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ALCOHOLIC LIVER DISEASE: VITAMIN B6 STATUS

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Thesis submitted for the degree of PhD
University of Glasgow
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February, 1983

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ABBREVIATIONS

AP	alkaline phosphatase
AST	aspartate aminotransferase (GOT)
ALT	alanine aminotransferase (GPT)
Am	aminopterin (4-amino-4-deoxyfolic acid)
ald	alcoholic liver disease
anld	alcoholic non-liver disease
B6	vitamin B6
B12	vitamin B12
BSS	balanced salt solution
Ci	curie
cpm	counts per minute
cont	control
4dPN (HCl)	4'-deoxypyridoxine (HCl)
DNA	deoxyribonucleic acid
dTMP	deoxythymidine monophosphate
dTDP	deoxythymidine diphosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dpm	disintegrations per minute
EDTA	ethylene diamine tetra-acetate
etOH	ethanol
fol	folate
5-fu	5'-fluorouracil
form	formate

GOT	aspartate aminotransferase (AST)
GPT	alanine aminotransferase (ALT)
HLA	histocompatibility locus antigen
H ₂ FR	dihydrofolate reductase
H ₂ F	dihydrofolate
H ₄ F	tetrahydrofolate
h	hour
INH	isonicotinic acid hydrazide
(¹²⁵ I)-UdR	5-(¹²⁵ I)-Iodo-2'-deoxyuridine
Ig	immunoglobulin
I.U.	international units
LAA	leucocyte ascorbic acid
MTX	methotrexate (4-amino-10-methyl-4-deoxyfolic acid)
5,10MeH ₄ F	N ⁵ , N ¹⁰ methylene tetrahydrofolate
min	minute
ml	millilitre
MEOS	microsomal ethanol oxidising system
MEM	minimal essential medium
MEMm	modified minimal essential medium
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NaCl	sodium chloride
n	number
n.s.	not significant
OHb12	hydroxycobalamin (vitamin B12)

P	probability
preinc'n	pre-incubation
PHA	phytohaemagglutinin
PLP	pyridoxal-5'-phosphate
PI (HCl)	pyridoxal (HCl)
PPO	diphenyloxazole
POPOP	bisphenyloxazolybenzene
RNA	ribonucleic acid
rpm	revolutions per minute
s.e.	standard error (of mean)
SDS	sodium dodecyl sulphate
SAA	serum ascorbic acid
ser	serine
$t_{\frac{1}{2}}$	half-life
TK	thymidine kinase
TCA	trichloroacetic acid
TdR	2'-deoxythymidine (thymidine)
TC II	transcobalamin II
TC I & III	transcobalamin I and III
UBBC	unsaturated vitamin B12 binding capacity
UdR	2'-deoxyuridine
WBC	white blood cell
- x	arithmetical mean

SUMMARY

The original purpose of this study was to investigate the clinical importance of nutritional deficiency and genetic markers in the aetiology and morbidity of alcoholic liver disease. Interest in this subject was based on the observation that the onset and severity of alcoholic liver disease was in part dependent on factors other than the period and extent of alcohol abuse. As the principal determinant factors, in addition to alcohol, were considered to be genetic or nutritional a survey of several of these factors in alcoholics was carried out. The genetic factors assessed were acetylator phenotype and alpha-1-anti-trypsin phenotype. The nutritional factors assessed were ascorbate, vitamin B12 and vitamin B6 status. The early results of this study indicated that the value of this approach was limited by the diversity of disease states represented by the term alcoholic liver disease and by the interdependence of the various factors involved. Therefore, it was decided to narrow down the field of research, to concentrate on one area, directly related to the human situation but more amenable to experimentation than the human subject. With a suitable system it would be possible to investigate the effect of specific perturbations under controlled conditions.

The alternative approaches considered included investigation of DNA synthesis in liver cell suspensions of biopsy samples, characterisation of the effects of

vitamin B6 deficiency in rats fed a vitamin B6 deficient diet and use of PHA stimulated lymphocytes. As the liver cell suspension and vitamin B6 deficient rat studies were unsuccessful it was decided to concentrate on PHA stimulated lymphocyte studies. It was considered that this was an appropriate model system for investigation of cell growth in the alcohol diseased liver, development of a functional assay for vitamin B6 status and characterisation of the effects of vitamin B6 deficiency on DNA synthesis and the effects of vitamin B6 antagonists. Two major advantages of the use of lymphocytes were that they represented a source of cells which under certain conditions might demonstrate the same biochemical abnormalities as other cell types of the same individual and that they would allow assessment of functional vitamin B6 status.

As vitamin B6 is the coenzyme of a large number of enzymes in several areas of metabolism, the effect of a vitamin B6 could be expressed in several ways. However, because of the high DNA synthetic requirement in PHA stimulated lymphocytes or in regenerating liver, it was considered that one of the most susceptible enzyme activities might be that of serine hydroxymethyltransferase, one of several enzymes involved in de novo thymidylate synthesis.

Even if a vitamin B6 deficiency did not affect de novo thymidylate synthesis directly, the effect of such a deficiency would still be expressed as a reduction in DNA synthesis. Therefore a principal parameter of interest in this study was the rate of DNA synthesis and the principal method of estimation was based on incorporation of the thymidine analogue (^{125}I)-UdR.

To avoid the possible confounding effect of medium pyridoxal on determination of vitamin B6 status, pyridoxal deficient medium was used. Because of the interest in this study on de novo thymidylate synthesis the effects of different medium folate concentrations on cellular adaptation to thymidine utilisation was assessed. To determine whether or not a vitamin B6 deficiency results in a specific restriction of de novo thymidylate synthesis, experiments using the de novo thymidylate synthesis inhibitors MTX and 5-fu were carried out. Several methods were used to determine whether or not PHA stimulated lymphocytes of alcoholic and control subjects differed with respect to functional vitamin B6 status. These included determination of sensitivity to culture in a pyridoxal deficient medium, sensitivity to the effect of the vitamin B6 antagonist 4-dPN and the effect of addition of UdR on (^{125}I)-UdR incorporation. The initial results of these studies indicated that the alcoholic and control groups were not markedly different in vitamin B6 status. This was despite the use of 4-dPN in an attempt to increase the

sensitivity of the test system. However, the sample size was small and it was not possible to exclude the possibility that a significant difference did exist. A survey of a much larger sample of the alcoholic population would be necessary to assess this. Because of the lack of availability of greater numbers of subjects suitable for inclusion in the study, further characterisation of the model and the effects of vitamin B6 antagonists was undertaken.

The results of experiments in media of different folate concentrations demonstrated that variation of the medium folate concentration has a significant influence on cellular adaptation to thymidine utilisation but no such effect could be demonstrated in the case of vitamin B6 deficiency induced by 4-dPN or in stimulated lymphocytes of alcoholics. This indicates that the primary lesion in 4-dPN induced vitamin B6 deficiency is not at the level of de novo thymidylate synthesis. Because the extent of UdR suppression of thymidine incorporation had been shown to differ in cells of different folate status and had been described as a diagnostic aid in determination of lesions of de novo thymidylate synthesis, its potential use in assessment of vitamin B6 status was investigated in this study. However, experiments directed at characterisation of UdR suppression, using MTX and 5-fu to produce a folate deficiency, indicated that the effect of UdR on de novo thymidylate synthesis was incompletely

understood and could not be used as a valid indicator of folate (or vitamin B6) status. The results of experiments carried out using (^{125}I)-UdR were substantiated by the reciprocal results of experiments where incorporation of the de novo thymidylate precursors 3-(^{14}C)-serine and (^{14}C)-formate was determined.

The clinical significance of this study was related to the possibility that alcoholics or an alcoholic subpopulation have a decreased DNA synthetic capacity and an increased, hepatic, DNA synthetic requirement. A vitamin B6 deficiency could reduce cell growth by affecting DNA synthesis or other area of metabolism and ethanol induced necrosis would increase the DNA synthetic requirement. The results reported in this study do not suggest that there is a general vitamin B6 dependent reduced cell growth capacity in the alcoholic liver disease population.

CHAPTER 1

INTRODUCTION

Section 1.1

Alcoholic Liver Disease

1.1.1. A Perspective

The three recognised hepatic complications of heavy alcohol consumption are alcoholic fatty liver, alcoholic hepatitis and cirrhosis (Lieber, 1977). Alcoholic fatty liver is an almost invariable finding in the early stages of alcohol abuse. It is manifested primarily by hepatomegaly, often in the absence of clinical symptoms and is reversible with abstinence. Alcoholic hepatitis is usually considered to be a transitional lesion in development of cirrhosis. It is probably reversible but with varying degrees of residual fibrosis. The essential lesion is focal or diffuse liver-cell necrosis with polymorphonuclear-leucocyte infiltration. Depending on the extent of necrosis it may cause acute liver failure with portal hypertension. Repeated episodes of alcoholic hepatitis develop into progressive micronodular cirrhosis which is usually irreversible (Baptista et al 1981).

The pathogenesis of liver disease in alcoholic patients is obscure. Many chronically alcoholic people have normal liver morphology and normal liver function (Klatskin, 1961). Statistics indicate that the prevalence of cirrhosis in patients with alcoholism is proportional to alcohol consumption (Smith, 1981). Accordingly, in a man of average size who drinks 170 g of ethanol per day for 25 years, there is 50 per cent chance that cirrhosis will develop (Lieber, 1978).

Other estimates of the incidence of cirrhosis in chronic alcoholics vary from 25 to 57 per cent (Lelbach, 1975; Brunt et al, 1974) but all agree that a substantial proportion of alcoholics do not develop cirrhosis. For example, Lelbach (1975) has calculated that in a total of over 5000 alcoholics analysed in 23 liver biopsy series, one-quarter had normal livers, one-third had uncomplicated fatty liver, one-fifth had alcoholic hepatitis without cirrhosis and one-quarter had cirrhosis. From this, it can be readily appreciated that the most important factors determining the development of alcoholic liver disease are the amount of alcohol, the pattern of consumption whether daily or intermittent, etc., and the period of abuse. However, superimposed on these primary factors are age, sex, weight, nutritional and other environmental variables as well as genetic and immunological determinants (Brunt and Mowat, 1975).

In approaching an understanding of how ethanol causes liver disease, it is perhaps most useful to concentrate on how the effects of ethanol and ethanol metabolism cause lesions at the cellular and intercellular levels, and then to consider how such a process, occurring repeatedly every day for a number of years, finally compromises the function of the liver and sets in chain the irreversible process leading to cirrhosis. A general model such as this is useful because it breaks the process up into a number of stages which can be

examined in turn to determine the role of various effectors throughout the course of the disease process. For example, the amount of ethanol would determine the extent of the local lesions, the periodicity would determine whether there was intermediate recovery or promulgation of extant lesions, and the cumulative effect of the localised lesions. Analysis of such variables has confirmed that there is no simple correlation between period and extent of abuse and incidence of disease (Lelbach, 1975) and therefore it may be reasoned that other parameters operate to augment or reduce the necrotic process at each stage in individual cases. It is the analysis and description of such variables that this project is concerned with and it is hoped that such an analysis will lead to identification of those most at risk to the medical consequences of heavy alcohol consumption and to the formulation of a more rational approach to therapy directed at reduction of morbidity in alcoholic liver disease.

By analogy with other diseases where a uniform challenge has invoked a disparate response, it is possible that genetic differences might account for variation in susceptibility to alcoholic liver disease. The common association of alcoholism and a reduced food intake has prompted many investigators to examine malnutrition as a contributory factor in alcoholic liver disease (Patek, 1979; Lieber, 1978). A more specific consideration of

the various stages of the disease process has directed attention on the role of collagen metabolism (Chen and Leevy, 1975), immunological processes (Mihas, Bull and Davidson, 1975) and specific nutritional deficiencies (Lumeng and Li, 1974).

In order to appreciate certain aspects of the complex interplay of factors in alcoholic liver disease, it seems appropriate to consider the primary causative agent, ethanol, its metabolism, the disease process, approaches to the study of the disease and the approach adopted in this project. Only the effects of ethanol on the liver are considered, other tissues being discussed only in so far as they affect the liver.

1.1.2. Ethanol

The ethanol molecule has weak properties of dissociation and polarisation and is freely miscible in water and lipids. One effect of this is that ethanol, in common with other pharmacologically active substances such as ether and chloroform, appears to have a fluidising effect on cell membranes (Chin and Goldstein, 1977a). A practical result of this is perhaps the disruption of neuronal membrane function, but other, less dramatic effects on cell membrane structure and function are probably at least as important in determining the overall effect of chronic alcohol consumption. This postulated mode of action of ethanol also suggests a mechanism for the development of tolerance. According to this mechanism, changes in membrane lipid composition would occur in order to reduce this fluidising

effect of ethanol and hence higher concentrations of ethanol would be required in order to produce the same disruption in physiological membrane function (Chin and Goldstein, 1977b). Tolerance to ethanol would also develop in other respects, for example, in enzyme systems associated with its metabolism.

Development of tolerance to ethanol is a crucial aspect of alcoholic liver disease. Lacking tolerance, the human body would not be able to withstand, physiologically, the onslaught of large quantities of alcohol, but as tolerance to the consumption of otherwise immediately toxic and incapacitating quantities of alcohol develops, the repeated consumption of such amounts over a number of years will result in severe functional disruption of many body tissues and especially the liver.

1.1.3. Metabolism of Ethanol

Ethanol is rapidly absorbed through the mucosal membranes of the gastro-intestinal tract. It is rapidly distributed throughout the body, diffusing across capillary membranes, tissue membranes and cell membranes. It has a destabilising effect on membrane structure with concomitant effects on the membrane's permeability and transport functions. Due to the lack of any storage mechanisms and the limited amount of pulmonary or renal excretion, ethanol must be removed by oxidative processes in the tissues (Brunt and Mowat, 1975). Because of its mass, its portal blood supply and its

functional metabolic specialization, the liver is the main tissue which metabolises ethanol, and which is most deleteriously affected by alcohol metabolism. The main oxidative process by which ethanol is metabolised is via the cytosolic enzyme, alcohol dehydrogenase (Li, 1977). This reaction requires the oxidised nicotinamide coenzyme NAD⁺, and it is the supply of this coenzyme which is the main rate limiting factor in ethanol metabolism. In addition, many of the disruptive effects of ethanol metabolism in cellular metabolism for example, accumulation of fat in the liver, hyperlactacidaemia and hyperuricaemia, are believed to be mediated through the cells' attempts to regenerate this coenzyme (Lieber, 1967; Lieber, 1975). The acetaldehyde produced is further oxidised to acetate and these two reactions constitute, quantitatively, the most important pathway for the removal of alcohol (Lundquist, 1975). The combination of organ specificity, the lack of a feedback mechanism to adjust the rate of ethanol oxidation to the metabolic state of the liver and the inability of ethanol to be stored or metabolised to a marked degree in peripheral tissues is seen as the main reason why ethanol metabolism, mediated through mechanisms aimed to maintenance of redox homeostasis, produces metabolic imbalances in the liver (Lieber, 1975).

It has been suggested that the increased serum lactic acid levels resulting from ethanol metabolism may reduce uric acid secretion and result in increased serum uric acid and that this may precipitate attacks of gout

(Lieber 1962, 1967). Krebs (1968) suggests that this is an interesting attempt to find a causal link between alcohol and gout. Overall, Krebs (1968) considers that the effect of alcohol on the regulation of cytoplasmic and mitochondrial NAD/NADH₂ ratios may be a major primary factor in the pathogenesis of alcoholic liver disease.

1.1.4. Production of Alcoholic Fatty Liver

Fatty liver, the earliest manifestation of alcoholic liver disease is characterised by a variety of changes, only one of which, the hepatic accumulation of lipids, gives rise to its descriptive name. At the cellular level, these include: accumulation of protein and swelling and disorientation of mitochondria and mitochondrial cristae (Baraona et al, 1975a; Baraona et al, 1975b; Svoboda and Manning, 1964). The effects of lipid and protein accumulation include retention of water and swelling of the hepatocyte. The main mechanisms whereby fatty acids of various sources can accumulate in the liver are: increased peripheral fat mobilisation; decreased hepatic lipoprotein release, decreased lipid oxidation in the liver and enhanced lipogenesis (Lieber 1975; Lieber, 1977). This disruption of hepatic fat metabolism is, at least initially, accompanied by hyperlipaemia (Lieber, 1967). A low fat diet will reduce the extent of lipid accumulation but where ethanol consumption is continued, the abnormality will persist (Lieber and Spritz, 1966).

Fatty liver may also result from protein malnutrition, the intestinal bypass operation for obesity, and, in rats, feeding of a choline deficient diet. However, the abnormality here is not accompanied by the ultrastructural changes of mitochondria and rough endoplasmic reticulum which occur in ethanol induced fatty liver, nor does this lesion in these cases develop into hepatitis or cirrhosis (Lieber, De Carli and Rubin, 1975).

1.1.5. Development of Alcoholic Hepatitis and Cirrhosis

Identification of the link between fatty liver and alcoholic hepatitis and between these conditions and cirrhosis is of considerable importance. Because alcoholic fatty liver is a common accompaniment of alcoholism and is usually fully reversible, it has been considered benign. However, the fact that it reflects a severe metabolic disturbance which is likely to result in irreversible damage to the hepatocyte and reduce its life span indicates that this lesion may represent a necessary precondition for the development of alcoholic hepatitis (Lieber, 1977).

Alcoholic hepatitis results when a number of liver cells die and this necrosis causes inflammation (Lieber, 1978). From the close similarity in the ultrastructural features seen in the hepatocytes of fatty liver and alcoholic hepatitis, it seems likely that the basic lesion precipitating cell death is the same in each condition. According to this hypothesis, because the rate of cell death is considerably lower in the fatty liver condition

and is likely to be within the hyperplastic response capacity of the liver, the degree of necrosis would be insufficient to cause an inflammatory response.

Up to the fatty liver stage of alcoholic liver disease, the effect of alcohol has been well described.

After this stage, the process is more complex. In humans, the occurrence of fatty liver is almost universal but the subsequent onset and severity of alcoholic hepatitis, if it occurs at all, is less certain.

The major factor is continuation of alcohol abuse but this alone is insufficient explanation for the variation in response to alcohol (Lelbach, 1975; Brunt and Mowat, 1975). As previously indicated, (section 1.1.1.) the main host susceptibility factors are believed to be genetic, nutritional, differences in immunological reactivity or differences in connective tissue metabolism. A major problem is the lack of a suitable animal model; the full spectrum of alcoholic liver disease cannot be reproduced in the rat and in what appears to be the most suitable animal, the baboon, the full development of alcoholic liver disease, as in humans, requires a period of several years (Lieber, 1975).

It is likely that the long incubation period required for the development of alcoholic liver disease reflects the degree of toxicity of ethanol and partly explains the difficulty in determining which factors potentiate or reduce the alcoholic liver disease process. It is feasible that initially, one of the determinants of the

disease process may be the balance between cell death and cell renewal. A reasonable strategy in an attempt to identify the critical determinants in such a multivariate disease is to focus attention on one particular parameter and determine its relation to the problem as a whole. However, after a certain stage, the process is self-perpetuating; the secondary changes induced by the disease process, in particular the excess of fibrous scar tissue, distort the normal architecture of the liver and inhibit blood flow and regeneration. This latter stage is termed cirrhosis.

Section 1.2.

Approaches to Study of Alcoholic Liver Disease

1.2.1. Nutritional Factors

It is clear from the work of Lieber, De Carli and Rubin (1975) that in an animal model of alcoholic liver disease, feeding a nutritious diet will not prevent development of alcoholic hepatitis and cirrhosis when excessive alcohol is chronically consumed. However, the relationship between malnutrition and the tissue injury seen in chronic alcoholics remains controversial. (Patek, 1979).

Evidence of malnutrition in chronic alcoholics is based on observation of weight loss and reduced circulating levels of vitamins including folic acid (Hines, 1969) pyridoxine (Lumeng and Li, 1974) and leucocyte ascorbic acid (Beattie and Sherlock, 1976). The causes of malnutrition in the alcoholic include reduced dietary intake, malabsorption, impaired nutrient metabolism, decreased hepatic storage of nutrients and increased nutrient requirements (Leevy, Zetterman and Smith, 1975).

However, the effects of reduced nutrient levels on the pathogenesis of alcoholic liver disease are difficult to establish. Some alcoholic liver disease subjects have apparently normal vitamin levels while some alcoholics without liver disease have reduced vitamin levels (Leevy, Zetterman and Smith, 1975; Lumeng and Li, 1974). This illustrates the multifactorial nature of the problem and the difficulty of separating the effects of alcohol itself from the effects of malnutrition. There are also the problems of relating nutritional

status at a particular time with tissue damage which develops over a number of years and individual susceptibility to tissue damage. Despite these difficulties it is clear that nutritional status is a major determinant of susceptibility to alcoholic liver disease. Therefore, it was decided to determine serum levels of several important nutrients in the alcoholic study population. The possibility that specific nutritional deficiencies enhance the susceptibility of certain alcoholics to alcoholic liver disease was also of particular interest. If this hypothesis could be sustained it would be possible to test whether specific nutritional supplements or dietary manipulations would have a protective effect.

In order to further characterise the role of ascorbate deficiency in alcoholic liver disease, it was also intended to investigate the effect of ascorbate status on drug metabolism and in particular metabolism of antipyrine. Antipyrine is a drug which is metabolised predominantly in the liver and whose metabolism and therefore elimination from the body has been shown to be delayed by liver disease (Branch, Herbert and Read, 1973). It has been reported (Beattie and Sherlock, 1976) that reduced ascorbate levels are associated with reduced antipyrine elimination and it was of interest both to further investigate this relationship and the effect of improved ascorbate status on antipyrine elimination.

1.2.2. Genetic Markers

It is suspected that individual susceptibility to many diseases including alcoholic liver disease is partly genetically determined. Evidence for this is based on observed association between certain genetically determined traits such as hair and eye colour, colour blindness, etc., and liver disease (Reid, et al, 1968). Functional association between genetic traits and liver disease has also been related to genetic determinants of the immune system (Bailey et al, 1976), drug metabolism (Rao et al, 1970) and protease inhibitor phenotype (Berg and Eriksson, 1972).

Two ways in which immune associated genetic markers could affect alcoholic liver disease are by a direct effect on the immune response or by linkage with a determinant of the immune response. The prevalence of certain histocompatibility locus antigens (HLA) in different groups of subjects suggests that these genetic markers may be determinants or may be linked to determinants of susceptibility to alcoholic liver disease (Bailey et al, 1976).

Recognition of genetic variation in drug metabolism e.g., of slow and fast acetylators (Rao et al, 1970) and of possible clinical implications such as reduced response to drug therapy and enhanced drug toxicity suggests that such markers could also be related to susceptibility to alcoholic liver disease.

Alpha-1-antitrypsin is a serum protein which comprises the majority of the alpha-1 globulin fraction and is the major protease inhibitor of human serum. Its biosynthesis is controlled by two allelic genes and homozygous deficiency has been associated with severe pulmonary emphysema in adults (Eriksson, 1965; Sharp, 1971) as well as liver disease in children (Johnson and Alper, 1970) and adults (Berg and Eriksson, 1972). Partial heterozygous deficiency may be associated with chronic lung disease (Kueppers, Fallat and Larson, 1969). Alpha-1 antitrypsin is a member of a group of proteins, acute phase reactants, which are synthesised in the liver and which increase in concentration in response to disease states involving acute inflammation and, or tissue necrosis.

Although homozygous deficiency of alpha-1 antitrypsin, with a United Kingdom frequency of about 1 in 4000 (Cook, 1975) is very rare and cannot be considered a significant factor in the incidence of alcoholic liver disease, it is possible that certain heterozygotes or persons with reduced levels of alpha-1 antitrypsin could have enhanced susceptibility to alcoholic liver disease. For this reason it was decided to determine alpha-1-antitrypsin levels in the study population.

One of the difficulties of characterising the genetic component of individual susceptibility to alcoholic liver disease is the possibility that alcoholism itself may have a genetic component and that this might confound

the analysis. However, because of the evidence of an association between certain genetic markers and alcoholic liver disease, it was decided to investigate the relation of several of these, i.e., alpha-1-antitrypsin and acetylator phenotype in the present study. Originally, it was also intended to determine HLA type but this was not possible.

1.2.3. Vitamin B6 Metabolism

Vitamin B6 is the generic name for a number of related compounds which all have the basic pyridine ring structure (fig. 1). Depending on which (-R) group is substituted, pyridoxine, pyridoxal or pyridoxamine is formed. The phosphate ester of pyridoxal, pyridoxal phosphate, is the active coenzyme.

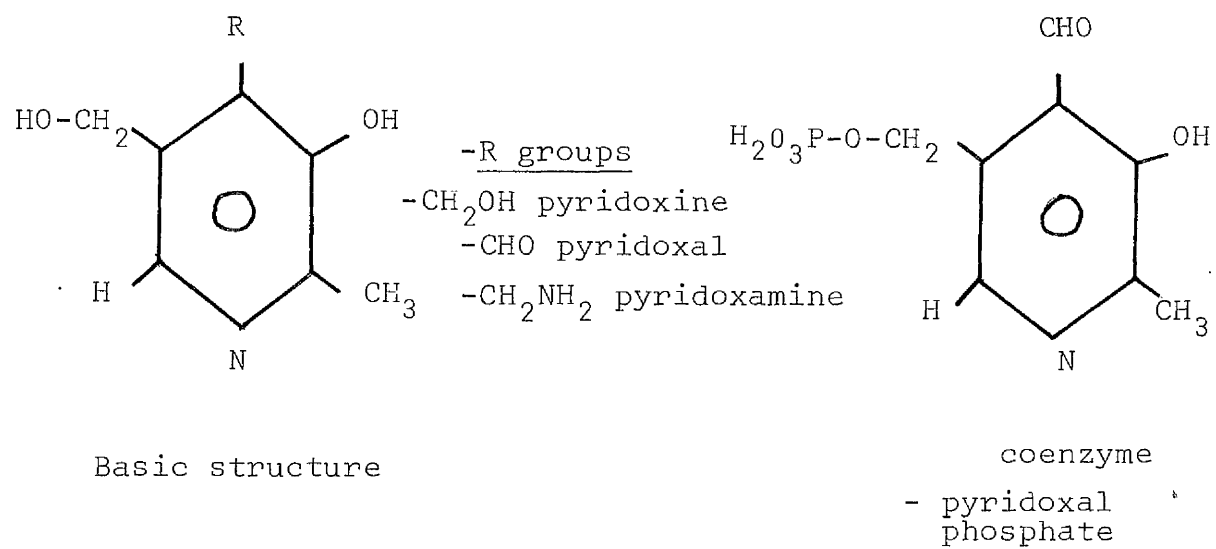
Pyridoxal phosphate is a coenzyme employed by many enzymes in many areas of metabolism and especially by those enzymes catalysing amino acid transformations. Among the enzyme reactions catalysed by pyridoxal phosphate are: transamination, decarboxylation, phosphorylysis of glycogen, formation of delta-aminolaevulinic acid and formation of 5, 10 methylene tetrahydrofolate. Thus vitamin B6 is an essential factor in such processes, as the maintenance of nitrogen balance, porphyrin synthesis and de novo thymidylate synthesis and it therefore plays an integral role in protein, carbohydrate, lipid, purine, pyrimidine and neurotransmitter metabolism. From this it can be readily appreciated that a B6 deficiency

would result in anaemia, growth retardation and alteration in neuronal function.

As the B₆ vitamins occur so widely in nature there is unlikely to be a primary B₆ deficiency in the human. However, there are a number of conditions from which a vitamin B₆ deficiency could arise. In general, malnutrition in chronic liver disease can result from: inadequate diet, malabsorption, diminished hepatic nutrient storage and disturbed nutrient metabolism. Thus patients with liver disease may have adequate total body levels of nutrient yet because of altered transport mechanisms or because of a nutrient metabolism block be unable to utilise the nutrient. A vitamin B₆ deficiency might also result from an increased metabolic requirement or due to the occurrence of a variant enzyme with an abnormally high pyridoxal phosphate requirement.

The non specific nature of the effects of B₆ deficiency and their occurrence only in severe deficiency masks marginal B₆ deficiency. In infants, vitamin B₆ deficiency produces convulsions, in the adult the most specific clinical manifestation is probably vitamin B₆ responsive sideroblastic anaemia (Hines and Cowan, 1970). Various reports have indicated a significant incidence of this abnormality in alcoholics (Hines and Cowan, 1970; Pierce, McGuffin and Hillman, 1976). However, the development of this abnormality is probably the end result of multiple contributing factors and cannot be considered a precise or sensitive index of vitamin B₆

Fig. 1. Structure of vitamin B₆



deficiency. For this reason, estimation of the incidence of vitamin B₆ deficiency in the alcoholic has relied either on the determination of the functional integrity of vitamin B₆ dependent pathways or the measurement of vitamin content of serum or erythrocytes (Li, 1976).

No completely satisfactory functional assay has been described and most recent studies have depended on tyrosine apodecarboxylase determination of pyridoxal phosphate (Chabner and Livingstone, 1970; Lumeng and Li, 1974). The range of serum PLP values determined using this method vary between 2.4 - 12.4 ng/ml, mean 7.1 ng/ml (Hamfelt, 1964); 5.0 - 26.3 ng/ml (Lumeng and Li, 1974); 18.5 ± 5.5 ng/ml (Chabner and Livingstone, 1970) and 11.3 ± 1.0 ng/ml (Rossouw et al, 1977).

In hepatic failure significantly higher serum PLP values are observed (Rossouw et al, 1977) which subsequently decrease to subnormal levels. It appears that serum PLP values are reduced in liver disease, including alcoholic liver disease, and that the normal range is different for each investigator. Furthermore, the presence of hepatic damage could lead to the release of PLP into serum and result in a false indication of satisfactory vitamin B₆ status.

Among the studies which have established that a high percentage of chronic alcoholics, whether with or without hepatic disease, have low serum pyridoxal phosphate

levels are those of Lumeng and Li (1974) and Pierce, et al (1976). The cause of these low levels has been related to diet but a more likely cause is probably the effect of ethanol metabolism (Lumeng, 1978). The main mechanism advanced to account for the disruption of vitamin B₆ metabolism is that acetaldehyde, the immediate product of ethanol oxidation, displaces or facilitates the dissociation of PLP from protein. This increases the availability of PLP for hydrolysis by alkaline phosphatase and this increased degradation of PLP results in low serum levels of the vitamin.

The functional significance of these low levels is not known. The possible effect on cell growth and DNA synthesis, suggested by Leevy (1966, 1975b) would, if confirmed, represent an important consequence. If this was further related to a limiting determinant in the pathogenesis of alcoholic liver disease, then vitamin B₆ therapy would have a more rational basis and a definite adjunctival role in the prevention and management of alcoholic liver disease.

In this study it was decided to determine serum vitamin B₆ levels in groups of normal, alcoholic and alcoholic liver disease subjects. This information would be used to investigate the relationship between vitamin B₆ levels and alcoholic liver disease.

The possibility that vitamin B₆ deficiency would result in an increase in the amount of apo- relative to holo-serum transaminase levels in alcoholic liver disease subjects was also investigated. It is possible that a relative reduction in holo- enzyme level would result in an underestimate of total serum transaminase levels in B₆ deficient subjects. It has also been suggested that the stimulation in serum transaminase activity after addition of PLP would provide an estimate of vitamin B₆ status (Sauberlich et al, 1972).

1.2.4. Vitamin B₁₂ Metabolism

The liver is the principal storage site for vitamin B₁₂ and assays of liver biopsy material for vitamin B₁₂ show a diminution in liver disease (Sherlock, 1963). This is paralleled by an increase in serum B₁₂ levels in liver disease, including alcoholic liver disease (Hines, 1969). In view of the assumption which is often made that serum levels of vitamins reflect cellular vitamin status, it is of particular interest that elevated serum B₁₂ levels should be associated with reduced hepatic B₁₂ levels.

Neither the cause of elevated serum B₁₂ levels in alcoholic liver disease nor the clinical significance of such changes have been established. Possible mechanisms include: increased absorption of vitamin B₁₂, an increase in serum vitamin B₁₂ binding proteins and consequent increase in the availability of binder sites, an increase in saturation of the binder sites and abnormal release

of vitamin B₁₂ from the liver. If elevated serum vitamin B₁₂ levels are associated with reduced cellular levels of vitamin B₁₂, and therefore vitamin B₁₂ deficiency, it is possible that they contribute to the neurological, haematopoietic and cell growth defects associated with alcoholic liver disease

In this study it was decided to further characterise certain aspects of vitamin B₁₂ metabolism in alcoholic liver disease by determination of vitamin B₁₂ absorption, serum vitamin B₁₂ levels and levels of unsaturated vitamin B₁₂ binding capacity of serum vitamin B₁₂ transport proteins.

1.2.5. Immunological Aspects

A variety of altered immunological parameters have been observed in alcoholic liver disease and these have been related to development and progression of the disease. It has been found that serum immunoglobulins are usually increased in patients with alcoholic liver disease and that they include some autoantibodies (Zimmerman and Levi, 1969). An altered cell mediated immunity to liver antigens has been described in patients with alcoholic hepatitis using either autologous liver, normal human liver or alcoholic hyaline (Sorrel and Leevy, 1972; Mihas, Bull and Davidson, 1975). It has been shown that lymphocytes from baboons with alcoholic hepatitis are cytotoxic against autologous liver cells in tissue culture (Paronetto and Lieber, 1976) and that a cell mediated autoimmune reaction may occur in patients with alcohol induced liver damage (Cochrane

et al, 1977).

This evidence suggests that necrosis of liver cells is accompanied by an inflammatory cell infiltrate which may mediate an autoimmune process leading to further liver cell damage. Further evidence suggestive of perpetuation by abnormal immunoreactivity is the demonstration of liver antigen induced migration inhibition in hepatitis but not in steatosis or cirrhosis (Mihas et al, 1975) and the increased levels of serum IgA in hepatitis and cirrhosis but not steatosis (Steigmann et al, 1974).

It has also been suggested that immunologic reactivity to collagen occurs in patients with alcoholic hepatitis and that such activity may be important in conversion of alcoholic hepatitis to cirrhosis (Zetterman, Liusada-Opper and Leevy, 1976). On the basis of a decreased lymphocyte response to phytohaemagglutinin (Lundy et al, 1975) and a pronounced decrease in peripheral blood lymphocytes in patients with alcoholic hepatitis (Bernstein et al, 1974), it was suggested that there is a basic impairment in cell-mediated immunity in alcoholic liver disease (Leevy et al, 1975).

These observations suggest that immunological mechanisms may be involved in the perpetuation of hepatic damage in alcoholic patients but they do not distinguish between immunological factors causing cell death and fibrosis and changes in immunological parameters resulting from the disease process.

1.2.6. The Role of Collagen Metabolism

It has been suggested that ethanol can trigger the development of cirrhosis not only through the scarring that results from necrosis and inflammation but also through a direct effect of ethanol on the metabolism of collagen (Feinman and Lieber, 1972). This possibility is suggested by the observation that after alcohol administration, in rats or baboons, an increase in liver collagen can be detected chemically at the fatty liver stage before necrosis, inflammation or fibrosis is present. It is questionable, however, if this observation can be extrapolated to man. For example, the relative amount of collagen in the adult rat is one-third that of man and in fully developed rat cirrhosis, hepatic collagen is about equal to that of the normal human liver (Popper and Piez, 1978).

Increases in hepatic collagen have been associated with increases in peptidyl proline hydroxylase activity (Chen and Leevy, 1975) and decreased degradation and accumulation of variant forms of collagen (Hutterer, Eisenstadt and Rubin, 1970). Hutterer et al (1970) have also suggested a decreased rate of collagen catabolism rather than increased rate of synthesis distinguishes the irreversible phase of fibrosis from the reversible phase. An immunological mechanism has also been advanced (Zetterman et al, 1976).

However, it is generally recognised that liver cell necrosis is the most important stimulus for hepatic fibrosis and that this stimulation is due to the release

of substances from the necrotic cell which stimulate fibrosis (Popper and Piez, 1978).

1.2.7. Use of Animal Models

Human studies are limited by the genetic and environmental heterogeneity of the human population and by ethical considerations of human experimentation. However, the advantages of animal studies such as uniform population, standard conditions and wider range of permissible experiments do not necessarily out-weigh the disadvantages of human studies. This is of particular importance in studies of alcoholic liver disease which has a long development period, is accompanied by many biochemical changes and is associated with numerous other variables. In such cases, the usual limitations of animal studies of human disease, e.g., the extent to which the human disease can be reproduced in animals and the validity of extrapolation from the animal to the human, assume greater significance.

This point can be illustrated with reference to the rat. In human alcoholic liver disease, an early manifestation is the 'fatty liver', a term used to describe a variety of biochemical abnormalities including altered fat metabolism. These abnormalities are believed to be progenitors of more serious lesions which would eventually result in alcoholic hepatitis and altered fat metabolism is central to these changes. However, under normal conditions there is a wide difference in the dependence of man and rat on fat in their diet and consequently in

their metabolic adaption to the metabolism of this substance (Stanko, Mendelow, Shinozuka & Adibi, 1978).

Other important considerations are differences in collagen metabolism (1.2.6.) and period of development of the disease. Human alcoholic liver disease takes at least four years and usually much longer to develop and this development period cannot be reproduced in the rat. Therefore, in several crucial respects, there is a wide species difference between man and rat which means the rat cannot be considered an appropriate animal model for study of alcoholic liver disease.

One animal which does appear to have some of the important attributes required for an animal model of alcoholic liver disease is the baboon. This species is longer lived, phylogenetically closer to man than the rat and on feeding with a nutritionally adequate diet containing alcohol, the entire spectrum of alcoholic liver injury is produced (Rubin and Lieber, 1974). However, the use of this model in the present study is not feasible for a variety of reasons, one of which is that a four year period is required for development of the full spectrum of disease. The lack of a suitable animal model of alcoholic liver disease does not however preclude the use of animals to study specific aspects of the disease process.

Section 1.3.

An Alternative Approach

1.3.1. The Need for an Alternative Approach

The original impetus for this project arose from the observation that the onset and severity of alcoholic liver disease was in part dependent on factors other than the period and extent of alcohol abuse. The prime consideration then was to determine the relationship of two major such factors: nutritional deficiency and genetic predisposition, to the aetiology and morbidity of the disease. Therefore, it was decided to investigate the frequency of various genetic markers and certain aspects of nutritional status in alcoholic liver disease and alcoholic non-liver disease populations. The rationale, methods and results of these studies are described in sections 1.2., 2.2. and 3.1. However, from an analysis of the preliminary results of this study and from a greater appreciation of the practical difficulties associated with such a study, it became apparent that a comprehensive, single-centre and short-term study was unlikely to produce significant results. Among the difficulties associated with this study were the general problems of research studies involving human subjects, the diversity of the disease states represented by the term alcoholic liver disease, the multiplicity and interaction of incident factors, the heterogeneity of the alcoholic liver disease population in respect of age, sex, social class, living conditions, smoking, co-operativeness, etc., and the more specific problems of integrating such a complex study into a clinical environment.

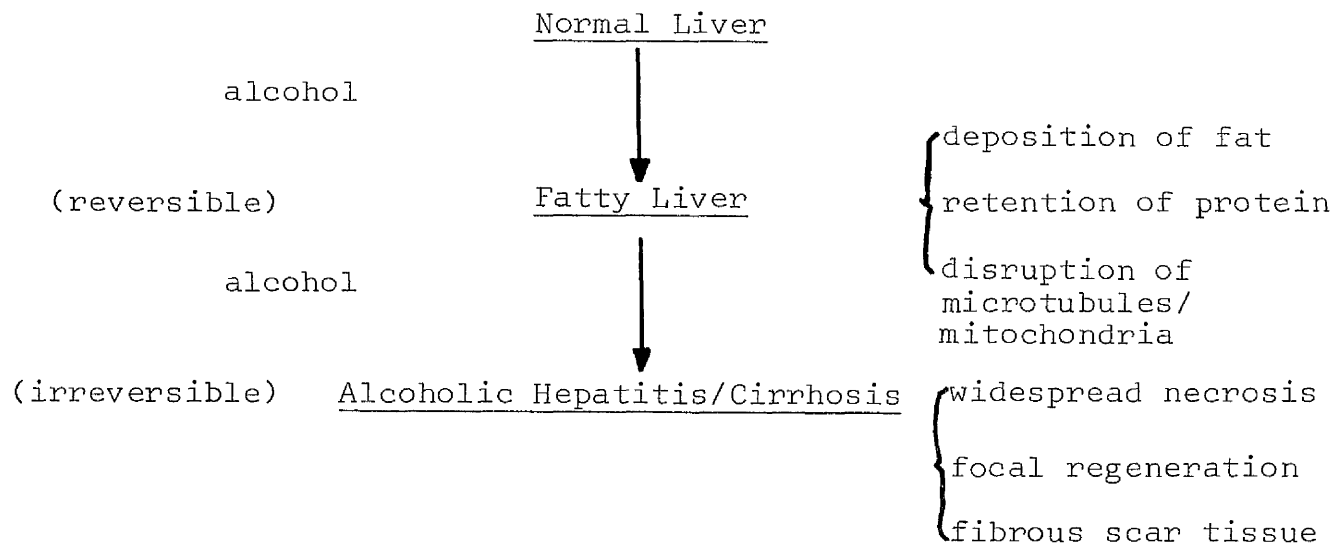
Such considerations determined that it would be more useful to narrow down the field of research to concentrate on one area directly related to the human situation but much more amenable to experimental manipulation than the human subject. With a suitable system it would be possible to investigate the effect of specific perturbations under controlled conditions. Such an approach would be concerned with elucidation of critical determinants in the development and progression of the disease rather than attempt to resolve the precise significance of major effector variables to the overall course of the disease.

Therefore, it was decided to choose one established variable observed in the alcoholic population, e.g., vitamin B₆ status, and study this intensively in order to determine cause and effect of variation in this parameter on a potentially crucial determinant of alcoholic liver disease. It was realised that the adoption of this fresh approach would limit the scope of the project but it was considered that this disadvantage would be outweighed by an increase in the power of the results.

1.3.2. A Simple Model of Alcoholic Liver Disease

In order to facilitate appraisal of alcoholic liver disease and determination of a suitable area for investigation, a simple model of the disease process was constructed. This is illustrated in fig. 2 and shows the progression from a normal liver which, on exposure to alcohol, develops into the fatty liver, a condition characterised

Fig. 2. A Simple Model of Alcoholic Liver Disease



at the cellular level by deposition of fat, retention of protein, disruption of microtubules and disruption of mitochondria.

The fatty liver thus represents a fairly serious state of cellular abnormality but it is a reversible condition, in contrast to the condition which develops on continued exposure to alcohol; alcoholic hepatitis or cirrhosis, which is irreversible. At some stage in the process, the reversible phase progresses into the irreversible phase and since this transition is crucial to the development of the disease, further attention was focused on the factors which might determine the transition. If we expand the 'fatty liver' stage of the model, fig. 3, and consider the factors which might govern, recovery, stability or progression of the disease process, it is apparent that the cellular disturbances present at this stage will result in increased cell death. Subsequent recovery or progression will depend on the response to this; either a normal increase in cell growth and regeneration, leading to recovery/stability, or an inadequate response leading to further necrosis and development of the disease process. Cell growth then, and in turn DNA synthesis is likely to be a key determinant in alcoholic liver disease.

1.3.3. DNA Synthesis in Alcoholic Liver Disease

Determination of DNA synthesis in alcoholic liver disease has been investigated using liver biopsy samples (Leevy, 1963, 1966), but the methods used limit interpretation of the results. With recent advances in techniques of primary

short term culture of hepatocytes (Demoise, Galambos and Falek, 1971; Gebhardt, Belleman and Mecke, 1978) it was hoped some of these limitations could be overcome. Accordingly, exploratory experiments were carried out to determine the feasibility of using such methods to determine parameters of cell growth in liver biopsy samples. The methods used in these experiments are described in section 2.2.9. Essentially, a suspension of isolated hepatic cells was prepared by disaggregation of finely chopped liver tissue of freshly sacrificed rats in medium containing collagenase and EDTA. Estimation of parameters of DNA synthesis in these freshly isolated cells was carried out by determination of acid-insoluble incorporation of the DNA precursor (^3H)-TdR using autoradiography of cytocentrifuge slide preparations. However, it was not possible from these experiments to determine appropriate conditions for obtaining or maintaining an adequate viable cell suspension which could be satisfactorily transferred to use of biopsy material.

As it was not possible to satisfactorily study cell growth and regeneration in the alcohol diseased liver directly, an indirect method was sought. This method was suggested by consideration of folate deficiency.

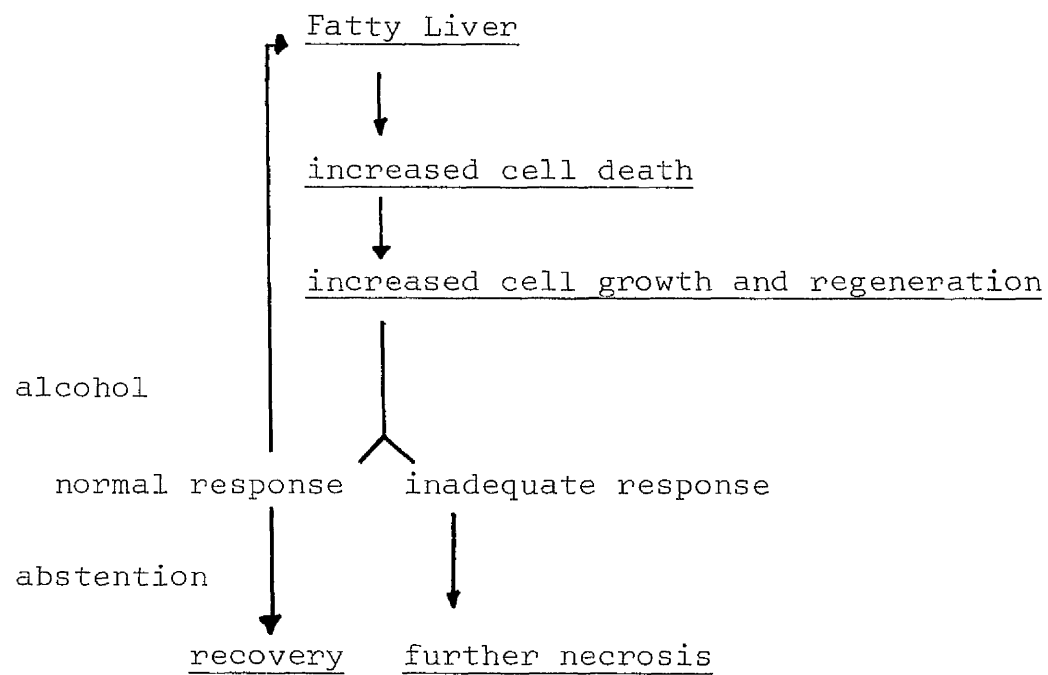
The main effect of folate deficiency is suppression of DNA synthesis. In the adult, the tissue most seriously affected is bone marrow and the most important clinical lesion is megaloblastic anaemia (Blakley, 1969). In folate deficient individuals the effects of deficiency

can be directly observed by examination of bone marrow tissue. However, Das and Hoffbrand (1970) have also demonstrated the effects of folate deficiency using a more accessible tissue, peripheral blood lymphocytes. The principle inherent in this observation, of using one cell type to investigate the effects of vitamin deficiency on DNA synthesis in another cell type was adopted and extended in the present study. This was to use peripheral blood lymphocytes as an indirect means of studying the effects of vitamin deficiency on cell growth and DNA synthesis in the alcohol diseased liver.

1.3.4. The Effect of Vitamin B₆ Deficiency on DNA Synthesis

The ubiquity of vitamin B₆ in cellular metabolism as well as the numerous manifestations of a B₆ deficiency has been mentioned previously (1.2.3.). If the limiting effect of a B₆ deficiency depends to a large extent on the B₆ requirement of a particular enzyme system in a specific tissue, then under different conditions the primary effect of a B₆ deficiency will be realised in different ways, reflecting the tissue and enzyme system most affected. Hence under different conditions the limiting effect of a B₆ deficiency might be; reduced activity of delta-aminolevulinic acid with reduced formation of protoporphyrin and the occurrence of sideroblastic anaemia or, reduced activity of serine hydroxymethyltransferase and consequent disruption of folate metabolism.

Fig. 3 The Fatty Liver



In the particular circumstances of alcoholic liver disease it is possible that the principal effect of a B₆ deficiency would be to reduce serine hydroxymethyltransferase activity and consequently, DNA synthesis and cell growth. Leevy (1966) has previously reported that chronic alcoholics rendered folate or pyridoxine deficient show defective incorporation of nucleic acid precursors into RNA and significantly impaired DNA synthesis and cell replication. This work utilized liver biopsy samples which are subject to sampling error and difficulties of obtaining even distribution of oxygen throughout the sampled tissue. In addition, it is not usually possible to carry out repeated biopsy sampling to assess progress of treatment. Therefore, development of a method which utilized peripheral blood lymphocytes to assess the effect of alcohol and vitamin deficiency on cell growth and DNA synthesis in the liver would offer significant advantages in investigation of alcoholic liver disease. As well as investigation of the role of reduced DNA synthesis in alcoholic liver disease, such a method would permit a more precise determination of the effect of B₆ deficiency on cellular metabolism.

Section 1.4.

Studies Utilizing the Phytohaemagglutinin Stimulated
Lymphocyte

1.4.1. Perspective

In 1960 Nowell demonstrated that an aqueous extract of the kidney bean, phytohemagglutinin (PHA) was able to produce large, dividing blast like cells in cultures of human peripheral blood. It was found that the particular cells stimulated were the small lymphocytes and that the morphological changes were preceded or accompanied by the induction of DNA synthesis and increased RNA and protein synthesis. Since then, a great deal of research has centred on the early events of lymphocyte activation with the aim of elucidating the physiological and biochemical mechanisms whereby the quiescent small lymphocyte is derepressed to become a metabolically active, dividing cell. Several of these early events are in fact not unique to lymphocytes but are known to occur in other systems in which gene derepression is taking place, e.g., regenerating liver cells.

The proliferative response of small lymphocytes to mitogens, such as PHA, or to foreign lymphocytes, as in the mixed lymphocyte reaction (MLR) has also been used as an in vitro model of the cell-mediated immune response (Bach, Segall and Zier, 1973). A further use has been to investigate the role of vitamin B₁₂ and folate deficiency in the development of pernicious anaemia and megaloblastic anaemia (Das and Hoffbrand, 1970; Hooton and Hoffbrand, 1977).

