub-class, it is considered that an adequate flow of bile salts is required for its absorption (Greaves and Schmidt,(1937)): that is, a concentration of di- and tri-hydroxy, conjugated bile salts (see Fig.2.1.1.) in the intestinal contents of at least 3 mM (Dawson, (1971)), which is necessary for the aggregation of these bile salts to form a micelle. The molecules are arranged in aggregates, the sterol back-bone of the bile salt molecule facing inwards, and, once formed, the micelles can solublise other lipids within their structure. Triglycerides are virtually insoluble in micelles, but their breakdown products, fatty acids and monoglycerides, are readily incorporated, thus producing an expansion of the structure. Fat soluble vitamins and cholesterol cannot enter a pure bile micelle, but readily enter the expanded "mixed" micelle and are thus able to partition themselves between the oil and micellar phases within the gut lumen (Borgström, (1967), Finkelstein, (1968)).

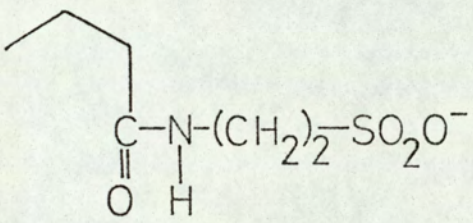
The absorption of fat soluble vitamins appears to take place principally in the proximal jejunum (Booth, (1968)). Overwhelming biochemical evidence suggests that lipids enter the intestinal epithelial cell by passive diffusion (Johnson and Borgström, (1964)), which probably involves direct contact between micelles and the cell surface and the entry of the lipids into the brush border membrane (Simmonds, Hofmann and Theoder, (1967)). (See Fig.2.1.2.). Schacter, Finkelstein and Kowarski, (1964) have shown that, in the rat, vitamin D is absorbed by a non-saturatable diffusion mechanism and that the rate of uptake into the mucosa is much more rapid than subsequent transfer

Fig. 2.1.1.

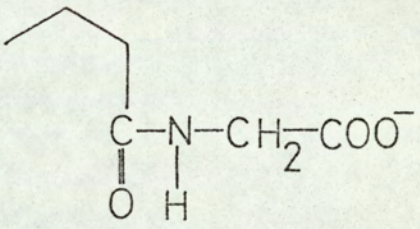
The main bile salts found in human bile.



Cholic Acid



Taurine Conjugate



Glycine Conjugate

	hydroxyl group	Bile Acid
Trihydroxy	3α 7α 12α	Cholic
Dihydroxy	3α 7α	Chenodeoxycholic
	3α 12α	Deoxycholic

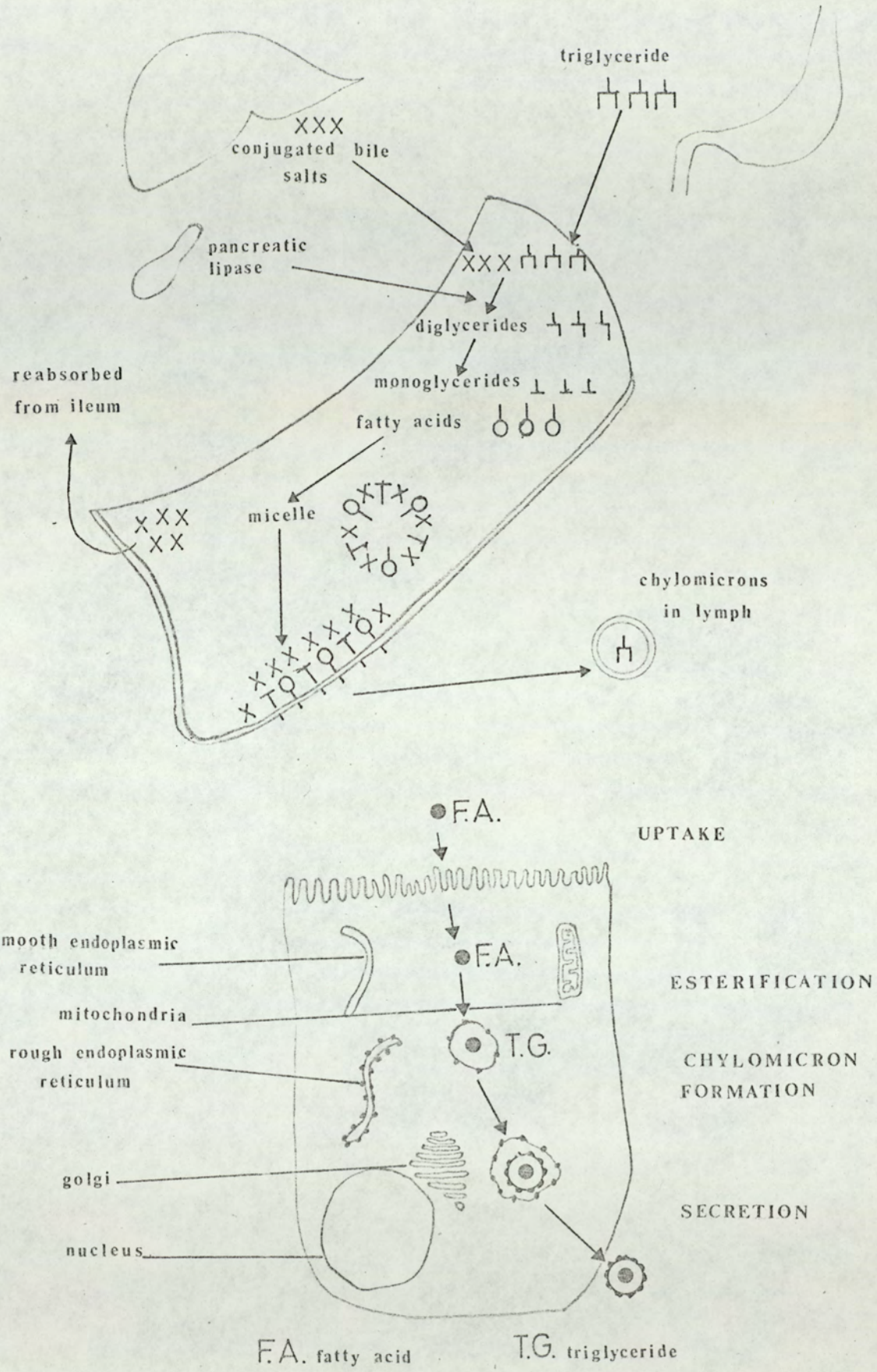


Fig. 2.1.2. A schematic representation depicting the major events of fat absorption from the lumen and subsequent esterification and secretion from the intestinal mucosal cell. (after Isselbacher (1967)).

through the cell. Vitamin E passes unchanged through the epithelial cell and is absorbed into the lymph (Johnston and Pover, (1962)).

The biological role of vitamin E has been the subject of controversy since the first International Congress on Vitamin E in 1939, when Drummond suggested that α-tocopherol might take part in specific redox changes of metabolic importance, by virtue of the oxidation-reduction properties inherent in its structure. In their recent review, Green and Bunyan, (1969), critically appraised the evidence for the two basic concepts of the biological role of vitamin E. These are:-

a) "that vitamin E functions in the animal solely as an anti-oxidant, being particularly involved in the protection of unsaturated tissue lipids against peroxidation."

b) "that vitamin E has a metabolic role which is specific or general, but which is not directly concerned with its properties as an anti-oxidant in vitro."

After an exhaustive analysis of the evidence available they found that the anti-oxidant theory rests on circumstantial evidence and hence must remain not proven. There was a striking lack of success in all attempts to establish, directly, the presence of increased lipid peroxide levels in the tissues of animals deficient in vitamin E and a general lack of correlation between tissue peroxidation and the onset of the deficiency diseases found in animals. Also, dietary stress with increased unsaturated fat or toxic agents, e.g. silver, lead, iron (daily intramuscular injections of iron dextran), carbon tetrachloride and ethanol, did not result in an increased catabolism of α-tocopherol. Bunyan, Green, Murrell, Diplock and Cawthorne (1968) have suggested that vitamin E may be concerned with the intestinal absorption and transport of certain long chain unsaturated fatty acids

and may thus play a role in the absorption of peroxidised fatty acids by the intestinal tract.

Excretion of vitamin E has been studied after administering varying doses of the vitamin by both oral and parenteral routes. Tocopheronic acid and its γ-lactone have been isolated from the urine of patients given an oral dose of 3-5 g α-tocopherol (Simon, Eisengart, Sundheim and Milhorat (1956)). When α-tocopherol labelled with ^{14}C or ^{3}H has been used to study excretion, it was found that 37.9% of the administered radio-activity was excreted in the urine of rats in 64 hours after dosing (Sternberg and Pascoe-Dawson (1959)). When a physiological dose (1.0 mg) of tritiated α-tocopherol was given to patients and control subjects MacMahon and Neale (1970) found a range of urinary excretion of radio-activity of 0-21.4%, which correlated with the extent of absorption from the intestinal tract. Simon, Gross and Milhorat (1956) found that a significant portion of intravenously administered d-α-tocopheryl-5-methyl-^{14}C-succinate was excreted in an unidentified form in the test rabbits' urines and also that considerable amounts of radio-activity were found in the faeces.

2.1.2. Chemistry of Vitamin E

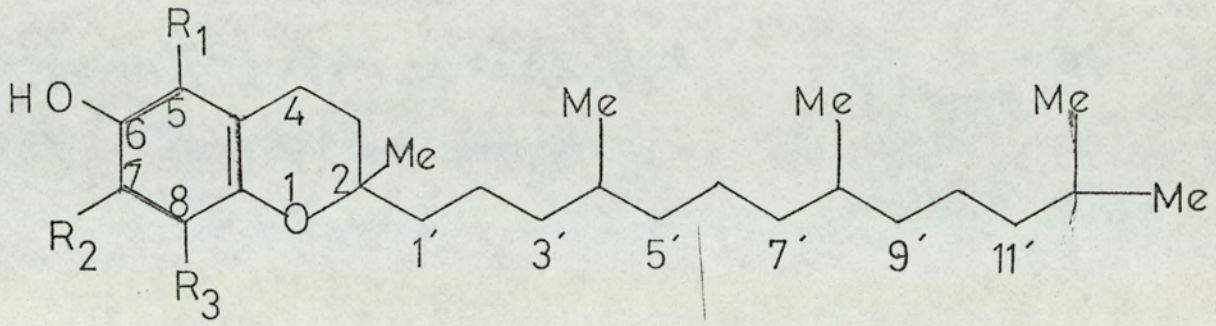
There are eight known naturally occurring tocopherols (See Fig.2.1.3. Nomenclature Policy (1972)). They are synthesised by green plants and a full biosynthetic pathway, from tyrosine through the toco-trienols to tocopherols has been published (Whistance and Threlfall (1968)). The tocopherols were first isolated from wheat germ oil and named by Evans, Emerson and Emerson (1936) and first synthesised in 1938 by Karrer.

A dimer and trimer of α-tocopherol have been isolated from rat tissue (Draper, Csallany and Shah (1962), Draper, Csallany and Chiu (1967)) and four further dimers were isolated from fresh cotton oil and identified by Nilsson, Daves and Folkers (1968). Dimers and trimers of α-tocopherol have also been found to be formed in rats and mice given parenteral α-tocopherol (Strauch, Fales, Pittman and Avignan (1969)) which are identical with synthetic products of oxidative condensation of α-tocopherol. The dimers and trimers were not found after oral administration of α-tocopherol to the rats and it was concluded that oxidative condensation did not represent a normal physiological metabolic pathway.

Tocopherols can act as hydrogen donors or hydrogen transferring systems, where the hydrogen of the 6-hydroxy group is the active component (Boguth (1969)), thus participating in a non-enzymatic hydrogen transfer (Nason, Garrett, Nair, Vasington and Detwiler (1964)). Fig.2.1.4. shows a suggested mechanism for α-tocopherol oxidation (Gruger and Tappel (1970)) in free radical reactions with preformed hydroperoxides of polyunsaturated fatty acid esters. Alpha-tocopherol quinone has been found in considerable amounts in the heart and spleen of rats given an oral dose of α-tocopherol (Pearson and Barnes (1970)) and α-tocopherol quinone has been shown to account for 10-15% of

Fig. 2.1.3.

The tocopherols isolated from natural sources.



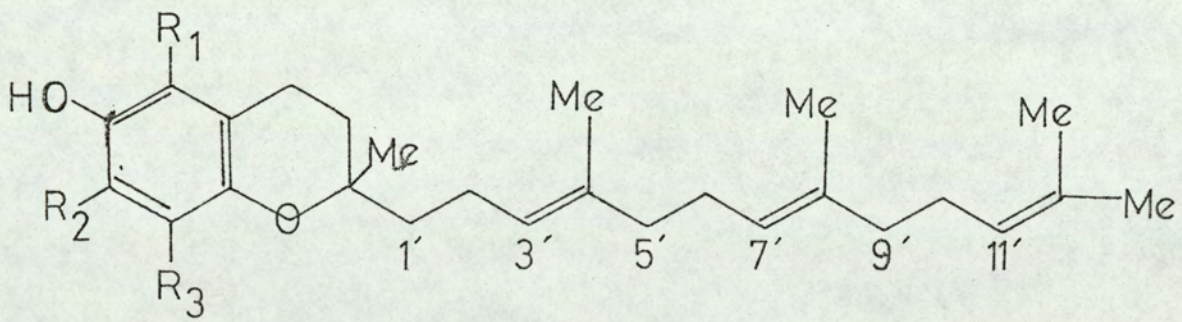
Tocopherol

$R_1 = R_2 = R_3 = -Me$ ∝Tocopherol

$R_1 = R_3 = -Me$; $R_2 = -H$ β Tocopherol

$R_1 = -H$; $R_2 = R_3 = -Me$ γ Tocopherol

$R_1 = R_2 = -H$; $R_3 = -Me$ δ Tocopherol



Tocotrienol

$R_1 = R_2 = R_3 = -Me$ ∝ Tocotrienol

$R_1 = R_3 = -Me$; $R_2 = -H$ β Tocotrienol

$R_1 = -H$; $R_2 = R_3 = -Me$ γ Tocotrienol

$R_1 = R_2 = -H$; $R_3 = -Me$ δ Tocotrienol

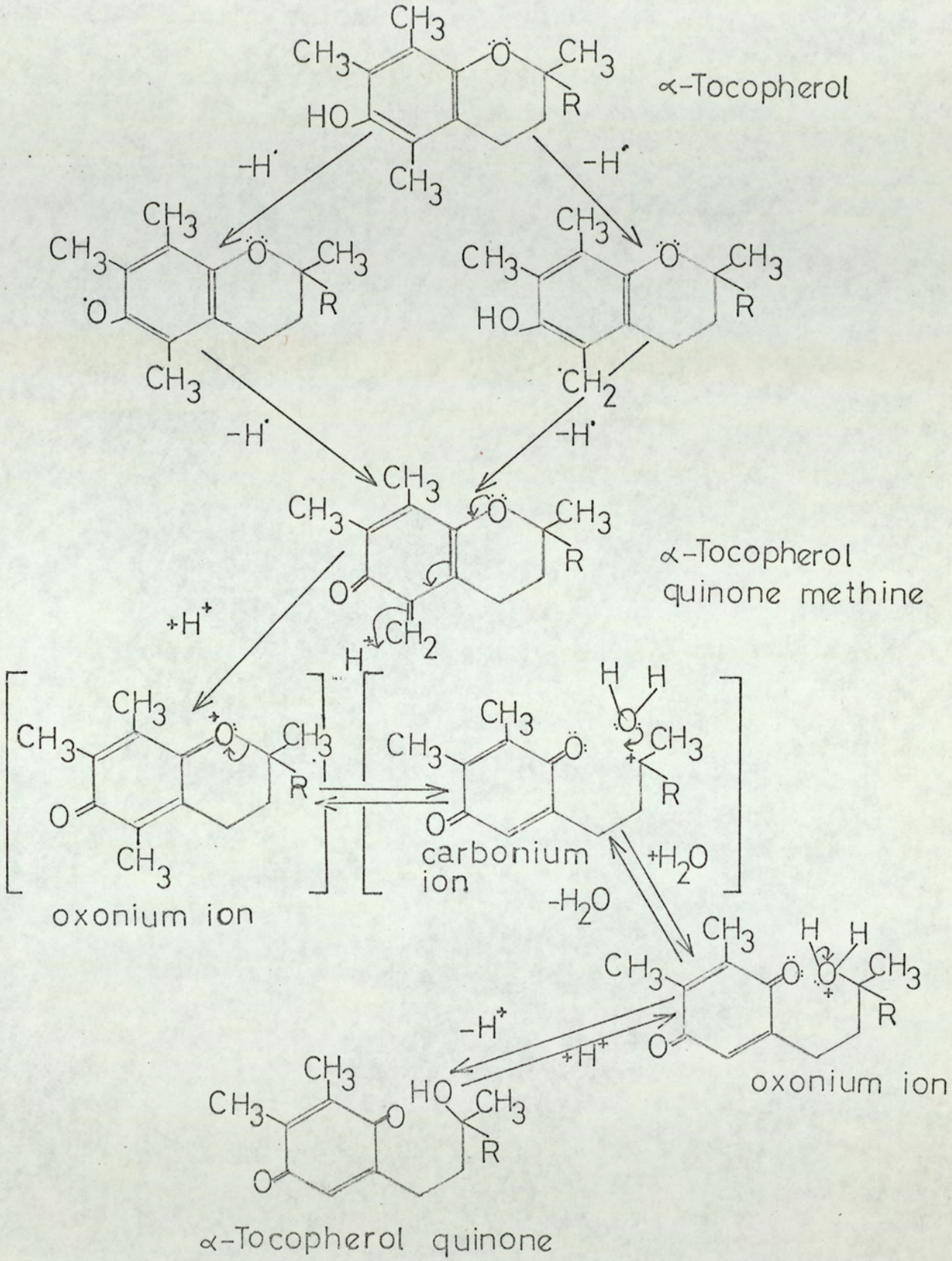


Fig.2.1.4. Suggested mechanism for α-tocopherol oxidation (after Gruger & Tappel (1970)).

radio-activity in human faeces after an oral dose of physiological amounts of DL(5-Me-^{3}H)α-tocopherol (Kelleher and Losowsky (1970)).

2.1.3. The Occurrence of Vitamin E in the Diet

Good sources of vitamin E in the normal diet are milk, eggs, meat, fish, cereals and leafy vegetables (Binder, Herting, Hurst, Finch and Spiro (1965)). Significant tocopherol deficiency in adults, secondary to nutritional deficiency, is considered unlikely in the more affluent sectors of world population (Bieri, Teets, Belvady and Andrews (1964)) but 21% of a rural population surveyed in East Pakistan had serum levels below 5.0 µg/ml. (Rahman, Hossain, Talukdar, Ahmad and Bieri (1964)). Bunnell, Keating, Quaresimo and Parman (1968) estimate that the α-tocopherol content of a typical days' food in the U.S.A. would, after cooking, range from 2.6-15.4 mg., with an average of 7.4 mg. This indicates the possibility of relatively low α-tocopherol intake in a portion of the population, depending on dietary habits. Smith, Kelleher, Losowsky and Morrish (1971) have found that the average British diet provides less than 5.0 mg. vit E/day, which is the lowest recommended intake.

Corn oil, cotton seed oil, margarine, peanut oil and soybean oil are the foods with the highest concentrations of tocopherols. Alfin-Slater, Morris, Aftergood and Melnick (1969) found that oils obtained after use in commercial frying operations contained sufficient vitamin E to meet the dietary requirements of test rats. The amounts of tocopherols in all natural plant oils are usually directly related to the level of polyunsaturation of the oil, although the relative amounts of tocopherols vary e.g. corn oil has 3.3 mg. α-tocopherol/100 g., and 33 mg. γ-tocopherol/100 g., and safflower oil has 33 mg. α-tocopherol/100 g. and 1.6 mg. γ-tocopherol/100 g. (Horwitt, Harvey and Harman (1968)). In plants the tocopherols have approximately equal potency, but in man tocopherols other than α-tocopherol have relatively much less potency (Horwitt et al (1968)). They estimate that

γ-tocopherol has approximately 10% of the potency of α-tocopherol.

The minimal daily requirement of vitamin E is estimated to be 5.0 mg by Bicknell and Prescott (1953). Horwitt (1960) estimated the daily requirement of α-tocopherol to range between 10 and 30 mg. Harris and Embree (1963) took into account the nature of lipids in the diet, especially the polyunsaturated fatty acids (PUFA) and designated a critical ratio of vitamin E (mg/day) : PUFA (g/day) = 0.6. Below this level it was expected that there would be detectable signs of deficiency. Recently Witting (1972) has argued that vitamin E requirement is related to tissue PUFA, which bears little relationship to the dietary PUFA level at any one instance in time. This is because stored dietary fat tends to reflect the average intake of lipids over a period of months, if not years. The one half turn-over rate of linoleic acid in human adipose tissue is approximately 26 months (Witting (1970)) and it is possible that patients who have been on a therapeutic diet, containing relatively large amounts of PUFA, may experience low tissue levels of vitamin E after stopping the special diets. For this situation Witting (1972) recommends that the vitamin E intake should be:-

mg d-α-tocopherol/day = 0.6 (g linoleate/100 g adipose tissue fatty acids).

2.1.4. The Effects of Vitamin E Deficiency

In their delineation of the effects of tocopherol deficiency in man Binder et al (1965) propose a graduation of deficiency:-

1) early and gradual depletion of body tocopherol, without physiological abnormalities;
2) a phase of moderately severe tocopherol depletion, characterised by increased erythrocyte hydrogen peroxide haemolysis and creatinuria;
3) prolonged and more severe depletion, characterised by the additional ocurrence of ceroid deposition in smooth muscle and lesions in skeletal muscles resembling muscular dystrophy.

Horwitt (1962) lowered the serum levels of adult male volunteers from an initial level of 8.0 µg/ml to a plateau of 5.0 µg/ml in a period of $2\frac{1}{2}$ years by restricting dietary intake to 3 mg/day. When the dietary source of lipids was changed from 30 g. of stripped lard to 30 g. of stripped corn oil, which was doubled (60 g.) 9 months later, the serum levels fell to 1.5 µg/ml after 7 years of restriction. At this stage of depletion, Horwitt, Century and Zeman (1963) reported a slight but significant decrease in the half survival times of erythrocytes. Binder et al (1965) found shortened survival of ^{51}Cr-tagged red cells in several vitamin E deficient patients but felt that this did not accurately reflect red cell life span, neither did they find any evidence of altered erythropoiesis. More recently, Leonard and Losowsky (1971) have presented evidence which suggests that the major effect of vitamin E in improving anaemia associated with low serum levels is obtained by an effect on erythropoiesis rather than by a reduction of haemolysis.

At birth normal infants have been found to have a serum vitamin E level of 2.3 $\pm$ 0.6 µg/ml (Hashim and Asfour (1968)). Leonard, Doyle and Harrington (1972) have found a direct relationship between the

plasma levels in the infant at birth and those of the mother, the mothers' levels averaging 9.2 ± 2.9 μg/ml and the infants' 2.4 ± 0.2 μg/ml for the first pregnancy (subsequent pregnancies did not give significantly different values). The levels appear to rise fairly rapidly to a mean level of 8.1 ± 1.7 μg/ml in children ranging in age from $2\frac{1}{2}$-10 years (Bennett and Medwadowski (1967)), but conventional artificial feeding does not result in as rapid a rise in body stores as does breast feeding (Darby (1968)). Premature infants, who are almost invariably artificially fed, appear to be at special risk from vitamin E deficiency, with an increase in *in vitro* H_2O_2 red cell haemolysis (Oski and Barness (1967), Hassan, Hasbim, Van Itallie and Sebrell (1966), Ritchie, Fish, McMasters and Grossman (1969)), although Leonard *et al* (1972) found no significant difference in the plasma levels of premature and full term infants. The tendency to *in vitro* H_2O_2 haemolysis is increased by therapeutic doses of iron given during the period of vitamin E deficiency (Milhorn and Gross (1971)). Children (aged 5 months to 4 years) suffering from anaemia associated with protein-calorie malnutrition have shown a consistent haematological response following administration of vitamin E (Whitaker, Fort, Vimokesant and Dining (1967)). This anaemia probably has a multiple aetiology, but it is significant that its treatment with folic acid, vitamin B_{12}, ascorbic acid or iron has not produced a consistent response (Majaj (1966)).

In adults vitamin E deficiency is found to be associated with an abnormal *in vitro* H_2O_2 haemolysis test and an increased urinary excretion of creatine (Losowsky and Leonard (1967)), although instances of an occasional lack of correlation between plasma vitamin E levels and peroxide haemolysis have been reported (Leonard and Losowsky (1967)). Auto-haemolysis does not appear to take place in patients with moderately

to extremely low serum tocopherol levels, secondary to prolonged steatorrhoea (Binder, Spiro and Finch (1966)).

Vitamin E deficiency in primates has been fully described (Fitch (1968)). Vitamin E may be virtually undetectable in the serum of young rhesus monkeys, fed a vitamin E deficient diet, for 2 years prior to the abrupt onset of the deficiency disease. There is a rapid loss of weight, anaemia and creatinuria, which is fatal in 1-2 months if left untreated. In the bone marrow there is an increased number of erythrocyte precursors, which have atypical chromatin, due to an increased production and a reduction in output. The creatinuria correlates with the extent of necrosis, fragmentation of fibres and nuclear proliferation in striated muscles. When the monkeys are treated with vitamin E there is a reticulocytosis in the first week, accompanied by the disappearance of abnormalities in erythrocyte precursors; creatinuria disappears in 48-72 hours. Porter and Fitch (1966) have reported that vitamin E is required for the normal synthesis of porphyrin and haem in monkeys. Also, co-enzyme Q_{10} administration can increase porphyrin synthesis in bone marrow cells of vitamin E deficient monkeys. Vitamin E and co-enzyme Q_{10} have been implicated in maintaining membrane stability in both monkeys (Fitch (1968)) and rabbits (Ludwig, Elashoff, Smith, Scholler, Farby and Folkers (1967)).

2.2. Materials and Methods

2.2.1. Introduction

The measurement of the concentration of vitamin E in serum is based on the method devised by Emmerie and Engel (1938). The determination rests on the ability of tocopherols to reduce ferric ions stoichiometrically to ferrous ions, the latter forming a colour complex with α,α'-dipyridyl. This original method required the extraction of about 10 ml serum, but a "micro" procedure was devised by Quaife, Scrimshaw and Lowry (1949) using the same complexing agent. The use of other complexing agents, to enable the ferrous ion concentration to be measured, has been proposed. These are:-

2,2',2",-tripyridine (Natelson (1960))

2,4,6,-tripyridyl-s-triazine (Martinek (1964))

4,7,-diphenyl-1,10,-phenanthroline (Tsen (1961))

The method used here is that of Fabianek, De Filippi, Rickards and Herp (1968). Photochemical reduction of ferric ions and interference from carotene have been obviated when using this method by the addition to the reaction mixture of orthophosphoric acid, which chelates the excess ferric ions after the reaction with tocopherols.

2.2.2. Reagents

The following reagents were used for the measurement of vitamin E in serum:-

(a) Purified absolute ethanol, obtained by redistillation of commercial absolute ethanol to which were added pellets of calcium chloride and crystals of potassium permanganate.

(b) ortho-xylene, Laboratory reagent grade, BDH.

(c) 4,7,-Diphenyl-1,10,-phenanthroline (bathophenanthroline BA) Sigma. 200 mg dissolved in 50 ml purified absolute ethanol.

(d) Ferric chloride, hexahydrate. ($FeCl_3.6H_2O$) Analar, Hopkin & Williams. 60 mg dissolved in 100 ml purified absolute ethanol.

(e) Alpha-tocopherol, DL α-tocopherol obtained from Sigma. Stock standard - 100 mg dissolved in 100 ml purified absolute ethanol. Working standards - 0.1 ml of stock standard was diluted with purified absolute ethanol to 20 ml and 10 ml in volumetric flasks, 0.2 ml was diluted to 10 ml in a volumetric flask, to give concentrations of 5, 10, and 20 µg/ml.

(f) Orthophosphoric acid, $\geq$ 88%, Analar, Hopkin & Williams. 0.5 ml diluted to 100 ml with purified absolute ethanol.

(g) Spectrophotometer. Uvispek, Hilger & Watts, for which micro-cuvettes, 1 cm light path length, were already available.

Solutions (c), (d) and (f) were stored in amber bottles as protection against light. The BA and $FeCl_3$ solutions were made up fresh each week and all three were stored at 4° when not in use. The stock standard was stored at 4° and was stable for six months; care was taken to avoid long exposure to light. Working standards were made up by dilution of the stock standard each week and were stored at 4° when not in use.

E-mil gold line, auto-zero, high precision micro-pipettes were used for measuring small volumes (0.1 and 0.2 ml): 1 ml graduated pipettes were used for measuring 0.4 ml volumes. Glassware was soaked in "Haemosol" solution overnight, rinsed in tap water, then distilled water and dried in an oven. The constriction pipettes were also soaked overnight in "Haemosol" solution and were cleaned by sucking this solution through the pipette. They were rinsed by sucking through distilled water, then acetone and dried in an oven.

2.2.3. Clinical Material

Serum was obtained from non-fasting patients attending the

out-patient clinics of the Gastrointestinal Unit. Serum vitamin E levels were also requested from some in-patients, but the number of these requests was small relative to those for out-patients. The blood samples were centrifuged and the serum separated within 2 hours of the sample being taken. All Sera were stored at -20° until analysed, within one month of sampling.

Specimens of normal serum were obtained from the Blood Transfusion Service within 24 hours of donation. The sera had been stored overnight at -20° and remained frozen whilst being transferred to the General Hospital from the Blood Transfusion Service laboratories. The samples were stored at -20° and were analysed within one week of receipt.

2.2.4. Procedure

Extraction (a) Samples of sera were thawed, mixed and a 0.4 ml aliquot was pipetted into a small test tube (10 cm x 1 cm).

(b) 0.4 ml purified absolute ethanol was added and the contents of the tube were mixed, thus precipitating the serum proteins.

(c) 0.4 ml ortho-xylene was added and the vitamin E was extracted into the o-xylene by mixing with a vortex mixer for 15 secs. The o-xylene layer was separated by centrifuging for 15 mins at 2,000 rpm in an MSE Mistral 2L at 4°.

Measurement 0.2 ml of the o-xylene extract was transferred to a test tube already containing 0.1 ml BA and the contents were mixed. 0.1 ml $FeCl_3$ was added and mixed, followed immediately by 0.1 ml orthophosphoric acid. The optical density (OD) at 535 nm was read between 15 and 30 mins after the addition of the reagents. 0.2 ml of working standards (5.0, 10.0 and 20.0 µg α-tocopherol/ml) were analysed in the same way as the xylene extracts; in this instance the sole solvent of the reaction mixture was ethanol. Two blanks were used: (a) an ethanol blank, where the 0.2 ml of xylene extract was replaced by 0.2 ml purified

absolute ethanol, which was used for zeroing the spectrophotometer.
(b) 0.4 ml distilled water was extracted in the same way as serum and the OD of this xylene extract was subtracted from those of the test sera, thus correcting for any slight impurity present in the xylene and also the differing optical properties of a xylene/ethanol mixture and ethanol alone. The extraction and measurement of vitamin E in serum was made in duplicate whenever possible.

2.2.5. Results

2.2.5.1. An investigation of the absorption characteristics of the reaction mixtures.

Fig.2.2.1. shows part of the absorption spectrum of the 20 µg and 10 µg α-tocopherol/ml standards, obtained when the reaction mixture was scanned on a Unicam SP 800. There is an absorption maximum at 535 nm. Fig.2.2.2. shows the absorption spectra obtained when the following reaction mixtures were used:-

(1) 10 µg α-tocopherol + 3 µg β-carotene/ml; no orthophosphoric acid.

(2) 10 µg α-tocopherol + 3 µg β-carotene/ml and orthophosphoric acid.

(3) 10 µg α-tocopherol/ml; no orthophosphoric acid.

(4) 10 µg α-tocopherol/ml and orthophosphoric acid.

(5) Serum extract and orthophosphoric acid.

(6) Serum containing 3 µg β-carotene/ml extract and orthophosphoric acid.

BA and $FeCl_3$ were present in all the reaction mixtures. The lower OD obtained when orthophosphoric acid was added (1 vs 2, 3 vs 4) are due to the diluting effect of the addition of a further 0.1 ml alcoholic solution. The absorption maximum remains at 535 nm when the vitamin E has been extracted from serum into xylene.

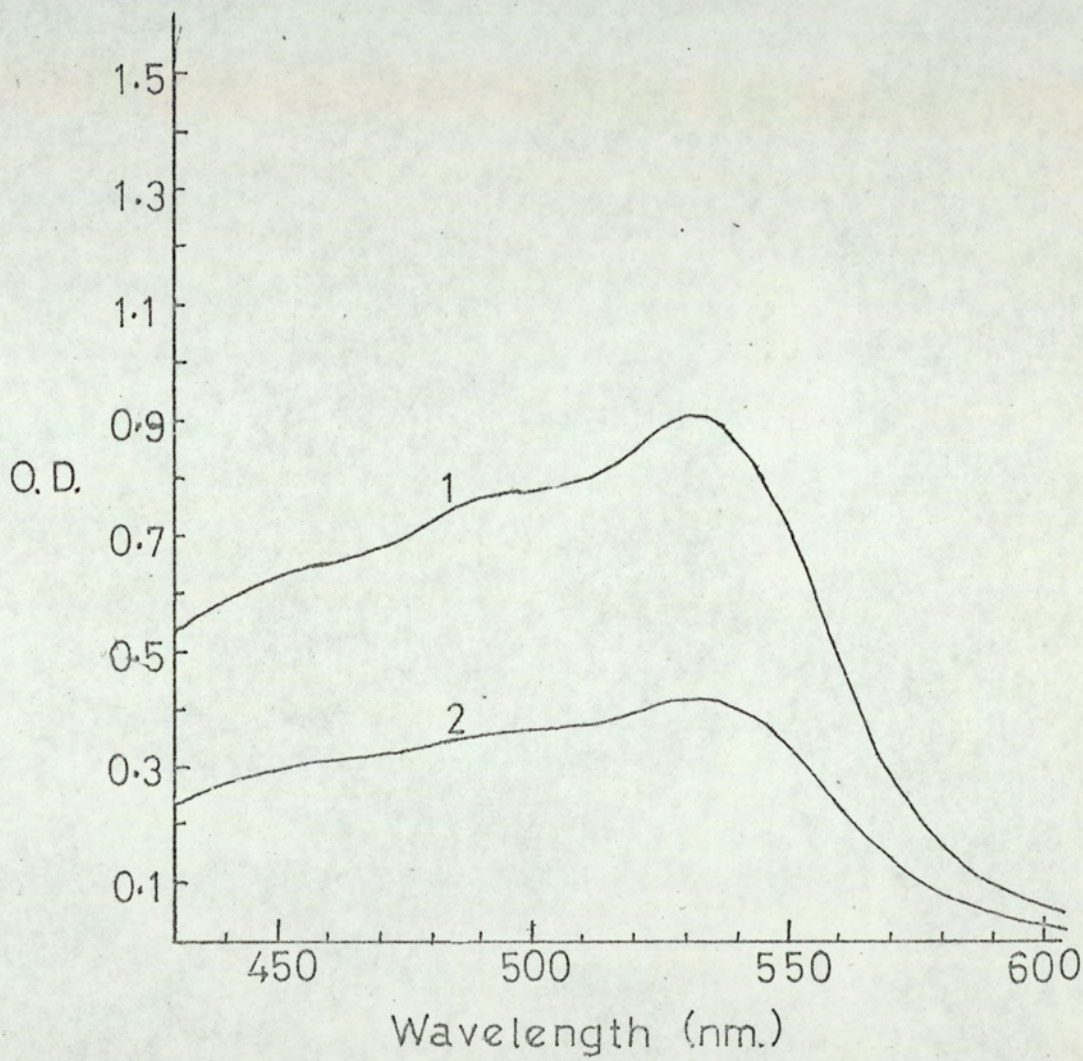


Fig. 2.2.1. The absorption spectra obtained when the 20 & 10 µg/ml working standards were scanned on a Unicam SP 800.

1) 20 µg/ml working standard.

2) 10 µg/ml working standard.

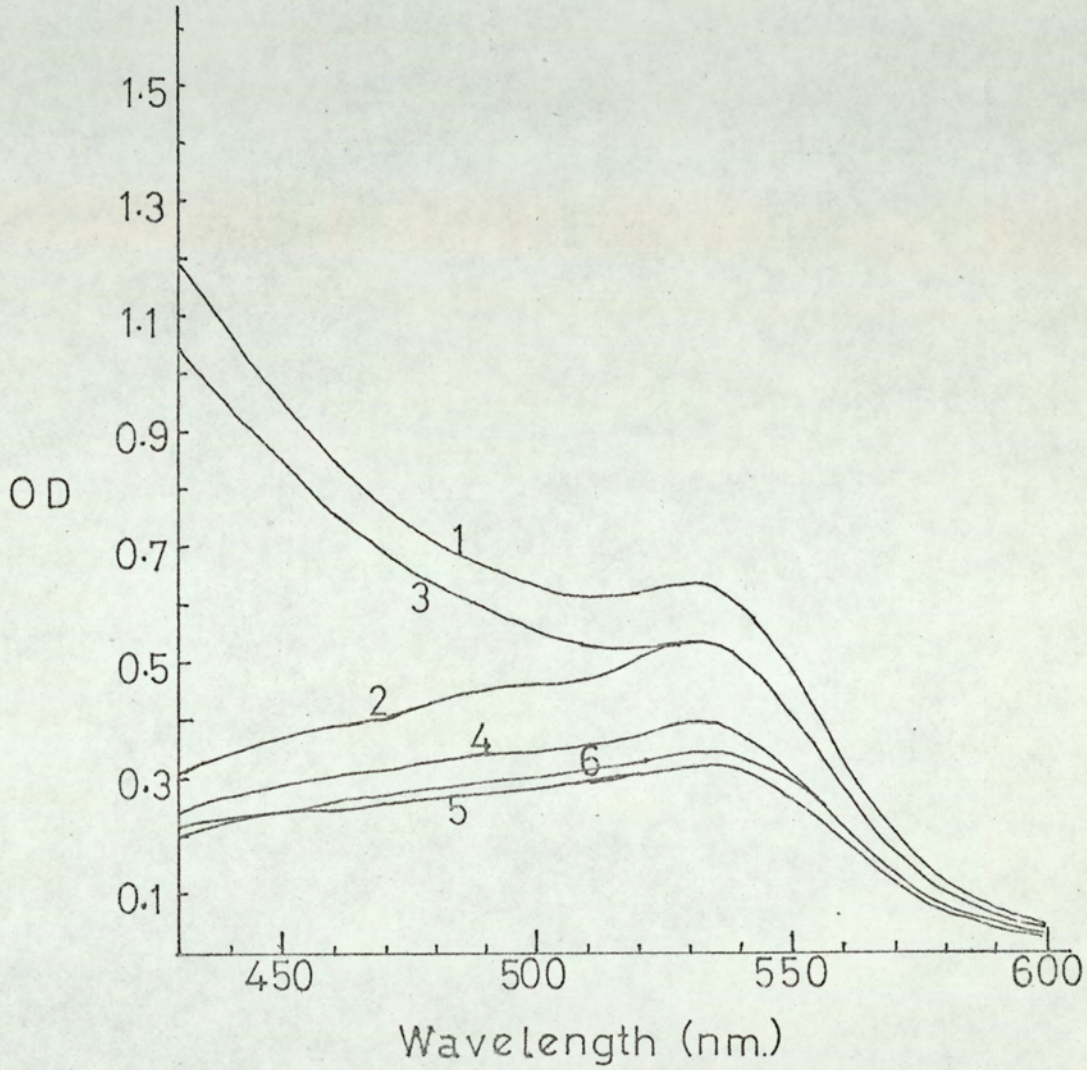


Fig 2.2.2. The absorption spectra obtained when the following reaction mixtures were scanned on a Unicam SP 800.

1) 10 µg α-tocopherol + 3 µg β-carotene / ml; no orthophosphoric acid.
2) 10 µg α-tocopherol + 3 µg β-carotene / ml and orthophosphoric acid.
3) 10 µg α-tocopherol / ml; no orthophosphoric acid.
4) 10 µg α-tocopherol / ml and orthophosphoric acid.
5) Serum extract and orthophosphoric acid.
6) Serum containing 3 µg β-carotene/ml extract and ortho-

2.2.5.2. Recovery of vitamin E from serum

Horse Serum Number 3 (Wellcome Reagents Ltd.) was used as a base from which to extract DL α-tocopherol. 0.1 ml of stock standard (100 µg/ml) was added to 20 ml horse serum and thoroughly mixed by gentle inversion for 10 mins. The serum was extracted and analysed for vitamin E content as described in 2.2.4.; horse serum, without added vitamin E was also analysed. The results are given in Table 2.2.1. and show that vitamin E is efficiently extracted from serum when using this method.

A secondary check on the efficiency of extraction of vitamin E from serum was made by re-extraction of the serum/ethanol residue. The vitamin E was extracted and analysed as described in 2.2.4., then the excess xylene was removed using a Pasteur pipette. A further 0.4 ml o-xylene was added to the residue; mixing and separation were carried out as before. This second xylene extract was analysed for vitamin E content. The results, given in Table 2.2.2., show that no more vitamin E can be extracted into o-xylene when the extraction is repeated.

2.2.5.3. An investigation of interference from carotene on vitamin E levels in serum.

β-carotene was obtained from Sigma (Type 111, crystalline, natural, from carrots) and a solution of 30 mg in 100 ml ethanol:xylene (1:1) was made. 0.1 and 0.05 ml of this solution were diluted to 10 ml in volumetric flasks with pooled human serum. The β-carotene solution was then diluted 1:5 with ethanol:xylene (1:1) and 0.1 and 0.05 ml of this solution were diluted to 10 ml as above. Similar dilutions of β-carotene were made with the 10 µg α-tocopherol/ml working standard; thus 3.0, 1.5, 0.6 and 0.3 µg β-carotene/ml were added to serum and a working standard of α-tocopherol. The serum was extracted as described in 2.2.4.; the carotene containing standard was treated in the same way as the

Table 2.2.1. The recovery of DLα-tocopherol from Horse serum No. 3.

Horse serum Vit E (μg/ml.)	Horse serum + DLα-tocopherol Vit E (μg/ml.)
0·1	6·8
0·3	5·0
0·9	4·8
0·1	5·4
1·0	5·4
1·9	5·9
0·5	4·9
0·4	4·9
0·7	7·0
$\bar{x}$ = 0·67	$\bar{x}$ = 5·57

Added 5 μg DLα-tocopherol / ml horse serum.

Recovered 5·57 - 0·67 = 4·90 μg/ml.

Recovery = $\frac{4·9}{5·0}$ x 100 = 98%.

Table 2.2.2. The effect on vitamin E levels obtained of extracting serum for a second time with o-xylene.

Serum	1st extraction µg vit. E / ml.	2nd extraction µg vit E / ml.
1	9·0	0·0
2	9·3	0·0
3	12·0	0·0
4	12·5	0·0

Table 2.2.3. The effect of β-carotene addition on the vitamin E levels obtained from serum extracts and the 10 µg/ml working standard . (Analyses in triplicate, mean and standard deviation).

µg β-carotene/ml added.	Vitamin E µg / ml.	
	Serum	Standard
Basal	5·9 ± 0·2	10·0
0·3	6·1 ± 0·6	9·9 ± 0·7
0·6	5·8 ± 0·4	10·1 ± 0·7
1·5	6·6 ± 0·3	10·1 ± 0·2
3·0	6·5 ± 0·7	10·1 ± 0·1

ethanol blank and working standards. Analyses were carried out in triplicate and the results given in Table 2.2.3., which shows the mean and standard deviation of the values obtained. There was no significant increase in the measured serum vitamin E level as a result of the addition of β-carotene.

The effectiveness of orthophosphoric acid in preventing interference from β-carotene was investigated. Pooled serum and pooled serum to which had been added 3 μg β-carotene/ml were extracted and analysed. The volumes used were increased by a factor of 5 so that standard size cuvettes could be used. The reactants were mixed in the cuvettes and placed in a Unicam SP 800 which was set to read the OD at 535 nm every 5 mins. The cuvettes were placed in such a way as to ensure that they were not exposed to the light beam between readings. In those reactions where orthophosphoric acid was omitted, the volume was adjusted by the addition of ethanol. A similar experiment was conducted on the 10 μg α-tocopherol/ml working standard, to which had been added 3 μg β-carotene/ml.

The results are presented graphically in Fig.2.2.3. and Fig.2.2.4. When orthophosphoric acid is added to the reaction mixture, there is a fall in OD of 0.02 and 0.03 for the serum extracts and 0.03 and 0.04 for the standards. This fall in OD corresponds with a change in colour of the reaction mixture from orange to pink. In the absence of orthophosphoric acid this colour change does not occur and there is an initial increase in the OD of the working standard solutions of 0.01 and 0.02. The OD of the serum extract continues to increase throughout the time course of the experiment. Since precautions were taken to ensure that none of the solutions was exposed to light between readings, it must be concluded that a continued reduction of ferric ions was taking place. It is unlikely that the continued reduction is due to the presence of vitamin E or β-carotene since standard solutions of these substances did not show this effect. The presence of

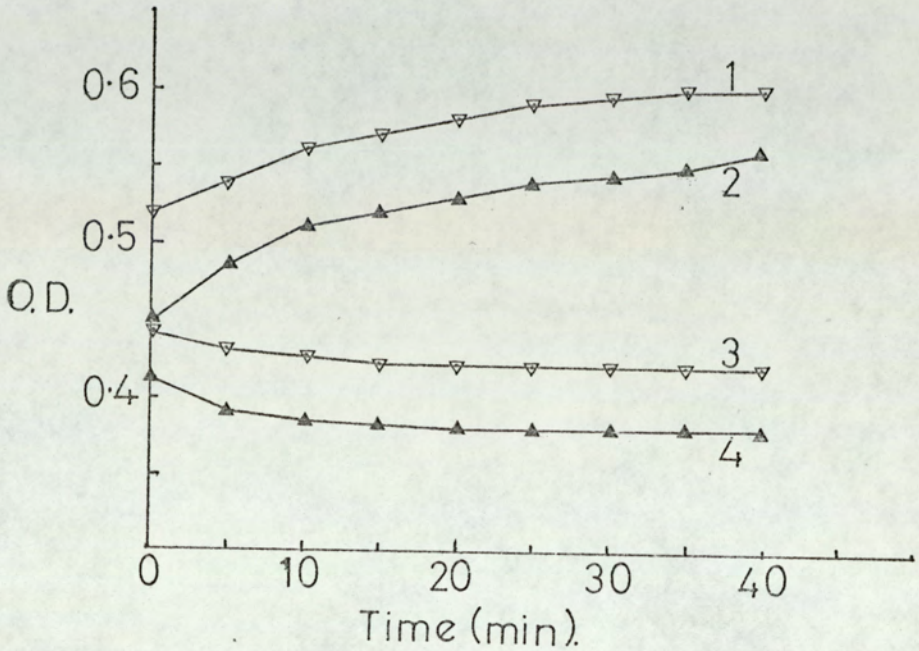


Fig.2.2.3. The effectiveness of orthophosphoric acid in preventing interference from β-carotene when measuring serum vitamin E levels.

1) Serum extract + BA + $FeCl_3$, no orthophosphoric acid.

2) Serum (containing 3 µg β-carotene/ml) extract + BA + $FeCl_3$, no orthophosphoric acid.

3) Serum extract + BA + $FeCl_3$ + orthophosphoric acid.

4) Serum (containing 3µg β-carotene/ml) extract + BA + $FeCl_3$ + orthophosphoric acid.

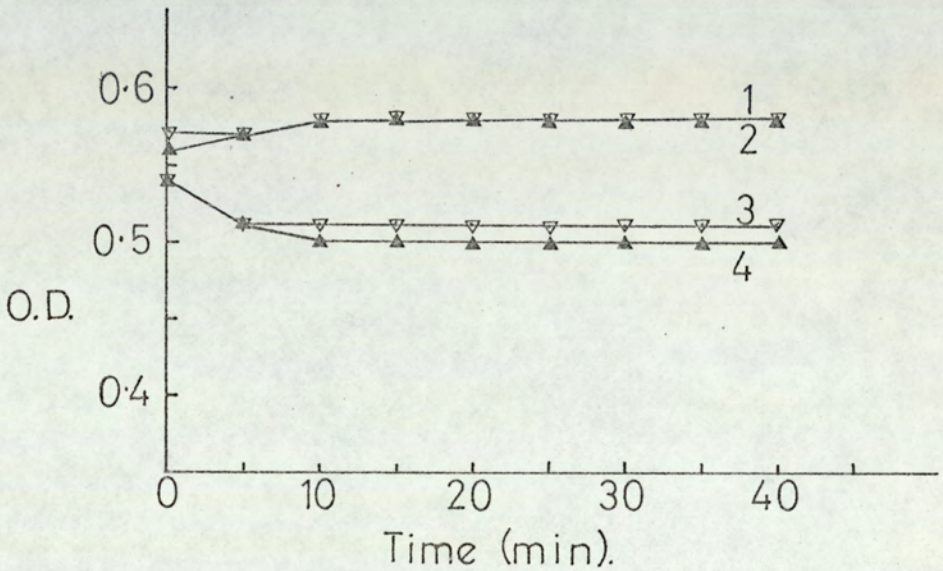


Fig.2.2.4. The effectiveness of orthophosphoric acid in preventing interference from β-carotene when measuring vitamin E levels.

1) 10 µg/ml vit.E working standard +BA +$FeCl_3$, no orthophosphoric acid.

2) 10 µg/ml vit.E working standard containing 3 µg/ml β-carotene + BA + $FeCl_3$, no orthophosphoric acid.

3) 10 µg/ml vit.E working standard + BA + $FeCl_3$ + orthophosphoric acid.

4) 10 µg/ml vit.E working standard containing 3 µg/ml β-carotene + BA + $FeCl_3$ + orthophosphoric acid.

orthophosphoric acid effectively inhibits the continued reduction.

2.2.5.4. An investigation of the effectiveness of orthophosphoric acid in preventing photo-chemical reduction of ferric ions

Extracts of pooled serum, pooled serum to which had been added 3 µg β-carotene/ml, working standard and standard containing 3 µg β-carotene were prepared as described in 2.2.5.3. OD's at 535 nm were read at 5 min intervals using a Unicam SP 600. Between readings the carrier containing the cuvettes was removed from the instrument and the reaction mixture was exposed to the light of the laboratory. Lids were placed on the cuvettes to minimise evaporation losses.

The results are shown graphically in Fig.2.2.5. and Fig.2.2.6. Photo-chemical reduction of the serum extract is reduced by an average of 87.5% and is eliminated in the case of the working standards. The working standard solutions again show an initial slight drop in OD, which is accompanied by the change in colour described in 2.2.5.3. The most rapid photo-chemical reduction of ferric ions takes place in the first five minutes.

2.2.5.5. Reproducibility

The within batch reproducibility has been calculated in two ways:-

(a) A pool of human serum was collected and deep frozen. This was thawed, mixed and 20 aliquots were removed for analysis of vitamin E content. From these 20 values the mean, standard deviation and coefficient of variation were calculated. The values are given in Table 2.2.4.

(b) The standard deviation of pairs was calculated from the formula

$$SD = \sqrt{\frac{\sum d^2}{(2N - 1)}}$$

where 'd' is the difference between the pairs and 'N' is the number of pairs used. For this calculation the duplicate measurements of sera

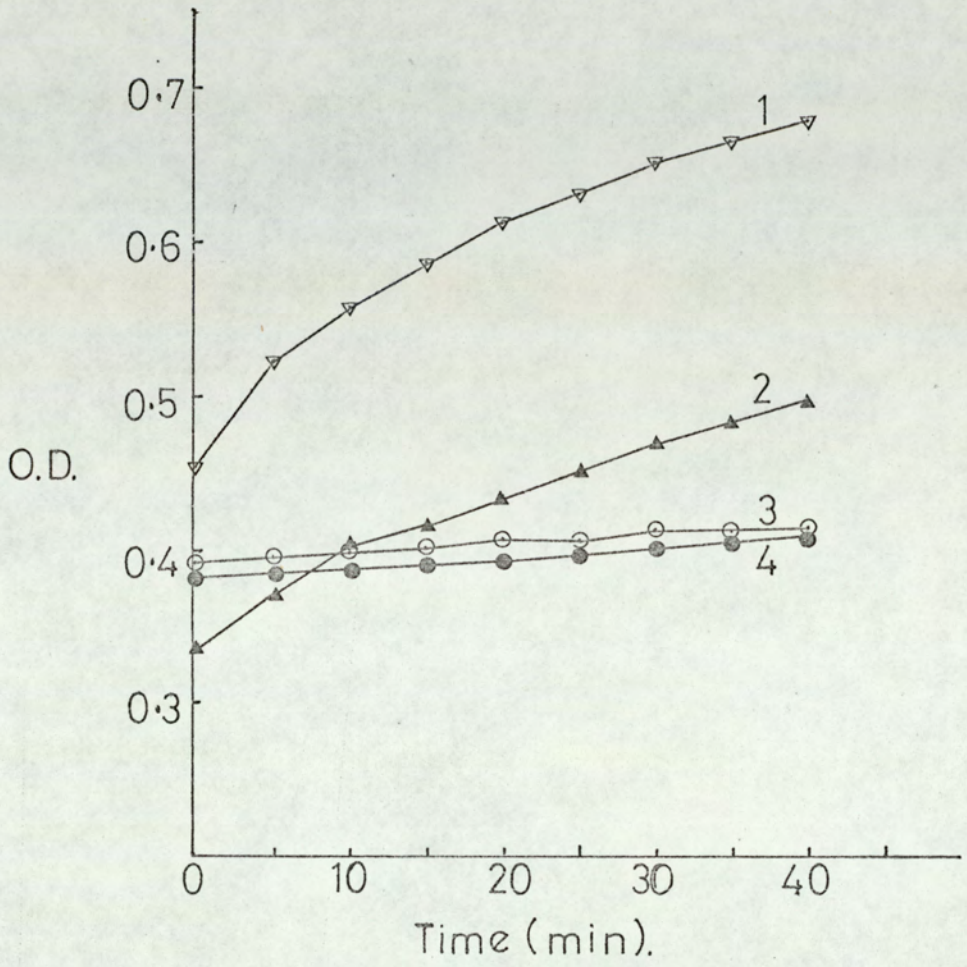


Fig.2.2.5. The effect of exposure to light of the following reaction mixtures.

1) Serum extract + BA + $FeCl_3$, no orthophosphoric acid

2) Serum (containing 3µg β-carotene/ml) extract +BA + $FeCl_3$, no orthophosphoric acid.

3) Serum extract +BA + $FeCl_3$ + orthophosphoric acid.

