

TABLE OF CONTENTS

INTRODUCTION AND ACKNOWLEDGEMENTS

SUMMARY

VITAMIN B₁₂ AND FOLIC ACID IN THE
SYNTHESIS OF METHIONINE BY MICRO-ORGANISMS

The pathway of methionine biosynthesis
in micro-organisms

THE UTILIZATION OF METHIONINE - THE
GROWTH OF THE METHYL GROUP

A THESIS

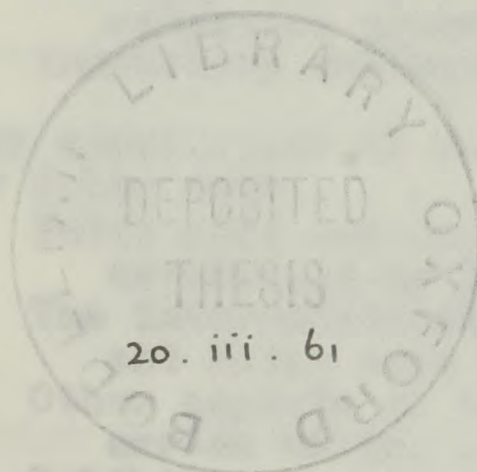
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TABLE OF CONTENTS

	Page
<u>NOMENCLATURE AND ABBREVIATIONS.</u>	1
 <u>INTRODUCTION</u>	
<u>CHAPTER 1</u>	
THE BIOSYNTHESIS OF METHIONINE.	3
Introduction.. . . .	3
The Pathway of methionine biosynthesis in micro-organisms.	4
<u>CHAPTER 2</u>	
THE METHYLATION OF HOMOCYSTEINE - THE ORIGIN OF THE METHYL GROUP.	8
Methionine formation by suspensions of organisms and growing cultures.	9
Methylation of homocysteine by soluble extracts of <u>Escherichia coli</u>	13
Methionine formation by soluble extracts of other organisms.	15
<u>CHAPTER 3</u>	
THE METHYLATION OF HOMOCYSTEINE - THE ROLE OF VITAMIN B ₁₂	17
Cobalamin and methionine formation by micro-organisms.. . . .	17
The function of cobalamin in other metabolic processes.. . . .	20
Inhibitory analogues of cobalamin.	26
<u>CHAPTER 4</u>	
THE METHYLATION OF HOMOCYSTEINE - THE ROLE OF FOLIC ACID.. . . .	28
Folic acid and the synthesis of methionine by <u>Escherichia coli</u>	29
The interrelationship between cobalamin and folic acid in methionine synthesis.	31
Other aspects of the metabolism of one- carbon units.	33
Inhibitory analogues of folic acid.. . . .	39
<u>CHAPTER 5</u>	
THE PRESENT INVESTIGATION.. . . .	41
Other components of the reaction mixture.	51
Effect of added methionine.	53
Summary.. . . .	54

EXPERIMENTAL RESULTSEXPERIMENTAL SECTION ITHE SYNTHESIS OF METHIONINE BY ULTRASONIC EXTRACTS OF
Escherichia coli

<u>CHAPTER 1</u>	METHODS.	45
	General techniques.	45
	Growth of organisms and preparation of cell-free extracts.	47
	Test for methionine synthesis.	49
	Chemicals (Pth ₄ G, CH ₃ O-Pth ₄ G; Cobalamins)	52
<u>CHAPTER 2</u>	SURVEY OF STRAINS OF Escherichia coli AND CONDITIONS FOR METHIONINE SYNTHESIS.	59
	Survey of strains of <u>Escherichia coli</u>	59
	Conditions for methionine synthesis.	62
	Summary.	65
<u>CHAPTER 3</u>	COBALAMIN IN METHIONINE SYNTHESIS.	66
	Cobalamin concentration.	66
	Analogues and derivatives of cobalamin.	67
	5,6-Dimethylbenzimidazolylcobamide coenzyme.	68
	Growth of organisms in the presence of cobalamin.	70
	Summary.	71
<u>CHAPTER 4</u>	FOLIC ACID IN METHIONINE SYNTHESIS.	73
	Requirement for EHC.	73
	Requirement for tetrahydropteroylglutamic acid.	75
	Inhibition of methionine synthesis by tetrahydropteroylglutamic acid.	75
	Interconversion of serine and glycine.	76
	Summary.	77
<u>CHAPTER 5</u>	OTHER FACTORS CONCERNED WITH METHIONINE FORMATION.	79
	Source of one-carbon units.	79
	Homocysteine.	80
	Other components of the reaction mixture.	81
	Effect of added methionine.	83
	Summary.	84

<u>DISCUSSION OF EXPERIMENTAL SECTION I.</u>	85
The relationship between cobalamin and the folic acid cofactor.. . . .	85
Cobalamin and cobalamin derivatives.. . . .	87
Folic acid.. . . .	88
Other factors.. . . .	89

EXPERIMENTAL SECTION II

THE FUNCTION OF COBALAMIN IN THE SYNTHESIS OF METHIONINE

<u>PREFACE.</u>	92
<u>CHAPTER 1</u> <u>METHODS.</u>	93
Extracts of acetone-dried <u>Escherichia coli</u> PA15.. . . .	93
Formation of the factor (X).. . . .	94
Assay of the factor (X).. . . .	95
Experiments with growing cultures of <u>Escherichia coli</u>	97
Assay of cobalamin.. . . .	98
Cobalamin-amide analogues.. . . .	98
<u>CHAPTER 2</u> <u>THE INHIBITION OF METHIONINE SYNTHESIS BY</u> <u>ANALOGUES OF COBALAMIN.</u>	100
Inhibition of methionine synthesis in ultrasonic extracts.. . . .	100
Inhibition in the presence of cobamide coenzyme.. . . .	103
The anilide analogue and ultrasonic extracts of cobalamin-grown organisms..	104
Inhibition of methionine synthesis in extracts of acetone-dried organisms..	105
Summary.. . . .	106
<u>CHAPTER 3</u> <u>THE ANILIDE ANALOGUE AND GROWTH OF</u> <u>Escherichia coli</u>	107
Summary.. . . .	109

<u>CHAPTER 4</u>	COBALAMIN AND THE ENZYMATIC FORMATION OF A FACTOR (X) CONCERNED IN THE SYNTHESIS OF METHIONINE.	110
	Indirect evidence for the synthesis of X..	110
	Synthesis of X by ultrasonic extracts. . .	111
	Properties of the factor (X)..	115
	Summary.	117
<u>CHAPTER 5</u>	THE EFFECT OF ANALOGUES OF COBALAMIN ON THE FORMATION AND ACTIVITY OF FACTOR (X) AND THEIR USE IN THE ASSAY OF FACTOR FORMATION. .	118
	Effect of analogues of cobalamin.. . . .	118
	Use of analogues of cobalamin in the assay of factor (X)..	121
	Summary.	124
<u>CHAPTER 6</u>	PROPERTIES OF THE SYSTEM FORMING FACTOR (X)..	126
	Effect of the concentration of cobalamin..	126
	5,6-Dimethylbenzimidazolylcobamide coenzyme.	127
	Requirements for the synthesis of factor (X).	128
	Other properties of the system forming factor (X).	130
	Summary.	131
<u>DISCUSSION OF EXPERIMENTAL SECTION II..</u>		132
	The formation of factor (X) and its role in methionine synthesis.. . . .	132
	Inhibitory analogues of cobalamin.	136
	5,6-Dimethylbenzimidazolylcobamide coenzyme.	143
	<u>EXPERIMENTAL SECTION III</u>	
	<u>THE FUNCTION OF FOLIC ACID IN THE SYNTHESIS OF METHIONINE</u>	
<u>PREFACE.</u>		145

	Page
<u>CHAPTER 1</u>	
METHODS.	146
Microbiological assay of folic acids. . .	146
Chromatographic methods.	151
Preparation and properties of folic acid derivatives.	153
<u>CHAPTER 2</u>	
THE ACTIVITY OF SEVERAL DERIVATIVES OF FOLIC ACID IN METHIONINE SYNTHESIS.	159
One-carbon unit donor and cofactor activities.	159
Inhibition of methionine synthesis by tetrahydropteroylglutamic acid and derivatives.	161
Summary.	164
<u>CHAPTER 3</u>	
DIHYDROPTEROYLGLUTAMIC ACID AS A COFACTOR IN THE SYNTHESIS OF METHIONINE.	165
Introduction.	165
Dihydropteroylglutamic acid and the inhibition of methionine synthesis by antimetabolites of folic acid.	167
The effect of folic acid antimetabolites on the inhibition of methionine synthesis by reduced pteroylglutamic acids.	171
Dihydropteroylglutamic acid reductase. . .	171
Summary.	172
<u>CHAPTER 4</u>	
CONJUGATED FORMS OF FOLIC ACID IN THE SYNTHESIS OF METHIONINE BY ENZYMIC EXTRACTS OF <i>Escherichia coli</i>	174
Introduction.	174
Conjugated forms of folic acid as one-carbon unit donors and cofactors for the synthesis of methionine.	176
Inhibition of methionine synthesis by folic acid derivatives.	179
Comparison of folic acid cofactor activities of pteroyltriglutamic acid compounds and the heated extract. . . .	180
Summary.	182
<u>CHAPTER 5</u>	
PROPERTIES AND PURIFICATION OF THE EXTRACT OF HEATED <i>Escherichia coli</i>	183
Physical and chemical properties of the heated extract (EHC).	183
Purification of the heated extract (EHC). .	185
Summary.	191

DISCUSSION OF EXPERIMENTAL SECTION III. 193

The specificity of the requirement for a folic acid cofactor in methionine synthesis.	193
The inhibition of methionine formation by tetrahydropteroylglutamic acid..	197
The mechanism of methionine methyl group formation.	198

Folic acid = A general term for the family of compounds including pteroylglutamic acid.

CONCLUDING DISCUSSION

The role of cobalamin in the synthesis of methionine.	202
Comparison of the function of cobalamin in methionine synthesis with the other known functions of cobalamin. .	206
The pathway of methionine biosynthesis..	210

P_{45} = Phospho-1-glycerate-3-phosphatase
acid, phosphatidase or phosphatidase.

Cobalamin = Vitamin B₁₂ compounds of which the biologically active form is methylcobalamin.

DPN = Diphosphopyridine nucleotide; DPN_2 = reduced diphosphopyridine nucleotide.

<u>APPENDIX 1</u>	Chemicals.. . . .	1
<u>APPENDIX 2</u>	Media and composite solutions.. . . .	111
<u>APPENDIX 3</u>	Organisms.. . . .	xiii
<u>REFERENCES.</u>		xiv
<u>ACKNOWLEDGEMENTS.</u>		xxviii

ABSTRACT

DPN = Diphosphopyridine nucleotide; DPN_2 = reduced diphosphopyridine nucleotide.

DPN_2 = Reduced diphosphopyridine nucleotide; DPN_2 = reduced diphosphopyridine nucleotide.

CoA = Coenzyme A.

HDP = Fructose-1,6-diphosphate.

NOMENCLATURE AND ABBREVIATIONS

For convenience the following abbreviations and conventions are used throughout this thesis:

Folic acid	= A general term for the family of compounds containing pteroylglutamic acid.
p-AB	= p-Aminobenzoic acid.
Pt	= Pteric acid.
PtG, Pth ₂ G, Pth ₄ G	= Pteroylglutamic acid, dihydro, and tetrahydropteroylglutamic acids.
N ⁵ -formyl Pth ₄ G	= <u>Leuconostoc citrovorum</u> factor natural or synthetic (leucovorin).
PtG ₃	= Pteroyl- γ -glutamyl- γ -glutamylglutamic acid, pteroyltriglutamate or teropterin.
Cobalamin	= Vitamin B ₁₂ , cyanocobalamin or α -(5,6-dimethylbenzimidazolyl)-cobamide cyanide (cobalamins or cobalamin analogues refer to the family of compounds related to cobalamin).
EHC	= A soluble extract prepared by heating a washed suspension of <u>Escherichia coli</u> of the appropriate strain.
ATP	= Adenosine triphosphate; ADP = Adenosine diphosphate; AMP = Adenosine monophosphate; P = inorganic phosphate.
DPN	= Diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide.
DPNH ₂	= Reduced diphosphopyridine nucleotide; TPNH ₂ = reduced triphosphopyridine nucleotide.
CoA	= Coenzyme A.
HDP	= Fructose-1,6-diphosphate.

Although solutions of all the acidic compounds used in this research were neutralised before use, they will be referred to as solutions of the acid concerned.

The structures of compounds to which frequent reference is made are shown in Figure 1.