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These and other properties combine to make this system particularly attractive for studying certain aspects of alcoholic liver disease. Previously, the main application in this area has been to investigate immunological factors, the main finding being the identification of a depressed immunocapacity in alcoholic liver disease (Lundy et al, 1975). This deficiency has been related to the immunosuppressive effect of a B₆ deficiency (Robson and Schwarz, 1975; Axelrod, 1971). This work and a novel approach to alcoholic liver disease utilising the PHA stimulated lymphocyte are discussed in later sections. Essentially the novel approach involves the concept of a systemic, marginal, B₆ deficiency resulting in an acute or chronic B₆ deficiency in a particularly stressed organ. In this case, the lymphocyte subjected to PHA is considered as a model for the liver subjected to alcohol. The main distinction between this and previous work is that here, the lymphocyte is employed as an in vitro model of the dividing liver cell, rather than of the immune response, and the effect of a B₆ deficiency is related to a specific cellular function, DNA synthesis, rather than to overall blastogenesis.

1.4.2. Advantages and Disadvantages

As previously indicated, (section 1.3.3.), the main reason for using the PHA stimulated lymphocyte to study the relationship between vitamin B₆ deficiency and DNA synthesis in alcoholic liver disease is that the value of this system has previously been demonstrated in studying the relationship of folate and vitamin B₁₂ deficiency to haematosuppression. The principal objection to the use of

lymphocytes as a means of investigating the alcohol diseased liver is the wide disparity in differentiation and function of these tissues. Therefore, there is no direct reason why changes in hepatic tissue should be reflected in the small lymphocyte cell population. However, the changes which accompany growth stimulation in lymphocytes are similar to those which occur during a switch to a more rapid rate of growth in other cell types, e.g., in the remaining hepatocytes after partial hepatectomy, in kidney cells after the removal of the opposite kidney or in confluent fibroblasts after subculture from a confluent monolayer (Ling and Kay, 1975). This suggests that although there are significant differences between hepatic tissue and lymphocytes, there are sufficient common characteristics to support the use of PHA stimulated lymphocytes as a valid model system.

Further advantages of this PHA stimulated lymphocyte cell culture system include the facility to control the culture medium concentration of serum and specific nutrients, etc., and therefore investigate the effect of specific perturbations under controlled conditions, and, the possibility of obtaining serial samples from an individual either to monitor a course of treatment or to compare pre- and post-treatment responses. These advantages, allied to the reproducibility of PHA stimulation of lymphocytes and the availability of autologous serum extends the range of investigations that it is possible to carry out. In particular, the use of autologous serum provides the opportunity to investigate the effect of cytotoxic or

immunogenic factors in alcoholic liver disease

(Lieber, 1977).

One limitation of the PHA stimulated lymphocyte as a model of alcohol diseased liver regenerative capacity and DNA synthesis is that it excludes the effect of the immediate cellular and intercellular environment as it affects regeneration. For instance, it does not preclude the possibility that a vitamin B₆ deficiency might arise and be expressed only in the liver due to the local effect of a metabolite of ethanol such as acetaldehyde. Experiments could be designed to determine the effect of such a factor but control of other extracellular factors is more intractable. In the presence of active liver cell damage where the proliferative response is impaired, instead of formation of new tissue there is deposition of fibrous, collagen scar tissue which disrupts the normal vascular architecture and reduces hepatic blood flow (Lieber, 1977). Even if alcohol assault ceases and nutritional deficiency corrected, any subsequent regenerative response is necessarily inhibited by the enveloping fibrous tissue and reduced blood supply. The same limitation, however, strengthens the model as it relates to a systemic vitamin sufficiency being expressed as a local deficiency in a stressed organ.

This study is primarily concerned with investigation of the effect of vitamin B₆ deficiency on DNA synthesis and thus on hepatic regenerative capacity. However, the association of vitamin B₆ deficiency and impairment

of the immune response (Robson and Schwarz, 1975) has also been linked to restriction of nucleic acid synthesis (Axelrod, 1971). Thus use of the PHA stimulated lymphocyte system in this study may be considered a means of investigating both hepatic and immune response aspects of alcoholic liver disease. Overall, it is clear that with certain qualifications, in particular an appreciation of the limitations, the model system imposes on interpretation of results and given that more direct methods such as use of biopsy material are not available, the indirect approach adopted in this study is justifiable.

1.4.3. Use of Vitamin B₆ Deficient Rats

As the reported vitamin B₆ deficiency of alcoholics is relative rather than absolute, as there are no clearly defined symptoms which can be specifically attributed to vitamin B₆ deficiency and since the alcoholic population is so heterogeneous with respect to period and extent of alcohol abuse, diet and other effector variables, a test system was sought where an unequivocal vitamin B₆ deficiency could be investigated. As a number of reports suggested that such a deficiency state could be produced in rats by feeding a diet deficient in pyridoxine HCL (Reynolds, 1978; Sloger, Scholfield and Reynolds, 1978; Lumeng, Ryan and Li, 1978) and since rat lymphocytes are also responsive to PHA (Ling and Kay, 1975) it was decided to carry out parallel experiments using vitamin B₆ deficient rat lymphocytes.

It was hoped to use such experiments to test the validity of certain aspects of the PHA stimulated lymphocyte model. In the rat, the effect of B₆ deficiency could be assessed in both lymphocytes and liver, perhaps by use of a double-labelling technique, and this information could be used to assess the validity of the lymphocyte model in man. Stressing the vitamin B₆ deficient rat liver by drug induced hepatic regeneration was also considered as a closer approximation to the alcohol stressed human liver. While it was realised that experimental manipulation of the whole animal to study parameters of growth in a specific cell population introduces uncontrolled variables such as alteration in blood chemistry and hormonal balance (Ling and Kay, 1975), which may interfere with or confound the effects of vitamin or other nutritional deficiency on the system, it was considered that such studies would still provide valuable information. For this reason, a group of rats were fed a vitamin B₆ deficient diet for a period of six weeks.

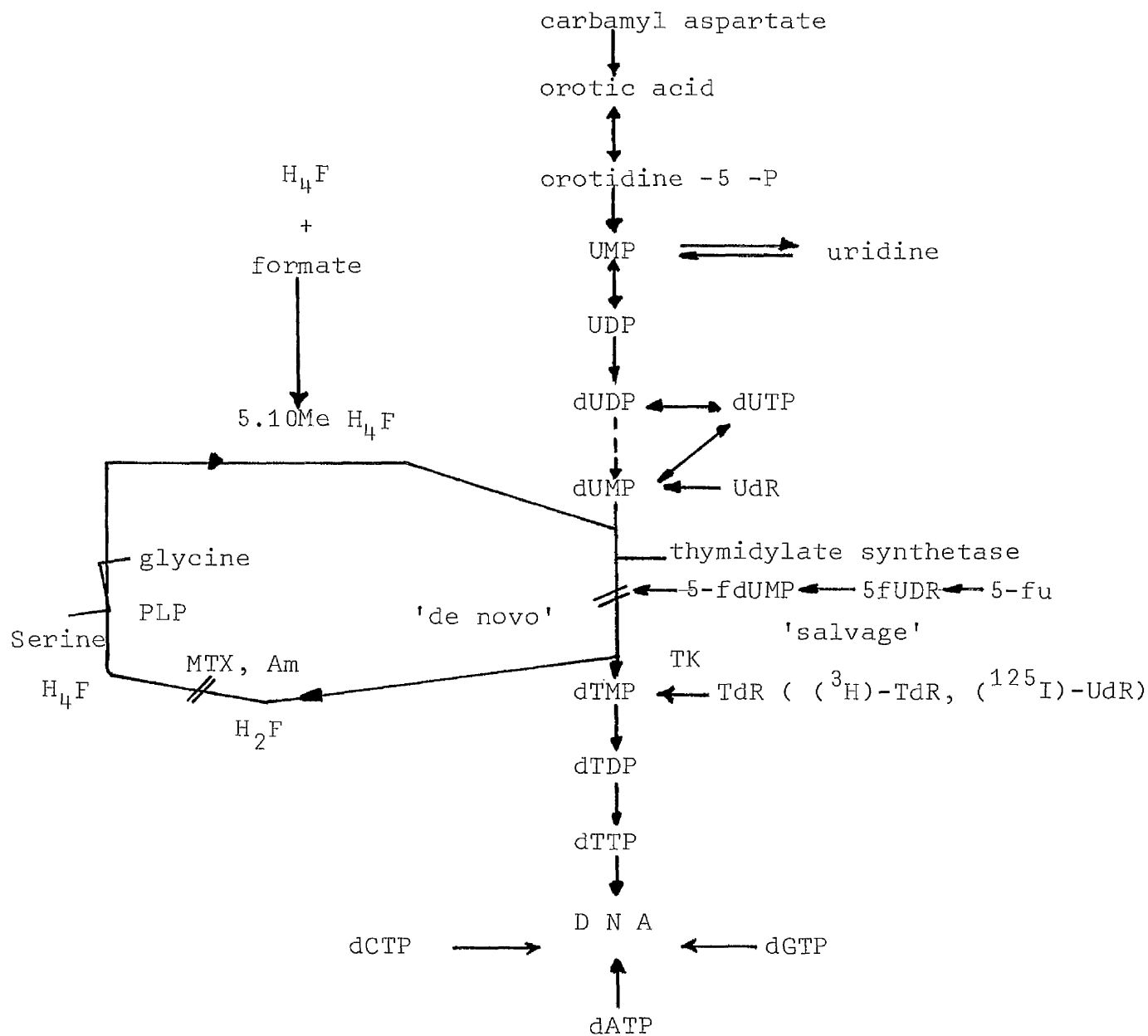
Unfortunately, for a number of reasons, it was not possible to satisfactorily complete these experiments. These reasons included the difficulty of obtaining serial aseptic blood samples from the rat and the problem of establishing the most appropriate culture conditions to obtain a satisfactory response of rat lymphocytes to PHA. When blood samples were collected from the cervical blood vessels, or from the rat tail vein, it was not possible to obtain a satisfactory culture due

to bacteriological contamination. This problem was not solved by washing the lymphocyte suspension in a balanced salt solution containing high concentrations of antibiotic. Therefore, microculture of rat lymphocytes from whole blood was unsuccessful and it was not possible to obtain a sufficient number of lymphocytes from a small blood volume for successful culture of a lymphocyte preparation. In addition to these problems, it became apparent that, probably due to gastrointestinal bacteria (Sumi, et al, 1977), the pyridoxine HCL deficient diet failed to produce a vitamin B₆ deficiency state. It is possible that a more successful approach to the production of an absolute vitamin B₆ deficiency state might involve the use of a vitamin B₆ inhibitor such as 4¹-deoxypyridoxine.

1.4.4. Determination of DNA Synthesis

A scheme illustrating known reactions in the biosynthetic pathway of DNA thymine is given in fig. 4. Two pathways are shown; the de novo pathway and the salvage pathway. Also shown is the site of action of the inhibitors Am, MTX and 5-fu and the involvement of folate and vitamin B₆ coenzymes. Although all four deoxyribose nucleoside triphosphates must be available for the synthesis of DNA chains to proceed, thymidylate is a unique component of DNA and therefore the formation of thymidine phosphates is a strategically important part of the complex metabolic system which provides nucleotide building blocks for DNA replication. In most systems studied, the activity of thymidine kinase is low in non-growing cells but increases just before the onset of DNA synthesis. Although the physiological significance of thymidine kinase and the salvage pathway has not been absolutely defined, thymidine kinase has many of the attributes of a rate-determining enzyme for DNA biosynthesis (Adams et al, 1976) including sensitivity to feedback inhibition by dTTP, and this has led to the idea that thymidine kinase is involved in the control of initiation of DNA synthesis. However, it is more likely that these controls are concerned with regulation of DNA precursor synthesis rather than DNA synthesis itself.

Fig. 4 Pathways for Synthesis of DNA Thymine *



* modified from Das et al (1978); Wolberg (1971);
Blakley (1969) and Hurst (1980)

In animal cells TdR is specifically incorporated into DNA thymidylate contributing neither to DNA deoxycytidylate nor to pyrimidine ribonucleotides. Since incorporation into DNA is the only major anabolic fate of labelled thymidine, DNA synthesis can be measured without isolation of DNA; only the removal of unused substrate and acid-soluble metabolites being necessary. Thymidine is incorporated into DNA via the salvage pathway which employs the enzyme thymidine kinase. Although the rise in activity of this enzyme prior to DNA synthesis and its sensitivity to allosteric controls indicates that thymidine kinase is important in the deoxyribonucleotide economy of cells (Taylor-Papadimitriou and Rozengurt, 1979), thymidine is not an obligatory intermediate in the biosynthesis of the thymidine phosphates and, under certain circumstances, the mutational loss of this enzyme is not lethal.

Despite this apparent redundancy, in certain tissue culture conditions, the ubiquity of thymidine kinase in proliferating cells, mitochondria, and in viral infection suggest that the role of thymidine kinase is not solely that of an enzyme which enables the cells in the body to synthesise DNA more economically by maximising efficient utilisation of endogenous nucleotides which arise from nucleic acid degradation or from the diet.

Some indication of a more extensive role may be derived from the observation that exogenous thymidine is required

in certain cell lines and in some freshly explanted cells when the medium folate concentration is comparable to that of serum (Taylor-Papadimitriou and Rozengurt, 1979). It was also demonstrated that the growth stimulating effect of 'feeder cells' is partly due to their supplying exogenous thymidine. The culture medium used in these experiments was medium 199 which has a folate concentration of 0.01 mg/l compared with MEM which has a folate concentration of 1.0 mg/l.

The importance of thymidine kinase, other than as a means for the incorporation of (^3H)-thymidine to measure DNA synthesis, has been considered because it is realised that salvage thymidylate synthesis competes with de novo thymidylate synthesis and therefore that the relative contribution of each pathway to total thymidylate synthesis must be considered in experiments in which the incorporation of (^3H)-thymidine is used to measure DNA synthesis. This is particularly relevant when the possible effect of a folate or vitamin B₆ deficiency on the de novo pathway of thymidylate synthesis is considered.

In bacteria and higher organisms de novo synthesis of DNA thymine proceeds by methylation of the uracil derivative, dUMP. This reaction requires the enzyme thymidylate synthetase and the methyl group is supplied by the folate coenzyme (5, 10) methylene tetrahydrofolate (5, 10 MeH₄F). The methyl group of this folate

compound is derived from precursors such as formate, formaldehyde or the hydroxymethyl group of serine. The relative contribution of each of these precursors is not known either under normal conditions or under vitamin B₆ deficient conditions. However, certain observations noted by Blakley (1969) are relevant. He reports that cells of E Coli growing exponentially in a simple synthetic medium derive the methyl group of thymine exclusively from carbon-3 of serine when this compound is supplied in the absence of other extracellular supplements. When 3-(¹⁴C)-serine or 2-(¹⁴C)-serine or 2-(¹⁴C)-glycine is administered to rats, there is extensive incorporation of labelled carbon into the methyl group of DNA thymine and this incorporation does not involve formate or formyl derivatives as obligatory intermediates. Formate and formaldehyde have also been shown to be precursors of thymine methyl in rats, rabbit bone marrow in vitro and Ehrlich ascites cell in culture.

Although formaldehyde is formed by the oxidation of sarcosine and dimethylglycine in liver preparations and formate can arise from the thioclastic fission of pyruvate (Blakley, 1969), neither can be regarded as major metabolites and so it is likely that the major physiological source of the thymine methyl group is the 3-C of serine. As the formation of 5, 10 methylene H₄F from serine requires the pyridoxal phosphate dependent enzyme serine hydroxymethyltransferase and the formation of 5, 10 methylene H₄F is crucial to de novo

thymidylate synthesis, it is possible that the activity of this enzyme and hence DNA synthesis would be specifically reduced by vitamin B₆ deficiency.

The significance of the enzyme serine hydroxymethyltransferase is further illustrated by the observation that during the log phase of growth of human fibroblast cells in culture it was shown that the specific activity of each of the enzymes dihydrofolate reductase, serine hydroxymethyltransferase and thymidylate synthetase rose by a factor of 20, 4 and 2 (Rosenblatt and Erbe, 1973). The implication being that these enzymes form a cycle supplying methyl groups for de novo thymidylate synthesis. Serine hydroxymethyltransferase was also found to be present in non stimulated human lymphocytes and its activity in these cells rose several fold following PHA stimulation (Haurani and Masse, 1977), and, when 3-(¹⁴C)-serine and (¹⁴C) formate were used as precursors for DNA thymine in PHA stimulated human lymphocytes it was found that changes in the uptake of each label were similar in magnitude and occurred at the same time (Wolberg, 1971).

1.4.5. Effects of Vitamin Deficiency

In stimulated lymphocytes, or bone marrow cells, of patients with megaloblastic anaemia due to folate or vitamin B₁₂ deficiency, there is an increase in (³H) TdR incorporation into DNA compared with corresponding cells of these patients treated with the appropriate vitamins either in vivo or in vitro (Das and Hoffbrand, 1970).

From experiments carried out on isolated nuclei, it was shown that this increase was not just the result of an increased specific radioactivity of the dTTP pool due to inhibition of de novo thymidylate synthesis and an increase in thymidine kinase activity but was also partly due to a real increase in DNA synthesis (Hooton and Hoffbrand, 1977).

When deoxyuridine is added to normal PHA stimulated lymphocyte cultures prior to incubation with (^3H)-TdR it is found that incorporation of (^3H)-TdR into DNA is reduced, or suppressed, to less than 10% of that in comparable control experiments. However, when UdR is added to PHA stimulated lymphocyte cultures derived from patients with folate or vitamin B12 deficiency, this UdR suppression of (^3H)-TdR incorporation is less marked (Das and Hoffbrand, 1970).

Although a precise biochemical basis for this phenomenon has not been described, it has been utilised as the basis of a clinical test for determination of vitamin B₁₂ and folate deficiency (Herbert et al, 1973).

The most commonly cited explanation of this UdR suppression effect is that, under normal conditions, addition of UdR stimulates synthesis of dTMP and hence dTDP and dTTP. The consequent increase in dTTP levels resulting in allosteric inhibition of thymidine kinase, the enzyme responsible for salvage synthesis of thymidylate and hence incorporation of (³H)-TdR (Herbert and Das, 1976). However, in the presence of a folate deficiency, or a vitamin B₁₂ deficiency leading to a folate deficiency, it is believed that the efficiency of the de novo pathway is reduced such that addition of UdR fails to stimulate dTMP synthesis to the same extent as in normal circumstances and consequently allosteric inhibition of thymidine kinase is reduced and (³H)-TdR incorporation is suppressed to a lesser extent (Herbert and Das, 1976). Thus a difference in the UdR suppression effect was related to differences in the efficiency of the de novo pathway caused by variation in folate coenzyme status.

Despite the uncertainty surrounding the biochemical basis of this test its use was justified on the basis that it did discriminate between vitamin sufficiency/deficiency on a functional basis and gave similar results to other tests which were more invasive, more indirect and less

convenient (Wickramasinghe and Longland, 1974).

Interest in the possible occurrence of a vitamin B₆ deficiency in alcoholic liver disease and in the hypothesis that such a deficiency would be expressed primarily through an effect on cellular DNA synthesis led to an examination of how such an effect might be mediated. From a consideration of known metabolic pathways of deoxyribonucleotide synthesis and particularly of thymidylate synthesis it was apparent that the pyridoxal phosphate dependent enzyme, serine hydroxymethyltransferase might represent a limiting metabolic block in a vitamin B₆ deficiency state. The central role of this enzyme in the supply of active folate coenzymes for the de novo thymidylate pathway is apparent from fig. 4.

If a vitamin B₆ deficiency did occur and was mediated through an effect on serine hydroxymethyltransferase it would most likely be expressed as a folate deficiency and would result in alteration of UdR suppression characteristics similar to that of an a priori folate deficiency. Thus it was hoped to be able to extend the use of the UdR suppression test for determination of folate and vitamin B₁₂ deficiency to the study of vitamin B₆ deficiency.

Acceptance of the validity of the UdR suppression test has been restricted by several observations which do not appear to be compatible with the concept of a stimulus in de novo thymidylate synthesis leading to

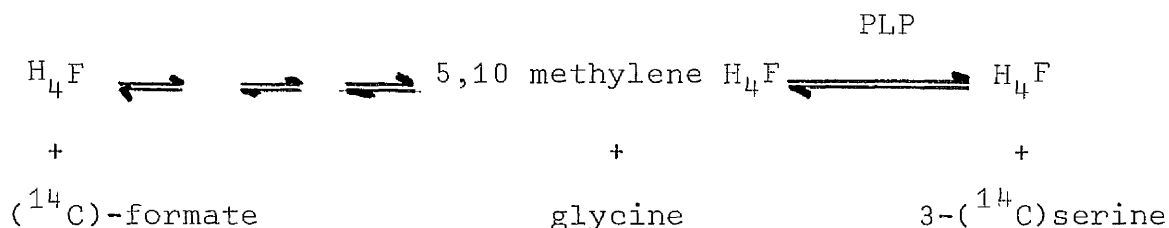
allosteric inhibition of thymidine kinase. For example, no consistent rise in dTTP concentration was observed in normal cells after exposure to deoxyuridine (Ganeshaguru and Hoffbrand, 1978) and, the addition of non radioactive TdR failed to decrease the incorporation of (^3H)-UdR into DNA of normal bone marrow cells (Beck, 1975). However, in other experiments, non radioactive TdR decreased the incorporation of (^3H)-UdR into DNA of PHA stimulated lymphocytes by more than 98% and it has been suggested that this discrepancy reflects the minor role of the salvage pathway in normal cells (Herbert and Das, 1976; Herbert and Das, 1979). It has also been shown that radioactive deoxyuridine is actively incorporated into DNA by normal bone marrow cells and that this process is inhibited in megaloblastic bone marrow cells (Metz et al, 1968; Ganeshaguru and Hoffbrand, 1978). The observation that increasing concentrations of added thymidine caused progressive inhibition of subsequently added (^3H)-UdR incorporation into DNA (Herbert and Das, 1979)(without inhibition of DNA synthesis, because decreasing (^3H)-UdR incorporation was paralleled by increasing incorporation of (^{14}C)-TdR into DNA) was considered to demonstrate reciprocity of the salvage and de novo pathways of DNA-thymine synthesis with control being mediated by dTTP, the common end product of both pathways, via feedback inhibition (Herbert and Das, 1976).

The fact that no consistent hypothesis has been established indicates that there has been a lack of full appreciation of the factors involved, e.g., cell type, medium folate concentration and the absolute rate of DNA synthesis. In addition, other possibilities such as increased degradation of thymidine and competitive substrate inhibition of thymidine kinase by deoxyuridine do not appear to have been fully considered.

The effects of a folate deficiency can also be produced using the inhibitors methotrexate and 5-fluorouracil. As in the case of folate deficiency, interruption of the flow of thymidylate by methotrexate (see fig. 4) has been shown to cause an increase in (^3H)-TdR incorporation and of (^3H)-TTP incorporation in isolated nuclei (Hooton and Hoffbrand, 1977) and radioactive deoxyuridine incorporation into DNA is inhibited by methotrexate in PHA stimulated normal human lymphocytes (Hoffbrand et al, 1973). In human tumour tissue in vitro, 5-fluorouracil was shown to cause a decrease in formate incorporation which was associated with a reciprocal increase in thymidine incorporation (Wolberg, 1969).

The identification of a functional folate deficiency in various disease states has also been investigated, using non stimulated human lymphocytes, by measurement of the incorporation of (^{14}C)-formate label into serine in the presence of excess glycine (Ellegaard and Esmann, 1972; Ellegaard and Esmann, 1973).

A functional folate deficiency being diagnosed by a decreased rate of (^{14}C)-serine synthesis according to the following scheme.



Conceivably this assay system could equally well be adapted to investigate the possibility of a functional vitamin B₆ deficiency. However, in two major respects, the stimulated lymphocyte is a more appropriate model. Like the normal adult liver, lymphocytes normally represent a quiescent cell population (Norman, et al, 1968) hence a vitamin deficiency affecting DNA synthesis would not be expressed in such a cell population. However, in cells actively synthesising DNA such as regenerating liver cells or stimulated lymphocytes a vitamin deficiency affecting DNA synthesis could be more readily identified. Similarly, the stimulated folate deficient lymphocyte has been shown to manifest the same biochemical abnormalities as the megaloblastic cell (Das and Hoffbrand, 1970) and therefore seems a more appropriate cell system for investigation of a possible vitamin deficiency in the stressed alcohol diseased liver. Furthermore, determination of a functional parameter of such strategic significance as DNA synthesis seems a more valid indicator of vitamin

status than determination of a parameter of more limited significance such as amino acid inter-conversion.

In folate deficient, DNA synthesising, cells, the inhibition of the de novo pathway due to the reduced supply of active folate coenzyme for thymidylate synthetase is likely to result in an adaptive shift in the relative utilisation of de novo and salvage pathways for dTMP synthesis. Therefore, when (3H)-TdR incorporation is used to assess the rate of DNA synthesis, the apparent rate will be higher than an equivalent rate of DNA synthesis under folate sufficient conditions. If a vitamin B₆ deficiency affected DNA synthesis at the level of serine hydroxymethyltransferase similar considerations regarding the assessment of the rate of DNA synthesis using (³H)-TdR under vitamin B₆ deficient conditions would apply.

A possible approach to determination of the respective contributions of the de novo and salvage pathways to thymidylate synthesis and hence to determination of the actual effect of a B₆ or other vitamin deficiency on DNA synthesis is to uncouple the de novo contribution using aminopterin, methotrexate or 5-fluorouracil or to suppress the salvage contribution using deoxyuridine. Aminopterin and methotrexate are folate analogues which effectively block all reactions involving the enzyme dihydrofolate reductase (Blakley, 1969; Paul, 1975). This enzyme is involved in the regeneration

of H_4F and hence 5, 10 methylene H_4F which is the coenzyme employed by thymidylate synthetase in the methylation of dUMP to form dTMP. The concerted operation of dihydrofolate reductase, serine hydroxymethyltransferase and thymidylate synthetase as a cycle producing folates for pyrimidine synthesis is indicated by the co-increase of these enzymes in the log phase of the culture cycle of human fibroblasts (Rossenblatt and Erbe, 1973). It may also be deduced from the observation that whereas dTMP synthesis in the presence of excess H_4F is unaffected by aminopterin, synthesis in the presence of a catalytic amount of H_4F and a supply of NADH is greatly decreased by aminopterin due to inhibition of the aminopterin sensitive enzyme dihydrofolate reductase (Blakely, 1969).

In the presence of this block the cells are made dependent on exogenous thymidine and its phosphorylation by thymidine kinase. Since this enzyme is thought to be involved in the control of DNA synthesis (Herbert and Das, 1979) and since its activity is usually associated with the rate of growth of an organism or organ, it is conceivable that thymidine incorporation in the presence of aminopterin would be a more appropriate index of growth than thymidine incorporation alone, where the efficiency of the de novo pathway for thymidylate synthesis is affected by a B_6 or other vitamin deficiency, or excess.

1.4.6. Other Uses of the PHA Stimulated Lymphocyte

The majority of studies using either PHA stimulated peripheral blood lymphocytes or the mixed lymphocyte reaction have focused on their immunological significance (Leevy et al, 1975 a, b; Robson and Schwarz, 1975). Very often these studies have relied on the acid insoluble incorporation of labelled thymidine to arrive at a stimulation index or an index of DNA synthesis (Ling and Kay, 1975) and the possibility that such studies have a wider application or that the assay system is sometimes inappropriate is not always realised. From studies of the incorporation of various precursors into DNA-thymine or stimulated lymphocytes it has been found that the incorporation of labelled thymidine does provide an adequate assessment of total precursor incorporation (Wolberg, 1971). However, consideration of the alternative pathways of DNA thymine synthesis and their respective dependence on folate, B₁₂ and B₆ coenzymes indicates that the thymidine incorporation index might not reflect total DNA synthesis in cells deficient in one or more of these coenzymes. Another factor not sufficiently appreciated in most studies is that standard cell culture medium contains pyridoxine HCl or pyridoxal HCl and hence would be expected to correct any pre-existing B₆ deficiency and so confound observations based on B₆ status.

When previous work relating to vitamin B₆ deficiency and lymphocyte stimulation was surveyed, certain inconsistent observations and anomalous results became apparent. Herbert et al (1973) detailed the analysis of an alcoholic patient (representative of a particular group of such patients) whose serum levels of vitamin B₁₂ and folate were not unequivocally low yet whose bone marrow showed megaloblastic change and whose UdR suppression test was abnormal. In addition this test was markedly corrected by in vitro addition of vitamin B₁₂. These observations indicated that the patient had true tissue vitamin B₁₂ deficiency despite normal serum B₁₂ folate levels. The authors suggested that the serum vitamin elevation in the face of tissue deficit was brought about by release into the serum of abnormal vitamin binding proteins from the damaged liver and that this abnormal binding reduced the availability of vitamin for normal cellular metabolism (Herbert and Tisman, 1975). While this hypothesis may be correct, the data is also consistent with a vitamin B₆ deficiency in these patients. The fact that an abnormal UdR suppression test was corrected by in vitro addition of vitamin B₁₂ is not necessarily in conflict with this latter hypothesis as additional vitamin B₁₂ might well be expected to cryptically correct a vitamin B₆ deficiency.

The effect of vitamin B₆ deficiency on cellular immunity has been investigated in rats (Robson and Schwarz, 1975).

The method used was to measure the incorporation of (^3H)-TdR in the mixed lymphocyte reaction. The reduced incorporation in the reaction between thoracic duct lymphocytes of B_6 deficient rats compared with that of normal rats indicated that B_6 deficient rats had a reduced cell-mediated immune response. While the overall conclusion may be correct, and is consistent with in vivo correlates of lymphocyte function, consideration of the known involvement of PLP in de novo thymidylate synthesis indicates that it would be inappropriate to rely solely on (^3H)-TdR incorporation via the salvage pathway to assess the immune response. Axelrod (1971), also from animal studies, concluded that a vitamin B_6 deficiency resulted in a marked reduction in the production of antibody-forming cells following antigenic stimulation and that this was secondary to a restriction of nucleic acid synthesis due to a reduced supply of C_1 units from serine.

In studies of alcoholic liver disease patients, Leevy et al (1975), assessed cell mediated lymphocyte reactivity at different stages of the disease process and found only slight differences. However, Lundy et al (1975), reported an impaired immunologic status in alcoholic patients which was reflected in a reduced response of lymphocytes of alcoholics to PHA stimulation. Both studies used (^3H)-TdR incorporation to determine reactivity or stimulation and the possible effect of specific vitamin deficiencies on this test method do

not appear to have been considered.

1.4.7. Principal Objectives of Study

From a consideration of alcoholic liver disease, the metabolism of ethanol, other contributory factors and of possible approaches to a more precise elucidation of the role of these factors, it was decided to adopt a novel approach. This novel approach utilised the phenomenon of PHA stimulation of lymphocytes and it was hoped that lymphocytes of alcoholic liver disease subjects, thus stressed, might reflect, to a certain extent, changes in the alcoholic liver. The role of one possibly significant factor, vitamin B₆ deficiency, was of particular interest because of its potentially crucial role in nucleic acid biosynthesis. The occurrence of a relative B₆ deficiency in the alcoholic liver disease population had been determined from measurement of serum levels of B₆ coenzymes but such methods were not completely reliable and it was not possible to attribute a functional defect to this postulated B₆ deficiency.

Because lymphocyte PHA responsiveness is reduced in disease and in alcoholic liver disease and is also very variable between individuals, experiments were carried out with an internal control, where lymphocyte response to culture in different conditions, essentially B₆ sufficient or B₆ deficient, was assessed. Although vitamin B₆ is important in a number of areas of metabolism, by analogy with the effect of a folate deficiency, it was thought that a particularly susceptible enzyme would be serine hydroxymethyltransferase; a reduction in the

activity of which would result in a cryptic folate deficiency. Therefore, various experiments were designed to determine such an effect.

As the reported B₆ deficiency of alcoholics is relative rather than absolute, as there are no clearly defined symptoms which can be specifically attributed to vitamin B₆ deficiency and since the alcoholic population is so heterogeneous with respect to period and extent of alcohol abuse, diet and other effector variables, a test system was also sought whereby an unequivocal absolute vitamin B₆ deficiency could be investigated. Two methods were used to achieve such a result, one was to feed rats a vitamin B₆ deficient diet and the other was to use the vitamin B₆ inhibitor 4-deoxypyridoxine.

The final objective of this study was to determine the effect of vitamin B₆ deficiency on aspects of cellular metabolism other than thymidylate synthesis. For example, vitamin B₆ coenzymes are essential for the interconversion of and the biosynthesis of certain amino acids and it is possible that limitation of growth and regeneration could occur at the level of protein synthesis rather than nucleic acid synthesis, though one might expect this only to occur as a result of combined vitamin and protein malnutrition. Similar considerations could apply to a vitamin B₆ deficiency affecting purine synthesis, porphyrin synthesis or lipid synthesis.

The principal objectives of this study then were to determine the occurrence and the effect of vitamin B₆ deficiency in alcoholic liver disease and to determine the effect of vitamin B₆ deficiency on certain aspects of cellular metabolism.

CHAPTER 2

MATERIALS AND METHODS

Section 2.1.

Materials

2.1.1. Experimental Subjects

Three groups of subjects were investigated in this study. The clinical, biochemical and histological characteristics of each of these groups were as follows.

Alcoholic Liver Disease Group

1. Several years' history of excessive alcohol consumption. Histories of alcoholics' alcohol consumption are known to be unreliable (Brunt et al, 1974). This means that it is impossible to obtain accurate estimates of alcohol consumption. Nevertheless, it is usually possible to establish an approximate history of excessive consumption, e.g., more than 80g of ethanol per day for a period of four years.
2. Marked hepatomegaly.
3. Histological evidence of fatty change, alcoholic hepatitis or cirrhosis.
4. Abnormally high serum transaminase levels, i.e., AST and ALT levels greater than 40 I.U. per litre.

Alcoholic Non-Liver Disease Group

1. Several years' history of excessive alcohol consumption.
2. Where a liver biopsy was carried out, no evidence of fatty change, alcoholic hepatitis or cirrhosis.
3. None or only slight hepatomegaly.
4. Normal or only slightly raised serum transaminase levels, i.e., AST and ALT levels less than 40 I.U. per litre.

Control Group

Members of this group included both hospital patient and hospital staff volunteers with no evidence of excessive alcohol consumption or vitamin deficiency associated disease. The clinical diagnoses of the patients in this group included: chronic obstructive airways disease, nervous dyspepsia, abdominal pain, gastric ulcer, nephrotic syndrome, hiatus hernia and irritable bowel syndrome.

General exclusion criteria for all groups were: prior treatment with vitamin supplements, evidence of recent myocardial infarction, primary biliary cirrhosis, cryptogenic cirrhosis, gastric or bronchial carcinoma and age less than 21 years. The clinical decisions concerning diagnosis of alcoholic liver disease, requirement for liver biopsy and diagnosis of alcoholic non-liver disease, etc., were made by Dr. A.D. Beattie, or other member of the hospital medical staff.

2.1.2. Cell Culture Media, Materials

Gibco Bio-Cult Ltd., Paisley

a) Modified MEM (1)

Minimal Essential Medium, with Hanks' Salts,
with L-Glutamine without Pyridoxal HCl.

b) Modified MEM (2)

Minimal Essential Medium, with Hanks' Salts, with
L-Glutamine without Pyridoxal HCl and with a
folate concentration of 0.01 mg/l.

c) Complete Medium

MEM - Minimal Essential Medium with Hanks' Salts,
with L-Glutamine.

d) Balanced Salt Solution

HBSS, Hanks' Balanced Salt Solution (10X)

e) Amino Acid Concentrates

MEM Amino acids solution (50X) with L-Glutamine
MEM Non essential amino acids (100X)

f) Vitamin Concentrates

MEM Vitamins Solution (100X)

g) Antibiotic-antimycotic Solution (100X)

h) Sodium Bicarbonate Solution (NaHCO_3) 7.5%

i) Folic Acid

j) Pyridoxal HCl

k) Phytohemagglutinin (M Form) Lyophilised

l) Tubes for Lymphocyte Culture (38 x 12.5 mm)

Evans Medical Ltd., Speke, Liverpool

Heparin, preservative free (1000 units/ml)

2.1.3. Chemicals, Biochemicals and Miscellaneous Items

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Aminopterin (4-aminofolic acid; 4 aminopteroyl
glutamic acid)

Bovine Serum Albumin

2 - deoxyuridine

4 - deoxypyridoxine HCl

5-fluorouracil

formic acid

histopaque

trypan blue

tyrosine apodecarboxylase

isonicotinic acid hydrazide (ISONIAZID)

hemin (Bovine, Type 1)

deoxyribonucleic acid, sodium salt Type III from
Salmon testis

Vestric, Alma Street, Glasgow

Sodium Metrizoate Sol'n (32.8% w/v)

Pharmacia Fine Chemicals, AB - Uppsala, Sweden

Ficoll 400

Millipore U.K. Ltd., London

Swinnex filters (0.22 um)

Millex sterile disposable filter units. (0.22 um)

BP Nutrition (UK) Limited, Stepfield, Witham, Essex

Pyridoxine deficient rat diet

Sterilin Ltd., Teddington, Middlesex

Universal containers (30 ml)

disposable test tubes (10 ml) round base with stoppers

disposable test tubes (12 ml) conical base with stoppers
(sterile)

plastic insert vials

Boehringer Mannheim GMBH

UV test kits for GOT (aspartate aminotransferase) and
GPT (alanine aminotransferase)

Lederle Laboratories, Gosport, Hampshire

methotrexate sodium

BDH Chemicals Ltd., Poole, Dorset

toluene (scintillation grade)

POPOP-2, 5-diphenyloxazole

Glaxo Laboratories Limited, Greenford, Middlesex

streptomycin sulphate

benzyl-penicillin

Packard Instrument Ltd., Caversham, Berks.

Insta-gel

Polyethylene Scintillation Counting Vials

hyamine hydroxide

Miles Laboratories Ltd., Slough, England

Alpha-1-antitrypsin test kit

All other materials and reagents used were of the appropriate grade as supplied by BDH Chemicals Ltd., Sigma Chemical Co. Ltd., the Department of Haematology, S.G.H., or the Pharmacy, S.G.H.

2.1.4. Radiochemicals

All radiochemicals used in the present study were obtained from the Radiochemical Centre, Amersham, Bucks.

5-(¹²⁵ I) Iodo - 2 - deoxyuridine	5Ci/mg, 0.5-1.0mCi/ml
L-(3- ¹⁴ C) serine	56mCi/m mol 50Ci/ml
(¹⁴ C) formic acid, sodium salt	60.3mCi/m mol solid
L-(U- ¹⁴ C) leucine	339mCi/m mol 50Ci/ml
(methyl- ³ H) thymidine	24Ci/m mol 1.0mCi/ml
Cyano(⁵⁷ Co) cobalamin	180-300 uCi/ng 10uCi/ml
L-(carboxyl- ¹⁴ C)-tyrosine	59mCi/mmol solid

Section 2.2.

Methods

2.2.1. Determination of Leucocyte and Serum

Ascorbic Acid

The method used was based on that described by Denson and Bowers (1961).

Sample Preparation

a) Leucocytes

4 ml venous blood was added to 12.5 ml of diluent (0.9% NaCl, 6% dextran, 10% EDTA; 100: 25 : 1). The diluent blood was left to stand for 30 min., during which time the majority of the red cells sedimented by gravity. The supernatant, containing the leucocytes was then removed by pasteur pipette and mixed. A 0.08 ml sample was removed and added to 20 ml Isoton. After addition of 8 drops dilute Zaponin (1:9, Isoton) the leucocyte cell concentration of the supernatant was determined using a Coulter, model D, electronic cell counter. 10 ml supernatant was then centrifuged (800 g, 15 min). The resulting supernatant was then discarded and the leucocyte cell pellet ground with 1.3 ml 5% TCA. After centrifuging (800 g, 10 min), 1 ml of the acid supernatant was retained for ascorbic acid assay.

b) Serum

2 ml of 5% TCA was added to 1 ml of serum and centrifuged (800 g, 10 min). Two 1 ml samples of the acid supernatant were retained for ascorbic acid assay.

Ascorbic Acid Assay

0.3 ml of reagent dye (2.2% 2, 4 di-nitro phenyl hydrazine in 10 N H_2SO_4 , 5% thiourea and 6% cupric sulphate ($\text{Cu SO}_4 \cdot 5\text{H}_2\text{O}$), 100:5:5) was added to 1 ml of sample and incubated in a water bath at 36°C for 4 hours. After cooling in ice water and addition of 1.5 ml 65% H_2SO_4 in ice water, each reagent sample was read against a similarly treated reagent blank (1 ml 5% TCA) at 520 nm on an S P 600 spectrophotometer.

A standard curve was drawn using the spectrophotometer readings from similarly treated 0.5, 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 ug/ml ascorbic acid standard solutions in 5% TCA. The ascorbic acid concentrations in the leucocyte and serum samples were determined from the standard curve. The leucocyte ascorbic acid (LAA) was expressed as ug/ 10^8 WBC and the serum ascorbic acid as ug/ml serum.

2.2.2. Determination of Serum Pyridoxal-5 -phosphate

The method use was based on that described by Chabner and Livingstone (1970) and involved determination of enzymatic, PLP dependent decarboxylation of 1- (^{14}C) -tyrosine using tyrosine apodecarboxylase.

Preparation of Apoenzyme Solution (Based on method described by Lumeng and Li (1974))

Tyrosine apodecarboxylase solution, 250 ml, was prepared by mixing:

60 ml (24%) glycerol
35 ul 2mM mercaptoethanol
62.5 mg tyrosine apodecarboxylase
and 0.3 M sodium citrate buffer pH 6.0 to 250 ml.
7 ml samples were stored at -20°C .

Sample Preparation

Deproteinised serum samples were prepared by mixing 0.1 ml 50% TCA with 1.0 ml serum and centrifuging (800 g, 15 min). The deproteinised serum supernatant was neutralized (pH 6-7) with 4.5 N NaOH (0.02 - 0.025 ml).

Assay

The PLP dependent decarboxylation was carried out in a 30 ml glass universal vial in a shaking water bath at 32°C . Reactions were initiated by injection of 200 ul of 1-(^{14}C)-tyrosine solution (final concentration, 5 mM sp.act 20 uCi/mmol) to a 15 min. pre-incubated vial containing 1.0 ml sodium citrate buffer pH 6.0, 200 ul apoenzyme solution and 200 ul deproteinised serum or standard PLP solution. Each vial also contained a scintillation counter minivial holding 0.2 ml Hyamine OH. Reactions were terminated after 15 min. by injection of 1 ml 5 M HCl. After a further 15 min. incubation, 5 ml of toluene scintillant was added to each minivial and ^{14}C activity counted.

Specimens and reactants were protected from exposure to light in order to avoid photolysis of PLP. All

assays were performed in duplicate. Recovery of added PLP was $90 \pm 8.5\%$. Reproducibility was $\pm 4.7\%$. The concentration of standard pyridoxal phosphate solutions was determined by absorbance at 388 nm (Peterson and Sober, 1954).

2.2.3. Determination of Serum AST and ALT Activity in the Presence and Absence of Added PLP

Two 0.5 ml serum samples from alcoholic liver disease subjects were prepared. To one 0.5 ml sample, 0.5 ml phosphate buffer (100 mmol/l, pH 7.4) was added and to the second 0.5 ml sample, 0.5 ml phosphate buffer (100 mmol/l, pH 7.4) containing PLP (5 mg/l) was added. The serum samples were then incubated in the dark at room temperature for 30 min. prior to duplicate determination of AST or ALT activity according to the test kit manufacturer's recommended method (Boehringer Mannheim UV Test Kits for GOT and GPT).

2.2.4. Determination of Acetylase Status

The method used was based on that described by Rao et al (1970) and involved administration of a 1.0 g oral dose of sulphadimidine to each subject followed by assay of free and total sulphonamide in a 5 - 6 hour post-dose urine sample. Sulphadimidine was supplied by the hospital pharmacy. The method of estimation of free and total sulphadimidine was based on the Bratton and Marshall technique (Varley, 1962) and the acetylated sulphadimidine (total-free) was expressed as a percentage of the total sulphadimidine. Classification

of acetylators status was based on Rao's finding that in slow acetylators, the proportion of acetylated sulphadimidine in urine samples was less than 70%.

The sulphadimidine estimation technique was based on diazotisation of sulphadimidine in urine followed by coupling with N-(1-naphthyl) ethylene-diamine dihydrochloride and spectrophotometric estimation of the product.

Determination of Total Sulphonamide

25 μ l of the 5 - 6 hour urine sample was mixed with 3 ml H_2O , 1 ml 20% TCA and 0.5 ml 5 M HCl. After heating ($100^{\circ}C$, 1 h), cooling, mixing with 0.1 ml 0.2% sodium nitrite, leaving to stand for 3 min., mixing with 0.1 ml 0.5 M ammonium sulphamate leaving to stand for 2 min., mixing with 0.5 ml 0.05% N(1-naphthyl) ethylenediamine dihydrochloride, and leaving to stand for 10 min., the optical density of the solution at 540 nm was determined. For determination of free sulphonamide, heating with HCl ($100^{\circ}C$, 1 hour) was omitted. The sulphonamide concentration was calculated by comparison with a similarly treated, standard sulphadiazine solution. Estimations were carried out in duplicate and the mean value was used to calculate results.

2.2.5. Determination of Serum Alpha-1-Antitrypsin

The method used, radial immunodiffusion, was based on that described by Mancini, Carbonara and Heremans (1965) as modified in the commercial kit supplied by Miles Labs.

The principle of radial immunodiffusion is that a soluble antigen, when placed into a round cylindrical well cut into an agar gel, will radially diffuse into the gel at a speed which is determined by many factors including the size of the molecule, the concentration of agar in the gel and the temperature of incubation. If monospecific, high titred antibody has been incorporated uniformly into the gel, immune precipitation will occur as the antigen and antibody reach equilibrium. The end point is reached when all of the antigen has reacted with the available antibody. The result is the formation of a precipitation ring around the antigen well which can be visualised and whose diameter can be easily measured. Since the diameter of this ring is directly proportional to the antigen concentration, a standard curve of diameter versus antigen concentration can be plotted using a series of reference standards of known antigen concentration. Measurement of the ring diameter produced by an unknown serum sample will allow the direct determination of antigen, and hence alpha-1-antitrypsin concentration, from the standard curve.

The immunodiffusion plates used in this method consisted of plastic dishes containing a monospecific alpha 1-antitrypsin antiserum incorporated into a layer of agar. The serum alpha 1-antitrypsin assay involved pipetting 5.0 ul of each serum sample or alpha 1-antitrypsin reference standards into wells cut in the agar layer. After incubating at room temperature for 18 hours the ring diameter of each of the serum samples and reference standards was measured. The ring diameters of the five reference standards ranging from 27 to 330 mg/100 ml were then plotted on semilogarithmic graph paper and the alpha 1-antitrypsin concentration of each serum sample determined from the standard curve. Although measurement of alpha-1 antitrypsin may be accomplished by functional i.e., anti-protease assays, immunochemical measurement such as radial immunodiffusion is the most specific approach.

2.2.6. Determination of Unsaturated Serum Vitamin B₁₂

Binding Capacity

The method used was that described by Gottlieb et al (1965) and is based on the finding that charcoal pre-coated with a large molecule can adsorb only free B₁₂, whereas uncoated charcoal can adsorb both free B₁₂ and B₁₂ bound to a large molecule.

Assay of unsaturated B₁₂ binding capacity of serum (UBBC) involved sequential addition of 0.5 ml unknown serum sample and 2.5 ng (⁵⁷C_O) labelled B₁₂ to a 10 ml plastic test tube containing 2 ml 0.9% NaCl, mixing for 10 sec and adding 2 ml albumin coated charcoal

for 10 sec and adding 2 ml albumin coated charcoal suspension. This was prepared by mixing equal volumes of 5% aqueous suspension of Norit 'A' charcoal and a 1% aqueous solution of bovine serum albumin (BSA). The short incubation period of serum and B₁₂ alone reduces equilibrium between native and added B₁₂. After centrifugation of the charcoal mixture the radioactivity of the supernatant was counted in a gamma scintillation counter.

This procedure was repeated once with omission of serum and once with the omission of albumin coated charcoal. The minus serum control determined the background radioactivity count and this was subtracted from all other determinations. The minus coated charcoal control determined the radioactivity equivalent to 2.5 ng B₁₂ and allowed calculation of the UBBC of the unknown serum sample (pg/ml) i.e., that quantity of B₁₂ prevented from adsorbing to charcoal by the serum. All determinations were carried out in duplicate.

2.2.7. Determination of Serum Transcobalamin Levels

The method used; differential precipitation of serum transcobalamins by ammonium sulphate, is based on that described by Carmel (1974). Serum (0.5 ml), was incubated with 0.1 ml (⁵⁷Co)-labelled-B₁₂ (20 ng/ml) and 1.4 ml potassium phosphate buffer (0.4M, pH 6.3) for 30 min., prior to addition to the pellet of 1 ml albumin coated charcoal. After mixing and centrifugation (15 min., 800 g) the supernatant was added to tubes

containing 4 ml, 3M ammonium sulphate. After a second incubation (30 min) and centrifugation (15 min., 800g) the supernatant was decanted into a separate tube. The radioactivity in the supernatant and precipitate was then determined in a gamma scintillation counter. Background adsorption was determined by repeating this procedure with omission of serum and, after the appropriate correction, the radioactive counts were converted to pg/ml by comparison to a known standard, tubes to which no coated charcoal was added. All determinations were carried out in duplicate. The precipitated transcobalamin was transcobalamin II and the supernatant contained the R, B₁₂ binders, transcobalamins I and III.

2.2.8. Determination of Antipyrine $t_{\frac{1}{2}}$

The method used was based on that described by Beattie and Sherlock (1976). Each subject received 1.2 g antipyrine orally and blood samples were taken at 0, 0.5, 2, 5, 8, 12 and 24 hours. Serum antipyrine concentration was measured by the method of Brodie et al (1949). From these estimations, the $t_{\frac{1}{2}}$ was determined by regression analysis.