4) Serum (containing 3µg β-carotene/ml)extract + BA + $FeCl_3$ +orthophosphoric acid

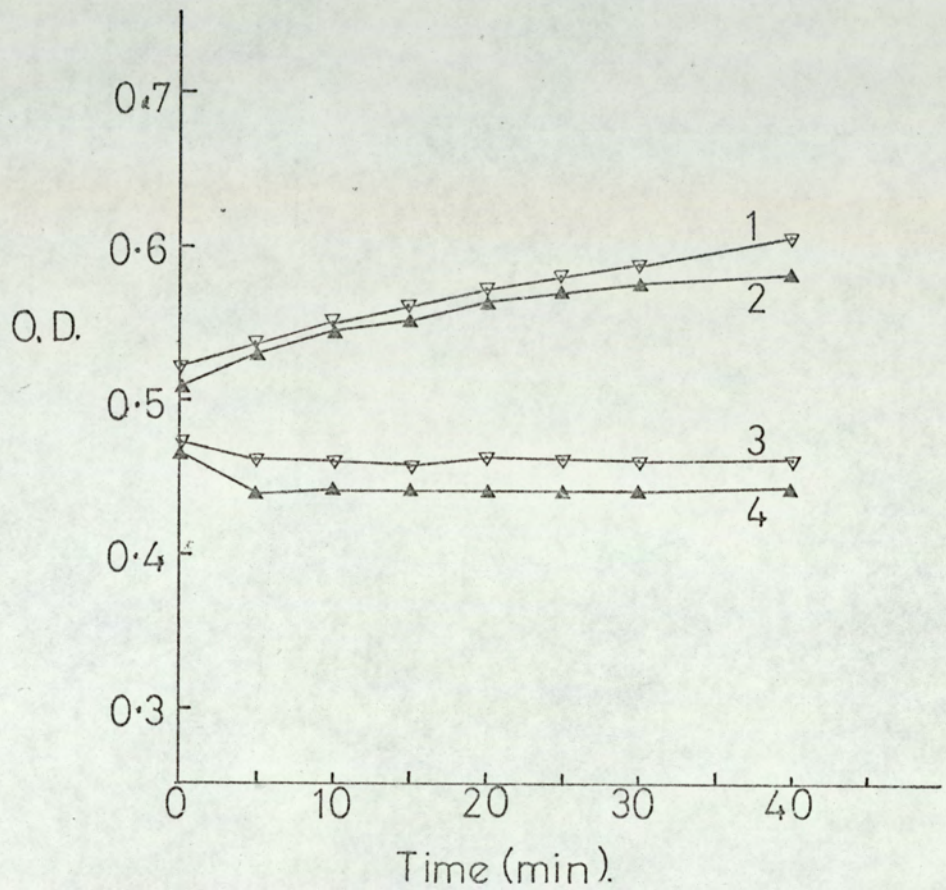


Fig. 2.2.6. The effect of exposure to light of the following reaction mixtures.

1) 10 µg/ml vit. E working standard + BA + $FeCl_3$, no orthophosphoric acid.

2) 10 µg/ml vit. E working standard containing 3 µg/ml β-carotene +BA + $FeCl_3$, no orthophosphoric acid.

3) 10 µg/ml vit. E working standard +BA +$FeCl_3$+orthophosphoric acid.

4) 10 µg/ml vit. E working standard containing 3 µg/ml β-carotene +BA +$FeCl_3$ + orthophosphoric acid.

obtained for establishing a normal range of vitamin E values were used. The standard deviation and range of values found are given in Table 2.2.4.

The between batch reproducibility has been calculated in two ways.

(c) The pool of human serum used for calculating (a) above was split into 4 ml portions and stored at -20° until required for use. One portion of this pool was thawed and analysed with each batch of serum assayed over a period of 6 months.

Baxter Normal Serum (Hyland) has also been used to measure between batch variation. Contained within the 25 repeat analyses there are 6 reconstitutions of different bottles of dried serum q.v. Table 2.2.4.

(d) The standard deviation of pairs for serum analysed in consecutive batches has been calculated as in (b) above. The standard deviation and the range of values found are given in Table 2.2.4.

There is very little difference in the standard deviation of pairs; neither is there a significant difference in the coefficient of variation when comparing the within batch pooled serum and Baxter Normal repeats. The relatively large coefficient of variation of the between batch pooled serum may be due to the gradual deterioration of the serum when stored at -20° over a period of months.

2.2.5.6. <u>The effect on vitamin E levels of freezing and thawing serum</u>

5 ml of pooled human serum was stored at -20° and thawed and analysed with six successive batches. The results are given in Table 2.2.5. and show that freezing and thawing of the serum sample had no significant effect on the measured level of vitamin E.

2.2.6. <u>Discussion</u>

The inclusion of orthophosphoric acid in the reaction mixture effectively eliminates reduction of ferric ions by substances other than vitamin E. In view of the photo-chemical reduction, which takes place very

Table 2.2.4. Reproducibility of method used.

Serum description	n	$\bar{x}$	SD	CV/Range.
a) Pooled serum (within batch)	20	5·9	0·35	5·9%
b) SD Pairs (Normal Range)	86	—	0·48	3·3-16·2
c) Pooled serum (between batch)	9	5·4	0·48	9·1%
Baxter Normal	25	9·4	0·57	6·1%
d) SD Pairs (next day repeats)	12	—	0·44	7·5-17·7

$$\text{Coefficient of variation (CV)} = \frac{SD}{\bar{x}} \times 100$$

Table 2.2.5. The effect of freezing and thawing on the measured level of vitamin E in serum.

Times frozen and thawed	Vitamin E µg/ml.
1	11·7
2	12·3
3	10·0
4	11·9
5	11·2
6	10·5

rapidly in the first 5 mins after the addition of $FeCl_3$, the accuracy of the method is improved by the addition of the orthophosphoric acid immediately after the $FeCl_3$ has been mixed with the BA and xylene extract already in the tube. The numbers of specimens analysed in each batch are such that the development of the pink colour has occurred in the first tube before the reagents have been added to the last tubes. It is important to add the $FeCl_3$ before the orthophosphoric acid. If the order is reversed no colour development occurs, since all the ferric ions are chelated before they can be reduced to ferrous ions by the vitamin E. This phenomenon can be used to obtain a blank reading for any extract which is coloured. With serum extracts this happens infrequently. The blank values obtained for 10 extracts from serum are given in Table 2.2.6. All samples were read against distilled water. In all instances the serum extract blank had an OD less than that of the ethanol blank which is routinely used for zeroing the Uvispek.

The range of β-carotene concentrations added to serum to assess the efficiency of orthophosphoric acid in the prevention of interference from carotenes, spans the range of serum levels given by Binder et al (1965). The mean value for serum carotene for normal subjects (i.e. those with no gastrointestinal disease) was 179.8 µg/100 ml with a standard deviation of 17.6 and range of 60-323 µg/100 ml. Bieri et al (1964) give a mean value for serum carotene in 71 men of 133 ± 47 µg/100 ml and in 61 women of 158 ± 62 µg/100 ml.

Criteria for acceptable limits of error for an analysis were first proposed by Tonks (1963). These are that the limits for analysis should be set at $\pm \frac{1}{4}$ of the "normal range" or $\pm$ 10% of the true value. If the maximum allowable error for a method of analysis be 'E', the between batch standard deviation 'S' and the "normal range" 'W' (where 'W' is the difference between the upper limit and lower limit of the range),

Table 2.2.6. The effect of adding orthophosphoric acid before $FeCl_3$ on the ODs of serum extracts. Distilled water was used as a blank.

Specimen	OD 1	OD 2	µg Vit E / ml.
Blank (EtOH)		0·098	0·0
Blank (xylene)	0·071	0·165	2·1
Serum extract 1	0·040	0·382	8·4
" " 2	0·030	0·427	9·7
" " 3	0·045	0·554	13·3
" " 4	0·027	0·515	12·2
" " 5	0·023	0·462	10·7
" " 6	0·044	0·348	7·3
" " 7	0·048	0·492	11·7
" " 8	0·039	0·641	15·9
" " 9	0·043	0·485	11·3
" " 10	0·033	0·601	14·7

OD 1 orthophosphoric acid added before $FeCl_3$.

OD 2 $FeCl_3$ added before orthophosphoric acid.

from Tonks' first criterion

$$E = \frac{W}{4}$$

Assuming that analytical error obeys the Gaussian law of distribution, then the error of the method should exceed 2S on less than one occasion in 20, and we may, therefore, write:-

$$2S = \frac{W}{4} \quad \text{or} \quad S = \frac{W}{8}$$

For the normal range found for serum vitamin E levels here, the maximum allowable error would be $\pm$ 1.6 µg/ml or, as a percentage of the mean, $\pm$ 15.7%.

From the second criterion, i.e. the maximum allowable error is less than $\pm$ 10% of the best estimate of the true value, we can write:-

$$2S = \frac{V}{10} \quad \text{or} \quad S = \frac{V}{20}$$

where 'V' is the best estimate of the true value.

$$\text{But} \quad \frac{S}{V} \times 100 = \text{Coefficient of variation} = 5.$$

From Table 2.2.4. the between batch variations are 6.1% and 9.1%, which exceeds the second criterion. The theoretical limits of error based on the first criterion $\pm$ 1.6 µg/ml reflects the rather broad nature of the "normal range". In 1968, Tonks revised his limits of allowable error to $\pm$ 10% coefficient of variation between batches, since experience had shown that the original criteria could not always be achieved in practice. The coefficient of variation for within batch repeats is almost identical to that quoted by Quaife et al (1949).

The method has proved to be satisfyingly robust. There has been only one major problem during three years use, namely, an impure batch of BA. The first indication of trouble was a fall in the measured value of vitamin E in the pooled serum which was used as a check on accuracy.

On that occasion two batches of BA had been mixed to give a solution of the required concentration. For the next batch of analyses only newly purchased BA was used and it was noticed on visual inspection that the OD of the blanks and standards was much higher than usual. The OD of the blank, read against distilled water, was 0.657 as opposed to the normal 0.07. A new sample of BA was acquired which gave an OD of 0.069 for the blank when read against distilled water. The presence of an impurity which reduces ferric ions was immediately noticeable when the contaminated reagent was used on its own, but forewarning was given by a change in the measured level of vitamin E for the repeating pooled serum.

Bieri and Prival (1965) have found that direct measurement of tocopherols, as occurs when any method based on the reducing powers of tocopherols is used, gives a result 10-12% greater than when α-tocopherol only is determined. The increase was due to the presence of β- and γ-tocopherol, which were measured together with α-tocopherol in the direct method. Since the objective of this project was to find the incidence of patients who were deficient in vitamin E, as measured by low serum levels, it was considered that chromatographic losses would have been too great to allow accurate determination of serum α-tocopherol. The ratio of red cell to plasma α-tocopherol has been reported as 0.244 for human blood (Bieri et al (1970)) and was found to be constant, the serum level of vitamin E was assumed to bear a constant relationship to that of the red cell.

2.3. Clinical Results

2.3.1. Normal Range

The normal range for serum vitamin E for the method of Fabianek et al (1968) was established by analysing sera samples obtained from blood donors via the Blood Transfusion Service (see section 2.2.2.2.). The samples were analysed in duplicate, where possible, (86 out of 97) in 5 batches, over a period of 10 days. The results are given in Table 2.3.1. and the relative cumulative frequency of the values obtained is shown in Fig.2.3.1. There was no statistical significance between the means of the groups of normals shown in Table 2.3.1. The vitamin E levels of those blood donors below 5 µg/ml were 3.3 (man), 3.5 and 4.8 (women) µg/ml.

The sera of 86 non-gastrointestinal in-patients were also analysed for vitamin E content, these results are included in Table 2.3.1. The relative cumulative frequency of these values is shown in Fig.2.3.1. There was no statistically significant difference between the mean serum vitamin E when the non-gastrointestinal in-patients were compared with the blood donors.

The normal range of serum vitamin E has been defined as the blood donor mean $\pm$ 2 standard deviations from that mean i.e. 5.2-15.2 µg/ml serum.

2.3.2. Adult Coeliac Disease

Vitamin E levels in the sera of 85 patients have been measured. The majority of the samples were obtained from patients attending the out-patient clinics and an effort was made to obtain serum from patients who had had blood taken for other analyses but for whom serum vitamin E had not been requested. The mean level of serum vitamin E for all the patients studied is given in Table 2.3.1.

Table 2.3.1. Serum vitamin E levels obtained from normal subjects and patients with gastro-intestinal disorders.

Category	Serum vitamin E (µg / ml). n	$\bar{x}$	SD	Range	t	Significance
Normal M+F	97	10·2	2·50	3·3 - 16·2	a) 0·83	NS
Normal M	52	10·1	2·59	3·3 - 16·2	a) 0·83	not significant
Normal F	45	10·3	2·43	3·5 - 15·0		
Normal 25	15	9·3	2·64	5·9 - 16·2	b) 1·51	not significant
Normal 25	82	10·4	2·46	3·3 - 15·0		
Non G-I in-patients	86	9·6	3·88	1·7 - 18·7	c) 1·35	not significant
ACD (all)	85	7·0	3·29	0·0 - 15·2	d) 7·35	p = <0·001
RE (all)	89	8·6	3·64	0·0 - 17·6	d) 3·48	p = <0·001
UC (all)	36	9·2	2·35	3·0 - 12·7	d) 2·07	p = 0·05
Gastrectomy	10	6·1	1·89	3·3 - 8·9	d) 4·96	p = <0·001

M = male F = female

a) 't' test between normal males and females.

b) 't' test between those normals aged 25 years and those aged 25 years.

c) 't' test between the means obtained for blood donor normals and non-gastro-intestinal (non G-I) in-patients.

d) 't' test between the mean of blood donor normals and the means of various groups of gastro-intestinal patients.

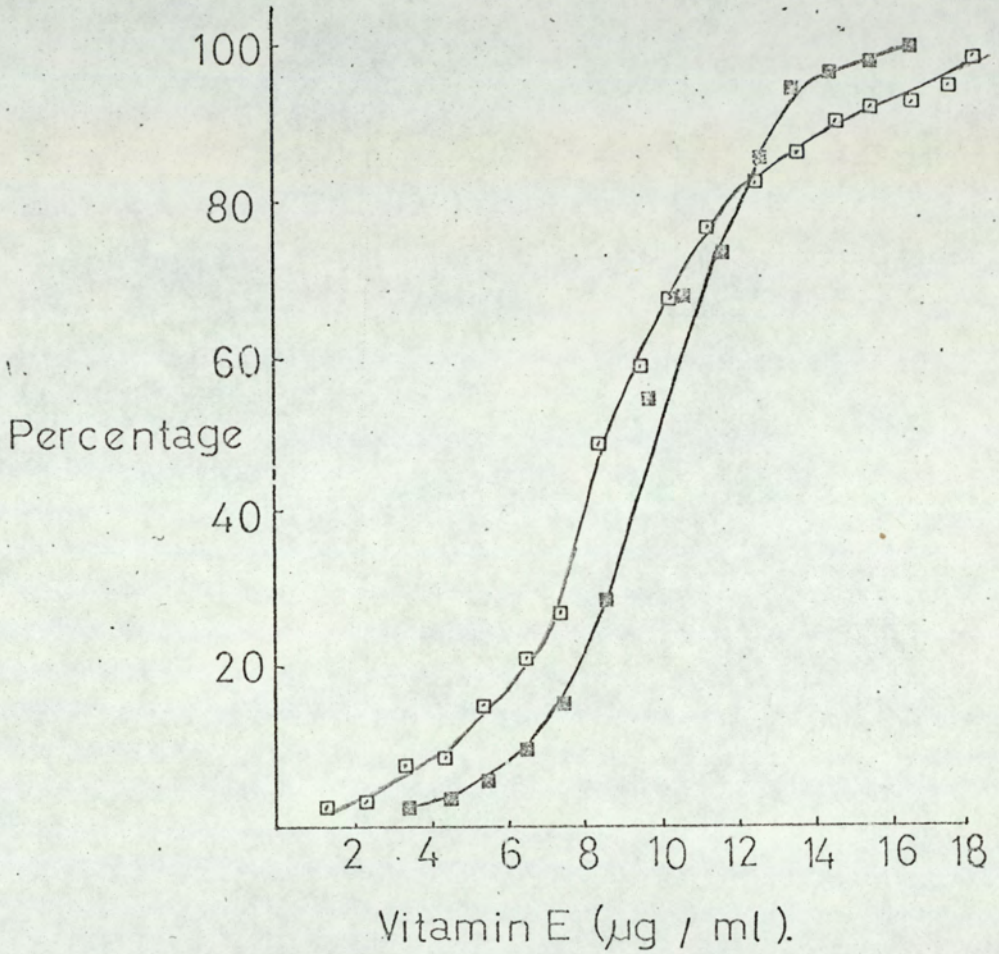


Fig. 2.3.1. Relative cumulative frequency of serum vitamin E for blood donors and non-gastro-intestinal in-patients.

Where the serum of a patient has been analysed on more than one occasion the first measurement has been included in all calculations. Table 2.3.2. gives the mean vitamin E levels of different categories of patients. Comparisons of the means obtained have been made using the Students 't' test, the results are shown in Table 2.3.3. Fig.2.3.2. shows the relative cumulative frequency of the values obtained for the ACD patients compared with the blood donor normals. 29% of patients were found to have serum vitamin E levels of less than 5 µg/ml.

There is a significant lowering of serum vitamin E levels in all groups of ACD patients compared with the blood donor normals. The influence of treatment with a gluten free diet is seen with the rise in serum levels of those on a strict gluten free diet when compared with those who admit to not adhering strictly to their diet and those whose diet still contains gluten; the difference in levels of those on a strict gluten free diet and those on a normal diet is statistically significant. Those patients who have been found to need supplementation of their diet with either folic acid and/or calciferol have statistically significantly lower vitamin E levels than those who do not need supplementation with these two other vitamins.

Correlation coefficients have been calculated between vitamin E levels and 9 other compounds measured in the same blood sample. These other determinations were performed as part of the routine screening of the patients which takes place whenever they attend the out-patient clinic. Patients on supplements of folic acid were excluded from the correlation of vitamin E and folate. For the correlation of vitamin E and faecal fat, only those measurements of fat excretions performed within one month of the measurement of serum vitamin E level were included. The results are presented in Table 2.3.4. A significant correlation was found between serum

Table 2.3.2. Mean serum vitamin E levels obtained for various groups of patients with ACD.

Group	Serum vitamin E (μg / ml).		
	n	$\bar{x}$	SD
All	85	7·03	3·29
Males	31	7·23	3·76
Females	54	6·92	3·02
GFD (strict)	41	7·76	3·54
GFD (not strict)	8	6·54	2·37
GFD ('yes' + strict)	61	7·69	3·47
Not on GFD	18	5·50	2·72
On folate	37	5·45	3·00
Not on folate	51	8·28	3·03
On calciferol	24	5·70	3·41
Not on calciferol	65	7·70	3·23

Table 2.3.3. Students 't' test on the means obtained for the different groups of ACD patients.

Comparison	t	DF	Significance
ACD males vs females	0·41	83	not significant
GFD (strict) vs no GFD	2·33	57	$p = 0·02$
No vs on calciferol	2·52	87	$0·02 \geq p > 0·01$
No vs on folate	4·29	86	$p = < 0·001$
Normals vs ACD (all)	7·35	180	$p = < 0·001$
Normals vs GFD (strict)	4·66	136	$p = < 0·001$
Normals vs GFD (not strict)	3·97	103	$p = < 0·001$
Normals vs GFD (strict+yes)	5·26	156	$p = < 0·001$
Normals vs not on GFD	7·17	113	$p = < 0·001$
Normals vs no folate	4·11	146	$p = < 0·001$
Normals vs on folate	9·24	132	$p = < 0·001$
Normals vs no calciferol	5·52	160	$p = < 0·001$
Normals vs on calciferol	7·25	119	$p = < 0·001$

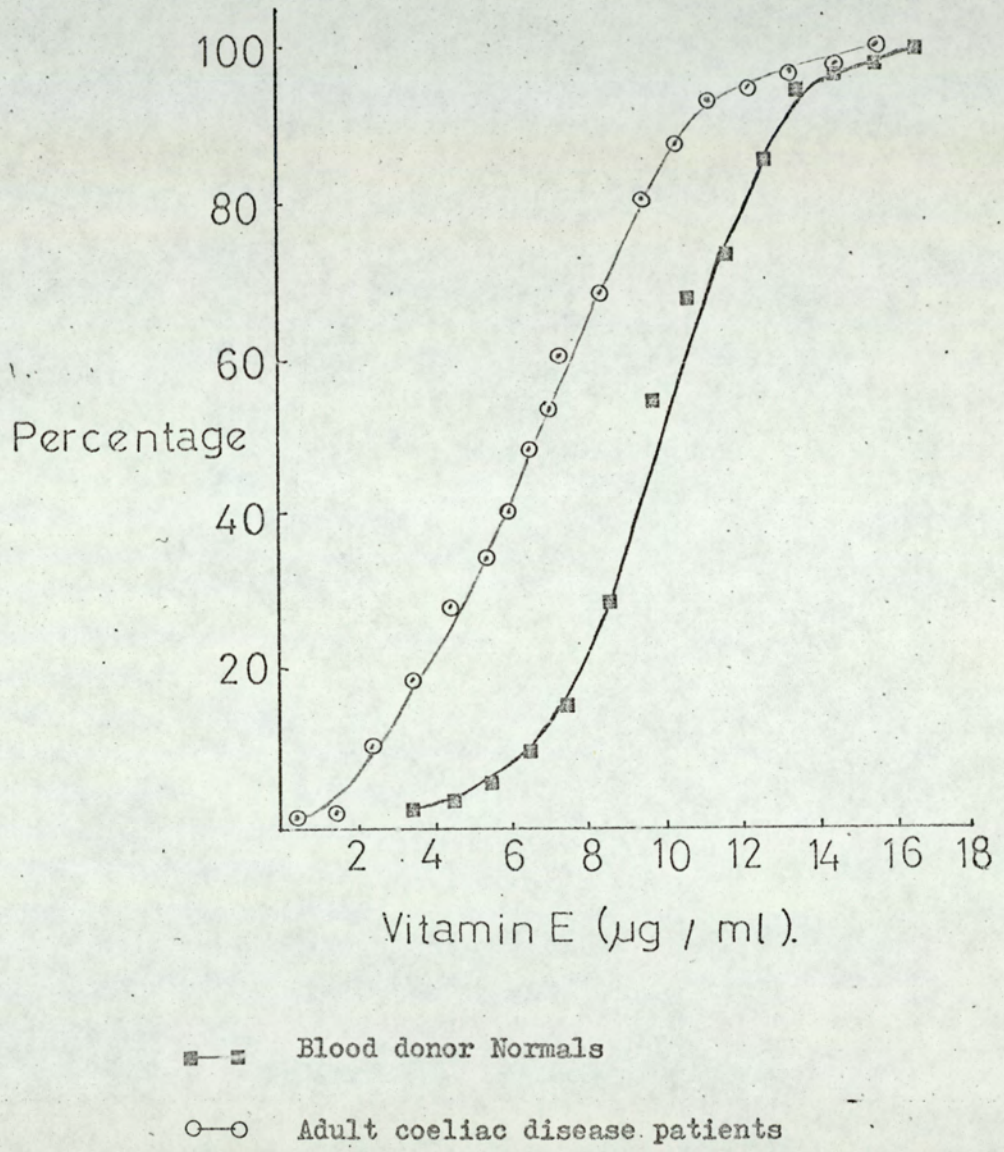


Fig. 2.3.2. Relative cumulative frequency of serum vitamin E for blood donors and ACD patients.

Table 2.3.4. Correlation coefficients of serum vitamin E and various other parameters measured in the same blood sample. (Faecal fats were measured within 1 month of the vitamin E measurement.)

Compound	r	DF	t	Significance
Faecal fat	-0·535	61	9·26	$p = < 0·001$
Heamoglobin	0·115	85	1·07	not significant
MCV	0·011	85	0·10	not significant
Folic acid	0·270	55	2·08	$0·05 > p > 0·025$
Albumin	0·351	85	3·44	$p = < 0·001$
Seromucoids	0·272	61	2·21	$p = 0·025$
Iron	0·360	81	3·46	$p = < 0·001$
IBC	0·226	80	2·07	$0·05 > p > 0·025$
Alkaline phosphatase	0·072	83	0·66	not significant
Potassium	0·029	85	0·27	not significant

vitamin E and

(a) faecal fat excretion
(b) serum folic acid
(c) serum albumin
(d) serum seromucoids
(e) serum iron
(f) serum iron binding capacity

2.3.3. Regional enteritis

Vitamin E levels in the sera of 89 patients have been measured. The sera were obtained in circumstances similar to those described above. The number, mean and standard deviation of all the RE patients is shown in Table 2.3.5., together with those of various categories of these patients. The relative cumulative frequency of the values obtained is shown in Fig.2.3.3. 16.5% of the patients with RE were found to have serum vitamin E levels of less than 5 µg/ml.

There was a statistically significant lowering of the mean serum vitamin E when the RE's as a whole were compared with the blood donor normals but, amongst the women RE patients, there was no significant lowering of the level (see Table 2.3.6. for the results of the Students 't' tests performed). Although those patients judged to be in remission had a higher mean serum level than those in relapse, there was no statistical difference between the two groups. Those patients with ileal involvement had significantly lower mean serum vitamin E levels than those with terminal ileal and colonic involvement. When the various categories of RE patients were compared with the blood donors there was a significantly lowered mean for all groups except the women patients, those with terminal ileal and colonic involvement and those who had had no operation.

Correlation coefficients between vitamin E and 8 other compounds measured in the same blood sample have been calculated. The results

Table 2.3.5. Mean serum vitamin E levels of various groups of patients with RE.

Group	Serum vitamin E (μg / ml).		
	n	$\bar{x}$	SD
All	89	8·62	3·64
Males	41	7·71	3·96
Females	48	9·40	3·17
Remission	75	8·94	3·22
Relapse	27	7·75	4·42
Area of involvement			
1) ileum	36	7·82	3·87
2) terminal ileum + colon	41	9·46	3·50
3) colon	14	8·25	2·64
Operations			
1) None	29	9·31	3·73
2) terminal ileal resection	39	9·02	3·35
3) ileal resection	12	6·92	3·72
4) ileostomy	10	8·32	3·32

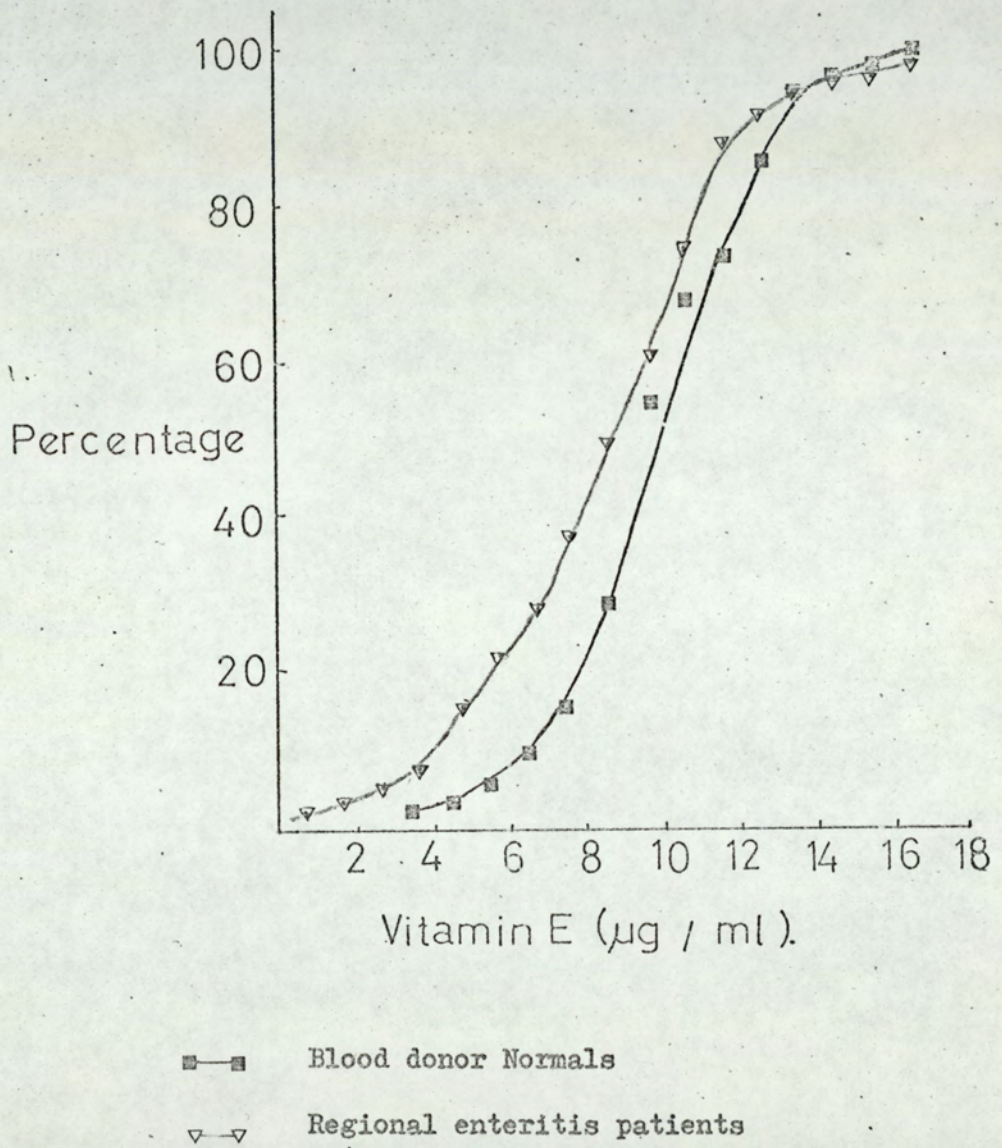


Fig. 2.3.3. Relative cumulative frequency of serum vitamin E for blood donors and RE patients.

Table 2.3.6. Students 't' test on the means obtained for the different groups of RE patients.

Comparison	t	DF	Significance
RE males vs females	-2·21	87	$0{\cdot}025 \geq p > 0{\cdot}0125$
Remission vs relapse	1·47	100	$0{\cdot}10 > p > 0{\cdot}05$
Normals vs RE (all)	3·48	184	$p = < 0{\cdot}001$
Normals vs RE (male)	4·43	136	$p = < 0{\cdot}001$
Normals vs RE (female)	1·66	143	$0{\cdot}10 > p > 0{\cdot}05$
Normals vs remission	2·90	170	$0{\cdot}05 > p > 0{\cdot}01$
Normals vs relapse	3·71	122	$p = < 0{\cdot}001$
Normals vs area of involvement			
1) ileum	4·14	131	$p = < 0{\cdot}001$
2) terminal ileum + colon	1·41	136	not significant
3) colon	2·70	109	$0{\cdot}01 > p > 0{\cdot}005$
Normals vs operations			
1) None	1·49	124	not significant
2) terminal ileal resection	2·25	134	$0{\cdot}025 > p > 0{\cdot}02$
3) ileostomy	2·18	105	$0{\cdot}05 > p > 0{\cdot}025$
4) ileal resection	3·95	107	$p = < 0{\cdot}001$

(see Table 2.3.7.) show that there is a significant correlation between serum vitamin E and

(a) faecal fat excretion

(b) serum albumin

(c) serum iron

Only those patients for whom faecal fat measurements had been made within one month of vitamin E analysis were included.

2.3.4. Other gastrointestinal disorders

(a) Ulcerative colitis

Ulcerative colitis (UC) is a disease confined to the colon in which patients suffer from watery, bloody diarrhoea which varies in severity. In a number of cases there is an effect on liver function. The sera of these patients was collected in the same manner as described in section 2.3.2. The mean, standard deviation and range of values obtained for 37 patients are presented in Table 2.3.1. and the relative cumulative frequency of the results is presented in Fig.2.3.4. A Students 't' test was performed on the UC's mean and that of the blood donors and there was a statistically significant difference between the two means.

(b) Gastrectomy

Serum vitamin E levels have been measured in 9 patients with partial gastrectomies and one patient with a total gastrectomy. The mean, standard deviation and range of values for all 10 patients are given in Table 2.3.1. There was a very statistically significant lowering of serum vitamin E in patients who have had part or all of their stomach removed compared with the blood donors.

Table 2.3.7. Correlation coefficients of serum vitamin E levels of RE patients and various other parameters measured in the same blood sample. (Faecal fats were measured within 1 month of the vitamin E measurement.)

Compound	r	DF	t	Significance
Faecal fat	0·315	49	2·32	$p = 0·025$
Haemoglobin	0·193	87	1·84	$0·10 > p > 0·05$
MCV	0·111	87	1·04	not significant
Folic acid	0·084	68	0·69	not significant
Albumin	0·373	87	3·77	$p = < 0·001$
Seromucoids	-0·170	87	1·60	not significant
Iron	0·265	86	2·55	$0·02 > p > 0·01$
IBC	0·177	86	1·65	not significant
Potassium	-0·133	87	1·25	not significant

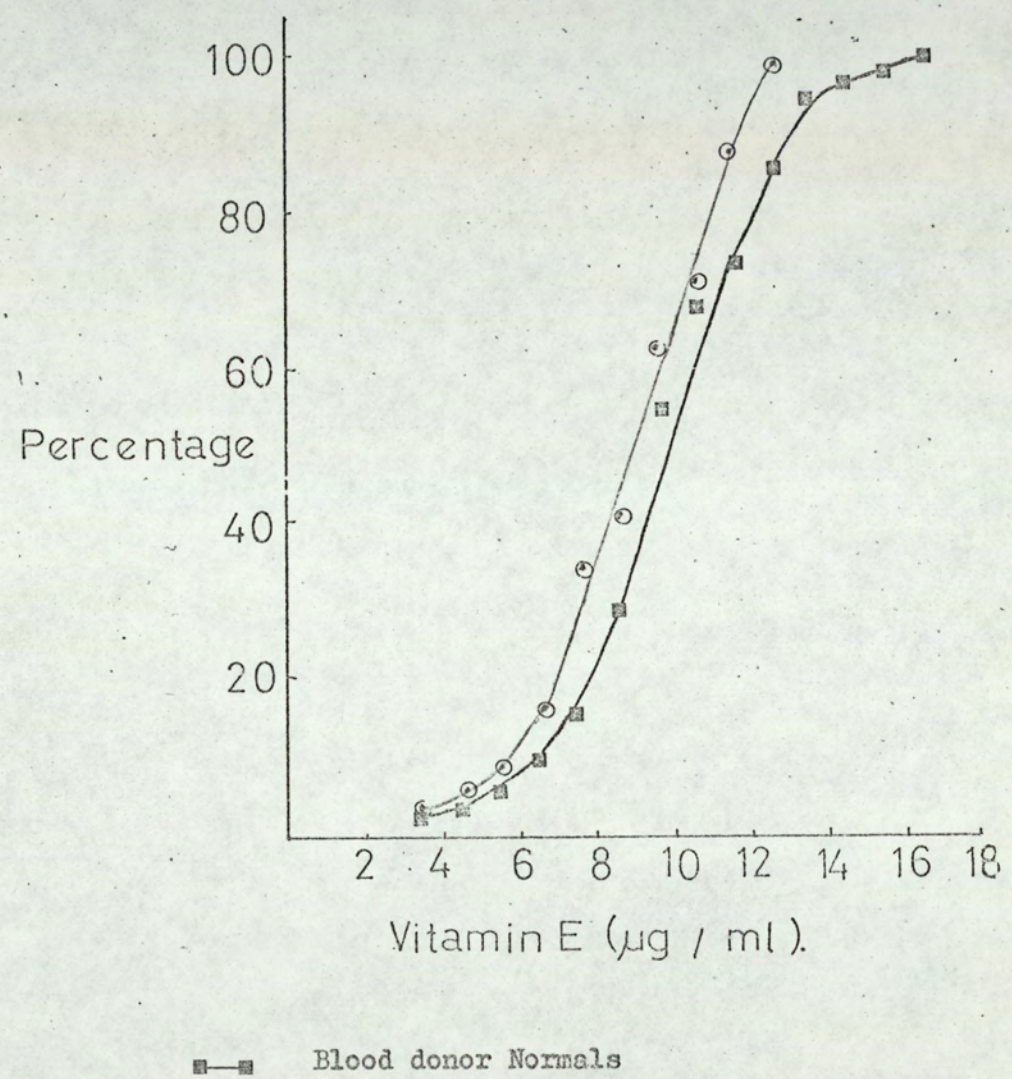


Fig. 2.3.4. Relative cumulative frequency of serum vitamin E for blood donors and UC patients.

2.3.5. The absorption of an oral dose of vitamin E (α-tocopherol acetate)

(a) Absorption over a 6 hr period

An oral dose of 200 mg α-tocopherol acetate was given to 5 patients and 2 normal volunteers. Samples of blood were taken before and 2, 4 and 6 hrs after dosing. All subjects were fasted over night prior to dosing but were permitted normal food after administration of the vitamin. The vitamin E levels of the serum taken during the test are shown in Table 2.3.8. As far as the normals are concerned there was an initial rise in the serum vitamin E levels which was sustained for the period of the test. For patients 2, 4 and 5 there was a delay in the rise of the serum level until 4 hrs after dosing, for patient 1 there was an initial rise then fall in the serum level. Patient 3 showed no appreciable change in serum levels of vitamin E.

(b) Absorption over a 2 hr period

It was felt possible that an early rise in serum vitamin E may have taken place which had been missed by delaying sampling until 2 hrs after the ingestion of the oral dose. Consequently, further tests were performed on patients and volunteers using the same dose of vitamin E (200 mg) but taking blood samples before and 15, 30, 60 and 120 mins after dosing. In this test the subjects were fasted over night and were not permitted to eat until the end of the test. The results are given in Table 2.3.9. and Fig.2.3.5. There was a rise in serum vitamin E in 15 mins following the administration of the oral dose in all subjects; in 2 of the 3 RE patients there was an overall fall in the serum level in the 2 hr period, although there was a general trend towards reduction of levels by the end of the 2 hr period compared with the highest level obtained during the test period.

Table 2.3.8. The absorption of an oral dose of vitamin E (200 mg ∝-tocopherol acetate).

Number	Diagnosis	µg vitamin E / ml serum at stated time(h)			
		0	2	4	6
1	RE	1·4	2·3	2·5	1·4
2	RE	3·0	3·0	3·7	3·2
3	ACD	2·7	3·1	3·1	2·8
4	ACD	3·8	3·9	4·4	4·6
5	ACD	4·2	4·0	4·6	5·2
6	Normal	13·4	14·9	14·6	14·9
7	Normal	11·1	12·2	12·1	11·9

Table 2.3.9. The absorption of an oral dose of 200 mg ∝-tocopherol acetate over a 2 h period.

Number	Diagnosis	vit E (µg / ml serum), time (min)					Increase in level	
		0	15	30	60	120	0 - 15 min	0 - 120 min
1	Normal	4·1	5·1	5·7	5·7	6·1	1·0	2·0
2	Normal	5·1	5·4	5·5	5·1	5·6	0·3	0·6
3	Normal	7·4	8·0	9·3	9·2	8·8	0·6	1·4
4	Normal	11·5	13·5	12·9	13·0	13·1	2·0	1·6
5	Normal	10·3	10·9	*10·6	9·5	11·7	0·6	1·4
6	RE	7·4	7·8	7·2	7·2	7·1	0·4	-0·3
7	RE	3·6	5·6	6·3	6·2	5·7	2·0	2·1
8	RE	4·4	4·9	4·9	4·6	4·1	0·5	-0·3

* sample taken after 45 min

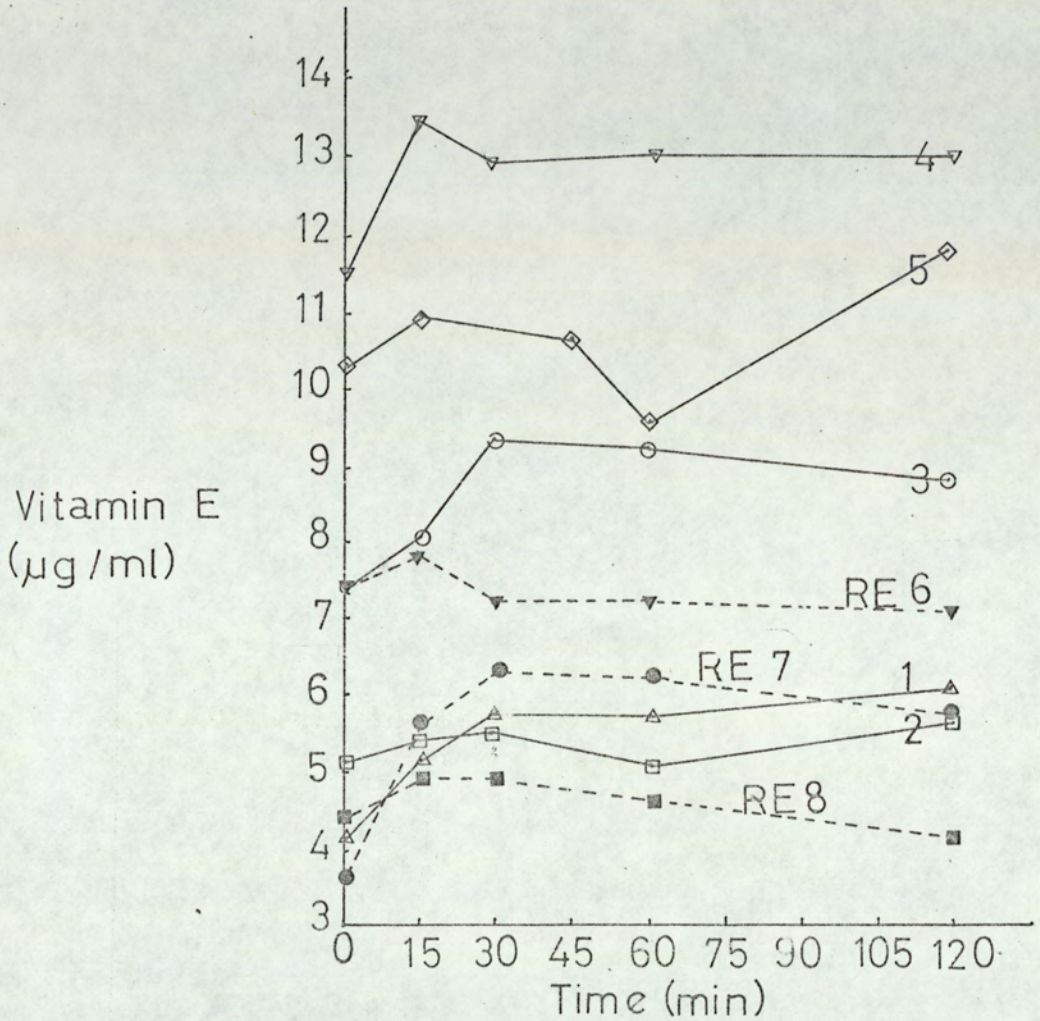


Fig. 2.3.5. The absorption of an oral dose of 200 mg α-tocopherol acetate.

2.3.6. Variation of vitamin E levels in serum over a period of time

Nineteen patients have had their serum vitamin E measured on more than 2 occasions. Table 2.3.10. shows the number of measurements made on each patient, the time span over which the measurements were made and the range of levels obtained. Of the 12 patients who were initially deficient, 7 had had rises in their serum level to the extent that they were no longer deficient. The results of Patient 19 have been presented in a separate Table, i.e. Table 2.3.11., which shows the increase in serum levels after a course of supplementation and their decline after reduction in the level of the supplement.

2.3.7. Vitamin E and cholesterol levels in serum obtained from the hepatic vein, the hepatic portal vein and the antecubital vein

The sera used were supplied by Dr. V. Melikian. The patients were suffering from a variety of liver disorders (see Table 2.3.12. and Table 2.3.13.) and had catheters placed in either the hepatic vein or the hepatic portal vein for the purpose of liver perfusion. When the blood was withdrawn for vitamin E and cholesterol determination the patient has been fasting for at least 6 hrs. The serum was separated and stored at -20° until analysed. The cholesterol was measured by the method of Huarg, Chen, Wefler and Raftery (1961), as part of a routine batch on an Auto-analyzer. The vitamin E measurements were made in duplicate, the standard deviation of pairs = 0.5 µg/ml. The results of the 6 patients in whom the catheter was placed in the hepatic portal vein are shown in Table 2.3.12. There was no consistent change in the levels of either vitamin E or cholesterol between the hepatic portal blood

Table 2.3.10. Variation of vitamin E levels in serum over a period of time.

Patient	Diagnosis	Number of measurements	Time span	Range
1	RE	3	8/69 - 3/70	2•8 - 4•9
2	ACD	8	9/69 - 9/70	0•0 - 6•4
3	ACD	7	10/69 - 9/70	0•0 - 4•6
4	ACD	5	10/69 - 5/70	4•3 - 8•4
5	ACD	7	10/69 - 9/70	1•7 - 3•8
6	ACD	3	9/69 -11/70	5•4 - 5•7
7	ACD	5	12/69 - 7/71	4•6 - 6•5
8	RE	5	10/69 - 6/72	1•0 - 4•3
9	ACD	3	10/69 - 7/71	3•3 - 10•0
10	ACD	3	6/69 - 6/72	5•3 - 7•4
11	ACD	3	11/69 - 7/70	9•2 - 16•3
12	RE	7	8/69 - 9/70	0•0 - 4•6
13	ACD	4	5/71 - 6/72	0•0 - 2•9
14	ACD	3	6/70 - 7/71	5•4 - 6•2
15	ACD	4	8/69 - 7/71	4•3 - 7•7
16	ACD	3	10/69 - 7/70	2•8 - 6•4
17	ACD	3	12/69 - 11/70	5•5 - 7•2
18	RE	5	12/69 - 5/72	5•5 - 8•7
19	ACD	10	10/69 - 4/72	1•8 - 14•2

Table 2.3.11. Variations of vitamin E levels in serum of patient 19 over a period of time.

Date	µg vitamin E / ml	Therapy
10/69	2·5	
12/69	1·8	Started oral vitamin E, dose - 250 mg twice daily
1/70	14·2	
2/70	9·2	Discharged from hospital 2/70 on 200 mg twice daily
3/70	10·2	
16/4/70	6·7	
27/4/70	4·9	4/71 supplement reduced to 50 mg daily
5/71	3·0	
6/71	2·9	Oral vitamin E, dose 50 mg twice daily
4/72	8·8	

Table 2.3.12. Vitamin E and cholesterol levels in serum taken simultaneously from the hepatic portal and ante-cubital veins.

Number	Hepatic portal(P)		Venous (V)		Difference (V-P)	
	Vit. E	Cholesterol	Vit. E	Cholesterol	Vit. E	Cholesterol
1	5·5	60	5·6	55	+0·1	-5
2	4·9	165	6·1	185	+1·2	+20
3	4·7	105	1·7	90	-3·0	-15
4	8·3	380	6·4	355	-1·9	-25
5	13·6	175	13·9	175	+0·3	0
6	12·6	145	20·1	205	+7·5	+60

Patients 1-3,5 & 6 had carcinoma of colon with secondaries in the liver.

Patient 4 had carcinoma of the rectum.

Table 2.3.13. Vitamin E and cholesterol levels in serum taken simultaneously from the hepatic (H) and ante-cubital (V) veins.

	Hepatic vein (H)		Venous (V)		Difference (V-H)	
Number	Vit. E	Cholesterol	Vit. E	Cholesterol	Vit. E	Cholesterol
1	2·7	120	2·4	130	-0·3	+10
2	10·6	295	13·5	315	+2·9	+20
3	6·0	460	5·8	475	-0·2	+15
4	1·4	——	2·8	145	+1·4	——
5	10·5	230	10·0	250	-0·5	+20
6	7·7	175	6·5	140	-1·2	-35
7	8·2	170	7·9	165	-0·3	-5
8	0·5	140	0·7	145	-0·2	+5
9	8·9	180	7·6	185	-1·3	+5
10	11·9	200	13·2	198	+1·3	-2
11	14·4	165	12·8	170	-1·6	+5
12	11·4	175	7·9	170	-3·5	-5
13	8·2	125	8·2	130	0	+5
14	5·5	180	5·3	140	-0·2	-40

Patients 2-4, 6, 7, 10-14 had cirrhosis.

Patients 1 & 8 had alcoholic cirrhosis.

Patient 9 had cirrhosis and hepatoma

Patient 5 had infectious hepatitis.

and the peripheral circulation, neither was there a consistent change in the levels found in the hepatic vein and the peripheral circulation (see Table 2.3.13.). The correlation coefficient between vitamin E and cholesterol in the peripheral circulation has been calculated, $r = 0.228$, and is not statistically significant.

2.3.8. Discussion

The task of adequately assessing what values one might expect to find for a single serum constituent in healthy subjects is fraught with difficulty (Robinson (1971)). No attempt was made to assess the general health of the "normal" blood donor, other than is routinely performed by the Blood Transfusion Service itself. Leitner, Moore and Sherman (1960) measured serum vitamin E levels in more than 500 subjects in whom no serious disease had been detected. They found considerable variations for both sexes but seasonal variations were small and their mean value for equal numbers of each sex was given as 1.05 mg/100 ml. Bieri et al (1964) found the mean serum vitamin E level of 132 normal adults resident in Washington D.C. to be 1.05 ± 0.26 mg/100 ml. Vitamin E levels of rural and urban Vancouver populations have been compared (Desai (1968)); the mean vitamin E level of both communities was found to be 0.97 ± 0.29 mg/100 ml. There was no difference between the sexes or between the two communities. A survey carried out in Ottowa on 125 subjects, using the method of Fabianek et al (1968), gave a mean of 1.29 ± 0.34 mg/100 ml (Hoppner, Phillips, Murray and Campbell (1970)). Both Desai (1968) and Hoppner et al (1970) found a lower mean value for subjects aged 25 years or less which was statistically significant. This was not found in this series (see Table 2.3.1.). O'Halloran, Studley-Ruxton and Wellby (1970) found that the "normal ranges" for healthy individuals and non-renal out-patients agreed well. The results presented here (Table 2.3.1.) show that there is good agreement between healthy volunteers and non-gastrointestinal in-patients.

When a normal range is expressed as the sample mean ± 2 standard deviations from the values found in blood donors, the lower

limit of normal for serum vitamin E is 5.2 µg/ml and the upper limit is 15.2 µg/ml. It has been suggested by Goldbloom (1960), Leitner et al (1960) and Horwitt (1962) that patients with a serum level of less than 5 µg vitamin E/ml should be considered as being deficient in the vitamin. Grimes and Leonard (1969) found that tissue (rectus abdominis muscle, heart and liver) concentrations of greater than 4 µg/g were always associated with plasma concentrations of more than 5 µg/ml and concluded that plasma concentrations of less than 5 µg/ml reflected low vitamin E concentrations in the tissues studied. Underwood, Siegel, Dolinski and Weisell (1970) have reported a correlation between blood concentrations and liver stores of vitamin E/g of (wet) tissue but the correlation coefficient was of relatively low magnitude ($r = 0.39$). The authors suggest that serum α-tocopherol levels within the range 4-12 µg/ml can be used to estimate relative liver storage of the vitamin since the ratio of liver to serum tocopherol is not significantly different over the range.

Low vitamin E levels were predicted in adults suffering from steatorrhoea (Badenoch (1960)) and have been reported in patients with sprue (Darby, Cherrington and Ruffin (1946)), steatorrhoea (Binder et al (1966)), malabsorption and alcoholism (Losowsky and Leonard (1967)) and after gastric surgery (Leonard, Losowsky and Pulvertaft (1966)). After upper intestinal surgery disruption of bile salt metabolism may occur. The principal feature of this disruption is deconjugation of bile salts by bacteria. Tabaqchali and Booth (1966) have shown that jejunal fluid, from patients with a "stagnant loop" syndrome or partial gastrectomy, which contained more than 100×10^6 bacteria/ml, also contained free bile acids. These patients also had pronounced steatorrhoea which was reduced by treatment with antibiotics. Inadequate mixing of food with bile

and pancreatic juice following gastrectomy has been put forward as an explanation of the malabsorption which may occur following gastric surgery (Butler and Eastham (1965)).

Bile salts are known to undergo an entero-hepatic circulation. Dowling (1972) has summarised intestinal bile acid transport as follows:-

(a) active transport from the ileum alone (the major route of bile salt re-absorption)

(b) passive absorption from the stomach, jejunum, ileum and colon, non-ionic diffusion being more important than ionic diffusion.

Interruption of the entero-hepatic circulation of bile salts has been reported in patients with RE (Meihoff and Kern (1968)) and ACD (Meittinen (1968), Low-Beer, Heaton, Heaton and Read (1971)). That ileal disease or resection, in the dog, could cause bile salt malabsorption has been clearly demonstrated (Playoust, Lack and Weiner (1965)). An increased loss of bile salts has been demonstrated in patients, which resulted in many of them having a deficiency of bile salts in the jejunal lumen (Austad, Lack and Tyor (1967), Hardison and Rosenberg (1967)).

Any bile salt deficit, which may occur as a result of diseased or resected ileum, or as a result of any other gastric or other small intestinal surgery, leads to impairment of micelle formation. Greaves and Schmidt (1937), Gallo-Torres (1970) and MacMahon and Thompson (1970) have all reported on the necessity of the presence of adequate amounts of bile salts (therefore mixed micelles) for the efficient absorption of vitamin E. Patients who had had gastric surgery were found to have low vitamin E levels in serum (mean 6.1 µg/ml) which is in accord with the findings of Leonard et al (1966). Ileal involvement in RE has the most marked

effect on vitamin E levels found in these patients (see Tables 3.2.5. and 3.2.6.). The bile salt concentration in the lumen of the jejunum of these patients may vary throughout the day. van Deest, Fordtran, Morawski and Wilson (1968) and Dowling and Campbell (1971) (cited in Dowling (1972)) have both found that there is a diurnal variation in jejunal bile salt concentration after ileal resection, the levels being normal after the first meal of the day, but subsequently were found to be deficient. An explanation of this phenomenon is that, over night, the liver has time to synthetise bile salts which are then stored and concentrated in the gall bladder, then released following ingestion of breakfast. The bile salts are malabsorbed to the extent that the liver is unable to resynthesise enough bile salts in the relatively short intervals between subsequent meals to redress the faecal losses. Thus, in the absence of a normal ileum, the jejunal bile concentration depends on:-

(a) increased bile salt loss in faeces due to malabsorption

(b) the ability of extra-ileal sites to maintain the enterohepatic circulation of bile salts, coupled with adaptive increases in hepatic bile salt synthesis.

The major site of fat absorption in man is the upper small intestine (Borgstrom, Dahlqvist, Lundh and Sjovall (1957)). When considering the mechanism of fat absorption there is no evidence to suggest that intact micelles enter the mucosa, rather, the lipids are in some way discharged and enter the cell in a molecular form (Isselbacher (1967)). Particulate absorption, by means of pinocytosis, is thought to be unlikely (Shiner (1966)). The activation energy required for the passage of single molecules of lipid from a watery medium to the lipid interior membrane may be low and thus the rate of entry rapid. The results of the absorption

experiments described in section 2.3.5. (b) show that there was an increase in serum levels of vitamin E 15 mins after an oral dose of the vitamin. This would indicate that absorption occurs rapidly from the jejunum.

Entry of mono-glycerides and fatty acids into the mucosa appears not to be energy dependent. Electron microscopy demonstrates dense droplets which are larger than micelles and which are presumed to be lipid in character, within the micro-villous membrane (Shiner (1966)). As soon as fat is seen beneath the terminal web area, it is almost always found to be surrounded by membranes which appear to be components of endoplasmic reticulum (Isselbacher (1967)). Impaired fat absorption has been produced in rats by treating them with puromycin and acetoxycycloheximide, which inhibit protein synthesis within the intestinal cell. Lipids, in the form of triglycerides, accumulate within the intestinal mucosa and hence the normal post-prandial increase in serum triglycerides is depressed (Sabesin and Isselbacher (1965)). This interference in lipid transport appears to be a consequence of impaired chylomicron formation.

In untreated ACD a decrease in the rate of triglyceride synthesis within the mucosal cell has been shown to occur (Brice, Owen and Tyor (1965), Dawson and Isselbacher (1960)), but this reduction in esterifying enzyme activity is thought to be a secondary phenomenon. The major deficit seems to be an inability to transport the resynthesised triglyceride into the lateral intercellular spaces (Rubin et al (1966), Samloff, Davis and Schenk (1965)) and the resulting retention of large droplets of lipid. Chylomicrons can be seen within the Golgi body and the lateral intercellular spaces of the cells contain lipid droplets (Rubin et al (1966)) hence, chylomicron synthesis would appear to be too slow to allow efficient

export of absorbed lipids. The large droplets of lipids are lost into the lumen when these cells desquamate (Rubin (1971)). The abnormal lipid distributions have been found to return to normal within a few days of commencement of a gluten free diet. Healthy subjects on a normal diet excrete, on average, less than 5 g fat per day in the faeces (Hendry (1960)). Patients with untreated ACD may excrete 30-40 g fat/day, some of which may never have been absorbed, the rest being derived from an accelerated rate of epithelial turnover (Trier and Browning (1970)). Correlations between serum vitamin E levels and faecal fat excretion have been reported by Binder et al (1966), Darby et al (1949) and Losowsky and Leonard (1967). There was a statistically highly significant correlation between serum vitamin E and faecal fat excretion in both RE ($r = 0.315$) and ACD ($r = 0.535$) patients.