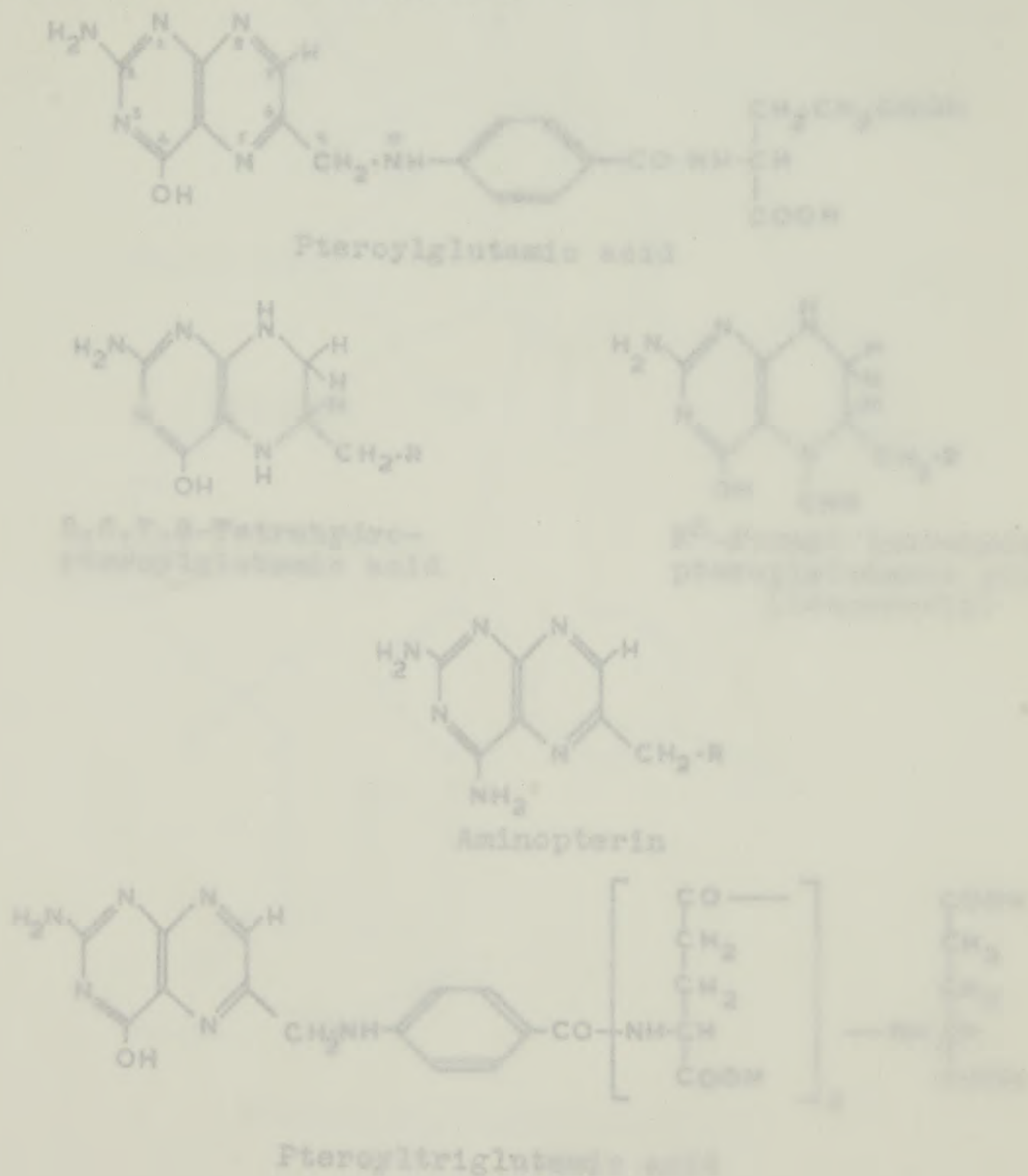
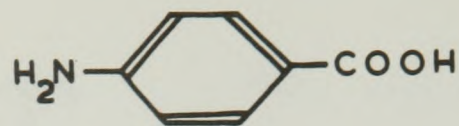
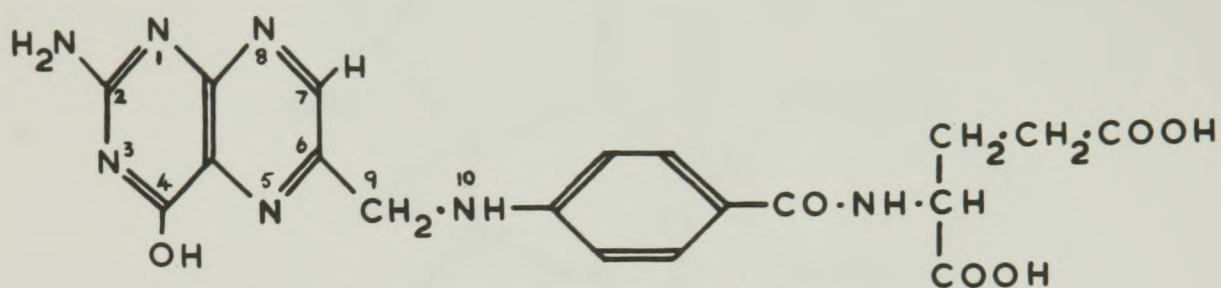


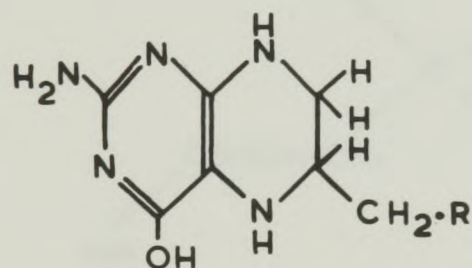
FIG. 1



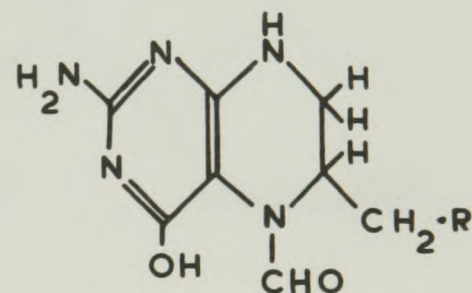
p-Aminobenzoic acid



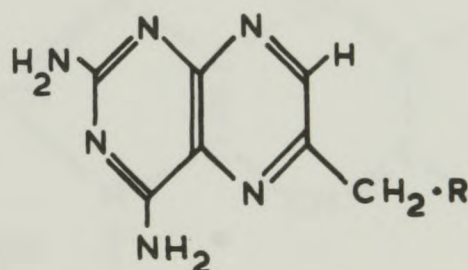
Pteroylglutamic acid



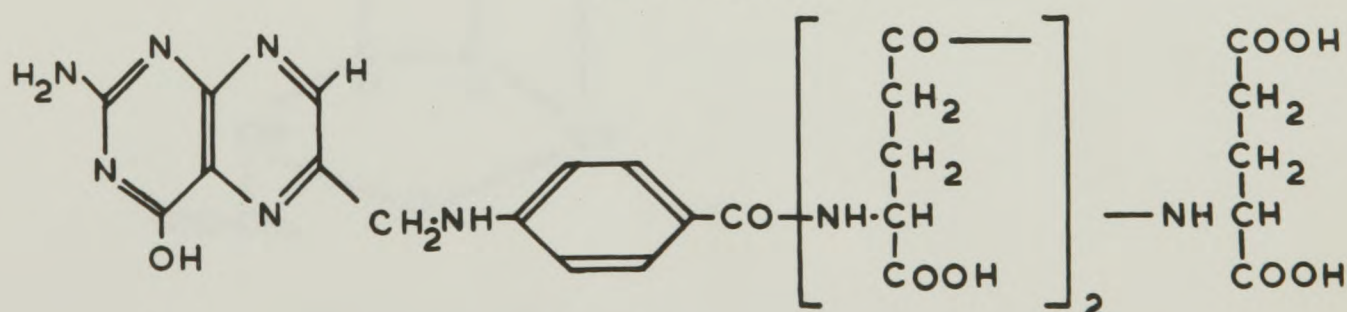
5,6,7,8-Tetrahydro-
pteroylglutamic acid



N⁵-formyl tetrahydro-
pteroylglutamic acid
(Leucovorin)

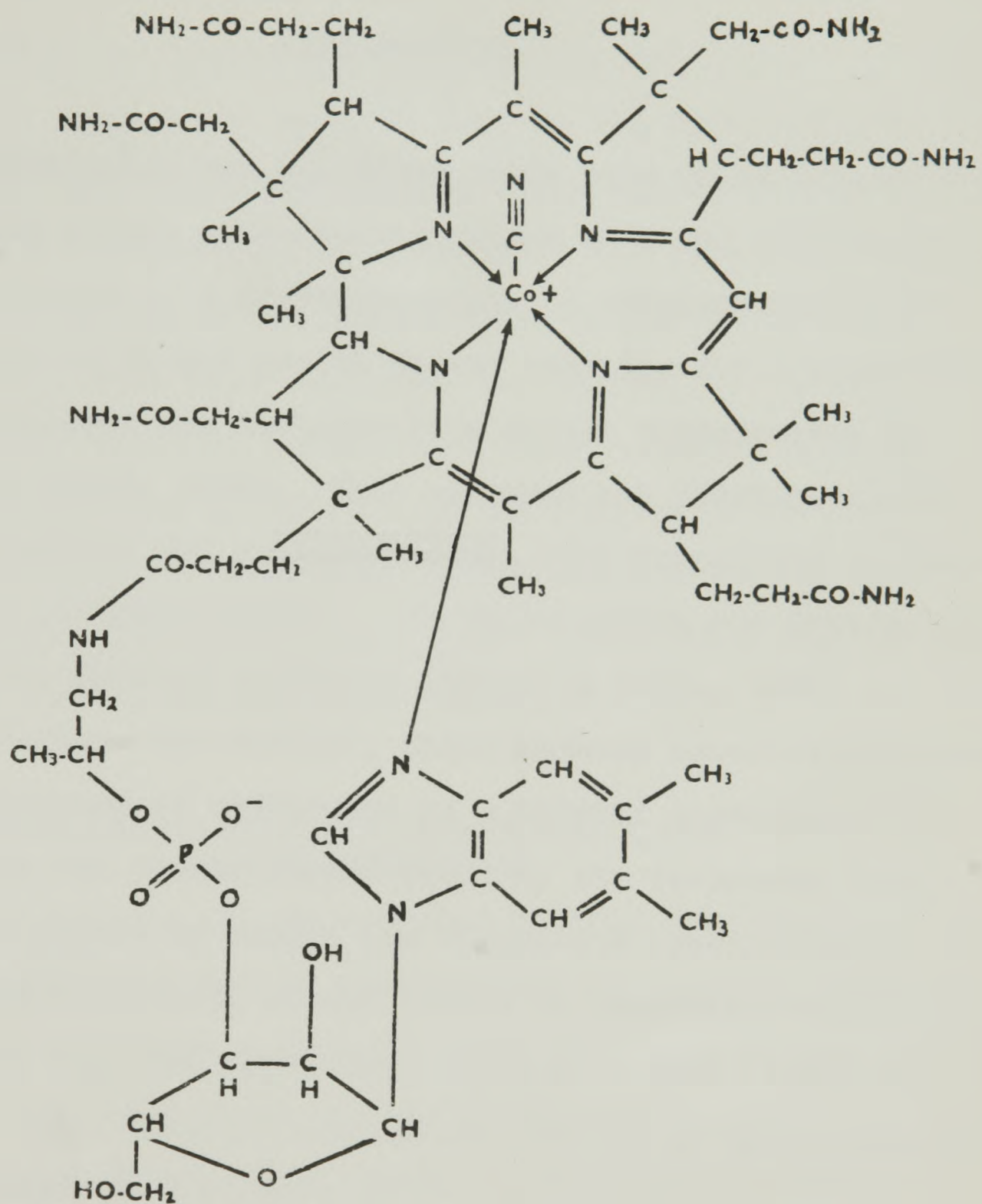


Aminopterin



Pteroyltriglutamic acid

FIG. 1 (continued)



Vitamin B₁₂ (cyanocobalamin)

CHAPTER 1

THE BIOSYNTHESIS OF METHIONINE

Introduction

Methionine was the first amino acid to be discovered as a direct result of an investigation into the nutritional requirements of a micro-organism. A sulphur-containing compound which had growth factor activity for a haemolytic streptococcus was isolated from casein hydrolysates by Mueller (1922, 1923). This compound was distinguishable from cysteine and cystine and was also discovered in yeast extract by Odake (1925). Its constitution was determined later by chemical synthesis (Barger & Coyne, 1928) and after consultation with Mueller, these authors named it methionine. The existence of methionine as a natural constituent of proteins was proved beyond doubt by its isolation from a tryptic digest of casein (du Vigneaud & Meyer, 1931).

The importance of methionine in mammalian metabolism was soon realised by Womack, Kemmerer & Rose (1937) who found that methionine and not cystine is an indispensable constituent of the diet.