2.2.9 Preparation of Liver Cell Suspension

Preparation and Incubation

Healthy young male rats killed by a blow to the head were used. Sections of liver (50 mg) were removed and placed in dissection medium (balanced salt solution with added penicillin 1000 units/ml and streptomycin 1000 ug/ml). In sterile conditions, the liver sections were cut into fine, 1 mm cubes. After rinsing three times with dissection medium the pieces of liver were resuspended in 5 ml Hams F10 medium containing 1000 units/ml collagenase and incubated with gentle shaking for 30 min., 37°C in a universal container. After centrifugation (100 g, 2 min) the pellet was resuspended in 10 ml Hams F10 medium with added fetal calf serum (20%), glutamine (1%), penicillin (100 units/ml) and streptomycin (100 ug/ml). Fragments were allowed to settle, 6 ml supernatant was collected, placed in a separate glass universal vial containing 30 ul (³H)-TdR (5 uCi/ml) and incubated with gentle shaking for up to 2 hours at 37°C. Samples 2 x 1 ml were collected at 30 min, 60 min and 2 hours and placed in ice.

Processing of Samples

0.2 ml volumes of the incubated samples and 0.2 ml volumes of 1:1, 1:2, 1:4 and 1:8 diluted samples were cytocentrifuged, and the slide preparations were dried rapidly in air and fixed in methanol for 10 min. After further fan assisted drying, the slides were examined using phase contrast microscopy to determine the most

appropriate dilution. These slides were then washed, three times with 10% ice-cold TCA and with ice cold water, rinsed with methanol, dried and stored dry at 4°C.

2.2.10. Autoradiography

The principle of this technique is that radioactive precursors which have been incorporated into cellular nucleic acid may be detected in slide cell preparations by overlaying the preparation with a radiosensitive emulsion. Beta-particles from radioactive decay of the (^3H) penetrate the emulsion and when stopped ($\sim 1 \mu\text{m}$) give up their energy to form a 'latent image' in the silver halide of the emulsion. Subsequent development reduces the silver halide to metallic silver where latent images have been formed. This produces a black dot in transmitted light microscopy, 0.1 - 0.5 μm in diameter. The technique therefore involves coating with emulsion, exposure, development, fixing, staining, and, examination.

In red safelight, the prepared slides were dipped into melted emulsion, (prepared by incubation in a water bath, (30 min, 45°C), the excess emulsion was drained off and the slides allowed to dry. The slides were then placed in a light tight box with dessicant and exposed for one week at 4°C.

Again in red safelight, the slides were allowed to come to room temperature and humidity, then placed in Kodak D19, developed for 10 min, washed briefly, placed in hypo for 4 min, rinsed and washed for 2 min in clearing agent, washed in tap water for 5 min, then rinsed in distilled water and allowed to dry.

The slides were stained to 10 min with dilute Giemsa stain (1:10), then rinsed in tap water, then distilled water, allowed to dry and then examined.

The experiments described in section 2.2.9. and 2.2.10. were carried out at the Beatson Institute for Cancer Research, Gartnavel Estate, Glasgow.

2.2.11. Vitamin B₆ Deficient Rat Studies

Six adult standard laboratory rats (Charles River strain), three male and three female, were fed the following pyridoxine deficient diet for a period of six weeks.

Vitamin, fat free casein	22.0%
Sucrose	69.5%
Beef tallow	2.0%
Cod liver oil	2.0%
Salt mix	4.0%
Vitamin supplement (B ₆ free)	0.5%

A further six rats from the same colony, three male and three female, were housed in identical conditions and were fed a standard laboratory diet. Use of a pyridoxine deficient diet to induce a vitamin B₆ deficiency state was suggested by the work of Ribaya and Gershoff, (1977); Hunter and Harper, (1977); and Reynolds, (1978).

The animals used in this study were supplied by and housed in the animal house of the Institute of Neurological Science, Southern General Hospital, Glasgow.

2.2.12. Collection of Blood Samples for Lymphocyte Studies

From each subject, 20 ml of venous blood was collected aseptically by venepuncture, in a sterile syringe, by a member of the medical staff of the Southern General Hospital. Of the 20 mls, 10 ml was added to 6 ml BSS containing 60 units preservative free heparin and mixed. The remaining 10 ml was placed in a sterile glass container, allowed to clot, centrifuged (800 g, 15 min) and the serum, collected by sterile pasteur pipette, retained.

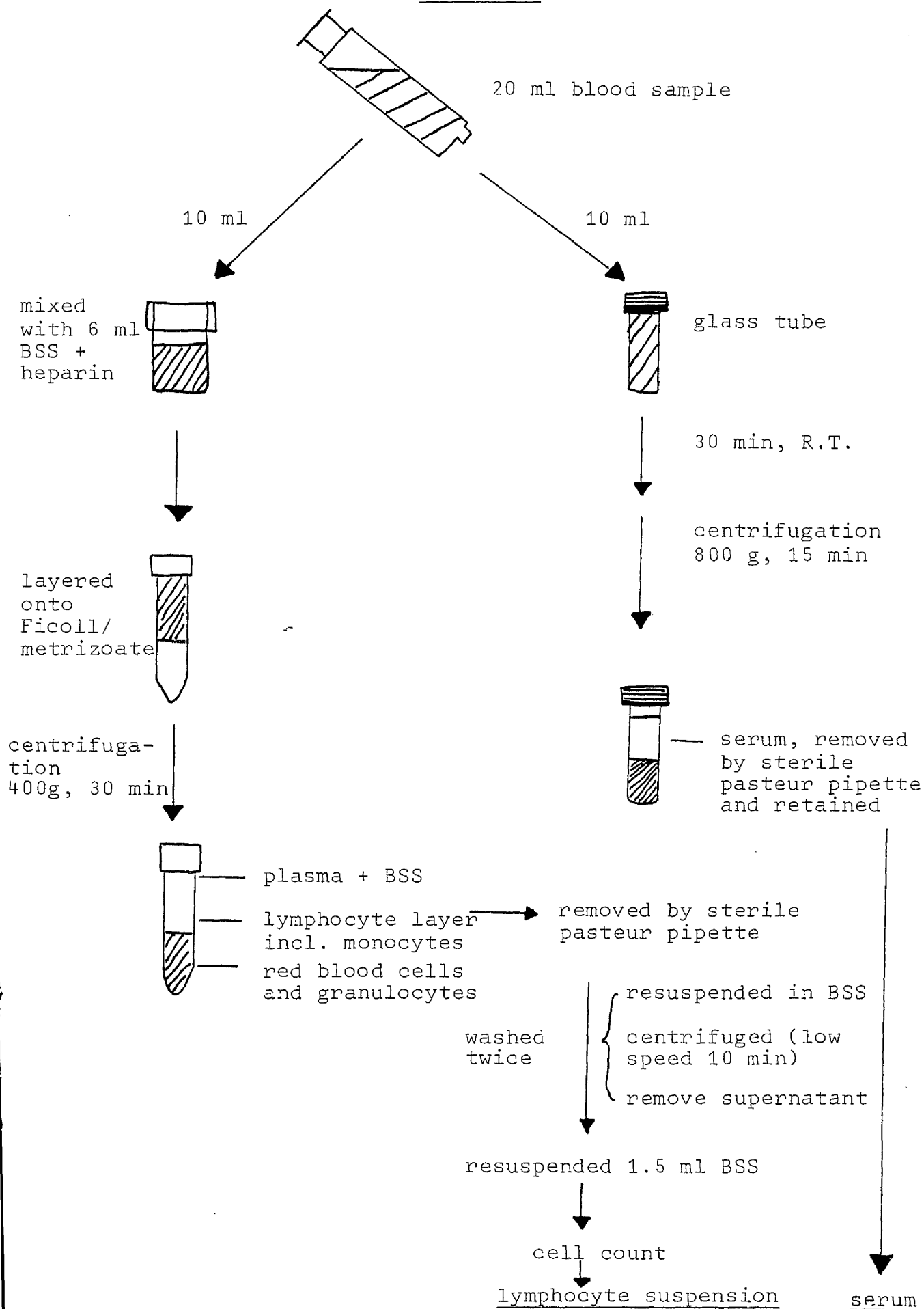
2.2.13. Preparation of a Lymphocyte Suspension

The isolation method is essentially that of Boyum (1968, 1976), who described methods for isolation of mononuclear cells from circulating blood and bone marrow. In general, these procedures employed mixtures of polysaccharide (ficoll) and a radiopaque contrast medium (sodium metrizoate, adjusted to a density of 1.077 ± 0.001 g/l. This medium allowed the rapid recovery of viable lymphocytes from small volumes of blood. The method involved layering dilute, anticoagulated blood onto the ficoll/sodium metrizoate mixture. During centrifugation erythrocytes and granulocytes are aggregated by ficoll and rapidly sediment; whereas, lymphocytes remain at the dilute plasma-ficoll/sodium

diatrizoate interface.

In this study an equivalent ficoll/sodium metrizoate solution was used. The composition of this mixture was: per 100ml; 30 ml 32.8% w/v sodium metrizoate + 5.6g Ficoll 400 + sterile distilled H₂O, and this medium was then sterilised by filtration prior to use. To prepare a lymphocyte suspension, aliquots (8 ml) of the whole blood/BSS/heparin mixture were carefully layered onto 2 ml of the ficoll/sodium metrizoate solution in a sterile centrifuge tube. After centrifugation (400 g, 30') the upper layer was removed and discarded, and the opaque interphase layer collected. After pooling the separated lymphocytes of individual subjects, the cells were washed twice by resuspension in BSS and centrifugation at 800 g for 15min. After the final wash the cells were resuspended in 1.5 ml BSS. This cell suspension was then counted using an electronic cell counter (the Coulter Counter, Model 4D). This method of cell counting is more reproducible and more convenient than the manual method using a haemocytometer. A differential white cell count, using Leishmans stain, showed that over 95% of this cell suspension consisted of small lymphocytes and viability, using trypan blue exclusion, was also shown to be over 95%. The method of preparation of the lymphocyte suspension is illustrated in fig. 5.

Fig. 5. Preparation of Lymphocyte Suspension
and Serum



2.2.14. Preparation of Cell culture Media

Complete MEM (minimal essential medium), with Hanks' salts, with L-glutamine, was supplied by Gibco. Prior to initiation of PHA stimulated lymphocyte culture, the following additions were made; per 100 ml medium.

2 ml rehydrated PHA-M (Gibco)

2 ml preservative free heparin, 2000 units (Evans)

0.1 ml streptomycin sulphate, 1000 μ g (Glaxo)

0.1 ml benzyl penicillin, 1000 units (Glaxo)

Two types of modified MEM, with Hanks' salts, with L-glutamine, were also used (Gibco). The modifications to these media from complete MEM, were as follows.

In modified MEM (1) pyridoxal HCl was omitted.

In modified MEM (2) pyridoxal HCl was omitted and the folic acid concentration was reduced from 1.0 mg/l to 0.01 mg/l. Further modifications to these media were as specified in the results section. Prior to use in PHA stimulated lymphocyte culture, PHA, heparin, streptomycin and penicillin were added, as for complete MEM.

Where appropriate for the requirements of particular experiments, folate, pyridoxal HCl, 4-dPN, INH, non-essential amino acids and hemin were added at this stage; to complete or modified MEM, to the specified concentration. Hemin was dissolved in NaOH and sterilised by filtration prior to addition to the culture medium. (Freedman, Geraghty and Rosman, 1974).

2.2.15. PHA Stimulated Lymphocyte Culture

PHA stimulated lymphocyte cultures were initiated as follows: to (38 x 12.5 mm) lymphocyte culture tubes the following additions were made:

0.85 ml MEM: complete, modified or as specified, containing PHA, heparin, streptomycin and penicillin

0.1 ml autologous serum

0.02 - 0.05 ml lymphocyte suspension, containing 2.5×10^5 cells.

Where variations to this procedure were used, the changes are specified in the appropriate results section.

Up to 30 PHA stimulated lymphocyte cultures were prepared from individual 20 ml blood samples, allowing up to 15 determinations to be made on lymphocyte cultures from each subject.

The choice of conditions used in preparation of these PHA stimulated lymphocyte cell cultures was partly determined by the size of the culture tube, i.e., a total volume of 1 ml was required in order to leave a sufficient gas phase and the experience of other authors determined the most appropriate cell concentration (Dupont, et al, 1977). The amount of PHA used was based on the supplier's recommendation and the use of heparin, streptomycin, penicillin and 10% autologous serum was based on the recommendations of Ling and Kay (1975). The preparation

of cell culture media and lymphocyte cultures are illustrated in fig. 6.

2.2.16. Determination of Acid-insoluble (¹²⁵I)-UdR Incorporation

In this study, estimates of the rate of DNA synthesis in PHA stimulated lymphocyte cultures were required both as a measure of the rate of cell growth and in order to further characterise aspects of DNA metabolism. The use of labelled thymidine as a means of quantitating DNA synthesis has been mentioned previously (1.4.4.). The principle of the use of a labelled precursor to follow the synthesis of a macromolecule is that the measured radioactivity incorporated into the macromolecule is proportional to the rate of synthesis of the macromolecule. Since thymidine is a specific precursor for DNA synthesis, the rate of incorporation of radioactively labelled thymidine has been used as an index of the rate of DNA synthesis in growing cells (Cleaver, 1967).

There are, however, several limitations inherent in methods which use labelled thymidine incorporation into PHA stimulated lymphocytes to quantitate the rate of DNA synthesis or degree of stimulation. Even if culture conditions are standardised with respect to medium, stimulant, duration of culture, temperature, pH and O₂ tension, and even if the proportion of viable cells and cells undergoing stimulation is comparable, the fact that incorporation of exogenous labelled thymidine is dependent on a variety of factors including

Fig. 6. PHA Stimulated Lymphocyte Culture

0.85 ml cell culture medium

- 100 ml MEM (standard or modified)
- + penicillin, streptomycin
- + 2 ml heparin
- + 2 ml PHA
- + where appropriate folate, pyridoxal, hemin etc.

0.02 - 0.05 ml lymphocyte suspension (2.5×10^5 cells)

0.1 ml autologous serum

lymphocyte culture tube app. 30/20 ml
blood sample
(i.e., 15 duplicate cultures)



Sealed, incubated 37°C (or 5°C, control)

competition between de novo and salvage thymidylate pathways, the size of endogenous pools of thymidylate precursors as well as the absolute rate of DNA synthesis, requires that results of experiments dependent on determination of such incorporation be carefully inter-related.

Ling and Kay (1975) discuss two factors which affect the validity of the assumption that changes in the rates of incorporation of precursor into a macromolecule reflect changes in the rate of synthesis of that macromolecule. The first of these is that the amount of unlabelled material present in the culture medium competing with the labelled precursor for uptake into the cell and incorporation into the macromolecule must be constant, or any changes in it brought about by stimulation must be known. The second is that the rate at which the added precursor saturates the pool of nucleotide triphosphates from which nucleic acid is synthesised must also be independent of stimulation.

Ideally, the time required for saturation of the nucleoside triphosphate pool should be short relative to the period of the radioactive pulse and the specific activity of the pool should remain constant throughout the pulse. Should stimulation prove to affect the rate of saturation of the nucleoside triphosphate pool significantly, it is extremely difficult to make the requisite corrections. These require accurate determinations of the specific activities of the pool throughout

the labelling period, an extremely difficult procedure when dealing with the amount of material available from lymphocyte cultures. Simple determinations of the rate of uptake of the precursor into the pool of triphosphates are not adequate. Furthermore, in all conditions which deviate from the ideal, the assumption that the nucleotide triphosphate pools of all the cells in the culture can be treated as non-compartmentalised becomes critical. Taheri, Wickremasinghe and Hoffbrand (1981) have in fact presented evidence that nucleotide pools in PHA stimulated lymphocytes are compartmentalised.

In considering the effect of the endogenous pool size on the validity of thymidine labelling experiments, the pre-existing pool is generally regarded as an expandable pool such that addition of a small amount of high specific activity precursor will not fully expand the pool (Cleaver, 1967). Under these conditions most of the thymidine nucleotides entering DNA are derived from the endogenous pathway and the relative rates of incorporation into DNA by different cultures will only indicate the relative rate of DNA synthesis if the proportion of nucleotides entering DNA derived from the labelled thymidine is constant. With a long pulse, one can offset the effect of changing pool size but the effect of a variable pool size complicates interpretation of data based on the changes in the incorporation of labelled thymidine and thus it is necessary that experiments based on such incorporation should include an internal control.

It is relevant to note that Ganeshaguru and Hoffbrand (1978) have presented evidence that the thymidine triphosphate pool in PHA stimulated lymphocyte cultures derived from folate deficient subjects is not significantly different from that observed in normal cells.

While there are limitations in the use of radio-labelled thymidine to assess DNA synthesis, there is considerable empirical evidence that the rate of thymidine incorporation does correlate quite well with other parameters of lymphocyte stimulation, and in practice several different methods, e.g., Ling and Kay (1975), Hirschhorn, Hirschhorn and Waithe (1974) and Bain (1970), appear to provide a relatively reproducible measure of DNA synthesis.

Certain other variables must be considered in the interpretation of experiments using PHA stimulated lymphocytes and labelled thymidine. It is known that the small lymphocyte population is functionally heterogeneous and that responsiveness to activators may be restricted to one or another of functionally defined cell types. The interaction of lymphocytes in culture and the kinetics of the reaction of activators with lymphocyte membranes is complex and poorly understood. The degree of reaction of cultures varies with the area of the culture surface, the cell concentration, the volume of medium and with both the concentration of activator and the ratio of activator molecules to cells.

The time of peak response, or of detectable response, varies not only with cell type but also with the particular activator. In cultures incubated for more than two days simultaneous cell death and cell division also complicate analysis of the response. Radiation effects, reutilisation, low thymidine kinase activity, the amount of radioactive label added, the specific activity, the presence of cold thymine, exposure time, effect of contaminating cells and the fact that different cell populations contain different levels of thymidine phosphorylase activity which may profoundly affect their uptake of labelled thymidine (Marsh and Perry, 1964). These considerations emphasise the requirement for standardised conditions and the use of an internal control.

The use of (^{125}I)UdR as an analogue of TdR is discussed by Cleaver (1967), and its use in PHA stimulated lymphocyte studies has been validated by Herbert et al (1973). The TdR analogue properties of (^{125}I)-UdR are attributable to the steric similarity of the iodine atom and the methyl group of thymidine, (^{125}I)-UdR therefore competes with thymidine for uptake and incorporation into DNA. In tracer studies, the serious toxicity of internuclear radiation from (^{125}I) (Hofer and Hughes, 1971) is a considerable disadvantage, but this problem is less critical in short-term labelling of activated lymphocytes (Ford, 1978). The principal advantages of using a gamma-emitter, (^{125}I)-UdR, rather than a beta-emitting nuclide are that radioactivity

counting characteristics are improved, sample preparation is simplified and hence determination of incorporation much more convenient. An additional advantage is that because it is released from dead cells as (^{125}I), reutilisation is minimal (Ford, 1978). The method used in this study was based on the work of Herbert et al (1973).

At a specified time after initiation of the PHA stimulated lymphocyte cell culture, 0.5 uCi of (^{125}I)-UdR was added to the appropriate cell culture tubes. Following a 3h incubation at 37°C cultures were terminated by addition of 1 ml cold saline. After centrifugation (800 g, 15'), removal of supernatant, two washes with cold 10% TCA and one rinse in H_2O , the radioactivity of the acid-insoluble residue was determined directly by placing the cell culture tubes in a Packard automatic gamma scintillation spectrometer. An outline plan of a PHA stimulated lymphocyte experiment is illustrated in fig. 7., and the details of the cell culture analysis are illustrated in fig. 8.

The principal labelling control employed in order to determine background contamination or the degree of non-specific incorporation of label was to determine the radioactivity retained in cell cultures to which cold saline was added, immediately prior to addition of (^{125}I)-UdR label. Zero time controls and analysis of cultures maintained at 5°C were also carried out. The radioactivity in these control tubes was found to

Fig. 7. A PHA Stimulated Lymphocyte Experiment

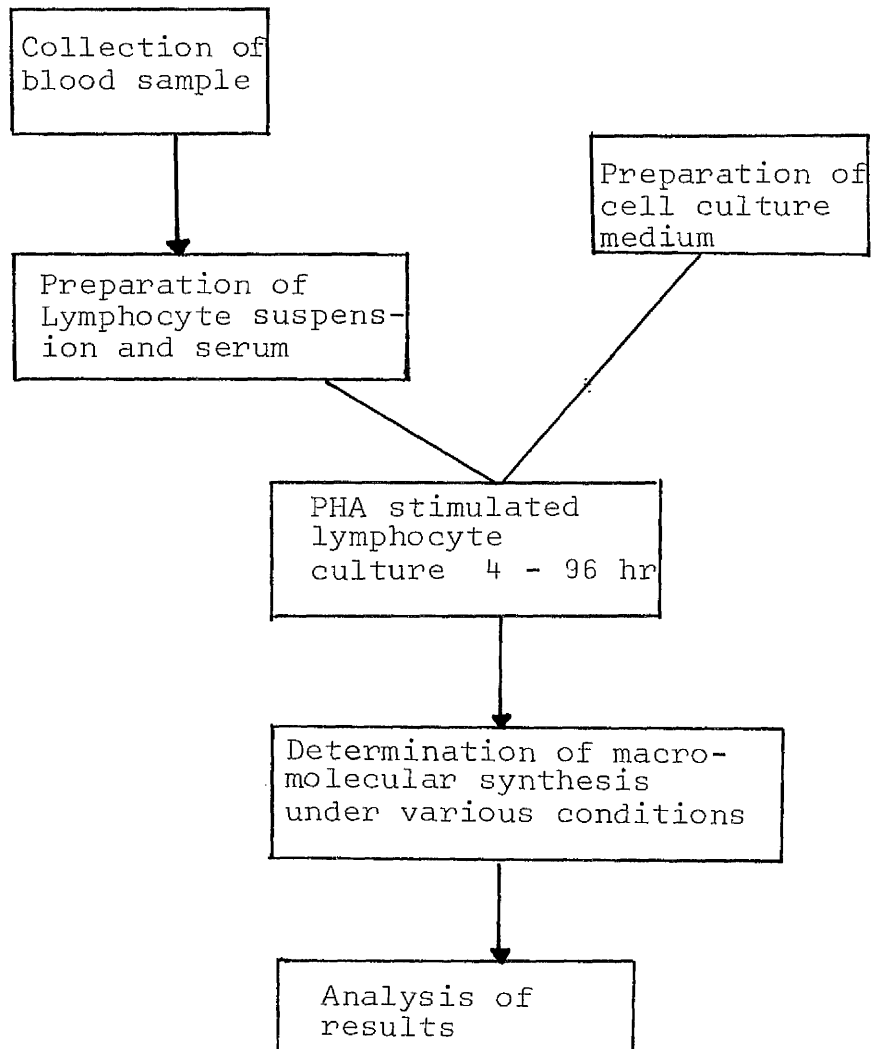
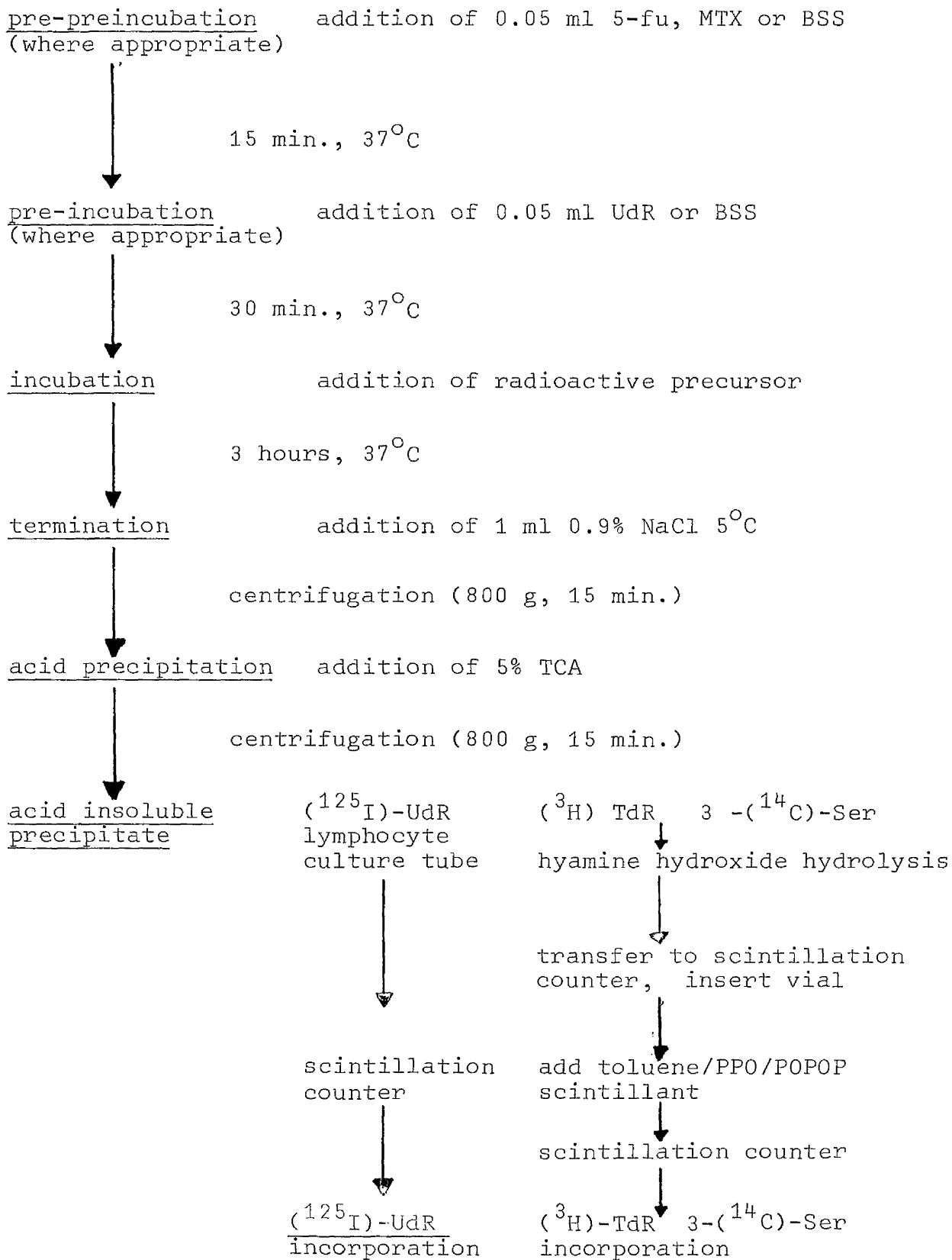


Fig. 8. Cell Culture Analysis

Determination of acid-insoluble (^{125}I)-UdR, (^3H)-Tdr
or 3-(^{14}C)-serine incorporation at 4, 24, 48, 72 and
96 hours.



be 5% or less than that of comparable untreated cultures. This background activity was subtracted from the activity determination of each culture tube. Results were calculated from the mean cpm of at least duplicate analyses. The standard error of the mean for duplicate analyses was usually between 1 and 5%.

2.2.17. Determination of the Effect of UdR, MTX, 5-fu, Formate and 4-dPN on Acid-insoluble (^{125}I)-UdR Incorporation

In order to determine the effect of prior treatment with UdR, MTX 5-fu, formate and 4-dPN on subsequent DNA synthesis in PHA stimulated lymphocyte cultures, specified amounts of these compounds were added to cultures 1 h prior to incubation with radioactive label. In control cultures, BSS (50 ul) was added and after this preincubation period, determination of DNA synthesis was carried out as described for each radioactive label.

2.2.18. Determination of the Effect of Pre-incubation with MTX and 5-fu on the Subsequent Effect of UdR on Acid-insoluble (^{125}I)-UdR Incorporation

In order to determine the effect of addition of MTX and 5-fu to PHA stimulated lymphocyte cultures on the subsequent effect of preincubation with UdR on DNA synthesis, specified amounts of MTX and 5-fu were added to cultures 15 min. prior to preincubation with UdR. In control cultures, BSS (50 ul) was added and after this pre-preincubation period, preincubation with UdR and determination of radioactive label

incorporation was carried out as appropriate.

2.2.19. Determination of DNA Synthesis Using

3-(^{14}C)-Serine or (^{14}C)-Formate

There are two pathways for the synthesis of thymidylate DNA precursor; the de novo pathway and the salvage pathway (fig. 4). It is convenient to make use of TdR to label DNA because it is a specific precursor of DNA and is not incorporated into either RNA or protein. However, it is incorporated via the salvage pathway which is probably of lesser physiological significance than the de novo pathway. There are a number of possible approaches to labelling DNA other than making use of TdR but all require some degree of separation of DNA from other cellular macromolecules before determination of incorporation.

The method chosen in this study of labelling DNA independently of the salvage pathway, was to use 3-(^{14}C)-serine or (^{14}C)-formate. Use of either of these radioactive precursors is likely to result in labelling of a wide variety of cellular constituents including: protein DNA, RNA and lipid. Therefore, determination of incorporation of radioactive label into DNA requires preliminary purification of harvested cell cultures. The principal route of incorporation of 3-(^{14}C)-serine, and (^{14}C)-serine and (^{14}C)-formate label into DNA is via the carbon-1 (C-1) pool. Entry of 3-(^{14}C)-serine label into this pool is largely dependent on PLP and the serine hydroxymethyltransferase reaction and hence might

be expected to be susceptible to a vitamin B₆ deficiency. Entry of (¹⁴C)-formate label into the C-1 pool is usually independent of vitamin B₆. Labelling of the C-1 pool results in labelled DNA via de novo thymidylate synthesis, via labelling of the purine ring at carbons 2 and 8, and also via post-synthetic methylation of specific sites.

The labelling procedure adopted in this study paralleled that of (¹²⁵I)-UdR labelling. At a specified time after initiation of the PHA stimulated lymphocyte culture, 0.25uCi L-(3-¹⁴C)-serine, specific activity 56mCi/mmol or 1.0uCi (¹⁴C)-formic acid, specific activity 60.3mCi/mol, was added to the appropriate cell culture tubes. Following a 3 hour incubation at 37°C, cultures were terminated by addition of 1 ml cold saline.

After centrifugation (800 g, 15 min.) and removal of supernatant, the following DNA purification procedure, based on that described by Turnbull and Adams (1976), was adopted. The cell pellet was resuspended by mixing with 1 ml of a solution containing sodium dodecyl sulphate (1%), EDTA (2mM), 4-amino salicylic acid (3%), n-butanol (5%), NaCl (0.5M) and salmon testis DNA (0.5mg/ml) to act as a carrier. Protein was removed by extraction with phenol 88% m-cresol 12%/8-hydroxyquinoline (0.1%). After centrifugation (800 g, 15 min.) the upper aqueous layer was removed and DNA spooled out after overlaying with 2 volumes of absolute ethanol. The DNA was then digested in 0.2 ml of 0.5N NaOH (3 hours,

37°C) to remove RNA. After two washes with cold 10% TCA and drying, the acid-insoluble material was solubilised by addition of 0.2 ml hyamine hydroxide and heating at 50°C for 15 min. This DNA extract sample was then transferred, with scintillant cocktail and rinsing, to a scintillation counter insert vial prior to counting in a Packard liquid scintillation spectrometer.

The purpose of treatment with phenol/m-cresol and alkaline hydrolysis was to exclude measuring incorporation into protein or RNA.

2.2.20. Determination of Protein Synthesis

Using (^{14}C)-leucine

An increase of protein synthesis is one of the major metabolic changes caused by PHA stimulation of lymphocytes. The rate of amino acid incorporation has been shown to be a reasonably accurate index of the rate of lymphocyte protein synthesis (Ling and Kay, 1975) and because l-leucine is one of the most specific amino acid precursors of protein, use of this amino acid is particularly convenient in experimental determination of protein synthesis. L-leucine however, cannot be considered an absolutely protein specific label because in its metabolism it is converted to acetyl coenzyme A and acetoacetate. A major advantage of this specificity is that if a radioactive metabolite is a precursor of a particular class of macromolecule then the incorporation of that

metabolite is likely to give a more accurate estimate of the synthesis of the particular macromolecule. A further advantage is that the determination of incorporation of radioactive label does not require prior removal of other cellular macromolecules.

The method used in this study was based on that described by Hirschhorn et al (1974). At a specified time after initiation of the PHA stimulated lymphocyte cell culture, 1.0uCi of uniformly labelled (^{14}C)-l-leucine, specific activity 339mCi/mol, was added to the appropriate cell culture tubes. Following a 3 hour incubation at 37°C cultures were terminated by addition of 1 ml cold saline. After centrifugation (800 g, 15 min.) removal of supernatant, one wash with cold 10% TCA, addition of 0.2 ml 1M NaOH, heating at 50°C for 15 min, a second wash, with cold 10% TCA the acid-insoluble material was finally solubilised by addition of 0.2 ml hyamine hydroxide and heating at 50°C for 15 min. prior to transfer, with scintillant cocktail and rinsing to a scintillation counter insert vial.

Controls for the labelling of protein included addition of saline prior to addition of label and preincubation with puromycin (20ug/ml, 30 min.) before addition of the label. The radioactivity of these controls was 5% or less than that of comparable untreated cultures.

2.2.21. Sterilisation of Solutions and Equipment

All materials and equipment used in lymphocyte cell culture studies were either sterile, e.g., lymphocyte cell culture tubes, centrifuge tubes, sterile water or were sterilised using one of a variety of methods. For example, glass pipettes were sterilised in a hot-air oven, plastic pipette tips and test-tubes were sterilised by ethylene oxide and solutions to be added to cell cultures were sterilised using sterile disposable Millex filter units (0.22 μm).

Preparative work and cell culture manipulations were performed using aseptic technique on a bench, swabbed with 70% alcohol, in a room separate from the main laboratory.

2.2.22. Radioisotope Counting

Counting of gamma ray emitting nuclides, i.e., (^{125}I) and (^{57}Co) was performed using a Packard automatic gamma scintillation spectrometer with a NaI crystal. Samples were counted directly by placing the processed cell culture tubes or test-tubes in scintillation counter vials.

Counting of beta-emitting nuclides, i.e., (^{14}C) and (^3H) was performed using a Packard model 3320 liquid scintillation spectrometer. In determination of ($^{14}\text{CO}_2$) (2.2.2.), the scintillant mixture was added to the hyamine

hydroxide, containing absorbed ($^{14}\text{C}\text{O}_2$), in the scintillation counter insert vial. In determination of radioactive incorporation into acid-insoluble precipitates, the samples were solubilised with hyamine hydroxide prior to transfer, with scintillant and rinsing, to scintillation counter insert vials.

Two scintillation fluids were used, each of which gave similar results. One was based on the use of diphenyloxazole (PPO) as primary phosphor and bis-phenyloxazolylnbenzene (POPOP) as secondary phosphor. This was made by dissolving 12.5 g PPO and 0.75 g POPOP in 2.5 l of scintillation grade toluene (Packard Technical Bulletin no. 1.). The second system used was Insta-gel (Packard). The channels ratio method was to estimate the degree of quenching and hence efficiency of counting of radioactive samples. This was between 70 - 85% for (^{14}C) and 15 - 30% for (^3H). As the degree of quenching was constant, within experiments, it was not necessary to convert from cpm to dpm.

2.2.23. Treatment of Results of PHA Stimulated Lymphocyte Experiments

For each determination of acid-insoluble incorporation of (^{125}I)-UdR, cpm, and (^{14}C)-leucine, cpm, into harvested cells, or of acid-insoluble incorporation of 3-(^{14}C)-serine, cpm, and (^{14}C)-formate, cpm, into a DNA extract of harvested cells, the mean value, derived

from replicate cultures, less background incorporation in control cultures, was calculated. These are the results of experiments on cells of individual subjects which are presented in tables of results. In analysis of group results, presented in figures, the mean and standard error of the mean of individual results was frequently used. However, as there was often considerable variation in incorporation into cultures of different subjects, individual results were, where appropriate, calculated as a percentage of incorporation in a control culture. For example, incorporation into cultures preincubated with Udr was compared with and expressed as a percentage of incorporation in replicate cultures to which no Udr was added. Similarly, incorporation into cultures at 48 hours was expressed as a percentage of incorporation into comparable cultures at 72 hours. This approach provides an internal control in each experiment and allows a valid comparison to be made of results of different groups of subjects. Use of this approach was suggested by the work of Herbert et al (1973).

In determining the significance or otherwise of group differences, the two sample student t-test or equivalent non-parametric test for testing equality of means of two random samples was used. The arithmetical mean, \bar{x} is defined as: $\sum x/n$. The sample standard deviation,

$\sigma (n - 1)$ is defined as: $\sqrt{\frac{\sum x^2 - \sum x^2/n}{n - 1}}$

The standard error of the mean is defined as

$$\sigma (n - 1) / \sqrt{n}.$$

CHAPTER 3

RESULTS

Section 3.1.

Preliminary Studies

3.1.1. Ascorbic Acid Levels

Results of determinations of leucocyte and serum ascorbic acid levels of control and alcoholic liver disease subjects are summarized in Table 1. The leucocyte ascorbic acid level in the alcoholic liver disease group is statistically significantly lower than that observed in the control group ($p < 0.05$, two sample t-test). The mean serum ascorbic acid level in the alcoholic liver disease group is also statistically significantly lower (at the 5% level) than that observed in the control group.

The entry criteria for the study required the exclusion of subjects who had recently received vitamin supplements. In practice it was found that in the alcoholism treatment unit at the Southern General Hospital, which was the main source of subjects for the alcoholic non-liver disease group, treatment often included vitamin supplements and this precluded their entry to the study. The leucocyte and serum ascorbic acid levels in a sample of these alcoholic non-liver disease subjects were: LAA ($\mu\text{mol}/10^8 \text{ WBC}$), 204.2 ± 13.4 ; SAA ($\mu\text{mol}/\text{l}$), 74.9 ± 10.4 ; ($n = 13$, $\bar{x} \pm \text{s.e.}$). The leucocyte ascorbic acid level but not the serum ascorbic acid level is statistically significantly higher ($p < 0.05$) than that observed in control subjects.

Table 1

Leucocyte and Serum Ascorbic Acid Levels in Alcoholic
Liver Disease and Control Subjects

	Alcoholic Liver Disease		Control		P
	Mean \pm s.e.	n	Mean \pm s.e.	n	
LAA ($\mu\text{mol}/10^8$ WBC)	96.1 \pm 10.4	27	148.2 \pm 13.2	13	< 0.05
SAA ($\mu\text{mol}/\text{l}$)	23.5 \pm 4.4	27	44.3 \pm 5.4	13	< 0.05

3.1.2. Serum Pyridoxal-Phosphate Levels

Results of determinations of serum PLP levels of control and alcoholic liver disease subjects are summarized in Table 2. The mean serum PLP level in the alcoholic liver disease group is not statistically significantly lower (at the 5% level) than that observed in the control group.

3.1.3. Effect of Added PLP on Serum Transaminase Activity

AST and ALT activities were determined in the presence and absence of added PLP. It was found that AST activity was stimulated to a greater degree than ALT activity. The results of determinations of AST activity in the presence and absence of added PLP in serum samples from alcoholic liver disease subjects are illustrated in Table 3. Using these values the percentage stimulation in AST activity due to added PLP was calculated and compared with the measured serum PLP levels. Product moment correlation analysis suggests there is no significant association between serum PLP and stimulation of AST activity due to added PLP.

Table 2

Serum PLP Levels in Alcoholic Liver Disease and Control Subjects

	Alcoholic Liver Disease		Control		P
	Mean \pm s.e.	n	Mean \pm s.e.	n	
PLP (ng/ml)	7.8 \pm 1.5	25	10.9 \pm 1.7	13	n.s.

Table 3

Effect of Added PLP on AST Activity in Serum Samples of
Alcoholic Liver Disease Subjects

Subject	AST (Iunits/l)		% Stimulation	Serum PLP (ng/ml)
	- PLP	+ PLP		
1	44	46	5	1.0
2	76	80	5	1.0
3	116	120	3	2.7
4	80	116	45	3.4
5	96	116	21	3.5
6	36	48	33	3.6
7	80	108	35	4.5
8	116	156	34	6.0
9	44	60	36	6.3
10	220	328	49	13.7
11	36	44	22	30.0

3.1.4. Acetylator Phenotype

The results of determinations of acetylator phenotype in alcoholic liver disease and control subjects are given in Table 4. Also included in this table are the results of determinations of acetylator phenotype in alcoholic non-liver disease subjects, some of whom had received vitamin supplements. In each of the groups, the distribution of fast and slow acetylators was not significantly different, at the 5% level, χ^2 test, from that expected in a normal Caucasian population, i.e., 40% fast, 60% slow.

3.1.5. Alpha-1-Antitrypsin Phenotype

The number of alcoholic liver disease subjects in which alpha-1-antitrypsin phenotype was assessed was 32, comprising 24 males and 8 females. Alpha-1-antitrypsin phenotype was also assessed in 6 control subjects (3 males, 3 females) and 7 alcoholic non-liver disease subjects (5 males, 2 females). In each case the alpha-1-antitrypsin level was within the normal range (200 - 400 mg/100 ml serum). There were no subjects with an abnormally low level which might indicate alpha-1-antitrypsin deficiency due to a homozygous or heterozygous alpha-1-antitrypsin genotype. In addition, there were no subjects with an abnormally high level of alpha-1-antitrypsin which might indicate acute inflammation. The results do not exclude the possibility that a heterozygous alpha-1-antitrypsin genotype is masked by raised levels due to disease.

Table 4

Acetylalator Phenotype

Group	n	Acetylalator Phenotype							
		Fast				Slow			
		male	female	total	%	male	female	total	%
Alcoholic Liver Disease	19	6	2	8	42.1	5	6	11	57.9
Control	7	1	2	3	42.9	3	1	4	57.1
Alcoholic Non-liver Disease	12	4	1	5	41.7	5	2	7	58.3

3.1.6. Unsaturated Vitamin B12 Binding Capacity and Serum Transcobalamin Levels

Results of determinations of serum levels of vitamin B12 (carried out by the Department of Haematology, Southern General Hospital), serum UBBC and transcobalamin levels of alcoholic liver disease and control subjects are summarized in Table 5. The apparently higher level of serum B12 observed in alcoholic liver disease subjects compared with control subjects, though not statistically significantly different, is consistent with several reports reviewed by Bonjour (1979). Product moment correlation analysis suggests that serum B12 levels are not statistically significantly correlated with UBBC in either the alcoholic liver disease or control group. Although there is no significant difference between the alcoholic liver disease and control group in either the transcobalamin II component of UBBC or total UBBC, the transcobalamin I and III component of UBBC in the alcoholic liver disease group is significantly higher than that observed in the control group.

Table 5

Serum B12 UBBC and Transcobalamin Levels in Alcoholic
Liver Disease and Control Subjects

	Alcoholic Liver Disease		Control			P
	Mean \pm s.e.	n	Mean \pm s.e.	s.e.	n	
Serum B12 (pg/ml)	897 \pm 105	17	607 \pm 75		9	n.s.
UBBC (pg/ml)	1444 \pm 75	17	1272 \pm 119		9	n.s.
TC II (pg/ml)	1185 \pm 60	17	1131 \pm 115		9	n.s.
TC I & III (pg/ml)	253 \pm 30	17	147 \pm 20		9	p<0.05

3.1.7. Antipyrine Clearance

The results of determination of antipyrine $t_{1/2}$ and SAA in alcoholic liver disease and control subjects are given in Table 6. The results of two-sample student t-tests indicate that antipyrine $t_{1/2}$ and SAA values are statistically significantly different in the alcoholic liver disease and control groups ($p < 0.001$ and $p < 0.01$). Product moment correlation analysis suggests there is no statistically significant correlation between antipyrine $t_{1/2}$ and SAA values in either the alcoholic liver disease or control group. Also there is no statistically significant correlation between antipyrine $t_{1/2}$ and serum AST activity or LAA values in the alcoholic liver disease group. However, seven of the eleven alcoholic liver disease subjects with an antipyrine $t_{1/2}$ greater than 23 h had histological evidence of cirrhosis.

3.1.8. Preparation and Autoradiography of a Liver Cell Suspension and Vitamin B6 Deficient Rat Studies

The preliminary experiments to assess DNA synthesis in a suspension of finely divided rat liver tissue using (^3H)-TdR and autoradiography were unsuccessful. The main difficulty was obtaining and maintaining a viable suspension of undamaged cells. Various approaches were used to try and overcome this problem, e.g., reduction of processing time, gentler processing of cells, etc., which were also unsuccessful. Use of a liver tissue slice technique was considered but this would have introduced further difficulties in interpretation of

Table 6

Antipyrine $t\frac{1}{2}$ and SAA in Alcoholic Liver Disease and

Control Subjects

Alcoholic Liver Disease Subjects				Control Subjects		
No.	Antipyrine $t\frac{1}{2}$ (h)	SAA (mmol/l)	No.	Antipyrine $t\frac{1}{2}$ (h)	SAA (mmol/l)	
1	49.9	6.2	1	21.1	37.1	
2	41.0	10.0	2	18.2	16.0	
3	39.4	20.5	3	14.1	42.9	
4	37.0	34.8	4	13.5	18.5	
5	36.7	3.4	5	12.8	57.3	
6	35.6	14.0	6	12.6	87.2	
7	31.5	23.6	7	12.0	87.0	
8	30.3	38.7	8	10.5	64.0	
9	23.5	6.1				
10	23.1	54.5				
11	22.3	11.0				
12	16.0	11.3				
Mean	32.2	19.5	Mean	14.3	51.3	
s.e.	2.8	4.5	s.e.	1.2	9.8	

results and would not have been suitable for use in studies of human liver biopsy material.

The experimental feeding of a vitamin B6 deficient diet to rats was also unsuccessful. After a period of 6 weeks feeding of the deficient diet, none of the rats showed signs of vitamin B6 deficiency symptoms or a reduction in serum PLP values. In contrast, Reynolds (1978) reports the occurrence of vitamin B6 deficiency after four weeks' feeding with a vitamin B6 deficient diet. The most likely explanation for the failure to produce a vitamin B6 deficient state in the present study is the presence of a bacterial flora in these rats which provided an alternative source of vitamin B6. This explanation is suggested by Sumi et al (1977) whose study of germ-free and conventional rats suggested that the presence of gut microflora made some contribution to the conventional rats' vitamin B6 nutrition.

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Section 3.2.

Preliminary PHA Stimulated Lymphocyte Studies

3.2.1. Acid-insoluble (^{125}I)-UdR Incorporation During PHA Stimulated Lymphocyte Culture

The results of determination of acid-insoluble (^{125}I)-UdR incorporation during PHA stimulated culture are illustrated in Fig. 9. The lymphocytes used were from control subjects and the culture medium was complete MEM.

3.2.2. Effect of Variation of Cell and Serum Concentration on Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures

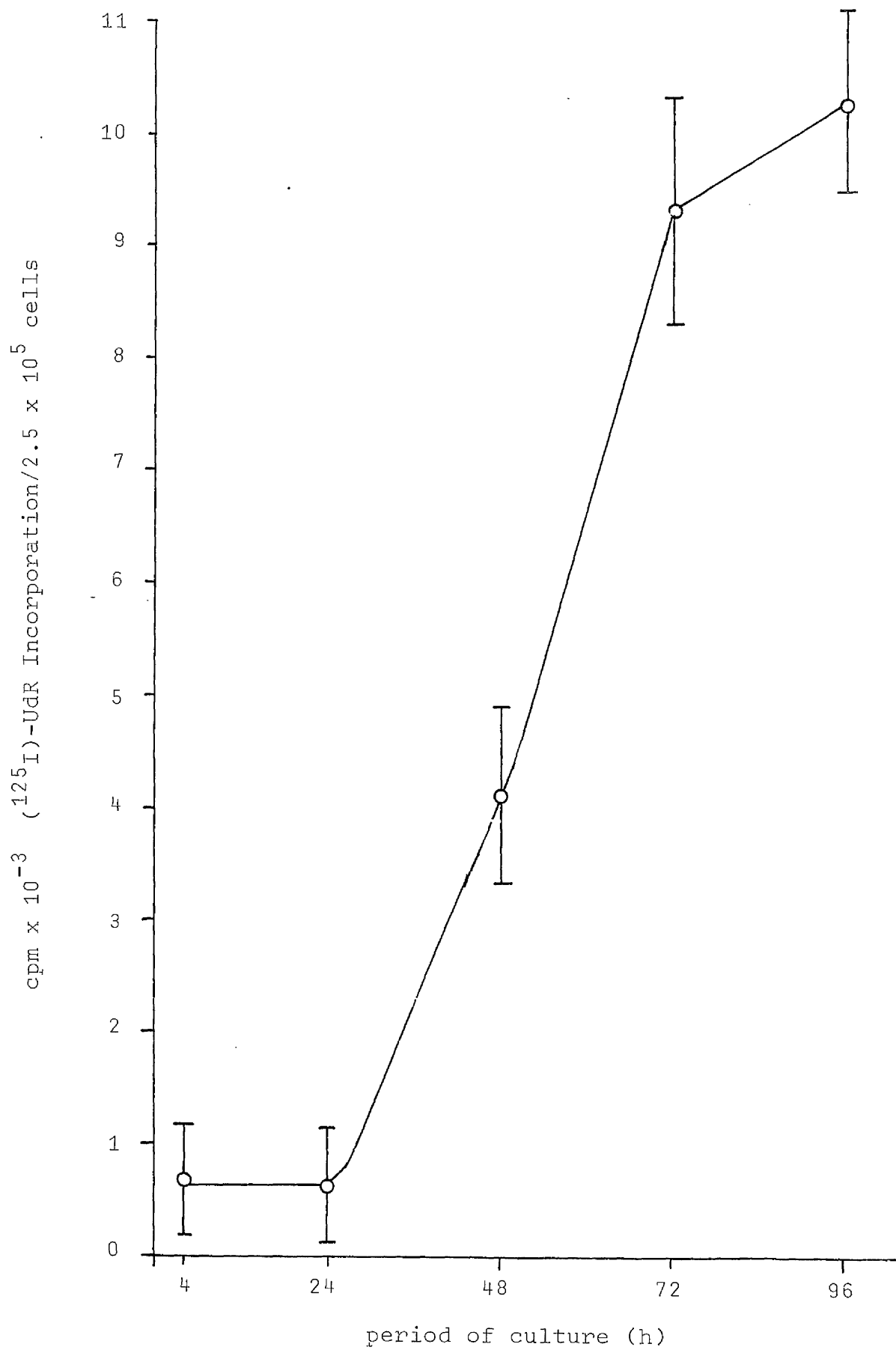
The results of determination of the effect of variation of cell and serum concentration on acid-insoluble incorporation into PHA stimulated lymphocyte cultures are given in Table 7. The results suggest that acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures is sensitive to increases in serum and cell concentration and that this sensitivity may be used to assess the effect of different sera on PHA stimulation. Table 7 also illustrates the use of calculating results as a percentage of incorporation in a standard culture for each subject. This reduces inter-individual variability and allows between subject comparisons to be made.