The majority of ACD patients in this series (89%) were on a gluten free diet. It is possible that the withdrawal of gluten and, therefore, wheat germ from the diet is sufficient to reduce the amount of vitamin E in the diet to below that necessary to meet physiological requirements. This factor is especially pertinent, since the average daily intake of vitamin E in Britain has been reported (Smith et al (1971)) as less than 5 mg/day, but they also found that the daily intake of polyunsaturated fats is less than in North America therefore it is possible that the requirement for vitamin E is reduced.

The absorption of radio-active vitamin E has been studied in humans. Blomstrand and Forsgren (1968) gave 2 female patients (one with gastric carcinoma and the other with an acute exacerbation of chronic lymphatic leukaemia) labelled tocopherols in a test meal. The thoracic duct of both patients had been previously cannulated and serial collections of lymph were made. They found that absorption of radio-activity occurred mainly 2-8 hrs after administration

and that the radio-activity was located primarily in the chylomicrons. MacMahon and Neale (1970) have found that the net absorption of radio-active vitamin E, as calculated by expressing the difference between administered radio-activity and that appearing in the faeces as a percentage of the administered dose, was severely impaired in patients with biliary obstruction. Absorption was less severely impaired in patients with exocrine pancreatic insufficiency and in patients with intestinal mucosa abnormalities, absorption could be correlated with the degree of steatorrhoea. Control subjects absorbed between 55% and 77% (mean 67%) of the test dose; there was a delay of 1-3 hrs in the appearance of radio-activity in the plasma. A peak level of plasma radio-activity was reached after 5-9 hrs. Six patients with ACD were studied, one of whom had had intermittent treatment with a gluten free diet; all absorbed less than 60% of the dose. Mild malabsorption of vitamin E was also found in 5 of 6 patients who had had a partial gastrectomy.

Using a similar experimental approach, Kelleher and Losowsky (1970) have found that the net absorption of radio-activity was correlated with the severity of steatorrhoea. Subjects with steatorrhoea absorbed, on average, 59% (range 31-83%) of the test dose and those with no steatorrhoea absorbed 72% (range 51-86%). Plasma radio-activity was maximal 6-12 hrs after dosing, but the curves were significantly lower in patients with steatorrhoea. Five of the patients studied had ACD, two were on a gluten free diet (2 and 3 years), had no steatorrhoea and absorbed more than 70% of the test dose. The other three, with steatorrhoea, absorbed less than 60% and had a satisfactory response when subsequently placed on a gluten free diet. Of four patients with RE and steatorrhoea, three absorbed less than 70% of the test dose.

The correlation between faecal fat excretion and serum vitamin E

levels found here can be explained on the basis of reduced absorption from the gastrointestinal tract. The delay in appearance of radio-activity in the lymph of 2 hrs found by Blomstrand and Forsgren (1968) and in plasma of up to 3 hrs found by MacMahon and Neale (1970) was not found here when using a relatively large test dose of non-radio-active ∝-tocopherol. Indeed, absorption measured in this way was found to be rapid and a peak serum level was reached in 30 mins or less (except for normal 5. see Fig.2.3.5.). RE is a disease which has an acute phase(s) and also quiescent spells. In the series reported here the 27 patients with active disease had lower serum vitamin E levels than those in the quiescent phase of the disease, but the difference was not statistically significant. In 6 of 11 patients a rise in serum vitamin E levels accompanied clinical improvement (see Table 2.3.14.).

Most of the ACD patients were on a gluten free diet (89%), some for longer than 10 years. The evidence presented by Kelleher and Losowsky (1970) indicates that, once on a gluten free diet, vitamin E absorption returns to normal, thus it is possible that some other factor(s) are affecting the serum level of vitamin E. The possibility of the ACD patients' diets being deficient in vitamin E has already been discussed. It is also possible that minor dietary indiscretions would be sufficient to damage the epithelial cells to the extent of preventing the efficient export of chylomicrons into the intercellular spaces (Rubin et al (1966), Samloff et al (1965)), although not enough to produce overt steatorrhoea. A graduation in serum vitamin E levels was found in the means of those patients on a strict gluten free diet (7.76), those not adhering strictly to the diet (6.54) and those not on the diet (5.50). Even those patients on a strict gluten free diet may fail to absorb dietary vitamin E efficiently.

Table 2.3.14. Changes in serum vitamin E levels of RE patients when in remission and relapse.

Patient Number	Remission	Relapse
20	12•2	11•5
25	4•9	3•9
39	14•6	13•2
45	0•7	1•5
46	5•0	7•5
57	7•6	12•8
58	12•0	4•0
62	6•3	8•7
77	12•0	10•6
79	9•2	7•8
84	9•7	10•3

The effects of low blood levels of vitamin E on the haematological status of patients has been discussed already (2.1.4.). For neither ACD nor RE patients was there any correlation between vitamin E levels and haemoglobin or mean corpuscular volume; there was a significant correlation between vitamin E and serum iron concentration for both groups. The haematological situation of these patients is complex. Out of a total of 174 patients, 57 were receiving folic acid supplements, 71 iron supplements, 23 vitamin B_{12} supplements.

The electron microscopic appearance of the jejunal epithelial cells of two children suffering from abetalipoproteinaemia have been reported (Molenaar, Hommes, Braams and Polman (1968)). Plasma vitamin E levels were initially 0.30 and 0.50 mg/100 ml and the jejunal biopsies taken at this time showed a lack of positive contrast from the intracellular membranes when examined under the electron microscope. The appearances were those of altered staining of the membranes, rather than a complete lack of membranes. Supplementation with vitamin E, over a period of 4 months, produced serum levels of 1.35 and 0.85 mg/100 ml which were accompanied by a return to normal cellular ultra-structure of the jejunal epithelial cell. Biopsies taken 10 weeks after the commencement of supplementation showed that the mitochondrial membranes responded earlier than the endoplasmic reticulum. It is possible that vitamin E deficiency could have a direct effect on the functioning of the epithelial cell (Bunyan et al (1968)).

β-lipoprotein, which is synthesised predominantly in the liver (Fredrickson, Levy and Lees (1967)) has been shown to be the principal carrier of vitamin E in serum. Kelleher and Losowsky (1969) have shown a very close correlation between total serum α-tocopherol

and that portion carried by the low density lipoproteins. This correlation has also been reported by Rubenstein, Dietz and Srinavasen (1969). Kater, Unterecker, Kim and Davidson (1970) have suggested that, in severe liver disease, synthesis of β-lipoprotein may be reduced to the extent that serum vitamin E levels would fall. The ability of the liver to synthesise protein may be reflected in the levels of serum albumin but the major reason for lowered albumin is protein loss into the intestinal tract. This loss correlates with disease activity. In both RE and ACD patients there was found to be a positive correlation between albumin and vitamin E levels in serum.

A relationship between plasma cholesterol and vitamin E has been demonstrated in patients with thyroid disorders (Postel (1956)) and coronary artery disease (Pelkonan (1963)). Nitowsky, Hsu and Gordon (1962) have shown that changes in serum cholesterol levels resulting from treatment of thyroid disorders were paralleled by changes in vitamin E levels. On the basis of the evidence presented by Kelleher and Losowsky (1969) these authors have suggested that serum concentrations of low density lipoproteins are an important factor in controlling the level of α-tocopherol, although the presence of high levels of vitamin E does not affect the levels of low density lipoprotein or cholesterol. In patients with a congenital lack of low density lipoprotein (abetalipoproteinaemia) there is an absence/severely restricted absorption of vitamin E, (Kayden and Silber (1965)). Limited absorption does occur in these patients when large doses of water miscible vitamin is given and there was some response in plasma levels (Dodge, Cohen, Kayden and Phillips (1967), Muller and Harries (1969)), thus indicating the possibility of a portal route of absorption of vitamin E if the lymphatic route is blocked for any reason.

The lack of significant correlation between the levels of cholesterol and vitamin E given in 2.3.7. may be due either to the small sample size or the extent of the liver disease. There was a lack of consistency when vitamin E levels of serum derived from the peripheral circulation were compared with those of serum derived either from the hepatic portal or the hepatic vein. This made comparison with the results of the absorption tests of MacMahon et al (1971) impossible. These authors found evidence of a portal route of absorption for both cholesterol and vitamin E in the rat. The serum used here was derived from fasting patients and, as such, could not represent the absorptive state. The presence of liver disease raises the possibility that vitamin E was leaching out of the damaged liver (serum levels were higher in the hepatic vein than in the peripheral serum). The mean venous serum level was significantly lower than that of the normal population ($\bar{x}$ = 7.9 $\pm$ 4.8, t = 3.05). Kater et al (1970) have reported that patients with severe cirrhosis and fulminant hepatitis had significantly lowered levels.

Storage of vitamin E takes place in both liver and adipose tissue. Underwood et al (1970) found that when a patient was receiving a dietary supplement of vitamin E of 300 I.U./day for 3 months, the liver was capable of storing large quantities of α-tocopherol, e.g. 180 µg/g (wet weight) (mean value for normals was 11.2 µg/g). The liver content of vitamin E has been found to vary least when expressed in terms of ug vitamin E/g lipid (Dju, Mason and Filer (1958), Underwood et al (1970)), which suggests that the vitamin is stored in the cell lipid fraction. However, Dju et al (1958) found that, in 5 alcoholic patients with fatty degeneration of the liver, there was no proportional increase in the tocopherol content.

There appears to be little correlation between the amount of tocopherol stored and the level of polyunsaturated fatty acids in the diet. Prolonged feeding of an unsaturated fat diet to humans revealed no apparent relationship between tocopherol levels in adipose tissue and its fatty acid composition (Imaichi, Cox, Kinsell, Schelstraete and Olcott (1965)). The supplementation of the diet of healthy adults with 1 g vitamin E/day for 14 days increased the levels in adipose tissue from 30 to 80 µg vitamin E/g and of the plasma from 4 to 11 µg/ml. The plasma levels fell to 7 µg/ml 14 days after supplementation had stopped and the adipose tissue levels were then 55 µg/g (McMasters, Howard, Kinsell, Van der Veen and Olcott (1967)). A similar rate of fall was found for patient 19 (see Table 2.3.11.).

2.4. The absorption of radio-active DL-α-tocopherol by the rat small intestine

2.4.1. Introduction

The specific site of absorption of vitamin E is not well established (Wiss, Bunnell and Gloor (1962)). The disappearance of tocopherols from rat small intestinal loops, *in vitro*, has been reported (Pearson *et al* (1970)) but no attempt was made to identify any specific site of absorption. Benson, Chandler, Vansteenhuyse and Gajnon (1956) found that the third quarter of the rat small intestine had the optimum absorption of an oral dose of olive oil.

In order to investigate the absorption of vitamin E, with special reference to position along the length of the intestine, it was decided to give normal laboratory rats an oral dose of radio-active DL-α-tocopherol; the dose being one of a physiological amount. The effect of feeding on the uptake of the vitamin into the intestinal mucosa was investigated by comparing the results of rats fed after administration with those of rats whose food was withheld after dosing. By varying the time of killing after dosing it was hoped that some information as to whether passage along the lumen would be accompanied by a movement in the region of maximal uptake into the mucosa. Some of the rats were not killed until 24 and 48 hrs after dosing in order to investigate the possibility of recirculation of the vitamin.

2.4.2. Materials and Methods

(a) Materials

(1) Adult Wistar rats were used throughout the experiment. Each was weighed prior to the administration of DL-α-tocopherol. The rats had been maintained on 4 lb (Pillsbury) cube diet, which was withheld on the evening prior to the administration of the vitamin. Water was freely available. The animals were not housed in metabolic cages.

(2) DL-α-tocopherol (5 methyl-T) 1mCi/ml, specific activity 2,720 mCi/mM was obtained from the Radio Chemical Centre, Amersham.

(3) DL-α-tocopherol, Sigma.

(4) Tween 80 (polyoxyethylene sorbitan mono-oleate) Hopkin & Williams Laboratory Reagent.

(5) Chloroform "Analar" B.D.H.

(6) Methanol G.P.R. B.D.H.

(7) Toluene (sulphur free) B.D.H.

(8) Saline 0.9N sterile.

(9) Butyl PBD, Koch Light qv 2-(4'-tert-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole.

(b) Methods

(1) Treatment of isotope.

The benzene:ethanol (9:1) solvent of the tritiated DL-α-tocopherol was allowed to evaporate at room temperature. Care was taken to exclude light from the ampoule by wrapping it in metal foil. The residue was redissolved in 10 ml 5% Tween 80 and stored at -20° in a plastic container until used. It was thawed and diluted with non-radio-active DL-α-tocopherol dissolved in Tween 80 to give a final solution containing 1 mg DL-α-tocopherol

per ml with radio-activity of 2 μCi/ml.

(2) Administration to rats

The rats were fasted over night before administering the tritiated DL-α-tocopherol. They were weighed and then given 0.5 ml DL-α-tocopherol solution directly into the stomach through a polythene tube attached to a 2 ml syringe. The dose contained 500 μg DL-α-tocopherol and 1 μCi tritium. The rats were returned to their separate cages, all were given free access to water and some were allowed to feed. After the required time periods the rats were anaesthetized with nitrous oxide, killed by decapitation and the blood collected directly into a centrifuge tube The serum was separated by centrifugation after clotting had occurred and was stored at -20° until analysis. The livers were removed from the carcass and stored at -20° until analysis. The stomachs were removed, weighed, washed out with 4 ml saline then re-weighed. The contents and washings were stored together at -20°; the stomach was deep frozen separately. When the small intestine, duodenum to ileo-caecal valve, was removed it was cut into portions approximately 10 cm long. Care was taken to collect all the contents from each segment. The segment and the contents were weighed together, the contents were washed out with 4 ml saline and the segment was reweighed. The contents and washings were stored together at -20°; the intestine was deep frozen separately. The carcass was incinerated.

Each treatment was given a colour code before storage. The intestinal segments were numbered S (stomach) and 1-8; each rat within a given treatment was designated by a number. The intestinal contents and washings were given the same number as their parent segment followed by the letter W. For example, the

third intestinal segment of rat 2 which was killed 2 hrs after oral dosing and whose food was withheld, was identified as:- white ii 3; the contents and washings being designated by white ii 3 W.

(3) Extraction and counting

(a) Serum was analysed for vitamin E by the method of Fabianek et al.(1968).

(b) The radio-activity of the serum was measured by extracting from 1 ml of serum into 20 ml chloroform:methanol (2:1). All extractions were carried out in Quickfit glass stoppered tubes which were large enough to allow good mixing when shaken on a Griffin flask shaker. After 15 mins shaking the layers were separated by the addition of 4 ml distilled water, the methanol layer being discarded. The chloroform layer was evaporated under reduced pressure at 25°. Scintillation fluid (10 ml toluene + butyl PBD (12g/2½ l)) was added to each vial and the radio-activity was measured for 10 mins, each vial, in a Phillips liquid scintillation counter.

(c) The contents and washings of the intestinal tract were thawed, the volume measured, then homogenised in an all glass homogeniser. A 1 ml aliquot of the homogenate was extracted in the same manner as serum (see (b)).

(d) The measured portions of the intestinal tract were cut into small pieces and placed in an all glass homogeniser. Saline (1 ml) was added prior to homogenisation. The homogenate was transferred to a glass stoppered tube and the homogeniser was washed out with a further 1 ml saline. This washing was combined with the homogenate and the radio-activity was extracted into 40 ml chloroform:methanol (2:1). 8 ml distilled water was

used to separate the layers, the chloroform was evaporated and the residue counted as described above (b).

(e) Approximately 1 g of liver (exact weight known) from each animal was homogenised in 2 ml saline. Two 1 ml aliquots were extracted into 20 ml chloroform:methanol (2 : 1); the layers were separated and the chloroform layer was treated as described in (b).

2.4.3. Results

2.4.3.1. Isotopic purity of DL-α-tocopherol (5 methyl-T)

30 µl of the solution administered to the rats was applied to a silica gel G thin layer plate. The solution applied contained 2 µCi and 1 mg DL-α-tocopherol dissolved in 1 ml 5% Tween 80. The plate was developed in 10% acetone in hexane for 90 mins. (McMahon and Neale (1970)). Areas 2 cm wide were scraped into scintillation vials; 10 ml scintillation fluid was added and the contents were counted for 10 mins. Fig.2.4.1. shows the distribution of the radioactivity. The percentage isotopic purity was calculated as the percentage of the counts per minute (C.P.M.) in segments 11-14 divided by the total C.P.M.; C.P.M. of less than 300 and the value for segment 18 (solvent front) were excluded. This gave a purity of 85% which is somewhat lower than that claimed by the suppliers (98%). There were two factors contributing to this; firstly, the tritium labelled DL-α-tocopherol was dissolved in 5% Tween 80, which has the effect of making the vitamin more soluble in any residual water within the silica gel. Secondly, the isotope had been stored deep frozen in 5% Tween 80 for 3 months before chromatography. A decomposition rate of 3% per month is given for storage at +2° and, if this rate of decomposition is applied, one would expect a purity of 89% after 3 months storage.

2.4.3.2. The effect of varying the volume of extractant

Horse Serum No 3 (Wellcome Reagents Ltd.) was used as a base from which to extract tritiated DL-α-tocopherol. To 20 ml horse serum was added 0.2 ml of the solution administered to the rats (i.e. 2 µCi and 1 mg DL-α-tocopherol/ml) and thoroughly mixed by gentle inversion for 10 mins. The volume of serum extracted into

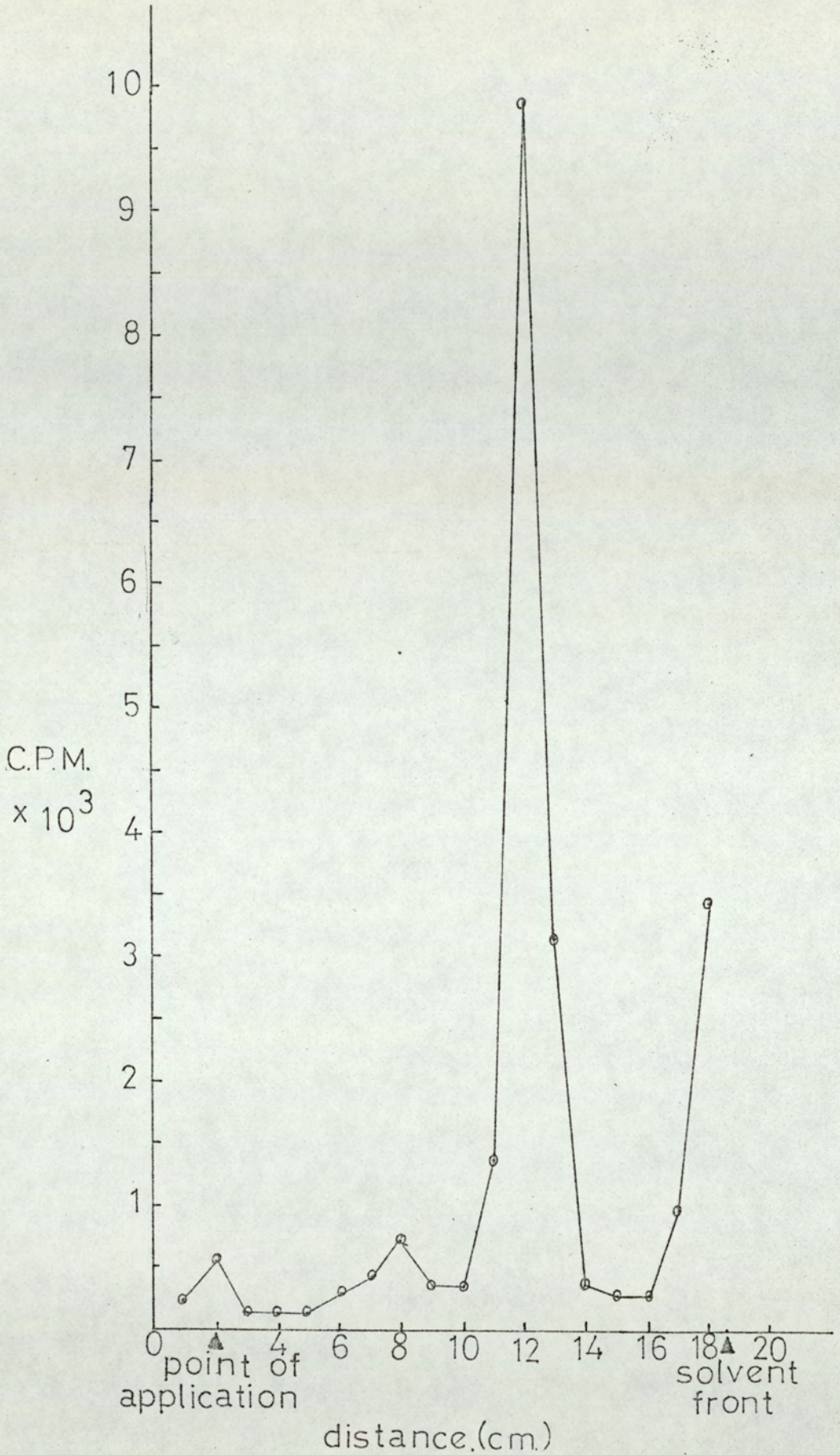


Fig. 2.4.1. Radio-chromatograph of DL-α-tocopherol (5-methyl-^{3}H), 30 µl of 2 µCi and 1 mg / ml on Silica Gel G, developed in 10% acetone in hexane.

chloroform:methanol (2:1) was 0.5 ml in each case and all tubes were shaken for 15 mins on a mechanical shaker. The layers were split by the addition of a volume of distilled water equal to 20% of that of the chloroform:methanol (2:1). The chloroform layer was evaporated and the residue counted as described in the methods section. The results are given in Table 2.4.1. An optimal extraction of radio-activity was obtained when 20 volumes of chloroform: methanol (2:1) were used in the extraction procedure.

2.4.3.3. The effect of varying the time of shaking

The serum containing DL-α-tocopherol used was the same as that in 2.4.3.2. 0.5 ml serum was extracted into 10 ml chloroform:methanol (2:1). The layers were separated after shaking for various times, ranging from 0 min to 180 mins. The chloroform layer was evaporated to dryness as described before. The results are given in Table 2.4.2. which shows that no more radio-activity is extracted when shaking is continued for longer than 15 mins. Therefore, all experimental samples were extracted into 20 volumes chloroform:methanol (2:1) by shaking for 15 mins on the mechanical shaker.

2.4.3.4. Rat serum levels of vitamin E

Table 2.4.3. shows the serum levels of vitamin E found in the rats after an oral dose of 500 µg DL-α-tocopherol. The chloroform extractable radio-activity in the same serum samples is also shown. There is a peak in the serum radio-activity 4 hrs after dosing but no corresponding peak in the serum levels of vitamin E.

2.4.3.5. Chloroform extractable radio-activity in gut contents and tissue

The results are expressed as C.P.M./g tissue (wet weight) and

Table 2.4.1. The effect on extraction efficiency of varying the volume of extractant.

Volume of serum extracted (ml.).	$CHCl_3$:MeOH (2:1) volume (ml.).	Volume distilled water (ml.).	C.P.M. x 10^3 from $CHCl_3$ layer.	
0·5	10	2·0	4·95	4·88
0·5	8	1·6	4·54	4·54
0·5	5	1·0	4·07	4·17
0·5	4	0·8	3·52	3·67
0·5	3	0·6	2·95	2·99
0·5	2	0·4	0·45	0·43

Table 2.4.2. The effect of varying time of shaking on extraction from serum.

Time of shaking (min.).	C.P.M. $\times 10^3$	
0	4·28	4·83
5	4·84	5·25
10	4·32	
15	5·92	5·48
20	5·64	5·54
30	5·27	5·20
45	5·76	5·65
60	5·90	5·99
120	5·54	5·68
180	5·66	5·79

Table 2.4.3. Mean serum levels of Vitamin E and the corresponding radio-activity for each treatment of rat.

Treatment.	Serum Vitamin E.			C.P.M./ml. serum.		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	2·5	1·1	3	464	278
+ 2h. fed	6	1·4	0·6	6	464	297
+4h. not fed	2	2·0	0·2	1	558	
+ 4h. fed	5	1·9	0·5	5	681	325
+ 6h. fed	4	1·7	0·3	4	492	113
+ 24h. fed	4	1·9	0·4	4	441	228
+ 48h. fed	3	1·6	0·6	3	262	200

as C.P.M. of total contents and washings for each segment. Each segment is listed on a separate Table numbers 2.4.4.-12. Against each treatment is entered the C.P.M. of contents, C.P.M./g tissue and the ratio of C.P.M. of contents:C.P.M./g tissue. The figures given are the mean and standard deviation for each treatment mentioned; the ratio was calculated for each segment, then the mean ratio for the treatment was calculated. The same results are also presented graphically, Figs.2.4.2.-5. show C.P.M. of contents, Figs.2.4.6.-9. show C.P.M./g tissue, Figs.2.4.10.-.6. show the ratio C.P.M. of contents:C.P.M./g tissue. The results for segments beyond 70 cm have been omitted from the graphs.

2.4.3.6. Chloroform extractable radio-activity in rats liver

The results are shown in Table 2.4.13. and are expressed as C.P.M./g (fresh weight) liver. The means and standard deviations are given for each treatment. Much more radio-activity accumulates in the liver when the animals are not fed even as early as 2 hrs after dosing.

Table 2.4.4. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Stomach for the treatments listed.

Treatment	C.P.M.x10^3 of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M. /g. Tissue).		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	14·81	15·20	4	2455	1418	4	8·3	6·2
+ 2h. fed	4	22·02	10·29	4	810	456	4	28·5	6·7
+ 4h. not fed	4	15·96	13·42	4	513	343	4	26·9	20·6
+ 4h. fed	3	15·39	19·92	3	247	346	4	76·6	31·6
+ 6h. fed	4	8·41	6·72	4	179	146	4	65·8	45·0
+ 24h. fed	4	0·71	0·52	4	107	54	4	47·2	78·5
+ 48h. fed	3	0·37	0·42	3	92	47	3	3·4	2·2

Table 2.4.5. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 1 (0-10 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M.contents : C.P.M./g. Tissue.		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	1342	618	4	1132	559	4	1·4	0·9
+ 2h. fed	4	552	238	4	640	325	4	1·3	1·1
+ 4h. not fed	4	1679	1021	4	737	357	4	2·2	1·1
+ 4h. fed	3	891	798	3	502	303	3	1·8	0·9
+ 6h. fed	4	296	171	4	107	376	4	2·7	0·6
+ 24h. fed	4	220	87	4	127	73	4	2·1	1·1
+ 48h. fed	3	129	12	3	64	31	3	2·3	0·9

Table 2.4.6. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 2 (11-20 cm.) for the treatments listed.

Treatment	C.P.M.of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	3162	1951	4	2963	1663	4	1·2	0·5
+ 2h. fed	4	890	613	4	1895	856	4	0·5	0·2
+ 4h. not fed	4	714	429	4	2290	2670	4	1·7	2·3
+ 4h. fed	3	873	417	3	910	452	3	1·0	0·1
+ 6h. fed	4	731	641	4	375	285	4	1·9	0·9
+ 24h. fed	4	370	254	4	523	622	4	1·3	1·1
+ 48h. fed	3	149	47	3	135	38	3	1·2	0·4

Table 2.4.7. Mean C.P.M.of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 3 (21-30 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	3648	2423	4	5254	3741	4	0·7	0·2
+ 2h. fed	4	2742	2280	4	3170	1117	4	0·8	0·4
+ 4h. not fed	4	3484	3943	4	2343	1454	4	1·6	1·2
+ 4h. fed	3	1761	875	3	1364	445	3	1·3	0·3
+ 6h. fed	4	1150	1106	4	444	426	4	2·5	1·2
+ 24h. fed	4	434	211	4	273	93	4	1·6	0·7
+ 48h. fed	3	179	100	3	234	160	3	0·8	0·3

Table 2.4.8. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 4 (31-40 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	8352	754	4	6282	4731	4	1·4	0·8
+ 2h. fed	4	2890	1392	4	4533	2078	4	0·7	0·3
+ 4h. not fed	4	4402	4799	4	3674	2545	4	1·3	1·1
+ 4h. fed	3	1609	791	3	1749	438	3	1·3	1·1
+ 6h. fed	4	1653	1819	4	674	781	4	2·4	1·0
+ 24h. fed	4	514	294	4	423	344	4	1·6	1·0
+ 48h. fed	3	252	209	3	201	93	3	1·2	0·6

Table 2.4.9. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 5 (41-50 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	8718	6388	4	5942	4720	4	1·9	1·1
+ 2h. fed	4	6157	2495	4	3504	1546	4	2·2	1·6
+ 4h. not fed	4	5360	4873	4	2804	1611	4	2·2	1·7
+ 4h. fed	3	3397	885	3	1737	1601	3	3·3	2·5
+ 6h. fed	4	2879	2318	4	695	641	4	4·8	4·2
+ 24h. fed	4	724	443	4	499	336	4	1·8	1·5
+ 48h. fed	3	223	172	3	166	106	3	1·3	0·4

Table 2.4.10. Mean C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 6 (51-60 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents : C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	7309	3278	4	3462	3016	4	5·3	5·2
+ 2h. fed	4	8881	3746	4	2346	867	4	4·2	2·3
+ 4h. not fed	4	9324	8624	4	1668	951	4	7·3	5·3
+ 4h. fed	3	5481	4171	3	1414	1331	3	4·4	0·9
+ 6h. fed	4	2513	2180	4	471	86	4	5·2	4·4
+ 24h. fed	4	982	617	4	504	527	4	2·7	1·9
+ 48h. fed	3	233	173	3	1156	186	3	2·3	1·1

Table 2.4.11. Mean C.P.M.of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 7 (61-70 cm.) for the treatments listed.

Treatment	C.P.M. of contents.			C.P.M./g. Tissue.			Ratio (C.P.M. contents :C.P.M./g. Tissue.)		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD
+ 2h. not fed	4	33134	18297	3	1286		4	19·7	17·4
+ 2h. fed	4	19295	18422	4	1148	894	4	25·1	15·1
+ 4h. not fed	2	4845	5621	2	1439	643	2	4·8	6·0
+ 4h. fed	3	8273	1811	3	1429	639	3	6·3	1·7
+6h. fed	4	1432	1783	4	234		4	10·0	9·9
+24h. fed	4	1440	1127	4	187	94	4	8·0	3·7
+ 48h. fed	3	408	372	3	150	99	3	2·5	0·8

Table 2.4.12. C.P.M. of contents (including washings); C.P.M./g. tissue (wet weight) and ratio (C.P.M. contents : C.P.M./g.) of Segment 8 (71-80 cm.) for the treatments listed.

Treatment	C.P.M.of contents.		C.P.M./g. Tissue.		Ratio (C.P.M.contents : C.P.M./g. Tissue.)	
+ 2h. not fed	——	——	——	——	——	——
+ 2h. fed	——	——	——	——	——	——
+ 4h. not fed	7448	27856	71	1521	104·9	18·3
+ 4h. fed	6680	——	1357	——	5·0	——
+ 6h. fed	740	1228	89	177	8·3	6·9
+ 24h. fed	3773	——	81	——	46·6	——
+ 48 h. fed	——	——	——	——	——	——

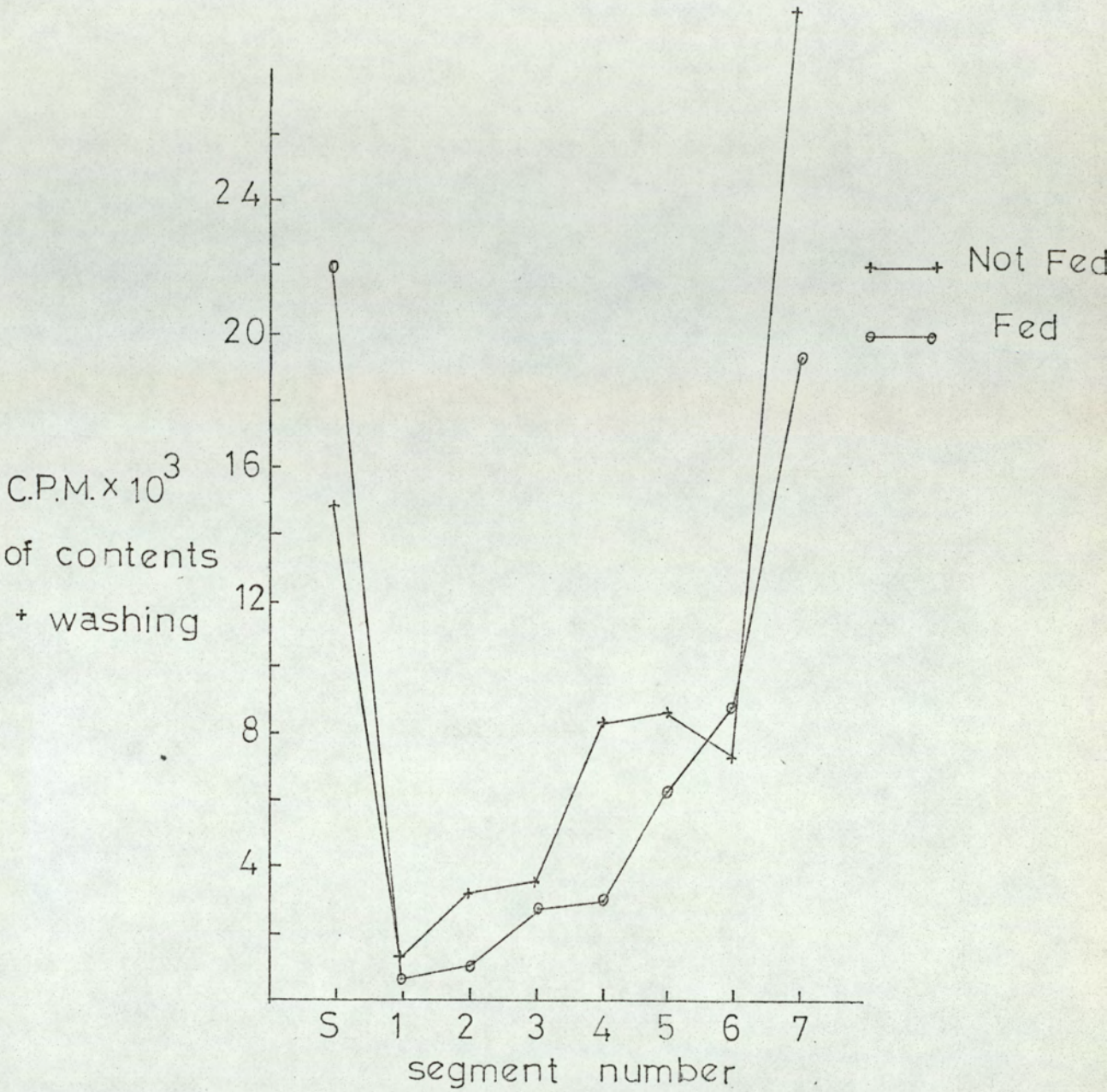


Fig. 2.4.2. C.P.M. of contents (including washings) 2 h after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

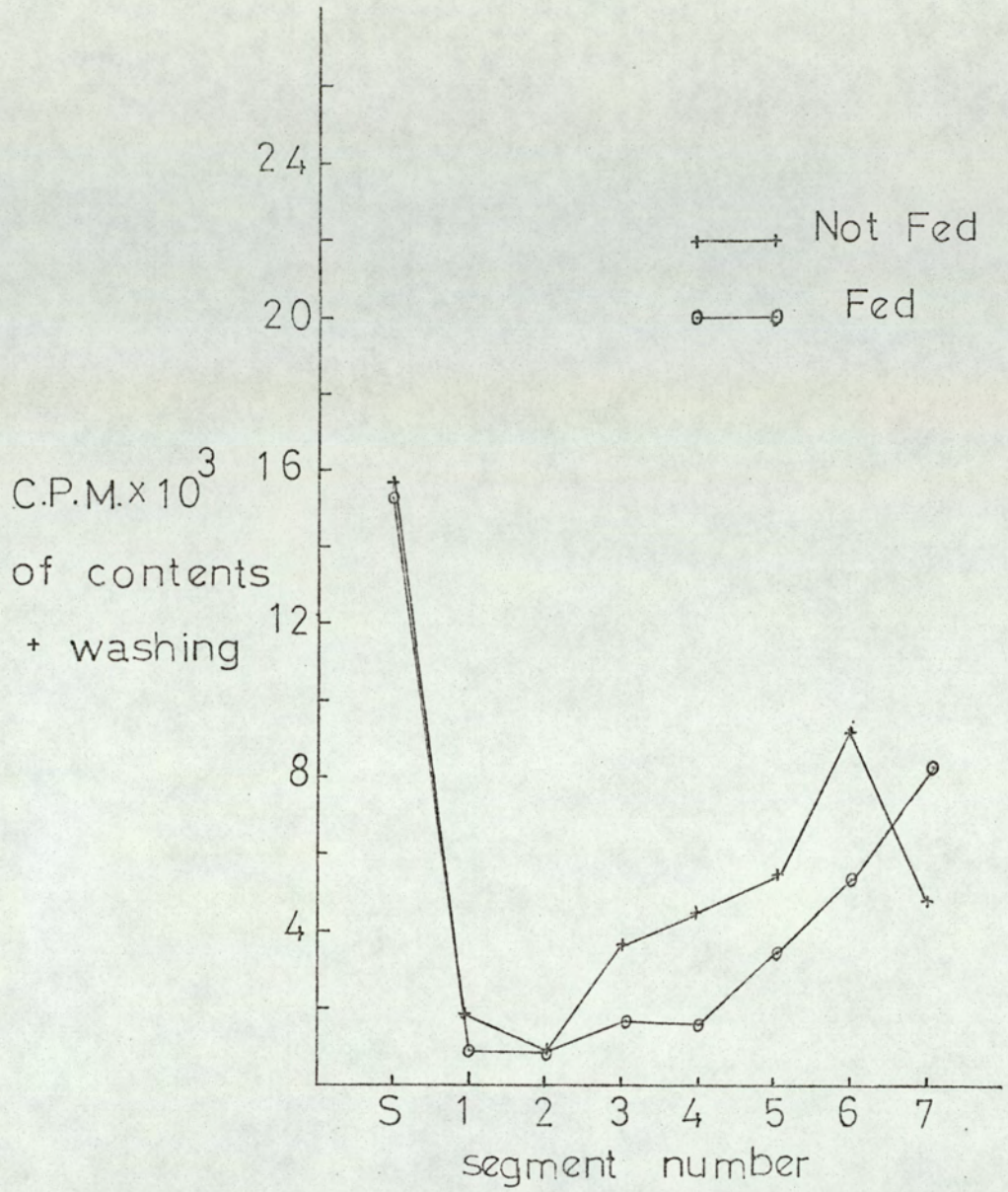


Fig. 2.4.3. C.P.M. of contents (including washings) 4 h after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

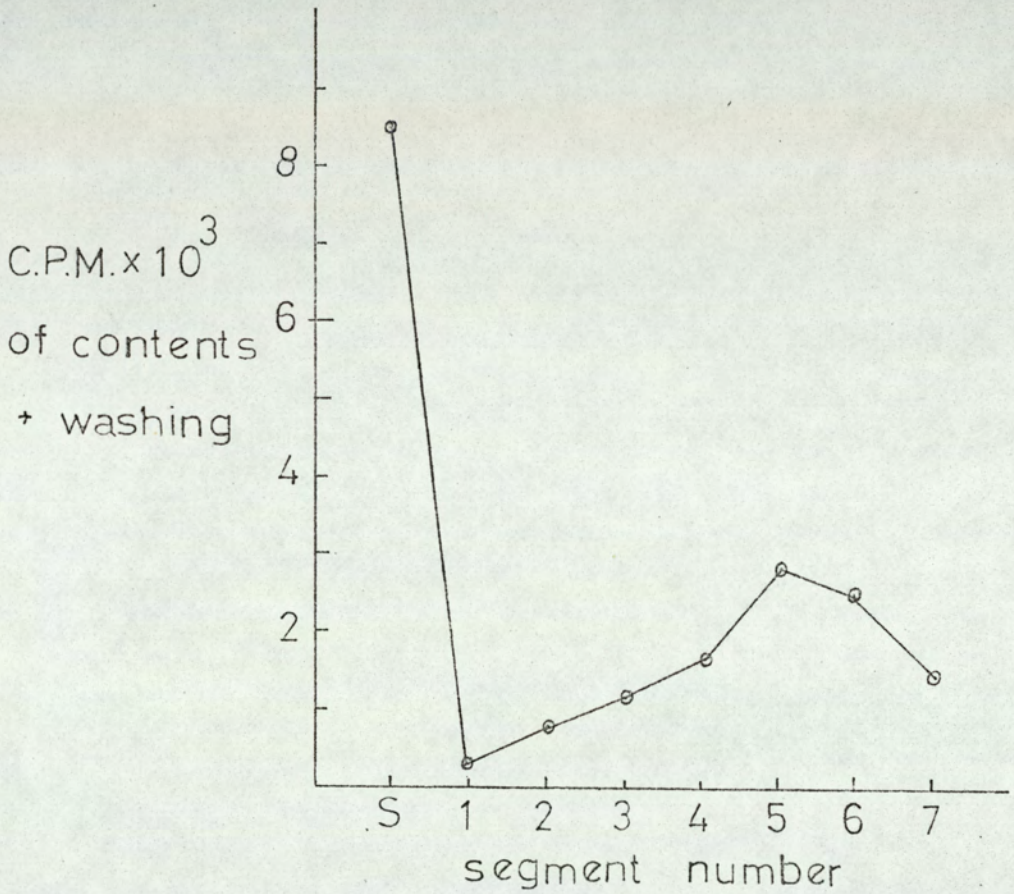


Fig. 2.4.4. C.P.M. of contents (including washings) 6 h after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

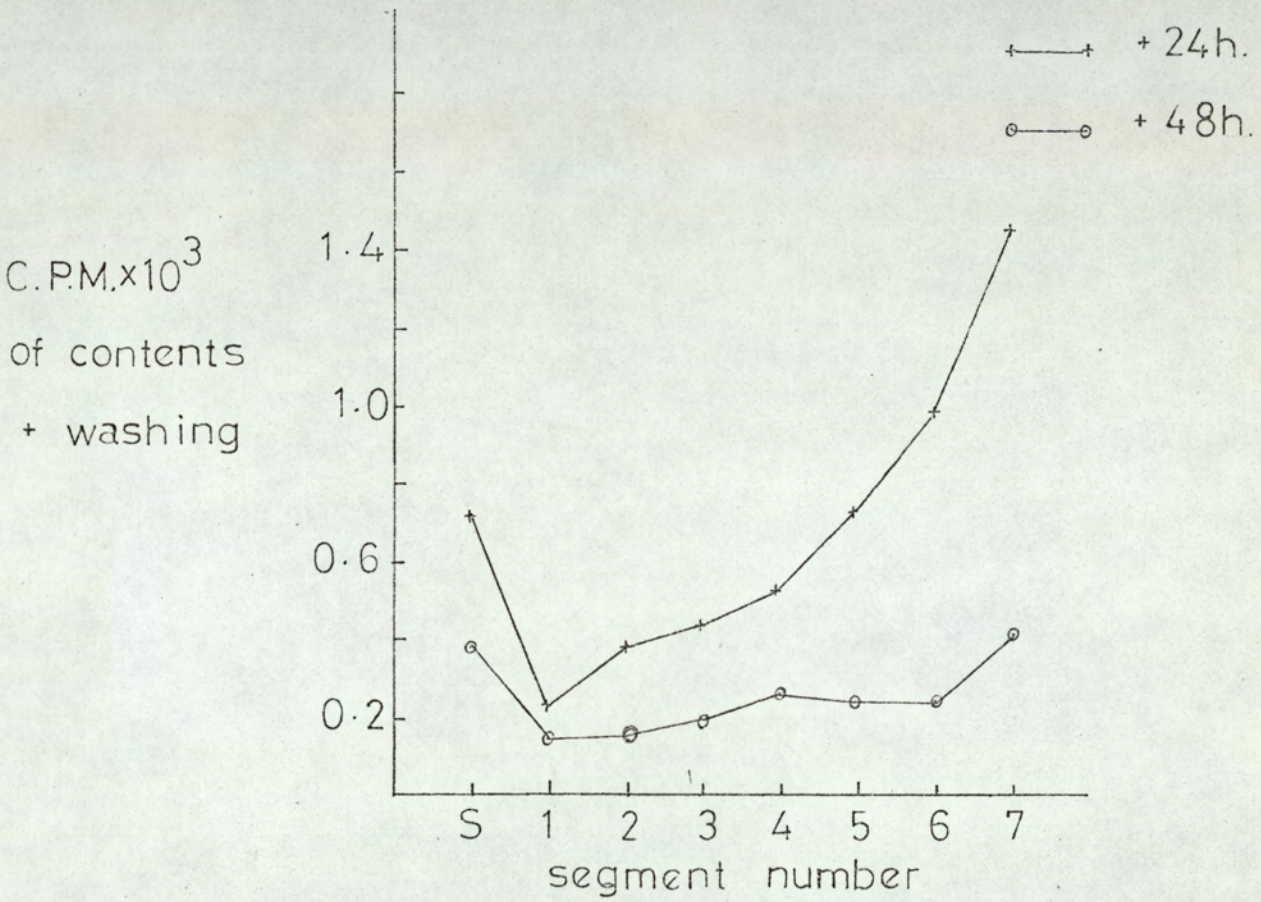


Fig. 2.4.5. C.P.M. of contents (including washings) 24 h and 48 h after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

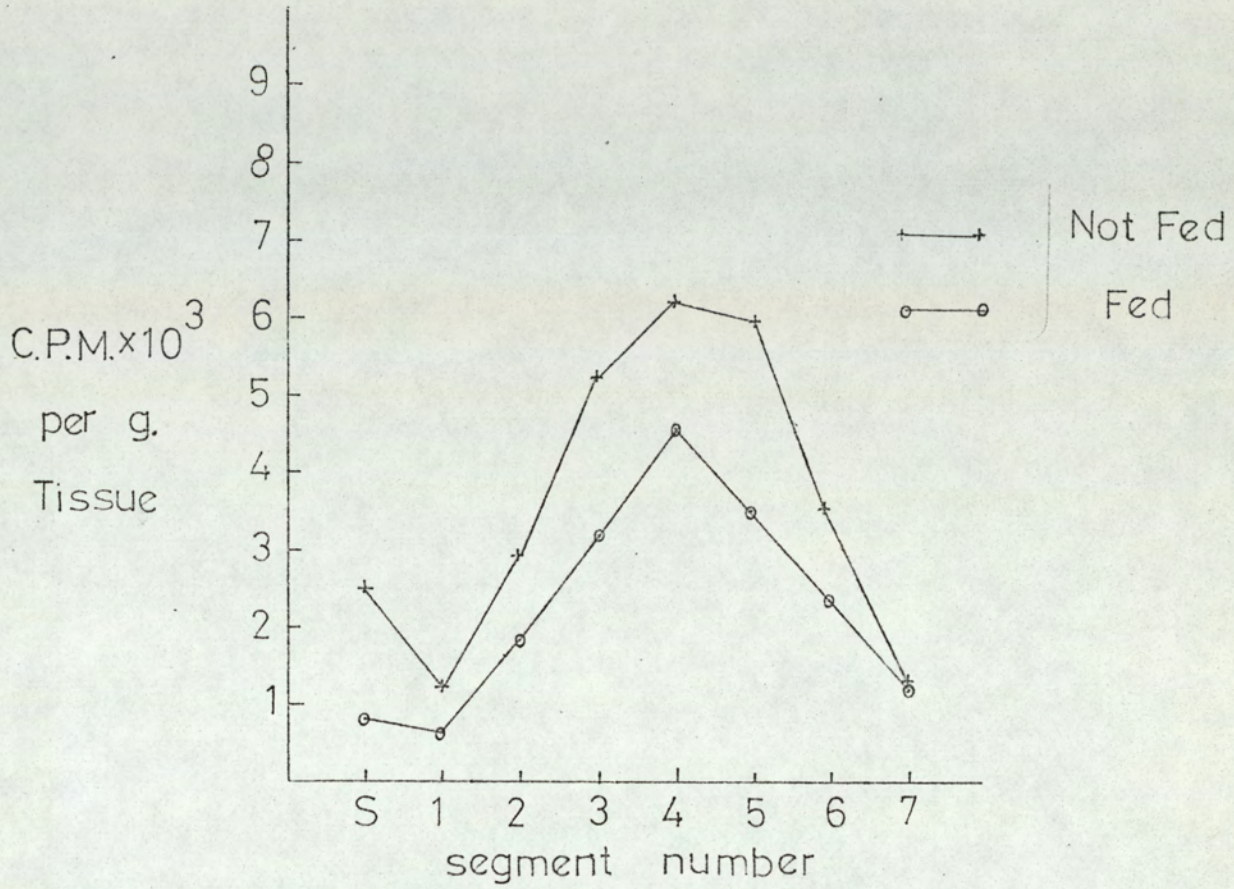


Fig. 2.4.6. C.P.M. / g tissue (wet weight) 2 h after dosing with 1 µCi and 500 µg DL-α-tocopherol (5-methyl-^{3}H).

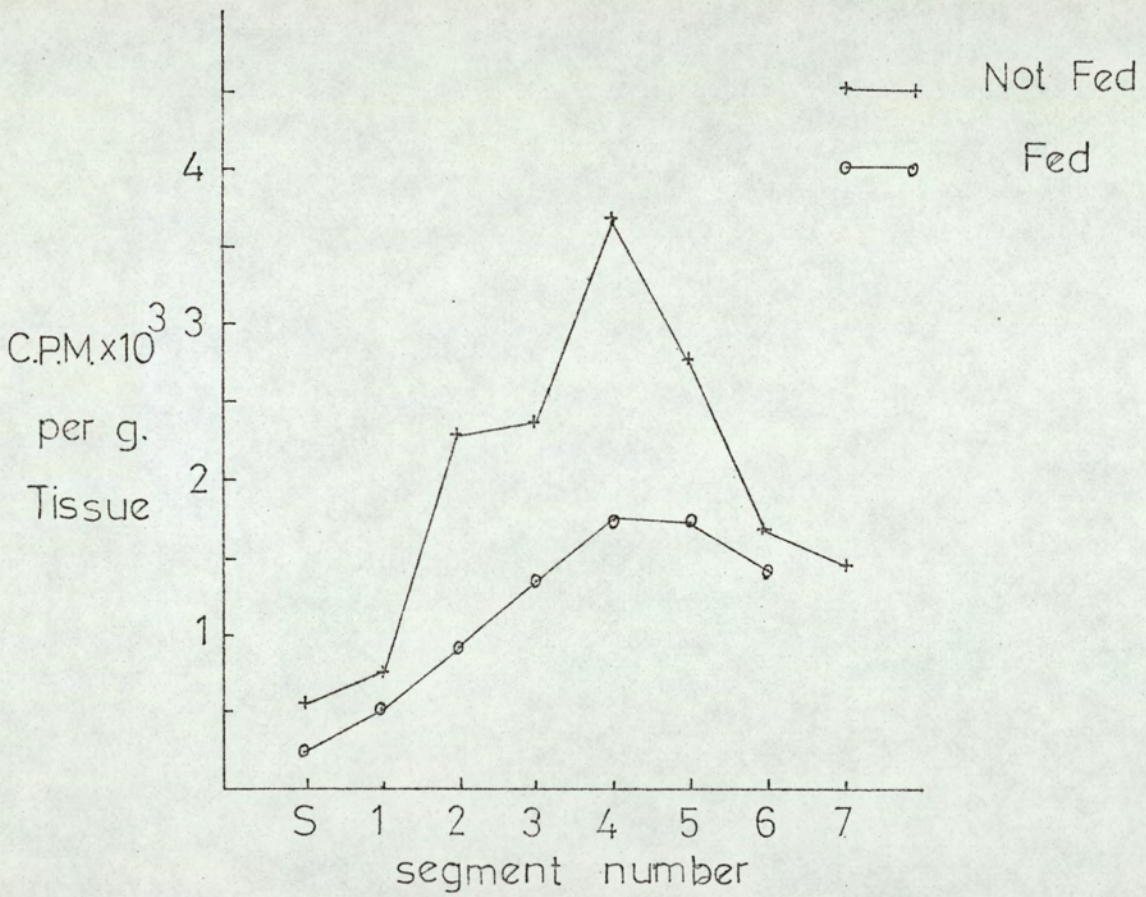


Fig. 2.4.7. C.P.M. / g tissue (wet weight) 4 h after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

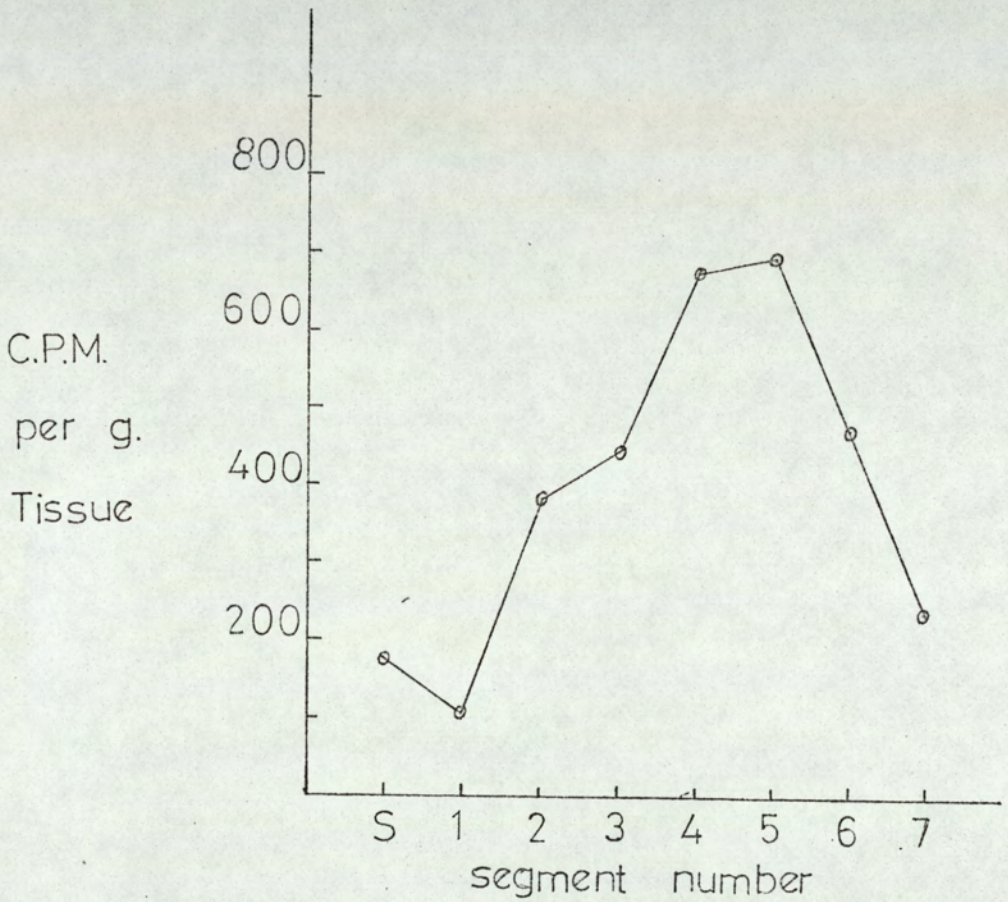


Fig. 2.4.8. C.P.M. / g tissue (wet weight) 6 h after dosing with 1 µCi and 500 µg DL-α-tocopherol (5-methyl-^{3}H).