Despite its comparatively recent discovery much information has accumulated concerning the role of methionine in living organisms. It is now established as the most important of the sulphur-containing amino acids. In

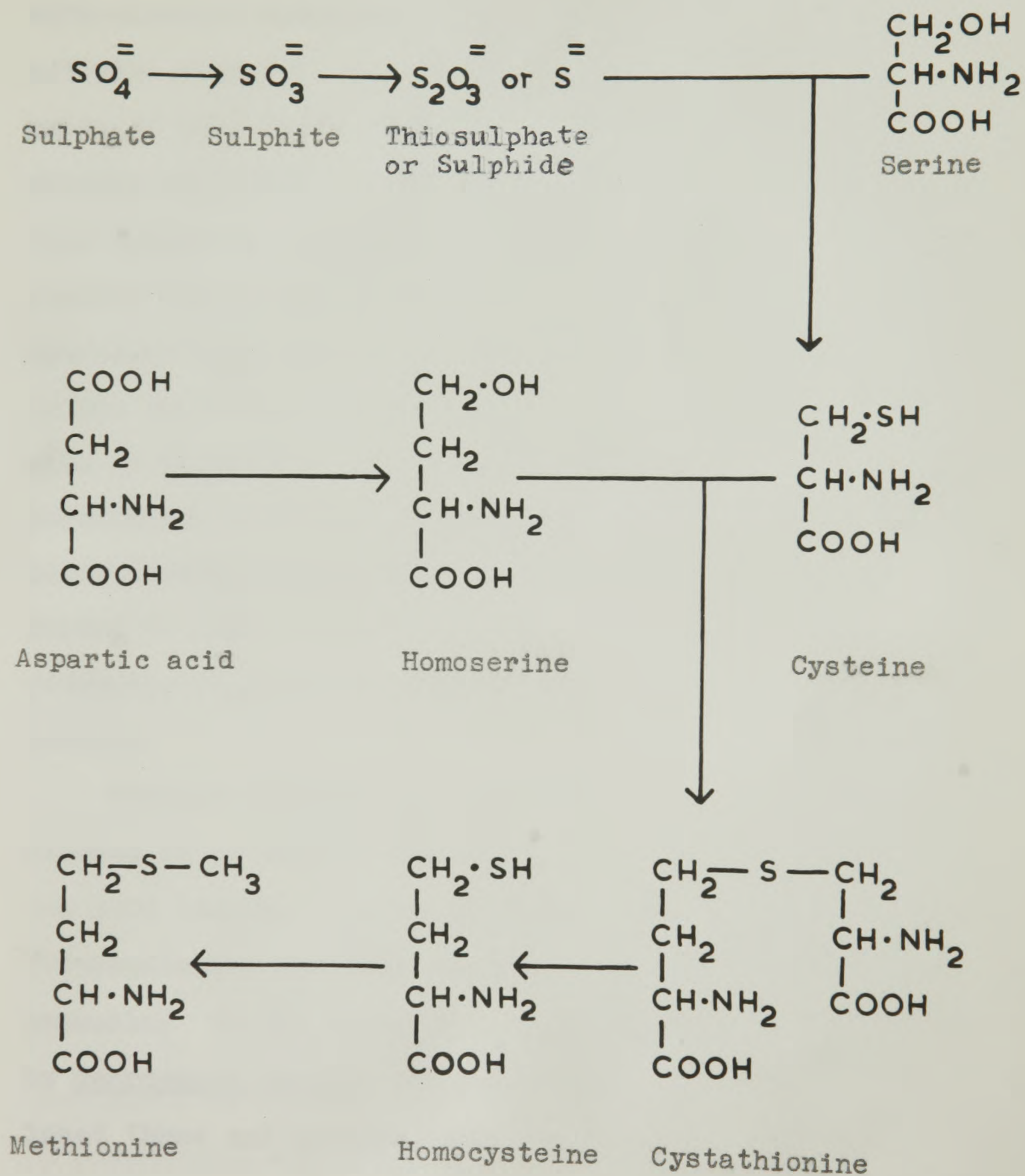
addition to its incorporation into protein, methionine is a source of cysteine and cystine in animals where it also plays a major role in metabolism as an intermediate in the formation and transfer (via S-adenosylmethionine) of methyl groups. Much work has been done on the inter-relationships between the sulphur-containing amino acids and other important metabolic intermediates. The biosynthesis of these amino acids and the vitamins concerned in these processes have been much studied, as well as the many products formed as a result of their catabolism. The biochemistry of methionine is the subject of a vast literature, several comprehensive reviews have however been published (Challenger, 1951; Bach, 1952; Cantoni, 1952, 1955; Stekol, 1955; Young & Maw, 1958).

The Pathway of Methionine Biosynthesis in Micro-organisms

An outline of the accepted pathway for the synthesis of methionine by a number of organisms is shown in Figure 2. Several different approaches have been used in the derivation of this pathway which has been reviewed in detail by Davis (1955) and Wijesundera (1954). Auxotrophic strains characterised by failure at some stage of the synthesis of methionine are amongst those most frequently isolated from Neurospora crassa (Horowitz, 1950), Escherichia coli (Lampen, Roepke & Jones, 1947), Aspergillus nidulans

FIG. 2

The pathway of methionine biosynthesis in micro-organisms



(Hockenhull, 1949) and Bacillus subtilis (Teas, 1950), by irradiating with X-rays or ultraviolet light or by treating with chemical mutagenic agents such as mustard gas and nitrogen mustard. An examination of the nutritional requirements of wild type organisms and many different auxotrophic strains has proved to be extremely valuable in elucidating this metabolic sequence of methionine biosynthesis. Moreover strains which fail to carry out a particular stage in the synthesis of a metabolite sometimes accumulate substances in the organisms or growth medium and these may be identical with or related to the substrate of the blocked reaction. In particular, a mutant of Neurospora crassa unable to form homocysteine accumulates cystathionine which in turn was formed to support the growth of another mutant with a metabolic block at an earlier stage of the biosynthetic pathway.

Genetic analysis of auxotrophic strains of the moulds blocked at several of the steps illustrated show that they are more complex, and consequently the existence of other intermediates, probably unstable compounds, is highly probable. In the conversion of homocysteine to methionine by Neurospora crassa, Emerson (1950) considers there are at least three and probably four distinct genetic blocks possible.

More recently this pathway has been confirmed by the

use of isotopic tracer techniques with substrates and intermediates labelled with ^{14}C and ^{35}S and also by the isolation of a number of enzymes concerned in the synthesis of methionine.

The main features of this biosynthetic pathway are common to many micro-organisms. However, small differences, particularly in the conversion of sulphate to cysteine may exist with different strains. In an auxotrophic strain of Aerobacter aerogenes (Schwartz & Shapiro, 1954) and in some yeasts transmethylation, the formation of methionine from α -amino butyric acid and 5'-methylthio-adenosine, has been suggested as an alternative route for methionine biosynthesis. The source of the sulphur of the nucleoside is however unknown.

Methionine formation in animal tissues.

The formation and fate of methionine in ~~xxx~~ animal metabolism is largely dependent upon the state of nutrition of the animal. Many of the reactions of the sulphur-containing amino acids in animal tissues are similar to those in micro-organisms. However the predominant pathway of sulphur metabolism in animals, the conversion of methionine to cystine (via homocysteine and cystathionine) is essentially the reverse of its biosynthesis in micro-organisms. Methionine is the source of methyl groups for the

formation of other important metabolic intermediates and products e.g. choline, creatine and N-methylnicotinamide. Such transmethylation reactions are mediated by S-adenosylmethionine (Cantoni, 1955). The methyl group can be derived from various sources containing preformed "labile methyl groups" e.g. glycine betaine, dimethylthetin and dimethyl- β -propiothetin (Dubnoff, 1949; Muntz, 1950). However in animals as well as micro-organisms the methyl group can be formed from compounds possessing one-carbon residues at a higher level of oxidation than the methyl group.

In this thesis the work described is concerned with this de novo synthesis of the methyl group of methionine by cell-free extracts of Escherichia coli and with the attendant relationships with the metabolism of certain vitamins. It is not intended to give a full account of the work in this field; wherever possible reference will be made to one of the many reviews on the subject. This introduction will be concerned mainly with work on the methylation of homocysteine by cell-free systems and, although many important contributions to the knowledge of the reaction have been obtained by studies using animals only those experiments having a direct bearing on the present topic will be mentioned.

1957, 1960). It is probable that methanol, acetone, methionine, tryptophan, purines and in fact all compounds which yield

CHAPTER 2

THE METHYLATION OF HOMOCYSTEINE - THE ORIGIN OF THE METHYL GROUP

Compounds containing preformed "labile methyl groups" do not seem to have an important role in methionine biosynthesis in micro-organisms. Choline is essential for the growth of Streptococcus pneumoniae and a mutant of Neurospora crassa; however adequate mixtures of choline and homocysteine would not replace methionine for the growth of a number of other organisms. Only two cases of the transfer of preformed methyl groups to homocysteine have in fact been reported; one was with a mutant of Neurospora crassa (Emerson 1950) while Dubnoff (1952) has claimed that a cobalamin or methionine requiring mutant of E. coli could grow in the presence of homocysteine and dimethyl- β -propiothetin. This latter finding has not however been confirmed.

Isotopic tracer techniques with intact animals or tissues have shown that formate, formaldehyde, the β -carbon atom of serine and the α -carbon atom of glycine are all precursors of the methyl group of methionine and also of thymidine and choline (see reviews by Bach, 1952; Arnstein 1957, 1960). It is probable that methanol, acetone, histidine, tryptophan, purines and in fact all compounds which yield

one-carbon units (C_1 -units) during their metabolism are also methyl group precursors albeit to a lesser extent. It is now accepted that a folic acid cofactor participates in the transfer of C_1 -units other than the methyl group itself (see Chapter 4). Some of the precursor and product relationships which have been determined both with animals and micro-organisms are shown in Table 1. Figure 3 shows the nature of the intermediates when tetrahydropteroylglutamic acid (PtH_4G) serves as the folic acid cofactor for C_1 -transfer. Serine, formaldehyde and formate are the most important sources of C_1 -units for the de novo synthesis of methyl groups

Methionine formation by Suspensions of Organisms and Growing Cultures

The greater part of our knowledge of the methylation of homocysteine by micro-organisms is the result of research carried out in this laboratory by Professor D.D. Woods and his colleagues.

Serine and glycine as the source of methionine methyl groups.

Washed suspensions of several strains of Escherichia coli convert homocysteine into methionine when incubated in phosphate buffer in the presence of glucose (Gibson & Woods, 1960; Gibson, 1952 a,b). A number of substances, known to act as precursors or sources of methyl groups in animals (e.g. formate, betaine, choline and glycine) failed to

TABLE 1

A.

One-carbon Unit Precursors

Oxidation level on entering the one-carbon unit pool

HYDROXYMETHYL

Formaldehyde

Transhydroxymethylation

Serine

Oxidation and transhydroxymethylation

Glycine

Threonine

Methanol

Acetone

Methionine

Choline, dimethylglycine sarcosine

Transmethylation, oxidation and
transhydroxymethylation

Thetins

FORMYL

Formate

Transformylation

N-formylglutamate

N-formylkynurenine (tryptophan)

N-formiminoglycine (purines)

N-formiminoglutamate (histidine)

N-formylaminoimidazole

carboxamide ribotide

1-C valine

B.

One-carbon Unit Products

Oxidation level on leaving the one-carbon unit pool

HYDROXYMETHYL

Formaldehyde

Transhydroxymethylation

Serine

5-hydroxymethylcytosine

Transhydroxymethylation and reductionMethionine (transmethylation
products via S-adenosylmethionine)

Thymidyllic acid

Pantothenic acid

FORMYL

Formate

Transformylation

N-formylglutamate

Glycinamide ribotide and

aminoimidazole carboxamide

ribotide (inosinic acid and
histidine)