Fig. 9. Acid-insoluble (^{125}I)-UdR Incorporation at 4,
24, 48, 72 and 96 hours PHA Stimulated Lymphocyte
Culture

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocytes (2.5×10^5 cells) was determined at the times indicated after;

- (I) culture in complete MEM
- (II) pre-incubation addition BSS (50 ul), 1 h prior to,
- (III) incubation 3 h (^{125}I)-UdR, 0.5 uCi

Results are given as mean (cpm) \pm s.e. of lymphocyte cultures of 5 control subjects.



Effect of Variation of Cell and Serum Concentration on Acid-insoluble (¹²⁵I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures derived from two control subjects was determined at 72 h after;

(I) culture in complete MEM at the specified serum and cell concentrations

(II) pre-incubation addition of BSS (50 μl) 1 h prior to;

(III) incubation 3 h, (^{125}I)-UdR, 0.5 μCi

Results given are the mean of duplicate analyses.

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3.2.3. Inter-individual Variation in Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures of Control Subjects

The results of determination of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures derived from control subjects are given in Table 8. These results demonstrate the wide variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures derived from a control group. The results are from analyses of cultures carried out over a period of several weeks using the same conditions and complete MEM in each case.

3.2.4. Inter-individual Variation in Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures of Alcoholic Liver Disease Subjects

The results of determination of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes derived from alcoholic liver disease subjects are given in Table 9. Also included in this table are the results of clinical laboratory investigation of these subjects. The results demonstrate the wide variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures of alcoholic liver disease subjects. Examination of Table 9 strongly suggests that there is no simple relationship between acid-insoluble (^{125}I)-UdR incorporation and the extent of liver damage as assessed by enzyme content of serum or histology of liver biopsies. This is confirmed by statistical,

Table 8

Inter-individual Variation in Acid-insoluble (^{125}I)-
UdR Incorporation into PHA Stimulated Lymphocyte
Cultures of Control Subjects

Subject	Acid-insoluble (^{125}I)-UdR Incorporation (cpm)
1	17147
2	50027
3	36291
4	12496
5	6443
6	14695
7	12393
8	7958
9	6566
10	9564
11	9543
12	16059
Mean	16599
s.e.	3820

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into
 PHA stimulated lymphocyte cultures derived from 12
 control subjects was determined at 72 h after;

(I) culture in complete MEM (2.5×10^5 Cells, 15%
 autologous serum)

(II) pre-incubation addition of BSS (50 ul) 1 h prior to;

(III) incubation 3 h, (^{125}I)-UdR, 0.5 μCi

Results given are the mean of duplicate analyses

Table 9

Inter-Individual Variation in Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures of Alcoholic Liver Disease Subjects

Sex M: Male F: Female	Subject	(^{125}I) -UdR incorp'n (cpm)	Serum				histology of liver biopsy
			AST	ALT	AP	bilirubin	
F	1	3293	103	25	245	77	fatty change
M	2	5006	389	104	165	400	alcoholic cirrhosis
M	3	5427	36	69	64	15	alcoholic hepatitis
M	4	5745	42	26	64	40	alcoholic cirrhosis
M	5	7647	34	30	76	8	fatty change
M	6	9048	290	146	90	101	alcoholic hepatitis
M	7	9471	39	39	114	12	alcoholic cirrhosis
M	8	10389	108	55	88	35	alcoholic hepatitis
M	9	11429	248	116	15	48	alcoholic hepatitis
M	10	11524	108	300	160	320	alcoholic hepatitis
M	11	11632	148	212	86	7	fatty change
F	12	30506	58	20	274	48	alcoholic hepatitis
M	13	32758	60	43	74	74	alcoholic cirrhosis
M	14	38776	104	46	62	68	fatty change
	Mean	13761					
	S.e.	3058					

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures derived from alcoholic liver disease subjects was determined at 72 h after;

- (I) culture in complete MEM (2.5×10^5 cells, 10% autologous serum)
 (II) pre-incubation addition of BSS (50 ul) 1 h prior to;
 (III) incubation 3 h (^{125}I)-UdR, 0.5 uCi. Results given are the mean of duplicate analyses.

Serum AST, ALT, AP and bilirubin determinations were carried out by the Department of Biochemistry and histology of liver biopsies was carried out by Dept. of Pathology, Southern General Hospital (AST, ALT, AP; I units/l; bilirubin; $\mu\text{mol/l}$)

product moment correlation analysis.

3.2.5. Relationship Between Serum PLP Concentration
and Acid-insoluble (^{125}I)-UdR Incorporation into PHA
Stimulated Lymphocyte Cultures of Alcoholic Liver
Disease Subjects

The results of determination of acid insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures and serum PLP levels of alcoholic liver disease subjects are given in Table 10. Product moment correlation analysis suggests there is no statistically significant association between acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures and serum PLP Levels of these subjects.

3.2.6. Absence of PHA Stimulated Lymphocyte Response
in Advanced Alcoholic Cirrhosis

In one subject with advanced alcoholic cirrhosis, a PHA stimulated lymphocyte response was absent when autologous serum was used in the culture medium. When homologous control serum was used however, a normal PHA stimulated lymphocyte response, as assessed by acid-insoluble (^{125}I)-UdR incorporation at 72 hours was observed. The results of Southern General Hospital clinical laboratory investigation of this subject were:

AST	389	I Units/l
ALT	104	I Units/l
AP	165	I Units/l
bilirubin	400	umol/l

Table 10

Serum PLP Concentration and Acid-insoluble (^{125}I)-
UdR Incorporation into PHA Stimulated Lymphocyte
Cultures of Alcoholic Liver Disease Subjects

Subject	Serum PLP (ng/ml)	(^{125}I)-UdR incorporation (cpm)
1	0.4	11524
2	0.4	30506
3	0.8	32758
4	1.7	7958
5	3.1	13687
6	4.3	6566
7	4.8	11496
8	5.0	14695
9	5.4	5427
Mean	2.9	14957
s.e.	0.7	3321

Serum PLP determinations were carried out using the method described in Section 2.2.2.

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures derived from alcoholic liver disease subjects was determined at 72 h after;

- (I) culture in complete MEM (2.5×10^5 cells,
10% autologous serum)
- (II) pre-incubation addition of BSS (50 ul)
1 h prior to;
- (III) incubation 3 h (^{125}I)-UdR, 0.5 μCi

Results given are the mean of duplicate analyses.

Subjects 1,2 and 3 are subjects 10, 12 and 13
in Table 9.

The observation of a normal PHA stimulated lymphocyte response in control serum suggests normal cellular function and abnormal serum. The most likely serum factor causing the inhibition of a PHA stimulated lymphocyte response is the high bilirubin content.

3.2.7. Effect of Culture in a Pyridoxine Deficient Culture Medium on Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

Initial experiments with a pyridoxine deficient medium, modified MEM (1), suggested that the absence of pyridoxine from MEM resulted in a significant reduction in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures. The results of determination of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes of three different groups of subjects cultured in both complete MEM and modified MEM 1 (without pyridoxine) are summarised in Table 11. The three groups of subjects were; control subjects, alcoholic liver disease subjects and alcoholic liver disease subjects whose recent treatment included vitamin supplements containing pyridoxine. In each subject group, acid-insoluble (^{125}I)-UdR incorporation in modified MEM (1) cultures was statistically significantly lower than in complete MEM cultures ($p < 0.05$; $p < 0.001$; $p < 0.01$ respectively). Acid-insoluble (^{125}I)-UdR incorporation in complete MEM cultures was not statistically significantly different between the control and alcoholic liver

disease groups ($p > 0.05$). The mean acid-insoluble (^{125}I)-UdR incorporation in modified MEM (1) cultures, expressed as a percentage of incorporation in complete MEM cultures, was statistically significantly lower in both alcoholic liver disease groups compared to the control group ($p < 0.05$) and was also lower in the vitamin treated alcoholic liver disease group compared to the non-vitamin treated alcoholic liver disease group ($p < 0.05$).

Several experiments were conducted to characterise this observed effect of culture in pyridoxine deficient modified MEM (1) on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocyte cultures. These included changing the culture medium from complete MEM to modified MEM (1) at different times during culture, the effect of addition of pyridoxine and other forms of vitamin B6 to modified MEM (1) and the effect of varying the proportion of complete MEM and modified MEM (1) in the PHA stimulated lymphocyte culture medium. The results of these experiments suggested that the observed reduction in acid-insoluble (^{125}I)-UdR incorporation in PHA stimulated lymphocytes cultured in modified MEM (1) was not solely due to the absence of pyridoxine. This was confirmed by later experiments in which acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes cultured in modified MEM (2), without pyridoxine and with a folate concentration

of 1.0 mg/l was not significantly different from that in lymphocytes cultured in complete MEM. It was concluded that the observed differences between modified MEM (1) and complete MEM could not be wholly attributed to an effect of the pyridoxine concentration and therefore that the results summarised in Table 11 could not be interpreted on this basis.

3.2.8. Effect of Ethanol on Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocyte Culture

Results of determinations of the effect of different amounts of ethanol added either at initiation of culture or 1 hour prior to addition of (^{125}I)-UdR on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures are shown in Fig. 10. The results suggest that the presence in lymphocyte cultures of progressively larger amounts of ethanol has a significant inhibitory effect on PHA stimulation of lymphocytes. When ethanol is added 1 hour prior to addition of radioactive label, a marked inhibitory effect is only apparent at the 5% concentration level.

Table 11

Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes Cultured in Complete MEM and in a Pyridoxine Deficient Medium (Modified MEM 1)

	Control Subjects			Alcoholic Liver Disease Subjects treated with Vitamins			Alcoholic Liver Disease Subjects		
	Complete MEM	Modified MEM (1)	%	Complete MEM	Modified MEM (1)	%	Complete MEM	Modified MEM (1)	%
n	12	12		7	7		8	8	
Mean	16599	6537	49	8549	2781	33	19520	3414	20
s.e.	3820	693	4.7	612	265	2.4	3715	682	4.6

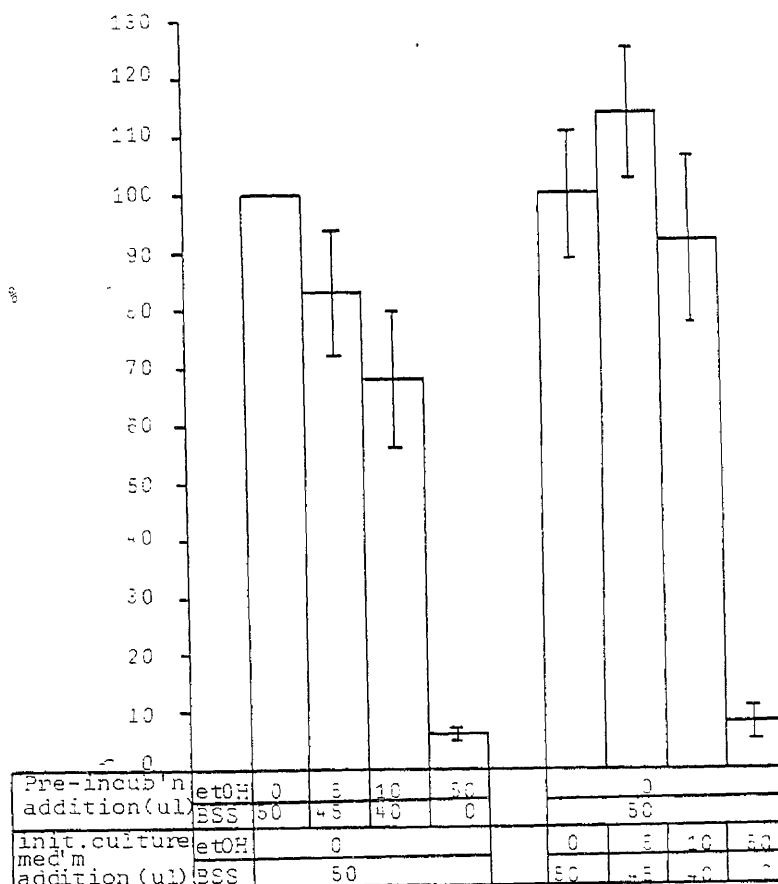
Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated Lymphocyte cultures was determined at 72 h after;

- (I) culture in complete MEM (2.5×10^5 cells, 15% autologous serum)
or in modified MEM (1) without pyridoxine HCl (2.5×10^5 cells, 15% autologous serum)
- (II) pre-incubation addition of BSS (50 ul) 1 h prior to;
- (III) incubation 3 h (^{125}I)-UdR, 0.5 uCi

Results are given as the mean and standard error of the mean of incorporation into duplicate cultures of n subjects.

Fig. 10

Effect of Ethanol on Acid-insoluble (125 I)-UdR Incorporation into PHA Stimulated Lymphocyte Cultures



Acid-insoluble (125 I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells, 10% serum) was determined at 72h after;

- (i) culture in complete MEM with specified addition of BSS or ethanol to a concentration of 0.5%, 5% or 5%
- (ii) pre-incubation addition of specified amounts of BSS or ethanol, 1h prior to;
- (iii) incubation, 3h (125 I)-UdR, 0.5μCi.

Results are given as the mean \pm s.e. of incorporation into duplicate cultures of 3 control subjects, expressed as a percentage of incorporation into cultures without added ethanol.

Section 3.3.

Development of a Functional Assay for
Determination of Vitamin B6 Status
In Alcoholic Subjects

3.3.1. Effect of Pre-incubation with Aminopterin on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes

Acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocyte cultures was determined at 4, 24, 48, 72 and 96 h after initiation. In experimental cultures, aminopterin, to a final concentration of 114 u M was added 1 h prior to incubation with (^{125}I)-UdR. In control cultures, 50 μl of BSS was added. The culture medium used was complete MEM and the results are shown in Fig. 11. Group results as illustrated here, are expressed as mean \pm standard error of individual results.

As previously indicated (section 1.4.4.) the effect of pre-incubation with aminopterin is to inhibit dihydro-folate reductase activity such that the de novo contribution to thymidylate synthesis is substantially reduced. Subsequent incorporation of thymidylate precursor via the salvage pathway is then largely free of competition with the de novo contribution. This use of aminopterin allows an assessment to be made of the relative contribution of the de novo and salvage pathways to thymidylate synthesis. Longer term use of aminopterin would, in the absence of an exogenous supply of salvage pathway substrate, result in cessation of DNA synthesis.

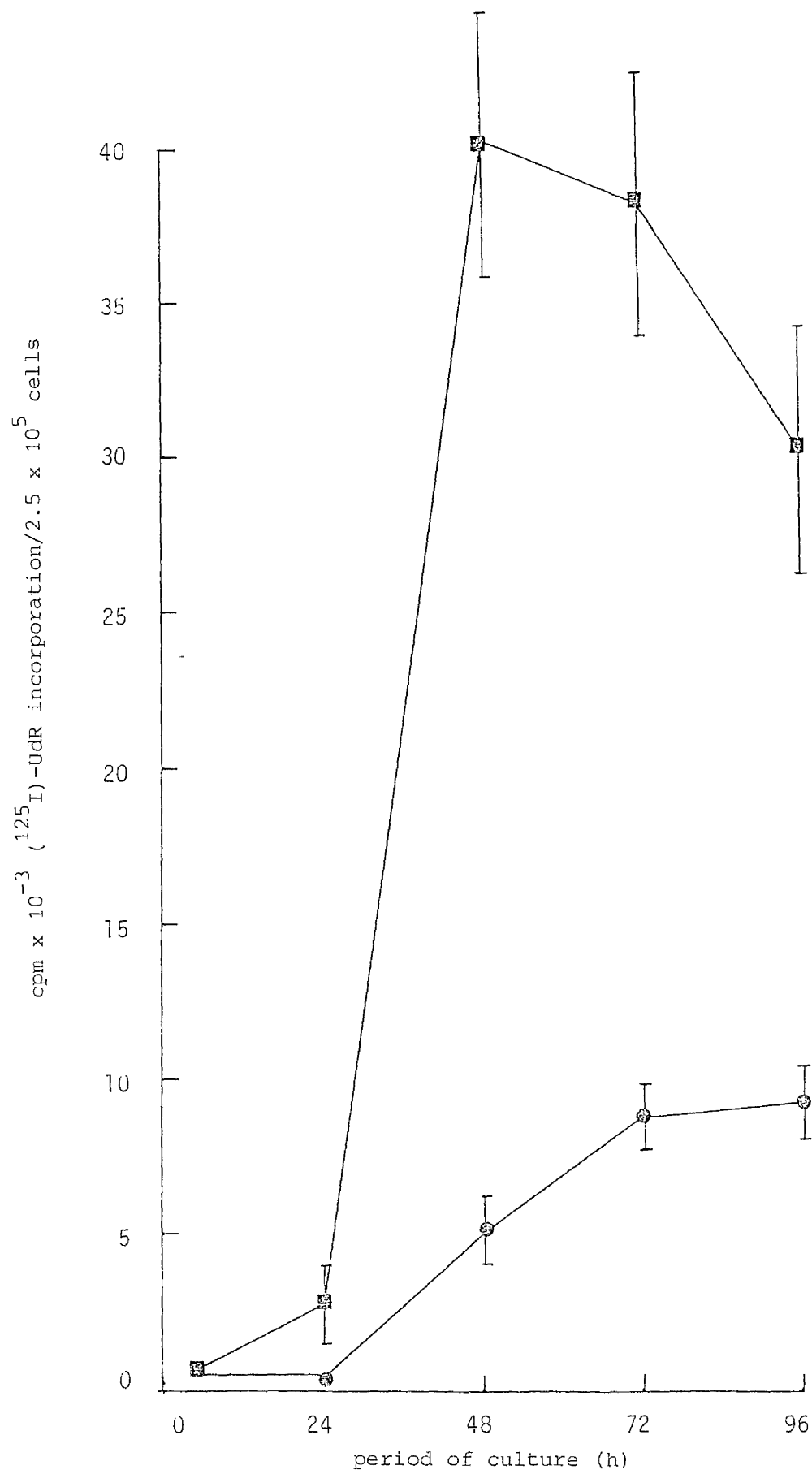
In Fig. 11, acid-insoluble incorporation of (^{125}I)-UdR is shown as various times during the course of PHA stimulated human lymphocyte culture, after prior addition of either aminopterin or BSS. The results show

Fig. 11 Effect of Pre-incubation with Am on
Subsequent Acid-insoluble Incorporation of (^{125}I)-
UdR into PHA Stimulated Lymphocytes Cultured in
Complete MEM

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocytes (2.5×10^5 cells) was determined at the times indicated, after;

- (i) culture in complete MEM
- (ii) pre-incubation addition of BSS (●), or Am, $114\mu\text{M}$ (■), 1h prior to
- (iii) incubation 3h, (^{125}I)-UdR, $0.5\mu\text{Ci}$

Results are given as mean (cpm) \pm s.e. of lymphocyte cultures of 6 control subjects.



that in complete MEM, which has a folate concentration of 1.0 mg/l, the salvage contribution to thymidylate synthesis is low relative to that of the de novo contribution. Furthermore, it appears from these results that the contribution of each of these pathways to total thymidylate synthesis varies during the course of stimulated lymphocyte culture such that, while the salvage contribution increases through 96 h, the de novo contribution is maximal at 48 h and declines slowly thereafter. The significance of the folate concentration in complete MEM is related to its likely effect on the de novo contribution to thymidylate synthesis and this relationship is examined in the following experiment.

3.3.2. Effect of Variation of the Medium Folate Concentration on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes

Modified MEM (2) was used to prepare two media identical in component specification to complete MEM except that one had a folate concentration of 0.01 mg/l and the other a folate concentration of 2 mg/l. Acid-insoluble incorporation of (^{125}I)-UdR was determined at 72 h, in PHA stimulated lymphocytes cultured in each of these media, after pre-incubation with BSS or aminopterin (114 μM). Individual results, the mean of incorporation into duplicate cultures, were used to calculate the group results, shown in Fig. 12, which are given as the mean \pm standard error of individual results.

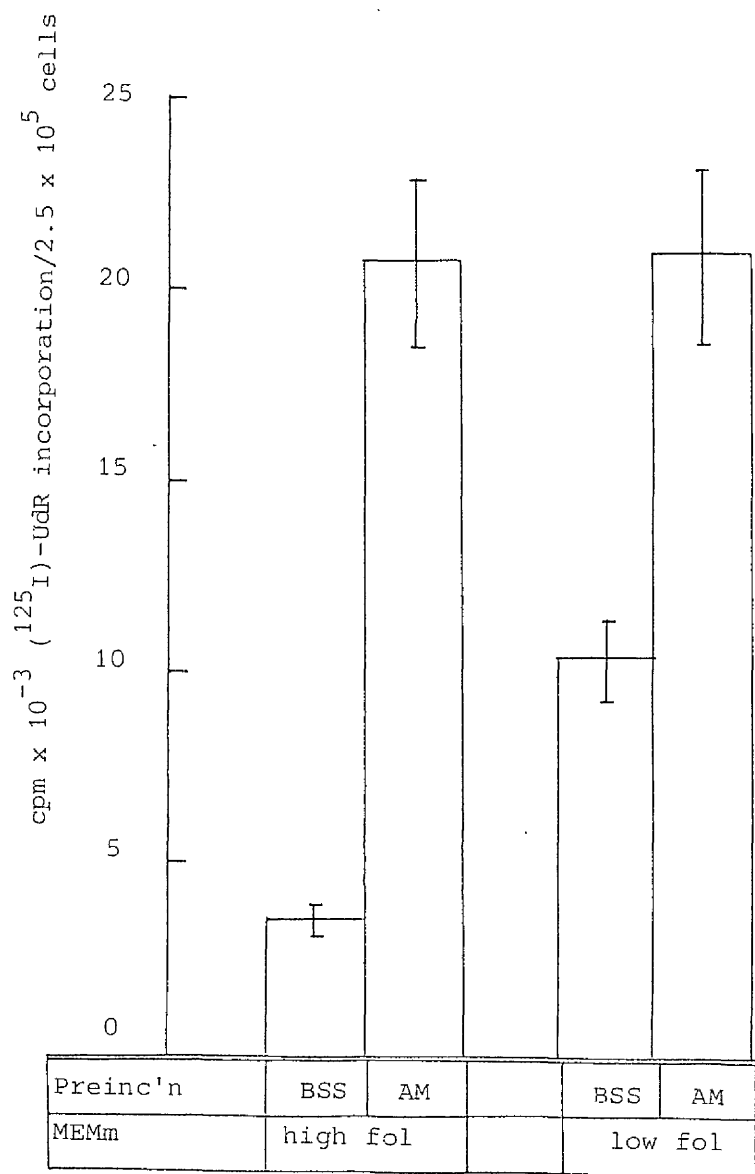
Fig. 12. Effect of Variation of the Medium Folate
Concentration on Acid-insoluble Incorporation
(¹²⁵I)-UdR into PHA Stimulated Lymphocytes

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into
PHA stimulated lymphocytes (2.5×10^5 cells) was
determined at 72h, after;

- (i) culture in modified MEM (2) of high folate
concentration (2.0mg/l), Pl (0.01mg/l)
or low folate concentration (0.01mg/l)
Pl (0.01mg/l)
- (ii) pre-incubation addition of BSS, or Am 114μM,
1h prior to,
- (iii) incubation 3h (¹²⁵I)-UdR, 0.5μCi

Results are given as mean (cpm) ± s.e. of lymphocyte
cultures of 5 normal subjects.

The culture and pre-incubation conditions for each experiment
are as indicated at the base of each column.



The results show that as the medium concentration of folate is reduced from 2 mg/l to 0.01 mg/l, the incorporation of (^{125}I)-UdR, after pre-incubation with BSS more than doubles, while after pre-incubation with aminopterin, there is no significant difference in incorporation into lymphocytes cultured in either medium. This result is consistent with the hypothesis that the medium folate concentration has a marked effect on the de novo pathway's contribution to total thymidylate synthesis. The closeness of the extent of incorporation in the two media, after pre-incubation with aminopterin, could either reflect similar levels of DNA synthesis in these cultures irrespective of the incident folate levels or it could be due to coincident levels of enzyme activity at that particular time.

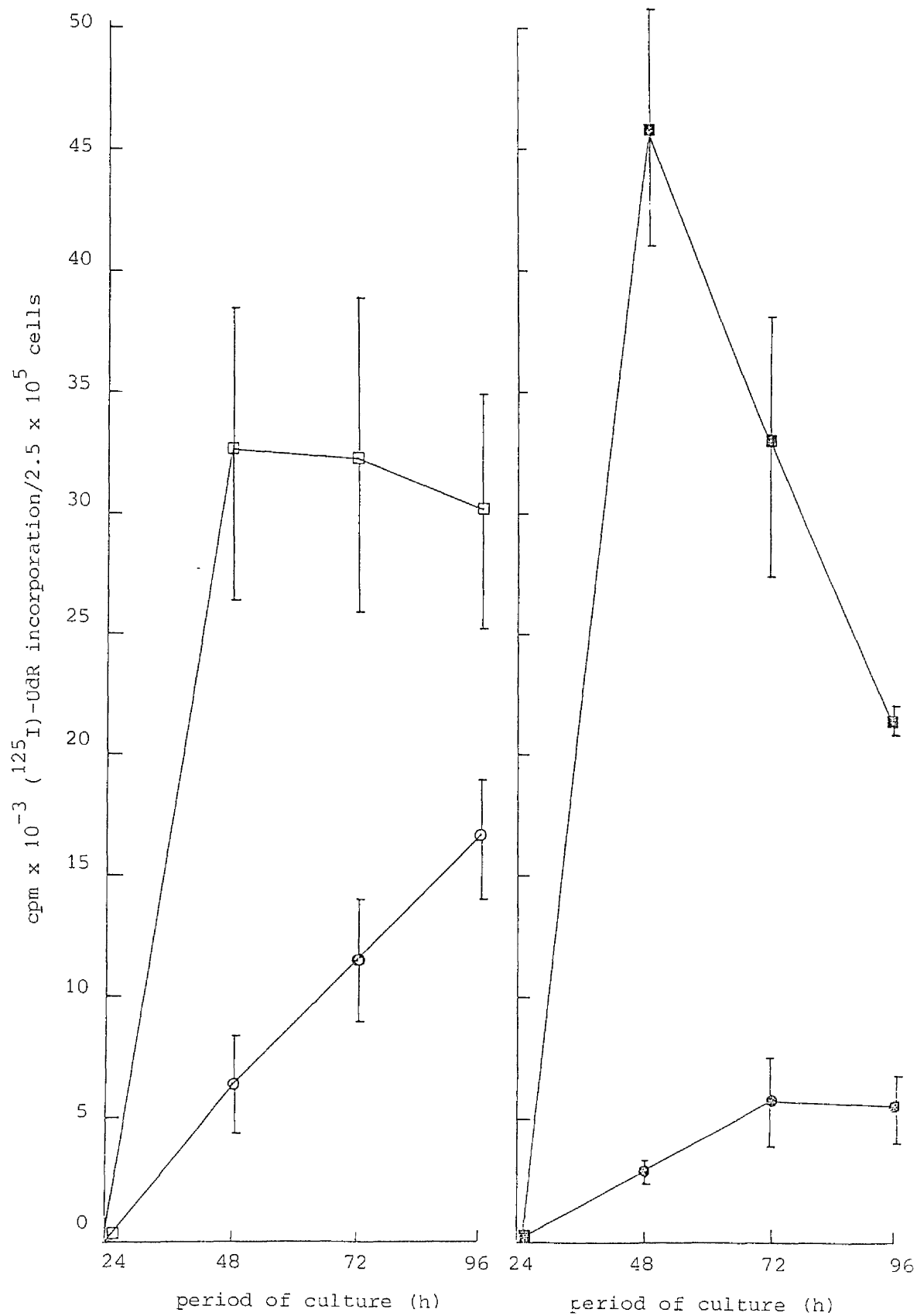
In order to determine the effect of variation of the medium folate concentration on acid-insoluble (^{125}I)-UdR incorporation after pre-incubation with BSS or MTX during the course of PHA stimulated lymphocyte culture, determination of such incorporation was carried out at 24, 48, 72 and 96 h after initiation of culture. The culture medium used was modified MEM (2) with a folate concentration of either 1.0 mg/l or 0.01 mg/l. Determination of incorporation was carried out after a 1h pre-incubation with either BSS or MTX (110 μM) and the results are illustrated in Fig. 13.

Fig. 13 Effect of Variation of the Medium Folate
Concentration on Acid-insoluble Incorporation
of (^{125}I)-UdR into PHA Stimulated Lymphocytes

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at the times indicated, after;

- (i) culture in modified MEM (2) of low folate concentration (0.01mg/l) and Pl (1.0mg/l) (0/□) or high folate concentration (1.0mg/l) and Pl (1.0mg/l) (●/■)
- (ii) pre-incubation with BSS (0/●) or MTX $110\mu\text{M}$ (□ / ■), 1h prior to;
- (iii) incubation 3h (^{125}I)-UdR, $0.5\mu\text{Ci}$

Results are given as mean (cpm) \pm s.e. of lymphocyte cultures of 4 control subjects.



The results show that in modified MEM (2) with a high folate concentration (1 mg/l) and after pre-incubation with either BSS or MTX, incorporation follows a similar pattern to that observed in complete MEM after pre-incubation with either BSS or aminopterin, Fig. 11. In modified MEM (2) with a low folate concentration (0.01 mg/l) however, a different pattern of incorporation is apparent. While incorporation after pre-incubation with MTX is essentially comparable to that in high folate medium, though the extent of incorporation at 48h is reduced and the decline thereafter less marked, (^{125}I)-UdR incorporation after pre-incubation with, BSS is greater in low folate medium and continues to increase up to 96 h. This is consistent with the result illustrated in Fig. 12 and indicates that the respective contribution of the de novo and salvage pathways to thymidylate synthesis is influenced by the medium folate concentration.

3.3.3. Effect of Pre-incubation with UdR on Acid-insoluble Incorporation of (^{125}I)-UdR or (^3H)-TdR into PHA Stimulated Lymphocytes

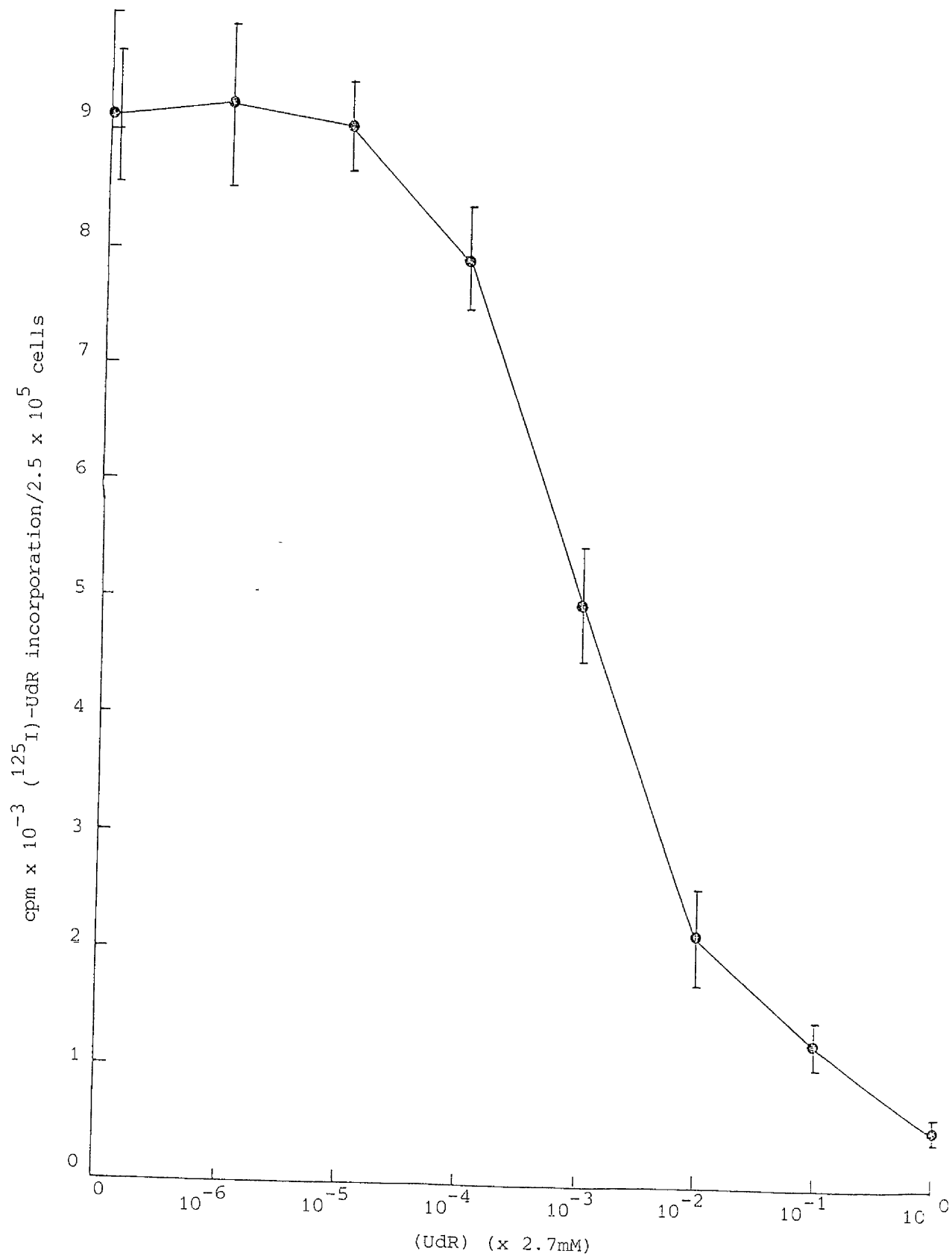
The addition of UdR to PHA stimulated lymphocyte cultures prior to determination of acid-insoluble incorporation of radio-labelled thymidine results in reduced incorporation relative to control cultures to which no UdR has been added. A similar effect was observed on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes cultured in complete MEM, Fig. 14.

Fig. 14 Effect of Pre-incubation with UDR on Acid-
insoluble Incorporation of (^{125}I)-UdR into
PHA Stimulated Lymphocytes

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocytes (2.5×10^5 cells) was determined at 72h after;

- (i) culture in complete MEM
- (ii) pre-incubation addition of UdR, to specified concentration, 1h prior to;
- (iii) incubation 3h (^{125}I)-UdR, $0.5\mu\text{Ci}$

Results are given as mean (cpm) \pm s.e. of lymphocyte cultures of 5 control subjects.



The observation that this normal deoxyuridine suppression was impaired in stimulated lymphocyte cultures derived from patients with folate or vitamin B₁₂ deficiency formed the basis of the UdR suppression test for determination of such a vitamin deficiency. The presumed rationale for such a test is discussed earlier in this report (1.4.5.) and also a rationale for the possible extension of this test to determination of a vitamin B6 deficiency.

In order to assess the validity of this test and also to determine whether or not (assuming the use of the test was justified) alcoholic liver disease subjects were vitamin B6 deficient relative to control subjects, experiments were conducted in which the effect of pre-incubation with UdR on subsequent radioactively labelled DNA precursor incorporation was determined in PHA stimulated lymphocyte cultures derived from control, alcoholic non-liver disease and alcoholic liver disease subjects.

Results of studies of lymphocytes of control and alcoholic liver disease subjects cultured in complete MEM are shown in table 12. There was no statistically significant difference in UdR suppression between lymphocytes of control and alcoholic liver disease subjects.

Table 12 Effect of Pre-incubation with UdR on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes Cultured in Complete MEM

Medium	MEM Complete							
Subjects	Control				Alcoholic Liver Disease			
preincubation	BSS		UdR		BSS		UdR	
	cpm	%	cpm	%	cpm	%	cpm	%
1	17147	100	1980	11.5	11524	100	299	2.3
2	14695	100	1183	8.1	32358	100	1323	4.1
3	12393	100	63	0.5	20161	100	1694	8.4
4	7958	100	772	9.7	30106	100	2489	8.3
5	6566	100	670	10.2	31628	100	2059	6.5
6	9564	100	467	4.9	13681	100	436	3.2
7					9409	100	449	4.8
8					7286	100	545	7.5
Mean	11387	100	856	7.5	19519	100	1162	5.6
s.e.	1668		270	1.7	3715		299	0.8

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) of control and alcoholic liver disease (ald) subjects was determined at 72h after:

- (I) culture in complete MEM (15% serum)
- (II) pre-incubation addition of BSS or UdR 2.7 mM, 1h prior to;
- (III) incubation 3h, (^{125}I)-UdR, 0.5 μCi

Results are given as the mean of duplicate analyses. Incorporation after pre-incubation with UdR is expressed as a % of incorporation in replicate cultures to which BSS had been added.

In view of the possible masking effect of medium pyridoxal on tests of vitamin B6 deficiency, experiments were also conducted in a basal BSS medium (table 14), and in modified MEM (2) with a low pyridoxal concentration and without pyridoxal (table 13 and 15). Also, in view of the role of vitamin B6 in de novo thymidylate synthesis and the effect of the medium folate concentration on incorporation of radioactively labelled DNA precursor, experiments were conducted in medium of both high (1.0 mg/l) and low (0.01 mg/l) folate concentration.

Incorporation was determined at 72 h after pre-incubation with BSS, MTX (Am), or UdR and the results are presented in tables 13, 14 and 17. (The UdR suppression results of table 15 are expressed in percentage terms in table 17). Incorporation after pre-incubation with UdR was expressed as a percentage of incorporation in control cultures to which no UdR had been added and this gives the percentage UdR value suppression value for a particular subject. Group values are given as mean \pm standard error of these individual results. Statistical analysis of these values (two sample student t-test) indicates that there is no significant difference between the effect of UdR on incorporation of (^{125}I)-UdR or (^3H)-TdR in lymphocytes of control subjects and lymphocytes of alcoholic non-liver disease and liver disease subjects irrespective of the culture medium used. The results of

Table 13

Acid-insoluble (³H)-TdR incorporation (cpm) into PHA stimulated lymphocyte cultures

(2.5 x 10⁵) cells of control subjects was determined at 72h after:

(I) culture in modified MEM (2) with folate 0.01mg/l and Pl 0.01 mg/l

(II) pre-incubation addition of BSS (50μl)

Am (0.114 mM)

UdR (2.7 mM)

UdR (2.7 mM) + folate (1.0 mg/l)

UdR (2.7 mM) + Pl (HCl) (1.0 mg/l)

or UdR (2.7 mM) + OH B12 (1.0 mg/l)

1h prior to;

(III) incubation 3h, (³H)-TdR, 20 μCi

Results given are the mean of triplicate analyses. Incorporation after pre-incubation with various additions is expressed as a % of incorporation in replicate cultures to which BSS had been added.

Table 13

Effect of Added Folate, Pyridoxal and Hydroxycobalamin on UdR
 Suppression of Acid-insoluble Incorporation of (³H)-TdR into
 PHA Stimulated Lymphocyte Cultures

Medium	Modified MEM(2): low folate 0.01 mg/l; low pyridoxal 0.01 mg/l											
preincuba- tion	BSS		Am		UdR		UdR+folate		UdR + Pl		UdR+OHB12	
Control Subjects	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
1	39285	100	53232	136	4648	12	3442	9	7417	19	8767	22
2	34678	100	33793	97	1319	4	1038	3	3239	9	3868	11
3	37109	100	59834	161	6476	17	2306	6	5459	15	4558	12
4	57661	100	67090	116	11714	20	6774	12	13767	24	15422	27
\bar{x}	42186	100	53487	128	6039	13	3390	7.5	7471	17	8154	18
s.e.	5243	0	7149	13.7	2172	3.5	1230	1.9	2266	3.2	2654	3.9

Table 14

Acid-insoluble (^3H)-TdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) of control and alcoholic liver disease subjects was determined at 72 h after:

- (I) culture in BSS medium with vitamin additives as specified in the table and 15% autologous serum
- (II) pre-incubation addition of BSS (50 μl), MTX (0.11 mM) or UdR (2.7 mM), 1h prior to;
- (III) incubation, 3h, (^3H)-TdR 20 μCi .

Results given are the mean of triplicate analyses. Incorporation after culture in the specified medium and after pre-incubation with MTX or UdR is expressed as a % of incorporation into lymphocytes cultured in BSS and pre-incubated with BSS.

Medium	BSS						BSS + folate (1.0mg/l)						BSS + pyridoxal HCl (1.0mg/l)						BSS + folate + pyridoxal (1.0mg/l)					
Preincubation	BSS	%	MTX	%	UdR	%	BSS	%	MTX	%	UdR	%	BSS	%	MTX	%	UdR	%	BSS	%	MTX	%	UdR	%
Control subs																								
1	36083	100	68529	190	2467	7	34115	95	81653	226	1474	4	37822	105	58577	162	4907	14	29391	81	55278	153	368	1
2	31859	100	50856	160	2210	7	7295	23	29703	93	350	1	11391	36	17176	54	808	3	5425	17	14276	45	335	1
\bar{X}		100		175		7		59			160	3		71		108			9		49			1
S.E.		0		15.0		0		36.1			67	1.5		34.6		54.1		5.5			32.1		54.2	0
Ald. subs.																								
1	5006	100	11650	233	782	16	5075	101	10031	200	537	11	4727	94	15687	313	1014	20	3064	61	9000	178	591	12
2	5130	100	12284	239	3156	62	7087	138	8902	174	546	11	5490	107	34607	675	854	17	9899	193	38687	754	307	6
3	38776	100	94953	245	3213	8	31298	81	77487	200	819	2	38232	99	88493	228	4356	11	33902	87	75029	193	734	2
\bar{X}		100		239		29		107		191		8		100		405		16			114			7
S.E.		0		3.5		16.8		16.7		8.7		3.0		3.8		137		2.6			40.4		189.8	2.9

Table 15 Effect of Pre-incubation with UdR on Acid-insoluble Incorporation of (¹²⁵I)-UdR into PHA Stimulated Lymphocytes Cultured in Various Media

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) of 8 control subjects and 4 alcoholic subjects was determined

- at 72h after;
- (i) culture in modified MEM (2) with folate 1.0mg/l, Pl, 1.0mg/l or folate 0.01mg/l, Pl 0.01mg/l or folate 1.0mg/l, Pl 0.0mg/l or folate 0.01mg/l, Pl 0.0mg/l
 - (ii) pre-incubation addition of BSS, MTX 110μM, or UdR 2.7mM, 1h prior to;
 - (iii) incubation 3h, (¹²⁵I)-UdR, 0.5μCi

Results are given as the mean of duplicate analyses.

Medium	MEMm (2) fol 1.0 mg/l, Pl 1.0 mg/l				MEMm(2) fol 0.01 mg/l, Pl 0.01 mg/l				MEMm(2) fol 1.0 mg/l, Pl 0.0mg/l				MEMm(2) fol 0.01 mg/l, Pl 0.0 mg/l			
	BSS	MTX	UdR		BSS	MTX	UdR		BSS	MTX	UdR		BSS	MTX	UdR	
Pre-inc'n																
Subjects																
Control																
1	9251	32814	2490		21930	30294	6018		11360	23046	4125		23340	31593	5518	
2	11440	26810	4309		25091	31095	7077		15592	26799	6042		25604	31811	7899	
3	3134	16390	1058		13946	15712	2386		1447	11875	918		14637	12470	1708	
4	9004	26330	2935		24607	25157	3552		7334	25336	1372		27347	32509	3959	
5	8629	20920	1767		7176	14266	1961		5150	11082	782		9283	14357	1838	
6	5115	25003	1186		7430	13026	516		3649	15835	389		16491	27364	3060	
7	7834	37285	2524		9647	25517	1170		9118	34354	3082		32980	41194	6962	
8	8815	31260	1471		18155	33145	2658		18239	56695	1826		53635	66059	6233	
and																
1	1974	12096	149		4240	9069	456		1225	4384	48		3396	8087	187	
2	3876	26722	915		22050	37051	2192		4159	41511	932		24595	29958	3131	
ald																
3	31615	57373	5147		55747	56650	8295		15972	43146	3764		44813	55356	8951	
4	7103	32796	2326		33045	35462	6132		5704	23469	952		31766	30333	6299	

Table 16. Results* of Biochemical Analysis of Serum Samples of the
Alcoholic Subjects in Table 15

Sex	Subject	Serum				histology of liver biopsy
		AST	ALT	AP	bilirubin	
M	anld 1	28	14	90	19	(no hepatomegaly, biopsynot done) (slight hepatomegaly, biopsy not done) fatty change alcoholic hepatitis
M	2	27	26	170	17	
	ald					
M	3	104	46	62	68	
M	4	290	146	90	101	

* Results supplied by Dr. A.D. Beattie. Analysis performed by
Department of Clinical Biochemistry, Southern General Hospital
(AST, ALT, AP; I units/l; bilirubin; umol/l)

Table (17) Effect of preincubation with UdR on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes

Medium	MEM m(2)			
	fol 1.0mg/l Pl 1.0mg/l	fol 0.01mg/l Pl 0.01mg/l	fol. 1.0mg/l Ø Pl	fol. 0.01mg/l Ø Pl
	Incorporn. after preincubation with UdR expressed as % of incorporation in control culture			
Subjects control				
1	27	27	36	24
2	38	28	39	31
3	34	17	33	12
4	32	14	19	11
5	20	27	15	20
6	23	7	11	19
7	32	12	34	21
8	17	15	10	12
\bar{x}	28	19	25	19
s.e.	3	3	4	2
anld				
1	8	11	4	6
2	24	10	22	13
ald				
3	18	15	24	20
4	33	19	17	20
\bar{x}	20	14	17	15
s.e.	5	2	5	3

Culture conditions as for table (15)

Incorporation after preincubation with UdR is expressed as a % of incorporation in replicate cultures to which BSS had been added

biochemical analysis of serum samples and histological analysis of liver biopsy samples of the alcoholic subjects in Table 15 are presented in Table 16. For the purpose of assessment of functional vitamin B6 status in alcoholics, the results of alcoholic non-liver disease and alcoholic liver disease subjects presented in Table 15, were combined in the analysis. It is apparent from the results presented in Table 16 that the wide variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures of different alcoholic subjects in Table 15 cannot be accounted for on the basis of variation in serum enzyme levels. The question of inter-individual variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes is also discussed in section 4.2.2.

3.3.4. Effect of Pre-incubation Addition of the Vitamin B₆ Antagonist 4-deoxypyridoxine on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes

Since the deoxyuridine suppression test did not discriminate between stimulated lymphocytes of control and alcoholic subjects, an alternative approach was adopted. This was based on the premise that lymphocytes of B₆ deficient subjects would be more susceptible to the effect of a vitamin B₆ antagonist than would lymphocytes of vitamin B₆ replete subjects.

This hypothesis was tested using the vitamin B₆ antagonist 4-deoxypyridoxine (4-dPN). The effect of pre-incubation addition of 4-dPN on acid-insoluble incorporation of (¹²⁵I)-UdR into PHA stimulated lymphocytes of control and alcoholic liver disease subjects cultured in modified MEM is shown in Table 18. In order to distinguish between the possible differential effect of such an antagonist on de novo and salvage thymidylate DNA synthesis, determination of incorporation was carried out after pre-incubation with BSS and aminopterin. The medium folate and pyridoxal concentrations were 0.01 mg/l.

The results indicate that 4-dPN, when added at the pre-incubation stage to PHA stimulated lymphocytes of control subjects does not significantly reduce (¹²⁵I)-UdR incorporation either in the presence of BSS or Am. However, when 4-dPN is added to PHA stimulated lymphocytes of alcoholic liver disease subjects there is a significant reduction in (¹²⁵I)-UdR incorporation in the presence of BSS ($P < 0.01$), but not after co-pre-incubation with Am.

When the effect of pre-incubation addition of 4-dPN on (¹²⁵I)-UdR incorporation into PHA stimulated lymphocytes of control subjects cultured in conditions of high or low folate concentration was studied, it was found that in high folate conditions incorporation was significantly reduced in the presence of BSS ($p < 0.01$) but not after co-pre-incubation with Am. The results of this experiment, shown in Table 19 also

Table 18

Effect of Pre-incubation with 4-dPN on Acid-insoluble Incorporation of (125 I)-UdR into PHA Stimulated Lymphocytes

Acid-insoluble (125 I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) of control subjects and alcoholic liver disease (ald) subjects was determined at 72h, after;

(i) culture in modified MEM (2), with a folate and P1 concentration of 0.01mg/l

(ii) pre-incubation addition of BSS, Am (114 μ M), UdR (2.7mM), 4-dPN (2.6mM)/BSS on 4-dPN (2.6mM)/Am (114 μ M)

(iii) incubation 3h (125 I)-UdR, 0.5 μ Ci.

Results are given as the mean of duplicate analyses.

Incorporation after pre-incubation with UdR, 4-dPN/BSS and 4-dPN/Am is expressed as a % of incorporation in replicate cultures to which BSS or Am had been added.

Medium	MEMm		fol. 0.01mg/l,		Pl 0.01mg/l			
Pre-inc'n	BSS	Am	UdR	%	4dPN/BSS	%	4dPN/Am	%
Subjects								
Control								
1	27114	28038	5153	19	24873	92	33238	119
2	12204	20262	2513	21	12099	99	17618	87
3	10786	23020	2520	23	12021	111	18063	78
4	12766	28772	3217	25	13222	104	31354	109
5	10722	15592	1911	18	8618	80	13298	85
6	6245	17469	903	14	5808	93	15660	90
\bar{x}				20		97		95
s.e.				2		4		6.5
ald								
1	29846	36805	4337	15	20657	69	17197	47
2	10389	14410	1875	18	9066	87	13332	93
3	17560	26188	3864	22	13118	74	22174	85
4	15547	21372	2925	19	10998	71	22541	105
5	6378	7920	101	2	4645	73	7221	91
6	6117	13187	1668	27	4956	81	12552	95
\bar{x}				17		76		86
s.e.				3.5		2.8		8.0

Table 19

Effect of Pre-incubation with 4-dPN on Acid-insoluble
Incorporation of (125 I)-UdR into PHA Stimulated
Lymphocytes

Acid-insoluble (125 I)-UdR incorporation into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h, after;

- (i) culture in modified MEM (2), folate and P1 concentrations, as specified, and 15% autologous serum
- (ii) pre-incubation addition of BSS, Am ($114\mu\text{M}$), UdR (2.7mM), 4-dPN (2.6mM)/BSS or 4-dPN (2.6mM)/Am ($114\mu\text{M}$)
- (iii) incubation 3h (125 I)-UdR, $0.5\mu\text{Ci}$.