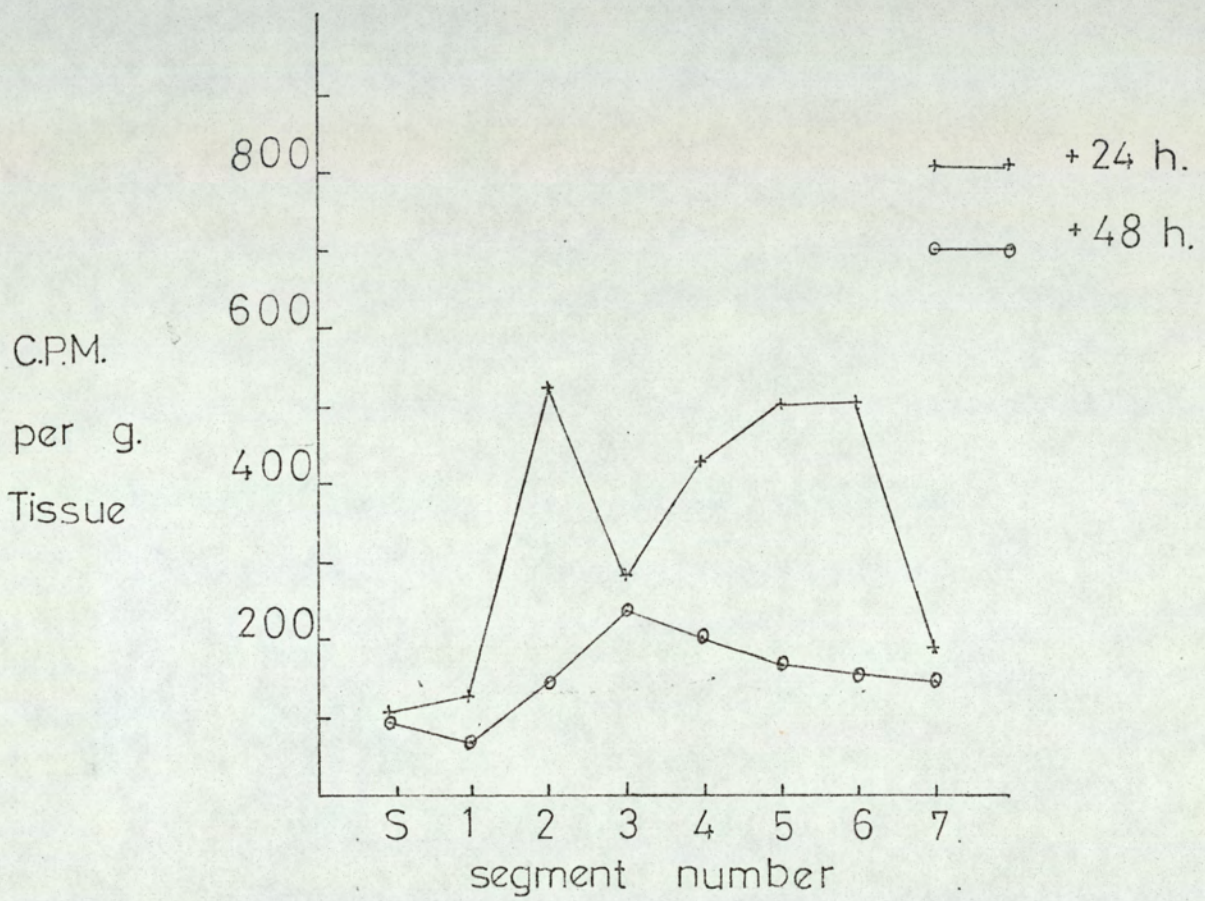


Fig. 2.4.9. C.P.M. / g tissue (wet weight) 24 h and 48 h after dosing with 1 µCi and 500 µg DL-α-tocopherol (5-methyl-^{3}H).

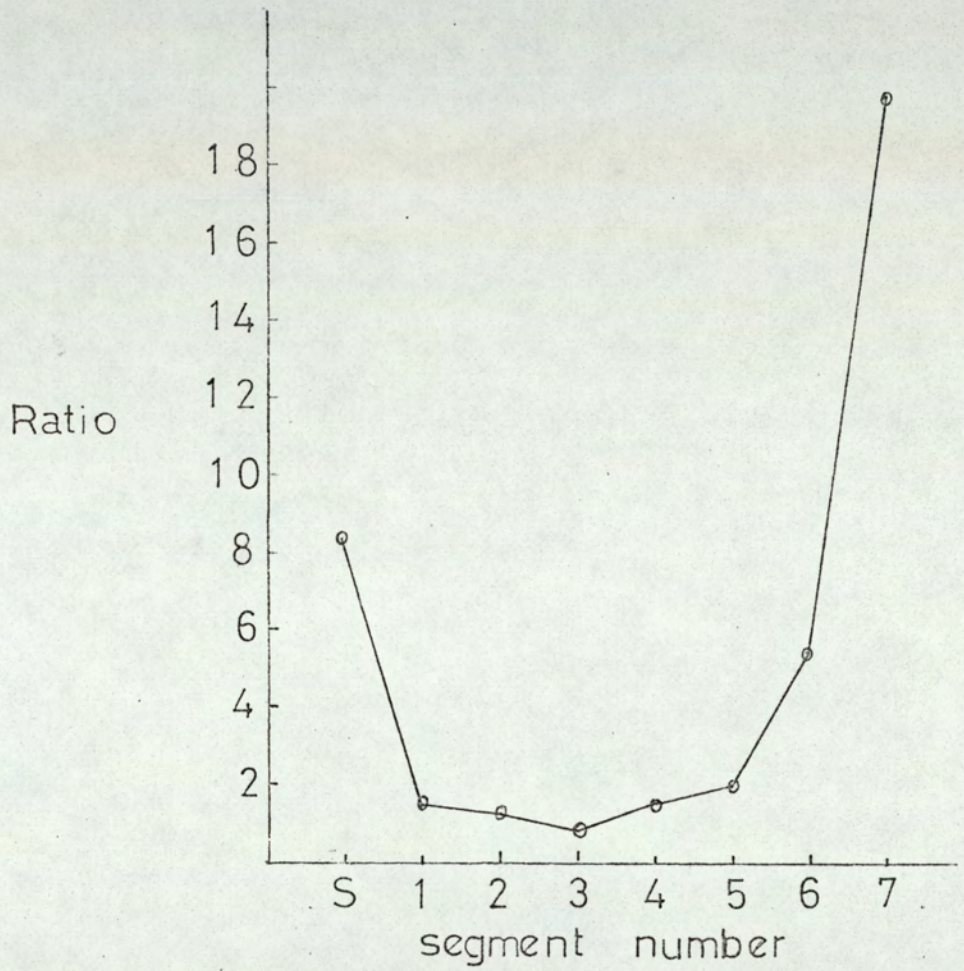


Fig. 2.4.10. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 2 h (not fed) after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

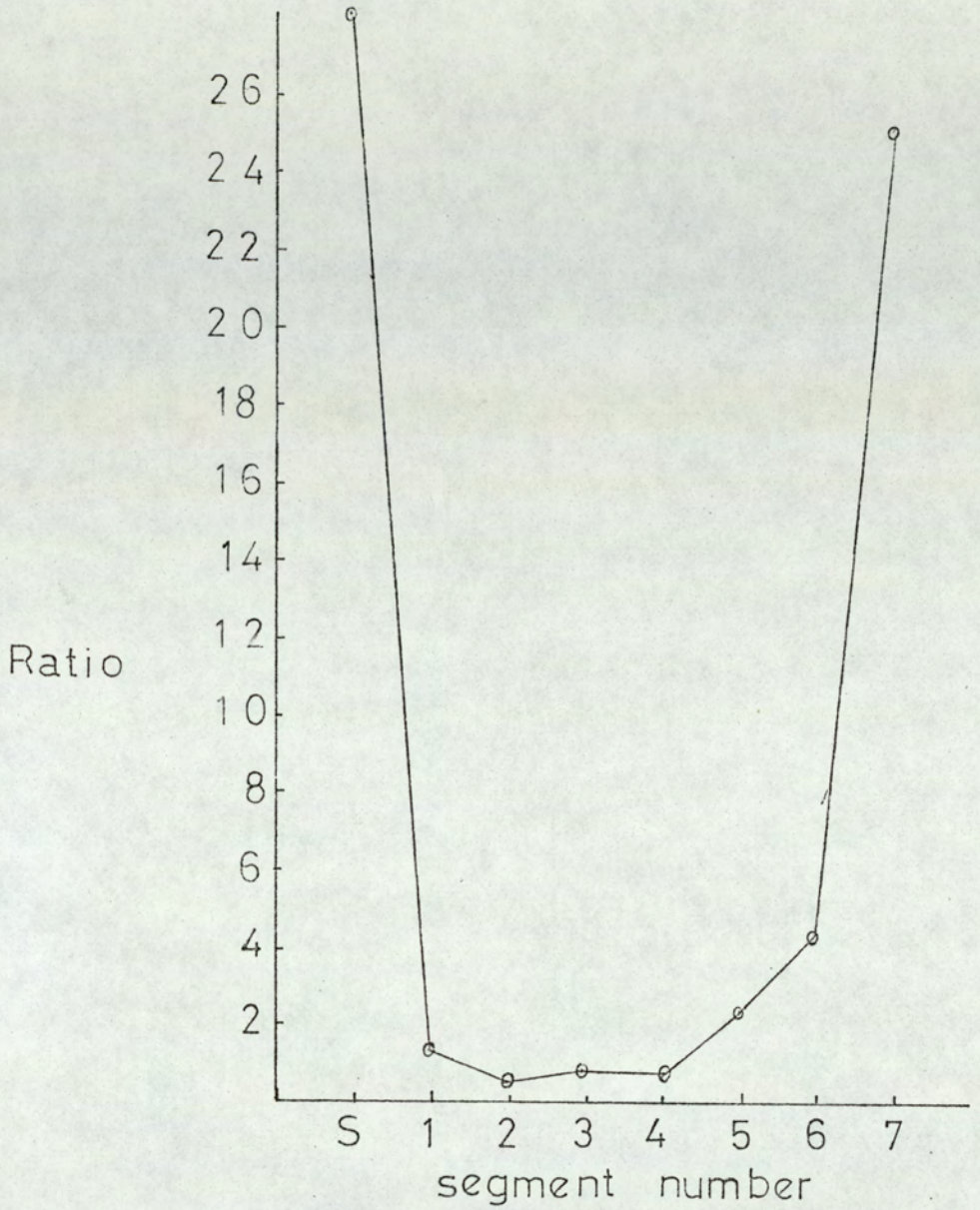


Fig. 2.4.11. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 2 h (fed) after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

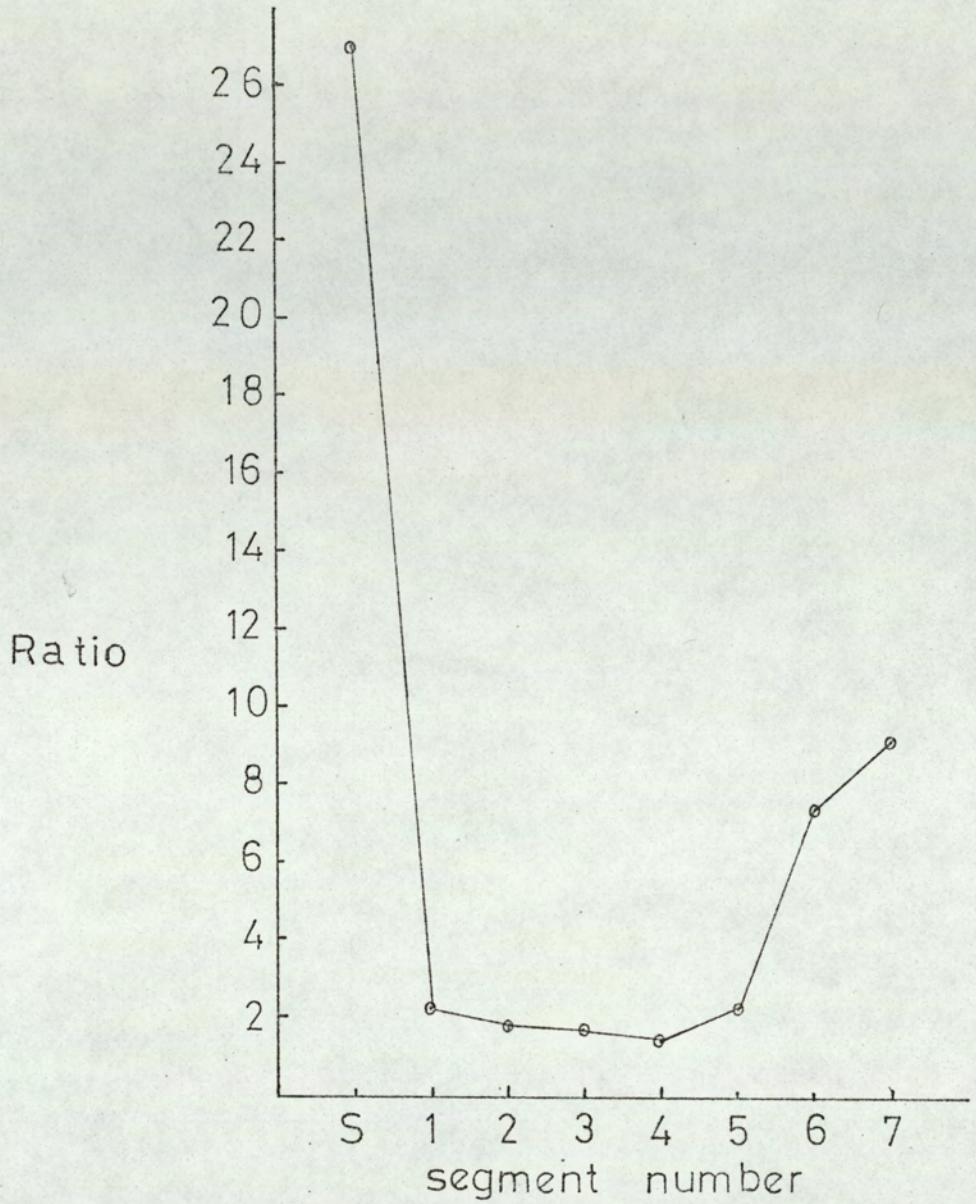


Fig. 2.4.12. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 4 h (not fed) after dosing with 1 µCi and 500 µg DL-α-tocopherol (5-methyl-^{3}H).

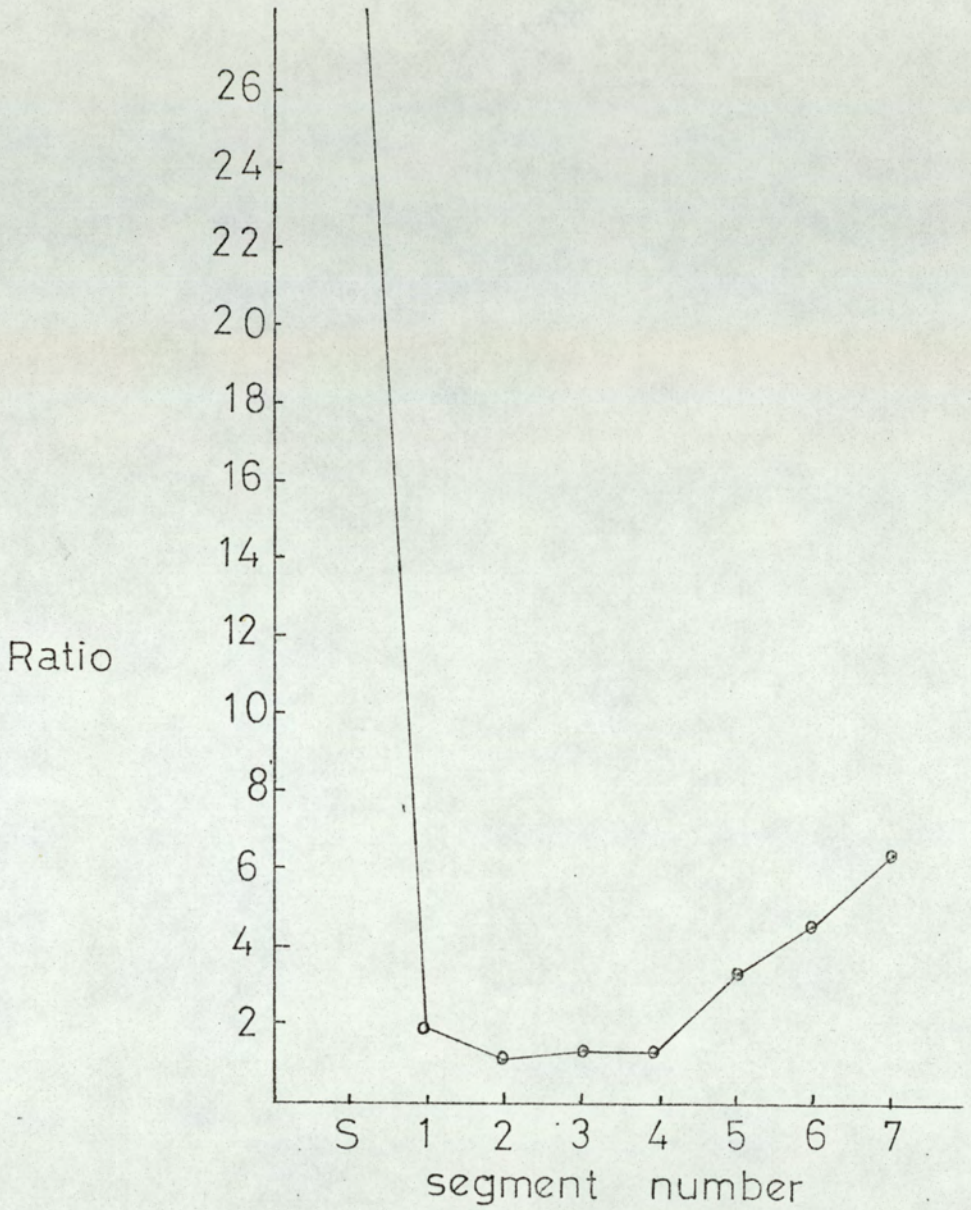


Fig. 2.4.13. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 4 h (fed) after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

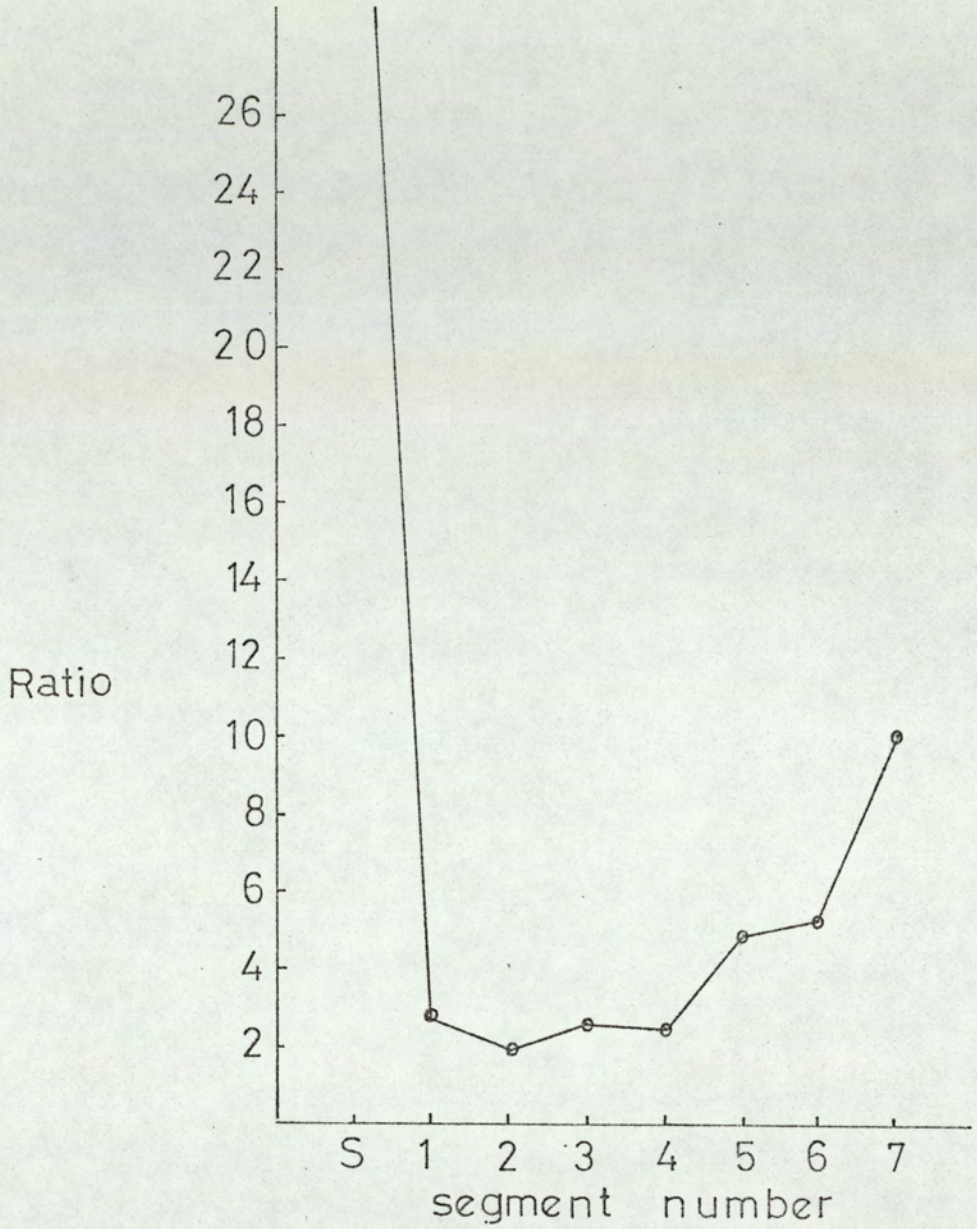


Fig. 2.4.14. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 6 h (fed) after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

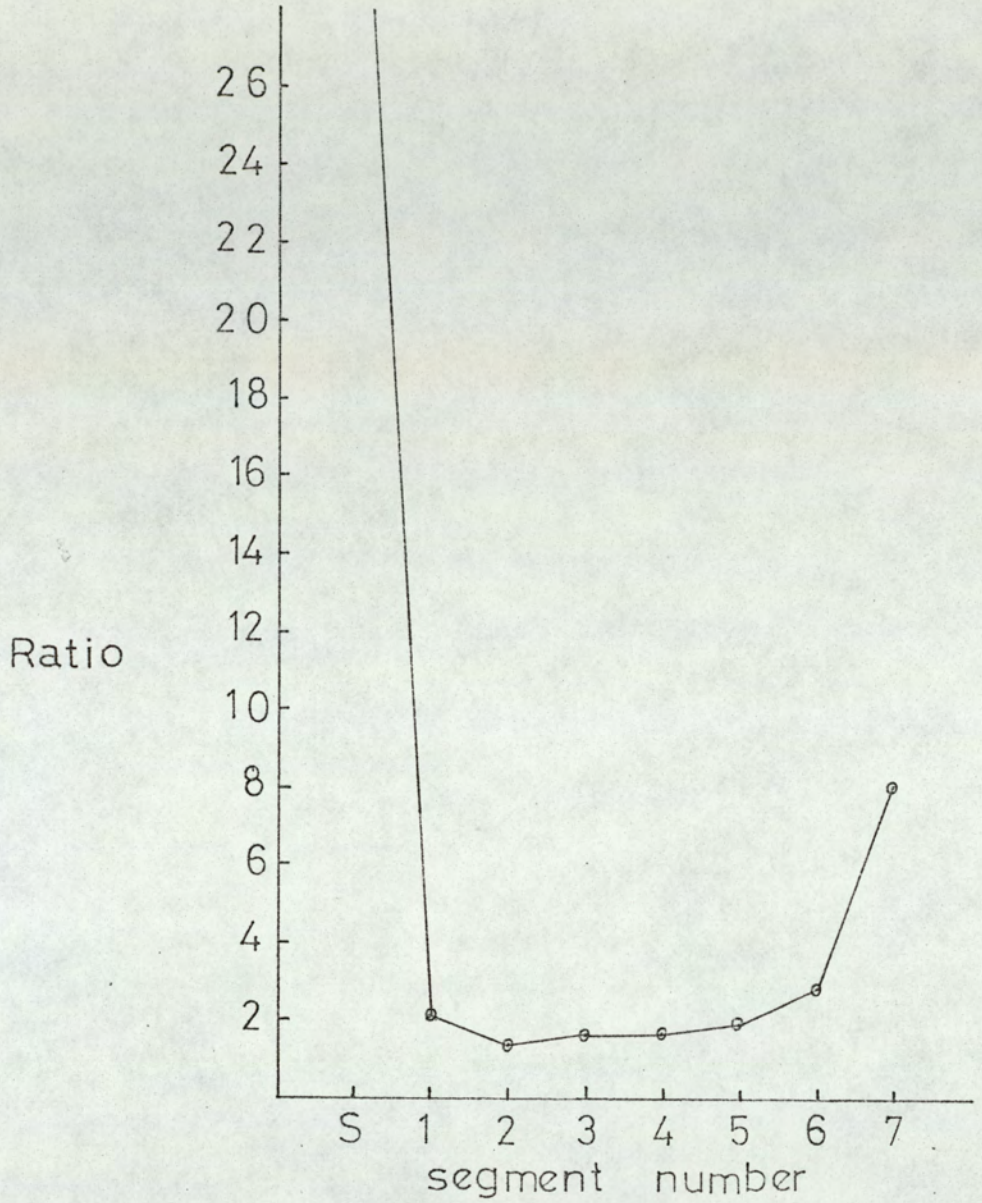


Fig. 2.4.15. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 24 h (fed) after dosing with 1 μCi and 500 μg DL-α-tocopherol (5-methyl-^{3}H).

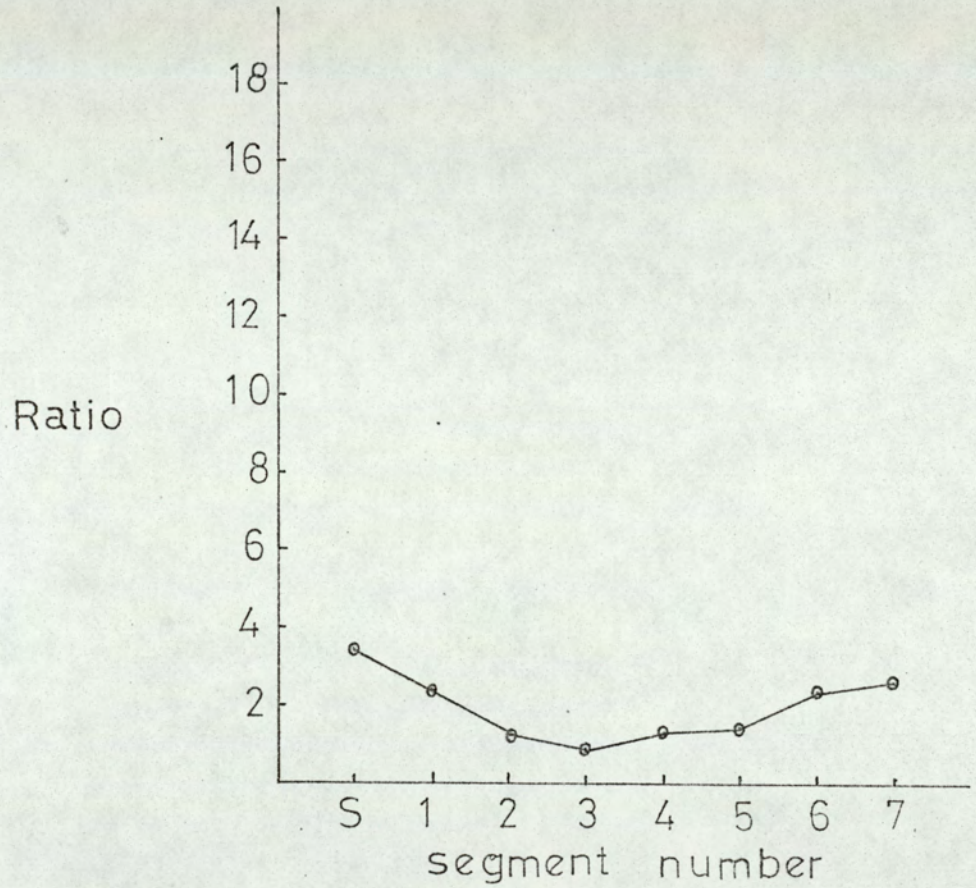


Fig. 2.4.16. Ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) 48 h (fed) after dosing with 1 µCi and 500 µg DL-α-tocopherol (5-methyl-^{3}H).

Table 2.4.13. Chloroform extractable radio-activity in rat livers after dosing with 1 μCi DL-α-tocopherol in 500 μg. DL-α-tocopherol.

Treatment	C.P.M./g. (fresh weight) liver.		
	n	$\bar{x}$	SD
+ 2h. not fed	6	1398	1166
+ 2h. fed	4	499	300
+ 4h. not fed	4	1740	108
+ 4h. fed	3	686	110
+ 6h. fed	4	971	575
+ 24h. fed	3	966	830
+ 48h. fed	2	162	64

2.4.4. Discussion

After giving the experimental rats an oral dose of 500 µg DL-∝-tocopherol their mean serum level of vitamin E was found to be 1.8 ± 0.6 µg/ml. The normal range for stock rat plasma tocopherol is given (Bieri et al (1970)) as 4-10 µg/ml, with a mean of 7.1 µg/ml and standard error of mean ± 0.63; these rats were maintained on Purina Lab Chow. The optimum dietary intake for rats is 50 mg/kg diet (Joubert (1967)); diet 41 was modified to 4 lb to prevent the appearance of symptoms of vitamin E deficiency in rats (Bruce and Parkes (1956)). Plasma levels of vitamin E in the range 3.9-5.6 µg/ml have been found (Bieri and Poukka (1970)) to be necessary to protect against significant (> 10%) red cell haemolysis in the presence of dialuric acid. It is concluded that the animals in this experiment were deficient in vitamin E. Both depleted (Krishnamurthy and Bieri (1963)) and presumed replete rats (Pearson and Barnes (1970)) have been used to study the absorption of vitamin E from the intestinal tract.

No attempt was made to identify the nature of the chloroform extractable radio-activity. Alpha-tocopherol has been shown to be absorbed into the lymph virtually unchanged (McMahon and Thompson (1970)). The object of this experiment was to find out whether any region of the small intestine showed an increased ability, relative to all other regions, to absorb vitamin E. The presence of chloroform extractable radio-activity in the intestinal segments was taken as evidence of the occurrence of absorption. The ratio of isotope present in the tissue relative to that within the lumen was used to measure the extent of absorptive competence. As shown in Figs.2.4.10.-16. the ratios were lowest in segments 2-4 inclusive, irrespective of whether or not the rats were fed. The high ratio found in the stomach after 24 hrs may be an indication that copro-

phagy had taken place, despite the fact that these rats were given a plentiful supply of food immediately after dosing with DL-α-tocopherol. High levels of radio-activity in the stomach contents of rats given an oral dose of radio-active α-tocopherol have been reported 16 hrs after ingestion by Sternberg and Pascoe-Dawson (1959). They attributed this persistence to re-secretion of vitamin E, possibly in the gastric juice.

The progression of the isotope along the lumen of the gut is seen in Figs.2.4.2.-5. A relatively large proportion of the isotope appears to remain within the lumen of the stomach; Figs.2.4.6.-9. show that there is not a correspondingly large amount of the isotope present in the stomach tissue. This elevated ratio shows that relatively little adsorption of the Tween 80 borne DL-α-tocopherol has taken place in the stomach, which is the most likely site for a non-specific reaction of this kind. The continued presence of chloroform extractable radio-activity in the gut lumen after 24 hrs and 48 hrs raises some important questions:-

(a) is the radio-activity residual i.e. has it never been absorbed,

(b) has coprophagy taken place,

(c) is the vitamin being recirculated?

From the results presented here it is not possible to tell, with any certainty, which of the above has taken place. The absorption of vitamin E is known to be incomplete when large doses are given to animals (Simon et al (1956), Dju et al (1950)). More recently it has been found (Pearson and Barnes (1970)) that 68% of a 600-800 µg dose of tocopherol was unabsorbed after 6 hrs in an in vivo small intestinal loop of rat. 10% of an intra-gastric dose of labelled tocopheryl acetate was collected from lymph fistula rats in 12 hrs (Gallo-Torres (1970)). Recirculation may have taken place

via an entero-hepatic mechanism but the relatively higher levels of chloroform extractable radio-activity found in the stomach after 24 hrs would seem to indicate that coprophagy had taken place.

Table 2.4.14. shows the mean ratios for each segment when all treatments are considered together and Table 2.4.15. gives the results of a Students 't' test performed between each successive segment. Fig.2.4.17. shows graphically the results given in Table 2.4.14. There is very little α-tocopherol absorbed from the rat stomach. (Sternberg and Pascoe-Dawson (1959) found that the stomach was as active as the jejuno-ileum in the absorption of radio-active α-tocopherol.) Some DL-α-tocopherol is absorbed from the duodenum (segment 1) but the major portion of the gut involved in its absorption is the jejunum and the proximal ileum (segments 2-4 inclusive).

There is an apparent difference between the uptake of DL-α-tocopherol in segments 2 and 3 when fed rats are compared with unfed ones, the time after dosing being the same (see Table 2.4.16 and Table 2.4.17.).

There is a statistically significant difference in the levels of chloroform extractable radio-activity 4 hrs after dosing when fed rats are compared with unfed ones. The apparently greater uptake of α-tocopherol when the rats are allowed to feed after the oral dose is probably due to the formation of mixed micelles during the process of digestion. It has been shown (McMahon and Thompson (1970)) that the absorption of ^{3}H-α-tocopherol by rats is greatly enhanced when it is administered in mixed micelles rather than emulsions. The rate of exit of vitamin E into the lymph has also been shown to be enhanced by simultaneous administration of exogenous triglycerides (McMahon, Neale and Thompson (1971)). In this experiment exogenous triglycerides were supplied in the cube diet mixture. The counts in

Table 2.4.14. Mean ratios for each segment (all treatments considered together).

Segment	n	$\bar{x}$	SD
Stomach	28	34·2	41·1
1	28	1·86	1·00
2	28	1·19	1·04
3	28	1·34	0·91
4	28	1·40	0·90
5	28	2·40	2·18
6	28	4·46	3·48
7	26	15·81	23·88
8	6	31·68	39·11
2-4	84	1·31	0·94

Table 2.4.15. Students 't' test (one tailed) on successive segments.

Segment	SE difference	t	DF	p
Stomach vs 1	7·91	4·09	27	0·0005
1 vs 2	0·28	2·41	54	0·01
2 vs 3	0·27	0·56	54	0·3
3 vs 4	0·25	0·24	54	0·4
4 vs 5	0·45	2·20	27	$0{\cdot}025 > p > 0{\cdot}0125$
5 vs 6	0·79	2·62	54	$0{\cdot}01 > p > 0{\cdot}005$
6 vs 7	4·65	2·44	27	0·01
1 vs 2-4	0·21	2·62	110	0·005
2-4 vs 5	0·30	3·65	27	0·0005

N.B. When SDs differ significantly then DF = n-1 where n is the number in the group with the larger SD.

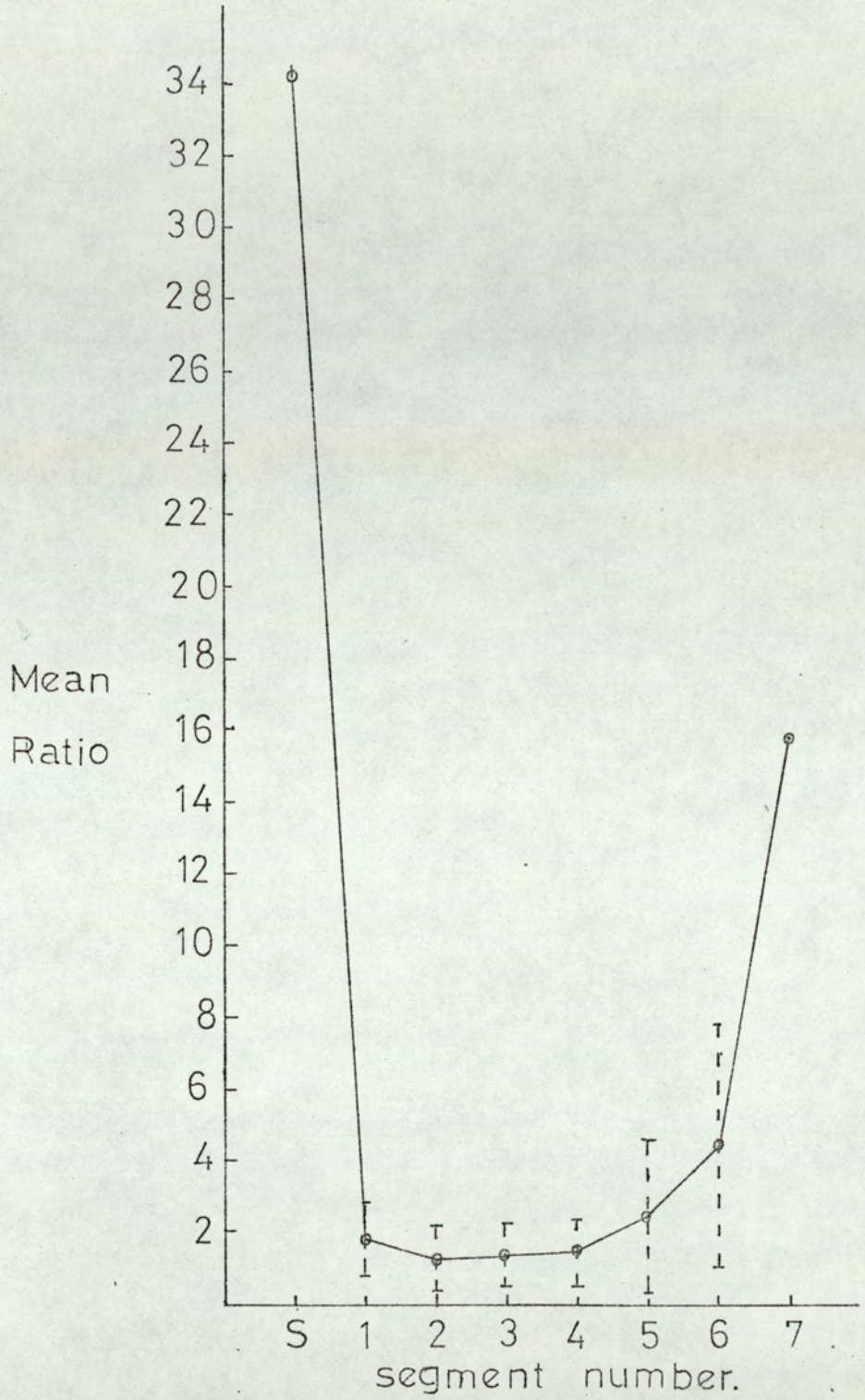


Fig. 2.4.17. Mean ratio of C.P.M. contents (including washings) : C.P.M. / g tissue (wet weight) for each segment (all treatments considered together).

Table 2.4.16. Comparison of absorption of DL-α-tocopherol two hours after dosing, between fed and unfed rats (1 tailed Students 't' test).

Segment	Fed			Not Fed			DF	t	p
	n	$\bar{x}$	SD	n	$\bar{x}$	SD			
2 Ratio	4	0·5	0·2	4	1·2	0·5	3	2·25	0·01>p>0·005
C.P.M. contents.	4	890	613	4	3162	1951	3	1·92	0·01>p>0·005
C.P.M./g. tissue.	4	1895	856	4	2963	1663	3	0·99	0·2
Liver. C.P.M./g. tissue.	4	499	300	6	1398	1166	5	1·35	0·15>p>0·10
3 Ratio	4	0·8	0·4	4	0·7	0·2	3	0·39	0·40
C.P.M. contents.	4	2740	2280	4	3648	2423	6	0·47	0·30
C.P.M./g. tissue.	4	3170	1117	4	5254	3741	3	0·92	0·20

Table 2.4.17. Comparison of absorption of DL-α-tocopherol four hours after dosing, between fed and unfed rats (1 tailed Students 't' test.)

Segment		Fed			Not Fed			DF	t	p
		n	$\bar{x}$	SD	n	$\bar{x}$	SD			
2	Ratio	3	1·0	0·1	4	1·7	2·3	3	0·44	0·35
	C.P.M. contents.	3	873	417	4	714	429	5	0·42	0·35
	C.P.M./g. tissue.	3	910	452	4	2290	2670	3	0·75	0·25
Liver. C.P.M./g. tissue.		3	686	110	4	1740	108	5	10·7	0·0005
3	Ratio	3	1·3	0·3	4	1·6	1·2	3	0·36	0·35
	C.P.M. contents.	3	1761	875	4	3484	3943	3	0·63	0·30
	C.P.M./g. tissue.	3	1364	445	4	2343	1454	3	0·95	0·20

the lumen and mucosa are lower throughout the length of the small intestine of the fed rats when compared with those of unfed rats (Figs.2.4.2.-9.). This points to a more efficient absorption into the mucosa and transport away from the intestine in conditions which facilitate the formation of mixed micelles in the lumen and chylomicrons within the mucosa (Thompson (1971)).

The administration of DL-∝-tocopherol in 5% Tween 80 and subsequent with-holding of the rats' food appears to increase the amount of radio-activity present in the liver when compared with rats who were fed after dosing. Kelleher, Davies and Losowsky (1969) found that administration of radio-active DL-∝-tocopherol in Tween 80 gave rise to higher tissue levels of the vitamin than did administration in arachis oil or alcohol, but the net absorption was the same in all cases. There is no evidence from this study that the site of absorption is altered whether the rats' fast is continued after the administration of DL-∝-tocopherol or not. All rats were fasted overnight prior to the commencement of the experiment. McManus and Isselbacher (1970) have reported a greater mucosal cell mass, amino acid and hexose transport <u>in vitro</u> in over night fed versus over night fasted rats. It is thought unlikely that the physical presence of food within the lumen would produce a marked increase in the mucosal cell mass within 4 hrs of feeding.

3. VITAMIN B_6

3.1.1. Introduction

Vitamin B_6 is the generic descriptor for the group of water soluble vitamins which comprise pyridoxine (pyridoxol), pyridoxamine and pyridoxal. Biological activity is confined to the phosphorylated forms of pyridoxal and pyridoxamine. Vitamin B_6 was first defined and delineated as a distinct entity from other members of the vitamin B_2 complex by György in 1934. The isolation of pure, crystalline vitamin B_6 was first reported by Lepkovsky in 1938. The structure of pyridoxine was established, independently, by Harris and Folkers (1939) and Kuhn, Westphal, Wendt and Westphal (1939), and its initial synthesis was effected in the same year (Harris, Stiller and Folkers (1939)).

Recognition of forms of vitamin B_6 other than pyridoxine was made by Snell, Guirard and Williams (1942) when using a microbiological assay technique on extracts of natural materials. In 1944, Harris, Heyl and Folkers were able to show that these substances were pyridoxal and pyridoxamine. It was later found that pyridoxal and pyridoxamine are more widely distributed than pyridoxine and account for most of the vitamin B_6 in natural materials (Snell (1945), Rabinowitz and Snell (1948) As a result of microbiological experiments performed by Gunsalus, Bellamy and Umbriet (1944) the existence of a highly active phosphorylated derivative of pyridoxal was deduced. The exact structure of this compound was not elucidated until Heyl, Luz, Harris and Folkers (1951) and Baddiley and Mathias (1952) performed a chemical synthesis of pyridoxal-5'-phosphate and were able to show that this compound had the same biological activity as the compound synthesised earlier by yeasts. A fifth naturally occurring form of vitamin B_6, pyridoxamine phosphate, was discovered and synthesised in 1947 by Rabinowitz and Snell, who heated pyridoxal phosphate and glutamic acid.

Human requirement for vitamin B_6 was not definitely established until Snyderman , Holt, Carretero and Jacobs (1953) reported on convulsions seen in a mentally retarded infant and an older child. Both children had been fed a vitamin B_6 deficient diet and their symptoms quickly resolved when given pyridoxine. Symptoms of this type had already been seen in vitamin B_6 deficient animals.

The role of vitamin B_6 in metabolism is that of a co-enzyme. The most active form is pyridoxal-5'-phosphate (PLP); pyridoxamine phosphate is only a co-enzyme in transamination reactions. PLP is involved in amino-acid transformations e.g. transamination, decarboxylation, racemisation and beta-, gamma- eliminations. These reactions have been reviewed by Guirard and Snell (1964). The discovery by Baranowsky, Illingworth, Brown and Cori (1957) that the presence of PLP was essential for the action of glycogen phosphorylase suggested a possible role for PLP, that of a stabiliser of protein tertiary structure.

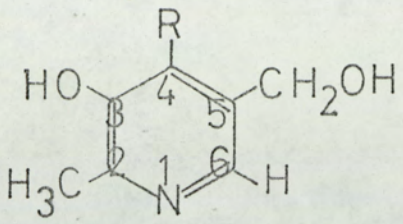
Absorption of vitamin B_6 has been investigated in rats and man by giving an oral dose of ^3H-labelled pyridoxine hydrochloride and measuring the rate of excretion of radio-activity in the urine. (Brain, Stewart and Booth (1963), Brain and Booth (1964)). They found that the absorption of ^3H-pyridoxine occurs rapidly from the upper small intestine of both man and rat. By using everted loops of rat small intestine it was shown that the mechanism of absorption was one of passive diffusion. Six out of 13 patients with adult coeliac disease (ACD) showed impairment of pyridoxine absorption (Brain and Booth (1964)) but the degree of impairment could not be correlated with results of other intestinal absorption tests. Absorption was also found to be unaffected by terminal ileal regional enteritis (RE) or resection of the ileum. Earlier, Girdwood (1956) had been unable to prove malabsorption of pyridoxine in 10 patients

suffering from steatorrhoea. The presence of subclinical deficiency of vitamin B_6 in patients with ACD has been suggested by Kowlessar, Haeffner and Benson (1964). Baker and Sobotka (1962) have shown that patients with ACD may have subnormal levels of pyridoxine in their serum.

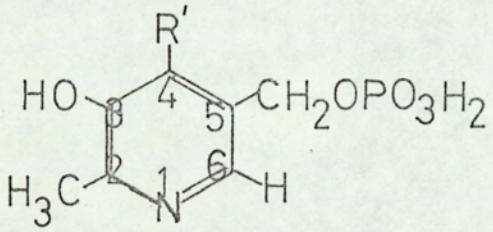
3.1.2. Chemistry and metabolism of vitamin B_6

The naturally occurring and biologically active forms of vitamin B_6 are shown in Fig.3.1.1. A simplified summary of the metabolic inter-conversions of vitamin B_6 is given in Fig.3.1.2. Snell (1964) has presented evidence which suggests that a lowered content or reduced activity of pyridoxal kinase may reduce the supply of PLP below that necessary for optimal enzymatic function even although vitamin B_6 intake is superficially adequate. Availability of PLP *in vivo* may also be impaired if levels of pyridoxine phosphate oxidase are reduced or absent. (Pyridoxine is the form of vitamin B_6 normally employed for therapeutic purposes.) When pyridoxine kinase from *E. coli* B was purified and its properties studied (White and Dempsey (1970)) it was noted that pyridoxal was a strong inhibitor of this enzyme. The *in vivo* conversion of pyridoxine to PLP by human red blood cells has been studied (Anderson, Fulford-Jones, Child, Beard and Bateman (1971)) and the authors proposed that PLP was formed in the red blood cells from pyridoxine and then converted to pyridoxal, the latter being gradually released into the plasma. A similar sequence of events had already been shown to take place *in vitro* with liver homogenates (Wada, Morisue, Nishimura, Morino, Sakamoto and Ichihara (1959)), as had the presence of pyridoxal kinase within red cells (Hamfelt (1967)).

The majority excretory product of vitamin B_6 metabolism is 4-pyridoxic acid. Isotopic techniques have been used to show that 40-50% of vitamin B_6 ingested in a normal diet is excreted in the urine in this form (Tillotson, Sauberlich, Baker and Canham (1966), Johansson, Lindstedt, Register and Wadstrom (1966)). Most of the remainder of the excreted vitamin B_6 is pyridoxal with lesser amounts of pyridoxamine;

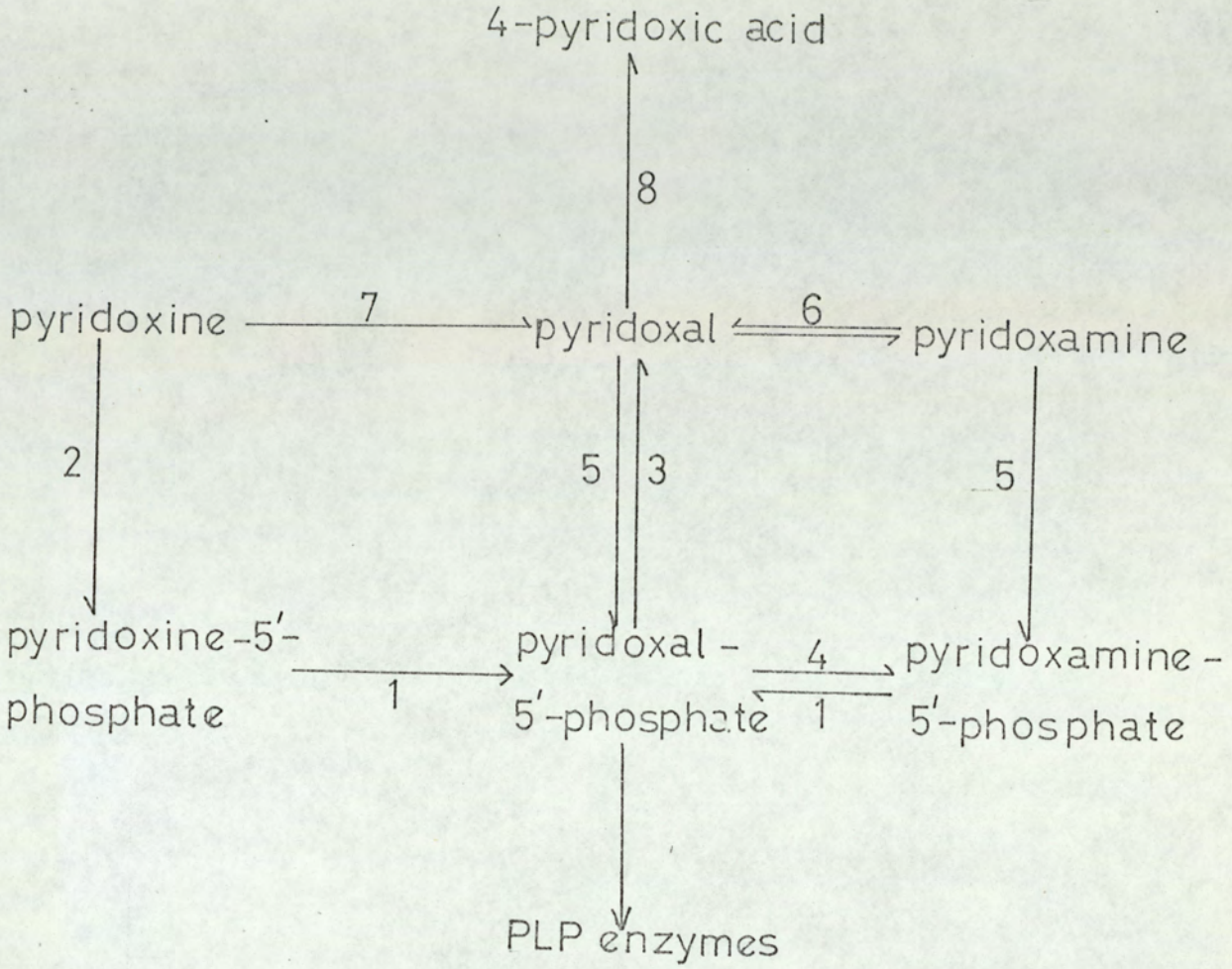


R =	CH_2OH	pyridoxine (pyridoxol)
R =	CHO	pyridoxal
R =	CH_2NH_2	pyridoxamine
R =	COOH	4-pyridoxic acid



R' =	CHO	pyridoxal-5'-phosphate
R' =	CH_2NH_2	pyridoxamine -5'-phosphate

Fig. 3.1.1. **The naturally occurring and biologically active forms of vitamin B_6.**



Enzyme 1 pyridoxine phosphate oxidase

2 pyridoxine kinase

3 pyridoxine phosphate phosphatase

4 *in vivo* transaminase dissociation

5 pyridoxal kinase

6 various apo-transaminases

7 pyridoxine oxidase

8 aldehyde oxidase

Fig. 3.1.2. The metabolic interconversions of vitamin B_6.

pyridoxine and phosphorylated forms have also been detected in the urine (Kelsay, Baysal and Linkswiler (1968)).

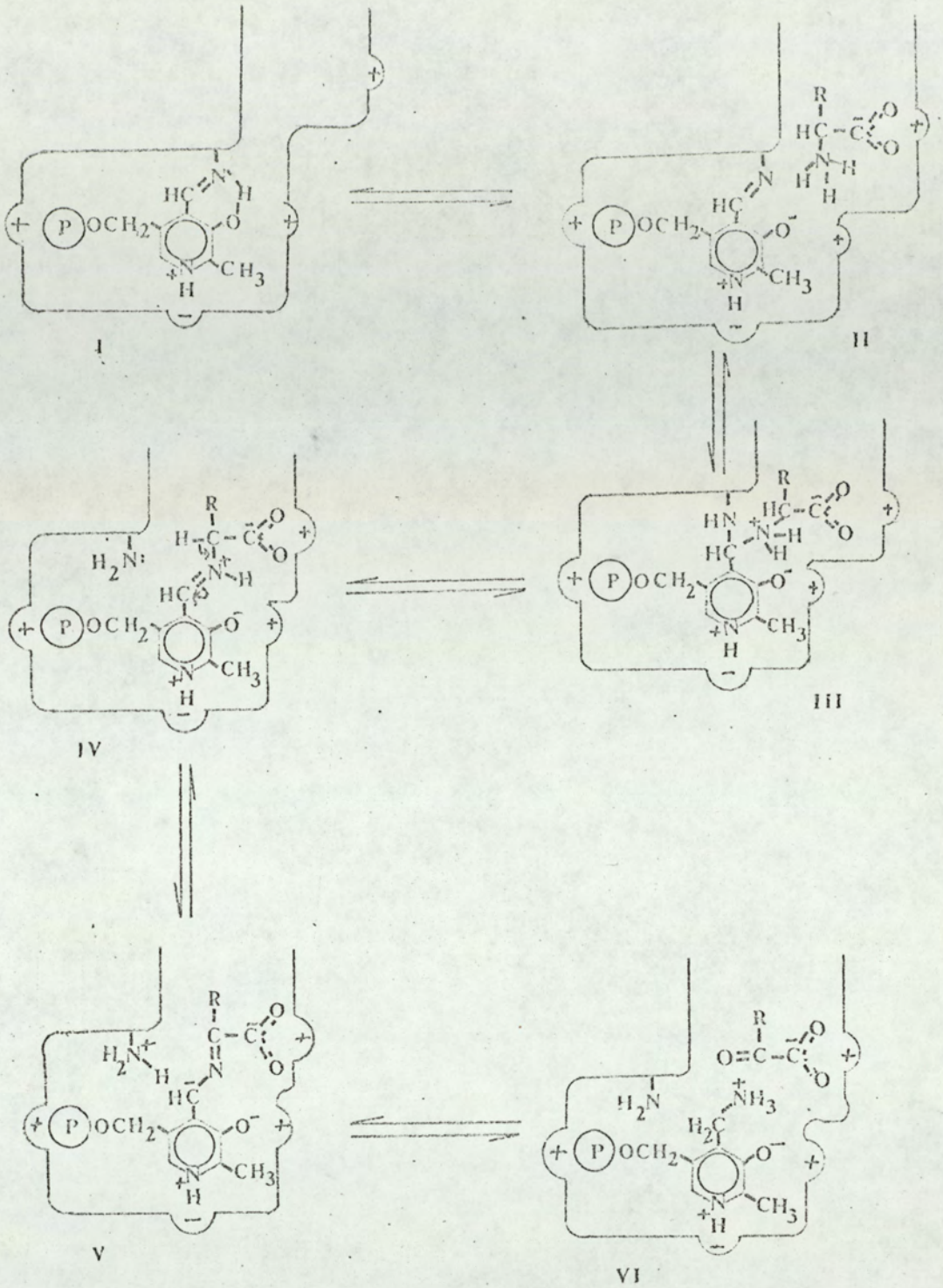
Marked differences exist in the mode of binding of PLP to apo-enzymes and these have been reviewed by Guirard and Snell (1964). PLP has been shown to have different affinities for 3 different decarboxylases capable of forming histamine (Udenfriend (1964)). Wada and Morino (1964) have shown that the immunologically distinct mitochondrial and extra-mitochondrial glutamic aspartate transaminases, which were isolated from single tissues, had a six-hundred-fold difference in affinity for PLP.