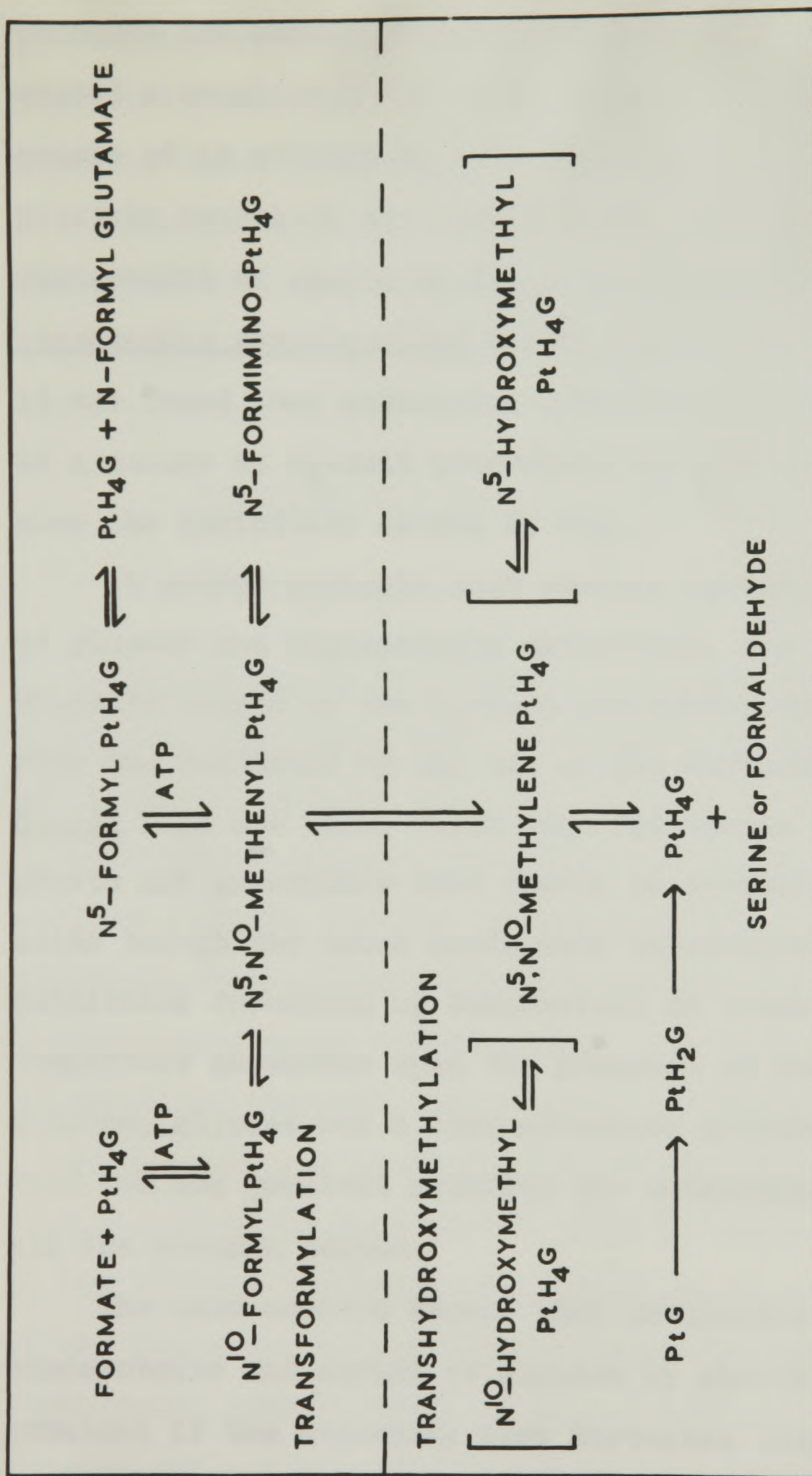


FIG. 3

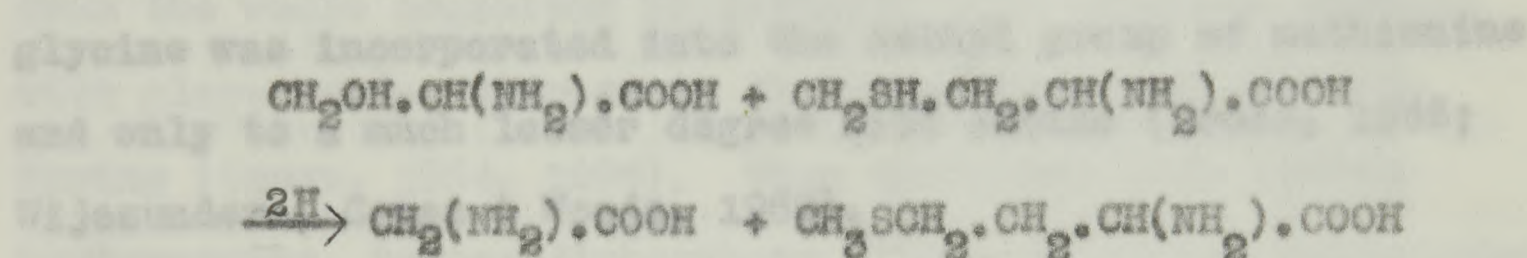
Interconversions of tetrahydropteroylglutamic acid derivatives

increase the amount of methionine formed. Serine however caused a consistent though variable increment. In the course of an attempt to determine the effect of CO_2 as a possible source of the methyl group, analogous with the replacement of serine by CO_2 and glycine for the growth of Leuconostoc mesenteroides (Lascelles, Cross & Woods, 1954), it was found that methionine synthesis was suppressed by CO_2 . Of a number of C_1 -unit precursors tested, only serine overcame the inhibition caused by CO_2 .

It seemed probable that serine, formed from the products of glucose and homocysteine metabolism, was serving as the ultimate source of the C_1 -unit for methionine formation. This was confirmed by the use of two auxotrophic strains, E. coli PA15 and 55/5, which required serine or glycine for growth and presumably were unable to synthesise these amino acids though the amino acids were interconvertible. Methionine formation by suspensions of these organisms was completely dependent upon the presence of serine as well as glucose; glycine was a less effective precursor. Strain PA15 had the greatest activity for methionine synthesis of all the strains tested.

The same authors showed that methionine formation from homocysteine and serine or glycine by strain PA15 was greatest if the organisms were harvested from a glycine-containing medium. However even under these conditions

glycine was only half as effective as serine. The other product of the reaction between serine and homocysteine was glycine, which was formed with methionine in approximately equimolar proportions. These results strongly support the view that the β -carbon atom of serine is the main source of the methionine methyl group in E.coli; the reaction was therefore represented thus



Suspensions of two strains of E.coli which required vitamin B₆ for growth were dependent upon the presence of pyridoxal or another member of the vitamin B₆ group (except pyridoxamine phosphate) for methionine formation from serine and homocysteine when the organisms had been grown in a manner rendering them relatively deficient in this vitamin (Wijesundera, Cross & Woods, 1960). This is presumably due to pyridoxal phosphate being required as the coenzyme for serine transhydroxymethylase (Blakley, 1955; Rabinowitz, 1960). It was also found that serine could be replaced by glycine as the C₁-unit donor and the latter was particularly effective if the organisms had been grown in the presence of glycine. Furthermore pyridoxal was essential for the utilisation of glycine but not when the organisms were harvested from a
into serine. Only traces of serine were incorporated into

glycine-containing medium. It was concluded that growth on glycine induces the formation of an alternative and more efficient mechanism of using glycine as a C₁-donor and this is independent of pyridoxal. The existence of a pathway for glycine utilisation independent of its conversion to serine was also demonstrated with suspensions of E. coli PA15 (glycine-grown) by using isotopic techniques; the α -carbon atom of glycine was incorporated into the methyl group of methionine and only to a much lesser degree into serine (Cross, 1956; Wijesundera, Cross & Woods, 1960).

Other sources of the methyl group of methionine.

Several other compounds including seventeen amino acids, choline, betaine, formate, glycollate; xanthine, ethanol and α -aminobutyrate were all tested as precursors of the methionine methyl group with suspensions of E. coli PA15 (Gibson & Woods, 1960). Only threonine and homoserine showed some effect and this was probably due to their preliminary conversion to glycine. The growth requirement for methionine of Streptobacterium plantarum 10S could be replaced by CO₂ and homocysteine; however tracer studies showed that no incorporation of CO₂ into the methyl group of methionine occurred (Cross, 1956). Isotopically labelled formate on the other hand was incorporated into the methyl group of methionine by growing cultures of this organism but not into serine. Only traces of formate were incorporated into

the methyl group of methionine by suspensions of E. coli PA15.

It was concluded that serine is the most important source of the C₁-unit for methionine formation in E. coli.

Methylation of Homocysteine by Soluble Extracts of Escherichia coli

Soluble extracts of E. coli strain PA15 were prepared from the whole organisms by grinding with alumina, shaking with glass beads, ultrasonic disintegration and acetone-drying (Cross, 1954, 1956). When incubated with serine, homocysteine, hexose diphosphate (HDP), adenosine triphosphate (ATP), pyridoxal phosphate, and magnesium sulphate in phosphate buffer at 37°, these extracts catalysed the formation of methionine. The most potent extracts were obtained by acetone-drying; these and ultrasonic extracts have been used extensively in later studies by Szulmajster & Woods (1960); Kisliuk & Woods (1960); Helleiner & Woods (1956); Helleiner, Kisliuk & Woods (1958). With dialysed extracts of acetone-dried organisms the presence of a source of energy and reducing power (glucose or HDP), ATP, diphosphopyridine nucleotide (DPN), Mg²⁺ and phosphate buffer were essential for optimal methionine formation in addition to serine, homocysteine, a source of folic acid cofactor (originally supplied by an extract of heated E. coli and later by Pth₄G under anaerobic conditions) and in certain circumstances cobalamin (Szulmajster & Woods, 1955, 1960;

Helleiner & Woods 1956; Helleiner, Kisliuk & Woods, 1958; Kisliuk & Woods, 1958). Serine was the most effective source of C₁-units for methionine formation with extracts obtained by mechanical disintegration and by acetone-drying. Glycine was 10 to 25 per cent as active as serine, and formate was virtually without activity as a source of C₁-units. Spontaneous formation of 1,3-thiazane-4-carboxylic acid from homocysteine and formaldehyde (Wriston & Mackenzie, 1957) prevented the use of the latter as a possible source of the C₁-unit in these investigations. This heterocyclic compound was not a precursor of methionine. However, in later work, the compound formed by mixing formaldehyde and Pth₄G (CH₂O-Pth₄G) was found to be an effective source of the methyl group of methionine.

Serine transhydroxymethylase.

Isotopic technique showed that the β-carbon atom of serine was incorporated into the methyl group of methionine (Szulmajster & Woods, 1960). The interconversion of serine and glycine by extracts of acetone-dried E. coli PA15 was demonstrated by two methods: glycine formation from serine was assayed microbiologically (Szulmajster & Woods, 1960) and serine formation from glycine and formaldehyde was assayed chemically (Kisliuk & Woods, 1960). A requirement for a folic acid cofactor and pyridoxal phosphate for serine

transhydroxymethylase was demonstrated when the extracts had been treated with Dowex-1 resin (Kisliuk & Woods, 1960). Furthermore, the latter authors also showed that pyridoxal phosphate was essential for the synthesis of methionine by Dowex-1-treated extracts when serine was the source of the C₁-unit but not with CH₂O-PtH₄G. It was therefore provisionally concluded that vitamin B₆ functions only in the serine transhydroxymethylase reaction. This work also suggested that the ultimate source of the methionine methyl group is probably an enzyme-bound hydroxymethyl derivative of a folic acid cofactor.

Methionine Formation by Soluble Extracts of other Organisms

Serine would appear to be the most important source of the methyl group of methionine in E.coli. However in Ochromonas malhamensis evidence that glycine is the source of the one-carbon unit has been obtained; cell-free preparations of this organism incorporated the α -carbon atom of glycine into the methyl group of methionine (Johnson, Holdsworth, Porter & Kon, 1957).

Several reports of the formation of methionine from homocysteine by extracts of animal tissues had been published prior to the commencement of this work. Soluble

extracts of pigeon liver homogenates incorporated formate into methionine (Berg, 1953). Extracts of chicken and sheep liver were also found to incorporate formaldehyde and the β -carbon atom of serine into methionine when incubated with homocysteine and several other cofactors e.g. ATP, DPN, Mg^{2+} and a folic acid cofactor (Doctor, Trunnell & Awapara, 1956; Doctor, Patton & Awapara, 1957; Nakao & Greenberg, 1955).

(de Vigneaud, Chandler, Meyer & Carroll, 1954; Tannen, 1946; Bennett, 1953). The function of cobalamin in the metabolism of labile methyl groups was later confirmed in several laboratories (Gillie & Davis, 1954; Johnson, Salmon & Strength, 1955; Stadel & Folmer, 1955). A number of investigations using isotopic techniques and normal and cobalamin-deficient animals have since shown that cobalamin is concerned in the synthesis of methyl groups from formate, the α -carbon atom of glycine and the β -carbon atom of serine; cobalamin does not appear to have a role in transmethylation. This subject has been well reviewed by Aronow (1956, 1957, 1958).

Methionine and Methionine Formation by Micro-organisms

The role of cobalamin in the synthesis of methionine by micro-organisms was first suggested by the isolation of

CHAPTER 3

THE METHYLATION OF HOMOCYSTEINE - THE ROLE OF VITAMIN B₁₂

Early nutritional studies with rats indicated that the dietary requirement for choline and methionine could be reduced by homocysteine and certain unidentified growth factors which were later found to be cobalamin and folic acid (du Vigneaud, Chandler, Moyer & Keppel, 1939; Bennett & Toennies, 1946; Bennett, 1950). The function of cobalamin in the metabolism of labile methyl groups was later confirmed in several laboratories (Gillis & Norris, 1949; Schaefer, Salmon & Strength, 1949; Stekol & Weiss, 1950). A number of investigations using isotopic techniques and normal and cobalamin-deficient animals have since shown that cobalamin is concerned in de novo synthesis of methyl groups from formate, the α -carbon atom of glycine and the β -carbon atom of serine; cobalamin does not appear to have a role in transmethylation. This subject has been well reviewed by Arnstein (1955, 1957, 1960).

Cobalamin and Methionine Formation by Micro-organisms

The role of cobalamin in the synthesis of methionine by micro-organisms was first suggested by the isolation of

mutants of E.coli requiring cobalamin or methionine for growth; homocysteine and homocystine were inactive (Davis & Mingioli, 1950). The relative concentrations of cobalamin and methionine required, indicated that cobalamin might function as a catalyst or coenzyme in the methylation of homocysteine. Similar mutants have also been isolated by Gots & Koh (1950); Kalan & Ceithaml (1954). Experiments with Chrysomonas (e.g. Ochromonas malhamensis) and Flavobacterium sp. confirmed the relationship between methionine and cobalamin (Hutner, Provasoli & Filfus, 1954; Lascelles & Cross, unpublished). Methionine, but no other compound tested, could replace the requirement for cobalamin for the growth of these organisms; the turbidity attained with the amino acid was however considerably less than with the vitamin. Cobalamin was also found to replace methionine as an antagonist of sulphonamides in prototrophic and cobalamin - requiring auxotrophic strains of E.coli (Shive, 1950; Alimchandani & Sreenivasan, 1955).