Results are given as the mean of duplicate analyses.

Incorporation after pre-incubation with UdR, 4-dPN/BSS and 4-dPN is expressed as a % of incorporation in replicate cultures to which BSS or Am had been added.

Medium	MEMm folate (0.01mg/l), Pl (0.01mg/l) 15% serum							
Pre-inc'n	BSS	Am	UdR	%	4dPN/BSS	%	4dPN/Am	%
Subjects								
Control								
1	12204	20762	2513	21	12099	99	17618	87
2	10786	32020	2520	23	12021	111	18063	78
3	12766	28772	3217	25	13222	104	31354	109
4	10722	15592	1911	18	8618	80	13298	85
5	6245	17469	903	14	5808	93	15660	90
\bar{x}				20		97		90
s.e.				1.9		5.3		5.2
Medium	MEMm folate (2.0mg/l), Pl (0.01mg/l) 15% serum							
1	2864	17776	82	3	1827	64	18237	103
2	4883	25478	173	4	2299	47	22168	87
3	4189	26566	188	5	2787	67	29225	110
4	3050	14604	0	0	1275	42	12805	88
5	3247	22034	77	2	1591	49	17583	80
\bar{x}				3		54		94
s.e.				0.9		4.9		5.5
Medium	MEMm folate (0.01mg/l), Pl (2.0mg/l) 15% serum							
1	10364	16629	2481	24	12023	116	18467	111
2	11733	25365	2155	18	10783	92	21214	84
3	11429	28593	3015	26	12572	110	27072	95
4	10395	15853	2352	23	8357	80	13593	86
5	5413	18488	848	16	5171	96	18941	102
\bar{x}				21		99		96
s.e.				1.9		6.4		5.0

indicate that this effect is independent of the pyridoxal concentration.

3.3.5. Effect of Culture in Medium without Pyridoxal (HCl) on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes

There were two particular questions of interest in determining the effect of the absence of medium P1 (HCl) on PHA stimulated lymphocyte culture. One was to assess the possible effect of a variation in the medium P1 (HCl) concentration on the relative contribution of the de novo and salvage pathways to thymidylate DNA, the second was to determine the sensitivity of lymphocytes of normal and alcoholic liver disease subjects to PHA stimulated culture in a vitamin B₆ deficient medium. The approach adopted in both questions was to determine acid-insoluble (^{125}I)-UdR incorporation after pre-incubation with BSS and MTX. Cognisant of the possible masking effect of a high medium folate concentration, in reducing the possible effect of a vitamin B₆ deficiency on serine hydroxymethyltransferase activity, experiments were carried out in media of high and low folate concentration. In order to determine the possible effect of the absence of P1 (HCl) during the course of PHA stimulated lymphocyte culture, determination of (^{125}I)-UdR was carried out at 24, 48, 72 and 96 h.

The results of this experiment, illustrated in Fig. 15, indicate that the absence of Pl (HCl) has little effect on acid-insoluble (^{125}I)-UdR incorporation at various times during PHA stimulated lymphocyte culture. This lack of effect of the absence of Pl (HCl) is independent of the medium folate concentration and the pre-incubation addition of BSS and MTX. The results of an experiment directed at determination of the sensitivity of lymphocytes of alcoholic subjects to PHA stimulated culture in a vitamin B₆ deficient medium are given in Table 15 and a comparison is presented in Table 20. Determination of incorporation was carried out at 72 h in lymphocytes cultured in medium of high or low folate concentration after pre-incubation with BSS and MTX. The results indicate that the absence of medium Pl (HCl) does not have a statistically significant effect on (^{125}I)-UdR incorporation in stimulated lymphocytes of normal or alcoholic subjects. This lack of effect is independent of the medium folate concentration and the pre-incubation addition of BSS or MTX.

3.3.6. Effect of Culture in Medium Containing 4-dPN on Acid-insoluble (^{125}I)-UdR incorporation into PHA Stimulated Lymphocytes

In order to obtain cells with an unequivocal vitamin B₆ deficiency and also to establish conditions likely to allow discrimination between cells of different vitamin B₆ status, experiments were carried out in which the effect of addition of the vitamin B₆ antagonist 4-dPN to the culture medium, prior to initiation of

Fig. 15 Effect of Culture in Medium Without Pl
(HCl) on Acid-insoluble Incorporation of
(¹²⁵I)-UdR into PHA Stimulated Lymphocytes

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) was determined at the times indicated after;

(i) culture in modified MEM (2) with low folate with or without Pl i.e., fol 0.01mg/l Pl 1.0mg/l (0/□)

fol 0.01mg/l Pl 0.0mg/l (●/■)

or

with high folate with or without Pl i.e.,

fol 1.0mg/l Pl 1.0mg/l (0/□)

fol 1.0mg/l Pl 0.0mg/l (●/■)

(ii) pre-incubation addition of BSS (0/●) or MTX 110μM (□/■) 1h prior to;

(iii) incubation 3h (¹²⁵I)-UdR, 0.5μCi

Results are presented as mean (cpm) ± s.e. of incorporation into lymphocyte cultures of 4 control subjects after culture in medium of low folate ± Pl and medium of high folate ± Pl.

low folate

high folate

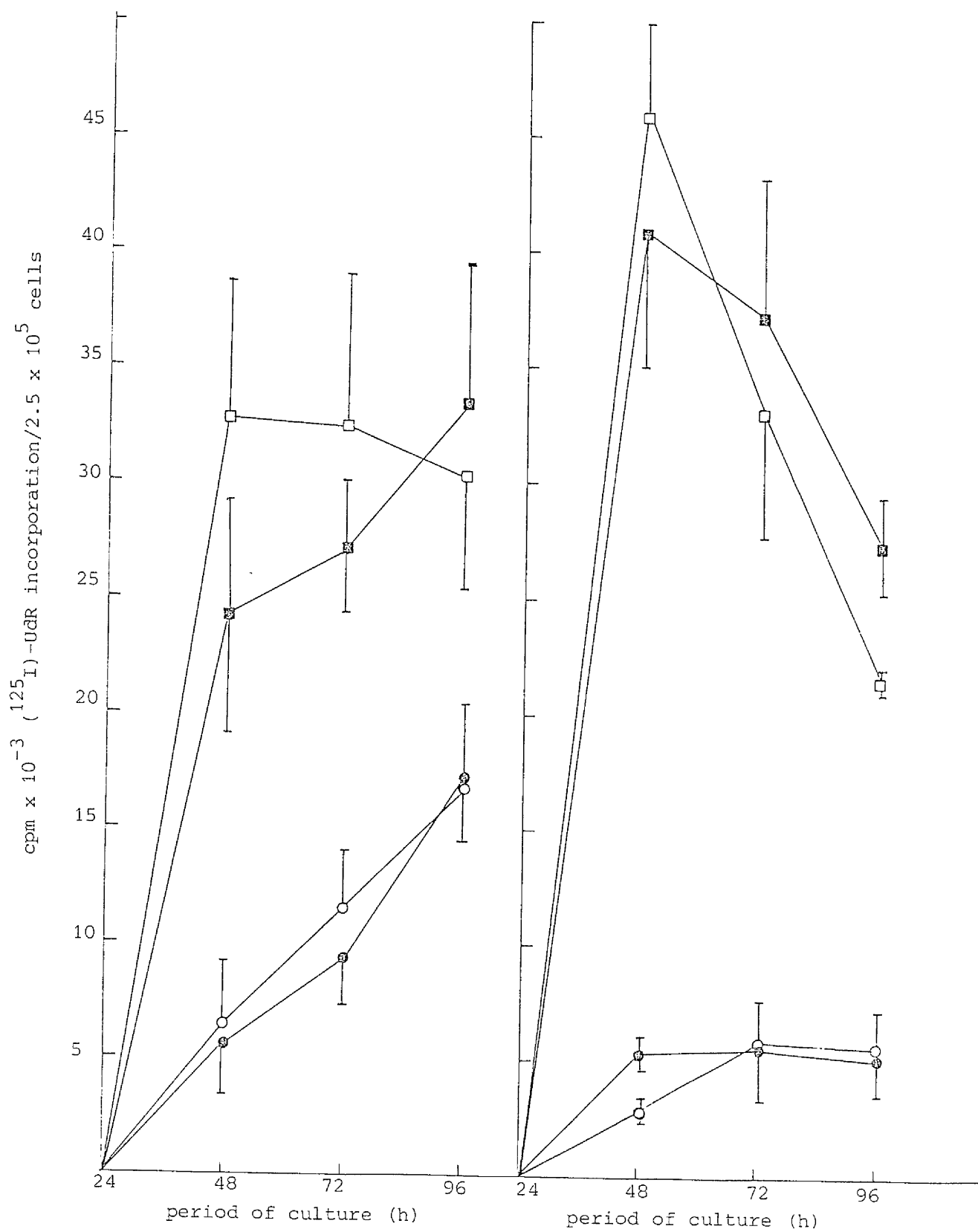


Table 20 Effect of culture in medium without P1 (HCl)
on acid-insoluble incorporation of (¹²⁵I)-UdR
into PHA stimulated lymphocytes

Medium	MEM m(2)			
	fol1.0mg/l (± Pl 1.0mg/l)		fol. 0.01mg/l (± Pl 0.01mg/l)	
Preincubn.	BSS	MTX	BSS	MTX
	Percentage (¹²⁵ I)-UdR incorporation in 0 Pl medium cf. 0 Pl medium cultures			
Subjects control				
1	123	70	106	104
2	136	100	102	102
3	46	72	105	79
4	81	96	111	129
5	60	53	129	101
6	71	63	222	210
7	116	92	342	161
8	207	181	295	199
\bar{x}	105	95	176	136
s.e.	18	13	34	17
anld				
1	62	34	80	89
2	107	155	112	81
ald				
3	51	75	80	98
4	80	72	96	86
\bar{x}	75	84	92	89
s.e.	12	25	8	4

Culture conditions as for table (15)

Incorporation after culture in medium without P1 (HCl) is expressed as a % of incorporation in replicate cultures in which P1 (HCl) was present

PHA stimulated lymphocyte culture, was determined.

In Fig. 16 the result of an experiment in which acid-insoluble (^{125}I)-UdR incorporation was determined at 24, 48, 72 and 96 h in stimulated lymphocytes cultured in the presence or absence of 4-dPN, is shown. The basal medium used was complete MEM and incorporation was determined after pre-incubation with BSS and MTX. The results of this experiment indicate that 4-dPN, when added prior to initiation of culture, results in a marked reduction in (^{125}I)-UdR incorporation throughout the subsequent course of PHA stimulation. This reduction in incorporation is of similar magnitude after pre-incubation with either BSS or MTX.

The sensitivity of PHA stimulated lymphocytes of normal and alcoholic liver disease subjects to culture in medium containing 4-dPN was assessed by determination of acid-insoluble (^{125}I)-UdR incorporation into 72h PHA stimulated lymphocytes. The results of this experiment are presented in Table 21; (a) control subjects and (b) alcoholic liver disease subjects. The media used was modified MEM with a folate concentration of 0.01 mg/l. The effect of culture in the presence of 4-dPN was determined in medium containing pyridoxal (1.0 mg/l) and in medium without pyridoxal. Determination of acid-insoluble (^{125}I)-UdR incorporation was carried out after pre-incubation with BSS, MTX or 5-fu. The use of 5-fluorouracil the reduction product of which, 5-fluoro-2'-deoxyuridine 5'-phosphate, is an inhibitor of thymidylate synthetase,

Fig. 16 Effect of Culture in Medium Containing
4-dPN on Acid-insoluble Incorporation
of (125 I)-UdR into PHA Stimulated
Lymphocytes

Acid-insoluble (125)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at the times indicated after;

- (i) culture in modified MEM (2) with folate
 1.0mg/l, Pl 1.0mg/l, without 4-dPN (○ / □)
or with folate 1.0mg/l, Pl 1.0mg/l, 4-dPN
 500mg/l (● / ■)
- (ii) pre-incubation addition of BSS (○/●) or MTX
 110μM (□ / ■) 1h prior to;
- (iii) incubation, 3h (125 I)-UdR, 0.5μCi

Individual results were expressed as a % of acid-insoluble (125 I)-UdR incorporation (cpm) in comparable lymphocytes cultured in medium free of 4-dPN, after pre-incubation with BSS, at 72h.

Group results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of 4 control subjects.

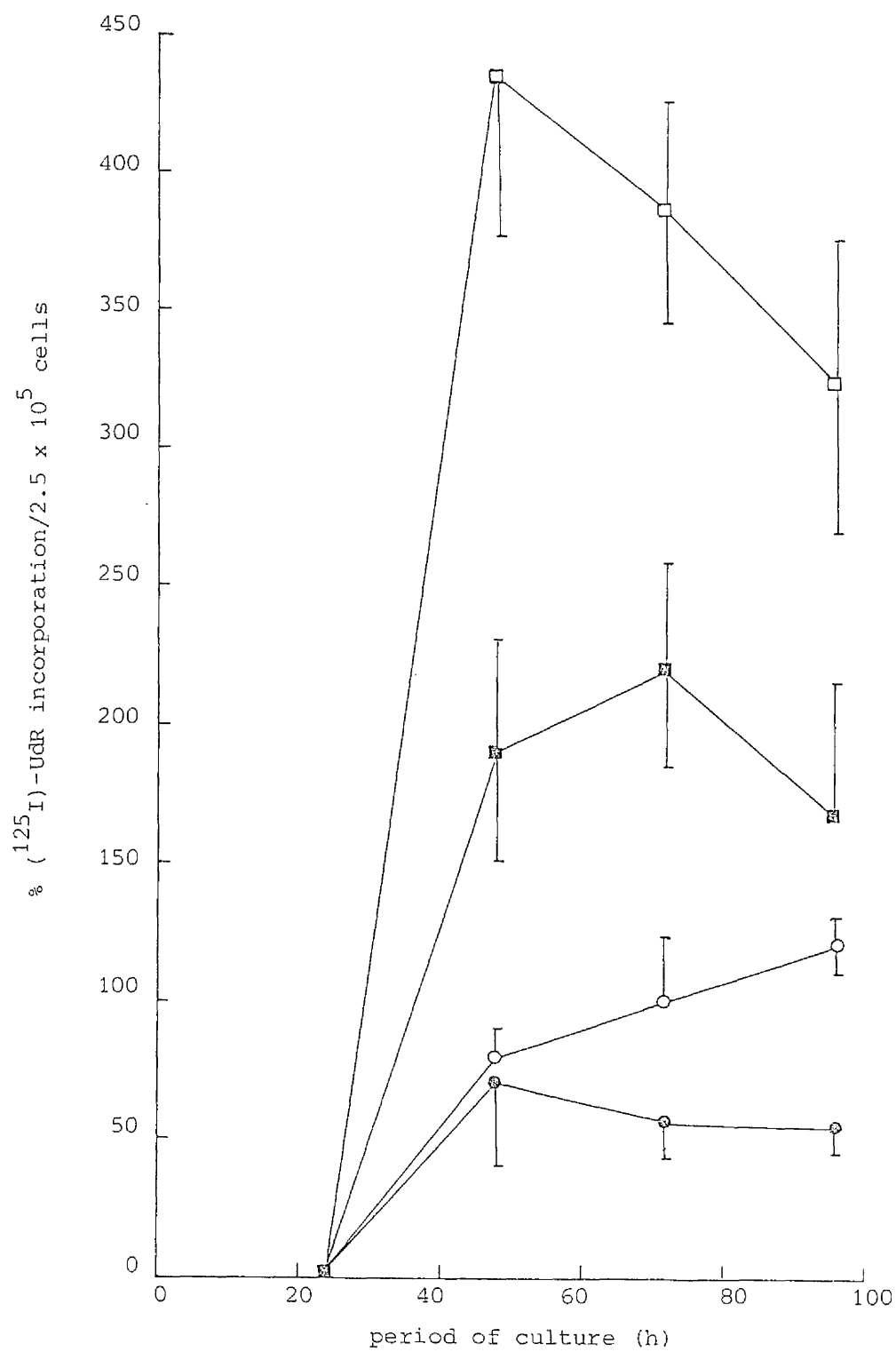


Table 21 Sensitivity of Acid-insoluble (^{125}I)-UdR
Incorporation into PHA Stimulated Lymphocytes
of Control and Alcoholic Liver Disease Subjects
to Initial Culture Medium Addition of 4-dPN

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) of normal (control) subjects (a) and alcoholic liver disease (ald) subjects (b) was determined at 72h after;

- (i) culture in modified MEM (2) with low folate 0.01mg/l and high Pl 1.0mg/l and with or without 4-dPN (500mg/l) or with low folate 0.01mg/l, without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-incubation addition of BSS, or MTX (110 μM) or 5-fu (1.0mM) prior to;
- (iii) incubation 3h, (^{125}I)-UdR, 0.5 μCi

Results are given as the mean of duplicate analyses. Incorporation into lymphocytes cultured in the presence of 4-dPN is expressed as a % of incorporation into lymphocytes cultured in medium free of 4-dPN.

Table 21

(a)

Medium	MEMm low fol / high Pl								
Pre-inc'n	BSS			MTX			5-fu		
Medium 4dPN	-	+	%	-	+	%	-	+	%
Subjects									
Control									
1	31946	20289	65	46057	42412	92	42896	35670	83
2	23722	18177	77	31366	25380	81	38541	31100	81
3	32685	31983	98	36832	39983	110	60300	47206	78
4	31676	18672	59	31040	27522	89	37086	23404	63
\bar{x}			75			93			76
s.e.			3.7			6.1			4.5
Medium	MEMm low fol / without Pl								
1	28393	9357	33	43405	28876	67	35814	23374	65
2	26724	15598	58	31719	22296	70	41338	30868	75
3	38774	32879	85	44910	41341	92	62229	43899	71
4	36230	18864	52	33198	17270	52	37844	14347	38
\bar{x}			57			70			62
s.e.			10.7			8.3			8.3

(b)

Medium	MEMm low fol / high Pl								
Pre-inc'n	BSS			MTX			5-fu		
Medium 4dPN	-	+	%	-	+	%	-	+	%
Subjects									
ald									
1	4901	1556	32	7811	2543	33	14265	6269	44
2	22240	9893	44	16767	12822	76	30090	26881	89
3	16870	10161	60	14589	10225	70	21518	9342	43
4	33587	11806	35	38596	43582	113	40338	16620	41
\bar{x}			43			73			54
s.e.			6.3			16.4			11.6
Medium	MEMm low fol/without Pl								
1	3555	2551	75	3365	3367	40	6346	3040	48
2	20902	13189	63	23422	14126	60	31885	20367	64
3	16214	6451	40	18555	6486	34	19781	18313	93
4	33172	35338	107	39317	42108	107	39660	10436	26
\bar{x}			71			60			58
s.e.			14.0			16.5			14.1

provides an alternative means of inhibiting de novo thymidylate synthesis (Paul, 1975).

Analysis of the results indicates that there is no consistent difference between stimulated lymphocytes of normal and alcoholic liver disease subjects in their response to culture in medium containing 4-dPN.

When the effect of 4-dPN on PHA stimulated lymphocyte culture in high folate conditions (1.0 mg/l) was determined, similar results were obtained and these are presented in Table 22. These results also indicate that the effect of 4-dPN in reduction of (^{125}I)-UdR incorporation is similar in medium with or without pyridoxal and after pre-incubation with either BSS or MTX.

The effect of increasing the medium P1 (HCl) concentration on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes cultured in the presence of 4-dPN is shown in Fig. 17. Increasing the medium pyridoxal concentration to a level of 10 mg/l was found to have little influence on the effect of 4-dPN in lymphocytes of either control or alcoholic liver disease subjects.

Table 22 Effect of Initial Culture Medium Addition
of 4dPN on Acid-insoluble Incorporation of (125I)-UdR
into PHA Stimulated Lymphocytes in High Folate Conditions

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) of 12 control subjects was determined at 72h after;

- (i) culture in modified MEM (2) with high folate 1.0mg/l and high Pl 1.0mg/l and with or without 4-dPN (500mg/l) or with high folate 1.0mg/l, without PL and with or without 4-dPN (500mg/l)

- (ii) pre-incubation addition of BSS or MTX (110 μM) 1h prior to;

- (iii) incubation 3h (^{125}I)-UdR, 0.5 μCi

Results are given as the mean of duplicate analyses.

Incorporation into lymphocytes cultured in various media and after pre-incubation with BSS and MTX, is expressed as a % of incorporation into lymphocytes cultured in high folate/high Pl medium, without 4-dPN and after pre-incubation with MTX.

Table 22

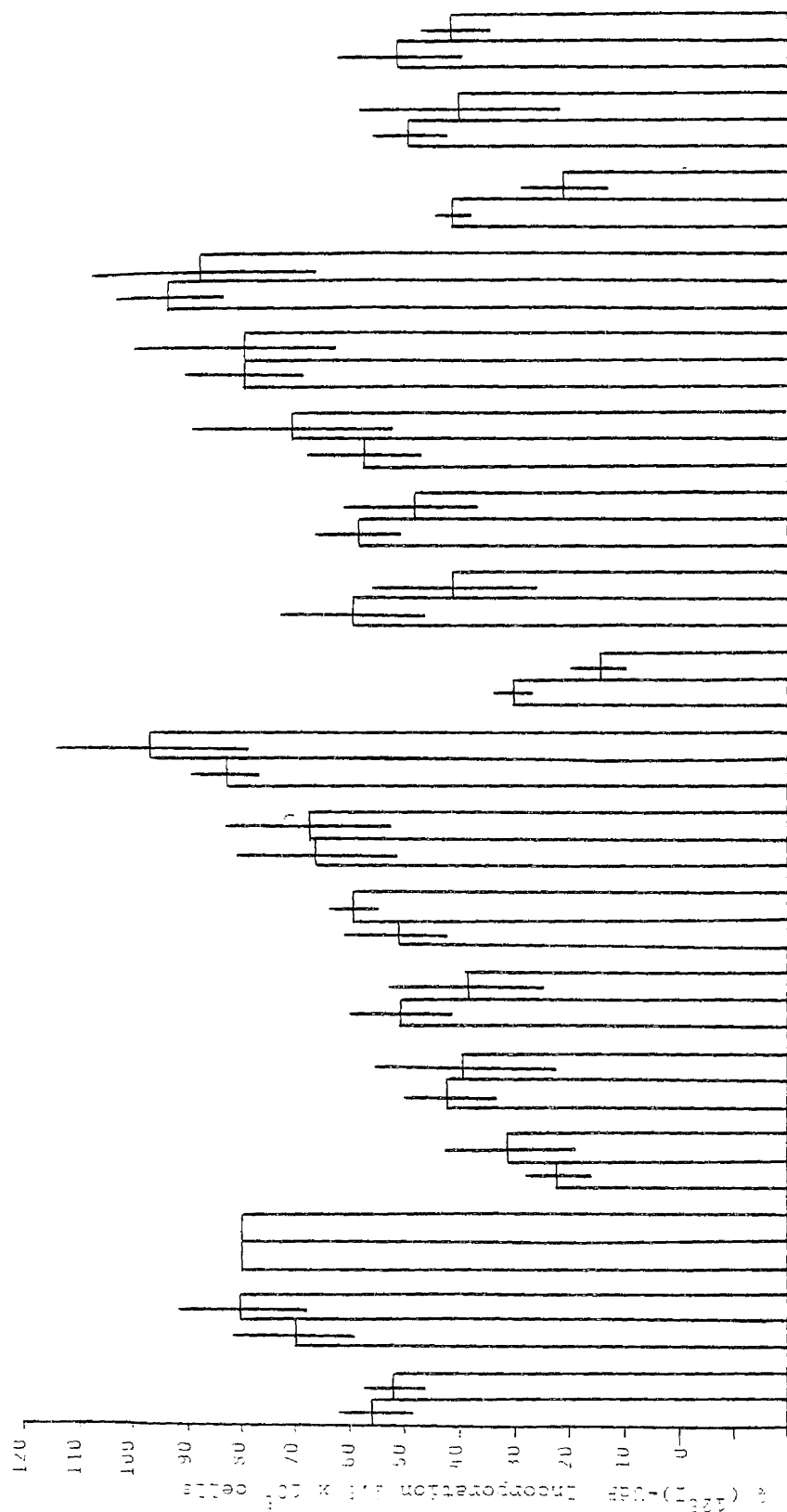
Medium	MEM high folate/high PI								high folate / without PI							
Medium 4dPN	4dPN (500mg/l)								4dPN (500mg/l)							
Pre-inc'n	BSS	%	MTX	%	BSS	%	MTX	%	BSS	%	MTX	%	BSS	%	MTX	%
Subjects Control																
1	10095	17	60271	100	5814	10	30881	51	9388	16	42774	71	7283	12	30708	51
2	3867	26	15025	"	2207	15	3858	26	3719	25	14609	97	1471	10	4453	30
3	4489	17	26376	"	1588	6	7592	29	5479	21	23028	87	2684	10	10730	41
4	8042	32	25187	"	5211	21	23468	93	6443	26	26413	105	10186	40	27593	110
5	5984	18	33700	"	2705	8	18182	54	6035	18	37427	111	3338	10	23565	70
6	5829	24	24745	"	2668	11	17019	69	5697	23	22277	90	2868	12	20448	83
7	3814	26	14783	"	1787	12	12909	87	2800	19	12828	87	2229	15	11908	81
8	5520	21	25837	"	2420	9	10594	41	5852	23	24147	93	3462	13	13117	51
9	5976	20	30410	"	3938	13	20622	68	4094	13	16497	54	1617	5	15706	52
10	5089	28	18358	"	4688	26	16065	88	6359	35	15229	83	4752	26	20471	112
11	5569	27	20826	"	1981	10	9196	44	4393	21	9097	44	2301	11	4580	22
12	2782	20	14211	"	2302	16	6509	46	2388	17	10678	75	1329	9	6957	49
\bar{x}		23		100		13		58		21		83		14		63
s.e.		1.4		-		1.7		6.6		1.7		5.7		2.7		8.4

Fig. 17 Effect of Increasing Medium Pl (HCl) Concentration on Acid-insoluble Incorporation of (¹²⁵I)-UdR into PHA Stimulated Lymphocytes Cultured in the Presence of 4-dPN

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 0.01mg/l a Pl concentration of 0, 1, or 10mg/l and with or without 4-dPN 500mg/l
 - (ii) pre-incubation addition of BSS, MTX (110μM) or 5-fu (1.0mM) 1h prior to;
 - (iii) incubation, 3h, (¹²⁵I)-UdR, 0.5μCi
- Incorporation into lymphocytes cultured in various media and after pre-incubation with BSS, MTX, or 5-fu, was expressed as a % of incorporation into lymphocytes cultured in low folate medium, without Pl or 4-dPN and after pre-incubation with 5-fu.

Group results are given as mean % (cpm) ± s.e. of lymphocyte cultures of 4 control subjects (C) and 4 alcoholic liver disease subjects (A). The culture and pre-incubation conditions for each experiment are as indicated at the base of each column.



Subject	0 mg/l				1 mg/l				500 mg/l				10 mg/l				500 mg/l			
	C	A	BSS	MTX	C	A	BSS	MTX	C	A	BSS	MTX	C	A	BSS	MTX	C	A	BSS	MTX
Pre-treatment																				
4dFN																				
PI (HCT)																				

Fig. 17

3.3.7. Effect of Variation of Cell Culture Medium Composition on Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

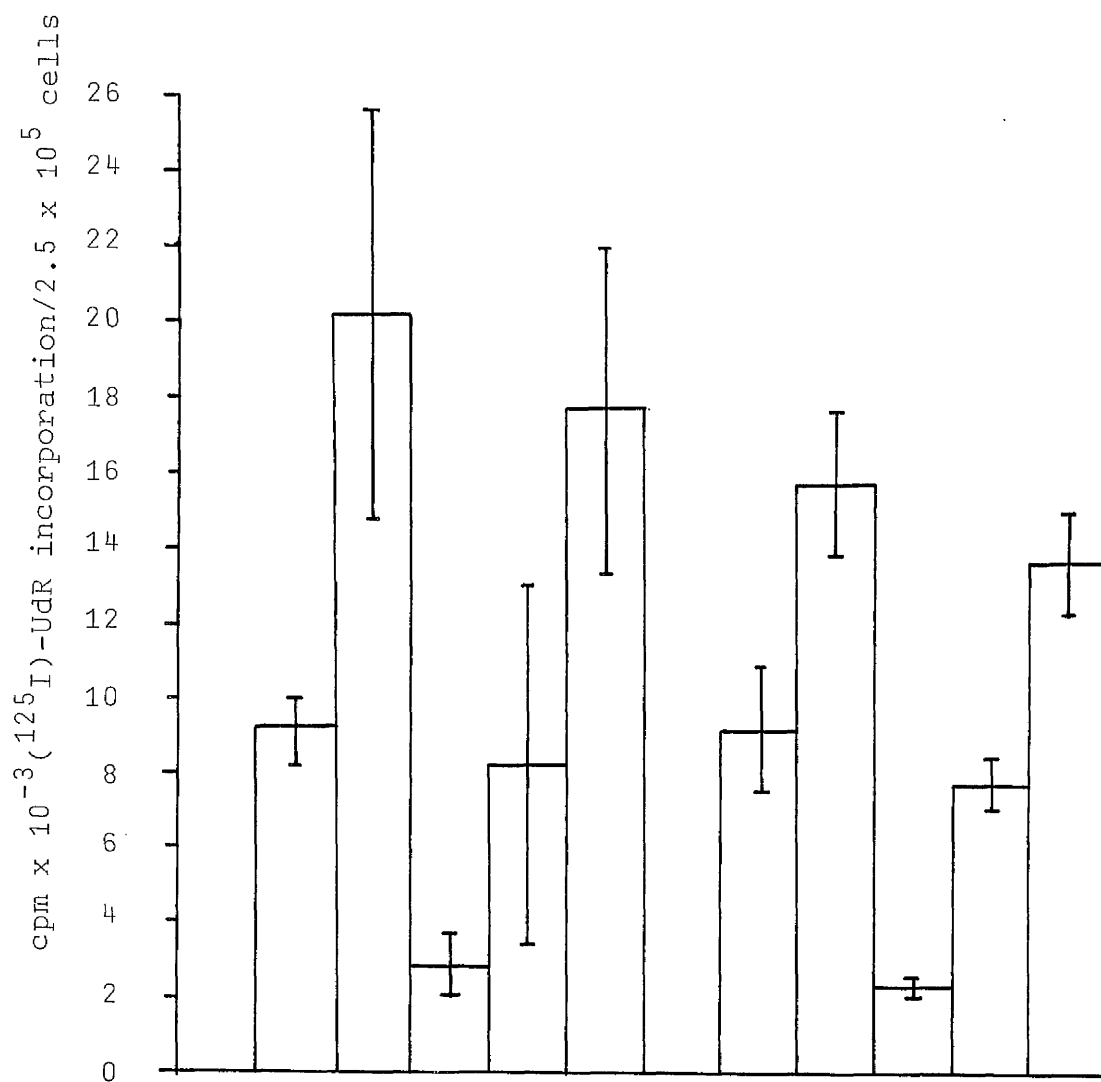
The experiments reported in this section were directed at characterisation of a selective medium for assessment of vitamin B6 status in PHA stimulated lymphocytes, of alcoholic liver disease subjects. The results of a comparison of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes cultured in modified MEM (2) or basal BSS medium are shown in Fig. 18, and the effect of variation of cell culture medium composition on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes is shown in Fig. 19.

Fig. 18 Comparison of Acid-insoluble (^{125}I)-UdR
Incorporation into PHA Stimulated
Lymphocytes Cultured in Modified MEM (2) or
Basal BSS Medium

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM(2) or basal BSS medium
 (15% autologous serum)
- (ii) pre-incubation addition of BSS (50 μ l), Am
 (0.114mM), UdR (2.7mM), 4dPN (2.6mM) or Am
 (0.114mM)/4dPN (2.6mM). 1 h prior to;
- (iii) incubation 3h (^{125}I)-UdR 0.5uCi

Results are presented as mean (cpm) \pm s.e. of incorporation into lymphocyte cultures of 2 ald subjects.



Pre-inc'n	BSS	Am	UdR	4dPN	4dPN/Am		BSS	Am	UdR	4dPN	4dPN/Am
Medium	MEM m (2)						BSS				

Fig. 19 Effect of Variation of Cell Culture Medium
Composition on Acid-insoluble (^{125}I)-UdR
Incorporation into PHA Stimulated Lymphocytes

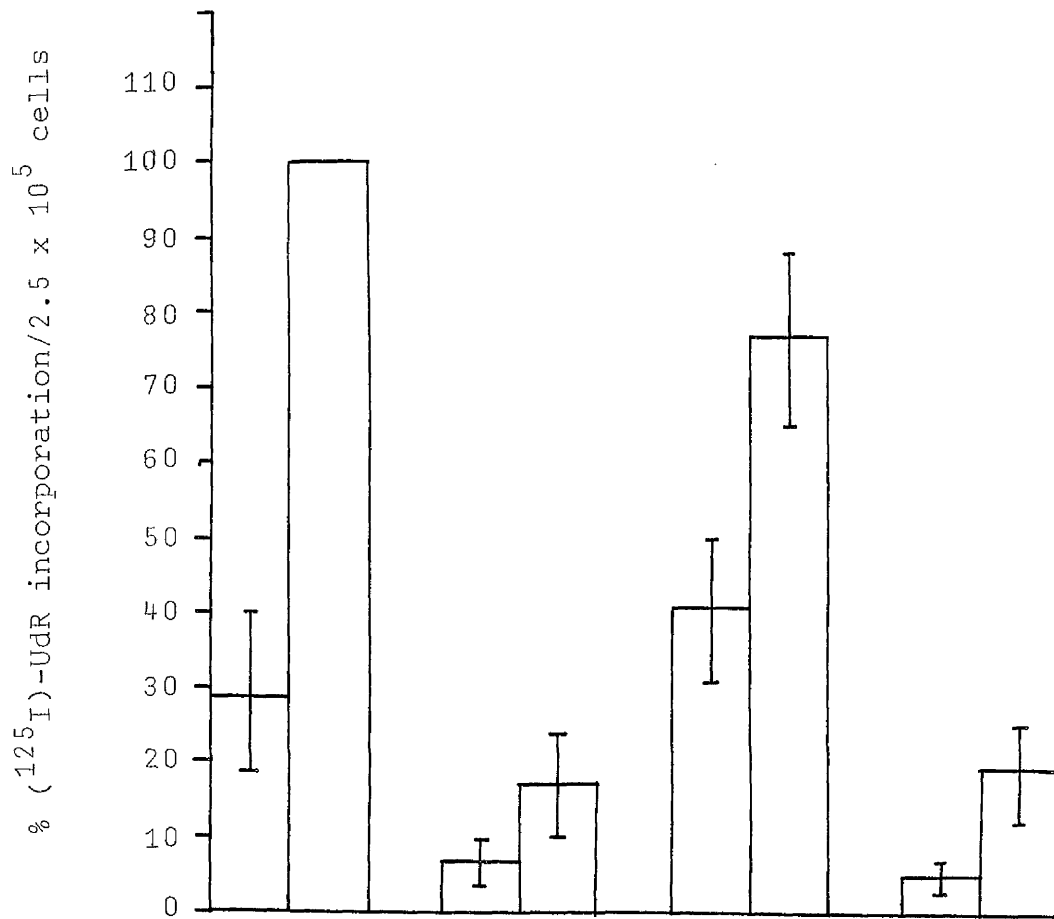
Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in basal BSS medium with MEM vitamin and MEM amino acid additives as specified. Where added, MEM vitamins and amino acids were present at the same concentration as in MEM. (10% autologous serum)
- (ii) pre-incubation addition of BSS or MTX ($110\mu\text{M}$) 1h prior to;
- (iii) incubation, 3h (^{125}I)-UdR, 0.5 uCi

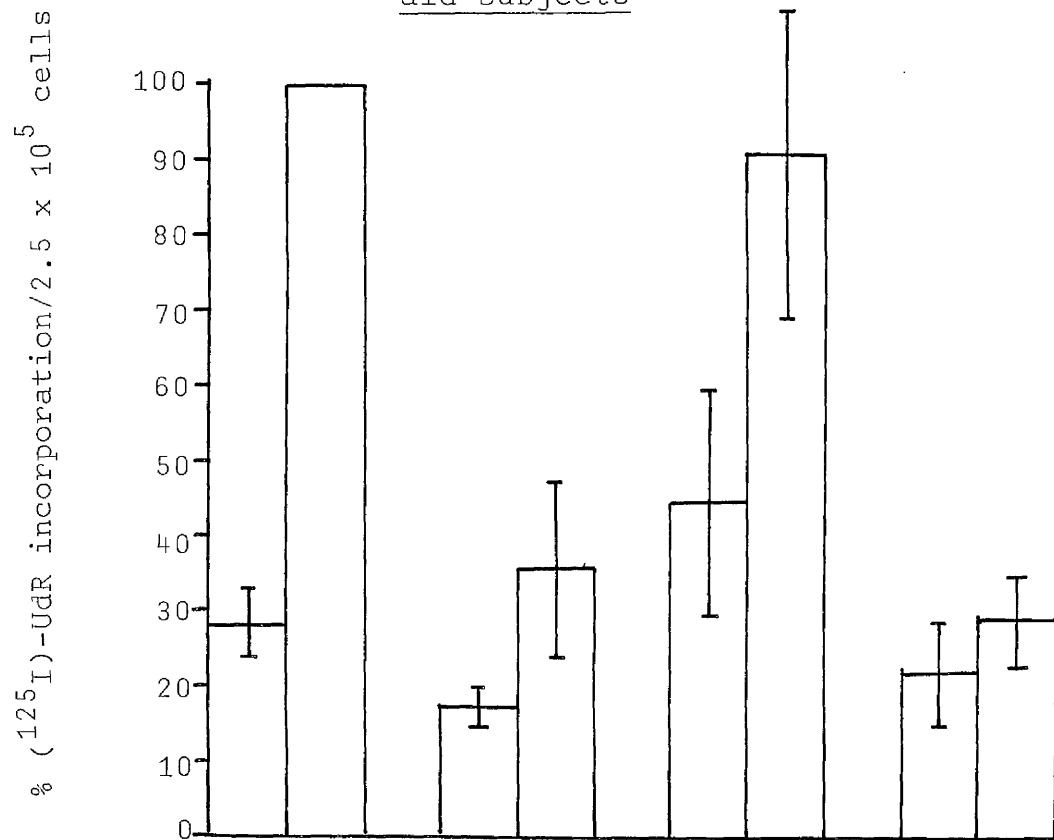
Incorporation into lymphocytes cultured in various media and after pre-incubation with BSS or MTX was expressed as a % of incorporation into lymphocytes cultured in basal BSS medium plus MEM vitamins and amino acids and after pre-incubation with MTX.

Group results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of 4 control subjects and 3 alcoholic liver disease subjects. The culture and pre-incubation conditions for each experiment are indicated at the base of the figure.

Control Subjects



ald Subjects



Pre-inc'n	BSS	MTX		BSS	MTX		BSS	MTX		BSS	MTX
Medium	+ MEM			+ MEM			+ MEM			BSS	
BSS	vitamins			vitamins			amino			alone	
	acids						acids				

Section 3.4.

Characterisation of the Effects of Vitamin B6
Antagonists on PHA Stimulation of Lymphocytes

3.4.1. Effect of Culture in Medium Containing the
Vitamin B6 Antagonist Isonicotinic Acid Hydrazide
on Acid-insoluble Incorporation of (^{125}I)-UdR
into PHA Stimulated Lymphocytes

The appropriate use of specific metabolic inhibitors in biochemical investigations has provided much useful information and insight into a number of areas of metabolism. Although the advantages of their use usually outweigh the disadvantages, it is necessary to apply due circumspection to interpretation of results of experiments based on their use. The principal assumption in a consideration of the effect of an inhibitor is its specificity. In particular it is assumed that the effects observed due to the use of an inhibitor result from inhibition of the target activity and are not due to a non-specific effect. A wide variety of compounds are known to interfere at one or another point with the metabolism of vitamin B6. In principle, such antagonists might exert their effects by interfering at any of the several points of importance in utilisation of vitamin B6 itself, i.e., in the absorption of the vitamin, in its transformation to PLP or by conversion to an analogue of PLP that could compete with the latter for combination with various apoenzymes. Demonstration that a particular inhibitor acts in one of these ways does not, however, eliminate the possibility that it also acts in another.

In considering 4-dPN as a specific vitamin B6 antagonist it is not possible to exclude a non-specific effect also resulting in a reduction of DNA synthesis, but its widespread employment by other investigators does

justify its use in this study. Further confirmation of the validity of its use is gained from a comparison of the effects of 4-dPN with the effects of an alternative vitamin B6 antagonist isonicotinic acid hydrazide (INH). This compound is one of a number of carbonyl reagents which are potent inhibitors of pyridoxal kinase and thus interfere with formation of PLP. The active inhibitors in this case are the condensation products of the carbonyl reagents with pyridoxal (Greenberg, 1970).

The results of such a comparison are shown in Fig. 20 and indicate that the effect of INH in reducing acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes is similar to that of 4-dPN.

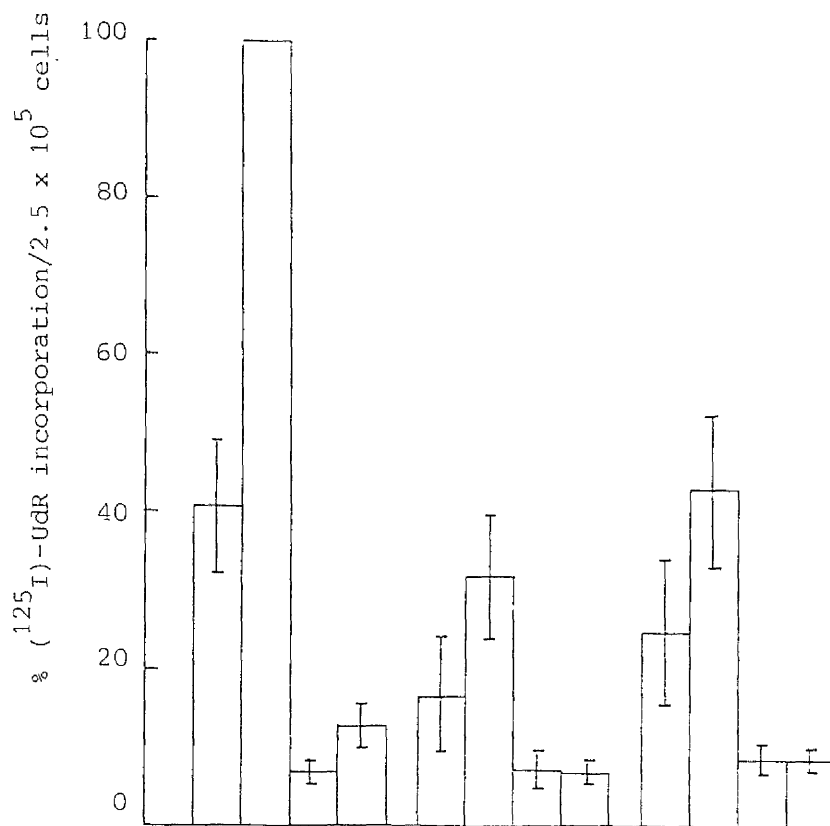
3.4.2. Effect of Pre-incubation with MTX and 5-fu on Subsequent Acid-insoluble (^{125}I)-UdR incorporation into PHA Stimulated Lymphocytes

The use of MTX and 5-fu in experimental determination of the respective de novo and salvage pathways' contribution to total thymidylate synthesis has been mentioned previously (sections 3.3.1. and 3.3.6.). Both these inhibitors, by their effect on de novo thymidylate synthesis, increase acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes. However, the increase in incorporation caused by 5-fu is greater than that caused by MTX. The reason for this difference is partly apparent from a consideration of the different modes of action of these two inhibitors.

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- Incorporation into lymphocytes cultured in the presence of INH and 4-dPN and after various pre-incubation additions was expressed as a % of incorporation into lymphocytes cultured in complete MEM with no addition and after pre-incubation with BSS and MTX.

The culture and pre-incubation conditions for each experiment are as indicated at the base of each column.



prepreinc	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX
pre incub.	BSS		UdR		BSS		UdR		BSS		UdR	
medium	MEM comp		MEMc + INH		MEMc + 4dPN		MEMc + 4dPN		MEMc + 4dPN		MEMc + 4dPN	

Methotrexate inhibits dihydrofolate reductase and therefore disrupts the regeneration of active one-carbon units necessary for methylation of dUMP by thymidylate synthetase. 5-fluorouracil, however, after conversion to 5-fdUMP, inhibits thymidylate synthetase directly. Therefore, it is likely that in the presence of methotrexate, de novo thymidylate synthesis will continue until the available pool of active one-carbon units is exhausted whereas in the presence of 5-fluorouracil inhibition of de novo thymidylate synthesis is more direct and is independent of the size of the active one-carbon folate pool.

Several factors can be expected to influence the size of the active one-carbon folate pool. Of particular interest in this study is the effect of vitamin B6 deficiency and the medium folate concentration. The effect of variation of these factors on the difference in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes pretreated with MTX and 5-fu is shown in Fig. 21. The results indicate that the greatest difference in (^{125}I)-UdR incorporation after pre-incubation with MTX and 5-fu occurs in PHA stimulated lymphocytes cultured in a high folate medium and that this difference is reduced either by culture in medium containing 4-dPN or in medium with a low folate concentration. Statistical analysis of these results, as presented in Table 23, confirms this interpretation. ($P < 0.01$).

Fig. 21 Effect of Pre-incubation with MTX and 5-fu on
Subsequent Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

(i) culture in modified MEM(2) with a folate 1.0 mg/l,
Pl 1,0mg/l

and with or without 4-dPN
(500mg/l)

or with a folate 0.01mg/l,
without Pl,

and with or without 4-dPN
(500mg/l)

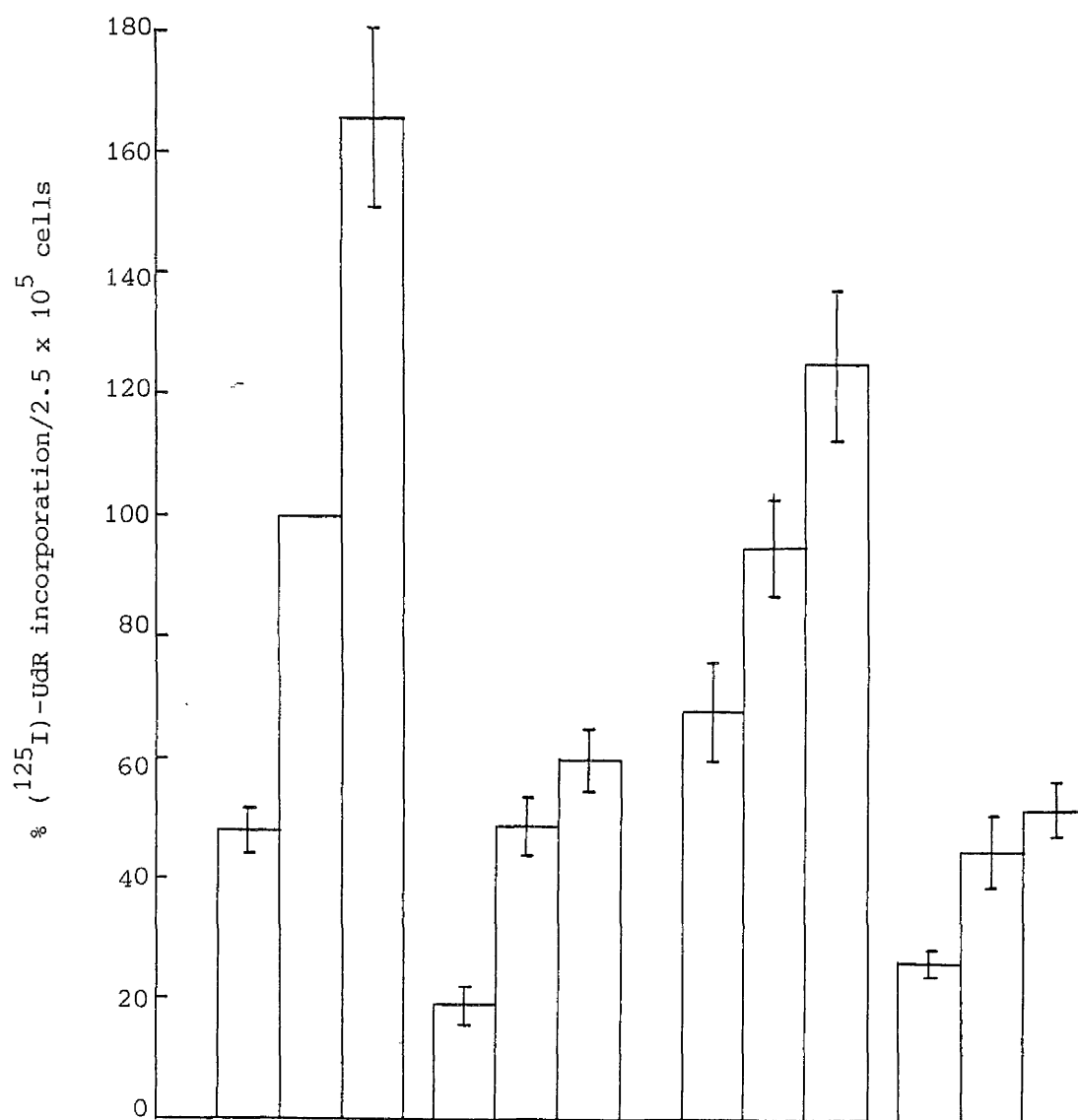
(ii) pre-incubation addition of BSS, MTX (110 μM or
5-fu (1.0mM) 1h prior to

(iii) incubation 3h (^{125}I)-UdR, 0.5 μCi

Incorporation into lymphocytes cultured in various media and after pre-incubation addition of BSS, MTX or 5-fu was expressed as a% of incorporation into lymphocytes cultured in MEMm (fol/Pl 1.0mg/l) without 4-dPN and after pre-incubation with MTX.