A general mechanism for vitamin B_6 catalysed reactions, based on theoretical considerations, was developed by Braunstein and Shemyakin (1953). This mechanism was the same as that independently, but slightly later, proposed by Metzler, Ikawa and Snell (1954) which was derived from model experiments involving non-enzymatic pyridoxal catalysed reactions of amino acids. Pyridoxal and metal ions e.g. Cu^{++}, Fe^{+++}, Al^{+++}, Ni^{++}, Mn^{++}, Zn^{++}, Mg^{++}, substituted for the apo-enzyme/PLP complex. Fig.3.1.3. shows the postulated mechanism for enzymatic transamination with PLP acting as a co-factor.

The binding of PLP to specific proteins has been investigated and reviewed (Braunstein (1964)). In all the enzymes studied the carbonyl group of PLP was found to be bound to an amino group of a protein. The NH_2 group involved in intramolecular bonding with the co-enzyme has been identified as the ϵ-amino group of a lysine side chain. Polyanovsky and Keil (1963) have elucidated the peptide sequence from the catalytic centre of aspartate transaminase. It is:-

PLP
|
-Lys-Lys-Ser-Asp(or $AspNH_2$)-Phe-

Recently, Dunitru, Iordachescu and Niculescu (1972) have found that the purified apo-enzyme L-alanine : 2 oxoglutarate amino transferase, from



I transaminase (inactive form)

II amino acid + transaminase (active form)

III hypothetical tetrahedral addition intermediate

IV enzyme-amino acid Schiff base

V enzyme-keto acid Schiff base

VI keto acid + transaminase

Fig. 3.1.3. Scheme of presumed molecular mechanism of enzymatic transamination (after Braunstein (1964)).

Glycine hispida var. Cheepewa seeds, contains 2 mol of PLP per mol of apo-enzyme. Snell (1958) found that, for the enzymes tested, all the substitutions on the pyridine ring which are found in PLP play an important role in permitting combination to yield a halo-enzyme.

Deficiency of the active form of vitamin B_6, namely PLP, either as a result of inadequate diet or inability to convert the dietary supply to PLP need not lead to a uniform decrease in the activities of PLP dependent enzymes. This is because these enzymes have differing decay rates and also differing affinities for the co-enzyme.

3.1.3. The occurrence of vitamin B_6 in the diet

Vitamin B_6 fulfills a fundamental metabolic role, hence, it is found in almost every raw food substance. Considerable losses of vitamin B_6 occur in the preparation and cooking of food e.g. in wheat flour most of the vitamin B_6 is found in the bran layers and 80-90% of it is removed in making white flour. Borsook (1964) has reported that 18-70% of the vitamin B_6 content of meat can be lost in cooking. These losses have recently been reviewed by Schroeder (1971) who based his figures on those supplied by Orr (1969).

Severe vitamin B_6 deficiency as a result of inadequacy of dietary intake is thought to be unlikely. However, there was an "outbreak" of pyridoxine deficiency symptoms in infants in the U.S.A. who had been fed an exclusively synthetic milk product (Coursin (1954)). Borsook (1964) has published a list of the vitamin B_6 contents of some common foods and, in general, diets found to supply high levels of vitamin B_6 contained most meat and/or legumes. By extrapolating the results of Deutsch, Duffy, Pillsbury and Loy (1963) for the nutrient content of diets consumed by 16-19 year old boys (which supplied 4,200 calories and 2.6 mg vitamin B_6) Borsook (1964) found the great majority of women and most men over 65 would, if they consumed the recommended allowance of calories, derive less than 1.5 mg/day of vitamin B_6 from dietary sources (assuming the dietary mix remained constant). Leitch and Hepburn (1961) have collected estimates of the national average vitamin B_6 intake per capitum, based on the national average food consumption. These are:-

(a) U.S.A. (1949) 1.7 mg vitamin B_6

(b) U.K. (1956) 1.6 - 1.9 mg vitamin B_6

(c) U.K. (1961) 2.3 mg vitamin B_6

Table 3.1.1. lists the estimates of the vitamin B_6 requirements of adult humans.

Table 3.1.1. Estimated daily requirement for vitamin B_6.

Reference.	Basis of estimate.	Requirement (mg).
Vilter, Mueller, Glazer, Abraham,	1,2.	0·5
Thompson & Hawkins. (1953)	3	2·0-3·0
Harding, Plough & Freidman. (1959)	3	1·93; not more than 2·76.
Berdjis, Greenberg, Rinehart & Fitzgerald. (1960)	4	50 µg / kg
Plough (1960)	3	2·5 ("maximal" minimum daily)
Cheslock & McCully. (1960)	3	0·5
Babcock, Brush & Sostman. (1960)	5	"considerably higher than 0·5
Coursin & Brown (1961)	1,6	2·0 ± 0·5
Efremov (1962)		2·0
Sauberlich (1963)	1,3	1·0-1·5 (30 g protein) 1·75-2·0 (100g protein)
Recommended dietary allowance (NRC)(1968)		2·0
Donald,McBean,Simpson,Sun & Aly (1971)	7,8	Women 1·5

Basis of estimate:-1. Clinical

2. B_6 antagonist

3.Tryptophan load

4. Optimum growth (Rhesus monkey)

5. Serum transaminase level

6. Metabolic

7. 4-pyridoxic acid excretion

8. Erythrocyte transaminases.

Vitamin B_6 requirement is affected by aging. Boxer, Pruss and Goodhart (1957) found that the PLP levels found in leucocytes decreased with increasing age. Ranke, Tauber, Horonick, Ranke, Goodhart and Chow (1960) found that serum transaminase activity declined with age. The addition of PLP to the sera of young and old people resulted in an increase of serum transaminase activity of the older group to the extent that both sets of *in vitro* stimulated sera had approximately the same activity. The authors felt that the difference could not be explained solely on the basis of differences in the dietary intake of vitamin B_6 since a variety of diets were consumed by both groups. Lowered levels of serum pyridoxal with increasing age have been reported by Hamfelt (1964), Walsh (1966), Anderson, Peart and Fulford-Jones (1970). Hamfelt (1964) suggested that defective nutrition, absorption, phosphorylation of the vitamin to its active form, or increased urinary loss could account for the lowering of serum PLP levels with age.

Evidence for an increased requirement for vitamin B_6 by women using oestrogen containing oral contraceptives has been presented by Toseland and Price (1969) and by Luhby, Davis, Murphy, Gordon, Brin and Speigel (1970). Rose, Strong, Adams and Harding (1972) have diagnosed sub-clinical vitamin B_6 deficiency in 7 of 31 women who had been taking oral contraceptives for 6-36 months. The diagnosis was based on a decreased excretion of 4-pyridoxic acid. Six of the 7 women judged to be deficient also had a raised 3-hydroxykynurenine:3-hydroxyanthranilic acid ratio after an oral dose of 2 g L-tryptophan. A total of 26 of the women studied had abnormal tryptophan metabolism after loading with the amino acid.

Since more than 30 enzymatic reactions are known in which PLP is a co-factor, individual variations in requirement for vitamin B_6 are likely to be greater than for other vitamins. There are large

differences in the affinities of co-factor for apo-enzyme since there are many loci for mutation and each mutation may result in a decrease of enzyme synthesis, or decreased affinity for the co-factor. The most marked of these genetic variations have been grouped together and termed "pyridoxine dependency syndromes" (Frimpter, Andelman and George (1969)). These patients need many times the normal requirement, as much as 200-600 mg daily, to produce the maximum alleviation of deficiency symptoms. Generally, there is only a single deficiency symptom e.g. cystathioninuria, anaemia or xanthurenicaciduria and not a multiplicity of symptoms one would expect in a true deficiency state.

3.1.4. The effects of vitamin B_6 deficiency

Spies, Bean and Ashe (1939) were first to report the occurrence of spontaneous vitamin B_6 deficiency in humans. Additional metabolic experiments were conducted (Spies, Ladisch and Bean (1940)) and it was found that subjects suspected to be pyridoxine deficient excreted little or no pyridoxine in the urine in the first hour after an intravenous dose of pyridoxine.

Pyridoxine deficiency can be produced relatively rapidly by experimentally restricting dietary intake. Manipulation of natural foods to give a diet yielding 0.34 mg vitamin B_6/day supplied to women (Donald et al (1971)) caused reduction in excretion of 4-pyridoxic acid in 32 days to levels at which a deficiency state existed (using the criteria of Baysal, Johnson and Linkswiler (1966)). Cheslock and McCully (1960) found biochemical evidence of vitamin B_6 deficiency after 28 days in subjects whose diets contained 0.4-0.5 mg vitamin B_6/day. Baker et al (1964) found that vitamin B_6 deficiency could be produced in 14 days in subjects given a liquid formula diet supplying 0.06 mg vitamin B_6 and 100 g protein per day. By the twenty-first day these subjects had become so depleted that supplementation with pyridoxine was deemed necessary. Subjects on a similar diet, but receiving 30 g protein/day, took twice as long to attain the same degree of depletion as those on a high protein diet. Anti-vitamin B_6 analogues such as desoxypyridoxine and isoniazid have produced vitamin B_6 deficiency symptoms (Sauberlich, Baker, Canham and Raica (1964)) as have penicillamine (Kuchinskas and du Vigneaud (1957)) and procarbazine (Chabner, DeVita, Considini and Oliverio (1969)).

The effect of vitamin B_6 deficiency on the human central nervous system (CNS) was first observed by Snyderman et al (1953) when convulsive seizures occurred in an infant who had been on a vitamin B_6 free diet. The symptoms responded to intravenous administration of 50 mg pyridoxine within hours. Coursin (1954) found that a seizure in an infant with

dietary deficiency of vitamin B_6 was abolished almost immediately, i.e. within 5 mins, after an intramuscular injection of pyridoxine. This indicated that, in normal individuals, conversion of pyridoxine to PLP and association of apo- and co-enzymes can be extremely rapid. Electro-encephalographic abnormalities have also been noted in adults on a vitamin B_6 deficient diet (Canham, Nunes and Eberlin (1963), Sauberlich et al (1964)). Reilly, Killam, Jenney, Marshall, Tausig, Apter and Pfeiffer (1953) found that administration of isoniazid in the treatment of tuberculosis could induce convulsive seizures or other neurological symptoms which were prevented or counteracted by vitamin B_6. Alcoholics with increased xanthurenic acid excretion after tryptophan loading and who also had convulsive seizures were found to respond to vitamin B_6 administration (Lerner, DeCarli and Davidson (1958)). Disordered metabolism of pyridoxine has been suggested as a cause of neurological disorders occasionally found in association with ACD (Cooke and Smith (1966)).

Weber and Wiss (1963) found that reduction in brain levels of 5-hydroxytryptophan decarboxylase was responsible for the cerebral lesions seen in vitamin B_6 deficient rats. Failure by normal brain to synthesise 5-hydroxytryptamine (5-HT) from L-tryptophan, with 5-hydroxytryptophan as an intermediate, may be important in the aetiology of depression (Curzon (1969)). Production of 5-HT may be reduced in women who develop depression whilst taking oral contraceptives (Winston (1969)). Rose (1972) has recently reviewed the biochemical evidence supporting the hypothesis that production of the amine is inhibited at the PLP dependent decarboxylase step and also that the increased turnover in the tryptophan nicotinic acid ribonucleotide pathway, due to oestrogen action, results in a lack of substrate for 5-HT synthesis.

Roberts, Wein and Simonsen (1964) have suggested a mechanism by which vitamin B_6 deficiency might lead to the appearance of convulsions. They

proposed that a chemical feed back inhibitor would be released in the post synaptic region (probably gamma-amino butyric acid). The enzymatic synthesis of this and several other neuro-reactive substances found in the CNS requires PLP as a co-factor. It was also suggested that PLP may act as a stabiliser of protein structure. Stephens, Havlicek and Dakshinamurti (1971) have found lowered brain levels of PLP and gamma-amino butyric acid in rats subjected to pyridoxine deprivation during the development of the CNS.

Vitamin B_6 plays an important role in haematopoesis. Harris, Whittington, Weisman and Horrigan (1956) were first to describe a pyridoxine responsive anaemia in man. Subsequent studies have characterised the syndrome as a microcytic, hypochromic anaemia with an elevated serum iron level, normoblastic bone marrow hyperplasia, xanthurenic acid excretion after a tryptophan load and a prompt response of the anaemia with reticulocytosis following the administration of vitamin B_6 (review by Sebrell (1964)). Garnick and Urata (1963) have found that delta-amino levulinic acid synthetase requires PLP as a co-factor and that the activity of this enzyme is the rate limiting factor in the synthesis of haeme. Bottomley (1962) has suggested that anaemic symptoms develop after prolonged shortage of vitamin B_6 and that neuropathy only develops when vitamin B_6 deprivation has been acute and severe.

Urinary oxalate excretion has been found to be increased in experimentally induced severe pyridoxine deficiency in man (Faber, Feitler, Bleiler, Ohlsen and Hodges (1963)). Vitamin B_6 administration has been found to be beneficial in preventing the formation of renal calcium oxalate stones (Gershoff and Prieri (1967)). On investigating primary hyperoxaluria Gibbs and Watts (1970) were unable to show that there was any abnormality of pyridoxine metabolism involved although large doses of pyridoxine lowered urinary oxalate excretion.

Experiments carried out on pyridoxine deficient rats indicate that

vitamin B_6 deficiency provides a state of immunological inertness required for the production of an immune tolerance (Axelrod and Trakatellis (1964)). Protracted periods of vitamin B_6 deprivation produced a marked deterioration in the teeth of Rhesus monkeys (Greenberg (1964)). Hillman, Cabaud and Schenbone (1962) have shown that pyridoxine produced partial protection against dental caries in pregnant women, although Hillman (1964) felt that the evidence for a relationship between vitamin B_6 and dental caries in man was inconclusive.

Vitamin B_6 plays a role in fat metabolism. The symptoms of deficiency of either essential fatty acid or vitamin B_6 in the rat are similar; pyridoxine administration will partially alleviate the dermal symptoms of essential fatty acid deficiency and the inclusion of essential fatty acids in the diet will delay the onset of symptoms in vitamin B_6 deficiency (Birch (1938)). Studies on humans who had a state of vitamin B_6 deficiency induced by the administration of desoxypyridoxine (Mueller and Iacono (1963)) produced significant decreases in red blood cell tetraenoic fatty acids accompanied by a rise in phospholipid and cholesterol levels. These results are in accord with the hypothesis that pyridoxine plays some role in the conversion of linoleic acid to arachidonic acid, but does not elucidate the mechanism involved. In his review of the inter-relationships of vitamin B_6 and fat metabolism, Mueller (1964) found that the lipid changes in pyridoxine deficient rats could result from insulin insufficiency or, equally indirectly, from altered protein metabolism affecting lipid transport. The vitamin B_6 content of the intima media layer of human vascular tissue has been measured (Kheim and Kirk (1967)). Lower vitamin B_6 levels were found in regions associated with lipid-arteriosclerotic changes than for normal vascular tissue.

Wiss and Weber (1964) have reviewed the correlations between the

actions of vitamin B_6 on a molecular level and the deficiency symptoms of the living animal. They have found that enzymes involved in the degradation of tryptophan and the sulphur amino acids are impaired at an early stage of deficiency, thus alterations in the metabolism of these amino acids provides a sensitive monitor of possible deficiency. Transaminases are more resistant to vitamin B_6 depletion, but enzymes of the cell sap are more sensitive to vitamin B_6 lack than those of the mitochondria. Greengard and Gordon (1963) have found that in the rat the presence of large amounts of PLP can induce production, in the liver, of tyrosine transaminase apo-enzyme.

3.2. Materials and Methods

3.2.1. Introduction

A variety of approaches have been made towards the measurement of vitamin B_6 levels in biological materials. The methods used for analysis of vitamin B_6 present in food stuffs have been reviewed by Toepfer and Polansky (1964) and, in blood and urine, by Storvick and Peters (1964). These can be placed in four major categories:-

(i) Microbiological methods. The micro-organisms used to measure total vitamin B_6 include strains of Saccharomyces carlsbergensis and Tetrahymena pyriformis. Strains of Lactobacillus casei, which responds principally to pyridoxal, and Streptococcus faecalis R, which responds to both pyridoxal and pyridoxamine have been used in conjunction with S. carlsbergensis to give a complete differential technique.

(ii) Enzymatic methods using a purified apo-enzyme with the addition of extracted PLP and substrate. For example, tyrosine decarboxylase (Umbreit, Bellamy and Gunsalus (1945)) and tryptophanase (Wada, Morisue, Sakamoto and Ichihara (1957)), or by using a linked apo-transaminase reaction in which PLP is the limiting factor. The transamination is linked to the oxidation of NAD; the disappearance of reduced NAD is a function of the amount of PLP present (Holzer and Gerlach (1963)).

(iii) Chemical methods based on the conversion of pyridoxine and pyridoxal to 4-pyridoxic acid, which, when heated in an acid medium, forms a highly fluorescent lactone (Fujita and Fujino (1955)). Pyridoxamine has to be converted to pyridoxine before it can be converted to the fluorescent lactone. A cyanohydrin reaction at the 4-formyl group of pyridoxal and

PLP has been used to measure the levels of these compounds.

(iv) Measurement of 4-pyridoxic acid in urine requires that it is efficiently separated from other fluorescent material present. This is effected by the use of ion exchange resins (Woodring, Fisher and Storvick (1964)). The 4-pyridoxic acid is converted to the lactone, whose fluorescence is measured.

Methods for biochemical assessment of the vitamin B_6 nuture in humans have been reviewed recently by Sauberlich, Canham, Baker, Raica and Herman (1972). The authors have listed five criteria which may be used for assessment.

(1) blood levels of vitamin B_6

(2) urinary excretion rates of vitamin B_6

(3) urinary metabolites of vitamin B_6

(4) urine or blood levels of abnormal metabolic products which may result from deficient or subnormal intakes of vitamin B_6

(5) changes in blood enzyme activities that can be related to intakes of vitamin B_6

The methods employed here are those involving an apo-enzyme type of reaction, used to measure the levels of PLP in serum. Some intermediate metabolic products of tryptophan metabolism excreted in urine after an oral dose of the amino acid have also been measured; these are xanthurenic acid, kynurenic acid, kynurenine, indole-3-acetic acid and 5-hydroxyindolyl-3-acetic acid.

3.2.2.1. The measurement of pyridoxal-5'-phosphate in serum

3.2.2.1.1. Principle of method

The principle of the method rests on the ability of PLP extracted from biological material to combine with an apo-enzyme to form a biologically active halo-enzyme. Tyrosine decarboxylase, L-tyrosine carboxy-lyase (E.C.4.1.1.25) is the enzyme used here. The extent of formation of the halo-enzyme is measured by the breakdown of tyrosine in a fixed interval of time, which is measured by the fall in radioactivity resulting from the decarboxylation of L-tyrosine-1-^{14}C present in the substrate. The reaction can be expressed by the following equations:-

tyrosine apo-decarboxylase + PLP $\longrightarrow$ tyrosine halo-decarboxylase

$$\text{L-tyrosine-1-}^{14}\text{C} \xrightarrow{\text{halo-enzyme}} \text{tyramine} + {}^{14}CO_2$$

The method used here is a modification of those described by Hamfelt (1967), Maruyama and Coursin (1968) and Chabner and Livingston (1970). (See Section 3.2.2.1.3.)

3.2.2.1.2. Materials

(1) Tyrosine decarboxylase apo-enzyme, prepared from Streptococcus faecalis grown on a pyridoxine free medium. Sigma. 100 mg were homogenised and suspended in 10 ml NaAc buffer (see (3) below)

(2) Sodium acetate (NaAc) anhydrous Analar (BDH). 3 g dissolved in distilled water and diluted to 100 ml

(3) 0.01 M NaAc buffer (pH 5.5). 1.2 ml 1N acetic acid + 1.0 ml 1N sodium hydroxide were diluted to 100 ml and the pH adjusted using a pH meter

(4) Substrate: L-tyrosine Sigma, L-tyrosine-1-^{14}C (0.25 mCi and 0.858 mg in 2.5 ml 1N HCl) New England Nuclear Corporation. 151.0 mg L-tyrosine were dissolved in NaAc buffer, 300 µl L-tyrosine-1-^{14}C were

added and the solution was diluted to 500 ml with NaAc buffer. Aliquots of approximately 100 ml were placed in polythene bottles and stored at -20° until required.

(5) Pyridoxal-5'-phosphate, Sigma. A stock standard was prepared by dissolving 3.0 mg in NaAc buffer and diluting to 100 ml. This solution was divided into 5 ml portions and stored at -20° until used. When used the concentration of the PLP was checked by measuring the OD of a 1:1 dilution in 0.2N sodium hydroxide at 388 nm. The molar extinction coefficient under these conditions is 6550 (Gunsalas and Smith (1957)). The concentration of the stock solution was calculated as follows:-[9]

$$\text{concentration } (\mu g/ml) = \frac{\text{OD x molecular weight x 2}}{6.55}$$

$$= \text{OD x } 755$$

Working standards:- 0.1 ml of stock standard was diluted to 10 ml with NaAc buffer in a 10 ml volumetric flask. This solution was then diluted 1 in 10 and further dilutions made from this to give a range of concentrations between 0.7 and 8.0 ng/ml.

(6) Trichloracetic acid (TCA) Analar (BDH). Solutions containing 30 g and 10 g TCA in 100 ml were made up.

(7) Sodium hydrogen carbonate ($NaHCO_3$) Analar (BDH) saturated solution (approximately 8 g/100 ml distilled water).

(8) 2-Methoxyethanol Laboratory reagent grade (BDH).

(9) Toluene + butyl PBD. 12 g butyl PBD in $2\frac{1}{2}$ l sulphur free toluene.

Solutions (1), (2) and (3) were made up fresh for each run, as were the working standards. Serum samples were pipetted using a 1 ml bulb pipette. All other reagents were pipetted using E-mil auto-zero constriction pipettes; these have now been superceded by Eppendorf pipettes, which were used at all stages except for the removal of 0.5 ml

of the final solution for counting. Zippettes were used for the addition of 10% w/v TCA and also for the addition of scintillation fluids to the vials. The serum extract was diluted and the pH adjusted using a Fisons automatic diluter, which picks up approximately 0.2 ml of the serum supernatant and delivers approximately 1 ml of supernatant + NaAc (3 g/100 ml w/v).

3.2.2.1.3. Procedure

(1) To 1.0 ml serum was added 0.2 ml 30% TCA (w/v) and the proteins were precipitated by mixing on a vortex test tube mixer ('buzzing') for 15 secs. The samples were then centrifuged at 5° for 15 mins in a Mistral 2L centrifuge.

(2) The supernatant was diluted approximately 1 in 5 and, at the same time, the pH adjusted to 5.5 by the addition of NaAc solution (3.0 g per 100 ml).

(3) 0.2 ml of this extract was added to 0.2 ml enzyme in a test tube. The enzyme + PLP extract was then incubated at 37° for 20 mins prior to the addition of 0.5 ml preheated substrate. Each rack of 36 tubes was shaken at 3 min intervals during the 15 min incubation. The reaction was stopped by the addition of 1 ml of 10% TCA (w/v) after exactly 15 mins incubation. Any $^{14}CO_2$ was displaced from solution by the addition of 0.5 ml saturated $NaHCO_3$, followed by 'buzzing' for 5 secs. The solutions were kept overnight at 4° before 0.5 ml was put in a vial for counting. Storage under these conditions caused precipitation of the enzyme protein, therefore only clear solutions were counted. Each vial was counted for 4 mins.

Each rack (36 tubes) contained the range of standards including a blank (0.2 ml enzyme + 0.2 ml buffer) and the first rack had an extra blank comprising solely buffer (0.4 ml). The extracts and standards were incubated with the enzyme in duplicate. Accurate timing of the

incubation was effected by starting the addition of the substrate to each row of 12 tubes at 1 min intervals. The reaction was stopped by adding the TCA (10% w/v) to each row 15 mins later. The numerical sequence was followed throughout and the times taken for the addition of substrate and TCA to each tube were approximately the same (maximum disparity/row was approximately 5 secs). A quality control serum was included with each run and, on some occasions, in each rack.

When the PLP content of red blood cells was measured a haemolysate was prepared as follows:-

(a) the cells and plasma were separated by centrifugation and the plasma stored at -20° until analysed. The cells were washed three times in an equal volume of 'normal' saline, the saline being removed after centrifugation following each washing.

(b) after the third wash and centrifuge the saline and buffy coat were removed. 2 ml of the packed cells were added to 8 ml distilled water and mixed. The haemolysate was stored at -20° until analysed.

The method of analysis was the same as that described for serum and the results were expressed as ng PLP/ml packed cells.

3.2.2.2. The measurement of xanthurenic acid and kynurenic acid in urine

3.2.2.2.1. Principle of method

When human urine is passed through a column of Dowex 50 (H^+) xanthurenic acid (XA) and kynurenic acid (KA) remain on the ion exchange resin together with aromatic amines. The XA and KA are eluted from the resin in a large volume of distilled water. The aromatic amines are not eluted using this procedure. Measurement of XA and KA in the eluates is based on the strong fluorescence exhibited by KA under acid conditions (particularly sulphuric acid) and the fluorescence of XA in alkaline conditions. The method used is that described by Price, Brown and Yess (1965).

3.2.2.2.2. Materials

(1) Resin, Dowex 50 W (H^+) 12% cross linked, 20-50 mesh coarse (BDH), 200-400 mesh, fine, Sigma. The resin was washed three times in twice its volume of distilled water, any "fines" being removed when the distilled water was sucked off. A further wash in 5N HCl was performed and the resin was stored under fresh 5N HCl.

(2) Columns, Quickfit CR12/10, nominal bore 10 mm, effective length 100 mm. A glass reservoir (capacity 75 ml) was placed on top of each column.

(3) Hydrochloric acid (HCl) 1N diluted from Volucon ampoule, 0.2N HCl prepared by diluting the 1N HCl.

(4) Sodium hydroxide (NaOH) Analar (BDH), saturated solution.

(5) Sulphuric acid (H_2SO_4) concentrated Analar (BDH).

(6) Phosphate buffer (0.5M) pH 7.4. 0.5M potassium dihydrogen phosphate, Analar (BDH) was adjusted to pH 7.4 by the addition of saturated NaOH.

(7) Kynurenic acid (KA), 4-hydroxyquinoline-2-carboxylic acid, Sigma. A stock standard (3.22×10^{-3}M) was prepared by dissolving 61 mg in distilled water and diluting to 100 ml. (2 drops of ammonia were added to allow the KA to dissolve.)

(8) Xanthurenic acid (XA), 4-dihydroxyquinoline-2-carboxylic acid, Sigma, Grade 1. A stock standard (3.22×10^{-3}M) was prepared by dissolving 66 mg in distilled water and diluting to 100 ml. (2 drops of ammonia were added as above.)

(9) Working standards of XA and KA were prepared in an identical manner. 1 ml of stock standard was diluted to 100 ml with 0.005M phosphate buffer (pH 7.4). 0.1, 0.5, 1.0 and 2.0 ml of this were further diluted to 5 ml (XA) or 6 ml (KA) which gave test solutions containing 3.2, 16.1, 32.2 and 64.4 n moles.

Stock standards were kept for not longer than four weeks at 4°. Fluorimetry was carried out on a Locarte, single sided semi-spectrum fluorimeter (Mark 5).

3.2.2.2.3. Procedure

(1) The 24-hour urine save was collected under the conditions described in 3.2.2.6. The urine volume was measured and a 250 ml aliquot removed and stored at -20° until analysis.

(2) Columns were set up the day before the urine was analysed. When the coarse resin was used the resin bed was 10 cm high; when the fine resin was used the resin bed was 3 cm high. The resin was washed in the column with 100 ml distilled water and the columns were left standing overnight in this condition. The urines were thawed by storing overnight at 4° and were filtered through Whatman No. 1 filter paper before use.

(3) 5% of the 24-hour volume was diluted to 120 ml with distilled water and 30 ml of 1N HCl were then added. 1 ml of each stock standard (XA and KA) was added to a duplicate of one of the urines of each patient.

This internal standard was usually added to the urine passed in the 24 hrs following the first loading with 2 g L-tryptophan (see Section 3.2.2.6.)

(4) The urines were run through the columns at a flow rate of approximately 2 ml/min. The colums were washed with 50 ml 0.2N HCL followed by 20 ml distilled water. Not less than 400 ml of distilled water was used to elute the XA and KA. The eluate was buffered by the addition of 4 ml 0.5M phosphate buffer (pH 7.0) and the precise volume of the eluate was noted.

(5) The KA content of each eluate and standard was measured in duplicate. To 4 ml eluate was added 2 ml 0.005M phosphate buffer. The test tubes containing these solutions were placed in an ice bath and 4 ml conc H_2SO_4 was slowly added to each tube (the contents of each tube were thoroughly mixed after each addition of a small volume of H_2SO_4). The test tubes were removed from the ice bath and the temperature of the solutions returned to room temperature before the fluorescence was measured. The fluorescence was measured at 435 nm after activation in the range 300-400 nm. A blank (phosphate buffer, 0.005M) was used to zero the instrument and the scale was adjusted in order that the 64.4 n moles standard gave a reading between 13 and 14 on the scale.

(6) The XA content of each eluate and standard was measured in duplicate. To 4 ml eluate was added 1 ml 0.005M phosphate buffer (pH 7.4) followed by 5 ml saturated NaOH (the solutions were mixed after the addition of each ml NaOH). The solution was allowed to stand for 1 hr before reading. The fluorescence was measured at 525 nm after activation in the range 300-400 nm. Calibration of the fluorimeter was effected in a manner similar to that described in (5) above. The fluorescence of the KA working standards which had been diluted to 5 ml were measured in these alkaline conditions.

(7) Calculation. The KA content of the eluate was calculated first. The n moles KA present in 4 ml eluate was read from the KA (acid) calibration curve. This value was then used to calculate the amount of fluorescence due to KA in alkaline conditions using the KA (alkali) calibration curve. The number obtained was then subtracted from the XA reading and the difference was used to measure the n moles XA present in 4 ml eluate.

The 24-hour excretion of XA and KA was calculated using the following formula:-

$$\text{24-hour excretion} = \frac{\text{XA/KA x eluate vol. x 20}}{4} \times 1000 \text{ } \mu \text{ moles}$$

3.2.2.3. The measurement of kynurenine levels in urine

3.2.2.3.1. Principle of method

Kynurenine (Kyn) is an aromatic amine present in human urine. It is adsorbed into Dowex 50 (H^+) and is eluted in 5N HCl. The quantity of kynurenine present in the eluate is measured by a diazotisation and coupling reaction. The method used is a modification of that described by Price et al (1965).

3.2.2.3.2. Materials

(a) The Dowex resin and the columns used were the same as those used for the separation of XA and KA.

(b) Sodium nitrite ($NaNO_2$), Analar (BDH). 250 mg dissolved in 100 ml distilled water.

(c) Ammonium sulphamate, laboratory reagent (BDH). 10 g dissolved in 100 ml distilled water.

(d) N (1-naphthyl)ethylenediamine dihydrochloride (BDH). 250 mg dissolved in 100 ml distilled water.

(e) Sodium hydroxide (NaOH), Analar (BDH). 9N solution.

(f) Hydrochloric acid (HCl) conc, Analar (BDH), diluted with distilled water to give solutions of the following concentrations:- 5N, 2.5N, 1N, 0.33N.

(g) DL-kynurenine sulphate, Sigma. Stock standard contained 40 mg per 100 ml distilled water. Working standards were prepared from this by diluting 1 ml to 25 ml with 0.33N HCl and further diluting the latter to give solutions containing 2.0, 4.0, 8.0 and 12,0 µg/ml.

3.2.2.3.3. Procedure

(1) Urine samples were taken from the same aliquots in which XA and KA had previously been measured.

(2) The Dowex resin was washed in the same way as described for XA and KA. Columns of bed length 10 cm were used on all occasions and the resin washed in situ with 100 ml distilled water.

(3) To 1% of the 24-hour urine volume was added 4 ml 1N HCl and the acidified urine was diluted to 40 ml with distilled water. 1 ml of stock standard was added to at least one 24-hour urine save per person in order to monitor the recovery of Kyn.

(4) The urine was applied to the column at a flow rate of approximately 1 ml/min. The resin was washed with 100 ml 2.5N HCl and the Kyn eluted with 100 ml 5N HCl. The eluate was collected into 100 ml volumetric flasks.

(5) 1 ml 9N NaOH was added to duplicate 2 ml aliquots of column eluate; duplicate 3 ml aliquots of working standards were also analysed. The diazotisation and coupling reactions were performed according to the following schedule:-

(i) Add 0.2 ml $NaNO_2$ solution, mix and stand for 3 mins.

(ii) Add 0.2 ml ammonium sulphamate solution, mix and stand for 2 mins.

(iii) Add 0.2 ml N (1-naphthyl)ethylenediamine dihydrochloride, mix and leave standing at room temperature for 3 hrs.

The optical densities were read against water at 550 nm on a Unicam SP 600.

(6) Calculation: The Kyn content of the eluate is read from the graph as µg kynurenine sulphate/ml. The eluate had been diluted by the addition of 9N NaOH and this was corrected for by multiplying by 1.5. There was 100 ml eluate and only 1% of the 24-hour urine volume was applied to the column, hence:-

$$\text{24-hour excretion of Kyn} = \frac{\mu g/ml \times 1.5 \times 100 \times 100}{306} \ \mu \text{ moles}$$

where 306 is the molecular weight of kynurenine sulphate.

3.2.2.4. The measurement of indole acetic acid levels in urine

3.2.2.4.1. Principle of method

Indole-3-acetic acid (IAA) is extracted from acid urine into chloroform and is then extracted from chloroform into a phosphate buffer pH 7.0. This buffer extract is then reacted with xanthrydol and a coloured derivative is formed due to the condensation of indoles and aldehydes (Dickman and Crockett (1956)). The procedure used is essentially that of Weissbach, King, Sjoerdsma and Udenfriend (1959); adjustments to volumes have been made.

3.2.2.4.2. Materials

(a) Chloroform, Analar (BDH).

(b) Xanthrydol (Hopkin & Williams). 100 mg dissolved in 100 ml glacial acetic acid, Analar (BDH). Made up freshly immediately prior to each colour reaction.

(c) Sodium hydrogen sulphite. 5 g in 100 ml distilled water, diluted from BDH Micro-analytical reagents (35% w/v).

(d) Phosphate buffer, 0.5M pH 7.0. 13.6 g potassium dihydrogen phosphate + 58 ml 1N NaOH diluted to 1 l with distilled water. The pH was checked using a pH meter.

(e) Hydrochloric acid (HCl) conc, Analar (BDH).

(f) Indole-3-acetic acid (IAA), Sigma. Stock standard: 25 mg dissolved in 100 ml distilled water. Working standards: stock standard diluted with distilled water to give concentrations of 5.0, 10.0, 25.0 and 50.0 µg IAA/ml.

When volumes of less than 1.0 ml were measured, a 0.5 ml constriction pipette was used to remove the buffer after the second extraction, otherwise Eppendorf pipettes were used.

3.2.2.4.3. Procedure

(1) To 4 ml urine in a glass stoppered tube was added 0.4 ml conc HCl, then 10 ml chloroform.

(2) The tubes were shaken for 5 mins on a mechanical shaker, then centrifuged at 2,000 rpm for 5 mins to separate the layers. The aqueous layer was discarded.

(3) 6 ml of the chloroform layer was then transferred to another tube and 1 ml phosphate buffer (pH 7.0) added. The tubes were shaken and centrifuged as described in (2) above.

(4) 0.5 ml of the buffer layer was removed and placed in another test tube and acidified by the addition of 0.5 ml conc HCl. 1.0 ml xanthrydol reagent was then added and the contents of the tube were mixed. After exactly 5 mins 0.5 ml sodium hydrogen sulphite was added and the contents of the tube were mixed. The OD of the pink compound was read at 520 nm within 20 mins of the addition of the last reagent.

(5) A recovery standard of 4 ml of 10 µg/ml working standard was extracted and measured; 4 ml distilled water was put through the whole procedure and acted as a blank. 0.5 ml of the working standards was reacted in duplicate as in (4) above and the readings obtained were used to construct a standard curve.

(6) Calculation. It was assumed that 100% extraction of IAA had occurred, thus:-

$$\text{IAA}(\mu\text{g/ml urine}) = \frac{X \times 10 \times 2 \times 1}{1 \times 6 \times 1 \times 4}$$

24-hour excretion = $X \times 0.833 \times$ 24-hour vol $\times 10^{-3}$ mg/24 hrs

where 'X' is µg IAA/ml extract.

3.2.2.5. The measurement of 5-hydroxyindolyl-3-acetic acid in urine

3.2.2.5.1. Principle of method

5-hydroxyindolyl-3-acetic acid (5-HIAA) is extracted from acid (pH 2.0), salt saturated urine into diethyl ether. An aliquot of the ether extract is evaporated to dryness and the residue dissolved in 4 ml distilled water. A violet chromogen, of unknown composition, is formed by the reaction of 5-HIAA with 1-nitroso-2-naphthol in dilute acid and in the presence of a trace of nitrous acid. The reaction is specific for indoles hydroxylated in the 5 position (Sadler (1963)). The method used here is that of Udenfriend, Titus and Weissbach (1955) and is used routinely in the laboratory.

3.2.2.5.2. Materials

(a) Sodium chloride (NaCl), Analar (BDH).

(b) Diethyl ether, Analar (BDH) washed with ferrous sulphate.

(c) Sulphuric acid (H_2SO_4) 2N. 1N Volucon H_2SO_4 diluted to 500 ml.

(d) Sodium nitrite ($NaNO_2$), Analar (BDH). 2.5 g dissolved in 100 ml distilled water, made up weekly and stored at 4°.

(e) 1-nitroso-2-naphthol (Hopkin & Williams). 100 g dissolved in 100 ml absolute alcohol, made up monthly and stored at 4°.

(f) Ethyl acetate, Analar (BDH).

(g) Nitrous acid, prepared freshly before use; 0.2 ml $NaNO_2$ solution + 5.0 ml 2N H_2SO_4.

(h) 5-hydroxyindolyl-3-acetic acid (5-HIAA), Koch-Light Laboratories. Stock standard: 25 mg dissolved in 25 ml distilled water; divided into aliquots of approximately 1.5 ml and stored at -20° until use. Working standards: dilute 1 ml stock standard to 100 ml with distilled water; 1.0, 2.0, 3.0 ml working standard were further diluted to 3.0 ml with distilled water. This gave standards containing 10, 20, 30 µg 5-HIAA/ml.

(j) 5-hydroxyindolyl-3-acetic acid carbonyl-1-^{14}C, New England Nuclear Corporation, 0.05 mCi. 1.28 mg in 0.5 ml acetic nitrile.

3.2.2.5.3. Procedure

(1) To 3.0 ml urine add 0.1 ml glacial acetic acid, approximately 2 g NaCl, 1 µl 5-HIAA-1-^{14}C and 25 ml diethyl ether. A blank of 3.0 ml distilled water was treated in the same way as was a blank of 3.0 ml distilled water without the addition of 1 µl 5-HIAA-1-^{14}C.

(2) The tubes were shaken for 5 mins and centrifuged at 2,000 rpm for 5 mins.

(3) 20 ml of the ether layer was evaporated in a hot water bath (60°). The residue was taken up in 4.0 ml distilled water and the tube shaken for 1 min.

(4) 3.0 ml of the extract, working standards or distilled water blank was transferred to test tubes. 1.0 ml 1-nitroso-2-naphthol solution was added followed by 1 ml nitrous acid solution; the contents of the tubes were mixed after each addition. The solutions were incubated at 55° for 5 mins and then washed twice in approximately 8 ml ethyl acetate. The OD of the aqueous layer was read at 540 nm.

(5) 0.5 ml of the aqueous extract was placed in a vial and 6 ml 2-methoxyethanol + 10 ml toluene butyl PBD were added. 1 µl 5-HIAA-1-^{14}C was added to two more vials to which had been added 0.5 ml distilled water and the same volumes of scintillation fluid were added. Each vial was counted for 1 min.

(6) The µg 5-HIAA/ml of each extract sample was read from the standard curve and the 24-hour excretion calculated as follows:-

$$\text{24-hour excretion} = \frac{X \times 25 \times 4 \times 1 \times \text{24-hour urine vol}}{1 \times 20 \times 3 \times 3 \times 1000} \text{ mg}$$

where 'X' is the µg 5-HIAA/ml of extract.

The recovery was calculated as follows:-

$$\text{Recovery \%} = \frac{\text{C.P.M. 0.5 ml extract x 25 x 4 x 100}}{\text{C.P.M. 1 µl 5-HIAA-1-}^{14}\text{C x 20 x 0.5}}$$

3.2.2.6. Clinical material

Normal serum was obtained through the Blood Transfusion Service. At the end of the donation approximately 10 ml blood was collected directly into a hospital biochemistry tube. The specimens were identified by writing the age, sex and donor reference number on the label of the tube. The specimen was then placed in a test tube rack which was wrapped in aluminium foil to protect against any possible deterioration due to the effects of light. At the end of each session (approximate length of session 3 hrs) the specimens were collected and taken to the General Hospital. The serum was separated and stored at -20° until analysed. In order to assess the general health of the donors, albumin, globulin, total protein, alkaline phosphatase, glutamic-oxaloacetic transaminase and bilirubin levels were measured in each serum as part of a routine batch on Technicon Autoanalyzers. Those donors with an alkaline phosphatase level of more than 13 King Armstrong units/100 ml serum and/or bilirubin levels of more than 1.2 mg/100 ml serum were not included in the normal range. All samples were analysed within 14 days of donation. Samples of serum were obtained from gastrointestinal patients as described in 2.2.3. for vitamin E.

Tryptophan load tests were carried out on a limited number of patients with ACD and also on normal subjects. All urine was collected for the four day period of the test, saved in 24-hour volumes and preserved by the addition of 25 ml 1N HCl. At the end of each 24-hour period the urine volume was measured and an aliquot (usually 250 ml, or 500 ml if the volume was more than 2,000 ml) was stored at -20° until analysis. Analyses were performed within one month of receipt of all urines from any one subject. On the first day of the test nothing was given to the subject in order to establish the basal excretion of the tryptophan metabolites measured; on the second day the

subject was given a load of 2 g L-tryptophan before breakfast. 50 mg pyridoxine hydrochloride, the dose used by Winston (1969) to treat depression associated with taking oral contraceptives, was administered orally on day 3 and, on day 4, another load of 2 g L-tryptophan was given. The urine collection times were arranged in such a way that the 24-hour save was completed before the administration of any drug. Blood samples were taken on each day of the test, approximately 3 hrs after dosing, and were placed in lithium heparin tubes. The plasma was stored at -20° until analysed. The cells were washed three times with normal saline, diluted 1:5 with distilled water and stored at -20° until analysed.

3.2.3. Results

3.2.3.1. The purity of L-tyrosine-1-^{14}C

The purity of the tyrosine-1-^{14}C was checked at intervals of three months. 2 µl of the stock tyrosine solution was applied to Whatman No. 1 chromatography paper and developed over night in ascending butanol:acetic acid:water (5:1:4). The chromatograms were air dried at room temperature and strips 2 cm wide x 1 cm were cut out of the chromatograms. These strips were placed in order in scintillation vials, 10 ml toluene butyl PBD were added and the radioactivity was measured by counting for 1 min. A typical graph is shown in Fig.3.2.1. The purity of the sample was calculated by adding the C.P.M. for the strips which included the peak (segments 13-16 in Fig.3.2.1.) and expressing this as a percentage of the sum of the C.P.M. for all the strips. The purity was found to range from 97.4% (1.12.70) to 89.7% (20.9.72).

3.2.3.2. Checks on the necessity for enzyme purification

The following procedures were used to purify the apo-enzyme:-

(a) Saturated alkaline ammonium sulphate was prepared by adding 760 g ammonium sulphate and 52 ml ammonia to 1 l distilled water. 70 mg apo-enzyme were suspended in 70 ml distilled water at 4° and 210 ml saturated alkaline ammonium sulphate were added. The mixture was left for 30 mins in an ice bath. The solution, with its precipitate, was transferred to 20 test tubes and centrifuged in an MSE Mistral 2L at 2,000 rpm for 30 mins. The supernatant was discarded, the precipitate resuspended in 70 ml cold distilled water and the procedure was repeated. After the second centrifuging the precipitate was resuspended in 21 ml 0.1N NaAc buffer (pH 5.5) which also contained 24% w/v glycerol, 10^{-3}M tyrosine and 2 x 10^{-3}M

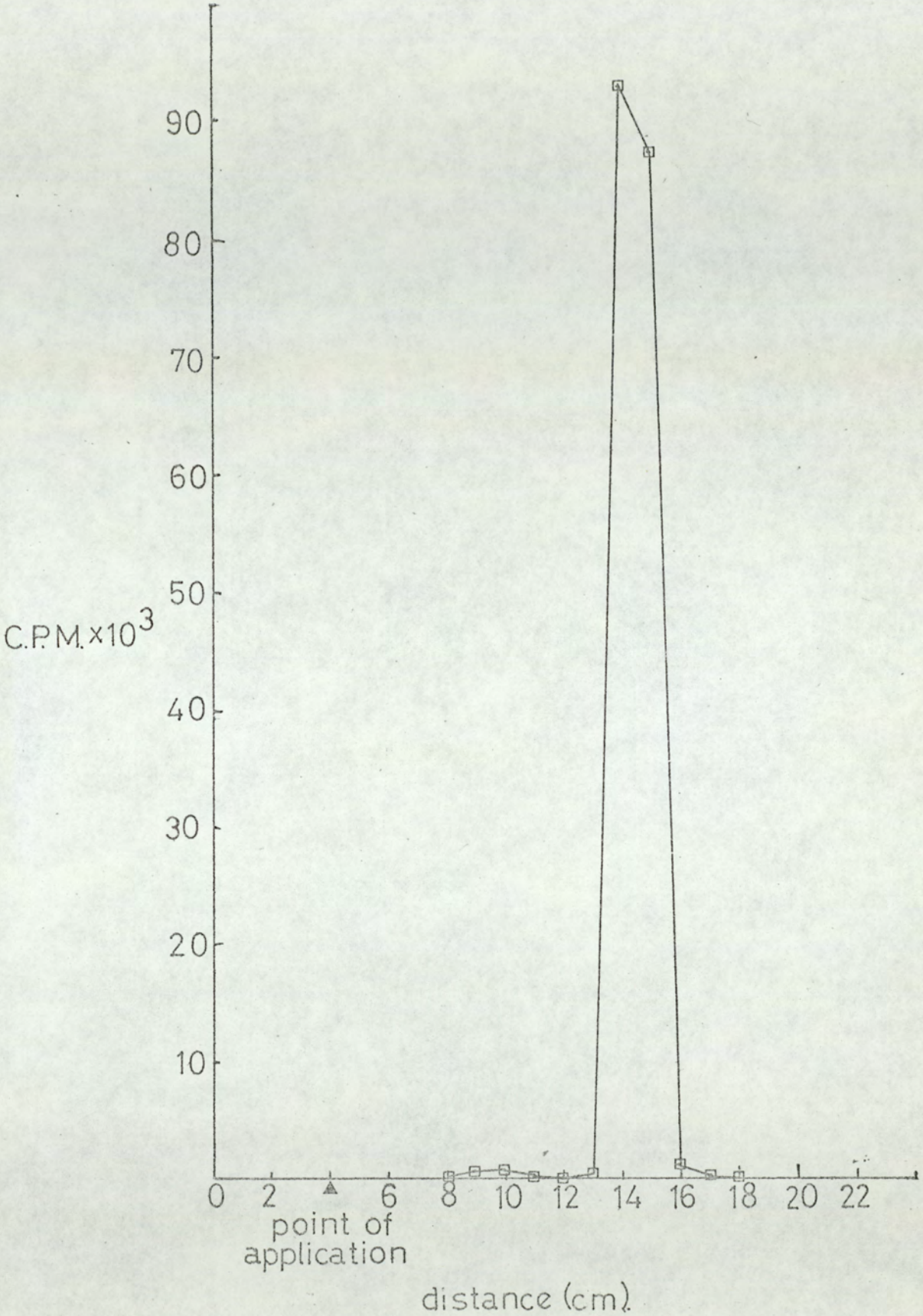


Fig. 3.2.1. Radio-chromatogram of L-tyrosine-1-^{14}C; solvent ascending butanol : acetic acid : water (5:1:4).

mercaptoethanol (enzyme buffer). The precipitation technique was that described by Hamfelt (1967) and the "enzyme" buffer was that described by Chabner and Livingston (1970). This preparation is referred to subsequently as enzyme 1.

(b) Enzyme 2. This enzyme was prepared as described above except that the preparative centrifugings were carried out on an International centrifuge at a speed of 9,000 rpm.

(c) Aliquots of enzymes 1 and 2 were further purified by gel filtration through a column of Sephadex G 200 0.9 cm x 10 cm. The Sephadex had been left in contact with the "enzyme" buffer for 3 days prior to the pouring of the column. Air was removed from the slurry by placing in a vacuum oven and producing a partial vacuum for 3 hrs. When the slurry was poured into the column, the first 2 cm were allowed to pack down under the influence of gravity before the packing was hastened by allowing the enzyme buffer to drip through. The packed column stood over night before its continuity was checked by running dextran 2000 through; the blue dye was eluted in 2 ml buffer. 2 ml enzyme 1 were applied to the column and eluted with 23 ml "enzyme" buffer which was collected in Auto-analyzer cups in 1 ml fractions. The column was regenerated by washing through with 10 ml "enzyme" buffer, then 10 ml distilled water and then 10 ml "enzyme" buffer, before the addition of enzyme 2. Enzyme 2 was eluted with 17 ml "enzyme" buffer, the eluate being collected in 1 ml fractions as above.

(d) Duplicate 2 ml samples of enzymes 1 and 2 were filtered through Whatman No. 1 filter paper. The filtrate was clear and a brown residue was retained.

The protein content of all fractions collected from the Sephadex column and also that of the filtrates was measured using the method of Rosenthal and Cundiff (1956). This method is based on the

Biuret reaction. Tables 3.2.1. and 3.2.2. show the results obtained for enzymes 1 and 2. They list the OD of test, blank and change in OD used to calculate the protein content of the eluate/filtrate and also the C.P.M. obtained when the eluate was used as the source of enzyme in a PLP reaction mixture. 30 µg and 7 ng PLP standards were supplied as co-enzyme and 20 min incubation at 37° preceded the addition of substrate. The reaction was stopped after 15 mins by the addition of 1 ml 10% TCA, and the $^{14}CO_2$ was displaced by adding 0.5 ml saturated $NaHCO_3$. 0.2 ml of the solution was counted for 1 min. Fig.3.2.2. and Fig.3.2.3. show the C.P.M. for each fraction when incubated with 30 µg PLP/ml standard and also the OD of the blanks obtained when measuring the protein content of the eluate for each enzyme. The results show that although enzyme activity is present, it is not sensitive enough to measure the small quantities of PLP expected to be present in a serum extract. The enzyme activity is split into two components after Sephadex gel filtration.

A further attempt was made to purify the enzyme. 210 mg of the crude enzyme was subjected to the ammonium sulphate purification procedure described in (a) above, the final precipitate being suspended in 10 ml acetate buffer (0.1M pH 5.5). This suspension was then applied to a column of Sephadex G 200 3 cm x 60 cm, the Sephadex having equilibrated in acetate buffer. The first 30 ml of eluate were collected and pooled. The solution was turbid. The activity of the eluate was measured as described above. At the same time the activity of the crude enzyme was measured, (170 mg had been suspended in 15 ml buffer). The results are given in Table 3.2.3. 0.5 ml of the reaction solution was counted for 1 min. There is very little enzyme activity left after ammonium sulphate precipitation. The losses probably occurred because it was not possible to centrifuge the suspension fast enough to precipitate the enzyme. Since a

Table 3.2.1. Protein content and enzyme activity of fractions collected after gel filtration on Sephadex G 200 or filtration through Whatman No. 1 paper of Enzyme 1.

Enzyme	O.D. Test	O.D. Blank	△ O.D.	Protein mg%	C.P.M. 30 µg PLP	C.P.M. 7 ng PLP
Blank					753 739	749 753
No filtration	1·504	1·502	0·002	1·8	549 569	740 724
Fraction 1	0·053	0·020	0·033	30·1	731	741
2	0·055	0·047	0·008	7·2	898	749
3	0·455	0·489	-0·034		656	731
4	0·677	0·716	-0·039		588	732
5	0·666	0·556	0·110	101·0	635	
6	0·198	0·108	0·090	82·0	719	757
7	0·145	0·078	0·067	61·0	728	761
8	0·073	0·041	0·032	29·1	734	769
9	0·065	0·042	0·023	20·9	738	753
10	0·067	0·028	0·039	35·6	748	758
11	0·062	0·042	0·020	18·3	742	756
12	0·085	0·033	0·052	47·5	727	
13	0·137	0·029	0·108	96·8	714	
14	0·074	0·000	0·074	67·2	753	771
15	0·728	0·037	0·691	630·0	662	
16	0·920	0·014	0·906	825·0	699	754
17	0·976	0·005	0·971	885·0	738	
18	0·826	0·000	0·826	752·0	738	
19	0·586	0·000	0·586	531·0	745	774
20	0·113	0·002	0·111	106·0	760	760
Filtered A	0·423	1·426	-1·003		732	744
B	0·439	0·775	-0·336		683	747

Fraction 1 contained 4 ml eluate, fractions 15 - 19 were yellow in colour.

Table 3.2.2. Protein content and enzyme activity of fractions collected after gel filtration on Sephadex G 200 or filtration through Whatman No. 1 paper of Enzyme 2.

Enzyme	O.D. Test	O.D. Blank	Δ O.D.	Protein mg %	C.P.M.	
					30 ng PLP	7 ng PLP
No filtration	3·0	2·04			734	767
Fraction 1	0·012	0·000	0·012	10·4	771	754
2	0·016	0·002	0·014	12·8	733	742
3	0·133	0·116	0·017	15·5	683	
4	0·741	0·831	-0·090		263	653
5	0·906	0·975	-0·069		203	
6	0·843	0·864	-0·021		206	
7	0·336	0·387	-0·051		452	707
8	0·166	0·162	0·004	3·7	651	726
9	0·147	0·135	0·012	14·8	631	708
10	0·112	0·110	0·002	1·8	646	
11	0·104	0·088	0·016	14·6	615	702
12	0·110	0·097	0·003	2·7	549	677
13	0·177	0·124	0·053	48·4	484	708
14	0·583	0·080	0·503	460·0	693	751
15	0·071	0·027	0·044	40·1	758	754
Filtered A	0·331	0·284	0·047	40·1	767	749
B	0·253	0·275	-0·022		762	749

Fractions 1 & 2 contained 2 ml eluate each, fractions 14 & 15 were yellow in colour.