Cobalamin and methionine synthesis in Escherichia coli.

Considerable advances have been made in defining the role of cobalamin by Gibson & Woods, (1952, 1960) and Gibson (1952a,b). The synthesis of methionine from homocysteine and serine by suspensions of several strains of E.coli was increased from two to six-fold by the addition of cobalamin; the greatest effect was obtained with strain PA15. Cobalamin

was essential for methionine formation by suspensions of cobalamin or methionine requiring strains (E.coli 113/3, 200 and 121/176 from the collection of Dr. B.D. Davis) made cobalamin-deficient by growth on methionine. The increase in methionine formation occurred independently of the C_1 -donor used (glucose, serine, glycine, homoserine or threonine).

Although necessary for optimal methionine formation by suspensions of E.coli PA15, cobalamin had no effect on methionine synthesis by soluble extracts of the organism prepared by mechanical disintegration or by acetone-drying (Cross & Woods, 1954; Szulmajster & Woods, unpublished). Helleiner and Woods (1956) however found that when extracts of E.coli 121/176, a cobalamin or methionine requiring auxotroph (grown with methionine) were prepared by ultrasonic disruption, addition of cobalamin caused a four or five-fold increase in methionine formation. Cobalamin had no stimulatory effect on the very low rate of methionine formation obtained with extracts of acetone-dried powders of this organism. A particular requirement for cobalamin metabolism was subsequently discovered with extracts of strain PA15 as well as strain 121/176 when PtH_4G served as the cofactor for C_1 -transfer. This will be discussed later in Chapter 4.

Cobalamin and methionine synthesis in other systems.

Ochromonas malhamensis responds to the presence of methionine in the growth medium whether or not cobalamin is also present (Johnson et al. 1957). Glycine, serine and to some extent threonine, histidine and homoserine showed the same effect as methionine but only in the presence of cobalamin. Furthermore, the α -carbon atom of glycine was incorporated into methionine by extracts of the organism only if it had been harvested from a cobalamin-containing medium. Although no direct effect of cobalamin on this reaction could be obtained, a role for cobalamin was indicated. With the soluble extracts prepared from animal tissues mentioned in Chapter 2, no requirement for cobalamin was observed, however no attempts to compare extracts from normal and cobalamin-deficient animals were reported.

(Rieske, 1955; Smith, 1955; Arnstein 1955, 1957, 1960; Smith, 1960).

The Function of Cobalamin in other Metabolic Processes

Cobalamin was first isolated from liver twelve years ago (Rieske et al. 1943; Smith & Parker, 1943) and its molecular structure was finally determined several years later (Hodgkin et al. 1955, 1956). It is an essential growth factor for animals and the anti-pernicious anaemia factor in humans. Many micro-organisms can synthesise the vitamin from simple precursors (Streptomyces aureofaciens, Propionibacterium shermanii, Clostridium tetanomorphum). A wide variety of

different forms of cobalamin depending on the nature of the nucleotide residue and other substituents have been recognised (Kon & Pawelkiewicz, 1960). Certain Lactobacilli have an alternative growth requirement for deoxyribosides and purines or cobalamin, just as some mutants of E.coli require methionine or cobalamin; for a number of other micro-organisms however, the nutritional requirement for cobalamin is not influenced by any known metabolite (Euglena gracilis, Arthrobacter: Lochhead Nos.12,38). A function for cobalamin in the metabolism of plants has not been conclusively demonstrated.

Despite its recent discovery cobalamin is the subject of an enormous literature and many detailed reviews of its biochemical functions have been published (Harris et al. 1954; Lascelles & Cross, 1955; Ford & Hutner, 1955; Heinrich, 1957; Arnstein 1955, 1957, 1960; Smith, 1960).

When the research to be described in this thesis was begun, the effect of cobalamin on the synthesis of methionine by extracts of E.coli demonstrated in Oxford was the only example of a direct action of the vitamin using a cell-free preparation. During the course of the present work several other examples have been discovered; these and other metabolic processes in which a role for cobalamin has been indicated will be mentioned briefly here.

Choline and thymidine methyl group synthesis.

Nutritional and isotopic tracer studies with animals and animal tissue preparations have suggested that cobalamin functions in the synthesis of the methyl groups of choline from a number of C_1 -unit sources. This could be due to transmethylation following the synthesis de novo of methionine methyl groups (Arnstein & Neuberger, 1953). Recently however, soluble extracts of rat liver which catalyse the synthesis of choline from formaldehyde and aminoethanol or dimethylaminoethanol independently of transmethylation have been obtained (Venkataraman & Greenberg, 1958; Peters & Greenberg, 1958). PtH_4G acts as a cofactor but no requirement for cobalamin has been reported.

Nutritional and isotopic studies also indicated a role for cobalamin in nucleic acid metabolism. There is however much conflicting evidence regarding its site of action. The methyl group of thymidine does not participate in transmethylation, it can be formed from serine or formaldehyde by a number of soluble preparations from animals and microorganisms (see Chapter 4) but apparently no attempt has been made to demonstrate a cobalamin requirement. Bolinder & Reichard (1959) have recently shown that minces of cobalamin-deficient chick embryos had a sub-normal ability to incorporate deoxycytidine and deoxyuridine into the thymidine of deoxyribonucleic acid; addition of cobalamin had a direct

stimulatory effect. Cobalamin also promoted the incorporation of formate, but not formaldehyde, serine or glycine, into thymine by suspensions of bone marrow cells from cobalamin-deficient chicks (Dinning & Young, 1959 a,b). Further evidence for a role of cobalamin in the synthesis of thymidine methyl groups was obtained with growing cultures of Lactobacillus leichmanii, again the effect of cobalamin on the incorporation of formate is the greatest. The activity of hydroxymethyl-tetrahydropteroylglutamic acid dehydrogenase of bone marrow and liver has recently been shown to be lower in cobalamin deficient chicks (Dinning & Hogan, 1960).

The mechanisms of choline and thymine methyl group formation appear to differ from methionine methyl group formation in E.coli. However they all demonstrate an intimate relationship between cobalamin, folic acid and one-carbon metabolism, albeit at different levels of oxidation. Evidence has also been obtained for a function of cobalamin in deoxynucleoside formation and in purine synthesis by the Lactobacilli (Lascelles & Cross, 1955).

Sulphydryl group metabolism.

The maintenance of the sulphydryl groups in the reduced state in biological environments appears to be controlled directly or indirectly by cobalamin. The reactivation by cobalamin of several sulphydryl-containing enzymes of suspensions of aged E.coli 113/3 has been directly related to

FIG. 4

the activation of the sulphhydryl groups using a cell-free system (Dubnoff & Bartron 1956 a,b). This work has not however been confirmed or extended.

Protein synthesis and amino acid activation.

Since this work was started, cobalamin was found to increase the incorporation of amino acids into the protein of microsomal preparations from the livers of cobalamin-deficient pigs and rats supposedly by its effect on the formation of aminoacyladenylates (Wagle, Mehta & Johnson, 1958, a,b). More active than cobalamin was 5,6-dimethylbenzimidazolylcobamide coenzyme (see below) for the effect on amino acid incorporation but the effect on amino acid activation was not confirmed (Mehta, Wagle & Johnson, 1959). However a direct function of cobalamin in protein synthesis has not been confirmed by Fraser & Holdsworth (1959), Arnstein & Simkin (1959) with chicks and rats or by Arnstein & White (1959) with suspensions of Ochromonas malhamensis.

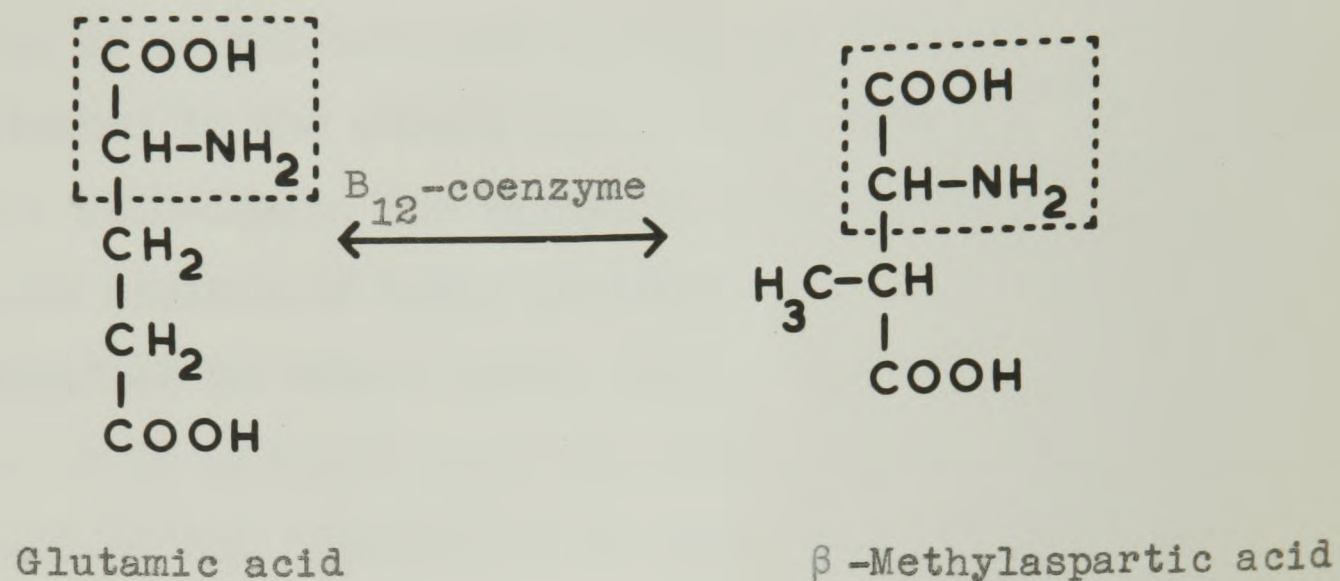
Glutamic acid metabolism.

Recently coenzyme forms of three cobalamin derivatives which are required for the interconversion of glutamate and β -methylaspartate (Fig.4) by soluble extracts of Clostridium tetanomorphum have been isolated by Barker, Weissbach & Smyth (1958) and Weissbach, Toohey & Barker (1959). These and several other cobamide coenzymes have subsequently been

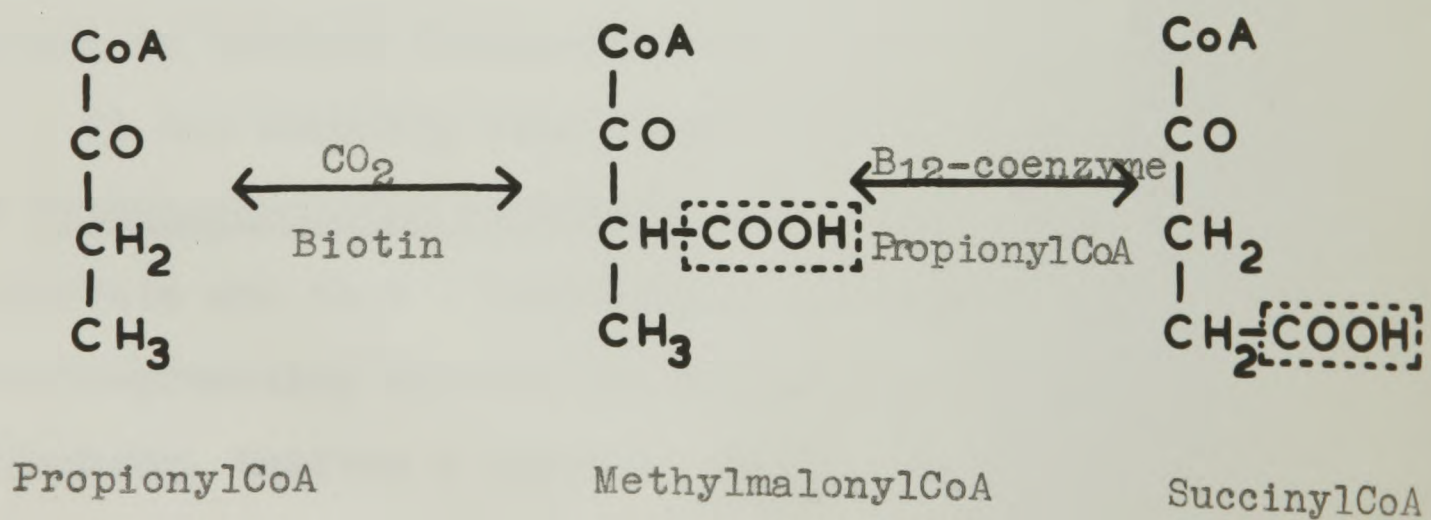
FIG. 4

Metabolic functions of cobamide coenzymes

Glutamic acid metabolism



Propionic acid metabolism



isolated also from Propionibacterium shermanii and their properties have been determined (Barker et al. 1960 a,b; Ladd, Hogenkamp & Barker, 1960; Pawelkiewicz, Bartosinski & Walerych, 1960). They all contain a second nucleoside group consisting of an unidentified pentose and adenine, the latter being attached to the cobalt atom. The coenzymes are light-sensitive, acid-labile and unstable in the presence of cyanide, the adenine nucleoside being removed in all cases. They have been identified in rabbit liver (Weissbach, Toohey & Barker, 1959) and it is probable that cobalamin is simply the stable end-product of the degradation of these more elaborate derivatives caused by the presence of acid and cyanide during its isolation. The cobamide coenzyme is concerned with the transfer of the glycine residue from carbon atom 3 to carbon atom 4 of the glutamate molecule, otherwise its mechanism of action is unknown (Munch-Peterson & Barker, 1958).