Group results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of 4 control subjects.

The culture and pre-incubation conditions for each experiment are as indicated at the base of each column.



preinc'n	BSS	MTX	5'Fu	BSS	MTX	5'Fu	BSS	MTX	5'Fu	BSS	MTX	5'Fu
initial med add				4dPN (500mg/l)						4dPN (500mg/l)		
medium	MEMm fol 1.0mg/l Pl 1.0mg/l						MEMm fol 0.01mg/l Pl 0.0mg/l					

Table 23 Effect of Pre-incubation with MTX and 5-fu on Subsequent Acid-insoluble (¹²⁵I)-UdR Incorporation into PHA Stimulated Lymphocytes

Medium	MEMm fol 1.0mg/l, Pl 1.0mg/l			MEMm fol 0.01mg/l, Pl 0.0mg/l		
Pre-inc'n	4dPN (500mg/l)			4dPN (500mg/l)		
Cont. Subjects	MTX	5-fu	%	MTX	5-fu	%
1	11185	15464	138	4415	5593	127
2	4939	11771	238	2837	3190	112
3	5736	12568	219	3634	3987	110
4	18247	28221	155	13259	14698	111
5	8740	10208	117	3751	4779	127
6	7958	14466	182	3370	4923	147
7	3620	5256	149	1127	1244	110
8	14255	18383	129	6560	8379	128
\bar{x}			166			122
s.e.			15.4			4.7
						7.9
						7.2

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) was determined at 72h after;

- (i) culture in modified MEM with a folate 1.0mg/l Pl 1.0mg/l and with or without 4-dPN (500mg/l) or with folate 0.01mg/l, without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-incubation addition of MTX (110μM) or 5-fu (1.0mM) 1h prior to;
- (iii) incubation 3h, (¹²⁵I)-UdR, 0.5μCi.

In each culture medium, incorporation into lymphocytes after pre-incubation with 5-fu is expressed as a % of incorporation into replicate cultures pre-incubated with MTX.

3.4.3. Effect of Prior Addition of MTX and 5-fu on UdR Suppression of Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

In section 1.4.5. the possible use of the UdR suppression test as a means of determining the efficiency of the de novo thymidylate pathway, in the presence of a vitamin B6 or folate deficiency, was described. In section 3.3.3. the results of experiments in which the UdR suppression effect was determined in PHA stimulated lymphocytes of both control and alcoholic subjects are presented. These experiments utilised medium of high and low folate concentration, with and without pyridoxal HCl, and the results indicated that there were no significant differences in UdR suppression of (^{125}I)-UdR incorporation into PHA stimulated lymphocytes of control or alcoholic subjects, irrespective of the culture medium.

A possible interpretation of these results would be that the lack of difference in the UdR suppression test indicates that there is no difference in the efficiency of the de novo thymidylate pathway in stimulated lymphocytes of control and alcoholic subjects cultured in these various conditions. However, in view of the doubts raised concerning the validity of this test, it seemed desirable to test its validity by an alternative approach.

Since MTX and 5-fu inhibit de novo thymidylate synthesis and thus mimic the effect of folate deficiency it was decided to determine the effect of pre-treatment with these two inhibitors on subsequent UdR suppression of (^{125}I)-UdR, incorporation into PHA stimulated lymphocytes.

As previously indicated the principal site of action of each of these inhibitors is different; MTX inhibits generation of H_4F from H_2F whereas 5-fdUMP, derived from 5-fu inhibits thymidylate synthetase. However, the overall effect of each is that subsequently added Udr would not be efficiently converted to dTMP and dTTP and could not therefore result in feedback inhibition of thymidine kinase.

The effect of prior addition of MTX and 5-fluorouracil on Udr suppression of acid-insoluble (^{125}I)-Udr incorporation into PHA stimulated lymphocytes was determined in medium of higher or low folate concentration (Table 24) and in the presence and absence of 4-dPN (Figs. 22 and 23).

3.4.4. Effect of Pre-incubation Addition of formate on Acid-insoluble (^{125}I)-Udr Incorporation into PHA Stimulated Lymphocytes

From the results of previous experiments concerning the effect of vitamin B6 deficiency and variation of the medium folate concentration on the respective contribution of the de novo and salvage pathways to total thymidylate synthesis, it was apparent that while vitamin B6 deficiency (as induced by 4-dPN) reduced overall DNA synthesis, but did not alter the relative contribution of the de novo pathway to total thymidylate synthesis, an increase in the medium folate concentration from 0.01mg/l to 1mg/l significantly reduced the salvage contribution but did not affect total thymidylate incorporation.

Table 24

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into
PHA stimulated lymphocyte cultures (2.5×10^5 cells)
was determined at 72h after;

(i) culture in complete MEM or in modified MEM (2)
with a folate concentration of 0.01mg/l,
without P1 (HCl)

(ii) pre-preincubation addition of BSS, MTX
(110 μM) or 5-fu (1.0mM) 15 min. prior to;

(iii) pre-incubation addition of BSS or UdR
(2.7mM) 4h prior to;

(iv) incubation, 3h, (^{125}I)-UdR, 0.5 μCi

Incorporation after pre-incubation with UdR is
expressed as a % of incorporation in replicate
cultures to which BSS had been added.

Table 24 Effect of Prior Addition of MTX and 5-fu on UdR Suppression of Acid-insoluble (125 I)-UdR Incorporation into PHA Stimulated Lymphocytes

(a)

Medium	(fol/Pl, 1.0mg/l)									
	MEM complete									
	BSS		MTX		5-fu					
Pre-preinc'n	BSS	UdR	%	BSS	UdR	%	BSS	UdR	%	
Cont. Subjects										
1	6283	935	15	32527	8192	25	46611	4942	11	
2	4325	799	18	21626	5280	24	25980	2488	10	
3	4522	995	22	17876	4618	26	21965	2232	10	
4	4578	656	14	21294	3261	15	23693	2633	11	
\bar{x}			17			23			11	
s.e.			1.8			2.5			0.3	

(b)

Medium	MEM fol (0.01mg/l), Pl (0.0mg/l)									
	BSS									
	BSS		MTX		5-fu					
Pre-preinc'n	BSS	UdR	%	BSS	UdR	%	BSS	UdR	%	
Cont. Subjects										
1	37692	12333	33	48443	9325	19	46332	5337	11	
2	30991	6551	21	30651	6441	21	31819	4034	13	
3	13167	1257	10	24372	2038	8	27240	2025	7	
4	12572	1053	8	15394	2515	16	18861	1857	10	
\bar{x}			18			16			10	
s.e.			5.8			2.9			1.3	

FIG. 22 Effect of prior addition of MTX and 5-fu on UdR suppression
of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated
lymphocytes cultured in the presence and absence of 4-dPN

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated
lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with folate concentration of
0.01mg/l without Pl and with or without 4-dPN (500mg.l)
- (ii) pre-preincubation addition of BSS, MTX ($110\mu\text{M}$) or 5-fu (1.0mM)
15 min. prior to;
- (iii) preincubation addition of BSS or UdR (2.7mM)
1h prior to;
- (iv) incubation 3h (^{125}I)-UdR, $0.5\mu\text{Ci}$

Incorporation into lymphocyte cultures was expressed as a % of
incorporation into comparable lymphocytes cultured in medium without
4-dPN and after preincubation with BSS.

Results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of
2 control subjects.

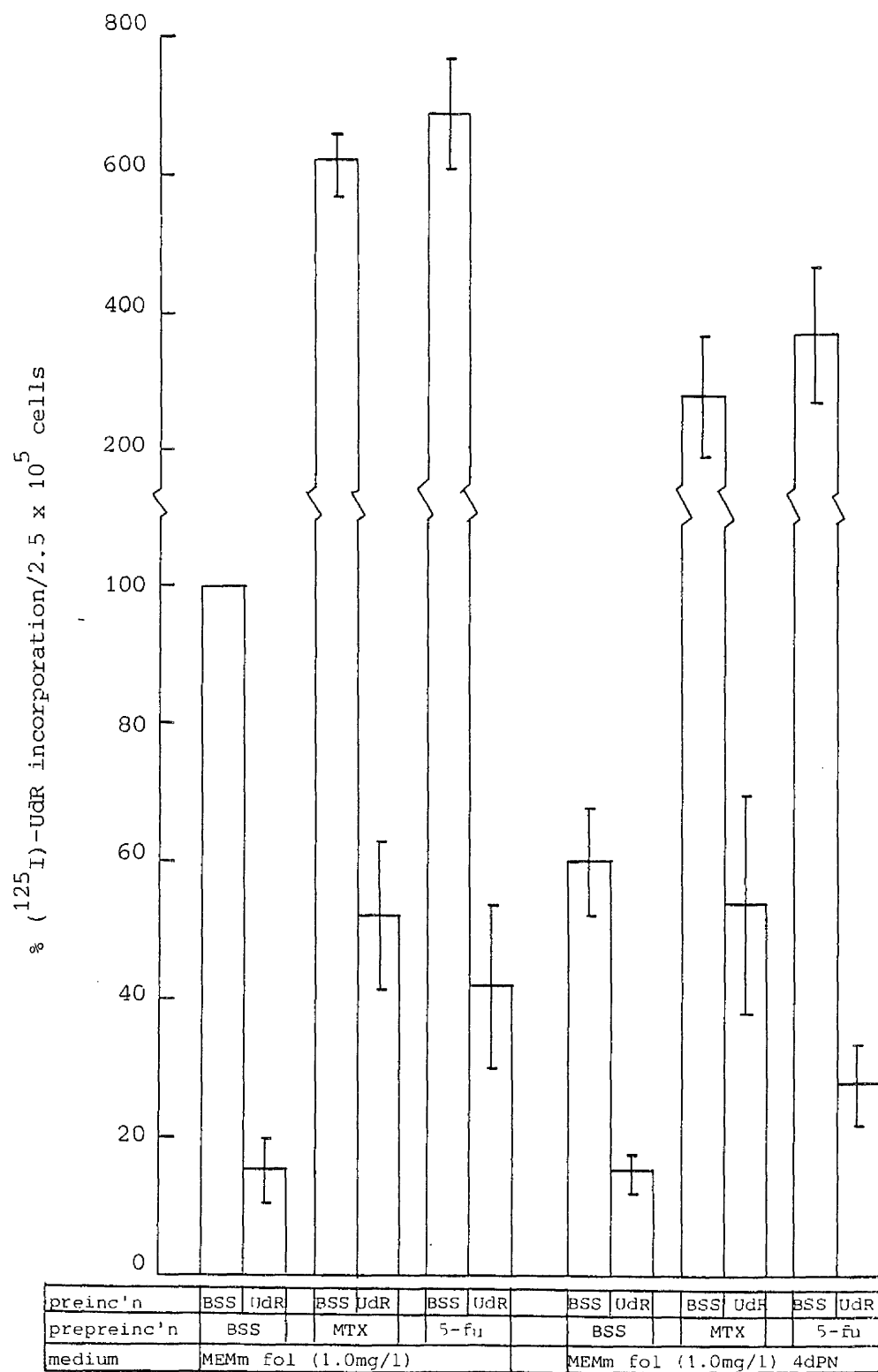
Fig. 23 Effect of Prior Addition of MTX and 5-fu on
UdR Suppression on Acid-insoluble (^{125}I)-
UdR incorporation into PHA Stimulated
Lymphocytes Cultured in the Presence and
Absence of 4-dPN

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 1.0mg/l without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-incubation addition of BSS, MTX (110 μM) or 5-fu (1.0mM) 15 min. prior to;
- (iii) pre-incubation addition of BSS or UdR (2.7mM) 1h prior to;
- (iv) incubation, 3h (^{125}I)-UdR, 0.5 μCi

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in medium without 4-dPN and after pre-incubation with BSS.

Results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of 5 control subjects.



These results indicated that whereas an increase in the folate concentration increased the contribution of the de novo thymidylate pathway, a decrease in vitamin B6 concentration had no effect. Efficient operation of the de novo pathway is dependent on availability of 5, 10 methylene H_4F . This is formed from H_4F and a carbon source such as serine by the PLP dependent enzyme serine hydroxymethyltransferase. Formation of 5, 10 methylene H_4F therefore requires H_4F , a source of one-carbon units and PLP. The influence of the medium folate concentration and the lack of influence of vitamin B6 deficiency on de novo thymidylate synthesis has previously been shown (3.3.2. and 3.3.5.). In this experiment the effect of variation in supply of one-carbon units was investigated. As formate has been shown to be a source of thymine methyl groups in various cell types (Blakely, 1969), it was decided to determine the effect of pre-incubation addition of formate on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes.

In Fig. 24 the results of an experiment in which the effect of formate was determined in stimulated lymphocytes cultured in medium of high and low folate concentration and in the presence and absence of 4-dPN are illustrated. In figs. 25 and 26, the effect of pre-incubation addition of formate on (^{125}I)-UdR incorporation was determined after prior addition of MTX and 5-fu. Again, medium of high and low folate concentration with and without 4-dPN was used.

Fig. 24 Effect of Pre-incubation with Formate on
Acid-insoluble (^{125}I)-UdR Incorporation into
PHA Stimulated Lymphocytes Cultured in Medium
of High and Low Folate Concentration and in
the Presence and Absence of 4-dPN

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM(2) with a folate/Pl concentration of 1.0mg/l and with or without 4-dPN (500mg/l) or with a folate concentration of 0.01mg/l, without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-incubation addition of BSS, formate (4mM), MTX (110 μM) or 5-fu (1.0mM), 1h prior to;
- (iii) incubation 3h (^{125}I)-UdR, 0.5 μCi

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in MEM (fol/Pl, 1.0mg/l) without 4-dPN and after pre-incubation with MTX.

Results are given as mean % (cpm) \pm s.e. of lymphocyte cultures of 4 control subjects.

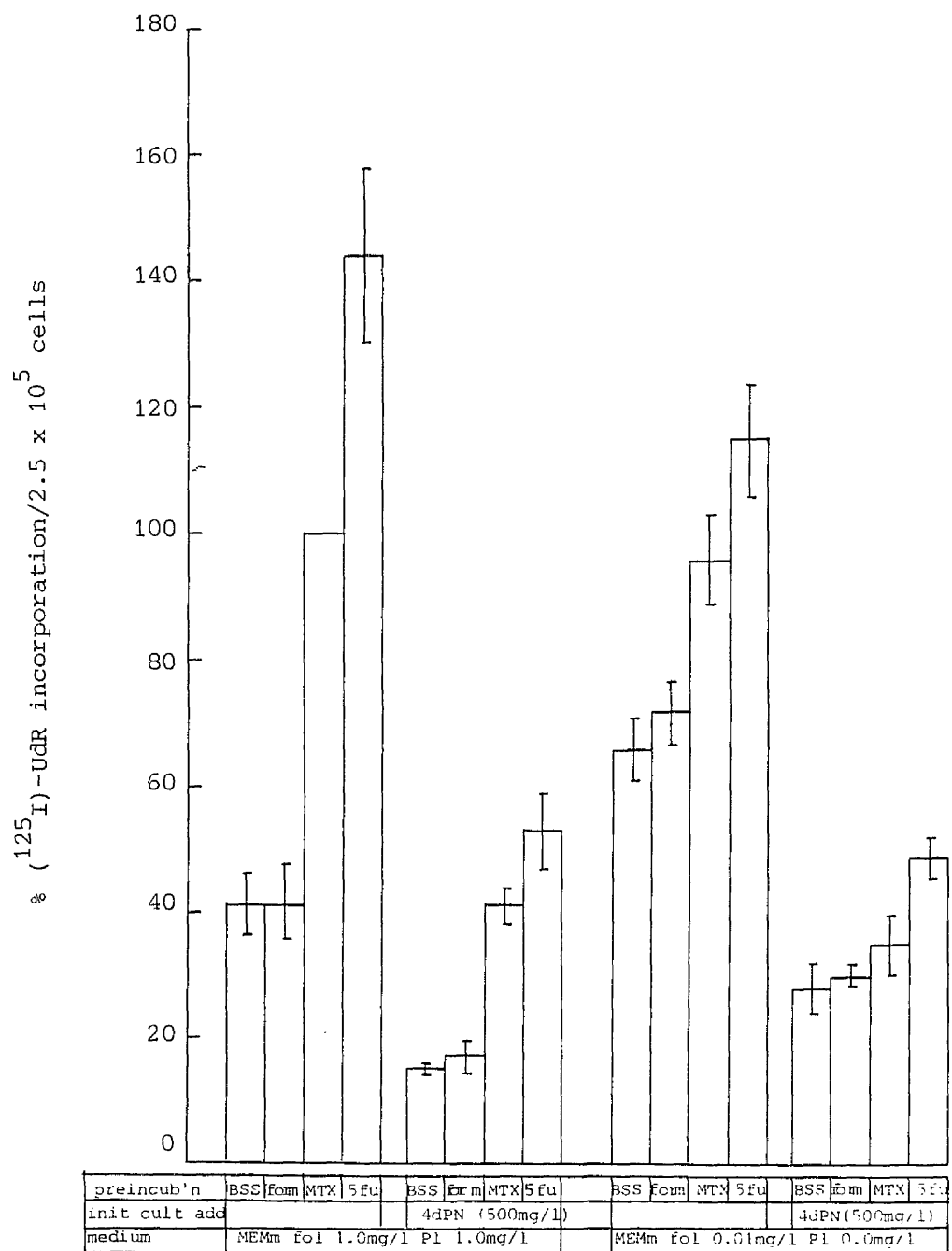


Fig. 25 Effect of Prior Addition of MTX and 5-fu, and
Pre-incubation with Formate, on Acid-insoluble
(¹²⁵I)-UdR Incorporation into PHA Stimulated
Lymphocytes Cultured in Low Folate Medium

Acid-insoluble (¹²⁵I)-UdR incorporation into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 0.01mg/l without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-preincubation with BSS, MTX (110μM) or 5-fu (1.0mM) 15 min. prior to;
- (iii) pre-incubation with BSS or formate (4mM) 1h prior to;
- (iv) incubation, 3h (¹²⁵I)-UdR, 0.5μCi

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in MEMm (folate 0.01mg/l) without 4-dPN and after successive pre-incubation with MTX and BSS.

Results are given as mean % (cpm) ± s.e. of lymphocyte cultures of 2 control subjects.

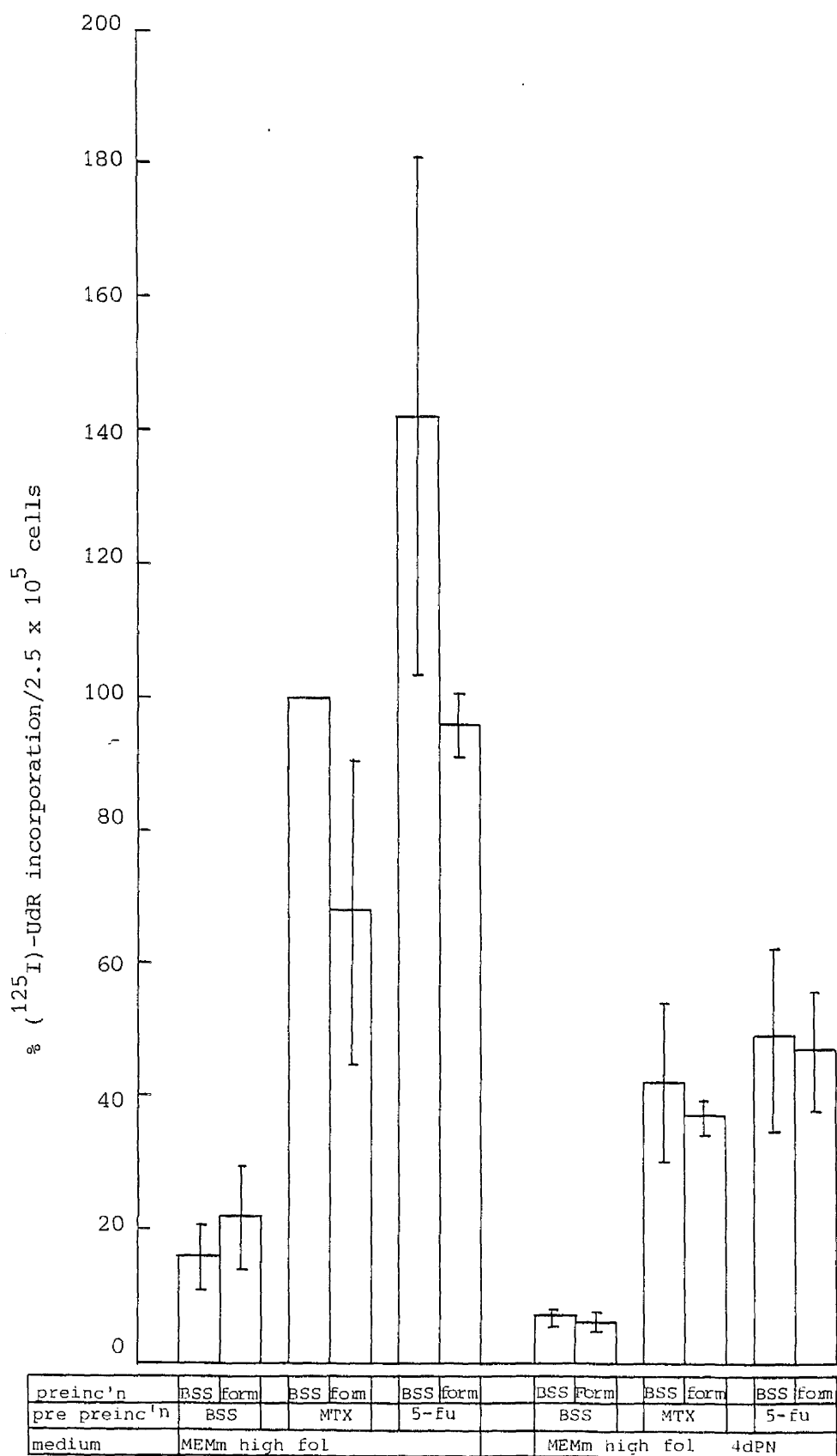
Fig. 26 Effect of Prior Addition of MTX and 5-fu,
and Pre-incubation with Formate on Acid-
insoluble (^{125}I)-UdR Incorporation into
PHA Stimulated Lymphocytes Cultured in
High Folate Medium

Acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 1.0mg/l without Pl and with or without 4-dPN (500mg/l)
- (ii) pre-preincubation with BSS, MTX (110 μM) or 5-fu (1.0mM) 15 min. prior to;
- (iii) pre-incubation with BSS or formate (4mM) 1h prior to;
- (iv) incubation, 3h, (^{125}I)-UdR, 0.5 μCi

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in MEMm (folate, 1.0mg/l) without 4-dPN and after successive pre-incubation with MTX and BSS.

Results are given as the mean % (cpm) \pm s.e. of lymphocyte cultures of 2 control subjects.



3.4.5. Incorporation of (^{125}I)-UdR and 3-(^{14}C)-Serine into PHA Stimulated Lymphocytes

Determination of the rate of DNA synthesis using a salvage pathway substrate such as (^{125}I)-UdR is independent of direct involvement of PLP dependent enzymes. In order to confirm that observed changes in acid-insoluble (^{125}I)-UdR incorporation do in fact reflect changes in total DNA synthesis and the respective contributions of salvage and de novo pathways to thymidylate synthesis, it is necessary to carry out parallel experiments in which incorporation of a de novo pathway substrate into DNA is determined. Such experiments are also necessary in order to characterise more directly the effect of vitamin B6 deficiency on DNA synthesis. Since a major source of the methyl group of thymine is carbon-3 of serine, 3-(^{14}C)-serine was used to label DNA via the de novo pathway. Incorporation of 3-(^{14}C)-serine label into DNA is dependent on both folate coenzymes and the PLP dependent enzyme, serine hydroxymethyltransferase, therefore one might expect such incorporation to be sensitive both to the medium folate concentration and to vitamin B6 status.

From a consideration of the various metabolic pathways in which serine is involved it is apparent that 3-(^{14}C)-serine label would be incorporated into a number of different cellular components including DNA, protein, lipid etc. Since the principal component of interest is DNA, the extent of 3-(^{14}C)-serine incorporation into a DNA extract of PHA stimulated lymphocytes was determined.

The pathway of incorporation of 3-(^{14}C)-serine label into DNA is via transfer of the labelled carbon to H_4F to form 5, 10 methylene H_4F . This step requires the PLP dependent enzyme serine hydroxymethyltransferase. The labelled carbon on 5, 10 methylene H_4F may then be incorporated into DNA via purine synthesis or via methylation of dUMP in de novo thymidylate synthesis. Thus incorporation of 3-(^{14}C)-serine label into a DNA extract is not absolutely specific for de novo thymidylate synthesis. This is not a serious limitation however, as such incorporation would still be dependent on vitamin B6 requiring enzyme activity, would still be expected to be sensitive to vitamin B6 deficiency and would still be expected to reflect the effect of vitamin B6 deficiency on DNA synthesis and cell growth.

In this experiment, acid-insoluble incorporation of the salvage thymidylate pathway, substrate analogue, (^{125}I)-UdR, into PHA stimulated lymphocytes is compared with incorporation of 3-(^{14}C)-serine label into a DNA extract of similarly cultured PHA stimulated lymphocytes. Determination of incorporation was carried out at 24, 48, 72 and 96h in lymphocytes cultured in modified MEM. The results are illustrated in Fig. 27, low folate conditions, and in Fig. 28, high folate conditions.

Fig. 27 Incorporation of (^{125}I)-UdR and 3-(^{14}C)-serine
label into PHA Stimulated Lymphocytes Cultured
in Medium of Low Folate Concentration

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) and incorporation of 3-(^{14}C)-serine label into a DNA extract of lymphocyte cultures derived from the same subject was determined at the times indicated, after;

- (i) culture in modified MEM (2) with a folate concentration of 0.01mg/l, (Pl, 1,0mg/l).
- (ii) for determination of (^{125}I)-UdR incorporation: pre-incubation with BSS (O) or MTX, 110 μM (\square), and for determination of 3-(^{14}C)-serine incorporation: pre-incubation with BSS (\blacksquare).
- (iii) incubation, 3h (^{125}I)-UdR, 0.5 μCi
or incubation 3h 3-(^{14}C)-serine, 0.25 μCi

Results are presented as mean (cpm) \pm s.e. of (^{125}I)-UdR incorporation or mean (cpm) \pm s.e. of 3-(^{14}C)-serine incorporation, in lymphocyte cultures of 2 control subjects.

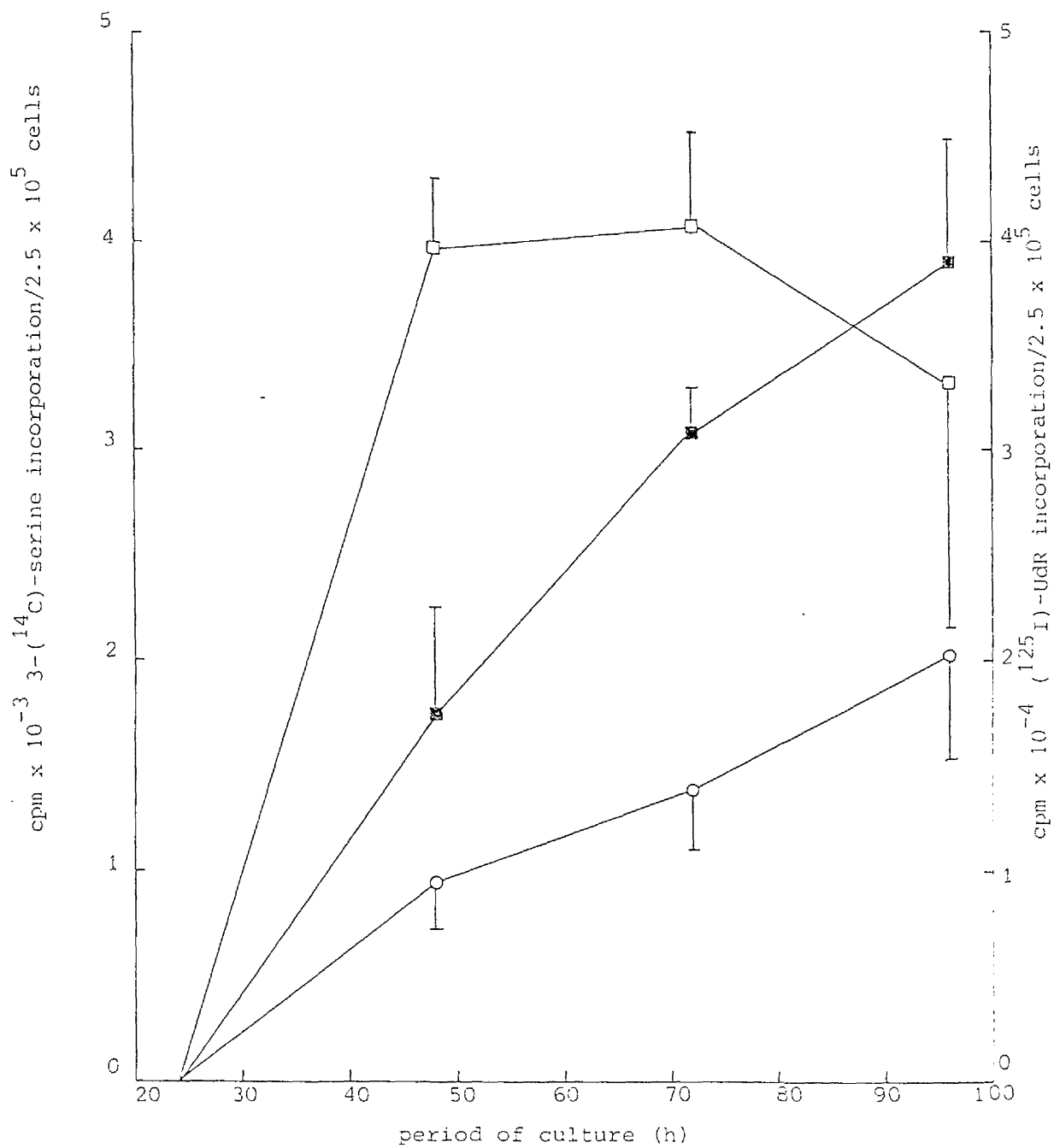


Fig. 28 Incorporation of (^{125}I)-UdR and 3-(^{14}C)-serine
Label into PHA Stimulated Lymphocytes Cultured
in Medium of High Folate Concentration

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) and incorporation of 3-(^{14}C)-serine label into a DNA extract of lymphocyte cultures derived from the same subject was determined at the times indicated, after;

(i) culture in modified MEM (2) with a folate concentration of 1.0mg/l, (Pl 1.0mg/l),

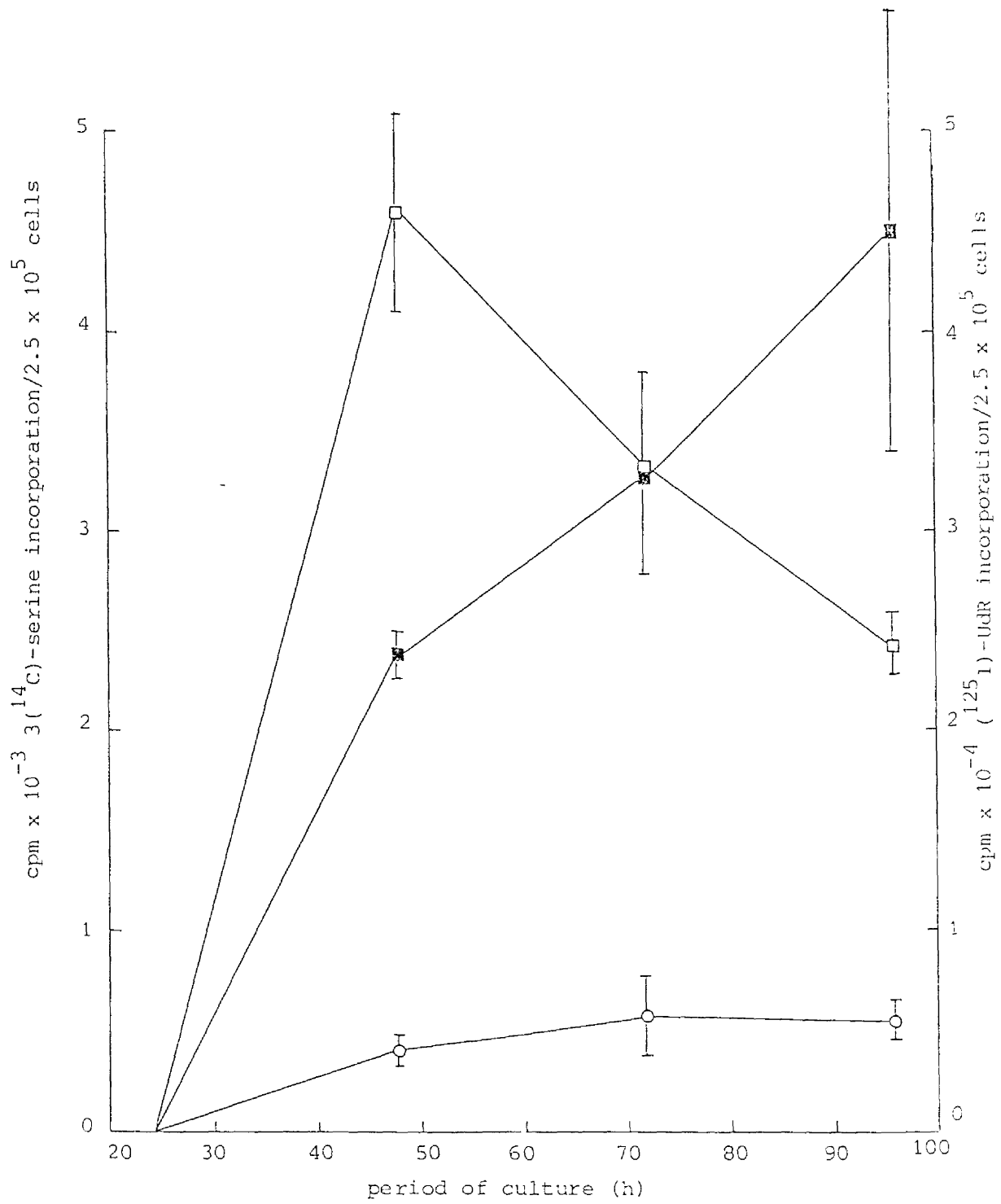
(ii) for determination of (^{125}I)-UdR incorporation: pre-incubation with BSS (○), or MTX, 110 μM . (□).

and for determination of 3-(^{14}C)-serine incorporation: pre-incubation with BSS (■).

(iii) incubation 3h, (^{125}I)-UdR, 0.5 μCi

or incubation 3h, 3-(^{14}C)-serine, 0.25 μCi

Results are presented as mean cpm \pm s.e. of (^{125}I)-UdR incorporation or mean cpm \pm s.e. of 3-(^{14}C)-serine incorporation in lymphocyte cultures of 2 control subjects.



3.4.6. Effect of Variation of the Medium Folate Concentration and Pre-incubation with MTX and 5-fu on Incorporation of 3-(¹⁴C)-serine label into a DNA Extract of PHA Stimulated Lymphocytes

In this experiment the effect of culture in medium of both high and low folate concentration, and pre-incubation with MTX and 5-fu, on subsequent incorporation of 3-(¹⁴C)-serine label into a DNA extract of PHA stimulated lymphocytes was determined. Determination of incorporation was carried out 72h after initiation of culture and the results are illustrated in Fig. 29. The results indicate that in a low folate medium, incorporation of 3-(¹⁴C)-serine label into a DNA extract is reduced relative to incorporation into lymphocytes cultured in high folate medium. The results also indicate that pre-incubation with MTX or 5-fu reduces 3-(¹⁴C)-serine label incorporation into DNA and that this effect is independent of the medium folate concentration.

3.4.7. Effect of Variation of the Medium Folate Concentration and Pre-incubation with MTX and Udr on Incorporation of 3-(¹⁴C)-serine Label into a DNA Extract of PHA Stimulated Lymphocytes

In this experiment the effect of culture in medium of both high and low folate concentration, and pre-incubation with MTX and Udr, on subsequent incorporation of 3-(¹⁴C)-serine label into a DNA extract of PHA stimulated lymphocytes was determined. Determination of incorporation was carried out 72h after initiation of culture and the results are illustrated in Fig. 30. As in the previous experiment (3.4.6.), incorporation

Fig. 29 Effect of Variation of the Medium Folate
Concentration and Pre-incubation with MTX
and 5-fu on Incorporation of 3-(¹⁴C)-
serine label into a DNA Extract of PHA
Stimulated Lymphocytes

Incorporation of 3-(¹⁴C)-serine label into a DNA extract
of PHA stimulated lymphocyte cultures (2.5×10^5 cells)
was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentra-
 tion of 1.0mg/l. (Pl 1.0mg/l) or 0.01mg/l,
 (Pl 1.0mg/l).
- (ii) pre-incubation addition of BSS, MTX (110μM) or
 5-fu (1.0mM).
- (iii) incubation 3h, 3-(¹⁴C)-serine, 0.25μCi

Results are presented as mean cpm ± s.e. of 3-(¹⁴C)-serine
label incorporation into a DNA extract of lymphocyte cultures
of 4 control subjects.

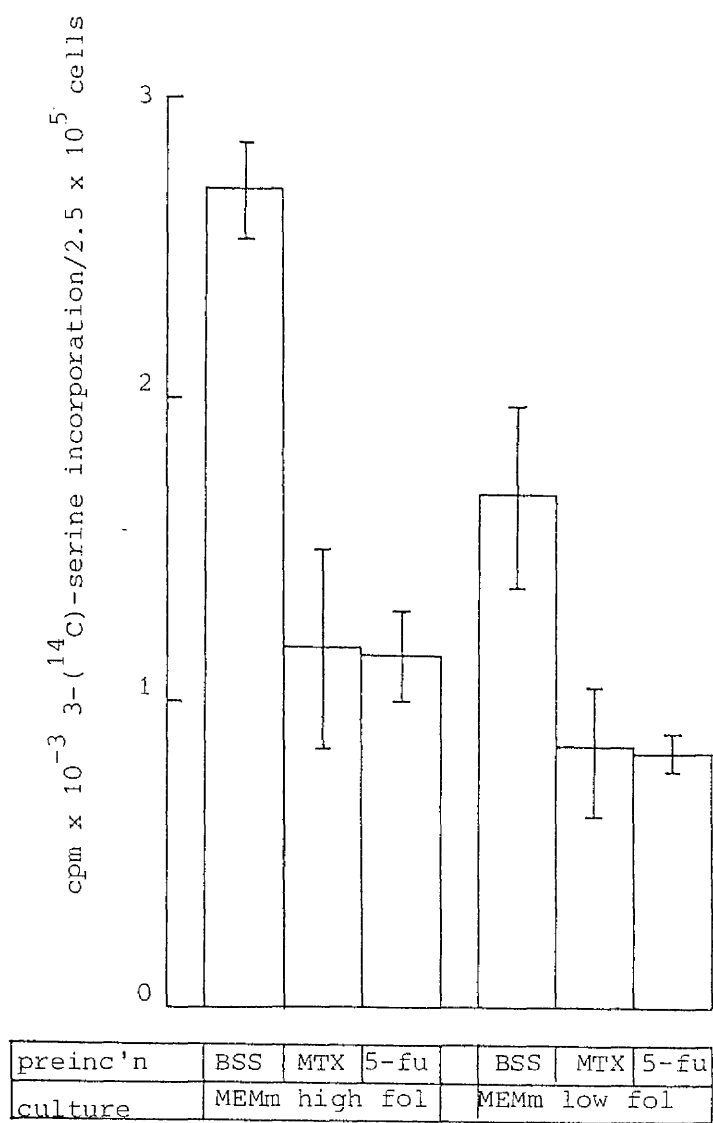
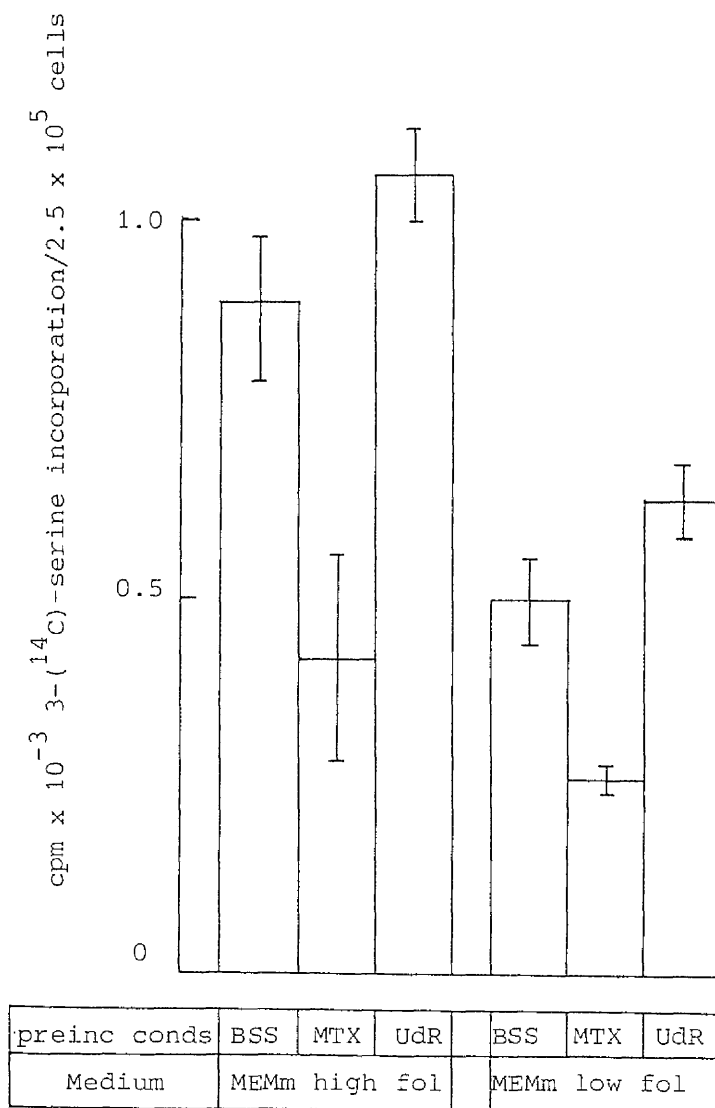


Fig. 30 Effect of Variation of the Medium Folate
Concentration, and pre-incubation with MTX and
UdR, on Incorporation of 3-(¹⁴C)-serine label
into a DNA Extract of PHA Stimulated Lymphocytes

Incorporation of 3-(¹⁴C)-serine label into a DNA extract of PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 1.0mg/l, (Pl 1.0mg/l) or 0.01mg/l (Pl 1.0mg/l)
- (ii) pre-incubation addition of BSS, MTX (110μM) or UdR (2.7mM)
- (iii) incubation, 3h 3-(¹⁴C)-serine, 0.25μCi

Results are presented as mean cpm ± s.e. of 3-(¹⁴C)-serine label incorporation into a DNA extract of lymphocyte cultures of 2 control subjects.



of 3-(^{14}C)-serine label into a DNA extract of PHA stimulated lymphocytes cultured in a low folate medium is reduced relative to that of incorporation into comparable lymphocytes cultured in a high folate medium. Similarly incorporation into lymphocytes cultured in either medium is reduced by pre-incubation with MTX. Pre-incubation with Udr however increases 3-(^{14}C)-serine label incorporation, relative to incorporation into lymphocytes pre-incubated with BSS. As before this effect occurs in lymphocytes cultured in medium of high or low folate concentration.

3.4.8. Effect of Addition of MTX and 5-fu Followed by Pre-incubation with Udr on Incorporation of 3-(^{14}C)-serine label into a DNA Extract of PHA Stimulated Lymphocytes

Several possible mechanisms may be proposed to account for the observed increase in 3-(^{14}C)-serine label incorporation into a DNA extract of PHA stimulated lymphocytes after pre-incubation with Udr. If it is assumed that addition of Udr does not cause an increase in the actual rate of DNA synthesis, and if it is further assumed that only local effects are involved then the most obvious implication is that pre-incubation addition of Udr results in a change in the relative contributions of the de novo and salvage pathways to thymidylate synthesis. Such a change could result from either an alleviation of a restriction in de novo synthesis, at the level of dUMP, or inhibition of salvage thymidylate synthesis, probably at the level of thymidine kinase. The possibility that the de novo thymidylate pathway was limited by availability of

dUMP and not by the availability of the folate co-enzyme, 5, 10, methylene H_4F , was investigated in the following experiment by using MTX and 5-fu to produce a de facto folate deficiency.

After 15 min. pre-pre-incubation with BSS, MTX or 5-fu, PHA stimulated lymphocyte cultures were pre-incubated with either BSS or Udr for a period of 1h prior to incubation with 3- (^{14}C) -serine and determination of radioactive label incorporation into extracted DNA. The results of this experiment are illustrated in Fig. 31. It is apparent from these results that limitation of folate coenzyme availability (MTX) is almost as effective as inhibition of thymidylate synthetase (5-fu) in preventing a Udr dependent increase in 3- (^{14}C) -serine label incorporation into a DNA extract of PHA stimulated lymphocytes.

3.4.9. Effect of Culture in Medium Containing 4-dPN on Incorporation of 3- (^{14}C) -serine label into a DNA Extract of PHA Stimulated Lymphocytes

In this experiment, the effect of culture in the presence of 4-dPN on incorporation of 3- (^{14}C) -serine label into a DNA extract of PHA stimulated lymphocytes was determined. The basal culture medium used was modified MEM with either a high (1.0mg/l) or low (0.01mg/l) folate concentration. Determination of incorporation was carried out at 20, 48, 72 and 96h of culture after incubation with 3- (^{14}C) -serine (0.25 μ Ci) for 3h. The results, illustrated in Fig. 32, indicate that culture in the presence of 4-dPN reduces 3- (^{14}C) -serine

Fig. 31 Effect of Addition of MTX and 5-fu, followed
by Pre-incubation with UdR, on Incorporation
of 3-(¹⁴C)-serine label into a DNA Extract of
PHA Stimulated Lymphocytes

Incorporation of 3-(¹⁴C)-serine label into a DNA extract of PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in complete MEM
- (ii) pre-preincubation addition of BSS, MTX (110 μ M),
 or 5-fu (1.0mM) 15 min. prior to;
- (iii) pre-incubation addition of BSS or UdR (2.7mM)
 1h prior to;
- (iv) incubation, 3h 3-(¹⁴C)-serine, 0.25 μ Ci

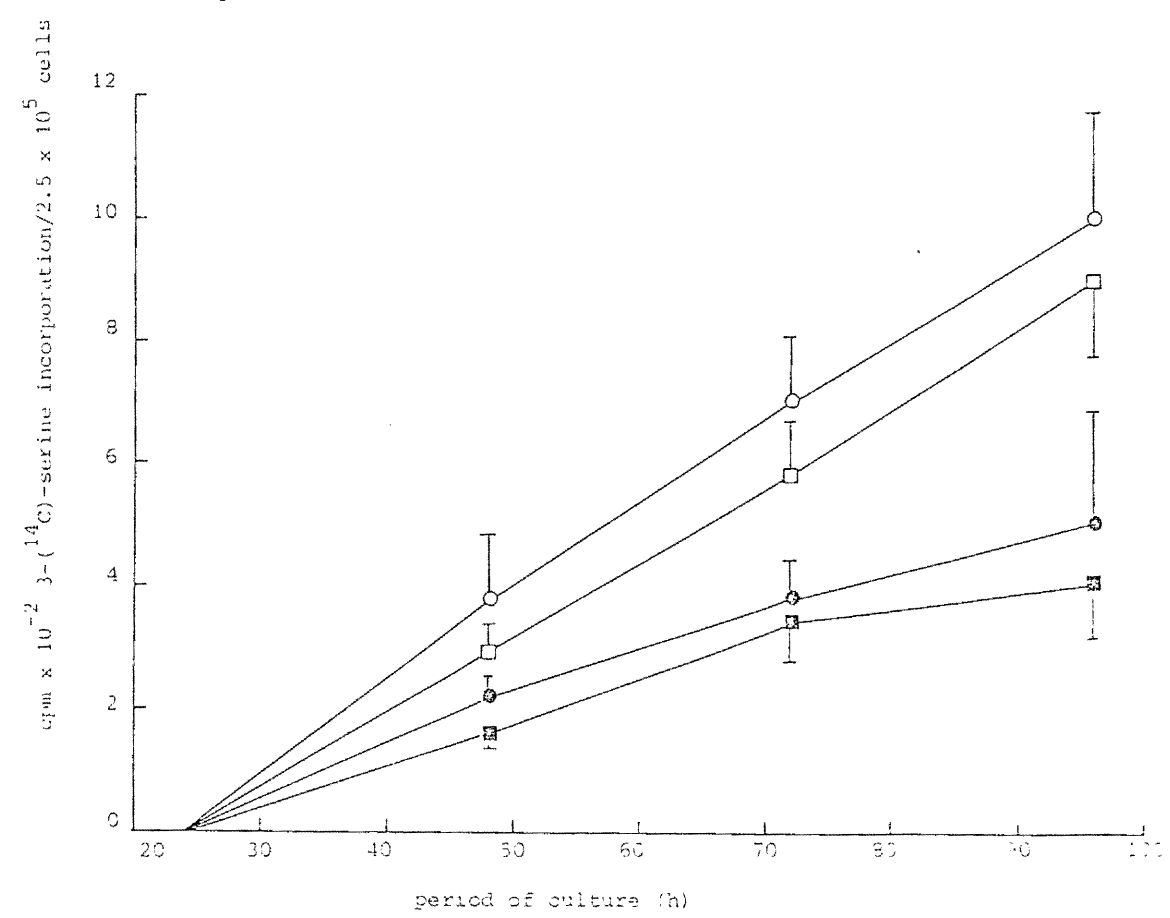
Results are presented as mean cpm \pm s.e. of 3-(¹⁴C)-serine label incorporation into a DNA extract of lymphocyte cultures of 4 control subjects.