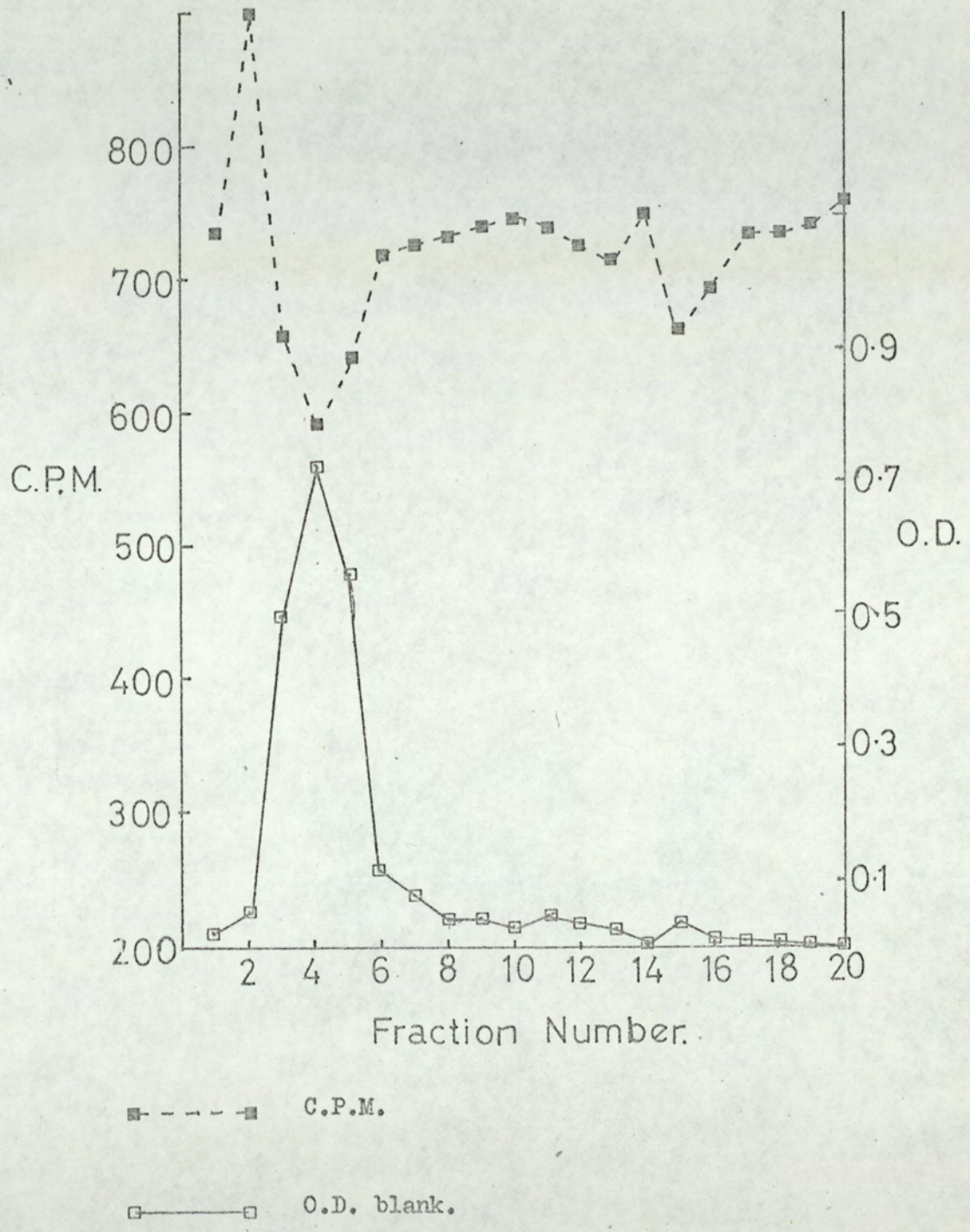


Fig. 3.2.2. O.D. blank obtained when measuring the protein content of fractions collected after gel filtration of Enzyme 1. The C.P.M. are those obtained when each fraction was incubated with 30 ug PLP / ml.

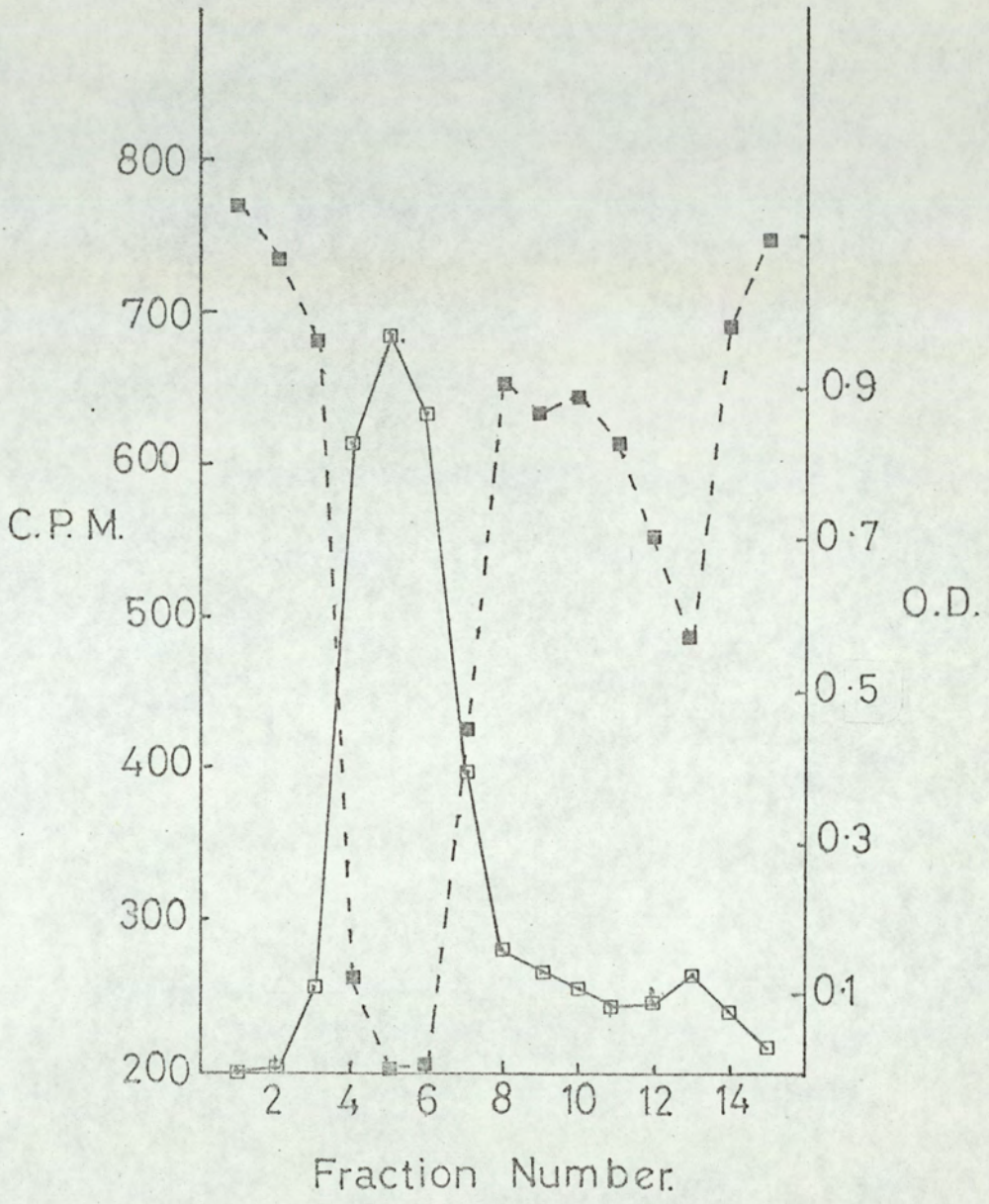


Fig. 3.2.3. O.D. blank obtained when measuring the protein content of fractions collected after gel filtration of Emzyme 2. The C.P.M. are those obtained when each fraction was incubated with 30 ug PLP / ml.

Table 3.2.3. The enzyme activity obtained after each stage of purification.

Treatment	PLP / ml	C.P.M.	
Crude enzyme	30 µg / ml	101	115
	8·6 µg / ml	151	153
After $(NH_4)_2SO_4$ treatment	30 µg / ml	1275	1219
	8·6 ng / ml	1503	1514
After $(NH_4)_2SO_4$ treatment	34·3 ng / ml	1480	1440
and sephadex gel filtration	8·6 ng / ml	1516	1482
	0·0 ng / ml	1537	1495
Blank, no enzyme	0·0 ng / ml	1568	1529

Table 3.2.4. The effect on enzyme activity of varying substrate concentration.

ng PLP / ml	Substrate 1 C.P.M.	Substrate 2 C.P.M.
0 enz. 0 PLP	1506	752
enz. 0 PLP	748	304
1·6	563	315
3·2	427	156
5·4	252	122
8·1	230	92
10·8	174	90
32·4	112	72

Substrate 1 contained 30·2 mg L-tyrosine + 60 µl L-tyrosine-1-^{14}C in 100 ml. Substrate 2 was a 1:2 dilution of substrate 1.

preparative ultra-centrifuge was not available in the laboratory, it was decided to use the enzyme as supplied by Sigma and simply homogenise and suspend the powder in buffer.

3.2.3.3. The effect of varying substrate concentration

Two concentrations of substrate were assessed; substrate 1 which contained 30.2 mg L-tyrosine and 60 µl of L-tyrosine-1-^{14}C in 100 ml buffer, and substrate 2 which contained 15.1 mg L-tyrosine and 30 µl L-tyrosine-1-^{14}C in 100 ml buffer. These substrates were added to the enzyme and a range of concentrations of PLP; the incubation conditions were the same as those described in 3.2.2.1.3. The incubations were performed in triplicate, 0.5 ml was counted for 1 min. The results are given in Table 3.2.4. and Fig.3.2.4. Substrate 1 gives a linear decay over the range 0-5 ng PLP/ml and the slope is such as to give a more precise measurement when compared with substrate 2.

3.2.3.4. The effect of varying pre-incubation time

The effect of varying the time of contact between apo- and co-enzyme before the addition of substrate was studied. Two standard solutions of PLP (2.6 and 13.0 ng/ml) were used and also a serum extract. The enzyme and PLP solutions were kept in an ice bath for 15 mins and then placed in a water bath at 37° for 0, 5, 10, 20, 30, 40 and 60 mins prior to the addition of substrate which was also pre-warmed at 37°. After 15 mins incubation the reaction was stopped in the usual manner and 0.5 ml of the final solution was counted for 1 min. The results are shown in Table 3.2.5., giving the absolute counts/min and in Table 3.2.6., giving the change in counts relative to the blank (no enzyme and no PLP). Fig.3.2.5. and Fig.3.2.6. present these results in graphic form.

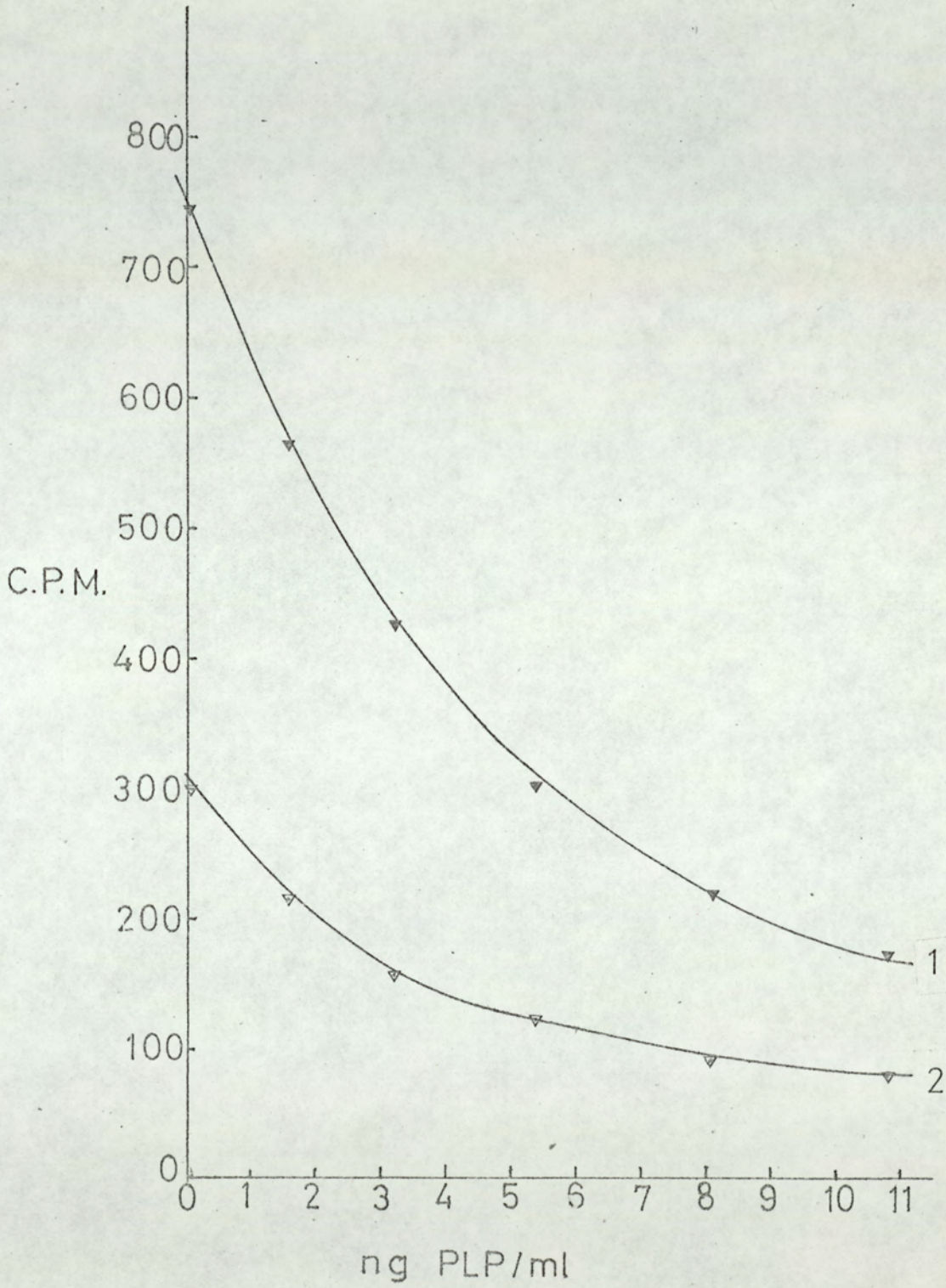


Fig. 3.2.4. The effect on enzyme activity of varying substrate concentration.

1) 30·2 mg L-tyrosine + 60 µl L-tyrosine-1-^{14}C.

2) 15·1 mg L-tyrosine + 30 µl L-tyrosine-1-^{14}C.

Table 3.2.5. C.P.M. obtained after varying times of pre-incubation of enzyme and PLP.

Time (min)	C.P.M x 10^3				
	Blank 1	Blank 2	Serum extract	2·6 ng PLP/ml	13·0 ng PLP/ml
0	15·21	11·13	10·50	8·58	2·69
5	15·16	11·04	10·52	8·35	2·35
10	14·95	10·55	9·45	7·20	2·41
15	14·61	10·35	9·37	7·40	1·94
20	14·94	9·19	8·24	5·56	1·85
30	14·75	7·71	7·80	6·41	1·50
40	14·81	9·54	8·24	6·55	1·46
60	14·95	7·47	7·75	5·80	1·55

Table 3.2.6. Change in C.P.M. relative to Blank 1 (above) after varying times of pre-incubation of enzyme and PLP

Time(min)	Change in C.P.M. x 10^3			
	Blank 2	Serum extract	2·6 ng PLP/ml	13·0 ng PLP/ml
0	4·08	4·71	6·63	12·52
5	4·12	4·64	6·81	12·81
10	4·40	5·50	7·75	12·54
15	4·26	5·24	7·21	12·67
20	5·75	6·70	9·38	13·09
30	7·04	6·95	8·34	13·25
40	5·27	6·57	8·26	13·35
60	7·48	7·20	9·15	13·40

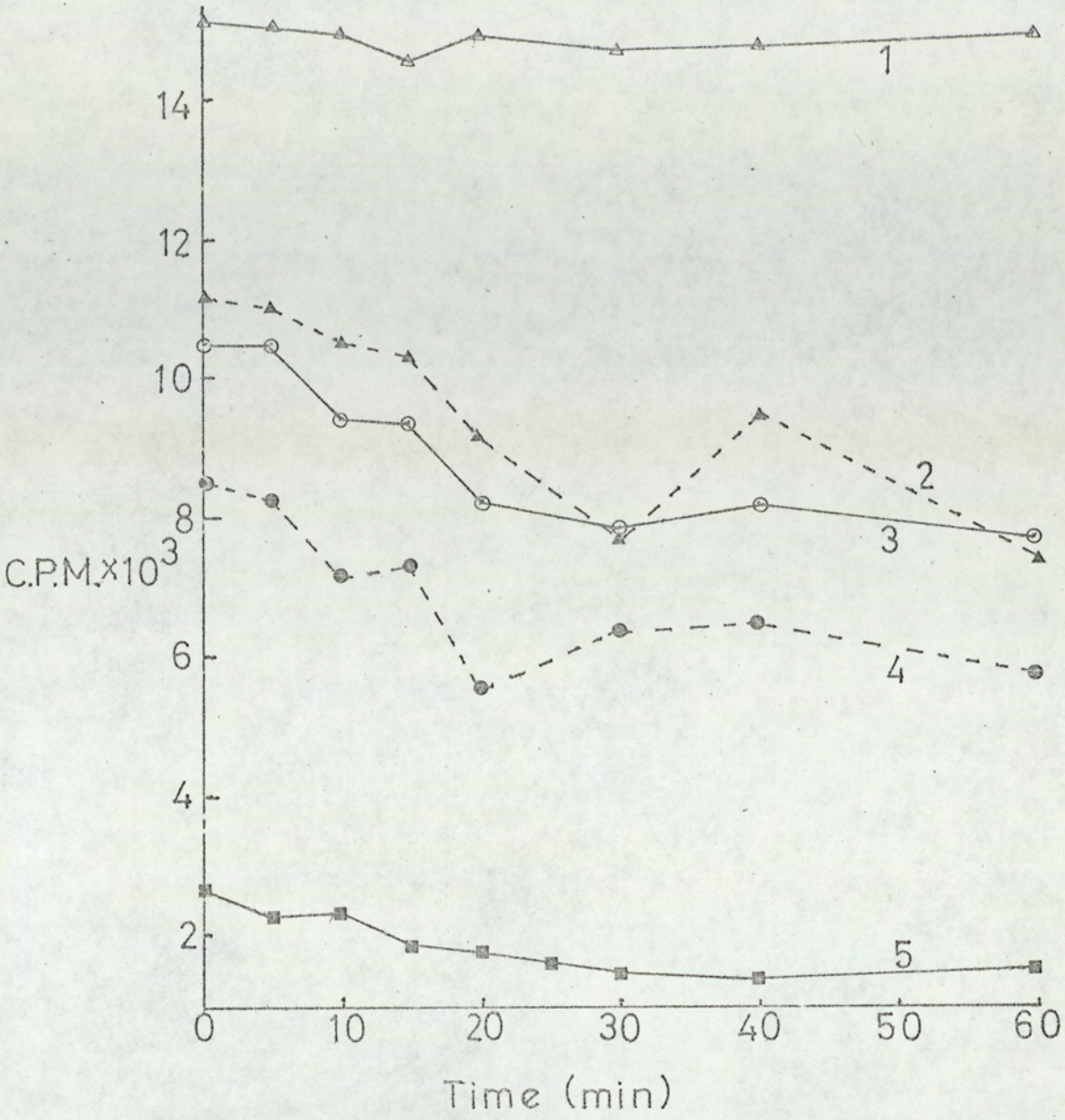


Fig. 3.2.5. The effect of varying time of pre-incubation expressed as absolute C.P.M.

1) 0 enzyme + 0 PLP.

2) 0·2 ml enzyme + 0 PLP.

3) 0·2 ml enzyme + serum extract.

4) 0·2 ml enzyme + 2·6 ng PLP / ml.

5) 0·2 ml enzyme + 13·0 ng PLP / ml.

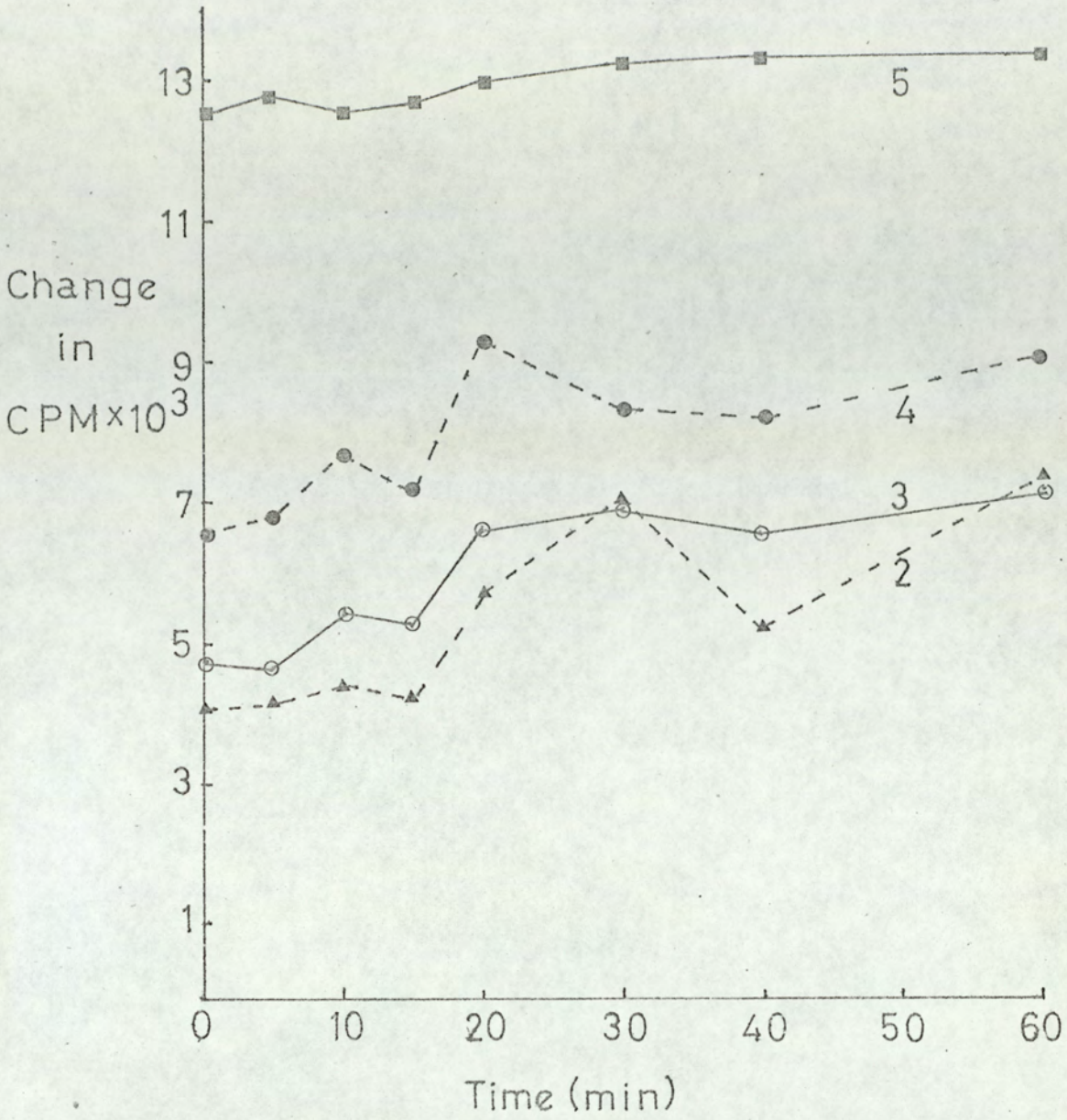


Fig. 3.2.6. The effect of varying time of pre-incubation, expressed as change of C.P.M. relative to a blank containing 0 enzyme + 0 PLP.

2) 0·2 ml enzyme + 0 PLP.

3) 0·2 ml enzyme + serum extract.

4) 0·2 ml enzyme + 2·6 ng PLP / ml.

5) 0·2 ml enzyme + 13·0 ng PLP / ml.

A pre-incubation period of 20 mins was chosen since this was the time at which the rate of change in counts was maximal.

3.2.3.5. The effect of varying the time of incubation

The effect of varying the time of incubation with substrate was studied using a serum extract, 0.8 ng PLP/ml and 8.0 ng PLP/ml. The reaction was stopped by the addition of 1 ml 10% TCA and the pre-incubation time was 20 mins. Table 3.2.7. shows the counts per 4 min of a 0.5 ml aliquot of the final solution and Fig.3.2.7. presents these results graphically. 15 min was chosen as the incubation time since this precedes the irregularity in the decline of counts found on numerous occasions which occurred around 20 mins of incubation.

3.2.3.6. The effect of varying concentration of enzyme

100 ml enzyme was homogenised and suspended in 10 ml buffer (0.01M NaAc pH 5.5). This was then diluted 1:2, 1:5, 1:10, 1:50 with the same buffer. These enzyme preparations were used to construct a standard curve and also to measure the concentration of PLP in a serum extract. The results are given in Table 3.2.8. and the standard curves obtained are shown in Fig.3.2.8. The incubation conditions were the same as those described in 3.2.2.1.3. It was concluded that the enzyme could not be made more dilute without loss of accuracy and precision. This concentration of enzyme was deemed adequate as judged by the recoveries obtained when PLP was added to serum to act as an internal standard (see section 3.2.3.10.).

3.2.3.7. The effect of the pH of the medium on the activity of the enzyme

The stock standard was diluted 1:100 with buffer pH 5.5.

Table 3.2.7. The effect of varying the time of incubation with substrate.

Time(min)	Counts / 4 min x 10^3				
	A	B	C	D	E
0	48·38	48·35	49·75	49·16	48·84
5	46·13	49·29	44·68	39·40	43·30
10	44·17	49·16	40·77	32·13	39·42
15	41·76	49·27	38·77	27·09	36·06
18	41·39	48·59	37·12	24·98	34·43
20	41·28	49·47	36·59	23·63	33·04
23	40·33	49·64	35·61	21·85	31·68
25	38·75	49·63	34·60	20·80	30·27
30	38·53	48·96	33·38	18·38	28·43
47	35·37	48·20	29·13	12·79	23·23
58	28·85	48·57	23·95	10·19	19·29

A contained 0·2 ml enzyme + 0·2 ml buffer.

B contained 0·2 ml buffer + 0·2 ml 8·0 ng PLP / ml.

C contained 0·2 ml enzyme + 0·2 ml 0·8 ng PLP / ml.

D contained 0·2 ml enzyme + 0·2 ml 8·0 ng PLP / ml.

E contained 0·2 ml enzyme + 0·2 ml serum extract.

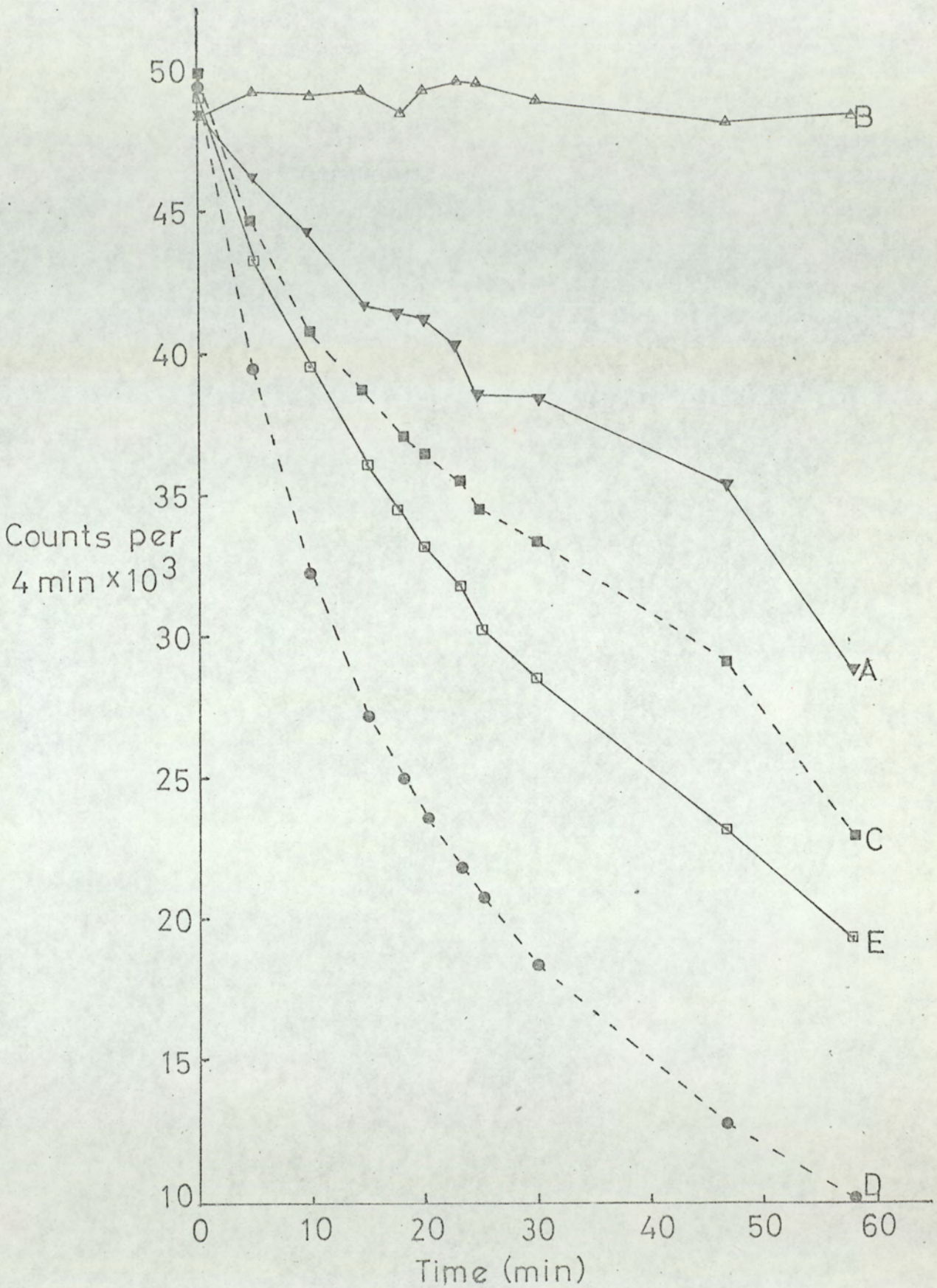


Fig. 3.2.7. The effect of varying time of incubation.

A contained 0·2 ml enzyme + 0·2 ml buffer.

B contained 0·2 ml buffer + 0·2 ml 8·0 ng PLP / ml.

C contained 0·2 ml enzyme + 0·2 ml 0·8 ng PLP / ml.

D contained 0·2 ml enzyme + 0·2 ml 8·0 ng PLP / ml.

E contained 0·2 ml enzyme + 0·2 ml serum extract.

Table 3.2.8. The effect of varying the concentration of the enzyme.

Enzyme concentration	C.P.M. x 10^3			
mg / ml	buffer	1·7 ng PLP/ml	5·5 ng PLP/ml	serum extract
10	9·16	8·23	6·21	8·64
5	9·39	8·64	7·36	9·41
2	9·50	9·18	8·48	9·42
1	9·59	9·33	9·14	9·59
0·2	9·46	9·53	9·59	9·56

Table 3.2.9. The effect of the pH of the extract on the activity of the enzyme.

pH	C.P.M. x 10^3
3·7	1·28
4·0	1·02
4·4	0·49
5·0	0·44
5·2	0·35
5·5	0·30
6·0	0·32

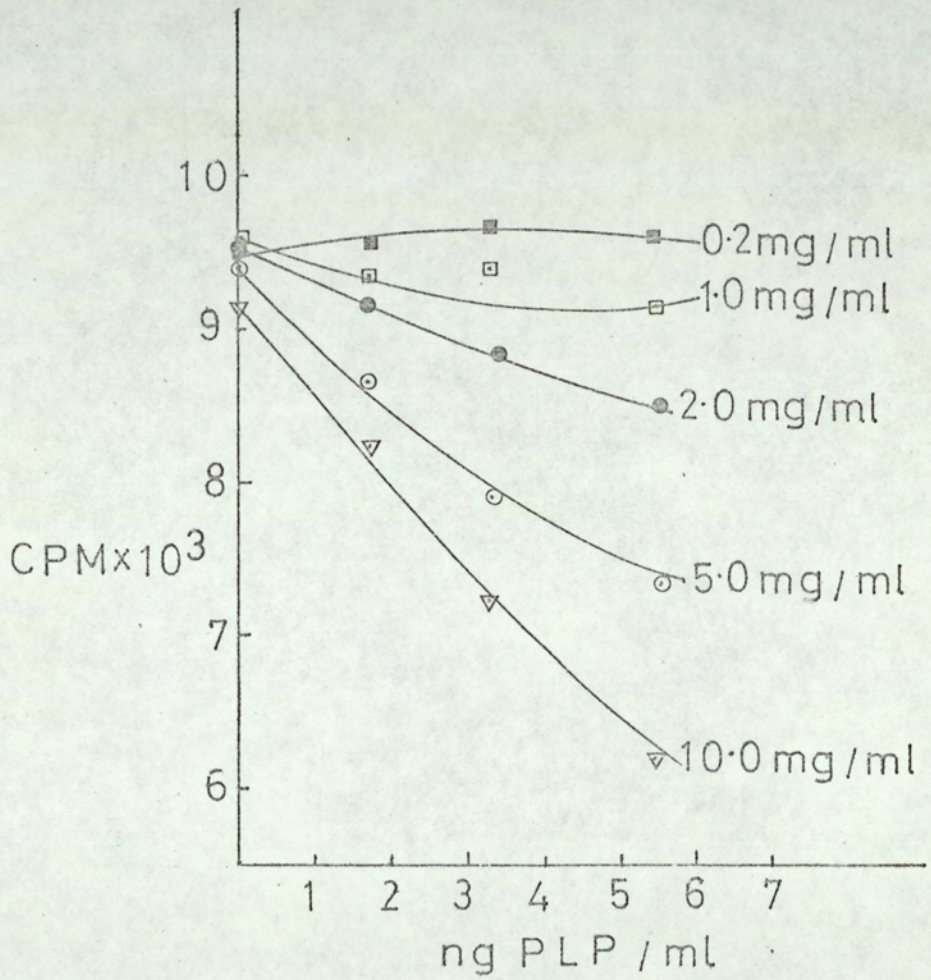


Fig. 3.2.8. The effect of varying enzyme concentration.

1 ml of this was then diluted with buffers of pH range 3.7-5.6. These standards which contained 3.4 ng PLP/ml were then incubated with apo-enzyme and then substrate as described in 3.2.2.1.3. Table 3.2.9. shows the C.P.M. of a 0.5 ml aliquot of each reaction mixture. Considerable enzyme inhibition occurs below pH 5.2.

3.2.3.8. Extraction of PLP from serum

A pooled serum and pooled serum to which had been added 13.1 ng PLP/ml were used throughout. The following procedures were carried out:-

(a) 1 ml serum + 0.2 ml 30% TCA were buzzed for 15 secs and centrifuged for 15 mins at 2,000 rpm. To 0.2 ml supernatant was added 0.25 ml freshly made up 0.2N NaOH (pH 5.0) and then 1.5 ml NaAc buffer pH 5.5. Dilution factor was x 7.2.

(b) 4 ml distilled water was added to 1 ml serum and mixed by buzzing. 0.5 ml 30% TCA was then added and the contents mixed by buzzing. After standing at room temperature for 10 mins the contents were centrifuged at 2,000 rpm for 15 mins. The supernatant was saved and the precipitate was re-extracted with 2.5 ml 2% TCA, mixed and centrifuged as before. To the pooled supernatants was added 1 ml 2.5M NaAc. The resulting solution had a pH of 5.1 and the serum had been diluted x 9.

(c) To 0.6 ml serum was added 2.75 ml 5% TCA and mixing was effected by buzzing for 15 secs. The tube was left to stand in the dark at room temperature for 30 mins before centrifuging at 2,000 rpm for 15 mins. 2.5 ml of the supernatant was removed and placed in a tube which already contained 0.3 ml 4M NaAc. The resulting solutions had pH's of 4.5-4.8 and the serum had been diluted x 6.2.

(d) To 1 ml serum was added 2 ml ice cold 0.4N perchloric acid. The solutions were mixed by buzzing for 15 secs and the mixture was kept

in an ice bath for 5 mins prior to the addition of 0.5 ml 1N KOH. Either 1 ml or 6 ml of NaAc buffer pH 5.5 was then added. The solutions were centrifuged for 15 mins at 2,000 rpm and the supernatant was used. The serum had been diluted x 4.5 or x 9.5.

Duplicate incubations of these extracts were then made with apo-enzyme and substrate, as described in 3.2.2.1.3. The pH of each extract was measured, as was the protein content (sulphosylic acid method using Gallenkamp standards). The results are shown in Table 3.2.10. Inhibition of the enzyme is severe when extraction procedures (b) and (c) are used. There is inhibition of the reaction between the apo-enzyme and the endogenous PLP since the counts obtained for these extracts are higher than those of the enzyme blanks. The presence of protein in the extract, (d), has an inhibitory effect although to a lesser extent than the inhibition due to pH found in extracts (b) and (c). Extraction (a) gives the highest results but there is a marked lack of reproducibility.

3.2.3.9. Reproducibility

Improvement in reproducibility relative to that of the preceding section (3.2.3.8.) was obtained using a Fisons Automatic diluter which picked up approximately 0.2 ml of the acid serum supernatant and then expelled and diluted it with approximately 1 ml 3% w/v NaAc. The precise volumes used were calculated by weighing distilled water picked up and expelled each time an adjustment had to be made to the instrument. The extracts were pooled after each run and the pH was checked; the range was found to be pH 5.5-pH 5.7.

The within batch reproducibility was measured on numerous occasions. The results are shown in Table 3.2.11. Where serum was a source of PLP 'n' represents the number of aliquots extracted and diluted; each dilution was incubated in duplicate. Possible sources

Table 3.2.10. Extraction of PLP from serum.

Extraction method	pH	protein	C.P.M. x 10^3	ng PLP / ml extract
Blank 1	5·5	Absent	2·03	
Blank 2			1·01	
Standards (ng PLP/ml)				
1·6			0·86	
3·2			0·61	
5·8			0·40	
8·0			0·37	
10·7			0·30	
32·7			0·22	
a) S	7·6	Absent	0·90	0·8
S	7·6		0·76	1·65
S	7·5		0·81	1·25
S + PLP	8·6		0·48	5·0
S + PLP	8·6		0·53	4·1
S + PLP	7·7		0·70	2·1
b) S	5·0	10 mg %	1·55	
S	5·0		1·56	
S	5·0		1·54	
S + PLP	4·9		1·48	
S + PLP	4·9		1·49	
S + PLP	4·9		1·42	

S = serum extract

S + PLP = extract of serum to which had been added 13·1 ng PLP / ml

Table 3.2.10. (continued)

Extraction method	pH	protein	C.P.M. x 10^3	ng PLP / ml extract
c) S	4·9	Absent	1·59	
S	4·9		1·68	
S	4·9		1·68	
S + PLP	4·9		1·66	
S + PLP	4·9		1·66	
S + PLP	4·9		1·65	
d) S	5·0	100 mg %	0·92	0·65 (x9·5)
S	4·4		1·00	0·30 (x4·5)
S	4·4		0·94	0·60 (x4·5)
S + PLP	4·9		0·79	1·40 (x9·5)
S + PLP	4·8		0·79	1·40 (x9·5)
S + PLP	4·7		0·67	2·40 (x4·5)

S = serum extract

S + PLP = extract of serum to which had been added 13·1 ng PLP / ml.

The factor following the ng PLP / ml extract for method d) is the dilution used.

Table 3.2.11. Within batch reproducibility.

Source of PLP	n	$\bar{x}$	SD	CV(%)	Dilution used
Serum	10	11·3	1·47	13·0	x 5·4
Serum	12	12·2	0·74	6·1	x 5·9
Serum	12	14·5	1·8	12·4	x 12·0
Serum	19	20·7	3·8	18·3	x 5·9
Serum	5	28·3	3·7	13·1	x 5·9
Serum	10	5·9	0·7	11·9	x 5·9
Serum	10	8·4	1·2	14·3	x 5·9
Serum	20	4·3	0·4	9·3	x 5·9
Standard	20	3·1	0·4	12·9	
Standard	18	4·4	0·2	4·5	

Table 3.2.12. Comparison of results obtained when aliquots are counted for 1 min and 4 min.

	Counted for 1 min			
Source of PLP	n	$\bar{x}$	SD	CV(%)
Standard(single)	22	2·4	0·27	11·5
Standard(paired)	11	2·4	0·17	7·3
Serum A	10	15·9	1·7	10·5
Serum B	12	2·1	0·5	23·8

	Counted for 4 min			
Source of PLP	n	$\bar{x}$	SD	CV(%)
Standard(single)	22	2·3	0·24	10·7
Standard(paired)	11	2·3	0·20	8·8
Serum A	10	16·1	1·80	11·0
Serum B	12	2·0	0·3	15·0

of variation were investigated. These were:-

(a) the effect of changing the counting time for each vial from 1 min to 4 min

(b) the standard deviation between the duplicate incubations of the same extract

(c) the influence of the position within the run i.e. whether the extract was incubated in rack 1, 2, 3.

(a) A working standard and also serum extracts were incubated with enzyme and substrate as described in section 3.2.2.1.3. 0.5 ml of the final solution was counted for 1 min and the same vials were re-counted for 4 min. The values of PLP obtained for the serum extracts were calculated from the mean counts obtained from the duplicate incubations, whilst those for the working standard were obtained from each incubation and also from the mean of the counts from consecutively paired incubations. The results are given in Table 3.2.12. Only for serum B is the coefficient of variation much reduced when counting is increased to 4 min, nonetheless, the counting time was increased to 4 min since this ensured that all counts within the range of the standards used were in excess of 20,000.

(b) and (c) Factors (b) and (c) were investigated in the same experiment. Twenty-seven aliquots of pooled serum were extracted and diluted, each dilution was incubated in duplicate. The standard deviation of pairs was calculated for each rack, for racks 1 + 2 and racks 1 + 2 + 3. The value of serum PLP was calculated for each pair and this was used when calculating the mean for each rack and also successive racks. The results are shown in Table 3.2.13.

Eppendorf pipettes were used for the addition of enzyme, PLP extract and substrate. The increase in variation in successive racks is not amenable to ready explanation. The strict time schedule was adhered to throughout, each rack was shaken the same number of

Table 3.2.13. The effect on reproducibility, of the position of the serum extract within the run.

Rack number	n	$\bar{x}$	SD	CV(%)	SD (pairs)
1	10	13·8	1·18	8·6	1·94
2	12	14·1	1·96	13·9	1·54
3	5	14·3	3·30	23·1	2·84
1 + 2	22	13·9	1·62	11·6	1·73
1 + 2 + 3	27	14·0	1·95	13·9	1·95

Table 3.2.14. Between batch reproducibility.

Pool	n	$\bar{x}$	SD	CV (%)
A	14	10.0	1·97	19·7
B (1)	14	19·6	5·03	25·6
B (2)	17	13·1	2·05	15·6

times and there was no temperature gradient across the bath. Rack 1 had a period of contact between PLP and enzyme of up to 5 mins at room temperature before being placed in the water bath for the 20 mins pre-incubation period, but the other racks were placed in the water bath at 3 min intervals thereafter. The temperature of the substrate on addition to the enzyme + PLP was lower for rack 4 than rack 1 since prior to addition a sufficient volume of preheated substrate was poured into a beaker to facilitate dispensing throughout the run.

The between batch variation has been measured by including within each batch a sample of pooled serum. This pooled serum had been collected, divided into 4 ml aliquots and stored at -20°. The results are given in Table 3.2.14. Pool A was used on 17 occasions. Runs giving values for the pool of (a) 19.4, (b) 5.5, 6.1 ng PLP/ml were rejected because in case (a) the temperature of incubation was found to be 42°, and in case (b) the enzyme had been frozen and thawed two and three times (see section 3.2.3.11.) Pool B has been used on 11 separate occasions, two of these were when within run reproducibility was being measured. Pooled serum was included in each rack of a run on 8 occasions and each value obtained is included here (see Table 3.2.14.). The between batch variation is large. When pool B (1) was being used sera with extremely high levels of PLP were included within the run. Carry over may have occurred when the serum was being pipetted out prior to the precipitation with 30% TCA. Carry over at the dilution stage is unlikely since the acid serum extract was washed out of the diluter probe with approximately 5 times its own volume of NaAc solution. When the diluted extract was added to the enzyme a fresh Eppendorf tip was used for each extract. Pool B (2) shows the change in reproducibility when a 1000 µl Eppendorf was used for pipetting the

serum (these repeats were performed with runs where absorption of an oral dose of pyridoxine hydrochloride was being monitored).

3.2.3.10. Recovery of PLP added to serum

0.1 ml of working standard was diluted to 10 ml with pooled serum in order that the recovery of varying amounts of PLP could be measured. The PLP added to the serum increased the levels of PLP present over the range of 1.5-13.4 ng PLP/ml. Table 3.2.15 shows the recoveries obtained for each addition of PLP and also the mean recovery for all additions. The additional PLP was efficiently extracted from serum when this method was used.

3.2.3.11. The effect of freezing and thawing the enzyme suspension

The effect of storage at -20° and alternate freezing and thawing of the enzyme has been investigated. The enzyme was homogenised and suspended in either NaAc buffer pH 5.5 or buffer which contained 50% glycerol. The enzyme concentration was 10 mg/ml. These two suspensions were each divided into four aliquots; one pair (buffer and 50% glycerol buffer) was stored at 4°, the others were stored at -20° and thawed 1, 2 and 3 times before use. Enzyme powder was also stored, desiccated, at -20° and made up on the day of the experiment. Sufficient enzyme from each treatment was prepared to enable a standard curve to be drawn for each treatment. The PLP content of extracts from 3 sera was measured using each of the enzymes and the values obtained are shown in Table 3.2.16. The highest levels of PLP in serum were obtained when the enzyme was homogenised and suspended in buffer on the day of the assay.

Table 3.2.15. Recovery of PLP added to serum.

PLP added (ng / ml)	Recovery (%)
8·3	95·8
9·6	106·0
6·5	98·8
8·4	97·7
13·4	87·0
6·7	98·3
1·5	108·0
10·0	96·0
Mean (± SD)	98·5 ± 6·5

Table 3.2.16. The effect of freezing and thawing the enzyme suspension.

Treatment	Serum A		Serum B		Serum C	
of enzyme	B	G	B	G	B	G
Fresh	13·4	10·1	13·4	9·5	13·7	10·1
4° for 48h	8·7	10·1	8·7	7·2	11·1	10·7
F & T x1	9·8	9·8	10·7	9·5	5·9	9·5
F & T x2	5·4	9·5	8·7	6·3	5·4	7·4
F & T x3	5·4	5·1	7·7	6·9	11·1	10·7

B = buffer G = 50% glycerol in buffer

F & T = freeze and thaw.

3.2.3.12. The effect of varying levels of alkaline phosphatase on the measured levels of serum PLP

The effect on PLP levels of the presence of varying levels of alkaline phosphatase in serum was studied. Serum containing varying levels of alkaline phosphatase was prepared by reconstituting Hyland Abnormal Serum (Wellcome Laboratories). The dilutions made were the same as those used when alkaline phosphatase standards were prepared for Auto-analyzer use. The dried serum was initially diluted to 6 ml with distilled water (dilution 7) and subsequent dilutions were made with normal saline as shown in Table 3.2.17. Stock standard PLP was diluted 1:500 with NaAc buffer pH 5.5 and 50 µl of this solution was added to 8 ml of the prepared serum, which then contained 4.2 ng PLP/ml. The sera containing the PLP were stored in the dark at room temperature (23°) for up to 4 hrs. At varying time intervals 1 ml of serum was transferred to another tube and the proteins were precipitated by the addition of 0.2 ml 30% TCA. One set of tubes was incubated in the light for 4 hrs and another set was incubated for 4 hrs with light excluded and then stored at -20° for 48 hrs prior to analysis. The PLP present in the serum was measured in the usual way (see section 3.2.2.1.3.). The sera were analysed on the day of incubation with alkaline phosphatase with the exception of the sera which had been kept in the dark for 4 hrs and then stored at -20° for 48 hrs. The results are given in Table 3.2.18. The experiment was repeated and on this occasion 3.3 ng PLP/ml were added to the serum. Samples were protected from light and incubated at room temperature for varying times. At intervals 1 ml portions of serum were removed, precipitated, centrifuged and diluted in the usual manner. The extracts were stored over night at 4° and analysed the next day. The 24-hour specimen was protected from light and incubated at room temperature

Table 3.2.17. Dilutions made to obtain a range of alkaline phosphatase activities from Hyland Abnormal serum.

Dilution number	KA (%)	Source
1	4	4 ml 2) + 4 ml saline
2	8	4 ml 3) + 4 ml saline
3	16	4 ml 5) + 4 ml saline
4	24	Dry serum diluted to 10 ml
5	31	4 ml 7) + 4 ml saline
6	46·5	3 ml 7) + 1 ml saline
7	62	Dry serum diluted to 6 ml

KA (%) = King Armstrong Units / 100 ml serum

Table 3.2.18. The effect of contact with varying activities of alkaline phosphatase for varying time on the measured PLP levels of sera.

Time of incubation (h)	ng PLP / ml serum						
	A	B	C	D	E	F	G
0	4·2	6·0	5·4	7·2	8·7	8·4	13·2
½	3·6	3·3	3·6	5·7	5·4	7·8	13·8
+ 4·2 ng PLP 1	3·3	2·1	6·3	6·6	7·2	8·4	14·4
/ ml serum 2	3·3	3·0	2·4	5·8	5·1	10·2	15·3
4	1·2	5·4		4·2	5·4	10·8	14·7
4 h in light	3·9	4·5	2·4	6·0	7·8	12·6	12·3
4 h in dark then 48 h at -20°	5·1	6·3	6·0	7·8	8·7	11·2	13·5
0	2·1	2·1	3·0	3·6	3·3	4·8	7·2
½	2·1	2·7	3·0	3·0	3·6	4·8	8·4
+ 3·3 ng PLP 1	2·1	2·1	2·1	4·8	4·5	5·1	7·5
/ ml serum 2		2·4	2·7	3·6	4·8	4·8	7·2
4	1·8	2·4	3·9	4·2	3·8	5·7	5·4
4 h in dark then 24 h at + 4°	0·7	1·5	2·1	3·6	4·5	5·4	7·8
Protein content of serum(g/ 100 ml)	0·6	1·2	2·5	3·8	4·9	7·4	9·8
King Armstrong Units / 100 ml serum	4	8	16	24	31	47	62

for 4 hrs and was then stored over night at 4° prior to extraction. The results are also shown in Table 3.2.18. There is a lack of consistency in the results obtained. Exposure to light for 4 hrs does not appear to have a deleterious effect on the measured level of PLP. Storage at -20° produced a rise in measured levels of PLP (pooled serum gave values of 7.6 and 7.2 ng PLP/ml in respective runs) whilst storage at 4° lowered the levels of PLP in sera containing 4-16 King Armstrong Units of alkaline phosphatase/100 ml serum. There was no trend towards lower PLP levels with increasing time of exposure to even the highest activities of alkaline phosphatase. On the basis of results presented here it was concluded that exposure to light, alkaline phosphatase and storage of serum at -20° had little effect on the measured levels of PLP. Wherever possible sera were not stored at 4° since there was some indication that losses may occur under these conditions.

3.2.3.13. A comparison of PLP levels measured in serum and plasma

On 11 occasions blood was taken and half placed in a plain glass tube and half in a lithium heparin coated tube. The serum and plasma were separated and stored at -20° until analysed. The results given in Table 3.2.19. show that there is no significant difference between the levels measured in plasma and serum.

3.2.3.14. Xanthurenic and kynurenic acids in urine. The effect of varying washing techniques and resin mesh size

Dowex 50W H^+ x 12% was used, the mesh sizes being 20-50 (coarse) and 200-400 (fine). Both resins were suspended in twice their volume of distilled water and then allowed to settle. The fine particles were removed by suction with the water and the washing was repeated twice. The coarse resin and half the fine resin were

Table 3.2.19. Comparison of PLP levels measured in serum and plasma.

Number	ng PLP / ml	
	Serum	Plasma
1	9·4	11·0
2	5·4	5·1
3	5·7	5·7
4	66·0	67·0
5	49·5	47·2
6	7·5	5·9
7	5·1	4·9
8	14·8	14·1
9	25·4	24·7
10	7·8	7·0
11	3·5	4·5

Table 3.2.20. The effect of washing of resin and variation in mesh size on the recovery of XA and KA.

Source of XA and KA	Resin A		Resin B		Resin C	
	XA	KA	XA	KA	XA	KA
Urine (μ moles / 24 h)	32·0	28·7	38·0	22·6	31·4	31·0
Recovery (%) Urine + Std.	83	93	45	52	77	82
Recovery (%) Std.	79	94	55	68	88	99

washed in 5N HCl, the washing solution was removed and the resins were stored under fresh 5N HCl until use. Approximately 500 ml of the fine resin were subjected to the washing procedure described by Price et al (1965) i.e. 2.3 l 2N NaOH, 1.7 l distilled water, 2 l 2N HCL, 11.7 l 8N HCl, 5.8 l 4N HCl and 5.8 l distilled water. The washing was performed by passing the reagents through the resin which was held in a large column.

Nine columns were used in this experiment, columns 1-3 contained 3 cm of the specially washed resin (A), columns 4-6 contained 10 cm of the coarse resin (B) and columns 7-9 contained 3 cm of the fine resin (C). Columns 4-9 were washed with 100 ml distilled water prior to the application of the test solutions. Test urine from a single 24-hour save, prepared as described in 3.2.2.2.3. was applied to columns 1, 4 and 7 and standard solutions were applied to columns 3, 6 and 9. Urine containing internal standards was applied to columns 2, 5 and 8. The samples and eluates flowed through the columns at gravity flow rates and the flow rates for columns 4-6 were adjusted to give the same flow rate. The procedures used were the same as those described in 3.2.2.2.3., the excretion of each metabolite and the recovery of the standards for each resin are given in Table 3.2.20. Percentage recovery of XA and KA was greater when the resin was washed with water then 5N HCl than when it was put through the more complex cleaning procedure. When resin B was used there was a much lower recovery of the standards, but there was not a pro rata reduction in the 24-hour excretion of XA or KA.

3.2.3.15. Change of fluorescence with increasing time

The following solutions were used to monitor the changes in fluorescence under the conditions used to measure XA and KA

concentrations of the column eluates:-

(a) blank, 0.005M phosphate buffer

(b) standard, 97.6 n moles XA/ml, 105.8 n moles KA/ml

(c) eluate from column to which had been applied 4.88 μ moles XA and 5.29 μ moles KA.

The volumes used were the same as those described in 3.2.2.2.3. The first readings were taken 10 mins after the addition of the reagents. This time lapse allowed the alkaline (XA) solutions to clear and also the acid (KA) solutions to warm to room temperature after removal from the ice bath. The solutions were placed in fluorimeter tubes for the first reading and the same solutions and tubes were used throughout the time course of the experiment. Table 3.2.21. shows that, in alkaline conditions, the fluorescence reaches a maximum after 30 mins and remains stable for more than 3 hrs. There is a gradual decline in the readings obtained under acid conditions, this is because KA is unstable to ultra violet irradiation under the conditions of the determination (Price et al (1965)). Normally the KA tubes are exposed only once to ultra violet irradiation. The fluorescence of test solutions was read within 90 mins of the addition of either acid or alkali.