It has recently been found that the cobalamin requirement of Poterochromonas stipitata can be replaced by β -methylaspartate and that β -methylasspartate has the same limited growth-promoting effects as thymine for a mutant of E.coli (Isenberg, Seifter & Berkman, 1960). The biological importance of β -methylasspartate has still to be elucidated; it was however suggested that its carbamyl derivative could be an intermediate in thymidine synthesis by a pathway analagous to that for uridine.

Propionic acid metabolism.

A requirement for cobalamin in propionate metabolism by ruminants was first investigated by Marston (private communication) and later using rat liver homogenates by Smith & Monty (1959). It is now known that 5,6-dimethylbenzimidazolylcobamide coenzyme but not cobalamin is a coenzyme for the interconversion of methylmalonyl CoA and succinyl CoA (Fig. 4) by methylmalonyl CoA isomerase in extracts of Propionibacterium shermanii (Stadtman, Overath, Eggerer & Lynen, 1960. a,b), fractionated ox liver (Stern & Friedman, 1960), and rat liver preparations (Gurnani, Mistry & Johnson, 1960).

Several other less defined functions of cobalamin have been reported e.g. the conversion of glycine to serine (Vohra, Lantz & Kratzer, 1956) and in p-aminobenzoic acid metabolism (Davis, 1951; Lascelles & Cross, 1955).

It is apparent that more research, particularly with cell-free systems, is required before the primary effects of cobalamin can be distinguished from that which is secondary and before a unifying hypothesis of cobalamin action can be presented.

Inhibitory Analogues of Cobalamin

Inhibitory analogues of several vitamins have proved to be of great value in chemotherapy and in studies of vitamin

metabolism. Weak antagonism of the growth factor activity of cobalamin for certain micro-organisms and of cobalamin absorption in animals has been obtained with the free heterocyclic bases which are often found in or can be incorporated into cobalamins. Several of the many natural analogues of cobalamin having positive microbiological activities can also have weak inhibitory activity (Smith, 1960).

The most effective antagonists have been synthesised in accordance with the principles of Woolley (1953) by Smith, Parker & Gant (1956). These were prepared by removal of the amino group from one or more of the reactive amide groups of cobalamin i.e. the propionamide groups (Fig.1). The carboxylic acids thus formed were converted into substituted cobalamin amide derivatives by reacting with several amines. These compounds were competitive inhibitors of the growth of E.coli 113/3 and rats (Cuthbertson, Gregory, O'Sullivan & Pegler, 1956).

Later, methionine has frequently been shown to be the most important component of mixtures which replace p-AB either for overcoming sulphamide inhibition or for supporting the growth of organisms which require p-AB for growth (Winkler & de Haan, 1948; Lampen, Jones & Bergman, 1950; Strehler, 1950; Cotte & Kohnen, 1950; Bird & Cotte, 1950). In a similar manner methionine was found essential for overcoming the inhibitory effects of aminopterin on the growth of Tarula arcuata (Broqvist, 1953). In animals the

CHAPTER 4

THE METHYLATION OF HOMOCYSTEINE - THE ROLE OF FOLIC ACID

The biological importance of p-aminobenzoic acid (p-AB) was first realised as a result of its ability to overcome competitively the inhibition of bacterial growth by sulphonamides (Woods, 1940). Convergent researches with micro-organisms and animals have since shown the presence of a p-AB residue in a number of other growth factors or vitamins, members of the folic acid family. A few of these compounds are shown in Figure 1.

The first indication that p-AB was concerned in the methylation of homocysteine by micro-organisms was the observation that methionine but not homocysteine overcame the inhibition of growth of E.coli by sulphonamides in a non-competitive manner (Harris & Kohn, 1941; Bliss & Long, 1941; Kohn, 1943). Later, methionine has frequently been shown to be the most important component of mixtures which replace p-AB either for overcoming sulphonamide inhibition or for supporting the growth of organisms which require p-AB for growth (Winkler & de Haan, 1948; Lampen, Jones & Roepke, 1949; Strehler, 1950; Cutts & Rainbow, 1950; Bird & Gots, 1958). In a similar manner methionine was found essential for overcoming the inhibitory effects of aminopterin on the growth of Torula cremoris (Broquist, 1952). In animals the function of

follic acid in methyl group synthesis was suggested by Bennett (1949, 1950) and since then the vast field of one-carbon unit and follic acid metabolism has emerged.

Follic acid and the Synthesis of Methionine
by Escherichia coli

More direct evidence for a function of p-AB in the methylation of homocysteine was obtained by Gibson & Woods (1952, 1960) using washed suspensions of p-AB-deficient strains of E.coli. Deficient organisms were obtained by harvesting a p-AB requiring auxotroph from a p-AB replacement medium (containing amino acids, purines and thymine); these organisms would synthesise methionine only if p-AB was added to the suspension. Sulphathiazole inhibited the action of p-AB. However, if the organisms were first incubated with p-AB, glutamic acid and glucose, the subsequent formation of methionine was insensitive to sulphathiazole. Concurrent formation of follic acids (active for the growth of Lactobacillus casei and Leuconostoc citrovorum) during methionine synthesis or during the preincubation in the presence of p-AB was demonstrated. Consequently it was concluded that p-AB functioned in methionine formation after it had been converted to a cofactor form of follic acid. Pteroylglutamic acid (PtG) and N⁵-formyl PtH₄G (leucovorin) would not replace p-AB for methionine synthesis or for the

growth of this organism; probably because they could not be elaborated into the cofactor. Even in the presence of optimal concentrations of cobalamin, p-AB was essential for methionine synthesis by this strain but the synthesis of methionine by suspensions of other strains of E.coli (not requiring p-AB for growth) was not increased by added p-AB.

The synthesis of methionine by soluble extracts of E.coli PA15 was increased by the addition of an extract of heated organisms, EHC (Cross & Woods, 1954). The requirement for the EHC was greater with dialysed enzymic extracts and EHC was essential for methionine formation by Dowex-1-treated extracts (Szulmajster & Woods, 1955, 1960; Kisliuk & Woods, 1960). Dialysed ultrasonic extracts of strain 121/176 also required EHC for methionine synthesis (Helleiner & Woods, 1956). Using enzymic extracts prepared from acetone-dried strain PA15, the EHC could not be replaced by p-AB, PtG, Pth₄G, N¹⁰-formyl Pth₄G and N⁵-formyl Pth₄G. A five-fold purification of the EHC was achieved by paper chromatography and evidence for the folic acid nature of the active component was obtained (Szulmajster & Woods, 1960). The EHC could be replaced by CoC (see below) and to some extent by a formylated derivative of pteroyltriglutamic acid (PtG₃) which was purified from the crude CoC by Wright (1955 b, 1956).

Fractionation of these enzymic extracts was attempted by

Szulmajster and by Helleiner but only very limited purification was obtained, probably due to the complexity of the reaction, and no evidence for any intermediate was obtained.

The Interrelationship between Cobalamin
and Folic acid in Methionine Synthesis

Tetrahydropteroylglutamic acid (Pth_4G) will serve as the folic acid cofactor for all the known C_1 -transfer reactions occurring in soluble extracts even though its instability and low intrinsic cofactor activity make it clear that it is not identical with the naturally-occurring cofactors. However, in this system for methionine synthesis it was inactive and later proved to be a potent inhibitor of the function of the EHC (Kisliuk & Woods, 1960).

Re-examining the role of cobalamin in methionine synthesis, Kisliuk & Woods (1958, 1960) discovered that enzymic extracts of acetone-dried powders of E.coli PA15 harvested after growth in the presence of cobalamin were endowed with the ability to use Pth_4G as the cofactor for C_1 -transfer in the methylation of homocysteine. Serine and the formaldehyde derivative of Pth_4G would serve as the source of the methyl group under these conditions; serine was not an intermediate in the utilisation of formaldehyde. Subsequently the cofactor activity of Pth_4G was tested with ultrasonic extracts of E.coli 121/176. It was active but only if

cobalamin was also added, however with this organism cobalamin was required even with EHC as the source of folic acid cofactor (Helleiner, Kisliuk & Woods, 1958).

At the same time as the work to be described in this thesis, a non-diffusible, heat-labile factor (X) which promoted the synthesis of methionine in the presence of PtH_4G by extracts of acetone-dried powders of strain PA15 was obtained from similar extracts of organisms grown on a medium containing cobalamin (Kisliuk & Woods, 1960). Cobalamin itself was inactive in this system. The factor (X) was partly-purified (five to ten-fold) from extracts of the organisms grown with cobalamin by treatments involving precipitation with protamine sulphate. The increase in rate of methionine formation by extracts of acetone-dried E.coli PA15 with PtH_4G as the cofactor for C_1 -transfer was used as an assay during the isolation of X. Kisliuk and Woods (1960) have presented a hypothetical pathway for the synthesis of methionine with the attendant vitamin functions on the basis of these results (Fig.5).

Folic acid cofactors were also required for methionine formation by soluble preparations from animal sources. Treatment of the pigeon liver preparation of Berg (1953) with Dowex-1 resin induced a requirement for an extract of heated liver or yeast presumably as a source of the folic acid cofactor. Soluble systems from chick and sheep liver required

the addition of PtG , PtH_4G or N^5 -formyl PtH_4G for methionine formation (Doctor, Patton & Awapara, 1957; Nakao & Greenberg, 1955).

Other Aspects of the Metabolism of One-carbon Units

The chemistry, biosynthesis, natural occurrence, coenzyme forms and metabolic functions of the folic acid group of vitamins have been extensively reviewed (Woods, 1960; Wright, 1960; Rabinowitz, 1960; Hutner, Nathan & Baker, 1959; Stokstad, 1954; Huennekens & Osborn, 1959; Greenberg & Jaenicke, 1957). There are numerous accounts of the isolation of monoglutamyl and polyglutamyl derivatives of folic acid from natural sources (Rabinowitz, 1960; Wright, 1960); many of these are probably the degradation products of more elaborate compounds, which have been produced during isolation. However, Wright (1955 a,b; 1956) has isolated several conjugated forms of folic acid from a heated extract of Clostridium cylindro-sporum (CoC). Some of these compounds were formylated, most had reduced pteridine residues and several contained additional amino acids (glycine or alanine) and pentose and phosphate residues. They were all coenzymes for the inter-conversion of serine and glycine by extracts of Cl. sticklandii. Such compounds have not been isolated in sufficient quantities to be used in enzymological investigations of one-carbon unit metabolism. Much information has however been

obtained using PtH_4G as a readily available substitute for the natural folic acid coenzymes. It is uncertain whether PtH_4G exists in biological materials because methods designed to prevent its oxidation during isolation have not been used. Thus it may be necessary to modify some of the theories concerning the metabolism of C_1 -units when natural coenzymes have been examined in detail. The functions of folic acid in the metabolism of one-carbon units are essentially the transfer of hydroxymethyl and formyl groups at the same level of oxidation, their interconversion between these oxidation levels and also their reduction to methyl groups. Only a brief and incomplete survey can be given here.

A. Methyl Group Formation and Hydroxymethyl Group Transfer

The synthesis of choline and thymidyllic acid.

PtH_4G serves as the folic acid cofactor for the synthesis of choline from formaldehyde and aminoethanol or dimethylaminoethanol apparently via formylated derivatives (Venkataraman & Greenberg, 1958; Nyc, 1956).

The participation of folic acid in the formation of thymidine from deoxyuridine by soluble extracts was first indicated by the inhibitory effect of aminopterin on this reaction and the stimulatory effect of N^5 -formyl PtH_4G (Friedkin & Roberts, 1956). Subsequently a requirement for

Pth_4G has been reported for the formation of thymidine or thymidylic acid from serine or formaldehyde and deoxyuridine or deoxyuridylic acid by soluble extracts of thymus gland, bone marrow, E.coli and Streptococcus faecalis (Phear & Greenberg, 1957; Greenberg & Humphreys, 1958; Blakley, 1957 a; Friedkin & Kornberg, 1957; Birnie & Crosbie, 1958; McDougall & Blakley, 1960); ATP , Mg^{2+} and DPNH_2 were also required. The overall reaction is similar to homocysteine methylation; it has been assumed that $\text{N}^5, \text{N}^{10}$ -methylene Pth_4G is the final C_1 -donor and there is evidence that, for thymidine formation, C_1 -transfer and reduction occur simultaneously with the liberation of dihydropteroylglutamic acid. The formation of 5-hydroxymethylcytidylic acid from formaldehyde and cytidylic acid by extracts of phage-infected E.coli also requires Pth_4G as a cofactor (Flaks & Cohen, 1958).