Fig. 32 Effect of Culture in Medium Containing 4-dPN
on Incorporation of 3-(¹⁴C)-serine label into
a DNA Extract of PHA Stimulated Lymphocytes

Incorporation of 3-(¹⁴C)-serine label into a DNA extract of PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at the times indicated, after;

- (i) culture in modified MEM (2) with folate 1.0mg/l, without Pl and with (●) or without (○) 4-dPN, 500mg/l
or with folate 0.01mg/l, without Pl
and with (■) or without (□) 4-dPN. 500mg/l
- (ii) incubation, 3h, 3-(¹⁴C)-serine, 0.25μCi

Results are presented as mean cpm \pm s.e. of 3-(¹⁴C)-serine label incorporation into a DNA extract of lymphocyte cultures of 4 control subjects.



label incorporation into a DNA extract of PHA stimulated lymphocytes throughout the course of culture and that the effect on incorporation into lymphocytes cultured in low folate medium is of similar magnitude to the effect on incorporation into lymphocytes cultured in high folate medium.

3.4.10. Incorporation of (^{14}C)-formate label into a DNA Extract of PHA Stimulated Lymphocytes

An alternative method of labelling DNA via the de novo thymidylate pathway is to make use of (^{14}C)-formate. Using (^{14}C)-formate, labelling of the active one-carbon folate pool can occur independently of vitamin B6 requiring enzymes but thereafter distribution of radioactive label is likely to be similar to distribution of one-carbon metabolites labelled from 3-(^{14}C)-serine. Thus (^{14}C)-formate label would be incorporated into protein, via formation of serine from glycine, and DNA, via formation of labelled thymidylate from dUMP. Labelling of the purine ring structure at carbons 2 and 8, and methylation of other bases would also contribute to labelling of DNA by (^{14}C)-formate. Therefore, although incorporation of (^{14}C)-formate into a DNA extract of PHA stimulated lymphocytes is not absolutely specific for labelling of DNA via the de novo thymidylate pathway, it does provide an alternative to use of 3-(^{14}C)-serine, which is independent of vitamin B6 requiring enzymes and which would therefore be expected to reflect DNA synthesis and cell growth independently of a direct effect of vitamin B6 deficiency on DNA synthesis.

In this experiment, the effect of pre-incubation with BSS, MTX, 5-fu, formate and UdR on subsequent incorporation of (^{14}C)-formate label, into a DNA extract of PHA stimulated lymphocytes cultured in modified MEM containing folate and pyridoxal (1.0mg/l), was determined. The results, illustrated in Fig. 33, are comparable with results of similar experiments where 3- (^{14}C)-serine label was used, (3.4.7.).

Principally, pre-incubation with MTX and 5-fu results in a reduction of incorporation, and pre-incubation with UdR results in an increase in incorporation, relative to incorporation into lymphocyte cultures pre-incubated with BSS.

3.4.11. Effect of Presence of Non-essential amino acids on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes Cultured in Medium Containing 4-dPN

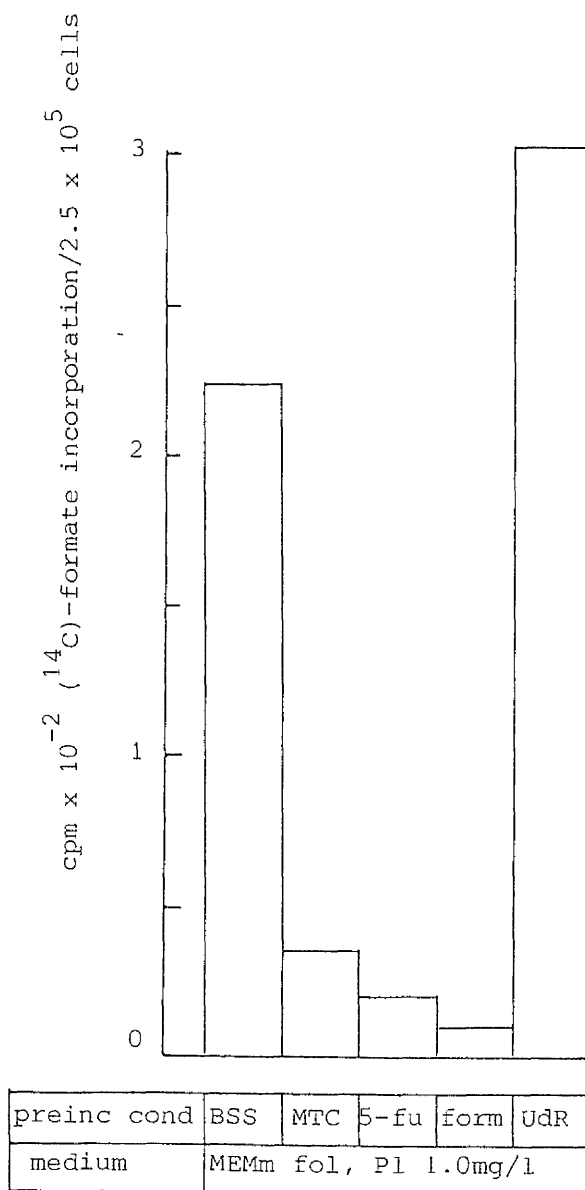
The possible causes of vitamin B6 deficiency and the likely effects of vitamin B6 deficiency in particular circumstances have previously been discussed (1.2.3.). Due to the increased rate of cell growth in PHA stimulated lymphocytes and the known involvement of PLP in de novo thymidylate synthesis, it had been imagined that one of the primary effects of vitamin B6 deficiency in such circumstances would be a restriction of DNA synthesis and particularly of de novo thymidylate synthesis. However, the process of PHA stimulation is accompanied by a variety of other biochemical activities which could equally be susceptible to the effects of vitamin B6 deficiency and which would also

Fig. 33 Incorporation of (^{14}C)-formate label into a
DNA Extract of PHA Stimulated Lymphocytes

Incorporation of (^{14}C)-formate label into a DNA extract of PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate/Pl concentration of 1.0mg/l
- (ii) pre-incubation addition of BSS, MTX ($110\mu\text{M}$), 5-fu (1.0mM), formate (4mM) or Udr (2.7mM), 1h prior to;
- (iii) incubation, 3h, (^{14}C)-formate, $1.0\mu\text{Ci}$

Results are presented as mean cpm (of duplicate analyses) of (^{14}C)-formate incorporation into a DNA extract of lymphocyte cultures of one control subject.



result in a restriction in cell growth and DNA synthesis, though not specifically at the level of inhibition of de novo thymidylate synthesis.

Among the many processes dependent on vitamin B6 co-enzymes, in addition to formation of 5,10,methylene H_4F , are the interconversion of numerous amino acids and the synthesis of delta-aminolaevulinic acid. Thus it is conceivable that a vitamin B6 deficiency could be primarily expressed through inhibition of protein or porphyrin synthesis, and also that such an effect could be alleviated by exogenous supply of the appropriate vitamin B6 dependent metabolite.

In these experiments, the possibility that the growth restriction caused by culture in the presence of 4-dPN could be alleviated by addition to the medium of non-essential amino acids, including glycine and serine, was investigated. In Fig. 34, the results of an experiment in which the effect of non-essential amino acids to PHA stimulated lymphocytes cultured in modified MEM, of high folate concentration, with or without pyridoxal and in the presence or absence of 4-dPN, was determined. Acid-insoluble incorporation of (^{125}I)-UdR was used as an index of cell growth and the analysis was carried out at 72h after pre-incubation with either BSS or MTX. In Fig. 35, the results of a similar experiment are illustrated. Here the medium used was complete MEM, as above, the effect of addition of non-essential amino acids to the initial culture medium, on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes cultured in the

Fig. 34 Effect of Presence of Non-essential amino-
acids on Acid-insoluble Incorporation of
(¹²⁵I)-UdR into PHA Stimulated Lymphocytes
Cultured in Medium Containing 4-dPN.

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5 x 10⁵ cells) was determined at 72h after;

- (i) culture in modified MEM (2); the composition of the medium used in each experiment was as indicated at the base of each column. The concentration of the medium components was as follows:

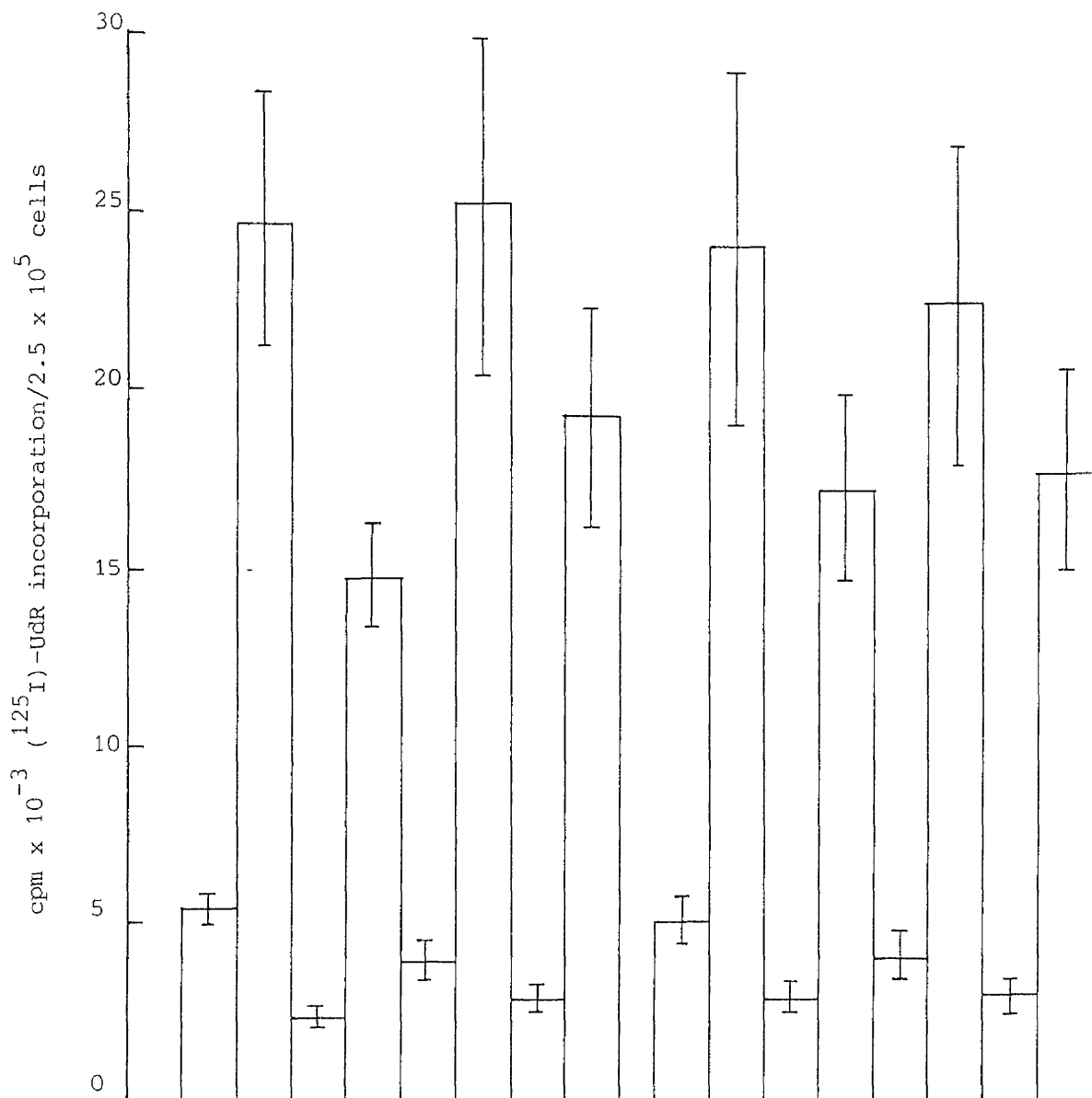
folate (1.0mg/l), Pl (1.0mg/l), non-essential amino acids (1 x MEM)* and 4-dPN (500mg/l).

- (ii) pre-incubation addition of BSS or MTX (110μM), 1h prior to;

- (iii) incubation, 3h, (¹²⁵I)-UdR, 0.5μCi

Results are presented as mean (cpm) ± s.e. of incorporation in lymphocyte cultures of 4 control subjects.

* MEM non-essential amino acids were added at a final medium concentration of; alanine (8.9mg/l), asparagine (15mg/l), aspartate (13.3mg/l), glutamate (14.7mg/l), glycine (7.5mg/l) proline (11.5mg/l) and serine (10.5mg/l).



preinc'n	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX	BSS	MTX
initial			4dPN			4dPN			4dPN			4dPN		
cult.adds														
			non ess amino acids						non ess amino acids					
medium	MEMm	high fol,	high Pl				MEMm	high fol,	-Pl					

Fig. 35 Effect of Presence of Non-essential amino
acids on Acid-insoluble Incorporation of
(¹²⁵I)-UdR into PHA Stimulated Lymphocytes
Cultured in Medium Containing 4-dPN

Acid-insoluble (¹²⁵I)-UdR incorporation (cpm) into PHA stimulated lymphocytes (2.5 x 10⁵ cells) was determined at 72h after;

- (i) culture in complete MEM; the composition of the medium used in each experiment was as indicated at the base of each column. The concentration of the medium components was as follows; non-essential amino acids (1 x MEM)* and 4-dPN (500mg/l)
- (ii) pre-incubation addition of BSS, MTX (110μM), 5-fu (1.0mM) or UdR (2.7mM), 1h prior to;
- (iii) incubation, 3h, (¹²⁵I)-UdR, 0.5μCi

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in complete MEM without non-essential amino acids or 4-dPN and after pre-incubation with MTX.

Results are given as the mean % (cpm) ± s.e. of lymphocyte cultures of 4 control subjects.

*MEM non-essential amino acids were added at a final medium concentration of; alanine (8.9mg/l), asparagine (15mg/l) aspartate (13.3mg/l), glutamate (14.7mg/l), glycine (7.5mg/l), proline (11.5mg/l) and serine (10.5mg/l).

presence and absence of 4-dPN, was determined at 72h. In this case however, the effect of pre-incubation addition of BSS, MTX, 5-fu and UdR was determined.

The results of these experiments indicate that the medium presence of non-essential amino acids has little effect on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes cultured in medium with or without pyridoxal and with or without 4-dPN.

3.4.12. Effect of Culture in Medium Containing 4-dPN on Acid-insoluble Incorporation of (^{14}C)-leucine Label into PHA Stimulated Lymphocytes

The principal method used in this study to estimate cell growth and DNA synthesis in stimulated lymphocyte cultures has been based on acid-insoluble incorporation of (^{125}I)-UdR. Such a determination has been shown to provide a reasonably accurate index of the rate of lymphocyte DNA synthesis, and, since DNA synthesis is an integral part of the cell cycle, of cell growth. This method has been used both to evaluate the cell growth response to PHA in lymphocytes of normal and alcoholic liver disease subjects and also to investigate the effect of various medium components and other reagents on this response.

Since increased protein synthesis is one of the major biochemical changes accompanying PHA stimulation it was decided to investigate the effect of 4-dPN induced vitamin B6 deficiency on protein synthesis in PHA stimulated lymphocytes. From this it was hoped to establish the relationship between the effect of 4-dPN on protein and DNA synthesis and also the

respective value of alternative methods of assessing cell growth response to PHA.

In this experiment acid-insoluble incorporation of (^{14}C)-leucine label incorporation into PHA stimulated lymphocytes cultured in complete MEM with or without added 4-dPN was determined at 72h after pre-incubation with BSS, MTX or UdR. The results, illustrated in Fig. 36, indicate that 4-dPN reduces protein synthesis by about 50%. This is similar to the effect of 4-dPN on DNA synthesis.

3.4.13. Effect of Presence of Hemin on Acid-insoluble Incorporation of (^{125}I)-UdR into PHA Stimulated Lymphocytes Cultured in Medium Containing 4-dPN

Vitamin B6 deficiency has been implicated in inhibition of porphyrin synthesis, and hence haematopoiesis, as a result of both reduced availability of glycine and reduced delta-aminolaevulinic acid synthetase activity. As in (3.4.11) it is conceivable that such inhibition could be alleviated by exogenous supply of the appropriate vitamin B6 dependent metabolite.

In this experiment, such a possibility was investigated by carrying out a PHA stimulated lymphocyte culture in modified MEM (fol, 1.0mg/l, Pl, 0.0mg/l) with added BSS, 4-dPN, hemin, or 4-dPN/hemin. After 67h of culture, 1h pre-incubation with BSS, MTX or 5-fu and 3h incubation with (^{125}I)-UdR (0.5 μCi), acid-insoluble incorporation of radioactive label into cultures was determined. The results of this experiment illustrated in Fig. 37, indicate that addition of hemin to cultures containing 4-dPN, does not markedly relieve the effect of 4-dPN,

Fig. 36. Effect of Culture in Medium Containing 4-dPN
on Acid-insoluble Incorporation of (^{14}C)-
leucine label into PHA Stimulated Lymphocytes

Acid-insoluble (^{14}C)-leucine incorporation (cpm) into
PHA stimulated lymphocyte cultures (2.5×10^5 cells)
was determined at 72h after;

- (i) culture in complete MEM with or without 4-dPN
(500mg/l)
- (ii) pre-incubation addition of BSS, MTX ($110\mu\text{M}$) or
5-fu (1.0mM) 1h prior to;
- (iii) incubation, 3h, (^{14}C)-leucine, $1.0\mu\text{Ci}$

Results are presented as mean (cpm) \pm s.e. of incorpora-
tion into lymphocyte cultures of 4 control subjects.

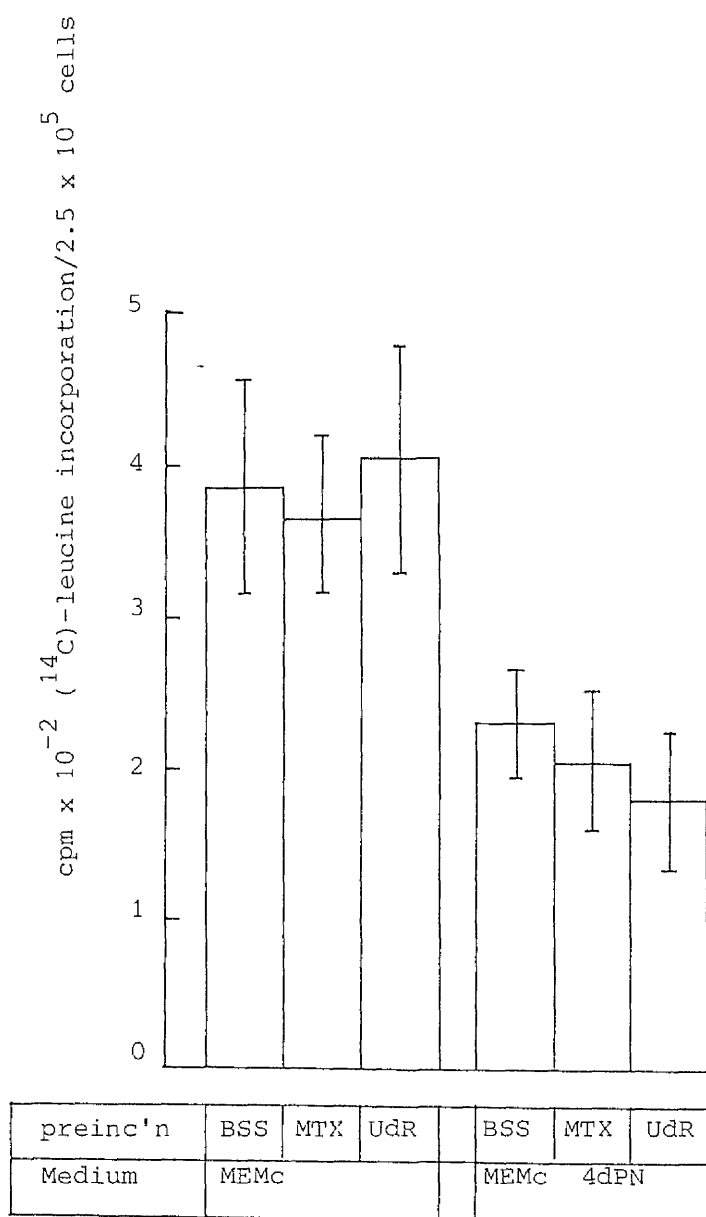


Fig. 37 Effect of Presence of Hemin on Acid-
insoluble Incorporation of (^{125}I)-UdR into
PHA Stimulated Lymphocytes Cultured in
Medium Containing 4-dPN

Acid-insoluble (^{125}I)-UdR incorporation (cpm) into PHA stimulated lymphocyte cultures (2.5×10^5 cells) was determined at 72h after;

- (i) culture in modified MEM (2) with a folate concentration of 1.0mg/l, without Pl and with added 4-dPN (500mg/l), hemin (0.1mM) or 4-dPN (500mg/l) and hemin (0.1mg/l)
- (ii) pre-incubation addition of BSS, MTX ($110\mu\text{M}$) or 5-fu (1.0mM) 1h prior to;
- (iii) incubation 3h (^{125}I)-UdR, $0.5\mu\text{Ci}$.

Incorporation into lymphocyte cultures was expressed as a % of incorporation into comparable lymphocytes cultured in modified MEM with no addition and after pre-incubation with MTX.

Results are presented as the mean % (cpm) \pm s.e. of lymphocyte cultures of 2 control subjects.

particularly when the effect of addition of hemin alone to cultures on subsequent (^{125}I)-UdR incorporation is considered.

CHAPTER 4

DISCUSSION

Section 4. 1.

Preliminary Studies

4.1.1. Ascorbic Acid Status

The results reported in section 3.1.1. suggest that ascorbic acid levels in alcoholic liver disease subjects are lower than those observed in control subjects.

This is consistent with previous reports, e.g., O'Keene, Russell and Goldberg, (1972); Beattie and Sherlock, (1976). The cause of lower ascorbic status in alcoholic liver disease is partly low dietary intake but the effects of alcohol may also be a factor (O'Keene et al 1972). The functional consequences of low ascorbate status may include interference with hepatic drug metabolism (Beattie and Sherlock, 1976); Zannoni, Flynn and Lynch, 1972) and anaemia (Goldberg, 1963). There is insufficient data in the present study to assess the relationship between low ascorbate status and the incidence of alcoholic liver disease.

The effect of reduced ascorbate status of alcoholic liver disease subjects on hepatic drug metabolism was considered in this study by comparing SAA levels with the rate of clearance of the drug antipyrine.

Antipyrine clearance was assessed by determination of the serum half-life ($t_{1/2}$) and the results are illustrated in Table 6. The results indicate there is no significant correlation between SAA level and antipyrine $t_{1/2}$.

Of eleven subjects with prolonged antipyrine $t_{1/2}$ (greater than 23 h) six had a low SAA level and five had a normal SAA level (greater than 20 mmol/l). A similar pattern of results was observed when LAA levels were considered.

As seven of the eleven alcoholic liver disease subjects with prolonged antipyrine $t_{1/2}$ had cirrhosis and as the severity of liver disease is known to be a major determinant of serum antipyrine $t_{1/2}$ (Branch et al, 1973) it is likely that in the present series the severity of the liver disease obscures any relationship between ascorbate status and drug metabolism. Beattie and Sherlock (1976) also noted that prolonged antipyrine $t_{1/2}$ can occur in the presence of normal ascorbate status. This suggests that ascorbate status is only one of several factors determining antipyrine clearance.

4.1.2. Pyridoxal Phosphate Status

The results reported in section 3.1.2. indicate that serum PLP levels in alcoholic liver disease subjects are not significantly lower than in control subjects. This contrasts with published reports by Lumeng and Li (1974), and Labadarios, et al (1977), which suggest that serum PLP levels are significantly lower in both alcoholic and alcoholic liver disease subjects than in control subjects. The reasons for the failure to detect a reduced vitamin B6 status in alcoholic liver disease subjects in this study may include the small sample population size and the wide variation in serum PLP values observed in this sample. For example, the serum PLP level of subject number 11 (Table 3) was 30 ng/ml. This suggests that release of vitamin B6 from damaged liver tissue may, in some cases, result in serum PLP values which do not necessarily indicate the true tissue vitamin B6 status. Such an effect of high serum

levels of vitamin B6 due to liver damage has been reported by Rossouw, et al (1978). The association of high serum levels of vitamin B12 and diminished hepatic levels has been observed in cases of liver disease (Sherlock, 1963) and reports of high serum B12 levels in alcoholic liver disease subjects have been reviewed by Bonjour (1979). These observations suggest that in some cases, usually dependent on the type, severity and stage of liver disease, serum levels of a vitamin cannot be considered indicative of tissue levels.

Measurement of cellular and serum transaminase activities in the absence and presence of added PLP has been suggested as a means of assessing vitamin B6 status and of providing information on the state of deficiency or the degree of depletion of vitamin B6 reserves (Sauberlich, 1972). The results reported in section 3.1.3 indicate that there is no statistically significant correlation between serum PLP levels and the degree of stimulation of AST activity by addition of PLP. A negative correlation might have been expected if low serum PLP levels accompanied low PLP saturation of serum AST and therefore a high degree of stimulation of AST activity in the presence of added PLP. The lack of any apparent correlation between these two measures of B6 status in this study may be due to the confounding effect of the wide range of AST activity observed, the serum half-life of AST and the effect of liver damage on serum PLP levels and AST activity. To avoid these confounding effects it may

be more appropriate to assess PLP stimulation of AST activity in erythrocytes (Sauberlich, 1972).

Although the results of the studies reported here do not provide evidence of reduced vitamin B6 status in alcoholics, perhaps because of the low number and type of subject, it is clear from the studies reviewed by Bonjour (1980) that there is a high incidence of low circulating levels of vitamin B6 in alcoholics. However, other than evidence of sideroblastic anaemia (Hines and Cowan, 1970) there are few reports of overt clinical manifestations of vitamin B6 deficiency. Therefore, to investigate whether or not the suspected vitamin B6 deficiency of alcoholic liver disease is functionally significant it seems appropriate to assess vitamin B6 status by a more functionally relevant method.

4.1.3. Genetic Markers

The results presented in sections 3.1.4. and 3.1.5. suggest that there is no difference in the distribution of fast and slow acetylators or alpha-1-antitrypsin phenotypes in the alcoholic or control populations studied. To establish with a greater degree of confidence whether or not such a difference does exist would require analysis of a much larger sample of the alcoholic population. Although there were no apparent sex differences in the results reported here, there was insufficient data to draw any conclusions.

4.1.4. Vitamin B12 Metabolism

This study was concerned with characterisation of several aspects of vitamin B12 metabolism and in particular the observation of raised serum vitamin B12 levels in liver disease (Hines, 1969). Preliminary studies by the department of Nuclear Medicine, Southern General Hospital suggested that intestinal vitamin B12 absorption remained normal in alcoholic liver disease subjects and the results of determinations of serum vitamin B12 levels in alcoholic liver disease subjects carried out by the Department of Haematology, Southern General Hospital were consistent with but did not confirm, the observation of raised serum vitamin B12 levels in these subjects. The most accepted explanation for raised serum vitamin B12 levels in alcoholic liver disease subjects is release of vitamin B12 from hepatic stores (Chanarin, 1982). It was expected that an increase in serum vitamin B12 levels would be accompanied by alteration of levels of serum vitamin B12 binding proteins and the object of this study, the results of which are presented in section 3.1.6. was to characterise these changes.

There appear to be three main types of vitamin B12 binding protein in serum, known as transcobalamins I, II and III (TC I, II & III) although some authors prefer the classification of TC II and R binders (TC I & III), (England et al, 1973). As the physiological role of each of these vitamin B12 binding proteins has not been established and since the main functional distinction

appears to be between TCII and TC I & III, this classification is adopted here. The prime role of TC II in vitamin B12 transport is illustrated by the appearance of severe megaloblastic anaemia in infants lacking this protein (Hakami et al, 1971). Neither the role nor the source of TC I and III have been established although England (1973) considers that all the transcobalamins are involved in vitamin B12 transport and the association of high TC I levels with disorders of granulocyte proliferation suggests that bone marrow is a major source (Herbert, 1968).

The physiological significance of the raised TC I and III levels in alcoholic liver disease subjects observed in this study (Table 5) is difficult to assess. A possible explanation is that the higher levels of TC I and III in serum from these subjects is indicative of release of vitamin B 12 binding proteins from damaged liver tissue which are, or behave similarly to TC I and III. However, Retief Vandenplas and Visser (1969), do not report increased unsaturated TC I levels in liver disease. Therefore, further studies are required to confirm the existence of raised TC I and III levels in alcoholic liver disease subjects, to establish the identity of these proteins and to assess their physiological significance.

4.1.5. Liver Cell Suspension and Vitamin B6 Deficient Rat Studies

The background and objectives of these studies are described in sections 1.3.3. and 1.4.3. The methods used are described in sections 2.2.9., 2.2.10 and 2.2.11. and the practical difficulties which were encountered and which prevented satisfactory results being obtained are described in section 3.1.8.

Section 4.2.

Preliminary PHA Stimulated Lymphocyte Studies

4.2.1. Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

The results of determination of acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes at different times after initiation of culture (Fig. 9), show that for the first 24 h there is little incorporation. The rate of increase of incorporation then increases to a maxima between 48 and 72 h and subsequently declines. This pattern of (^{125}I)-UdR incorporation is similar to the pattern of (^3H)-TdR incorporation observed by Cooper, Barkham and Hale (1963).

The results presented in Table 7 show the sensitivity of acid-insoluble (^{125}I)-UdR incorporation in PHA stimulated lymphocyte cultures to variation of serum concentration and cell number. It is apparent that increasing the serum concentration from 10 to 20% results in a marked increase in (^{125}I)-UdR incorporation. This indicates that serum concentration may be a limiting factor in PHA stimulation and suggests a possible approach to discrimination between serum of different subjects. Increasing the cell concentration from 2.5×10^5 cells to 1.0×10^6 cells also results in an increase in (^{125}I)-UdR incorporation though the percentage increase in (^{125}I)-UdR incorporation is smaller than the percentage increase in cell number. A further increase in cell number, to 2.0×10^6 cells, results in a decrease in (^{125}I)-UdR to a level comparable to that where the cell concentration is 2.5×10^5 cells.

4.2.2. Inter-individual Variation in Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes

Inter-individual variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures of control subjects is shown in Table 8 and of alcoholic liver disease subjects in Table 9. It is apparent that in both alcoholic liver disease and control subjects there is considerable variation in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures. Such variation has also been observed by other authors, e.g., Das and Herbert (1978) report a range of (^{125}I)-UdR incorporation in PHA stimulated lymphocyte cultures of seven folate/vitamin B12 deficient subjects of 9251 cpm to 46958 cpm and Metz et al (1968) report results in which the range of (^3H) TdR incorporation in bone marrow cells of five normal subjects was 4200 cpm to 45,500 cpm and in five vitamin B12 deficient subjects 9710 cpm to 83,680 cpm. The results of comparison of serum enzyme levels and other indicators of liver damage with (^{125}I)-UdR incorporation in PHA stimulated lymphocyte cultures of alcoholic liver disease subjects suggests that there is no simple relationship between (^{125}I)-UdR incorporation and the extent of liver damage.

Since similar numbers of cells are used in cultures of lymphocytes of different subjects, the variable incorporation of (^{125}I)-UdR would also be apparent if the results were expressed in terms of (^{125}I)-UdR per unit (ug) DNA of the cultured cells. This is because incorporation of radiolabelled nucleotide precursor

depends on the proportion of DNA synthesising cells in the lymphocyte culture which is known to vary considerably in different normal subjects and patients with disturbed haemopoiesis (Ling and Kay, 1975). Because of this variability, relating the amount of incorporation of (^{125}I)-UdR to the total number of cells in the cultures or the DNA content may be misleading. However, the comparison of the uptake of (^{125}I)-UdR may be made more meaningful by providing an internal control in the cell culture from each individual subject and expressing the experimental result relative to that of the control (Das, Manusellis and Herbert, 1980). This was the approach adopted in this study.

4.2.3. Relationship between Serum PLP Concentration and Acid-insoluble (^{125}I)-UdR Incorporation into PHA Stimulated Lymphocytes of Alcoholic Liver Disease Subjects

A principal objective of the PHA stimulated lymphocyte studies was to develop a functional assay of vitamin B6 status independent of reliance on determination of serum levels of vitamin B6. The commonly used methods for the determination of serum PLP, e.g., using tyrosine apodecarboxylase, have been criticised because of incomplete extraction of PLP from serum proteins (Srivastava and Beutler, 1973), and it is also possible that abnormal release of vitamin B6 and vitamin B6 dependent enzymes from damaged liver tissue, raised serum alkaline phosphatase levels and altered serum albumin levels could affect serum PLP levels and result in a false estimate of vitamin B6 status.

Nevertheless, it was of interest to compare the results of determinations of serum PLP concentrations with acid-insoluble incorporation of (^{125}I)-UdR in PHA stimulated lymphocyte cultures of alcoholic liver disease subjects. However, it is apparent from the analysis of such a comparison (Table 10) that there is no simple relationship between serum PLP concentration and acid-insoluble (^{125}I)-UdR incorporation. Other problems inherent in such a comparison are the possible effects of medium pyridoxine, the presumed folate sparing effect of a vitamin B6 deficiency and consequent increase in (^{125}I)-UdR incorporation and the possibility that reliance on a simple measurement of (^{125}I)-UdR incorporation, without reference to an internal control may be misleading.

4.2.4. Absence of PHA Stimulated Lymphocyte Response in Advanced Alcoholic Cirrhosis

The absence of a PHA stimulated lymphocyte response in a patient with advanced alcoholic cirrhosis is reported in section 3.2.6. Such cases have been documented by other authors, e.g., Hsu and Leevy (1971); Young, Dudley and Van der Weyden, (1979). Hsu and Leevy (1971) considered that this effect might result from toxic effects of unmetabolised products, abnormal protein produced by the diseased liver, the increase in immunoregulatory humoral factor in response to stimuli such as hypergammaglobulinaemia or inactivation of PHA by anti-PHA proteins in the cirrhotic serum. Young, et al (1979) presented evidence that the suppression of lymphocyte transformation was not due to nutrient deficiencies in the serum, increased serum thymidine phosphorylase levels or interference with lymphocyte-mitogen interaction. Young, et al (1979) also reported that inhibition was most marked in serum from patients with alcoholic hepatitis and that it correlated with bilirubin and AST levels. These observations suggest that hepatocyte damage is the principal determinant of serum inhibition of the PHA stimulated lymphocyte response and that the in-vivo significance of this phenomenon may be related to the observed impaired immunological response in alcoholic cirrhosis (Berenyi, Straus and Cruz, 1974).

4.2.5. Effect of Culture in a Pyridoxine Deficient
culture medium on Acid-insoluble (^{125}I)-UdR
Incorporation into PHA Stimulated Lymphocytes

As a result of the experiments reported in section 3.2.7. it was concluded that the observed effects of culture in modified MEM (1) could not be attributed to the absence of pyridoxine in this medium and were more likely to be due to some non-specific medium variable. All PHA stimulated experiments reported in other sections used complete MEM, modified MEM (2) or basal BSS medium and where necessary included appropriate controls for the effect of different media on the PHA stimulated lymphocyte response.

4.2.6. Effect of Ethanol on Acid-insoluble (^{125}I)-UdR
Incorporation into PHA Stimulated Lymphocytes

When the effect of ethanol on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes was investigated, Section 3.2.8., Fig. 10, it was found that whether ethanol was added at the start of culture or 1 h prior to determination of (^{125}I)-UdR incorporation, concentrations of absolute ethanol up to 1% v/v had little effect whereas a concentration of 5% v/v had a significant inhibitory effect, reducing (^{125}I)-UdR to less than 10% of that in comparable control cultures. This inhibitory effect of ethanol on (^{125}I)-UdR incorporation in lymphocytes may be compared with the cytotoxic effect of ethanol reported by Walker et al (1974). These workers reported that after a period of

3 days' exposure to different concentrations of ethanol, a significant cytotoxic effect on fibroblast and liver cells in stationary culture was apparent at an ethanol concentration of 0.25% v/v. At a 1% v/v ethanol concentration, the percentage of surviving liver cells was 75% and of fibroblast cells, 90%. Other acute effects of ethanol have also been investigated, e.g., Parker et al (1979) reported that acute high doses of ethanol in dogs, (3g ethanol/kg body weight) lowered plasma PLP. While it is not possible to extrapolate these results to the in-vivo clinical situation, it is apparent that in addition to the long term metabolic effects of ethanol ingestion, it is possible that there are significant acute effects on plasma PLP, cell viability and DNA synthesis.

Section 4. 3.

Development of a Functional Assay for
Determination of Vitamin B6 Status in
Alcoholic Subjects

4.3.1. Effect of Variation of the Medium Folate Concentration

In view of the demonstrable effect of variation of the medium folate concentration on the relative contributions of the de novo and salvage pathways to total thymidylate synthesis, it is surprising that little cognisance of this fact is noted in many studies of DNA synthesis. For example, in a study of the adaptation of thymidine utilisation to changing rates of DNA synthesis in the cell cycle (Miller, et al, 1979), the culture medium used was MEM which has a folate concentration of 1.0 mg/l. Under such conditions most of the DNA-thymine is derived from the de novo pathway and it is unlikely that the physiological role of the salvage pathway can be properly assessed under such conditions.

Since this project is concerned with the relationship between vitamin B6 deficiency and inhibition of cell growth, and with the effect of vitamin B6 deficiency on de novo thymidylate synthesis, the influence of the medium folate concentration on certain aspects of DNA synthesis was of particular significance. In this study, folate status was varied by altering the medium folate concentration and the effect of such variation on the relative contribution of de novo thymidylate synthesis to DNA was determined by comparing rates of incorporation of (^{125}I)-UdR into PHA stimulated lymphocyte cultures in the presence and absence of Am or MTX. The basis of this method

has previously been discussed (3.3.1.) and a similar method has also been described by Miller et al (1979), and Siegers et al (1974).

The effects of variation of the medium folate concentration on parameters of (^{125}I)-UdR incorporation into PHA stimulated lymphocytes, as observed in this study, may be summarised as follows:

There is an inverse relationship between (^{125}I)-UdR incorporation into DNA and folate status, such that in low folate conditions, (0.01mg/l), incorporation is high and in high folate conditions (1.0mg/l), incorporation is low. This effect is apparent from the results illustrated in Figs. 12. and 13. Using Am or MTX to inhibit the de novo pathway (^{125}I)-UdR incorporation into DNA is similar in both high and low folate conditions, Fig. 12.

These results indicate that in high folate conditions, the contribution of the de novo thymidylate pathway to DNA synthesis is high relative to that in low folate conditions and that the rate of DNA synthesis in comparable PHA stimulated lymphocyte cultures is similar in both high and low folate conditions.

In studies of lymphocytes of megaloblastic anaemic subjects, due to folate deficiency, it was found that the incorporation of (^3H)-TdR into DNA was significantly greater than that of incorporation into lymphocytes of normal subjects (Das and Hoffbrand, 1970). Originally this was thought not to be

due to increased DNA synthesis in the folate deficient lymphocytes but later studies using (^3H)-TTP incorporation into isolated megaloblastic nuclei compared with similar nuclei treated with folate in vitro suggested that this increased (^3H)-TdR incorporation into megaloblastic PHA stimulated lymphocytes was due to increased DNA synthesis and not just the result of an increased specific radioactivity of the dTTP pool due to inhibition of de novo synthesis and an increase in thymidine kinase activity (Hooton and Hoffbrand, 1977).

However, in the conditions used in this study where the folate concentration is at least 0.01mg/l, folate deficiency is obviated and changes in (^{125}I)-UdR incorporation cannot be attributed to compensatory increases in DNA synthesis. Therefore, although variation of the folate concentration within the range 0.01 - 2mg/l, is likely to result in some degree of adaptation of the de novo and salvage thymidylate pathways to the folate status, such adaptation is not likely to invalidate the use of Am or MTX in assessing the relative contributions of the de novo and salvage pathways to DNA-thymine synthesis.

Interest in the effect of variation of folate status on parameters of (^{125}I)-UdR incorporation into DNA, stems from the possible parallels in the effect of vitamin B6 deficiency and folate deficiency on de novo thymidylate synthesis. As the effects of variation of folate status on (^{125}I)-UdR incorporation are

clearly demonstrable, such effects, attributable to variation in vitamin B6 status, would provide clear evidence of the functional effect of vitamin B6 deficiency and also provide a means of assessing vitamin B6 status.

If a vitamin B6 deficiency occurs, but not at the level of a reduction in the de novo contribution to DNA-thymine, then the relative contributions of the de novo and salvage pathways to DNA thymine will not alter but a reduction in total DNA synthesis would be expected. The effect of variation of the medium folate concentration on this reduction would depend on the extent to which folate status either ameliorated or exacerbated the effect of vitamin B6 deficiency.

4.3.2. Use of the UdR Suppression Test

The use of the UdR suppression test to assess folate and vitamin B6 status in PHA stimulated lymphocytes, its presumed basis and certain reservations concerning its validity have been discussed (1.4.5.). The rationale for the extension of this test to assessment of vitamin B6 status is that if a vitamin B6 deficiency exists, or is produced as a result of PHA stimulation of lymphocytes in a pyridoxal deplete culture medium and if the primary effect of a vitamin B6 deficiency is to reduce the active one-carbon folate pool, then similar changes to those effected by culture in folate deficient conditions will be observed.

The results shown in Fig. 14 and Tables 12, 13, 14 and 15 demonstrate the basic phenomenon that incorporation of labelled thymidine (or an analogue) is reduced by prior treatment of cultures with UdR. However, these results do not indicate that there is a functionally significant vitamin B6 dependent difference between lymphocytes of control and alcoholic subjects. Furthermore, there is no apparent trend in the results which suggests such a difference exists. A variety of conditions including use of medium of high or low folate concentration with or without pyridoxal were used in these experiments.

The magnitude of the differences observed between normal and folate deficient subjects in UdR suppression tests is of the order of 30% (Wickramasinghe and Longland, 1974; Das and Herbert, 1978). The magnitude of the differences observed between serum PLP levels of normal and vitamin B6 deficient subjects is also of the order of 30% (Lumeng and Li, 1974; Labadarios et al, 1977; Bonjour, 1980). Differences of such magnitude were not observed in the present study. Because of the inter-individual variability observed in the present study, determination of significant differences of a smaller size would require study of larger groups.

Reduction of (^{125}I)-UdR incorporation by pre-incubation with UdR is consistent with the hypothesis that exogenous non-radioactive UdR leads to the intracellular formation of non-radioactive dTMP which dilutes the pool of radioactive dTMP (or (^{125}I)-dUMP)

arising from TdR (or (^{125}I)-UdR) and reduces labelling of DNA. The absence of a reduced UdR suppression effect in the lymphocyte cultures of the alcoholic subjects suggests that conversion of dUMP to dTMP is not reduced in lymphocyte cultures of these subjects. This indicates that these subjects are not vitamin B6 or folate deficient. However, alternative hypotheses are also consistent with these results and for this reason experiments were carried out to further characterise this effect (4.4.2.).

Other investigations have also examined the UdR suppression test in alcoholic liver disease though not specifically with regard to vitamin B6 deficiency. These studies also reported negative or inconclusive results, e.g., in a study of 17 alcoholic liver disease subjects with macrocytosis, results of UdR suppression tests could not be related to folate status as some had a normal UdR suppression test and low red cell folate status while others exhibited the reverse (Belaiche, Cattani and Zittoun, 1978). In a study of 16 alcoholic or chronic liver disease subjects, UdR suppression tests on bone marrow aspirates were all normal despite macrocytosis being present in each case (Wickramasinghe and Longland, 1974). Part of the reason for the apparent inconsistencies in these results may be the existence of indirect folate deficiency in primarily iron deficient subjects who despite apparently normal

folate status exhibited abnormal Udr suppression results (Das et al, 1978). Das and Herbert (1978) consider that the persistence of an abnormal Udr suppression result in a group of patients with past deficiency of folate and vitamin B12, up to 84 days after vitamin therapy, suggests that circulating unstimulated lymphocytes do not incorporate appreciable amounts of vitamin B12 or folate and therefore that results of studies on such lymphocytes reflect the vitamin status of the patient when the lymphocytes were generated. One consequence of this is that the recent history of the subject is of less importance than the underlying disease. It has been suggested that the existence of macrocytosis and megaloblastosis reversible by alcohol withdrawal but not by treatment with folate demonstrates that these effects result from the direct action of alcohol on the developing haematopoietic cells and not, in the first instance, to accompanying folate deficiency (Sullivan and Herbert, 1964; Wu et al, 1975). It is possible therefore that reports of vitamin B6 deficiency dependent changes are attributable to the effects of alcohol and liver disease rather than on a priori vitamin B6 deficiency.

4.3.3. Effect of Pyridoxal Deficient Medium

The most direct approach to determination of the cellular requirement for a particular nutrient, and the effects of a specific deficiency, is to culture cells in deficient medium and to observe the subsequent effects on specific areas of cellular metabolism. This was the approach adopted in this study.

The results shown in Fig. 15 suggest that the absence of pyridoxal from the culture medium has little effect on (^{125}I)-UdR incorporation during 96h PHA stimulated lymphocyte culture. The pattern of (^{125}I)-UdR incorporation in both low folate and high folate medium is similar to that shown in Fig. 13. The results do not suggest that the absence of pyridoxal increases salvage thymidylate incorporation. Such an effect was observed in low folate conditions. Therefore, there was no evidence that variation of medium pyridoxal concentration affected the relative contribution of the de novo and salvage pathways to total thymidylate synthesis. Furthermore, there was no evidence that high folate conditions potentiated the effects of vitamin B6 deficiency resulting from lack of medium pyridoxal. When the effect of culture in medium without pyridoxal was compared in lymphocytes of control and alcoholic subjects, Table 20, it was found that there was no statistically significant differences between these two groups of subjects in their sensitivity to such culture conditions.

Several factors may account for this result. If the test system is valid, the most direct conclusion is that alcoholic subjects are not vitamin B6 deficient relative to normal subjects. However, before such a conclusion can be accepted, several other possible explanations must be investigated. One possibility is that the conditions are insufficiently exacting to result in vitamin B6 deficiency and so discriminate between cultures of subjects differing in vitamin B6 status. For example, the presence of MEM amino-acids may decrease the requirement for PLP dependent activities such as decarboxylation and transamination and therefore the requirement for medium pyridoxal to sustain normal DNA synthesis. Other possibilities are that a longer period than 72h is required to produce vitamin B6 deficiency or that PLP is more tightly bound to PLP dependent enzymes involved in DNA synthesis than to other PLP dependent enzymes. Such variation in the effect of absence of pyridoxine from cell culture medium on the activity of vitamin B6 dependent enzymes has been observed by Lipson et al (1980). These investigators found that activity of pyridoxine kinase remained constant during all depletion and repletion experiments while that of aspartate aminotransferase fell to a mean of 50% of control values.

As the results of experiments with lymphocytes cultured in various media with or without pyridoxal did not demonstrate an effect of vitamin B6 status on acid-insoluble incorporation of radioactively

labelled DNA precursor, it was decided to investigate the effect of a vitamin B6 antagonist. It was felt that this approach would increase the sensitivity of the model system, facilitate the detection of a difference in functional vitamin B6 status if it existed and reduce the possible confounding effect of the relatively short culture period. Another factor which determined that these studies be extended at this stage, rather than consolidated, was the limited availability of suitable alcoholic liver disease subjects for inclusion in the study. This also determined, through the study, that a large number of observations and investigations be carried out on a relatively small group of subjects.

4.3.4. Effect of 4-dPN

Use of pyridoxal deficient medium did not, during 96h PHA stimulated lymphocyte culture, have a significant effect on DNA synthesis as determined by (^{125}I)-UdR incorporation. Nor did use of such media discriminate between lymphocytes of control and possibly vitamin B6 deficient alcoholic subjects. To exclude the possibility that this lack of difference was due to insensitivity of the test and also to assess the effect of vitamin B6 deficiency on DNA synthesis and cell growth, the use of more exacting vitamin B6 requiring conditions was investigated. The method adopted to attain such conditions was use of the vitamin B6 antagonist 4-deoxypyridoxine. The effect of this antagonist was investigated in two ways.

Firstly, by addition of 4-dPN to cultures 1h prior to determination of (^{125}I)-UdR incorporation, and secondly, by addition of 4-dPN to cell culture medium at start of culture.

In Table 18, the sensitivity of (^{125}I)-UdR incorporation, in the presence of BSS and Am to 4-deoxypyridoxine was determined in stimulated lymphocytes of control and alcoholic liver disease subjects cultured in low folate and low pyridoxal conditions. The extent of suppression of (^{125}I)-UdR incorporation by UdR was also determined.

In the sample group of control subjects, 4-dPN did not reduce either salvage incorporation ((^{125}I)-UdR incorporation in the presence of BSS) or total DNA synthesis ((^{125}I)-UdR incorporation in the presence of Am). However, in the sample group of alcoholic liver disease subjects, 4-dPN resulted in a significant reduction of salvage incorporation but not of total DNA synthesis. This result seems difficult to account for. If 4-dPN caused a vitamin B6 deficiency at the level of de novo thymidylate synthesis one would expect a relative increase in salvage incorporation comparable to that observed in low folate conditions, and total DNA synthesis would be either reduced or unaffected. If 4-dPN caused a vitamin B6 deficiency which did not specifically limit serine hydroxymethyltransferase activity but did result in a reduction of cell growth, and DNA synthesis, then one would expect a decrease in both salvage incorporation and total DNA synthesis. This is perhaps the most likely explanation for the results

reported here as there is a significant decrease in salvage incorporation (24%) and an apparent (14%) decrease in total DNA synthesis. Udr suppression was also determined and again no significant difference was found between lymphocyte cultures of normal and alcoholic liver disease subjects in this respect. A tentative conclusion therefore, is that there is a significant difference in the sensitivity of lymphocyte cultures of control and alcoholic liver disease subjects to variation in vitamin B6 supply. However, the sample size is small and such a result requires confirmation before a firm conclusion can be stated.

To further characterise the effect of pre-incubation addition of 4-dPN, this experiment was repeated in medium of high or low folate and high or low pyridoxal concentration in PHA stimulated lymphocyte cultures of control subjects (Table 19). It was found that in low folate medium (0.01mg/l), irrespective of the pyridoxal concentration (0.01mg/l of 2.0 mg.l), pre-incubation addition of 4-dPN did not significantly affect either salvage incorporation or total DNA synthesis.