3.2.3.16. The emission spectra of XA and KA

(a) Acid conditions. The emission spectra of the 48.8 n mole KA standard and a column of eluate which was derived from a urine sample containing 4.88 μ moles XA and 5.29 μ moles KA were recorded under the conditions used to measure KA. Fig.3.2.9. shows the spectra obtained when activated in the range 300-400 nm (filters OX 1 or LF 2). There is no interfering fluorescence present in the extract derived from the urine sample.

(b) Alkaline conditions. The emission spectra were recordéd on

Table 3.2.21. The change of fluorescence with time under the conditions used for reading the XA and KA content of column eluates.

Time	Alkaline (XA)				Acid (KA)			
(min)	a	b	c		a	b	c	
10	1·0	5·7	4·2	4·2	2·0	8·9	5·7	5·7
20	1·7	6·8	5·1	5·2	2·3	8·9	5·8	5·9
30	2·3	7·3	5·5	5·6	2·3	8·8	5·7	5·8
40	2·2	7·2	5·5	5·6	2·3	8·6	5·7	5·8
55	2·2	7·2	5·5	5·5	2·3	8·6	5·7	5·7
70	2·2	7·1	5·4	5·5	2·2	8·5	5·6	5·7
85	2·1	7·0	5·4	5·4	2·3	8·5	5·6	5·7
100	2·1	6·9	5·3	5·3	2·2	8·4	5·5	5·6
115	2·1	6·9	5·3	5·3	2·3	8·4	5·5	5·5
130	2·1	6·9	5·3	5·3	2·2	8·3	5·5	5·6
160	2·1	6·9	5·3	5·3	2·2	8·3	5·5	5·5
190	2·1	6·9	5·1	5·2	2·2	8·2	5·4	5·5

a) blank 0·005 M phosphate buffer.

b) Standard 97·6 n moles XA / ml, 105·8 n moles KA / ml.

c) Eluate from column to which had been applied 4·88 μ moles XA and 5·29 μ moles KA.

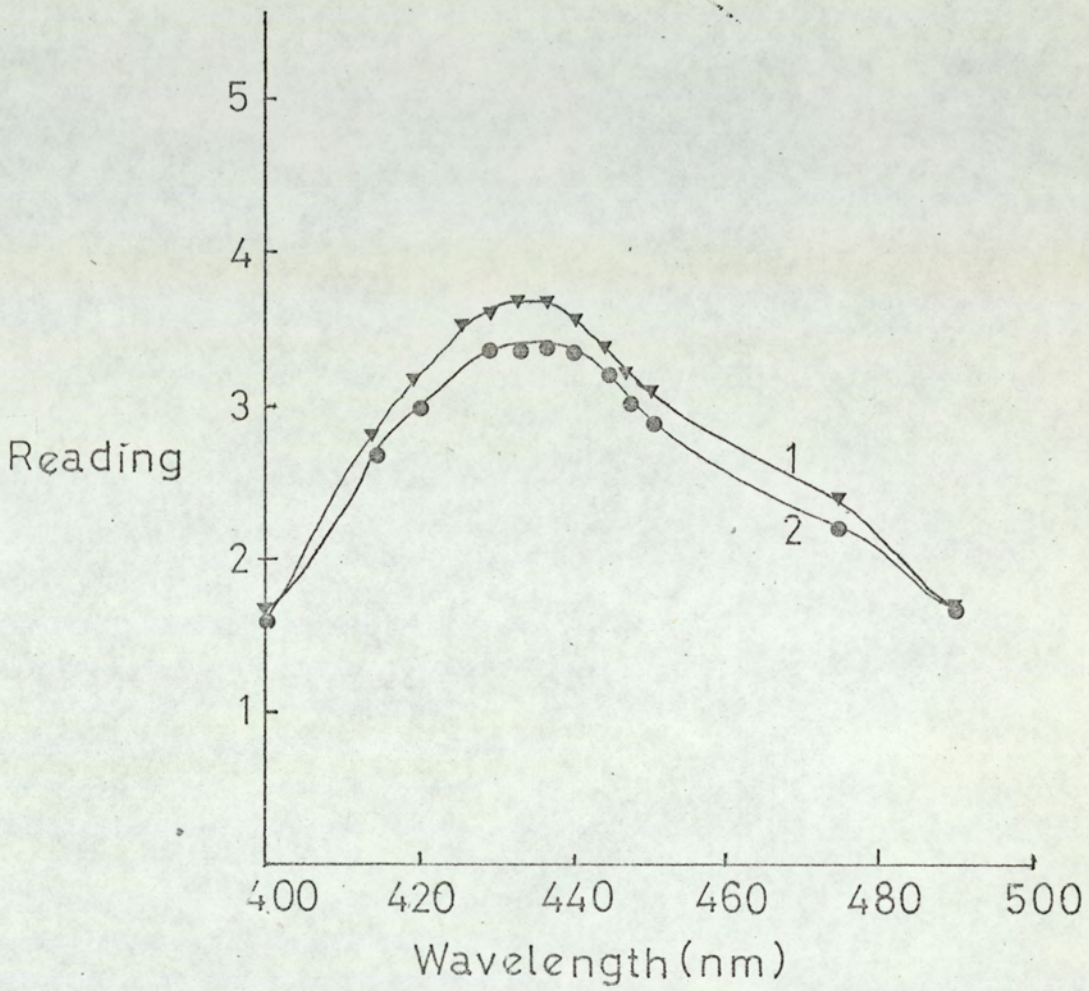


Fig. 3.2.9. The emission spectra of:-

1) 48·8 n moles KA.

2) Eluate from column to which had been added urine containing 4·88 μ moles XA and 5·29 μ moles KA as internal standards, under acid conditions.

the column eluate solution as above, (a), and also those of the 195.2 n mole XA and 211.6 n mole KA standards. An emission peak for the eluate was seen at 487 nm whilst that of the XA standard occurs at 530 nm. Under these conditions there was also an emission peak at 480 nm from the KA standard (see Fig.3.2.10.). Activation wavelengths were in the range 300-400 nm (filters OX 1 and LF 2).

In order to check the additive nature of the emission spectra obtained, those of the 48.8 n mole XA, 52.9 n mole KA and that of a solution containing 48.8 n mole XA + 52.9 n mole KA were scanned and drawn (Fig.3.2.11.), together with the predicted points derived from the summation of the readings obtained from the separate standards. At the wavelength used to measure the fluorescence due to XA (525 nm) addition of the readings from the two components gives a reading of 0.5 (units) higher than that actually measured. All measurements of XA presented here have been corrected for interference from KA.

3.2.3.17. The recovery of XA and KA from columns of coarse and fine resin

The resins used were Dowex 50W H^+ x 12%, of mesh size 20-50 (coarse) and 200-400 (fine). The procedure used was that described in 3.2.2.2.3. Columns 1-5 contained coarse resin of bed length 10 cm, columns 6-10 contained 3 cm of the fine resin. Standards of XA and KA were applied to each column of concentration range 0.32 n mole to 9.66 n mole. Table 3.2.22. shows the recoveries obtained. When 1.61 µg or less of either XA or KA are applied to the columns containing the coarse resin recoveries are 78% and 75% respectively. When the coarse resin was used in the analysis of XA and KA in human urine 4.88 µ mole and 5.29 µ mole were used as

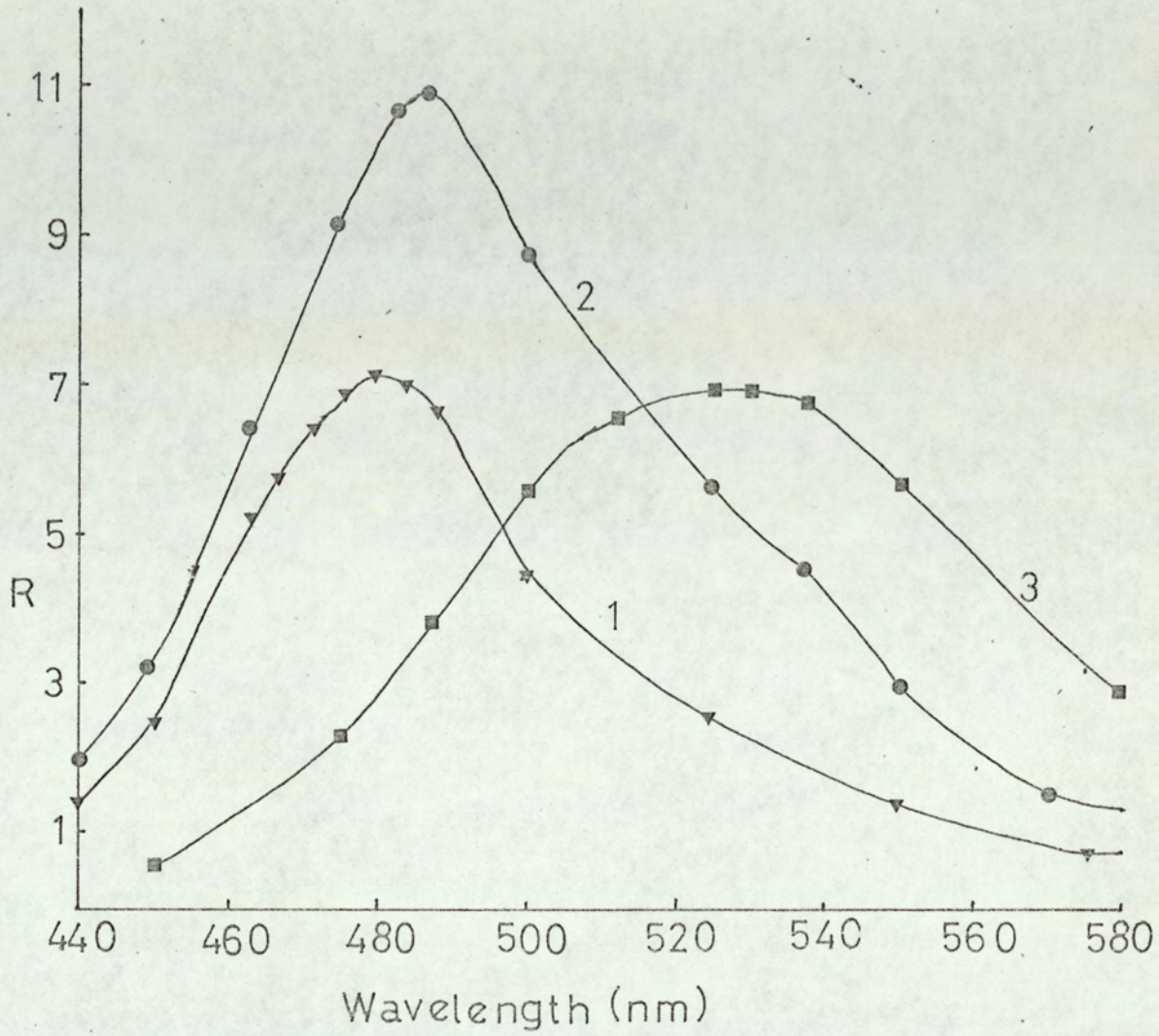


Fig. 3.2.10. The emission spectra of:-

1) 211·6 n moles KA.

2) Eluate from column to which had been added urine containing 4·88 μ moles XA and 5·29 μ moles KA as internal standards.

3) 195·2 n moles XA.

under alkaline conditions.

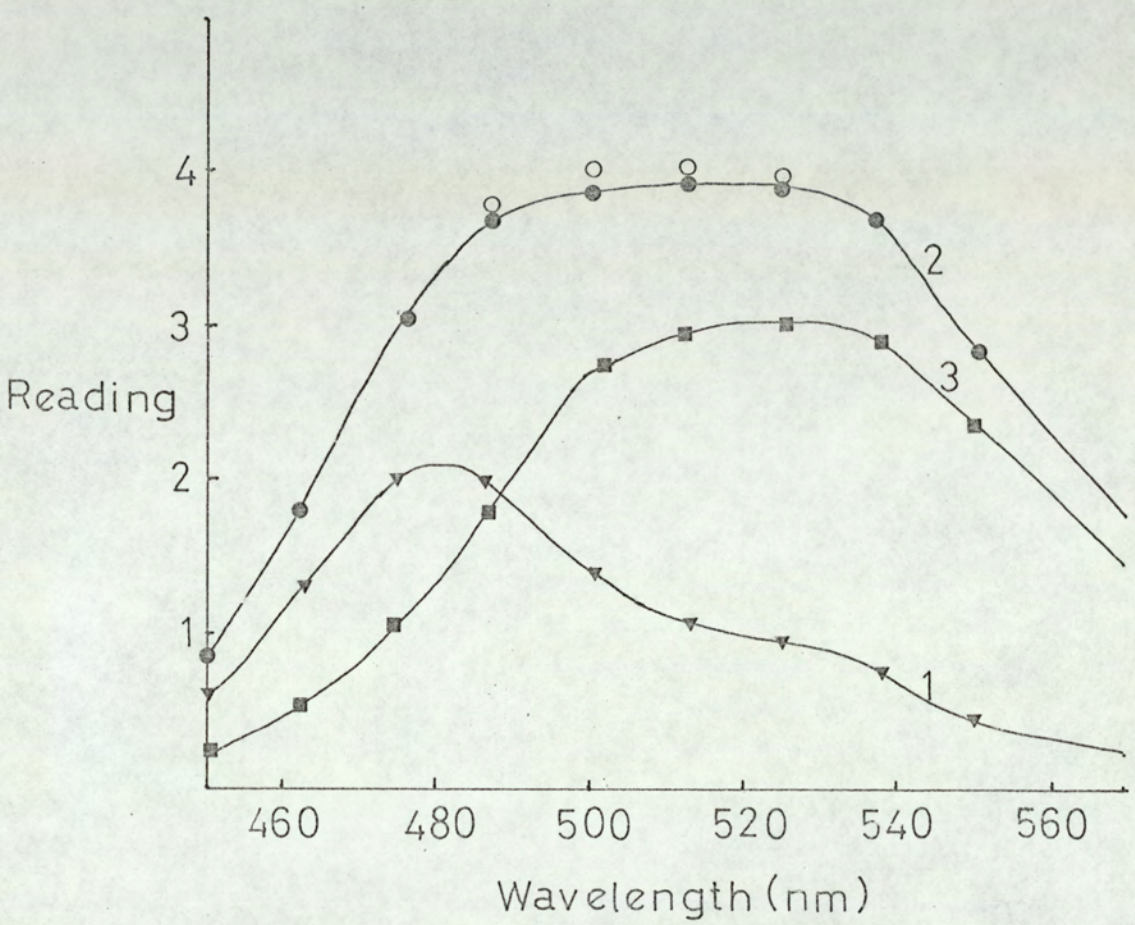


Fig. 3.2.11. The emission spectra of:-

1) 52·9 n moles KA.

2) 52·9 n moles KA + 48·8 n moles XA.

3) 48·8 n moles XA.

o Predicted points of 2) derived from the summation of 1) and 3).

under alkaline conditions.

Table 3.2.22. The recovery of varying amounts of XA and KA from coarse and fine resins.

Column number	μ moles added	XA		KA	
		Recovered μ moles	Recovery (%)	Recovered μ moles	Recovery (%)
1	0·32	0·28	87	0·37	115
2	1·61	1·25	78	1·21	75
3	3·22	1·96	61	2·20	68
4	6·44	4·20	65	4·35	68
5	9·66	5·96	62	6·10	63
6	0·32	0·40	129	0·34	106
7	1·61	1·60	99	1·52	94
8	3·22	3·21	100	2·85	89
9	6·44	6·56	102	6·66	103
10	9·66	8·61	89	9·34	97

internal standards for XA and KA respectively.

3.2.3.18. The recovery of 5-hydroxyindolyl-3-acetic carbonyl-1-^{14}C from human urine

The recovery of 5-HIAA-1-^{14}C from human urine was measured as described in 3.2.2.5.3. The results are given in Table 3.2.23. The concentration of 5-HIAA in the urine ranged from 1.1 mg/24-hour save to 225 mg/24-hour save, the mean recovery of 5-HIAA-1-^{14}C was 97.7%.

3.2.3.19. The effect of varying the time of contact of the reagents involved in the colour reaction for indole-3-acetic acid

(a) The effect of varying the time of contact with xanthrydol before the addition of sodium hydrogen sulphite.

The 50 µg IAA/ml standard was used and the experiment was performed in duplicate. To 0.5 ml of the buffered standard was added 0.5 ml concentrated HCl. These were mixed prior to the addition of 1 ml xanthrydol. At varying time intervals 0.5 ml sodium hydrogen sulphite was added and the OD was read within 10 mins of this last addition. The contents of the tubes were thoroughly mixed after each addition. The results are shown in Table 3.2.24. and they show that the OD varies with time of contact between IAA and the xanthrydol. When test urine extracts were analysed the sodium hydrogen sulphite was added exactly 5 mins after the xanthrydol.

(b) The effect of varying the time of reading OD after the addition of sodium hydrogen sulphite.

Duplicate 10 µg IAA/ml standards were treated as described in 3.2.2.4.3. The OD was read at varying times after the addition of sodium hydrogen sulphite (see Table 3.2.25.). There was little change in the OD in the first 30 mins. All OD's were read within

Table 3.2.23. Recovery of 5-hydroxy-3-indoleacetic carbonyl-1-^{14}C acid from urine samples.

mg 5HIAA / 24 h save	Recovery (%)
225 (neat urine)	94·6
urine diluted 1 : 20 before extraction	91·4
2·5	98·1
6·8	99·7
5·0	96·6
5·4	98·3
6·3	101·6
1·1	101·7
4·2	97·6
4·2	100·6
6·5	97·4
5·0	99·5
4·8	99·7
19·5	91·1

Table 3.2.24. The effect of varying time of contact with xanthrydol before addition of sodium hydrogen sulphite.

Time (min)	OD	
0	0·015	0·018
2	0·171	0·177
5	0·375	0·371
8	0·485	0·460
10	0·510	0·490
15	0·560	0·560
20	0·599	0·590
30	0·600	0·605

Table 3.2.25. The effect of varying time of reading after addition of sodium hydrogen sulphite.

Time (min)	OD	
1	0·062	0·075
2	0·067	0·075
4	0·067	0·075
6	0·068	0·074
8	0·064	0·072
10	0·063	0·072
15	0·061	0·068
20	0·063	0·071
25	0·065	0·075
30	0·062	0·072

20 mins of the addition of the last reagent.

3.2.3.20. The recoveries of the tryptophan metabolites measured

The mean recoveries of the internal standards for each set of test urines are listed in Table 3.2.26. The conditions under which these recoveries were carried out have been described in the appropriate procedures. For XA, KA and Kyn the recoveries are listed under coarse and fine resins. None of the results contained within the Clinical Results section have been adjusted to give 100% recovery.

Table 3.2.26. Recovery of metabolites of tryptophan from human urine expressed as a percentage of added internal standard.

Compound measured.	Recovery (%).			
	n	$\bar{x}$	SD	Range
XA 20-50 mesh resin	13	58	9·0	43 - 74
200-400 mesh resin	9	91	9·3	71 - 100
KA 20-50 mesh resin	13	52	10·1	33 - 66
200-400 mesh resin	9	101	9·0	92 - 115
Kynurenine	9	82	12·1	61 - 100
5HIAA	19	97	3·5	91 - 102
IAA	10	96	19·7	83 - 133

3.2.4. Discussion

3.2.4.1. Measurement of pyridoxal-5'-phosphate

The specificity of the method for pyridoxal-5'-phosphate (PLP) was tested by incubating the enzyme with excess pyridoxamine phosphate (1,800 ng/ml) and pyridoxal (3,000 ng/ml). These two compounds gave enzyme activities equivalent to 0.8 and 0.9 ng PLP/ml respectively. The highest concentration of pyridoxal in serum after acid hydrolysis reported by Anderson et al (1970) was 13.5 ng/ml, this would give a maximum concentration of 2.25 ng/ml extract for the procedure described here.

Loss of enzyme activity during preparation with saturated alkaline ammonium sulphate has been reported by Epps (1944) and Hamfelt (1962). The sulphate ion has been found to be a competative inhibitor of the reaction between PLP and the apo-enzyme (Sloane-Stanley (1949)) and this may have been the source of the loss of activity in the procedure used in 3.2.3.2. as well as dilution losses. After Sephadex gel filtration enzyme activity was present in two components when excess co-enzyme was supplied (30 µg PLP/ml) but the enzyme was not present in sufficient concentration to react with 7 ng PLP/ml. Chabner and Livingston (1970) have estimated the molecular weight of L-tyrosine apo-decarboxylase to be 200 000 or greater, which is similar to those of bacterial arginine (Boecker and Snell (1968)), histidine (Chang and Snell (1968)) and glutamic acid decarboxylases (Shukuya and Schwert (1960)).

Reversible inactivation of glutamic decarboxylase has been shown by Shukuya and Schwert (1960); PLP and albumin have afforded protection against inactivation. Alternate freezing and thawing of the enzyme reduced the enzyme activity in such a way as to alter the enzyme's affinity for PLP extracted from serum but not for the

working standards. This resulted in a lowering of the values obtained for the test sera, the extent of which was proportional to the number of times the enzyme had been frozen and thawed (see Table 3.2.16.). The resolution of the enzyme into two components by filtration through Sephadex C-200 and the partial inactivation resulting from freezing and thawing suggest that the enzyme is composed of sub-units which require the presence of PLP to confer full stability and activity. Baranowsky et al (1957) found that PLP stabilised the tertiary structure of glycogen phosphorylase.

The pre-incubation of PLP and apo-enzyme was performed in order to assure the complete association of halo-enzyme. Time of pre-incubation has little effect on the extent of decarboxylation taking place when PLP is present in relatively large quantities (13.0 ng PLP/ml). When smaller quantities of PLP are present (2.6 ng PLP/ml) the amount of decarboxylation increases with time of pre-incubation up to 20 mins (see Fig.3.2.5.). Hamfelt (1967) has reported that apo-decarboxylase requires 30 mins pre-incubation at 37° for complete formation of the halo-enzyme, but Maruyama and Coursin (1968) found that halo-enzyme formation was complete after 10 mins at 37°. These latter authors did not pre-incubate their PLP extract with the apo-enzyme although Hamfelt (1967) and Chabner and Livingston (1970) both pre-incubated for 30 mins.

An optimum pH of 5.5 for tyrosine decarboxylase has been given by Epps (1944) and this has been the pH of the reaction mixture used here and by Hamfelt (1967), Maruyama and Coursin (1968) and Chabner and Livingston (1970). Addition of PLP extract at pH 7.6-8.6 caused little enzyme inhibition, although complete inhibition was produced when extract at pH 5.0 was used (see Table 3.2.10.). The presence of protein in the extract (extraction (d) in Table 3.2.10.) protects the enzyme in some manner and permits limited

activity when the extract has a pH of 4.4.

The measurement of radio-activity after the termination of the reaction has been performed in a variety of ways:-

(1) Separation of tyrosine and tyramine by paper chromatography and scanning the radio-chromatogram (Hamfelt (1967)).

(2) Driving off the liberated $^{14}CO_2$ by bubbling with nitrogen (Maruyama and Coursin (1968)).

(3) Trapping the evolved $^{14}CO_2$ in KOH and counting that (Chabner and Livingston (1970)).

In the method used here the $^{14}CO_2$ is driven out of solution by the addition of excess CO_2. The incubation mixture contained 0.8 μ moles tyrosine, of which 0.8 n moles were tyrosine-1-^{14}C. If all the substrate had been decarboxylated, 0.8 μ moles CO_2 could have been released. The addition of 0.5 ml saturated $NaHCO_3$ solution released 500 μ moles CO_2 in the presence of 615 μ moles TCA; "buzzing" ensured the complete liberation of CO_2.

The within batch and between batch reproducibility are outwith acceptable limits (Tonks (1968)) (see section 2.2.6.). Hamfelt (1967) found that his method had a standard error of pairs, expressed as a percentage, over a range of concentrations, of 6.6-17.2%. The source of the lack of reproducibility has not been found.

Chabner and Livingston (1970) showed that alkaline phosphatase was able to dephosphorylate PLP present in deproteinised solution. The evidence presented in Table 3.2.17. shows that, when PLP is added to serum whose alkaline phosphatase activity is as high as 62 King Armstrong units/100 ml, there is no appreciable reduction in the level of PLP extracted after incubation for 4 hrs at 23°. Neither was there a consistent reduction in PLP levels when serum was exposed to light for 4 hrs. Hines, Love and Peart (1969) have

found that PLP level was unaffected by exposure of whole serum to light for 24 hrs.

3.2.4.2. Measurement of tryptophan metabolites in urine

The use of coarse resin (Dowex 50 (H^+) 20-50 mesh) for the separation of XA and KA gave a mean recovery of internal standards of 58% and 52% respectively. When these recoveries were being monitored the internal standards being used were 4.88 μ moles (XA) and 5.29 μ moles (KA) but the urine samples applied to the column contained less than 1.5 μ moles in the majority of cases. It was decided not to correct to 100% recovery of internal standard since the levels of XA and KA in the urines applied to the columns would probably have recoveries in excess of 75%.

The emission spectra of XA and KA have been investigated by Price, Kaihara and Howerton (1962) using 370 nm and 340 nm as excitation spectra of XA and KA solutions respectively, although the emission spectra of a solution containing both XA and KA were not studied. Satoh and Price (1958) have reported on interference by KA in alkaline conditions, but thought that it was of little significance in routine studies. The contribution made by a relatively wide range of wavelengths of the activating light (300-400 nm) has not been assessed.

3.3. Clinical Results

3.3.1. Normal Range

Sera were collected as described in 3.2.2.6. and were assayed within 14 days of collection in 3 batches. The relative cumulative frequency of the levels of PLP obtained for men and women are shown in Fig.3.3.1; the number, mean value, standard deviation and range for different age groups are given in Table 3.3.1. The Students 't' test (2 tailed) was used to ascertain whether the age and sex differences were statistically significant (see Table 3.3.2.).

Under the age of 40 years women have a statistically significant lower mean level of PLP in serum than the corresponding age group of men. When the women are further divided into those aged less than 30 and those aged 30-39 the difference in the mean levels of serum PLP in these two groups is not statistically significant. There was a statistically significant age related fall in the mean serum levels of PLP amongst men when age 30-39 was compared with those over 40. From the age of 40 to 60 (women) or 65(men) there is no statistically significant difference between the sexes with regard to serum levels of PLP.

On the basis of the results presented above the observed range of serum PLP for normal men was taken to be 5-18 ng PLP/ml serum and for normal women 4-16 ng PLP/ml serum and these values were taken as the limits of normal. Women with serum PLP levels of between 4 and 5 ng/ml were judged to be borderline cases.

3.3.2. Levels of PLP found in the sera of patients with a variety of gastrointestinal disorders

The sera of 38 patients with ulcerative colitis (UC), 75 with Regional Enteritis (RE) and 69 with Adult Coeliac Disease (ACD) have

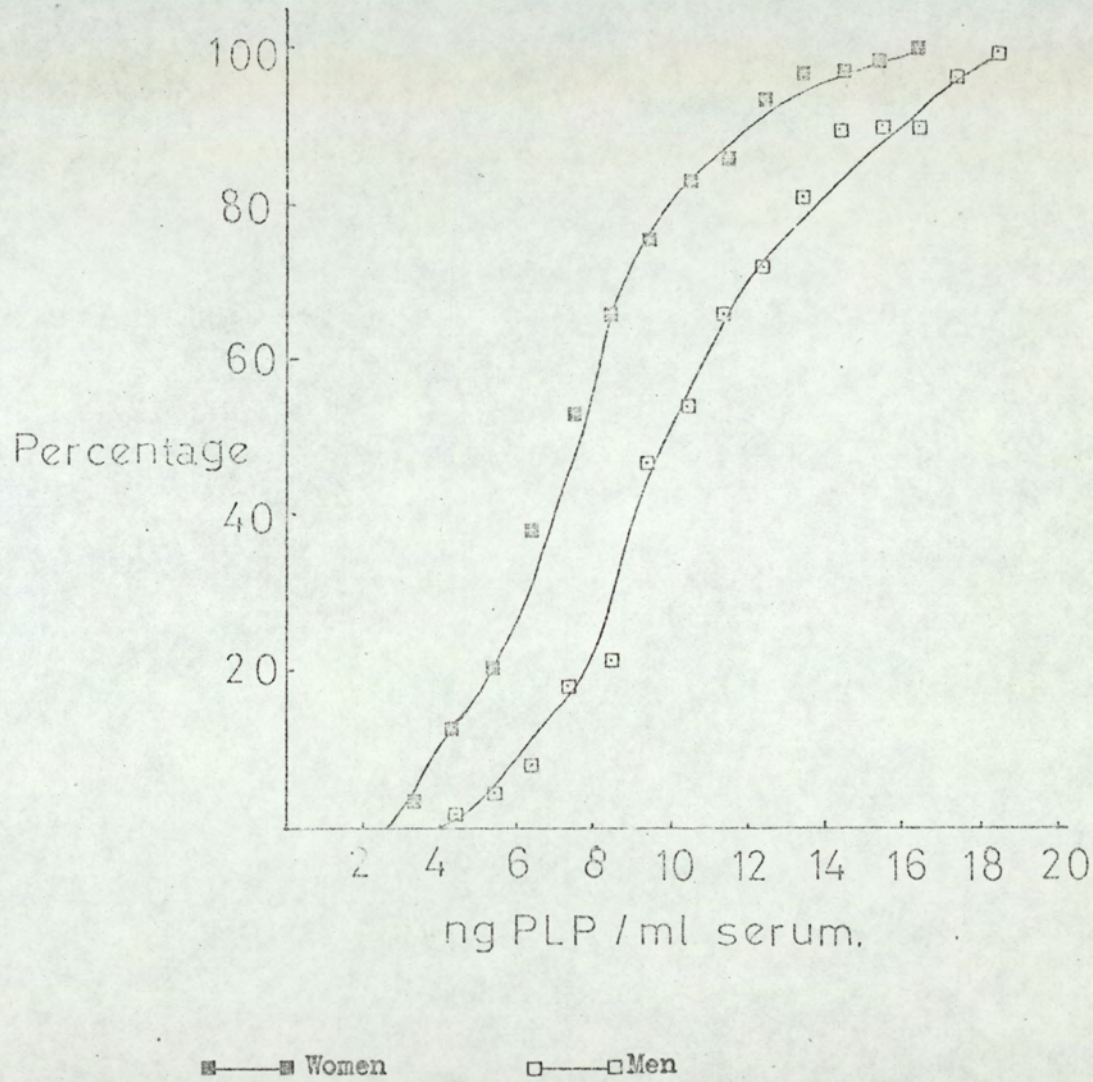


Fig. 3.3.1. The relative cumulative frequency of serum PLP (ng / ml) found in blood donor normals.

Table 3.3.1. Serum PLP levels found in a sample of blood donors.

Age group	ng PLP / ml serum (men)				ng PLP / ml serum (women)			
	n	$\bar{x}$	SD	Range	n	$\bar{x}$	SD	Range
18-29	31	11·0	3·26	5·8-18·2	27	8·5	3·35	4·1-16·1
30-39	15	12·1	2·73	7·4-17·9	18	6·8	1·89	3·4-12·6
18-39	46	11·4	3·11	5·8-18·2	45	7·8	2·95	3·4-16·1
Over 40	18	9·7	3·55	4·5-17·0	17	9·1	2·71	5·4-13·9
All	64	10·9	3·29	4·5-18·2	62	8·2	2·92	3·4-16·1

Table 3.3.2. Students 't' test on means obtained from a sample of blood donors.

Comparison	t	DF	Significance
M vs F 18-29 years	-2·84	56	$0·01 > p > 0·005$
M vs F 18-39 years	-5·51	89	$p < 0·001$
M vs F 30-39 years	-6·29	31	$p < 0·001$
M vs F 40 years	-0·55	33	NS
M vs F All	-4·86	124	$p < 0·001$
M 18-29 vs 30-39	1·87	43	NS
M 18-39 vs $\geq$ 40	1·56	60	NS
M 30-39 vs $\geq$ 40	2·83	33	$p = 0·01$
F 18-29 vs 30-39	-1·07	44	NS
F 18-39 vs $\geq$ 40	1·78	62	NS
F 30-39 vs $\geq$ 40	2·02	31	NS

been analysed for PLP content. The RE patients have been divided into a number of categories, namely those on ACTH, those with terminal ileal involvement and those described as having ileitis (diffuse involvement throughout the length of the small intestine). These groupings were not mutually exclusive. Table 3.3.3. gives the mean serum PLP levels for each sex for each of the categories described and also for the group as a whole. The Students 't' test has been used to compare these results with those of the normal population and also to compare categories. The results of these comparisons are given in Table 3.3.4. No subdivision into age groups was made and the comparisons with normal were made with the mean derived from all age groups of each sex. The relative cumulative frequency of the levels of PLP for RE men and women are shown in Fig.3.3.2. and Fig.3.3.3. together with those for the normals of each sex.

The difference in mean levels of PLP between the sexes has been abolished amongst the RE patients. The fall in serum PLP amongst men is statistically highly significant in all categories of men with RE but only the women with extensive disease in the small bowel have highly significantly lowered levels of PLP compared with the normal population, although there is a significant reduction of levels amongst women RE's as a whole. From the relative cumulative frequency graphs it can be seen that 27% of the men and 26% of the women have sub-normal serum levels of PLP. When women with borderline levels of PLP are included 41% of women with RE are found to be deficient.

The ACD patients have also been divided into categories; those on a gluten free diet, those not on a gluten free diet. Table 3.3.5. gives the means for each sex for each of the categories described and also for the group as a whole. Comparisons between each category and

Table 3.3.3. The levels of serum PLP found in patients with RE.

Category	ng PLP / ml serum.			
	n	$\bar{x}$	SD	Range
All men	26	6·9	3·39	2·2 - 13·7
All women	47	6·6	3·80	1·2 - 18·4
ACTH men	9	4·7	2·23	2·2 - 8·0
ACTH women	9	6·2	4·72	1·2 - 16·0
T-ileum + C men	10	6·9	3·29	2·2 - 12·4
T-ileum + C women	21	7·7	4·49	2·8 - 18·4
Ileitis men	14	7·1	3·59	2·6 - 13·7
Ileitis women	27	5·5	2·93	1·2 - 13·7

T-ileum + C = terminal ileal and colonic involvement.

Table 3.3.4. Students 't' test on the means obtained from RE patients.

Comparison	t	DF	Significance
N vs all men	5·19	88	$p = < 0{\cdot}001$
N vs all women	2·51	107	$0{\cdot}02 > p > 0{\cdot}01$
RE men vs RE women	0·33	71	not significant
N vs ACTH men	5·40	71	$p = < 0{\cdot}001$
N vs ACTH women	1·74	69	not significant
N vs T-ileum + C men	3·54	72	$p = < 0{\cdot}001$
N vs T-ileum + C women	0·54	81	not significant
N vs ileitis men	3·80	76	$p = < 0{\cdot}001$
N vs ileitis women	3·95	87	$p = < 0{\cdot}001$
T-ileum + C vs ACTH men	1·59	17	not significant
T-ileum + C vs ACTH women	0·82	28	not significant
T-ileum + C vs ileitis men	0·11	22	not significant
T-ileum + C vs ileitis women	2·02	46	$p = 0{\cdot}05$

T-ileum + C = terminal ileal and colonic involvement.

N = normal

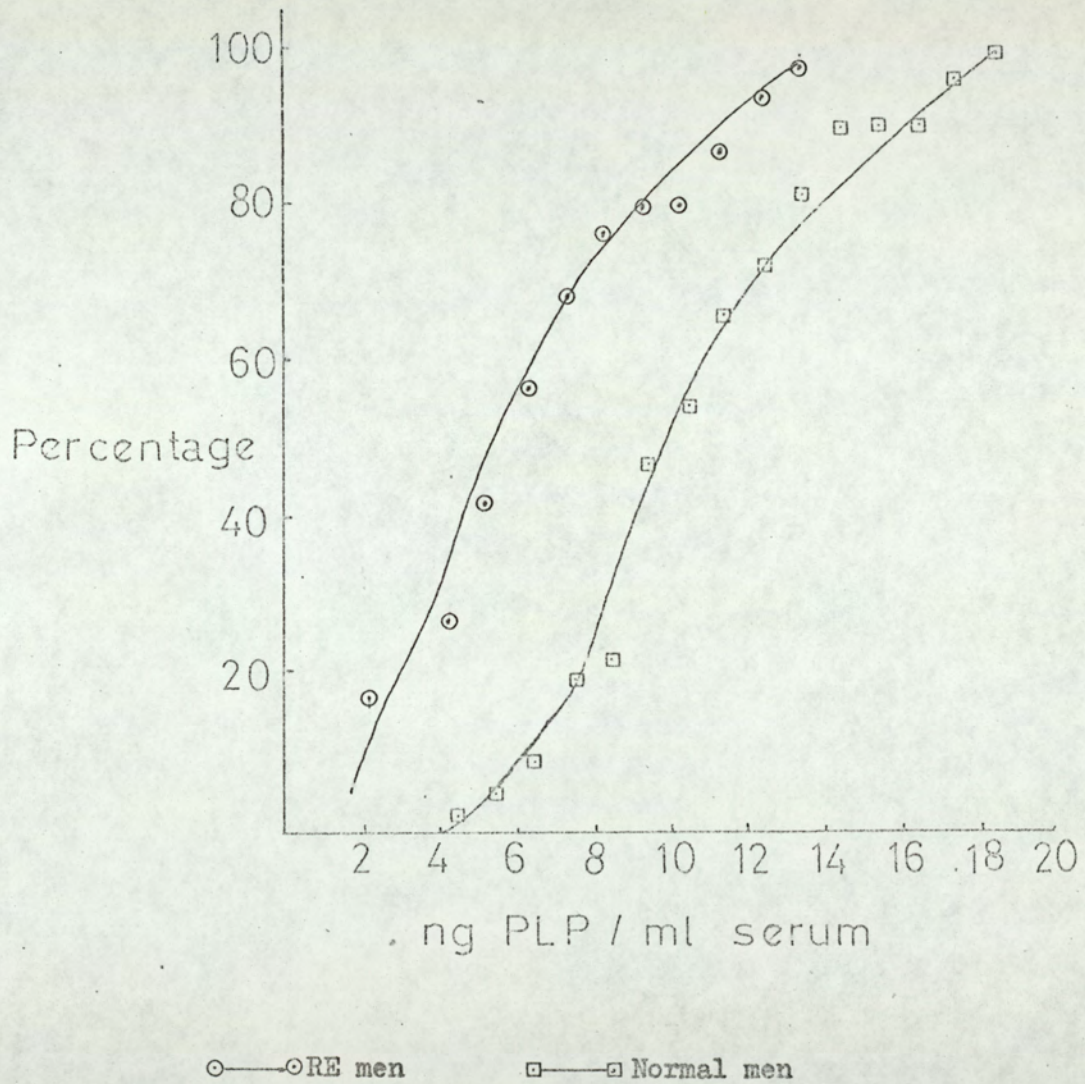


Fig. 3.3.2. The relative cumulative frequency of serum PLP (ng / ml) found in RE men and normal men.

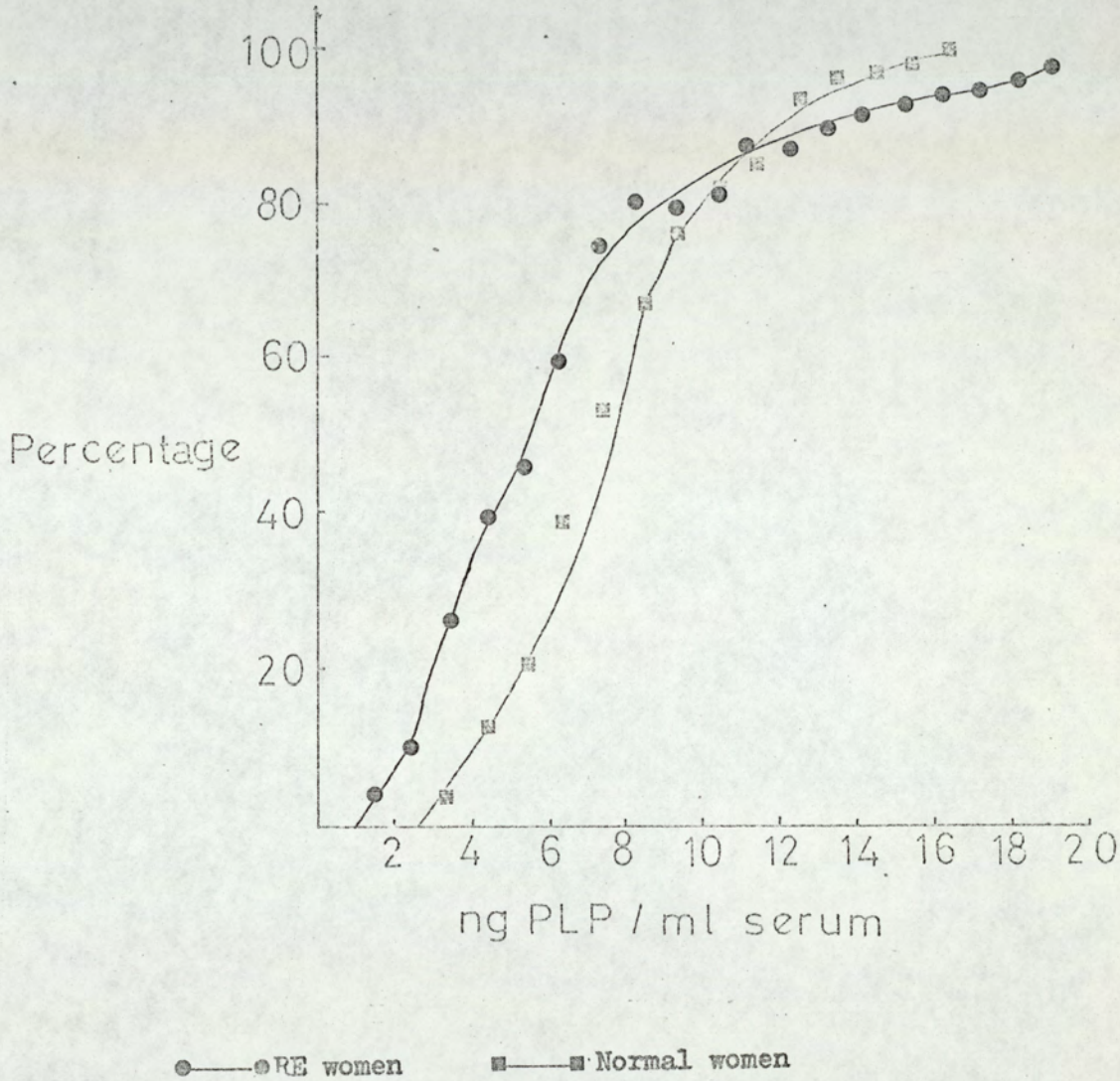


Fig 3.3.3. The relative cumulative frequency of serum PLP (ng / ml) found in RE women and normal women.

normals have been made using the Students 't' test and are given in Table 3.3.6. The relative cumulative frequency of the PLP levels found in the serum of men and women suffering from ACD is shown in Fig.3.3.4. and Fig.3.3.5. along with those for the normals of each sex.

The mean levels of serum PLP were significantly lowered for both men and women suffering from ACD. The statistically significant difference of the mean levels of PLP between the sexes was abolished in this disease state. Institution of a gluten free diet had the effect of significantly raising the mean serum levels of PLP. For the ACD women, the levels were raised to the extent that the mean serum PLP level was no longer significantly lower than that of the blood donor women. 35% of the men with ACD were found to have sub-normal levels of serum PLP, whilst 24% of the women had sub-normal levels and 39% had borderline or sub-normal levels.

The UC patients have not been divided into categories, apart from sexes. The mean levels for each sex are given in Table 3.3.7. and a comparison of the means with those of normals using Students 't' test is given in Table 3.3.8. Fig.3.3.6. shows the relative cumulative frequency of serum PLP levels obtained from women suffering from UC together with that of normal women. There was a statistically significant lowering of the serum level of PLP in men with UC when compared with normals but the mean serum level of PLP in women with UC was no different from that of the normal population of women. However, 17% of women with UC and 20% of men had sub-normal levels of PLP in their serum. When borderline levels are included 26% of women with UC could be judged to be deficient.

3.3.3. The absorption of an oral dose of pyridoxine hydrochloride

The subjects were fasted over night prior to an oral dose of

Table 3.3.5. The levels of PLP found in patients with ACD.

Category	ng PLP / ml serum			
	n	$\bar{x}$	SD	Range
All men	23	7·4	3·70	2·7 - 14·7
All women	46	6·0	2·81	0·7 - 12·3
Gluten free diet (men)	13	9:0	3·48	3·6 - 14·7
Gluten free diet (women)	20	7·3	2·74	1·0 - 12·3
Normal diet (men)	10	5·4	3·04	2·7 - 13·0
Normal diet (women)	26	5·4	2·47	0·7 - 11·2

Table 3.3.6. Students 't' test on means obtained from ACD patients.

Comparison	t	DF	Significance
Normals vs all men	4·18	85	p = < 0·001
Normals vs all women	3·77	106	p = < 0·001
All men vs all women	1·66	67	not significant
Normals vs GFD men	1·90	75	p = 0·005
Normals vs GFD women	1·21	80	not significant
Normals vs no GFD men	4·92	72	p = < 0·001
Normals vs no GFD women	4·27	86	p = < 0·001
GFD vs no GFD men	2·46	21	p = 0·025
GFD vs no GFD women	2·42	44	p = 0·02

GFD = gluten free diet.

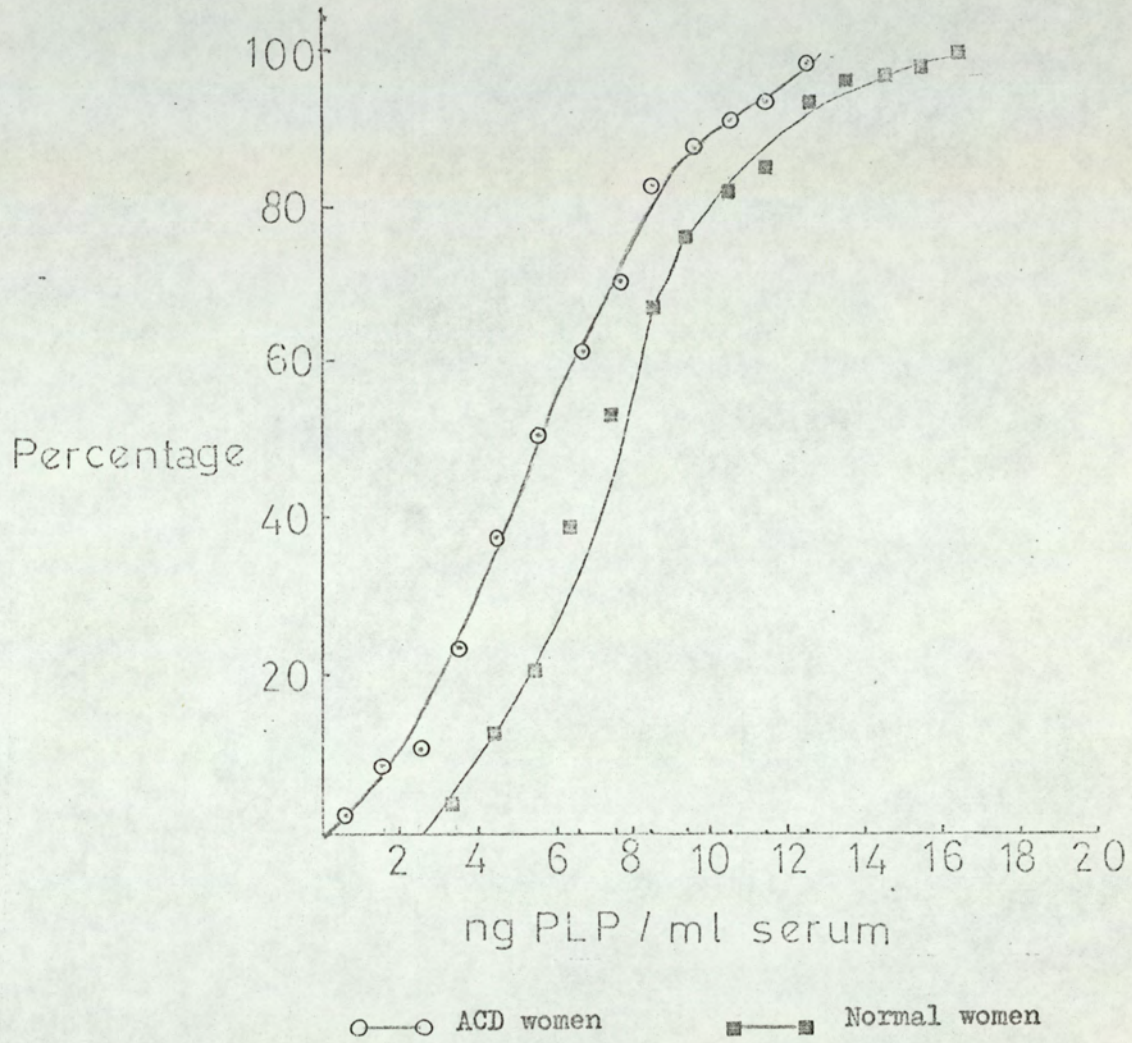


Fig. 3.3.4. The relative cumulative frequency of serum PLP (ng / ml) found in ACD and normal women.

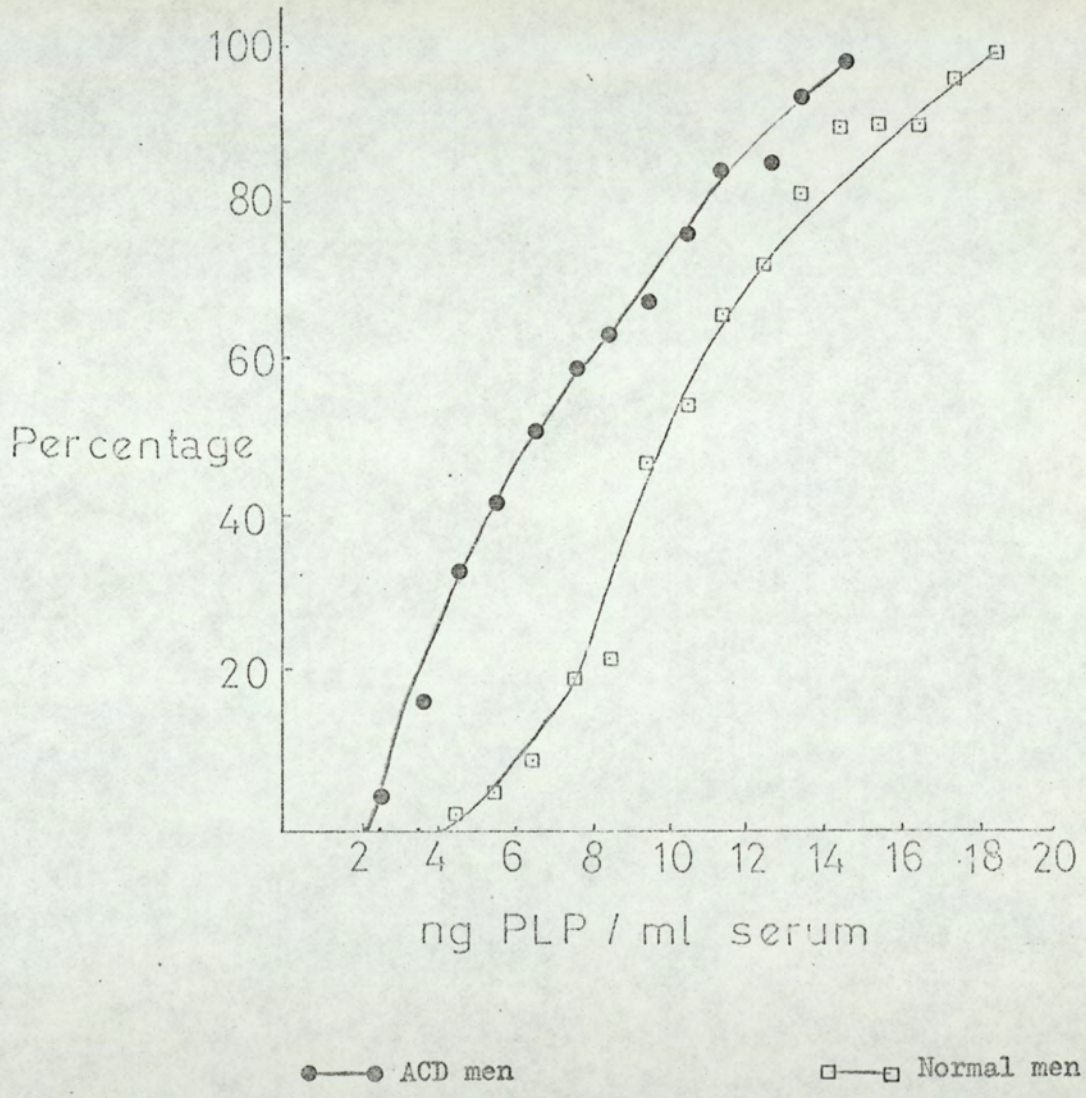


Fig. 3.3.5. The relative cumulative frequency of serum PLP (ng / ml) found in ACD men and normal men.

Table 3.3.7. The levels of serum PLP found in patients with UC.

Category	ng PLP / ml serum			
	n	$\bar{x}$	SD	Range
All men	15	7·9	3·67	3·2 - 16·4
All women	23	8·0	4·15	2·2 - 18·8

Table 3.3.8. Students 't' test on means obtained from UC patients.

Comparison	t	DF	Significance
Normals vs all men	8·08	77	$p < 0·001$
Normals vs all women	0·22	83	NS

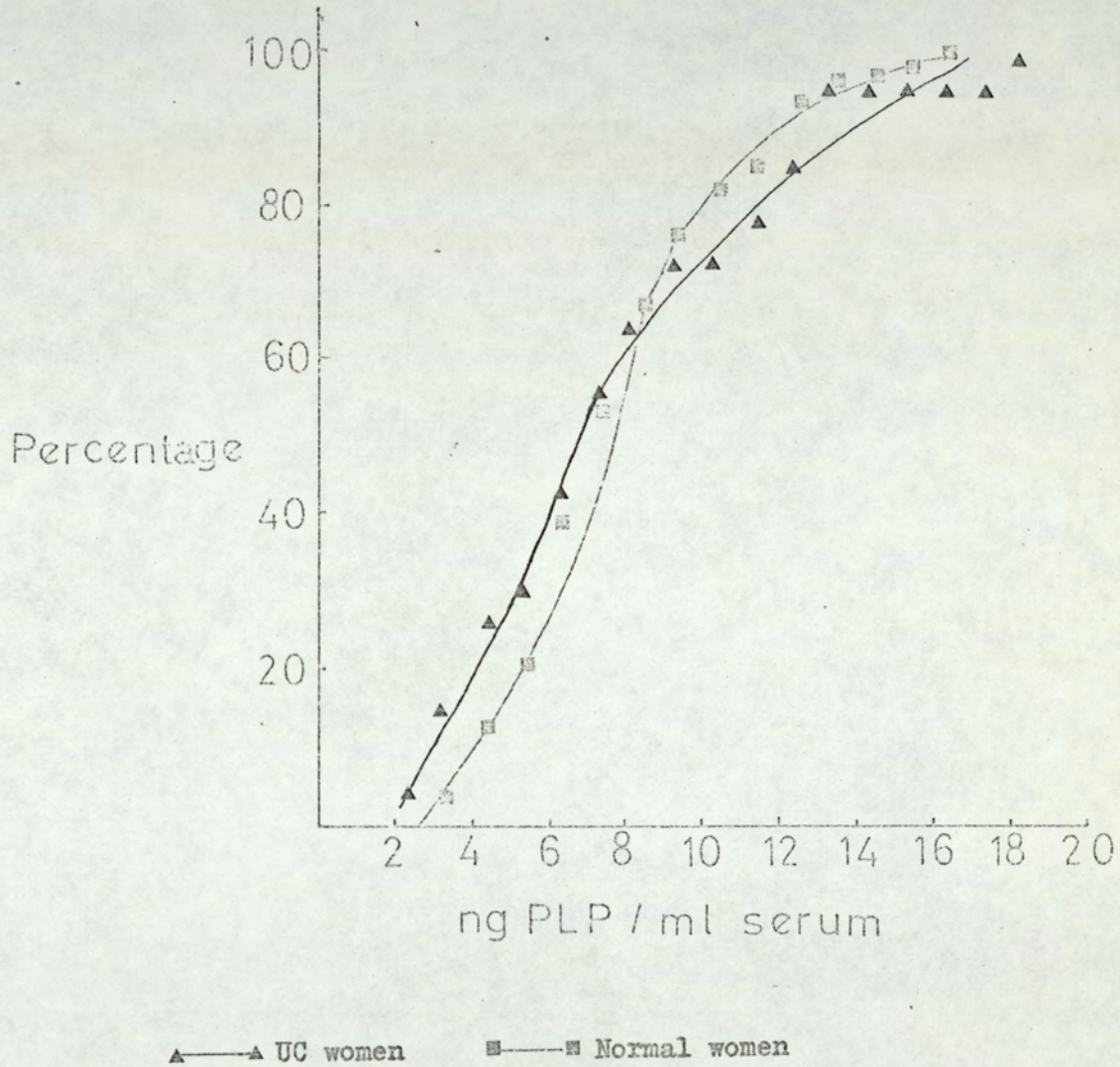


Fig. 3.3.6. The relative cumulative frequency of serum PLP (ng / ml) found in UC women and in normal women.