Carbons 8 and 9 of the purine ring arise from C_1 -methyl
Hydroxymethyl Pth_4G and serine biosynthesis.

The interconversion of serine and glycine was indicated by the isolation of a mutant of E.coli having an alternate requirement for one of these amino acids (Roepke, Libby & Small, 1944). Early nutritional and isotopic tracer studies with animals and micro-organisms confirmed the existence of this reaction and also the participation of folic acid. Preparations of serine transhydroxymethylase have now been purified from several sources; Pth_4G and certain other folic

acid derivatives and pyridoxal phosphate are essential cofactors and the reaction mechanism has been postulated (Kisliuk & Sakami, 1955; Blakley 1957b,c; Metzler, Ikawa & Snell, 1954). N^5, N^{10} -methylene PtH_4G and glycine are presumed to be the substrates for serine formation although N^5 - or N^{10} -hydroxymethyl PtH_4G could be the intermediate donors (Blakley, 1959).

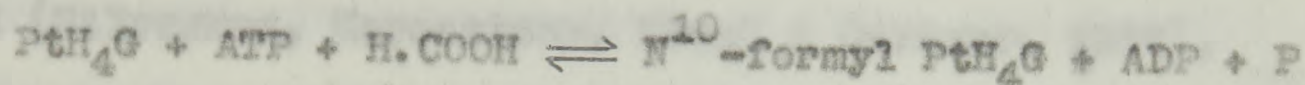
N^5, N^{10} -methylene PtH_4G is formed either spontaneously by the chemical reaction between formaldehyde and PtH_4G (Kisliuk, 1957; Blakley, 1959, 1960) or enzymically from the same reactants (Osborn, Vercamer, Talbert & Huennekens, 1957).

B. Reactions at the Formyl Level of Oxidation

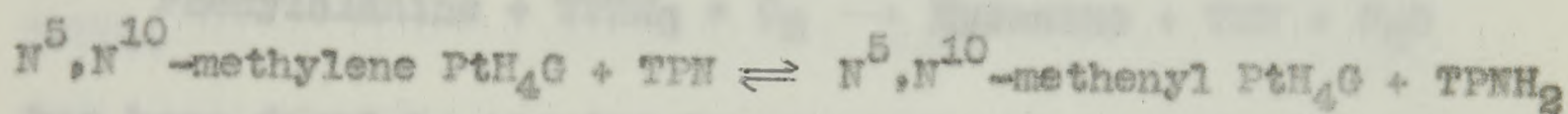
Biosynthesis and degradation of purines and histidine

Carbons 2 and 8 of the purine ring arise from C_1 -units (Buchanan, 1960; Greenberg & Jaenicke, 1957). The enzymes concerned with the incorporation of these C_1 -units have been extensively purified; they are glycinamide ribotide transformylase and amino-imidazole carboxamide ribotide transformylase (Warren, Flaks & Buchanan, 1957). The immediate C_1 -donors are N^{10} -formyl PtH_4G or N^5, N^{10} -methenyl PtH_4G ; N^5 -formyl PtH_4G , which is at a lower energy level, is active only after conversion to one of the former compounds with the aid of ATP, and N^5 -formyl PtH_4G isomerase or cyclodehydrase

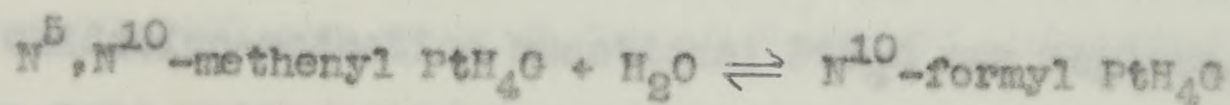
(Greenberg, 1954; Peters & Greenberg, 1958). The C_1 -units can also be obtained from formate with PtH_4G formylase (Greenberg & Jaenicke, 1957).



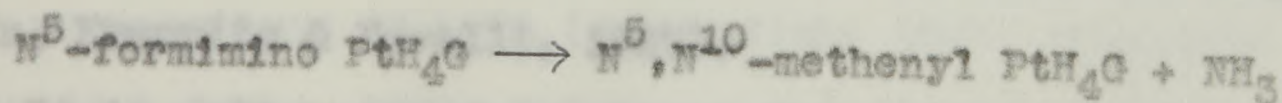
or by the interconversion of C_1 -units derived from other metabolic reactions (Table 1, Fig.3) e.g. N^5, N^{10} -methylene PtH_4G dehydrogenase (Osborn & Huennekens, 1957).



and N^5, N^{10} -methenyl PtH_4G cyclohydrolase (Rabinowitz & Pricer, 1956a)



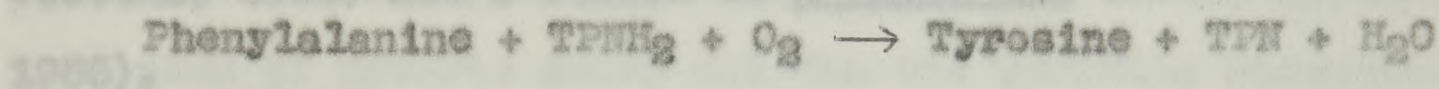
The degradation of histidine (by animals and bacteria) and purines (by bacteria) yields N -formiminoglutamic acid and N -formiminoglycine. In the presence of PtH_4G and formimino transferase, N^5 -formimino PtH_4G is produced (Tabor & Rabinowitz, 1956; Rabinowitz & Pricer, 1956b) and these formimino groups finally enter the formyl group pool by the action of N^5 -formimino PtH_4G cyclodeaminase.



Although N^{10} -formyl PtH_4G and N^5, N^{10} -methenyl PtH_4G are the most important formylating agents, N^5 -formyl PtH_4G functions directly in the reversible formation of N-formylglutamic acid (Silverman, Keresztesy, Koval & Gardner, 1957).

C. Other Metabolic Functions of Folic acid

The oxidation of phenylalanine to form tyrosine thus



has been found to require two enzymes and a pteridine cofactor which are present in liver (Kaufman, 1959; Kaufman & Levenberg, 1959). The function of the cofactor appears to be in linking the oxidation-reduction reactions; PtH_4G can replace, though it is less active than the natural cofactor which appears to be a pteridine. Folic acid derivatives also function in pantothenic acid synthesis and the biosynthesis of valine and leucine (see review by Hutner, Nathan & Baker, 1959). There is also evidence for a function of the vitamin in the catabolism of glycine (Nakada & Weinhouse, 1953; Nakada & Sund, 1958); and, PtH_4G has been found to stimulate the phospho-elastic cleavage of pyruvate by extracts of E. coli (Chin, Krampitz & Novelli, 1957).

It is well known from clinical observations of anaemias that folic acid and cobalamin have a close metabolic inter-

relationship. There is evidence for a function of folic acid in cobalamin formation and vice versa; examination of the structure of cobalamin (Fig.1) reveals the presence of many residues which might be products of folic acid function. Cobalamin is a sulphonamide reversing factor (Shive, 1950) and it spares the p-AB requirement of a mutant of E.coli (Davis, 1952), a thermophilic bacillus (Baker, Hutner & Sobotka, 1955) and a strain of Acetobacter (Brown & Rainbow, 1956).

and it is resistant to inhibition by aminopterin and

Inhibitory Analogues of Folic acid

Aminopterin and amethopterin (4-amino PtG and 4-amino, 10-methyl PtG) are amongst the most potent antagonists of folic acid which have been synthesised; they are inhibitors for both animals and micro-organisms and for a time they showed promise as an effective treatment for clinical and experimental leukemias (Stokstad, 1954). Specific inhibition of the biosynthesis of purines, thymidine, methionine and N^5 -formyl PtH_4G has been demonstrated in a number of systems.

Of particular interest is the formation of N^5 -formyl PtH_4G from PtG which occurs in animals, bacteria and cell-free preparations of both (Sauberlich, 1949; Broquist, Stokstad & Jukes, 1951; Heisler & Schweigert, 1955) and is inhibited by the folic acid antagonists (Nichol, 1956).

Pyridine-nucleotide-linked enzymes which convert PtG to PtH_4G via PtH_2G have now been isolated and both steps are sensitive to the antimetabolites (Futterman, 1957; Zakrzewski & Nichol, 1958; Peters & Greenberg, 1958; Osborn & Huennekens, 1958). An enzyme however which catalyses the conversion of PtG , N^{10} -formyl PtG , PtG_3 and N^{10} -formyl PtG_3 to the dihydropteridine level has been purified from Cl. sticklandii (Wright & Anderson, 1958). This reduction is linked to the oxidation of pyruvic acid, not via the pyridine nucleotides, and it is resistant to inhibition by aminopterin and amethopterin.

CHAPTER 5

THE PRESENT INVESTIGATION

The most important points relevant to the research to be described which emerge from the previous work are:

- 1) Washed suspensions of many strains of E.coli can synthesise methionine from homocysteine; serine is the most important source of the methyl group and using the appropriate auxotrophic strain requirements for p-AB (folic acid), cobalamin and vitamin B₆ can be demonstrated for this reaction.
- 2) Soluble extracts of acetone-dried E.coli PA15 (a serine or glycine requiring auxotroph) also catalyse the formation of methionine from homocysteine and serine. The folic acid cofactor can be supplied by an extract of the heated organism (EHC) but cobalamin has no effect on the rate of methionine formation.
- 3) The EHC could only be replaced by Pth₄G if the organisms had first been grown in the presence of cobalamin; the addition of cobalamin to the reaction mixture had no effect. A heat-labile, non-diffusible factor (X) can be isolated from organisms which have been grown in the presence of cobalamin and this factor (X) enables the utilisation of Pth₄G as a cofactor for the synthesis of methionine by extracts of acetone-dried organisms grown without added cobalamin.

4) The formation of methionine by ultrasonic extracts of E.coli 121/176 (a cobalamin or methionine requiring auxotroph) is dependent upon the addition of cobalamin to the reaction mixture with either EHC or PtH_4G as the cofactor for C_1 -transfer.

The object of the present work was to investigate further the role of cobalamin and folic acid in the synthesis of methionine by ultrasonic extracts of E.coli. Although much of the work was carried out concurrently, the experimental results have been divided into three sections.

In the first place (Section I), ultrasonic extracts of a number of prototrophic and auxotrophic strains of E.coli were examined for their ability to synthesise methionine under conditions similar to those described by Helleiner & Woods (1956) and Helleiner, Kisliuk & Woods (1958). Particular attention was paid to the effect of cobalamin on the reaction when the source of folic acid cofactor was PtH_4G or EHC; serine and formaldehyde were used as C_1 -donors. The other requirements for methionine synthesis were also investigated in detail.

Section II is concerned with the function of cobalamin and inhibitory analogues of cobalamin were employed in an attempt to elucidate the nature of its action. A system, independent of methionine synthesis, was developed for

studying the formation of factor (X) by ultrasonic extracts incubated in the presence of cobalamin.

Guest. The interrelationship between folic acid and cobalamin is examined in greater detail in Section III in an attempt to define more clearly the mechanism of methionine synthesis and to shed light on the role of cobalamin. Folic acid antagonists were used, several synthetic derivatives of PtG and PtG₃ were tested for their cofactor activities and the isolation and characterisation of the folic acid cofactors present in the EHC was attempted.

The first chapter of each experimental section deals with the general techniques and special chemicals used in that and subsequent sections.

The experimental results of each section will be discussed immediately and finally they will be correlated in a concluding discussion.

This research is a contribution to the study of the synthesis of methionine by Escherichia coli which has been in progress for several years in this laboratory; some of the present results have been published in the Biochemical Journal.

Guest, J.R. (1959). The Relationship between Cobalamin and Methionine Synthesis in Escherichia coli PA15. Biochem. J. 72, 5P.

- Guest, J.R. & Jones, K.M. (1960). Tetrahydropteroyltri-glutamate as a Cofactor of Methionine Synthesis. *Biochem. J.* 75, 12P.
- Guest, J.R., Helleiner, C.W., Cross, M.J. & Woods, D.D. (1960). Cobalamin and the Synthesis of Methionine by Ultrasonic Extracts of Escherichia coli. *Biochem. J.* 76, 396.
- Guest, J.R. (1960). Inhibition of Methionine Synthesis in Escherichia coli by Analogues of Cobalamin. *Biochem. J.* 76, 405.
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EXPERIMENTAL RESULTS

EXPERIMENTAL SECTION I

THE SYNTHESIS OF METHIONINE BY ULTRASONIC EXTRACTS OF *Escherichia coli*

Materials

Throughout this work *Escherichia coli* has been used exclusively for studying methionine formation. Several different strains both prototrophic and auxotrophic have been examined and these are listed with their growth factor requirements and origins in Table I.1. Details of the organisms used in microbiological assays are given in Appendix I. Stock cultures of all organisms were subcultured monthly and stored at 5° . No subculture was used for longer than one week.