However, when the folate concentration was increased to 2.0mg/l, it was found that 4-dPN significantly reduced salvage incorporation ($P < 0.01$) but had no significant effect on total DNA synthesis. Again, this result seems difficult to interpret. With regard to the earlier discussion in this section, of the likely effect on (^{125}I)-Udr incorporation of a vitamin B6 deficiency inhibiting de novo thymidylate synthesis,

and the high medium folate conditions (2.0mg/l), it is unlikely that the effect of 4-dPN seen here is due to reduced serine hydroxymethyltransferase activity. Furthermore, there is no significant reduction in total DNA synthesis. This result indicates that in high folate conditions, 4-dPN specifically reduces salvage incorporation. The Udr suppression effect was also determined in this experiment. It was found that in low folate conditions, irrespective of the pyridoxal concentration, Udr reduced salvage incorporation to 20% of that in control conditions, while in high folate conditions (2.0mg/l) Udr reduced salvage incorporation to 3% of that in control cultures. While the enhanced Udr suppression effect in high folate conditions is consistent with the conventional rationale for Udr suppression (1.4.5.), the specific sensitivity of salvage incorporation to the effect of 4-dPN in high folate conditions is less readily explained.

As total DNA synthesis, (^{125}I)-Udr incorporation in the presence of Am, is unaffected, this effect cannot be attributed to a specific effect on thymidine kinase activity, and this implies that in high folate conditions, 4-dPN actually increases the de novo contribution to thymidylate synthesis, resulting in a reduction of salvage incorporation. A rationale for this apparently anomalous result might be that excess folate corrects a vitamin B6 deficiency in one area of metabolism, thereby exacerbating its effects on another area of metabolism. This

explanation implies that high folate conditions potentiate the effect of vitamin B6 deficiency as induced by 4-dPN. An alternative explanation however, is that in high folate conditions, salvage incorporation is very much reduced relative to that in low folate conditions and this factor alone enhances the sensitivity of such incorporation to reduction by UdR or 4-dPN.

The effect of culture in medium containing 4-dPN on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes at various times during the course of culture is shown in Fig. 16. These results show that culture in medium containing 4-dPN results in a significant reduction of DNA synthesis in PHA stimulated lymphocytes. There is no evidence of a differential effect on incorporation after pre-incubation with BSS compared with incorporation after pre-incubation with MTX and this indicates that vitamin B6 deficiency, as induced by 4-dPN, although reducing total DNA synthesis does not affect the respective contributions of the de novo and salvage pathways to thymidylate synthesis. However, the medium folate concentration in this experiment was 1.0 mg/l and this might mask the effect of a vitamin B6 deficiency operating at such a level.

Therefore, in contrast to use of pyridoxal deficient medium and pre-incubation addition of 4-dPN, the inclusion of 4-dPN in the initial culture medium does result in a significant reduction in subsequent DNA synthesis, which, subject to the qualifications

regarding the specificity of inhibitors, may be attributed to the effects of an induced vitamin B6 deficiency. Attainment of such conditions offered a further approach to assessment of relative vitamin B6 status in different population groups. The basis of this approach was that sensitivity of PHA stimulated lymphocyte cultures derived from different subjects, to 4-dPN, would vary according to the cellular and serum vitamin B6 status of the donor. The results of an investigation of this possibility are presented in Table 21. The cell culture medium used was modified MEM of low folate concentration, with or without pyridoxal. Acid-insoluble (^{125}I)-UdR incorporation was determined at 72h after pre-incubation with either BSS, MTX or 5-fu, in PHA stimulated lymphocyte cultures of both control and alcoholic liver disease subjects. Low folate medium was used to avoid the possible effect of excess folate correcting a vitamin B6 deficiency at the level of serine hydroxymethyltransferase activity. 5-fu was used because of its inhibitory action on thymidylate synthetase. Such inhibition has a similar effect to that of antifolates in 'uncoupling' de novo thymidylate synthesis and thus permitting discrimination between de novo and salvage thymidylate synthesis. 5-fu is converted to the nucleotide 5-fdUMP which is the active inhibitor of thymidylate synthesis. As the fluorine atom is similar to that of hydrogen, 5-fdUMP competes with dUMP but the 5-position cannot be methylated and thus de novo thymidylate synthesis is inhibited.

Although 5-fdUMP does not become incorporated into DNA, the corresponding riboside is incorporated into RNA, disrupting the activity of this macromolecule. 5-fdUMP also inhibits orotic acid metabolism and the incorporation of phosphate into DNA (Hurst, 1980). In addition to these multiple biochemical effects, it is likely that in its conversion to an active inhibitor, 5-fu will compete with TdR, or an analogue of TdR, for uptake and phosphorylation. These considerations preclude the use of 5-fu in precise quantitative studies of DNA synthesis. However, its major effect, particularly in short term studies is inhibition of thymidylate synthetase and therefore its use in these circumstances is justifiable.

Although the results in Table 21 show considerable variation, it is possible to derive some valid conclusions from them. Firstly, it is apparent that in PHA stimulated lymphocyte cultures derived from either control or alcoholic liver disease subjects, inclusion of 4-dPN in the initial culture medium results in a significant reduction of DNA synthesis, and hence, cell growth. However, there is no consistent pattern of significant differences between the extent of this reduction, in cultures derived from control or alcoholic liver disease subjects. Similarly, the presence or absence of pyridoxal in the culture medium does not appear to significantly influence the effect of 4-dPN, and even in low folate conditions, it is apparent that vitamin B6 deficiency, as induced by 4-dPN, does not have a differential

effect on de novo and salvage thymidylate synthesis comparable to that of folate deficiency.

The validity of these conclusions is strengthened by analysis of the results presented in Table 22. These results, obtained using lymphocytes cultured in high folate medium, confirm the limited influence of medium pyridoxal in reducing the effect of 4-dPN and the lack of a differential effect on de novo and salvage thymidylate synthesis. The influence of the medium pyridoxal concentration on the effect of 4-dPN on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures derived from control subjects was further investigated (Fig. 17). It was apparent from the results shown in Tables 21 and 22 that pyridoxal at a concentration of 1.0mg/l had little influence on the effect of 4-dPN and this lack of influence is also apparent from Fig. 17 where an increase in the pyridoxal concentration to 10mg/l is also shown to be ineffective in modulating the effect of 4-dPN.

A possible reason for the apparent lack of differential effect of 4-dPN on de novo and salvage thymidylate synthesis has been described by Coburn and Mahuren (1976). In a study of the in-vivo metabolism of 4-dPN in rat and man these authors reported that in contrast to in-vitro studies where conversion of 4-dPN to 4dPN-phosphate resulted in competitive inhibition of PLP enzymes, this did not appear to take place in-vivo, nor were PLP concentrations reduced. The authors suggest that under some conditions the main effect of deoxypyridoxine may

be inhibition of vitamin B6 transport. Subsequently Coburn et al (1980) presented further evidence that the in-vivo effect of 4-dPN may involve more than simple competitive inhibition of PLP enzymes. The authors suggest that inhibition of PLP enzymes was very selective and that vitamin B6 may exert regulatory effects independently of its enzyme cofactor role.

4.3.5. Effect of Variation of Cell Culture Medium Composition

It had been noted (section 3.2.2.) that increasing the proportion of serum in the cell culture medium resulted in an increase in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes. This observation suggested that it might be possible, by altering the medium composition, to characterise a medium which could be used to assess the nutritional status of serum and lymphocytes of alcoholic liver disease subjects (4.2.1.). The results shown in Fig. 18 indicate that when a 15% serum concentration is used, there is little difference between acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures of two alcoholic liver disease subjects cultured in either modified MEM (2) or basal BSS medium. Similarly, the results shown in Fig. 34 indicate that the medium presence of non-essential amino acids has little effect on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocyte cultures. However, when the serum concentration used is 10%, the addition of MEM amino acids to a basal BSS medium results in a significant increase in acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures.

These results are illustrated in Fig. 19. It is also apparent from Fig. 19 that a similar pattern of (^{125}I)-UdR incorporation is observed in both the alcoholic liver disease and control subjects studied. The effect of addition of MEM vitamins to the basal BSS medium on (^{125}I)-UdR incorporation is much less than the effect of addition of amino acids. However, it is interesting to note that in the BSS plus MEM amino acid medium (^{125}I)-UdR incorporation (after pre-incubation with BSS) is higher than in the BSS plus MEM vitamins/amino acids medium. This observation is consistent with the effect of folate concentration of (^{125}I)-UdR incorporation observed in other experiments, e.g., Fig. 12.

These experiments illustrate certain aspects of the effect of serum concentration and medium composition on (^{125}I)-UdR incorporation into PHA stimulated lymphocyte cultures. Although no discriminatory effect of culture of lymphocytes of different subjects in different media was apparent in the results presented here, it is feasible that in nutritionally deficient subjects, use of such selective media could be used to assess nutritional status. However, in these studies, the principal determinants of (^{125}I)-UdR incorporation were the serum concentration and the presence of MEM amino acids. As such studies could not be considered to assess vitamin B6 status specifically, they were not pursued in the present study.

Section 4.4.

Characterisation of the Effects of
Vitamin B6 Antagonists on PHA Stimulation
of Lymphocytes

4.4.1. Use of Vitamin B6 Antagonists

A large number of substances as well as several useful drugs react with PLP requiring enzymes. For example, one of the drugs used in the treatment of tuberculosis, isonicotinic acid hydrazide (isoniazid) inhibits pyridoxal kinase, the enzyme that converts pyridoxal to PLP. Inhibition is believed to be mediated by the reaction of the drug and pyridoxal to form a hydrazone which blocks the enzyme. The rationale of the therapeutic use of this drug is that since the content of pyridoxal kinase in mycobacteria is low, its blockage effectively inhibits growth. However, patients on long-term isoniazid therapy sometimes develop symptoms of vitamin B6 deficiency.

Phosphorylation of 4-deoxypyridoxine by pyridoxal kinase is thought to produce an antagonistic analogue of PLP and administration of this substance induces death in chicks, and, during tests in man of the potential anti-cancer effects of 4-dPN, it was shown to cause convulsions and other symptoms of vitamin B6 deficiency. Other substances with known vitamin B6 antagonist activity include cycloserine, the L-isomer of which inhibits many PLP enzymes and is toxic to humans and, the substituted cysteine derivative, L-penicillamine which causes convulsions and low glutamate decarboxylase levels in the brain (Metzler, 1977).

From a consideration of the known involvement of PLP in enzyme activity in a variety of metabolic systems it can be appreciated that a deficiency of this coenzyme might result in a metabolic block in a number of areas

of metabolism. In the whole animal this will result in a variety of effects such as anaemia, nervous system dysfunction and skin complaints. It is unlikely that these symptoms can be attributed to a unique enzyme lesion but it is possible that one effect is associated with a limiting metabolic block which when relieved would alleviate the most serious defect.

Various studies in man and other animals have looked at the effects of various vitamin B6 antagonists on a number of areas of metabolism. For example: Carbidopa, which is a hydrazine derivative of the amino acid L-dopa, selectively inhibits the enzyme aromatic L-amino acid decarboxylase in peripheral tissues, and this makes it a useful adjunct in the treatment of Parkinson's disease. However, studies in rats suggest that large doses of carbidopa can, by depleting tissues of PLP, cause potentially undesirable non-specific changes in PLP dependent enzymes, though coadministration of pyridoxine with carbidopa can maintain tissue PLP levels and protect against such enzyme changes without reducing the effectiveness of carbidopa (Airoldi, et al, 1978).

Sideroblastic, pyridoxine responsive, anaemia, associated with alcoholism and isoniazid (INH) therapy has been attributed to a block in heme synthesis. In cell culture studies using murine erythroleukemia cells (MELC) and mouse fetal liver cells (MFLC), treatment with hemin overcome the inhibitory effect of INH and thus the vitamin B6 antagonist effect in

this case could be primarily attributed to inhibition of delta-aminolaevulinic acid synthetase (Hoffman & Ross, 1980).

In studies of the effect of acute alcohol ingestion on plasma PLP in dogs it was demonstrated that acute high doses of alcohol lower plasma PLP accumulation from precursor pyridoxine (Parker et al, 1979).

Although it is not possible to extrapolate this result to the human circumstances of long-term alcohol intake, it is apparent that alcohol, or a metabolite of alcohol, may have a specific effect on PLP dependent enzymes as well as a more direct effect on haematopoiesis (Wu et al, 1975; Sullivan and Herbert, 1964).

These studies illustrate some of the factors which determine the effects of alcohol and vitamin B6 deficiency on cellular metabolism. This complexity is further illustrated by the description of cases where impairment in reticulocyte globin synthesis, due to alcohol, was correctable by addition of haem and by other cases where abnormal haem synthesis was corrected by pyridoxine but not by delta-aminolaevulinic acid (Solomon and Hillman, 1979 a). Thus alcohol may well impair haem synthesis by a direct toxic effect only correctable by alcohol withdrawal, by other effects correctable by administration of pyridoxine and by a specific effect requiring treatment with the appropriate vitamin B6 dependent metabolite.

In this study, the incidence of vitamin B6 deficiency in alcoholic liver disease subjects as well as the effect of vitamin B6 deficiency on cellular metabolism

has been investigated using the vitamin B6 antagonist 4'-deoxypyridoxine. In view of the various effects of different vitamin B6 antagonists, it was of interest to compare the effect of 4-dPN with that of another vitamin B6 antagonist, INH. The results of such a comparison are shown in Fig. 20. These results suggest that the overall effect of each of these vitamin B6 antagonists on DNA synthesis in PHA stimulated lymphocyte cultures of control subjects is similar, and that neither has a differential effect on salvage or de novo thymidylate synthesis.

These results also indicate that the use of vitamin B6 antagonists represents a useful cell culture model system for investigating vitamin B6 deficiency.

From the effect of 4-dPN and INH on DNA synthesis (acid-insoluble (^{125}I)-UdR incorporation) in PHA stimulated lymphocyte cultures it is clear that cells cultured in such conditions have impaired ability to function and replicate and so it would be of interest to determine whether the biochemical abnormalities of such chronically exposed cells could be reversed by addition of exogenous hemin or other vitamin B6 dependent metabolite.

4.4.2. Characterisation of UdR Suppression

In view of the reported value of the UdR suppression test in determining folate and vitamin B12 status

(Wickramasinghe and Longland, 1974), its potential use in determining vitamin B6 status and also because of the uncertainty surrounding its biochemical basis (Beck, 1975), it was of interest to further

characterise this test. The approach adopted was

to determine the effect of UdR on (^{125}I)-UdR

3-(^{14}C)-serine and (^{14}C)-formate incorporation into

PHA stimulated lymphocyte cultured in medium of high or low folate concentration after pre-incubation with BSS, MTX or 5-fu. Such an approach allows assessment of UdR suppression in conditions of different folate status, i.e. In low folate medium (0.01 mg/l), the folate status is comparable to that in serum and in high folate medium (1.0 mg/l), folate is in excess of normal physiological levels (Lundquist, 1975).

Treatment with MTX may be considered to induce folate deficient conditions. In cell cultures treated with 5-fu the de novo thymidylate pathway is inhibited at the level of thymidylate synthetase and pre-pre-incubation with BSS represents a control of normal UdR suppression.

In all the experiments discussed in this section it is realised that competitive and metabolic relationships are not capable of precise interpretation when applied to such a complex system as the living cell, where the interrelation of metabolite and inhibitor can be affected by transport phenomena at membranes, pool size, equilibria

between products of consecutive reactions and so on. Nevertheless, it is possible to deduce certain qualitative information from analysis of such experiments.

Prior to determination of the effect of UdR, the effect of pre-incubation with MTX and 5-fu on subsequent acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes was determined. These results are illustrated in Fig. 21 and Table 23 and are consistent with the hypothesis that differences in (^{125}I)-UdR incorporation into PHA stimulated lymphocytes after pre-incubation with MTX and 5-fu reflect changes in the size of the active one-carbon folate pool available for de novo thymidylate synthesis. The results presented in Fig. 21 show that the difference between acid-insoluble (^{125}I)-UdR incorporation after pre-incubation with MTX and 5-fu is much greater in high folate conditions than in low folate conditions. This is consistent with a greater one-carbon folate pool in high folate conditions.

If this principle was established it would provide an indirect, but functional, means of estimating the relative size of the active one-carbon folate pool under different conditions. These results indicate that the size of an active one-carbon folate pool is affected by vitamin B6 deficiency, as induced by 4-dPN, and by varying the medium folate condition. As 4-dPN has not been shown to have a specific effect on de novo thymidylate synthesis in other experiments reported in this study, it is unlikely that the apparent effect of 4-dPN on the active one-carbon folate pool observed

here is due to specific inhibition of serine hydroxymethyltransferase. However, substantiation of this hypothesis would require further characterisation of various aspects of the observations. In particular it would be necessary to determine changes in this parameter throughout the course of PHA stimulated lymphocyte culture and to exclude the influence of indirect effects of both inhibitors. It would also be important to ensure that the level of each inhibitor present was such that each target enzyme was inhibited to a similar extent.

When the effect of UdR on acid-insoluble incorporation of (^{125}I)-UdR into PHA stimulated lymphocytes, cultured in medium of high or low folate concentration and in the presence or absence of 4-dPN, after pre-pre-incubation with MTX and 5-fu was considered (Table 24 and Figs. 22 and 23), it was apparent that the extent of UdR suppression was not significantly effected by the presence of MTX or 5-fu.

The principal conclusion from this result is that since the de novo thymidylate pathway is inhibited by the presence of either MTX or 5-fu, the major part of the UdR suppression effect must be mediated independently of the de novo pathway. This conclusion conflicts with the hypothesis that UdR suppression is mediated by feedback inhibition of thymidine kinase due to increased de novo synthesis of dTTP from dUMP (Herbert et al, 1973). It is therefore probable that the major part of the UdR suppression effect can be attributed to competitive substrate inhibition of thymidine kinase, the salvage pathway enzyme necessary for (^{125}I)-UdR

incorporation, by added UdR. Evidence for such a hypothesis can be adduced from the data of Cleaver (1967) concerning the substrate specificity of thymidine kinase. From this data it is apparent that UdR is 67% as effective as substrate of TK as is TdR, and therefore it is likely that this factor alone will account for significant suppression of (^{125}I)-UdR incorporation by added UdR. ((I)-UdR is 93% as effective a substrate of TK as is TdR.) Another observation which is inconsistent with UdR suppression being mediated by feedback inhibition of TK by dTTP is the finding that dTTP levels are not unequivocally raised in UdR treated, normal or folate deficient cells (Ganeshaguru and Hoffbrand, 1978).

To account for the observation that the UdR suppression effect is different in normal and folate deficient cells, and can therefore be used to discriminate between such cells, it is necessary to consider what other factors might affect the sensitivity of (^{125}I)-UdR incorporation to UdR. As it is known that (^{125}I)-UdR incorporation is dependent on TK, this question is primarily concerned with what factors might affect TK activity. From results of experiments discussed earlier in this report (e.g.4.3.1.) it is clear that a major factor affecting the relative contribution of the de novo and salvage pathways to thymidylate synthesis, and hence TK activity, is the medium folate concentration.

In low folate conditions TK activity($(^{125}\text{I})\text{-UdR}$ incorporation) is high, whereas in high folate conditions TK activity ($(^{125}\text{I})\text{-UdR}$ incorporation) is low, and in both high and low folate conditions, in the presence of MTX TK activity ($(^{125}\text{I})\text{-UdR}$ incorporation) is similar, (Fig. 12). As expected the effect of UdR is dependent on the level of $(^{125}\text{I})\text{-UdR}$ incorporation and where this is low, as in high folate conditions, UdR suppression will be more effective than in low folate conditions where $(^{125}\text{I})\text{-UdR}$ incorporation is high. In the results of experiments reported here e.g., Figs. 22 and 23, where the folate concentration is either 0.01 mg/l or 1.0 mg/l, the absolute level of $(^{125}\text{I})\text{-UdR}$ incorporation in the presence of UdR is higher in high folate conditions than in low folate conditions, though the percentage UdR suppression is of similar magnitude in both high and low, folate conditions.

This result, in common with the observed effect of MTX on $(^{125}\text{I})\text{-UdR}$ incorporation in high and low folate conditions indicates that TK activity is adapted to cellular folate status. If consideration of these circumstances is extended to folate deficient conditions, it is likely that the degree of adaptation to thymidine utilisation will be greater, that TK activity will be higher and that this would account for the reduced extent of UdR suppression in such conditions. Further evidence for such adaptation can be adduced from a report of a three fold increase in thymidine kinase

activity in extracts of peripheral blood lymphocytes of cobalamin or folate deficient patients (Ellims, Hayman and Van der Weyden, 1979).

Interest in characterisation of the UdR suppression test stemmed not only from its potential use in evaluating vitamin B6 status but also from the information it would provide concerning the possible effect of vitamin B6 deficiency on de novo thymidylate synthesis. It was postulated that such an effect would be mediated by inhibition of serine hydroxymethyltransferase with a consequent reduction of active one-carbon folate units available for de novo thymidylate synthesis.

However, as it does not appear that the UdR suppression effect is primarily mediated via thymidylate synthetase, it was not possible to use the UdR suppression effect to assess the effect of vitamin B6 deficiency on de novo thymidylate synthesis.

Determining the effect of pre-incubation addition of formate on acid-insoluble (^{125}I)-UdR incorporation into PHA stimulated lymphocytes (3.4.4.), offered an alternative means of assessing the role of the de novo thymidylate pathway. As 5,10-methylene tetrahydrofolate can be formed from formate and H_4F , independently of any PLP requiring enzyme (Blakley, 1969) it is likely that an exogenous supply of formate would relieve a restriction in 5,10 me H_4F availability due to a vitamin B6 deficiency. If a vitamin B6 deficiency did result in inhibition of serine hydroxymethyltransferase and this inhibition was relieved by addition of formate,

the expected result would be a reduction in (^{125}I)-UdR incorporation. The results of experiments directed at determination of such an effect are illustrated in Figs. 24, 25 and 26. These results indicate that formate does not significantly affect (^{125}I)-UdR incorporation in high or low folate conditions, in the presence or absence of 4-dPN, or after pre-preincubation with either BSS, MTX or 5-fu. These experiments therefore give further indication that vitamin B6 deficiency does not specifically inhibit 5,10 me H_4F synthesis from serine and H_4F .

The effect of UdR on incorporation of 3-(^{14}C)-serine and (^{14}C)-formate label into a DNA extract of PHA stimulated lymphocyte cultures is illustrated in Figs. 30, 31 and 33. These results indicate that incorporation of 3-(^{14}C)-serine is increased by pre-incubation with UdR in both high and low folate conditions, (Fig. 30) that incorporation of (^{14}C)-formate is also increased by pre-incubation with UdR in complete MEM (Fig. 33) and that this effect of UdR on 3-(^{14}C)-serine incorporation is inhibited by pre-preincubation with MTX or 5-fu (Fig. 31). It is apparent from these results that UdR increases incorporation of de novo thymidylate precursors into DNA but these experiments do not discriminate between such an effect being mediated by stimulation of de novo thymidylate synthesis or by inhibition of salvage thymidylate synthesis.

During this project a number of reports concerning the biochemical basis of the UdR suppression test have been published. One report (Van de Weyden, 1979), which recognises the competition of UdR and TdR as substrates for thymidine kinase but not the likely effect of adaptation to TdR utilisation or raised TK levels in folate deficient cells, concludes that; reduced deoxyuridylate conversion to (deoxy)-thymidylate by thymidylate synthetase accounts for the UdR suppression effect. A further report (Hayman and Van der Weyden, 1980) describes the use of PHA stimulated normal human peripheral blood lymphocytes cultured in folate depleted medium as an in vitro model for megaloblastic haematopoiesis. This was considered an appropriate system for analysis of biochemical events accompanying megaloblastic maturation and the biochemical basis of the UdR suppression test as it allowed a convenient means of obtaining deficient cells while avoiding the uncertain specificity involved in the use of metabolic inhibitors such as MTX or 5-fu. A study by Pelliniemi and Beck (1980) used PHA stimulated lymphocytes cultured in RPMI 1640 medium, which has a folate concentration of 1.0 mg/l, and MTX and 5-fUdR as inhibitors of thymidylate synthetase, to investigate the biochemical basis of the UdR suppression test. These authors also noted the significance of UdR as a competitive substrate inhibitor of TK and reports of raised levels of TK in megaloblastic cells and concluded that exogenous deoxyuridine has multiple

effects on (^3H)-TdR incorporation which must be considered in interpretation of deoxyuridine suppression test results.

4.4.3. Use of de novo Thymidylate Precursors

Interest in the use of de novo thymidylate precursors in PHA stimulated lymphocyte studies stemmed from the realisation that this would provide an alternative approach to assessing the effect of vitamin B6 deficiency on cell growth and DNA synthesis. This approach complements studies using (^{125}I)-UdR and also provides further information on aspects of de novo thymidylate synthesis.

In Figs. 27 and 28, the results of a determination of both acid-insoluble (^{125}I)-UdR incorporation, and 3-(^{14}C)-serine incorporation into a DNA extract, of PHA stimulated lymphocytes at various times during 96h of culture in medium of either high (1.0 mg/l) or low (0.01 mg/l) folate concentration are presented. From these results it is apparent that the pattern of (^{125}I)-UdR incorporation after pre-incubation with either BSS or MTX, in both high and low folate medium is similar to that previously observed in Fig. 13. Incorporation of 3-(^{14}C)-serine label into a DNA extract of replicate PHA stimulated lymphocyte cultures is shown to increase linearly throughout the course of culture in both high and low folate conditions. This pattern of 3-(^{14}C)-serine label incorporation is consistent with the expected de novo contribution to thymidylate synthesis as determined from the

incorporation of a salvage precursor in the presence and absence of an inhibitor of endogenous production of thymidine phosphates. This result therefore substantiates the use of such inhibitors in determination of rates of DNA synthesis.

The effect of culture in high and low folate conditions and pre-incubation addition of MTX and 5-fu on incorporation of 3-(^{14}C)-serine label into a DNA extract of PHA stimulated lymphocyte cultures was further examined in Fig. 29. It was apparent from these results that at a corresponding period during culture, incorporation of 3-(^{14}C)-serine label is reduced in low folate medium compared to culture in high folate conditions. As results of other experiments e.g., Fig. 12 indicate that total DNA synthesis is similar in both high and low folate conditions, this result further supports the concept of adaptation of de novo thymidylate synthesis to the prevailing folate status. It was also apparent from Fig. 29 that pre-incubation addition of MTX or 5-fu results in appreciable inhibition of 3-(^{14}C)-serine label incorporation. This inhibition of incorporation of de novo thymidylate precursor is also evident from analysis of the results presented in Figs. 30, 31 and 33. In Fig. 33 where the radioactive label used is (^{14}C)-formate this inhibition is even more marked. It is considered that sensitivity of 3-(^{14}C)-serine incorporation or (^{14}C)-formate incorporation to the effect of MTX or 5-fu is reduced by the incomplete specificity of such labelling for DNA-thymine. To exclude such non-specific labelling,

i.e., of purines and methylation, it would be necessary to carry out further purification. This might involve further hydrolysis to liberate the bases, separation of bases using a chromatographic technique and quantitation of radioactive label incorporation into specific bases (Wolberg, 1969). As this degree of resolution was not considered necessary for these experiments, this purification was not carried out. Similar considerations would also apply to other aspects of these experiments where de novo thymidylate incorporation per se is considered rather than total DNA synthesis and cell growth. The difference in the sensitivity of 3- (^{14}C) -serine and (^{14}C) -formate label to MTX and 5-fu may be attributed to differences in their metabolism. This might indicate that carbon-3 of serine is a preferred source of one-carbon units compared with formate but this is not substantiated by the work of Wolberg (1971) which indicates that these sources are incorporated to similar extents.

The effect of UdR and the effect of sequential treatment with MTX or 5-fu and UdR on 3- (^{14}C) -serine incorporation into a DNA extract of PHA stimulated lymphocyte cultures (Figs. 30 and 31), has previously been discussed (4.4.2.). The observation that the effect of UdR in increasing 3- (^{14}C) -serine incorporation is not further enhanced either in low folate conditions (Fig. 30) or in folate deficient conditions (as induced by MTX) (Fig. 31) indicates that stimulation of de novo thymidylate synthesis by addition of

exogenous UdR, as suggested by Van der Weyden (1979), is independent of the folate status and this supports the hypothesis that the effect of UdR is primarily mediated via inhibition of thymidine kinase. This result also indicates that de novo thymidylate synthesis is not restricted by the dUMP supply.

As previous experiments, the results of which are illustrated in Figs. 24, 25 and 26, indicated that de novo thymidylate synthesis was not limited by the availability of one-carbon metabolites, as supplied by exogenous formate, it is apparent that the availability of H_4 -folate coenzyme represents an important control element in de novo thymidylate synthesis. This conclusion is substantiated by the lack of effect of variation of pyridoxal status which contrasts with the marked influence of folate status on the relative contribution of the de novo thymidylate pathway to DNA synthesis.

The result of an experiment directed at determination of the effect of culture in medium containing 4-dPN on incorporation of 3- (^{14}C) -serine label into a DNA extract of PHA stimulated lymphocytes is shown in Fig. 32. The reduction in incorporation of 3- (^{14}C) -serine label is seen to be similar to the reduction in (^{125}I) -UdR incorporation caused by 4-dPN, (Fig. 16) and is also independent of the medium folate concentration. In other experiments, not reported here, it was further demonstrated that, as with studies using (^{125}I) -UdR, the presence or absence of medium pyridoxal (1.0 mg/l) did not affect 3- (^{14}C) -

serine incorporation into a DNA extract of PHA stimulated lymphocytes and nor did it affect the reduction in incorporation caused by 4-dPN.

The lack of influence of low or high medium folate concentration and the presence or absence of pyridoxal on the effect of 4-dPN is consistent with the results of previous experiments and confirms that sensitivity to vitamin B6 deficiency, as induced by 4-dPN is neither potentiated nor mitigated by variation of folate status. These observations also substantiate the previously advanced contention that vitamin B6 deficiency does not specifically affect de novo thymidylate synthesis.

Formate has also been shown to be a precursor of DNA-thymine. For example, Blakley (1969) has cited reports that formate is incorporated into the methyl group of DNA-thymine by rabbit bone marrow in vitro and that each incorporation is considerably increased when UdR or CdR is present in the suspending medium and is decreased by the inhibitor aminopterin.

And, Wolberg, (1971), has shown that incorporation of (^{14}C)-formate label into DNA-thymine of PHA stimulated lymphocytes is similar to incorporation of 3-(^{14}C)-serine label under comparable conditions. The results of determination of (^{14}C)-formate incorporation into a DNA extract of PHA stimulated lymphocytes presented here (Fig. 33) also show this pattern of incorporation, is reduced after pre-incubation with MTX or 5-fu and is increased

after pre-incubation with UdR. In other experiments, not reported here, incorporation was also shown to be reduced after culture in low folate conditions.

The results of these experiments indicate that incorporation of 3-(^{14}C)-serine or (^{14}C)-formate label into a DNA extract of PHA stimulated lymphocyte cultures, primarily via the de novo thymidylate pathway is a reciprocal reflection of (^{125}I)-UdR incorporation into an acid-insoluble residue of comparable cultures and these results further demonstrate that the primary lesion in vitamin B6 deficiency, in these conditions, is unlikely to be that of a restriction in serine hydroxymethyltransferase activity. Because the use of 3-(^{14}C)-serine label incorporation into a DNA extract of PHA stimulated lymphocytes provides a means of determining DNA synthesis independently of salvage incorporation, and therefore represents a more direct, functional means of determination of vitamin B6 deficiency, these results also support the validity of methods based on the use of (^{125}I)-UdR incorporation, via the complementary salvage pathway, to assess the effect of vitamin B6 deficiency on DNA synthesis and cell growth.

4.4.4. Effect of Medium Addition of Non-essential Amino Acids

Interest in the possible effect of additional, non-essential amino acids on the effect of vitamin B6 deficiency as induced by 4-dPN arose from observations, reported by Axelrod (1971) that the effect of 4-dPN in inhibiting antibody synthesis could be partially

reversed by glycine or serine supplementation, serine being much more effective than glycine on a molar basis. The results of experiments in this study directed at determination of the effect of medium addition of non-essential amino acids, including glycine and serine, on vitamin B6 deficiency as induced by 4-dPN are illustrated in Fig. 35.

These results indicate that medium supplementation with non-essential amino acids does not effect either an increase or a decrease in subsequent (^{125}I)-UdR incorporation irrespective of presence or absence of 4-dPN and pre-incubation with BSS, MTX, 5-fu or UdR. This suggests that the presence or absence of non-essential amino acids has no effect on vitamin B6 deficiency as it affects DNA synthesis and cell growth.

The possible effect of vitamin B6 deficiency on synthesis of specific cell products or specific proteins such as immunoglobulins was not considered in this project though it is conceivable that such a deficiency could have a differential effect on the synthesis of such products. If such a differential effect did arise it is possible that it could be detected by the application of an appropriate technique, particularly in the early stages of the vitamin B6 deficiency process. In this study, however, it is apparent that amino-acid supplementation does not affect 4-dPN induced inhibition of cell growth as expressed in reduced (^{125}I)-UdR incorporation.

The effect of supplementary serine and glycine on vitamin B6 deficiency in chicks was examined by Aboaysha and Kratzer (1980). These workers had previously demonstrated that glycine, but not serine depressed the growth of chicks when added to a diet marginal in folic acid. This was presumed to be due to the fact that folic acid is required for the interconversion of glycine and serine so that glycine toxicity was spared by the failure of serine to be converted to glycine in folate deficiency. Since PLP is also required for the interconversion of glycine and serine these workers then investigated the possibility that a vitamin B6 deficiency would act similarly to a folic acid deficiency in reducing the toxicity of glycine for young chicks. The results they in fact observed however, were that addition of serine to a diet containing isolated soybean protein caused growth depression with marginal levels of pyridoxine, and that this growth depression could be overcome by the addition of extra dietary pyridoxine. Since serine appeared to be readily converted to glycine, and also from the results of other studies, these authors attributed the metabolic defect, in this instance of pyridoxine deficiency, to a block in the conversion of phosphatidylserine to phosphatidylcholine.

Since addition of non-essential amino-acids caused neither an increase nor a decrease in (^{125}I)-UdR incorporation, there is no evidence in this study

that supplementary amino-acids either exacerbated or alleviated the effects of 4-dPN induced vitamin B6 deficiency. This work again indicates a possible relationship between the effects of folate and vitamin B6 deficiency and reaffirms the complexity of the interrelationships involved in this area of metabolism.

4.4.5. Protein Synthesis in Stimulated Lymphocytes

In this project, the principal means of assessing the effect of 4-dPN induced vitamin B6 deficiency on cell growth has involved the use of (^{125}I)-UdR incorporation to estimate the rate of DNA synthesis. In view of the possible differential effect of vitamin B6 deficiency on protein and DNA synthesis, the relationship between protein synthesis and the physiological role of lymphocytes, and the association of increased protein synthesis with PHA stimulation, it was of interest to establish whether or not the 4-dPN induced reduction in cell growth, as evidenced in reduced DNA synthesis, was reflected to a similar extent in reduced protein synthesis.

The results of an experiment in which the effect of culture in medium containing 4-dPN on acid-insoluble (^{14}C)-leucine incorporation into PHA stimulated lymphocytes was determined are presented in Fig. 36. These results indicate that the effect of 4-dPN on protein synthesis in 72h PHA stimulated lymphocytes is comparable to the effect on DNA synthesis under similar conditions, and therefore substantiate the validity of the methods used in so far as DNA synthesis is considered to equate with cell growth capacity.

4.4.6. Effect of Medium Addition of Hemin

It is known that deprivation of vitamin B6 from animals causes disorders of erythropoiesis characterised by hypochromic, microcytic, anaemia, elevation of serum iron with increased saturation of iron binding capacity, erythroid hyperplasia in the bone marrow and increased iron storage in body tissues (Soloman and Hillman, 1979b). The clinical condition is sideroblastic or pyridoxine-responsive anaemia and the primary lesion has been attributed to a block in heme synthesis. The role of PLP in heme synthesis is that of a cofactor for delta-aminolaevulinic acid synthetase; the enzyme which catalyses the condensation of succinyl CoA and glycine to form delta-aminolaevulinic acid and which is believed to be the rate limiting enzyme in the heme synthetic pathway.

Other symptoms of vitamin B6 deficiency include glossitis, dermatitis, and neuropathy but in certain circumstances this may not develop (Aoki et al, 1979), and the principal lesion is that of a block in haem synthesis. Although quantitatively the highest body requirement for haem synthesis is in erythropoiesis, because haem is a constituent of a number of enzymes and is a cofactor in oxidative phosphorylation, an adequate supply of haem is required for cell viability in other cell types. In folate deficiency the primary lesion is considered to be a restriction of DNA synthesis and this effect is most marked in those tissues with the highest turnover, i.e., haematopoietic cells. Similarly in vitamin B6 deficiency affecting

haem synthesis, the greatest effect is likely to be on those tissues with the highest haem requirement, i.e., maturing red cells. However, depending on the degree of hyperplasia in other tissues, perhaps due to alcohol, other cell types could also be susceptible to the effects of vitamin B6 deficiency induced haem restriction.

The relationship of pyridoxine deficiency and inhibition of haem synthesis has also been investigated using the vitamin B6 antagonist INH and on erythroid cell culture system (Hoffman and Ross, 1980). The levels of INH used were such as to reduce the capacity of these cells to synthesise haem without significantly affecting their viability or their ability to replicate. This study offered a further example, albeit in a specialised cell type, of an instance where a vitamin B6 deficiency, in this case induced by 4-dPN, specifically reduced haem synthesis, and hence globin synthesis, but did not reduce overall protein synthesis. This study also presented evidence that exogenous haem could overcome the inhibitory effects of INH.

Evidence that exogenous haem * may be incorporated into haemoprotein of cells other than those of the erythroid type is presented in a report by Correia et al (1979). These workers presented evidence that exogenously supplied haem had been incorporated into the hepatic microsomal haemoprotein of rats and they also suggested that exogenously supplied haem readily gains access

* In this report haem is considered to be interchangeable with hemin (iron-protophyrin - IX).

to an unassigned haem pool which provides haem for cytochrome P-450 formation.

These studies illustrate the potential value of using vitamin B6 antagonists to obtain a model, cell culture system for investigating the effects of vitamin B6 deficiency. They also describe an instance where a biochemical abnormality arising from chronic exposure of cells to a vitamin B6 antagonist could be reversed by pyridoxal, exogenous hemin or other vitamin B6 dependent metabolite.

In this project, the possibility that exogenous hemin could overcome the inhibitory effect of 4-dPN on DNA synthesis in PHA stimulated lymphocytes was investigated. The results of this experiment are illustrated in Fig. 37 and these results indicate that, in this instance, exogenous hemin has only a limited influence on the effect of 4-dPN on DNA synthesis. A number of hypotheses may be proposed to account for this lack of influence; the most obvious inference is that in PHA stimulated lymphocytes the primary lesion in 4-dPN induced vitamin B6 deficiency is unrelated to a block in haem synthesis, but it is also possible that hemin is not incorporated into cells in this system or that although hemin corrects inhibition of haem synthesis caused by 4-dPN, other effects of vitamin B6 deficiency still result in inhibition of DNA synthesis.

Section 4.5.

Conclusion

4.5.1. Preliminary Studies

The original purpose of this study was to investigate the clinical importance of nutritional deficiency and genetic markers in the aetiology and morbidity of alcoholic liver disease. The results of studies carried out for this purpose are described in section 3.1. and discussed in section 4.1. As it was apparent from these initial studies that this approach was unlikely to produce significant results it was decided to adopt an alternative approach.

4.5.2. Vitamin B6 Deficiency and the Stimulated Lymphocyte

In these studies acid-insoluble incorporation of (^{125}I)-UdR was measured in stimulated lymphocyte cultures of alcoholic and control subjects. The conditions and period of labelling, described in section 2.2.16., were based on the methods described by Das and Herbert (1978); Herbert, Tisman, Go and Brenner (1973); Das and Hoffbrand (1970); Das, Herbert, Colman and Longo (1978); Ling and Kay (1975) and McCluskey (1974). For a number of reasons discussed by Adams (1980) it is incorrect to equate acid-insoluble incorporation of a radioactively labelled precursor with the rate of DNA synthesis which means that acid-insoluble incorporation must be considered a relative rather than absolute index of DNA synthesis. However, it is apparent from Ling and Kay (1975) that by using carefully controlled and standard conditions a valid assessment of relative DNA

synthesis in PHA stimulated lymphocyte cultures of different subjects may be made by determination of acid-insoluble incorporation of an appropriately labelled DNA precursor.

One reason why acid-insoluble incorporation of radioactively labelled DNA precursor cannot be equated with the rate of DNA synthesis is that growth promoters affect pool sizes which in turn affect the specific activity of the DNA precursor pool. One approach to this problem is to measure the acid-soluble pool and apply an appropriate correction factor, Cowan and Leake (1979).

However, certain findings indicate that use of this method in the experiments reported in this study may not necessarily provide a more accurate index of DNA synthesis. These include the reported compartmentation of nucleotide pools in PHA stimulated lymphocytes which are not uniformly labelled (Taheri, Wickremasinghe and Hoffbrand, 1981), the possible effect of variation in thymidine kinase activity (Hooton and Hoffbrand, 1977), and evidence that the thymidine triphosphate pool in PHA stimulated lymphocyte cultures derived from folate deficient subjects is not significantly different from that observed in normal cells (Ganeshaguru and Hoffbrand, 1978). This finding suggests that variation in the acid-soluble pool size in folate deficient and normal cells does not explain the variation in acid-insoluble incorporation observed in cultures of these cells.

From this it is reasonable to expect that similar considerations would apply to pyridoxal deficient cells. Validation of the method of estimation of DNA synthesis in this study is also provided by the results of

experiments in which the de novo thymidylate synthesis inhibitors MTX and 5-fu were used.

Most means of determination of vitamin B6 status depend on measurement of one or more of the forms of vitamin B6 in body fluids or tissues. A variety of techniques have been used and there is no generally agreed optimal method (Sauberlich et al, 1972).

The range of results obtained from different investigators using the same method can vary considerably (Lumeng and Li, 1974; Labadarios et al, 1977).

Although most agree that alcoholics have lower circulating levels of vitamin B6 than control subjects (Bonjour, 1980), such reduced levels were not observed in the present study (4.1.2.). Because of the technical (Srivastava and Beutler, 1973) and clinical limitations of direct measurement of vitamin B6 it was decided to attempt to assess the functional significance of the relative vitamin B6 deficiency observed in alcoholic liver disease subjects.

Several approaches were used to determine whether a functional vitamin B6 deficiency existed in lymphocytes of alcoholic subjects, or of control subjects cultured in medium free of pyridoxal or exposed to vitamin B6 antagonists. Although a vitamin B6 deficiency might be expected to affect numerous enzymes in several areas of metabolism and to result in a variety of systemic and local disorders, the method of assessment of functional vitamin B6 deficiency adopted in this study was based on determination of DNA synthesis.

This method was used because inhibition of de novo thymidylate synthesis had been postulated as a primary site of action of vitamin B6 deficiency and because the principal interest was to determine the overall effect of vitamin B6 deficiency on cell growth.

The results of this study do not indicate that a functional vitamin B6 deficiency is present or is expressed in stimulated lymphocytes of alcoholic subjects, or in stimulated lymphocytes of control or alcoholic subjects cultured in medium free of exogenous pyridoxal or other PLP precursor. There was, however, a significant reduction in DNA synthesis in lymphocytes exposed to either of the vitamin B6 antagonists 4-dPN and INH. This latter observation formed the basis of using 4-dPN in PHA stimulated lymphocyte culture to study certain aspects of the effect of vitamin B6 deficiency on DNA synthesis. The appropriateness of this approach has been demonstrated by a report describing the use of PHA stimulated lymphocytes cultured in folate depleted medium as an in vitro model for megaloblastic haematopoiesis (Van der Weyden, 1979).

Several approaches were used to determine the effect of 4-dPN induced vitamin B6 deficiency on DNA synthesis and cell growth. The results of experiments involving the use of the inhibitors and metabolites MTX, 5-fu, Udr, folate and formate, and the radioactivity labelled precursors (125)-Udr, 3-(14 C)-serine and (14 C)-formate indicated that 4-dPN induced vitamin B6 deficiency,

although it reduced overall DNA synthesis did not have a specific effect on de novo thymidylate synthesis. The results of experiments where the effect of Udr on incorporation of salvage and de novo thymidylate precursors into cells pre-treated with MTX or 5-fu was determined, indicated that the presumed basis of the Udr suppression test was suspect and that the major part of the Udr suppression phenomenon could be attributed to competitive substrate inhibition of thymidine kinase. Results of experiments where the effect of variation of the medium folate concentration on subsequent incorporation of thymidylate precursors in the presence and absence of inhibitors of de novo thymidylate synthesis indicated that the medium folate concentration is a critical factor in determining the degree of cellular adaptation to thymidine utilisation. It was considered that this would have a significant bearing on many experiments in which DNA synthesis is determined.

Experiments were carried out to determine the effect of addition of non-essential amino acids and hemin on the reduction of DNA synthesis caused by 4-dPN induced vitamin B6 deficiency. In each case no appreciable effect was observed.

Although these experiments demonstrated the potential value of the use of 4-dPN and PHA stimulated lymphocyte culture in investigating aspects of the cellular effects of vitamin B6 deficiency, it is considered that in order to exploit the advantages of this system fully it would be necessary to refine the methods used

and to perhaps restrict the study to analysis of more specific enzyme lesions rather than overall DNA synthesis.

4.5.3. Vitamin B6 Deficiency and Alcoholic Liver Disease

The principal aim of this project was to determine whether or not a functional difference existed between the vitamin B6 status of control and alcoholic liver disease subjects. While blood level studies have demonstrated that vitamin B6 levels are lower in alcoholic or alcoholic liver disease subjects than in control subjects (Bonjour, 1980), the clinical significance of this finding or its relevance to the pathogenesis of alcoholic liver disease has not been established. For example, Labadarios et al (1977) have presented evidence that plasma PLP is significantly lower than normal in patients with liver disease but that there is no significant difference in plasma PLP between patients with alcoholic liver disease and other forms of liver disease.

The approach used in this study to assess vitamin B6 status and the functional significance of reduced vitamin B6 status employed peripheral blood lymphocytes from control and alcoholic subjects. Maximal vitamin B6 requiring conditions were created by culture in the presence of PHA and in medium free of pyridoxal.

Sensitivity of lymphocytes to culture in different folate concentrations and to the vitamin B6 antagonist 4-dPN was also determined. As DNA synthesis and cell growth was considered to be a possible determinant factor in the pathogenesis of alcoholic liver disease, determination of DNA synthesis was used to assess functional vitamin B6 status, and, as vitamin B6 deficiency had been reported to have a specific effect on de novo thymidylate synthesis (Axelrod, 1971), cultures were pre-incubated with MTX or 5'fu prior to incubation with a salvage thymidylate precursor, (^{125}I)-UdR, to detect such an effect.

The results of experiments carried out in this study do not indicate that there is a marked difference in functional vitamin B6 status between the alcoholic and control subjects studied. However, as the sample size of the population studied was small it was not possible to exclude the possibility that a smaller though real difference did exist. Because of the lack of subjects suitable for inclusion in the study, it was decided to concentrate on characterisation of the model system and on the effects of vitamin B6 antagonists. The failure to demonstrate a functionally significant lower vitamin B6 status in the alcoholic subjects surveyed in this study, the lack of overt clinical manifestations of vitamin B6 deficiency and the difficulty in distinguishing between the role of alcohol and the role of liver damage on vitamin B6 metabolism emphasise the difficulty in assessing whether the reduced vitamin B6 levels observed in

alcoholics are a necessary accompaniment or pre-condition for alcoholic liver disease. Soloman and Hillman (1979) observed that alcohol ingestion per se did not appear to impair either pyridoxine kinase activity or PLP activity and that as the anaemia of alcoholism is often complicated by the simultaneous presence of folate deficiency, inflammation, active liver disease and major dietary deprivations, the influence of each of these factors on vitamin B6 metabolism has yet to be determined.

4.5.4. Conclusions

This study reports;

an approach to assessment of functional vitamin B6 status

the use of PHA stimulated lymphocytes as a model system for study of cell growth

the use of a customised cell culture medium to differentiate between lymphocytes of alcoholic and control subjects

the application of the UdR suppression effect to assessment of vitamin B6 status

an approach to characterisation of the effects of vitamin B6 antagonists

The results of this study suggest that;

vitamin B6 deficiency is not always of functional significance in the aetiology and morbidity of alcoholic liver disease

the primary lesion in 4-dPN induced vitamin B6 deficiency in the PHA stimulated lymphocyte is not at the level of de novo thymidylate synthesis

the use of the UdR suppression test is not a valid method of assessing folate status

the UdR suppression effect is mediated primarily by inhibition of thymidine kinase rather than stimulation of de novo thymidylate synthesis

the medium folate concentration has a significant influence on cellular adaptation to thymidine utilisation

low medium pyridoxal concentration does not result in increased salvage thymidylate incorporation.

4.5.5. Future Work

The work described in this project may be developed and extended in several ways. Certain experiments could be carried out to further characterise the methods used and results observed in the present study. For example, determination of acid-soluble pool sizes and thymidine kinase activity in PHA stimulated lymphocytes of different subjects cultured in different conditions. The availability of a larger population of subjects suitable for inclusion in the study would allow assessment of larger sample sizes and greater clinical and demographic standardisation of subjects. If the lymphocyte cell population recovers from the effects of alcohol and nutritional deficiency quickly, the possible effect on vitamin B6 metabolism may be confounded. However, it has been suggested (Das and Herbert, 1978) that the lymphocyte is a marker of past nutritional status rather than nutritional status at the time of collection of the blood sample.

This suggests that PHA stimulated lymphocyte studies can be used for retrospective diagnosis of vitamin deficiency and are not affected by recent changes in nutrition or alcohol intake.

With reference to alcoholic liver disease, the PHA stimulated lymphocyte system could be used to study other possible nutritional factors in the aetiology and morbidity of this condition and certain aspects of immunological and other abnormalities in the alcoholic liver diseased subject.

With reference to determination of vitamin B6 status and to characterisation of the effects of vitamin B6 deficiency, there are a number of areas which could be investigated. For example, if use of the PHA stimulated lymphocyte system is retained, the actual vitamin B6 deficiency induced lesions could be considered rather than the overall effect on protein or DNA synthesis. Such a study could also be carried out in specialised cell culture systems such as Friend cells or cells derived from nerve or hepatic tissue where effects of vitamin B6 deficiency on specific protein synthesis or cell functions might more easily be determined.

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