50 mg pyridoxine hydrochloride. Blood samples were taken by venepuncture before dosing and 15,30,60 and 120 mins after dosing. The blood was placed in a plain tube and clotting took place in a dark cupboard before the serum was separated and stored at -20° until analysed. A second dilution of the extract with NaAc (3 g%) was necessary for all specimens taken after the oral dose. The absorption curves obtained from 6 healthy volunteers and 3 RE patients are shown in Table 3.3.9. and Fig.3.3.7.

The change in serum levels of PLP over the 2 hr period of the test range from 23-51 ng PLP/ml for normals and 21-45 ng PLP/ml for the RE patients. The average increase in serum PLP in 30 mins after dosing was found to be 11.2 ng/ml for normals and 11.3 ng/ml for the RE patients. Thus there was found to be no impairment of absorption of pyridoxine hydrochloride and conversion to PLP in the 3 patients suffering from RE.

3.3.4. Tryptophan load test

The procedure used was that described in section 3.2.2.6. which can be summarised as follows:-

(1) Basal, no tryptophan, no vitamin B_6

(2) 2 g L-tryptophan, no vitamin B_6

(3) no tryptophan, 50 mg pyridoxine hydrochloride

(4) 2 g L-tryptophan, no vitamin B_6

Patients 1-5 were given pyridoxine hydrochloride intramuscularly, whilst patients 6-10 and the normals were given it orally. The results are given in Table 3.3.10 (normals) and Table 3.3.11. (ACD patients). A variety of statistical analyses have been performed on these results. The mean plasma and red blood cell (RBC) PLP levels have been calculated for the ACD and normals, for days 1 and 2 of the test, also the mean ratio of ng PLP/ml packed RBC to ng PLP/ml

Table 3.3.9. The change in serum PLP levels at varying times after an oral dose of 50 mg pyridoxine hydrochloride.

Subject	ng PLP / ml serum					Change ng PLP / ml	
	0 min	15min	30 min	60 min	120 min	0 - 30 min	0 - 2h
N 1 M	11•6	16•7	21•8	25•3	34•7	10•2	23•1
N 2 F	9•6	13•2	19•5	45•6	60•8	9•9	51•2
N 3 F	13•9	17•7	23•0	40•2	45•7	9•1	31•8
N 4 M	13•9	14•5	22•5	36•7	43•0	8•4	29•1
N 5 M	11•9	12•7	27•1 *	41•4	65•8	15•2*	43•9
N 6 M	16•5	16•5	31•0	37•5	44•0	14•5	29•5
RE 1 F	2•1	8•8	18•0	33•2	47•3	15•9	45•1
RE 2 F	1•8	7•0	9•2	20•8	29•0	7•4	27•2
RE 3 F	1•0	8•0	11•5	13•8	21•8	10•5	20•8

* blood collected 45 min after oral dose

N = normal M/F = male /female

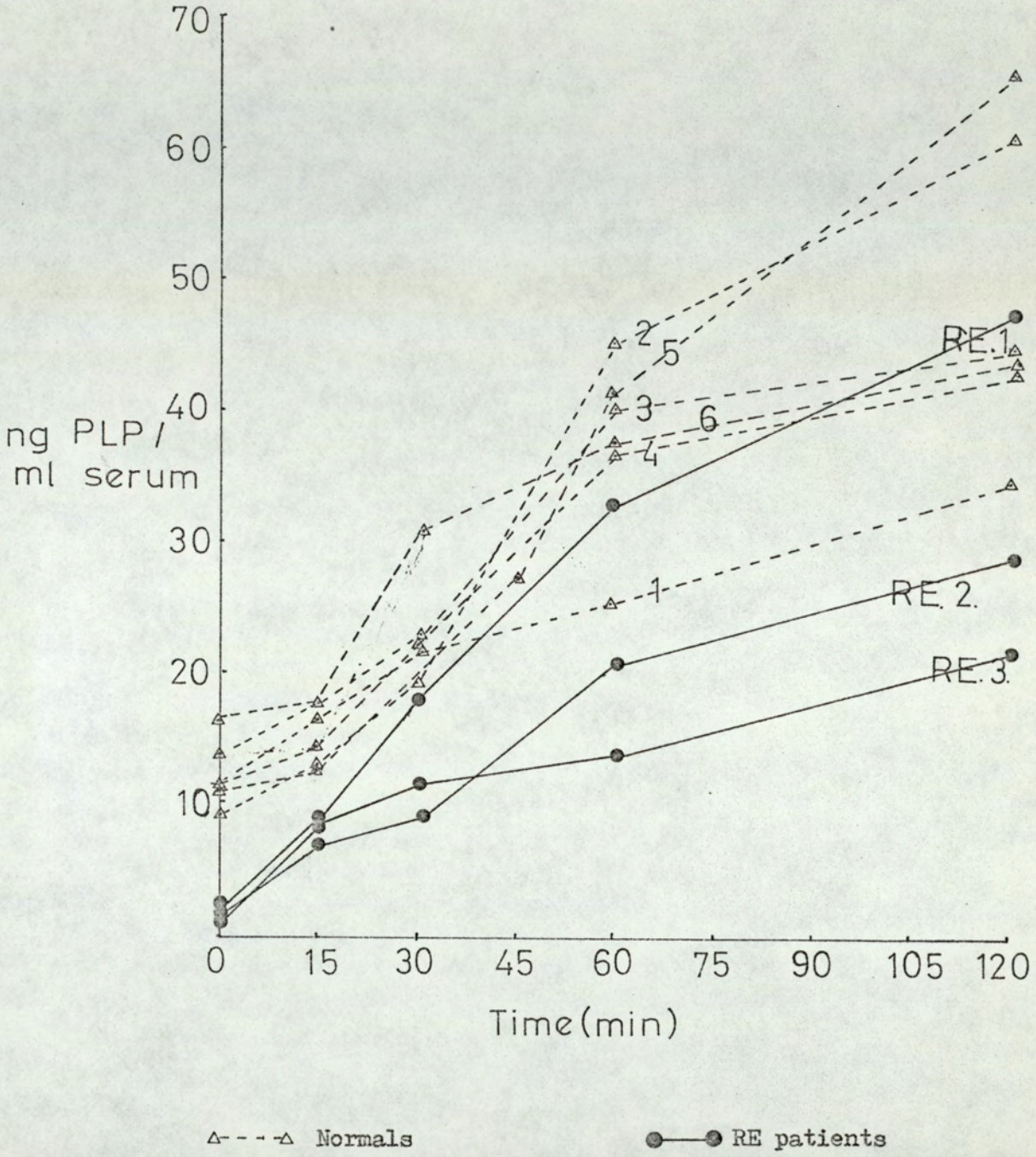


Fig. 3.3.7. The absorption of an oral dose of 50 mg pyridoxine hydrochloride.

Table 3.3.10 Results of Tryptophan load tests on normal volunteers

No	Subject	Volume		Creat	XA	KA	Kyn	5-HIAA	IAA	SPLP	HPLP	Ratio
1	A.B. (M)	1	2240	2755	45.6	31.8	13.1	11.8	12.6	25.5		
		2	1380	828	27.0	30.6	17.9	3.0	6.3	29.9		
		3	2480	3274	59.4	33.0	13.1	9.8	16.0	146.1		
		4	1500	1220	43.0	59.0	25.6	5.5	8.7	121.2		
		2+1	2020	1454	25.6	21.0	12.2	13.6	8.4			
		2+2	1940	1921	52.0	26.0	13.1	8.0	8.9			
		4+1	1480	1954	48.2	25.6	17.9	8.0	8.6			
	Recovery %				91	101	96					
2	R.H. (M)	1	1740	2088	20.8	31.2	33.6	6.3	11.6	8.5	20.8	2.34
		2	3560	1068	21.0	116.0	48.0	1.1	14.8	7.1	16.2	2.28
		3	1540	1617	23.6	18.2	36.0	4.2	12.8	20.9	65.2	3.23
		4	1880	4116	21.8	50.6	33.6	4.2	10.2	34.1	37.4	1.09
	Recovery %				96	94	61					
3	H.C. (F)	1	1620	1118	22.0	11.6	40.9	4.1	5.1	7.5	7.9	1.05
		2	1820	1256	51.4	86.6	84.2	7.4	6.8	7.8	11.2	1.44
		3	1480	1332	22.0	15.8	40.9	6.0	6.8	44.5	35.3	0.79
		4	1960	1235	34.6	75.0	57.7	8.0	11.8	29.6	20.8	0.95
	Recovery %				86	95	77					
4	C.P. (F)	1	750	1125	19.4	17.5	36.0	2.5	9.7	2.5	11.6	4.64
		2	920	1159	85.0	102.8	219.3	6.8	13.1	3.6	20.3	5.63
		3	860	1342	23.4	11.6	45.6	5.0	7.9	53.8	38.2	0.71
		4	1240	1190	39.0	68.4	52.8	5.4	14.4	43.3	23.2	0.54
	Recovery %				100	115	92					

Table 3.3.11. Results of Tryptophan load tests on ACD patients

No	Subject	Volume		Creat	XA	KA	Kyn	5-HIAA	IAA	SPLP	HPLP	Ratio
1	M.C. (F)	1	1225	625	20.5	15.8	12.2	2.2	7.2	4.8	14.9	3.51
		2	975	234	10.4	6.6	9.6	5.2	4.1	3.9	7.1	1.82
		3	1800	702	25.8	31.7	24.0	9.2	7.5	44.5	260.2	5.54
		4	2150	645	29.3	21.1	45.7	6.5	14.3	25.6	39.8	1.55
	Recovery %				56	67	75					
2	C.G. (F)	1	-	-	19.6	16.9	-	3.5	3.0	4.2	12.2	2.91
		2	-	-	14.6	7.4	-	2.2	2.6	4.7	15.8	3.36
		3	-	-	-	-	-	-	-	72.0	106.2	1.47
		4	-	-	22.4	14.3	-	7.6	2.4	34.6	25.7	0.74
	Recovery %				60	43	-					
3	V.R. (F)	1	2225	333	11.2	68.5	19.5	1.3	4.6	5.1	14.9	2.92
		2	1425	427	44.0	55.0	38.3	1.3	7.7	4.1	5.8	1.41
		3	1525	457	9.7	18.5	7.9	2.7	3.2	45.2	145.7	3.22
		4	1375	206	24.5	55.0	111.6	3.9	11.4	29.5	25.3	0.86
	Recovery %				48	54	56					
4	J.A. (M)	1	1150	380	2.4	14.7	24.0	20.7	18.2	3.6	7.9	2.19
		2	320	422	20.5	22.7	246.7	4.5	6.9	3.2	19.5	6.09
		3	1680	1008	20.5	10.6	35.3	12.8	44.1	9.4	65.1	6.92
		4	2020	788	21.9	18.5	124.7	23.1	60.6	7.6	21.6	2.84
	Recovery %				55	57	55					
5	D.B. (F)	1	-	-	16.6	4.2	-	4.3	7.7	2.7	19.9	7.37
		2	-	-	42.6	27.4	-	1.5	9.9	4.5	10.0	2.22
		3	-	-	-	-	-	-	-	68.1	153.6	2.25
		4	-	-	20.4	12.7	-	0.5	3.0	27.6	29.0	1.05
	Recovery %				65	55						

contd .....

Table 3.3.11.(contd) Results of Tryptophan load tests on ACD patients

No	Subject	Volume	Creat	XA	KA	Kyn	5-HIAA	IAA	SPLP	HPLP	Ratio
6	P.M. (M)	1 1380	1201	15.8	14.2	40.9	8.3	8.3	3.3	0.0	-
		2 2220	1465	132.0	63.0	120.2	7.3	8.3	8.0	0.0	-
		3 1620	1458	22.0	17.5	64.9	12.4	7.8	23.5	50.0	2.30
		4 2300	1794	66.0	66.6	74.3	4.1	10.1	38.5	57.5	1.49
	Recovery %			85	91	75					
7	D.M. (M)	1 1450	1170	17.6	12.4	24.1	5.4	8.6	6.9	12.5	1.82
		2 1050	1260	24.2	36.2	43.3	2.8	9.2	5.4	15.0	2.78
	Recovery %			99	92	79					
8	G.B. (F)	1 1580	760	17.8	15.2	16.8	5.7	6.6	11.0	12.5	1.13
		2 1400	760	30.8	25.0	21.6	4.1	9.3	9.6	8.5	0.89
	No B6	3 1660	1050	22.4	13.2	19.3	0.7	10.4	7.4	5.5	0.74
	+ B6	4 1000	1050	27.2	48.2	28.9	5.5	14.6	62.7	20.0	0.318
	Recovery %			71	107	84					
9	N.R. (F)	1 2670	800	10.2	11.8	37.5	6.7	4.4	5.4	22.0	4.07
		2 2720	810	30.4	67.4	93.9	8.0	7.9	0.6	9.1	15.20
		3 2390	720	18.4	12.2	69.9	25.6	3.4	33.8	123.7	3.65
		4 2360	850	33.6	49.2	102.3	29.2	6.9	26.7	110.4	4.14
	Recovery %			96	113	100					
10	J.C. (F)	1 2300	897	14.3	10.3	20.2	0.3	5.2	5.9	29.5	4.99
		2 2710	732	29.2	74.9	48.0	42.9	8.3	1.2	13.3	10.90
		3 1170	421	10.7	28.0	4.8	1.6	3.6	37.4	80.1	2.69
		4 2010	904	39.9	65.7	20.2	2.8	15.4	22.9	65.1	2.84
	Recovery %			97	106	76					

plasma. These results are presented in Table 3.3.12. Table 3.3.13 shows the results of a Students 't' test which compared the means obtained for day 1 and 2 within each group and also the means of normals and ACD's on day 1 and 2. Table 3.3.14. shows the correlation coefficient obtained when plasma PLP levels of the ACD patients were plotted against the 24-hour excretion of XA, KA and Kyn for days 1 and 2. The statistical significance of these correlation coefficients is also shown. The mean urinary excretions of XA, KA, Kyn, 5-HIAA and IAA for each 24-hour period of the test by the ACD patients are shown in Table 3.3.15.

The four normals were members of the laboratory staff. Normal 1 had been subject to tryptophan load tests held in another hospital on previous occasions and was a healthy control. Additional urine saves were made after day 2 and day 4 to check that the excretion of the metabolites measured was complete within 24 hrs of loading. For each of XA, KA and Kyn the level of excretion after loading with 2 g L-tyrptophan was very much reduced after supplementation with oral pyridoxine hydrochloride for normals 2-4. Normal 1 had reduced creatinine excretion on day 2 compared with the other days which suggests that the urine collection on that day may have been incomplete. The plasma levels of PLP for this subject are high and the relative ineffectiveness of the oral dose of pyridoxine hydrochloride in altering the level of excretion of the tryptophan metabolites points to some mechanism, other than PLP levels, which is controlling their excretion in this subject.

Normal 4 had plasma levels of PLP lower than those considered to be normal. There was a large rise in the levels of excreted XA, KA and Kyn following the 2 g L-tryptophan load, which was very considerably reduced after supplementation with pyridoxine hydrochloride. Normal 3 had been taking an oestrogen containing oral contraceptive

Table 3.3.12. Mean levels of blood PLP for normals and ACD patients on days 1 & 2 of the tryptophan load test.

Group	Day 1			Day 2		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD
Normals						
Plasma PLP ng/ml	4	11·0	10·02	4	12·1	12·01
ng PLP / ml RBC	3	13·4	6·64	3	15·9	4·56
Ratio	3	2·68	1·82	3	3·11	2·22
ACDs						
Plasma PLP ng / ml	10	5·3	2·35	10	4·5	2·74
ng PLP / ml RBC	10	14·6	8·01	9	11·6	6·57
Ratio	9	3·10	1·84	9	4·96	4·94

$$\text{Ratio} = \frac{\text{ng PLP / ml packed RBC}}{\text{ng PLP / ml serum}}$$

Table 3.3.13. Comparisons of blood PLP obtained on the first two days of the tryptophan load tests.

Comparison	t	DF	Significance
ACD D1 vs D2 plasma	0·64	18	NS
ACD D1 vs D2 RBC	0·94	17	NS
ACD D1 vs D2 Ratio	1·00	17	NS
Normals D1 vs D2 plasma	0·12	6	NS
Normals D1 vs D2 RBC	0·44	4	NS
Normals D1 vs D2 Ratio	0·21	4	NS
ACD vs Normals D1 plasma	-1·56	12	NS
ACD vs Normals D2 plasma	-1·74	12	NS
ACD vs Normals D1 RBC	+0·22	11	NS
ACD vs Normals D2 RBC	-1·30	10	NS
ACD vs Normals D1 Ratio	0·31	10	NS
ACD vs Normals D2 Ratio	0·57	10	NS

NS = not significant

Table 3.3.14. The correlation coefficients obtained for PLP (plasma) vs XA, KA, Kyn on days 1 & 2 of the tryptophan load tests on ACD patients.

Compound	Day 1				Day 2			
	r	DF	t	Sig	r	DF	t	Sig
XA	0·240	8	0·700	NS	0·433	8	1·36	NS
KA	0·029	8	0·082	NS	0·312	8	0·93	NS
Kyn	-0·417	6	1·12	NS	0·176	6	0·44	NS

NS = not significant.

Table 3.3.15. Mean levels of metabolites excreted during the tryptophan load test by ACD patients.

Compound	Day	n	$\bar{x}$	SD
XA	1	10	14·6	5·4
	2	10	37·9	34·8
	3	6	17·9	6·4
	4	9	31·7	14·3
KA	1	10	18·4	18·0
	2	10	38·6	24·9
	3	6	19·8	8·5
	4	9	39·0	22·3
Kyn	1	8	24·4	9·9
	2	8	77·7	77·5
	3	6	34·5	27·9
	4	7	72·5	41·8
5HIAA	1	10	5·9	5·8
	2	10	8·0	12·5
	3	7	9·3	8·8
	4	9	9·2	9·9
IAA	1	10	7·4	4·2
	2	10	7·4	2·4
	3	7	11·4	14·7
	4	9	15·4	17·6

Levels of XA KA & Kyn expressed in μ moles / 24 h, those of 5HIAA & IAA in mg / 24 h.

for three years up till 9 months prior to the loading test. In the light of these facts it was decided to use the results published by Leklem (1971) for the normal response to a load of 2 g L-tryptophan. (see Table 3.3.16.).

The urines of patients 1-5 were chromatographed on Dowex 50 (H^+) 20-50 mesh, those of patients 6-10 and the normals were chromatographed on the 200-400 mesh resin. Recoveries of XA, KA and Kyn are included for each subject. Amongst the ACD patients tested, only patients 7 and 8 could be considered to have normal levels of plasma PLP, all the others had less than 6.0 ng PLP/ml plasma and patients 4, 5 and 6 had levels of less than 4 ng/ml. Administration of pyridoxine hydrochloride to patients prior to a second load of 2 g L-tryptophan was effective in lowering the level of excreted XA in patients 3, 5, 6 and 8; KA in patients 4, 5, 9 and 10 and Kyn in patients 4, 6 and 10. Thus in none of these patients was the administration of pyridoxine hydrochloride effective in reducing the excretion of all these metabolites relative to the pre-supplementation levels. Abnormalities of excretion of 5-HIAA were seen in patients 4, 9 and 10 and no patient showed any abnormal excretion of IAA (normal range in man given as 5-18 mg/24 hrs by Weissbach *et al* (1959)).

Table 3.3.16. Normal values for the excretion of metabolites of tryptophan in healthy subjects (Leklem (1971)) using the methods of Price et al (1965).

Condition	μ moles / 24 h excretion					
	XA		KA		Kyn	
	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Basal (day 1)	15	9 - 36	23	10 - 62	14	4 - 28
Post 2 g load (day 2)	34	28 - 41	56	40 - 67	36	22 - 67

3.3.5. Discussion

Storvick and Peters (1964) have suggested that the major problem encountered in the measurement of vitamin B_6 content of blood, or other biological tissue, is the efficient extraction of the vitamin from any binding protein. Benson, Peters, Edwards and Storvick (1964) found a maximal release of vitamin B_6 after autoclaving blood for 10-24 hrs in the presence of 0.055N HCl. It is thus likely that the levels of vitamin B_6 reported here and in those series referred to subsequently reflect the levels of vitamin B_6 present in a free form in serum. The relatively mild hydrolysis procedures used here are unable to disrupt protein structure to the extent that intra-molecular PLP will be released. The method employed by Baker, Frank, Ning, Gellene, Hutner and Carroll (1966) which used the protozoa Tetra-hymena pyriformis as an assay organism, involved two autoclavings for 30 mins at pH 4.5 with an intervening incubation in an enzyme mixture. The authors found that, using this hydrolysis procedure, serum vitamin B_6 levels ranged 30-80 ng/ml with a mean of 37 $\pm$ 6 ng/ml.

When tyrosine decarboxylase apo-enzyme has been used in other series the normal ranges have been found to be:-

(a) 9.6 $\pm$ 2.8 ng PLP/ml (Wachstein, Kellner and Ortiz (1960))

(b) 9.9 $\pm$ 3.27 ng PLP/ml (Hamfelt (1962))

(c) men 20-34 yrs 18.5 $\pm$ 5.5 ng PLP/ml, men 35-49 yrs 15.8 $\pm$ 3.3 ng PLP/ml, women 20-34 yrs 16.8 $\pm$ 3.6 ng PLP/ml, women 35-49 yrs 11.4 $\pm$ 3.4 ng PLP/ml (Chabner and Livingston (1970)).

Other enzyme systems have been used to measure the concentration of PLP in serum. Hines, Love and Peart (1969) using skeletal muscle

apo-phosphorylase b extracted from rabbit, found that hospital control patients had a mean serum PLP level of 34.9 ng/ml (± 3.20 standard error of mean). Apo-tryptophanase extracted from E. coli (Wada et al (1957)) produced an average level of 23 ng PLP/ml human serum. A chemical method, measuring the fluorescence of cyanohydrin and lactone derivatives of the various forms of vitamin B_6 present in whole blood was used by Contractor and Shane (1968) and they found the level of PLP in whole blood to be in the range 8-17 ng/ml.

A micro-biological method, using Saccharomyces carlsbergensis (Baysal (1965)), which probably measured the free and phosphorylated derivatives of vitamin B_6, gave an average of 11.3 ng/ml whole blood. Anderson et al (1970), using Lactobacillus casei found the following concentrations of pyridoxal in serum (ng/ml)

Age	Men	Women
20 - 29	9.1	6.5
30 - 39	6.4	5.3
Over 60	3.9	3.5

There were highly significant differences between all the age groups of men and a significant difference between women over 40 and those over 60. The difference in levels of men and women in the 20-29 age group was also highly significant.

The results presented in 3.3.2. show that there is a significant difference in the levels of serum PLP when men are compared with women below the age of 40. There was no difference between the sexes in the over 40 age group. When the age groups of each sex were compared, there was a decrease in the PLP levels amongst men which was statistically significant in the case of the 30-39 vs over 40 age group. However, when these two age groups (30-39 vs over 40) for women were compared there was a non statistically

significant increase in the level of PLP. The significance of the lower levels of PLP amongst women is difficult to ascertain.

It was not possible to find out which of the women blood donors were taking oestrogen containing oral contraceptives. Hamfelt and Hahn (1969) found that there was a negative correlation between plasma PLP and xanthurenic acid excretion during pregnancy. The authors felt that the high excretion of XA was due to the combined influence of PLP deficiency and hormonal derangement. When a combination of oestrogen and gestogen was given to male subjects, Wolf, Price, Brown and Madsen (1970) found an increase in urinary metabolites of tryptophan after a loading dose. These levels were normalised by supplementation with vitamin B_6. Thus an increase in the levels of circulating oestrogens has been found to influence the requirement for and the serum levels of vitamin B_6. The increase in requirement is reflected by an increase in the spontaneous excretion of 3-hydroxyanthranilic acid (Price, Rose and Toseland (1972)), and other tryptophan metabolites after an oral dose (Rose (1966), Heilmann, Knap, Stolp and Sudhodolitz (1968), Aly, Donald and Simpson (1971), Rose et al (1972)). These increases in excreted metabolites of tryptophan are reduced or reversed by supplementing the diet with pyridoxine hydrochloride.

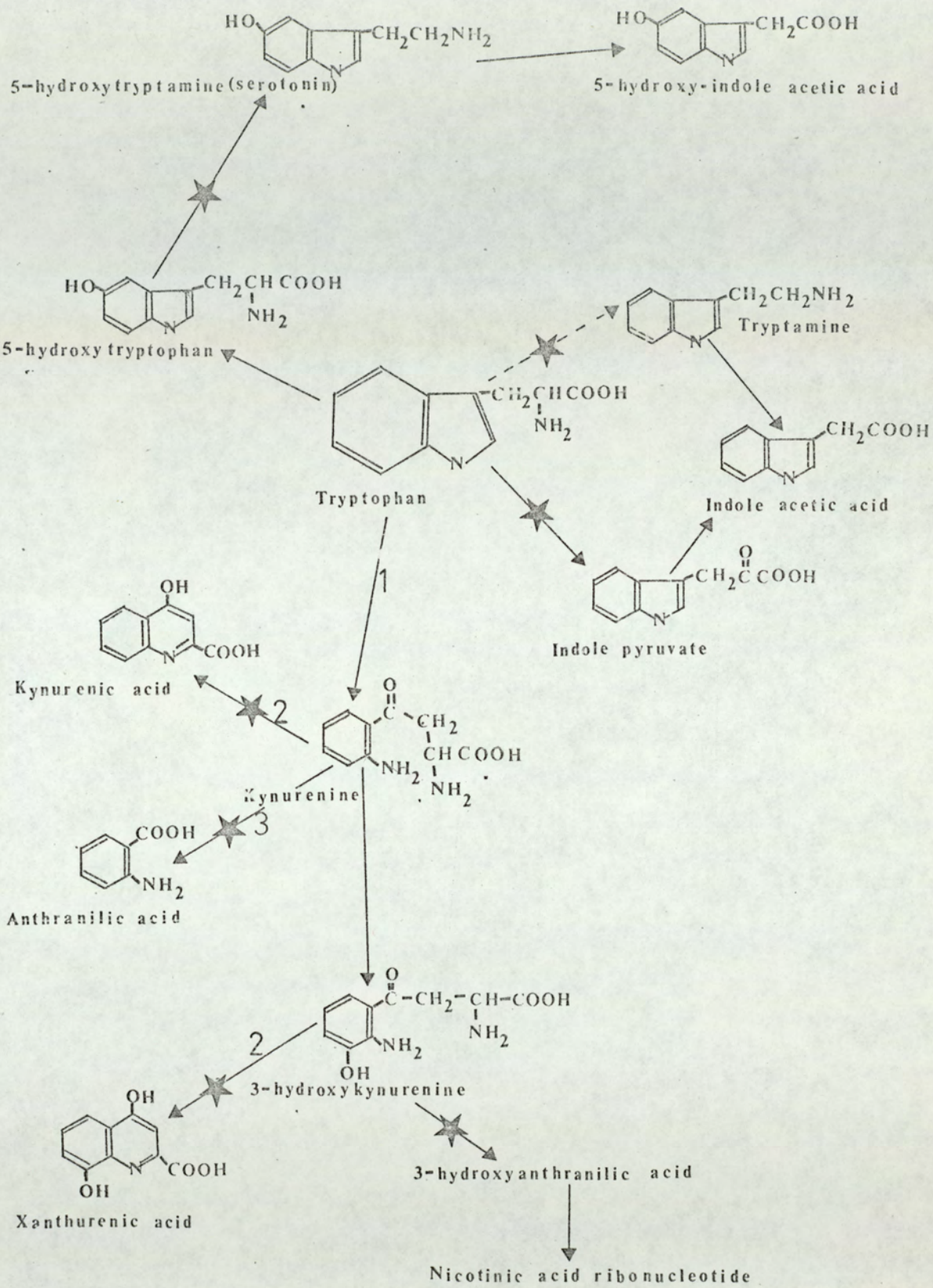
Depressive symptoms associated with the taking of oral contraceptives have been investigated recently by Herzberg, Johnson and Brown (1970) and they found that, of the group of women taking oral contraceptives, 6.6% were more severely depressed than the control group. Supplementation of the daily intake of vitamin B_6 by an oral dose of pyridoxine hydrochloride (50 mg) has been used to treat depression associated with oral contraceptives (Winston (1969)). This dose was reduced to a daily supplement of 25 mg after 1 month. Further investigations showed that supplementing with 50 mg pyridoxine for 2-5 days before menstruation relieved the premenstrual tension

experienced by 44 out of 58 women taking oral contraceptives. Curzon (1969) has suggested that the depression is caused by low brain 5-hydroxytryptamine levels which result from elevated plasma corticosteroids which, in turn, induce a high activity of tryptophan oxygenase (see Fig.3.3.8.). Thus, less tryptophan is available for the synthesis of 5-hydroxytryptamine. Coppin, Shaw, Herzberg and Maggs (1967) have found a therapeutic response by depressed patients to large oral doses of L-tryptophan. There is some evidence that the decarboxylation step may be the rate limiting step in the synthesis of 5-hydroxytryptamine in humans (Robins, Robins, Croninger, Moses, Spencer and Hudgens (1967)). In a recent review, Hodges (1971) found that there was an increased pysiological need for pyridoxine and that preliminary evidence tended to verify the relationship between this abnormality and certain side effects accompanying the use of oral contraceptives.

The statistically significant differences between the sexes was abolished when the serum PLP levels of patient suffering from UC, ACD or RE were analysed. When each condition is considered as a whole, the mean levels of serum PLP for men were significantly lower than that of the controls but, amongst the women patients, only the RE women had a significantly lowered mean serum PLP level. The chronic inflammatory disorder RE had a high incidence of patients with serum PLP in the deficient range (26% for each sex). Those with terminal ileal and colonic involvement had serum levels of PLP very little lower than normal, but those with diffuse disease and/or on ACTH had very low levels (see Table 3.3.3.). The lack of statistical significance for women on ACTH and between categories is probably due to the small sample size.

The percentage of RE patients found to be deficient is very much lower than that found by Anderson et al (1970), however, all

Fig. 3.3.8. Pathways of tryptophan metabolism in man.



Enzymes requiring PLP as a co-factor

1 Tryptophan oxygenase

2 Kynurenine aminotransferase

3 Kynureninase

their patients had active untreated disease whereas the patients in this series were attending out-patient clinics and were in receipt of treatment. No attempt was made to sub-divide the groups further into age ranges and statistical analyses were made between all the normal men and women.

17% of the women and 20% of the men with UC (a chronic inflammatory disorder of the colon) had serum PLP levels below the normal range.

In the case of ACD 39% of women had borderline or sub-normal serum levels of PLP (24% were deficient) and 35% of the men. These percentages are very much lower than those reported by Anderson et al (1970) i.e. 83% of the untreated patients (all four patients who had been treated with a gluten free diet had normal levels). Of the patients reported here, 16 of the 36 patients not on a gluten free diet had serum levels of PLP over 5.0 ng/ml. Baker and Sobotka (1962) have also shown that ACD patients have sub-normal levels of pyridoxine in the serum. Kowlessar et al (1964) found that ACD patients with steatorrhoea had evidence of insufficient levels of vitamin B_6, as judged by the excretion of abnormal levels of metabolites after an oral dose of tryptophan. The abnormal levels of these metabolites returned to normal, or near normal, after supplementation with vitamin B_6.

The absorption and conversion of pyridoxine hydrochloride to PLP occurs rapidly in normals and RE's with diffuse ileal involvement (see Fig.3.3.7.). Brain and Booth (1964) found evidence of reduced absorption of an oral dose of 1 mg tritiated pyridoxine hydrochloride in ACD patients. The mean excretion of radioactivity in the urine after saturation of the patients with 300 mg pyridoxine given intra-venously was very significantly less than

the mean excretion of similarly treated control subjects. They found that two patients with Crōhns disease of the terminal ileum absorbed pyridoxine normally. Woodring and Storvick (1970) found that the free vitamin B_6 present in whole blood increased from 4.0 ng/ml before supplementation to 200 ng/ml 2 hrs after an oral dose of 50 mg pyridoxine hydrochloride (Vitamin B_6 in forms other than PLP is not measured by the method used here). Anderson et al (1971) have shown (see Fig.3.3.9.) that, in vivo, pyridoxine is taken up by red cells, where it is converted to pyridoxal phosphate and then pyridoxal. A portion of the pyridoxal is then gradually released into the plasma. Any increase in PLP levels in the serum would have been masked in their procedure since large dilutions were made and PLP was measured indirectly. Wada et al (1957) found that the serum PLP level doubled within 10 min of an intra-venous injection of pyridoxal. Hines and Love (1969) found a peak in the serum level of PLP 3 hrs after an oral dose of 25 mg pyridoxine hydrochloride. In the subjects presented here a peak PLP level had not been attained 2 hrs after dosing.

The metabolism of pyridoxine to PLP has been found to be inhibited in two ways:-

(a) Hines and Cowan (1970) have suggested that the metabolism of pyridoxine to PLP can be affected in vivo by the inhibition of pyridoxal kinase resulting from excessive ingestion of alcohol.

(b) White and Dempsey (1970) have found that purified pyridoxine kinase derived from E. coli was strongly inhibited by the presence of pyridoxal. This points to a possible negative feed-back mechanism controlling the production of PLP.

No evidence was found in the three patients studied that there was any inhibition of conversion of pyridoxine to PLP, hence it is

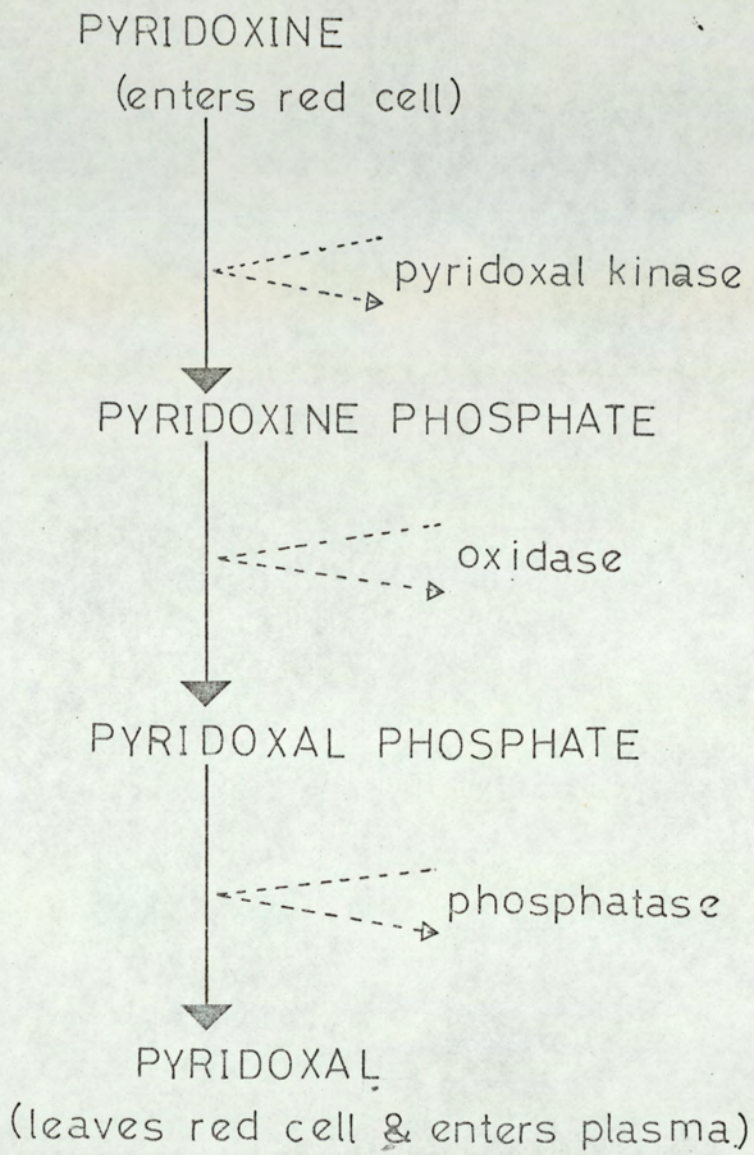


Fig. 3.3.9. The *in vivo* conversions of vitamin B_6 (after Anderson *et al* (1971)).

thought unlikely that this was a cause of the low serum PLP levels found in the patients tested.

Measurements of the turnover of vitamin B_6 in humans have been made using radio-actively labelled pyridoxine. Johansson et al (1966), using tritiated pyridoxine, proposed the presence of a small compartment with a rapid turnover rate and a larger storage compartment. The half life for labelled pyridoxine was 18-38 days, the body content of the vitamin was estimated to range 40-150 mg, with a daily excretion of 1.7-3.6 mg. Tillotson et al (1966), using ^{14}C labelled pyridoxine, found the half life of the labelled compound to be 15-20 days, depending on the level of dietary intake of vitamin B_6, and estimated the total body pool to be 22-27 mg (assuming a normal intake).

The inter-relationships of the metabolites of tryptophan measured in this study are shown in Fig.3.3.8. The metabolism of tryptophan has been the subject of a recent symposium (Brown R. R. Editor (1971)) and review article (Rose (1972)). Control over entry into the tryptophan-nicotinamide pathway is exerted by the first enzyme i.e. tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, E.C. 1.13.1.12) which is subject to control through a negative feedback mechanism by nicotinic acid derivatives (Cho-Chung and Pitot (1967)). The activity of the enzyme is also influenced by levels of adrenocorticoids, oestrogens and androgens. Rose and Braidman (1971) have proposed that elevated levels of oestrogen act via the pituitary adrenal axis to raise the levels of corticosteroids which, in turn, have an induction effect on the levels of tryptophan oxygenase. Accumulation of abnormal levels of metabolites of tryptophan after an oral load of the amino acid occurs when there are not sufficient levels of PLP present to effect the complete metabolism of tryptophan once the tryptophan-nicotinamide pathway has been entered (Rose (1972)).

Wolf and Brown (1971) found that the availability of PLP for the kynureninase catalysed production of anthranilic acid from kynurenine was the limiting factor for the pathway. Kynurenine transaminase is not so sensitive to shortage of PLP thus allowing the levels of XA and KA to increase when co-enzyme deficiency is not too severe.

Standardisation of the dose of L-tryptophan was recommended by Coursin (1964). The recommended dose of 2 g L-tryptophan was given, suspended in milk, in the morning. The test patients received their tryptophan just before, or with, their breakfast, the healthy controls received theirs at 9.00 a.m. The metabolites measured (XA, KA, Kyn, 5-HIAA and IAA) all require the presence of PLP for their formation from tryptophan.

Care is necessary when choosing "normal" volunteers to act as controls in tryptophan loading tests. Normal 1 had been the subject of tryptophan loading tests some years previously, the loading doses being 10 g DL-tryptophan and 5 g L-tryptophan. Induction of tryptophan oxygenase as a result of loading dose has been shown (Knox (1958)). Altman and Greengard (1966) found no induction of tryptophan oxygenase in human subjects when the 2 g L-tryptophan load was used, although Hankes, Brown, Lippincott and Schamaeller (1967) found that with a loading dose of 2 g L-tryptophan more of a tracer amount (17-78 mg) of tryptophan-2-^{14}C entered the kynurenine-CO_2 pathway than when the tracer dose was given alone. Wolf and Brown (1971) concluded that a 2 g L-tryptophan load was close to the limit of the capacity of the kynurenine pathway but, so far as the chemical measurements of the metabolites was concerned, there was no induction of tryptophan oxygenase when this load was used. It is proposed that Normal 1 had a raised activity of tryptophan oxygenase which was not fully compensated for by the presence

of relatively high serum levels of PLP.

The high basal excretion of Kyn and high post loading excretion of XA, KA and Kyn in normal 3 may be due to the fact that the subject had been taking oral contraceptives for 3 years but had ceased taking them 9 months prior to the test. Rose (1966) found that of the tryptophan metabolites excreted in abnormal amounts by women taking oestrogen containing oral contraceptives, XA appeared in the highest concentration, followed in decreasing amounts by 3-hydroxy kynurenine, kynurenine KA and acetyl kynurenine. This pattern of excretion was returned to normal, or near normal, by the administration of large doses of pyridoxine. In the case of Normal 3 the abnormality in excretory pattern returned to near normal after supplementation with 50 mg pyridoxine hydrochloride. Rose and Adams (1972) have found that abnormal tryptophan metabolism may persist for 3 months, or longer, after cessation of the taking of oral contraceptives. Normal 4 was found to be deficient in vitamin B_6 as judged by the serum level of PLP; this was probably due to the consumption of a diet devoid of red meat. Excretion of XA and Kyn returned to normal after repletion with vitamin B_6 and the excretion of KA was very much reduced.

The correlation coefficients between PLP levels and levels of excretion of XA, KA and Kyn have been calculated for the ACD patients studied for days 1 and 2 (see Table 3.3.14.). The lack of any statistically significant correlation for any of the metabolites is probably due to the small sample size. Hamfelt (1964) found that there was a significant correlation between μ moles XA excreted/Kg body weight in the 24 hrs after a load of 100 mg L-tryptophan/Kg body weight ($n = 58$), although there were a number of results where the inverse relationship did not hold.

When using a load of 4 g L-tryptophan, Kowlessar *et al* (1964)

found that patients suffering from ACD failed to metabolise tryptophan normally, since urinary levels of XA, KA and Kyn were significantly higher than those of normals (Table 3.3.17. gives these figures expressed as μ moles/24 hrs (XA, KA, Kyn) and mg/24 hrs (5-HIAA, IAA). Table 3.3.18. gives further particulars of the patients tested, relative to type of diet, type of biopsy found at time of diagnosis (and subsequently for some patients) and a recent faecal fat excretion figure. One of the two patients with normal plasma PLP levels has no steatorrhoea and a normal excretion of tryptophan metabolites (patient 7). Of the patients who excreted less than 6 g fat/day, patient 5 had low plasma levels of PLP and a raised excretion of XA after the first load, which was returned to normal after supplementation with vitamin B_6. Patient 3 had borderline plasma PLP levels, elevated excretion of XA after the first load of tryptophan which returned to normal following supplementation, however, there was also a rise in excreted Kyn on day 4.

Both patients with moderate steatorrhoea (less than 10 g/day) had sub-normal plasma PLP levels. Patient 2 excreted low levels of XA and KA on both days 2 and 4, whilst patient 6 had persistently raised excretion of Kyn on all occasions and raised XA on days 2 and 4. Supplementation of this patient (6) with vitamin B_6 caused a marked reduction in the levels of these metabolites.

Of those patients who excreted more than 10 g fat/day patient 4 had low levels of PLP and patients 1, 9 and 10 had borderline plasma levels. Patient 4 had very much raised excretion of Kyn, but lowered excretion of XA and KA both before and after supplementation: excretion of 5-HIAA was also abnormally high on days 1, 3 and 4. The elevated excretion levels of Kyn in patient 9 were not affected by supplementation with vitamin B_6, although the supplementation

Table 3.3.17 Results of Tryptophan load tests on ACD patients (Kowlessar et al (1964))

		XA*	KA*	Kyn*	5-HIAA‡	IAA‡	
Controls	Basal	36.5	46.5	5.6	5.6	13.2	n = 10
	+ 4 g L-Try	56.5	121.5	146.0	7.5	41.4	
	+ 4 g L-Try + B_6	52.7	101.0	110.0	7.6	43.0	n = 5
Untreated ACD	Basal	97	202	15	11.4	32.1	
	+ 4 g L-Try	377	565	415	13.9	60.3	n = 5
	+ 4 g L-Try + B_6	114	156	150	13.3	59.0	
GFD ACD with steat	Basal	72	133	7	10.7	16.4	
	+ 4 g L-Try	271	434	406	14.3	60.3	n = 13
	+ 4 g L-Try + B_6	85	140	154	14.5	69.8	
GFD ACD no steat	Basal	61	232	6	6.4	12.3	
	+ 4 g L-Try	274	654	645	9.7	46.5	n = 3
	+ 4 g L-Try + B_6	76	134	191	8.5	31.2	

* expressed in μ moles/24 hrs
‡ expressed in mg/24 hrs

Table 3.3.18. Clinical details of ACD patients taking part in the tryptophan load tests

No.	Faecal fat (g/day)	Diet	Biopsy
1	18·0	GFD (strict)	Partial villous atrophy '61 '62 Grade II III '68 Grade II '71
2	7·6	No GFD	Grade II III '63
3	3·0	GFD (not strict)	Grade III '66
4	11·0	No GFD	Carcinoma in situ in rectum.
5	5·2	GFD (strict)	Grade III '61 & '68
6	10·0	GFD (strict)	Grade II '71
7	4·3	GFD (strict)	Abnormal '66
8		GFD (strict)	Grade III '68
9	100·0	GFD (no milk)	Grade III '71
10	16·7	GFD	Grade III '68

GFD = gluten free diet

Grade III = sub-total villous atrophy

Grade II = partial villous atrophy

did have the effect of increasing the excretion of 5-HIAA. Patient 10 excreted abnormal levels of KA and 5-HIAA on day 2, but these were returned to normal after supplementation.

Patients 4, 9 and 10 were the only patients to show any abnormalities in excretion of 5-HIAA. Patient 4 was known to have a carcinoma *in situ* in the rectum, but the site of the primary has not been identified. Patient 9 had elevated 5-HIAA excretion after supplementing with vitamin B_6. The pre-supplementation levels were not low enough to indicate that there may be an inhibition of 5-hydroxytryptophan decarboxylase. Supplementation with vitamin B_6 had very little effect on the excretion of the other tryptophan metabolites by this patient, which suggests that, in this patient, there may be an augmented shunt of the serotonin-5-HIAA pathway as proposed by Kowlessar *et al* (1964). This shunt may also have operated in patient 10 when the first tryptophan load was given, although this is unlikely since the 5-HIAA excretion on the other days of the test was rather low.

The lack of consistent lowering of the levels of excreted metabolites after the second loading makes the assessment of the role played by vitamin B_6 in the metabolism of tryptophan by these patients difficult. The lowering of the levels of excreted XA, KA, Kyn and 5-HIAA in Normal 4 (who had a dietary deficiency of vitamin B_6) was not seen in these patients. For none of the ACD patients was the administration of pyridoxine hydrochloride effective in lowering the excretion of *all* these metabolites relative to the pre-supplementation levels, although only in patient 9 was there no significant lowering of any metabolites.

The ratio of ng PLP/ml packed cells to ng PLP/ml plasma was greater than 1 in the basal state for all normals and all but patient 6 of the ACD's. For this patient the plasma PLP level does

not appear to reflect accurately the intracellular levels of the vitamin. Supplementation with an oral dose of the vitamin had the effect of raising the RBC PLP content as well as that of the plasma and also lowering the level of excretion of XA and Kyn. After supplementation the plasma levels of PLP were found to vary considerably; this was probably due to the variety of routes of administration and also the fact that the blood samples were not taken at a constant time after dosing. The mean PLP level of packed RBC was found to be 13.4 ng/ml (normals) and 14.6 ng/ml (ACD's), which is very much lower than that reported by Donald et al (1971) who found 336 ng vitamin B_6/ml packed RBC, with a range 300-373 ng/ml. Baker et al (1969) have reported a mean of 20 ng PLP/ml packed RBC with a range of 13-31 ng/ml and the ratio of vitamin B_6 in RBC:plasma to be 0.32.

On the basis of the evidence presented in 3.3.2. there is a real deficiency of vitamin B_6 as judged by the serum levels (27% men, 26% women with RE: 20% men, 17% women with UC). Abnormalities in tryptophan metabolism have been reported in another chronic inflammatory disorder, rheumatoid arthritis (Bett (1962), Flinn, Price, Yess and Brown (1964), Pinals (1964)), urinary excretion of vitamin B_6 metabolites were found to be decreased (McKusick, Sherwin, Jones and Hsu (1964)) and sub-normal levels of serum pyridoxal have been reported (Anderson et al (1970)) in these patients. There could be two causes of sub-normal levels of PLP in both RE and UC. Firstly, the chronic inflammatory nature of the diseases. Secondly, the treatment of the condition with steroids, whether it be salazopyrine or ACTH. (The effect of steroid hormones on the requirement for vitamin B_6 has already been discussed).

The incidence of carcinoma of the large bowel amongst patients

who have had UC for 10 years or more is very much greater than in the normal population (Hinton (1966)). The presence of abnormal levels of tryptophan metabolites have been reported in cancer of the bladder, breast, prostate, kidney and Hodgkins Disease (see review Rose (1972)). All except cancer of the bladder may be due to endocrine disfunction. Chabner, DeVita, Livingston and Oliverio (1970) found lowered plasma PLP and abnormal tryptophan metabolites in patients with Hodgkins Disease which were absent in patients who had undergone a complete remission after chemotherapy. At the present time it is not possible to correlate the existence of low serum PLP levels amongst UC patients and the raised incidence of cancer in these patients.

In this series the incidence of vitamin B_6 deficiency in ACD patients has not been found to be as high as in the series reported by Kowlessar et al (1964) or by Anderson et al (1970). The levels of serum PLP amongst men and women ACD patients were significantly lower than the normal population. Brain and Booth (1964) found that there was no clear difference in absorption of ^{3}H-pyridoxine between ACD patients with sub-total villous atrophy and those with partial villous atrophy but 6 our of 13 patients tested showed abnormal absorption of the labelled vitamin. In patients 6-10 in the tryptophan load series there was a substantial increase in plasma PLP following an oral dose of 50 mg pyridoxine hydrochloride, but it is not possible to compare efficiency of absorption.

4. CONCLUSION

4.1. Vitamin E

The method used to measure serum vitamin E has proved to be precise and reproducible. There was no difference in mean serum vitamin E levels between sexes, neither was there a difference between blood donors and non-gastrointestinal hospital patients. The mean serum levels of vitamin E for various types of gastrointestinal patients were all significantly lower than those of the normals. Patients who had had partial gastrectomy operations had the lowest mean level; ACD, RE and UC patients had increasing mean levels of vitamin E. The greatest reductions in serum vitamin E were seen amongst those ACD patients not on a gluten free diet and those RE patients with ileal resection. Low mean levels of vitamin E were also found in patients who had been placed on supplements of folic acid and/or calciferol. That the mean serum level of the vitamin is correlated with the degree of intestinal disease can be seen in the increasing mean levels of those ACD patients on a normal diet, those not adhering strictly to a gluten free diet and those on a strict gluten free diet. The same situation is true for the RE patients; those in clinical relapse have lower mean serum levels than those in remission, involvement or resection of the ileum produces a greater lowering of the levels than involvement or resection of any other area of the small or large bowel. However, surgical intervention in any other area of the gastrointestinal tract produces a significant lowering of the serum levels of the vitamin, the most profound effect being produced by surgery to the stomach and ileum.

In both RE and ACD patients the serum vitamin E level was statistically significantly correlated with faecal fat excretion,

iron and albumin levels in serum. All of these parameters reflect the severity of gastrointestinal disease. Both albumin and iron (in the form of whole blood) can be lost from the body by seepage into the gastrointestinal tract lumen. Faecal fat excretion reflects the degree of disorganisation of the gastrointestinal tract. The absence of correlation between vitamin E and haemoglobin and MCV was probably due to the presence, and treatment of, other vitamin deficiencies.

When monitoring the absorption of an oral dose of vitamin E only changes of more than 0.5 µg/ml can be taken as real; thus the method is of limited use when testing for absorption of vitamin E from the gastrointestinal tract. The same limitation applies when measuring the difference in levels between venous and hepatic or hepatic portal vein blood in the fasting patient. If an oral dose of ^{3}H vitamin E were used and blood taken from hepatic or hepatic portal vein and simultaneously from the peripheral circulation, it would be possible to follow the absorption and physiological fate of physiological amounts of the vitamin.

The major site of absorption of DL-α-tocopherol from the rat small intestinal lumen corresponds to the jejunum and proximal ileum. Whether the animals are fed or not does not affect the site of absorption, but feeding appears to make uptake into and exit from the intestine more efficient. It seems clear that a certain amount of recirculation of the vitamin takes place, but the mechanism for this recirculation is unknown. The site of absorption does not alter as the DL-α-tocopherol progresses along the lumen of the gut, indeed, absorption is taking place in the jejunum and proximal ileum 24 hrs and 48 hrs after dosing.

4.2. Vitamin B_6

The method described in 3.2.2.1. is specific for pyridoxal-5'-phosphate (PLP) and gives a good recovery of PLP added to serum (87-108%). Within batch variation is relatively large (coefficient of variation (CV) = 6.1-18.3%), although counting of the final solution for 4 mins instead of 1 min resulted in a slight reduction in CV. Between batch reproducibility was improved when steps were taken to prevent contamination by carried over serum. Automation of dispensation of enzyme and substrate may result in a reduction of CV both within and between batches.

When used to measure serum or plasma levels of PLP, some interesting features have emerged. Amongst blood donor normals, women were found to have lower mean serum levels than men, when aged less than 40 years. This difference was abolished for ages over 40. Also, for men, there was a reduction in mean serum level when the 18-39 group was compared with the 40-65 group; amongst women there was an increase in levels when these age groups were compared. The significance of this has been discussed (3.4.).

Gastrointestinal disease, whether of the small or large bowel, has the effect of significantly lowering the serum PLP levels, except amongst women with UC. The significant difference between the mean levels for each sex was abolished. Diffuse involvement of the ileum (RE) and the continued presence of gluten in the diet (ACD) have the most profound effect on the serum levels of PLP. Treatment of RE with ACTH has the effect of markedly lowering the serum levels. Those ACD patients who had been put on a gluten free diet had significantly higher levels of serum PLP than those not on the diet.

Low serum levels and excretion of abnormal levels of metabolites after a 2 g load of L-tryptophan were found in both normal (4)

and ACD patients, although the presence of abnormal quantities of tryptophan metabolites in normals (1) and (3) was not accompanied by low serum PLP levels. There are factors which can affect the metabolism of tryptophan other than low serum levels of PLP. These other factors may produce a lowering of the levels of serum PLP. A double tryptophan load test, with a repleting dose of pyridoxine, as carried out here, could be useful in the assessment of whether there is an increased requirement for vitamin B_6 and whether dietary sources alone are sufficient to meet this requirement.

Absorption of pyridoxine hydrochloride and its conversion to PLP can be monitored (see Fig.3.3.7.) and could be extended to measure the effectiveness of adminstration of this form of vitamin B_6. Comparison of the rate of rise of serum PLP when pyridoxine hydrochloride is given intra-venously (rate of conversion to PLP) with that obtained after an oral dose (absorption and conversion) may give further information as to why serum levels are low.

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