CHAPTER 1

METHODS

General Techniques

The usual precautions necessary in microbiological studies were observed throughout, particularly with regard to the purity of chemicals and the cleanliness of the glassware. Chemicals were of the best quality available; any special details concerning individual chemicals and their preparation will be given in the appropriate section and in Appendix 1. Hot chromic acid was employed as cleaning agent, the glassware being rinsed thoroughly with resin-deionised water followed by glass-distilled water.

Organisms

Throughout this work Escherichia coli has been used exclusively for studying methionine formation. Several different strains both prototrophic and auxotrophic have been examined and these are listed with their growth factor requirements and origins in Table I.1. Details of the organisms used in microbiological assays are given in Appendix 3. Stock cultures of all organisms were subcultured monthly and stored at 3°. No subculture was used for longer than one week.

TABLE I.1

Strains of Escherichia coli used in the present work

<u>Strain of E.coli</u>	<u>Growth factor requirements</u>	<u>Parent strain</u>	<u>Source</u>
<u>Prototrophs</u>			
W	None	-	Prof.B.D.Davis
518 (A.T.C.C.No.9723)	None	-	Dr.R.Roblin
<u>Auxotrophs (produced by u.v.-irradiation)</u>			
PA15	Serine or glycine	Unknown	Dr.B.E.Wright
121/176	Cobalamin or methionine	W	} Prof.B.D.Davis (Davis & Mingioli, 1950)
113/3	Cobalamin or methionine	W	
3/62	Methionine	518	This laboratory

Media

Full details of the composition of all media used are given in Appendix 2. They were usually stored and dispensed at twice the final concentration and diluted to the desired volume with water and test solutions. Unless stated otherwise such additions were made before sterilization by autoclaving. A little carbon tetrachloride-toluene mixture (50% of each v/v) was added as preservative and media were stored at 5°.

Assessment of growth

Except where otherwise stated the extent of growth of cultures was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) in 6mm. tubes with a neutral density filter; the uninoculated medium was used to give the zero setting. The relationship between galvanometer reading and dry weight (mg./ml.) was determined for each strain of *E. coli* and found to be linear up to an EEL reading of 55. The dry wt./ml. corresponding to a reading of 10 varied only from 0.31 to 0.36mg. for the different strains of *E. coli*.

a 10hr. culture (37°) from the glucose and salts medium A supplemented with the appropriate growth factor. Both trays and Roux bottles were incubated for 48hr. at 37°. The organisms were harvested, washed twice with water (140ml./tray; 45ml./Roux bottle), and finally suspended in 0.05M-potassium phosphate buffer pH 7.2. The yield was generally 300mg. dry wt. organisms/Roux bottle or 1.8g. dry wt. organisms/tray.

Growth of Organisms and Preparation of Cell-free Extracts

Growth of organisms

The basal medium for bulk growth of strains of E.coli was the glucose, lactate and salts medium B₂. The medium was supplemented with DL-methionine (0.5 mM) for strains 121/176; 113/3 and 3/62 and with glycine (10 mM) for strain PA15. After addition of 3% (w/v) agar (BDH) and sterilisation (121°; 10 min.) the medium was dispensed either in Roux bottles (100ml.) or in enamelled trays (38cm. x 25cm. x 3cm. sterilised at 121° for 15 min.; 600ml.). In some experiments cobalamin replaced methionine as a growth factor for strain 121/176 or was added to the complete medium for strain PA15; it was added as a solution (sterilised by Seitz filtration) to the autoclaved medium after it had cooled to 60°.

The inoculum for a Roux bottle was about 1.5mg. dry wt. of organisms from a tryptic meat agar slope harvested after incubation for 16hr. at 37°. Each tray received about 3mg. dry wt. of organism from a 16hr. culture (37°) from the glucose and salts medium A supplemented with the appropriate growth factor. Both trays and Roux bottles were incubated for 44hr. at 37°. The organisms were harvested, washed twice with water (140ml./tray; 45ml./roux bottle), and finally suspended in 0.05M-potassium phosphate buffer pH 7.8. The yield was generally 300mg. dry wt. organisms/Roux bottle or 1.3g. dry wt. organisms/tray.

Preparation of the ultrasonic extract

25-30ml. samples of the washed suspensions containing approximately 75mg. dry wt. organisms/ml. of phosphate buffer were subjected to ultrasonic vibration (25 Kc./sec., 600 watts) for 4min. in a 50ml. stainless steel conical flask to the base of which the magneto-strictor was brazed. The exterior of the flask was cooled with running tap-water so that the contents remained below 20° throughout. The ultrasonic generator was type E7590B of Mullard Ltd., London, W.C.1. After centrifuging at 0° (25,000g. 10 or 15 min.) the supernatant fluid was dialysed overnight against 0.05M-phosphate buffer pH 7.8 at 3° using 50ml./ml. of supernatant fluid with two changes. The dialysed preparation was centrifuged to remove any precipitated protein. The ultrasonic extracts were normally used within two days of preparation since that from strain 121/176 progressively lost activity (50% in 3 weeks) when stored at either 2° or -15°; the preparation from strain PA15 was more stable (15% loss in 3 weeks).

Estimation of protein concentration

The protein concentration of extracts was measured spectrophotometrically at 280 and 260mμ according to Layne (1957).

Treatment of extracts with Dowex-1 resin

Residual folic acid coenzymes were removed from dialysed extracts by passing through a short column of washed Dowex-1

resin (chloride form, X8, 100-200 mesh) at 3° immediately before use. Approximately 1cm. of column (diameter 1.3cm.) was used per 50mg. of protein treated. The extract was washed through with water; there was a negligible loss of protein although the 280/260m μ ratio of the extract was slightly higher.

Extract of heated *Escherichia coli*

Washed suspensions (170mg. dry wt./ml.) of the strain under test were heated 3 min. at 100°, cooled and centrifuged (25,000g; 15 min.). The supernatant fluid, the extract of heated *E. coli* (EHC) which contains natural folic acid coenzymes was stored at -15°, for periods up to 8 weeks with no apparent loss of activity. Such preparations were standardised by adjusting the optical density at 260m μ to 100; this gave a dry wt. of 14mg./ml. and the solution contained 0.1 μ moles L-methionine/ml.

Test for Methionine Synthesis

Reaction mixture and conditions for methionine synthesis

The solution used for testing methionine synthesis by ultrasonic extracts (based on the work of Helleiner, Kisliuk & Woods, 1958) was Reaction Mixture I and contained (in 8ml. of 0.125M-phosphate buffer pH 7.8): L-serine, 10 μ moles; DL-

homocysteine, 10 μ moles; glucose, 20 μ moles; MgCl_2 , 10 μ moles; adenosine triphosphate (ATP), 10 μ moles; diphosphopyridine nucleotide (DPN), 1 μ mole; pyridoxal phosphate, 0.5 μ mole. In experiments with the formaldehyde derivative of tetrahydropteroylglutamate ($\text{CH}_2\text{O-PtH}_4\text{G}$) as the C_1 -donor the amount of DL-homocysteine was reduced to 5 μ moles and pyridoxal phosphate was omitted.

When added, EHC was an amount equivalent to 17mg. dry wt. of original organisms. The amount of ultrasonic extract used as a routine contained 10mg. protein. Small variations in the reaction mixture were occasionally made and will be mentioned in the appropriate experiments.

The complete reaction mixture was incubated at 37° under an atmosphere of H_2 . Originally, Thimberg tubes were used but later, adapted "Kilner" fruit-preserving jars containing pre-heated glycerol and fitted with sections to hold 125x16mm. hard glass test-tubes were found to be satisfactory. The period of incubation was 2 or 4hr. and methionine synthesis was stopped by heating at 100° for 3 min.; the reaction mixture was centrifuged to remove precipitated protein, and the supernatant fluid used for the assay of methionine.

Precautions were taken to exclude light in experiments with 5,6-dimethylbenzimidazolylcobamide-coenzyme.

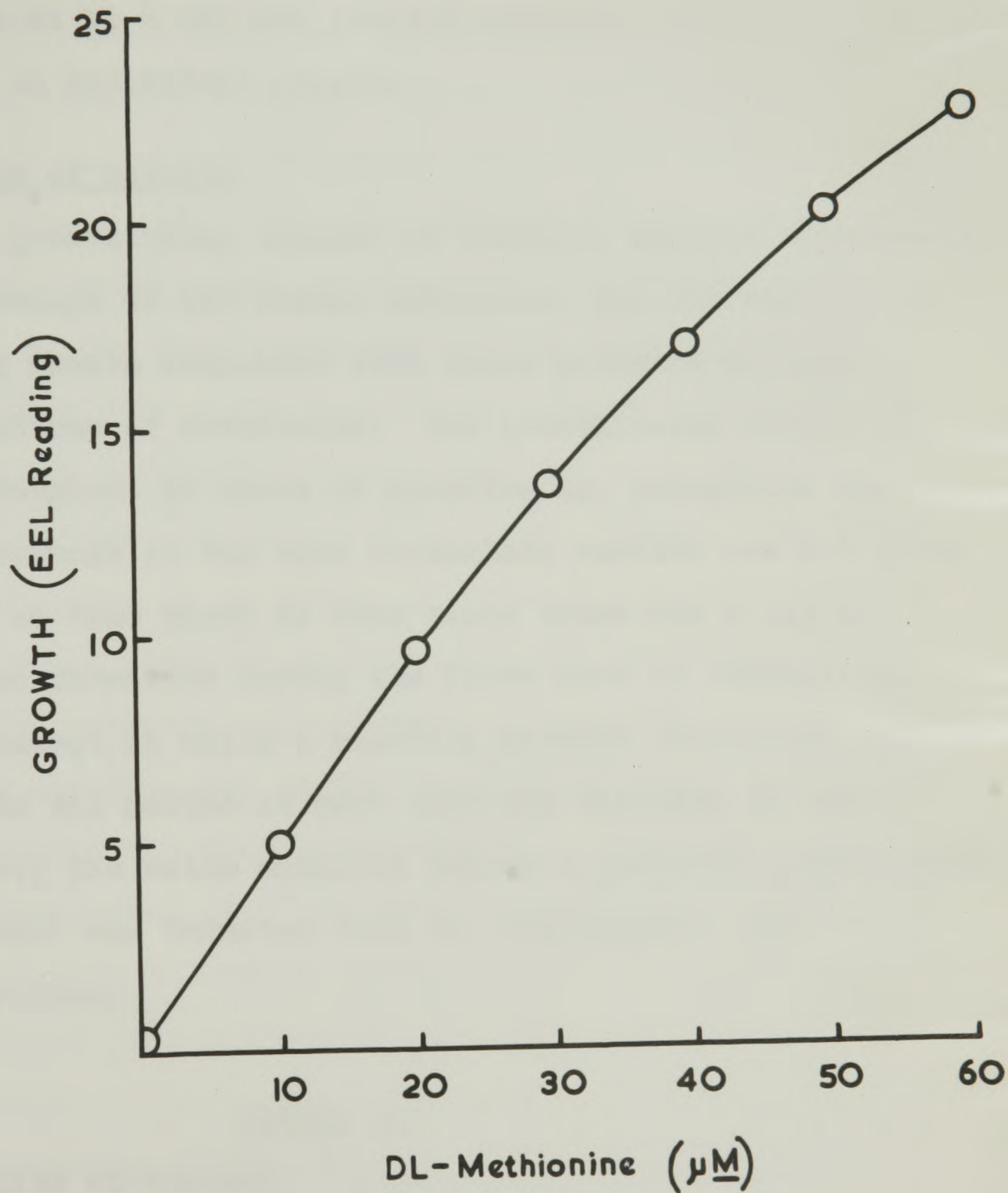
The microbiological assay of methionine with *Leuconostoc mesenteroides* P60

The method described by Gibson & Woods (1960) was followed. A linear reproducible growth response for the organism was obtained with DL-methionine ($10^{-5}M$ to $4 \times 10^{-6}M$) using medium C (amino acids, glucose, vitamins and salts) containing a final concentration of $3 \times 10^{-7}M$ pyridoxal (Fig.6). The double strength medium C was distributed in 1ml. quantities in sterile hardglass tubes (125x16mm.) and three or four samples of each reaction mixture supernatant added with water to 1.9ml. Duplicate standards were included in each assay containing 20,40,60,80,100 and 120 μ m-moles of DL-methionine. After autoclaving (115° ; 7 min.) the tubes were inoculated with organisms prepared from a tryptose glucose broth (Medium S_3) culture incubated 16hr. at 37° . The organisms were harvested, resuspended in water and diluted 50-fold in sterile pyridoxal ($4 \times 10^{-6}M$); to provide the inoculum (0.1ml./tube). The inoculated tests were incubated in air at 37° for 40-44 hr. and growth assessed with an EEL Colorimeter using the uninoculated medium to obtain the zero setting.

The specificity of this organism has been studied by Gibson & Woods (1960) and Kisliuk & Woods (1960) and it has been found to respond only to L-methionine. The components of Reaction Mixture I had no effect on the response of the organism to methionine, nor had any of the special chemicals

FIG. 6

Response of *Leuconostoc mesenteroides* P60 to DL-methionine



used in^{the} course of this research when tested at concentrations equivalent to those in the samples of the reaction mixture to be assayed. The over-all accuracy of the assay has been estimated to be $\pm 10\%$ but greater accuracy than this can be obtained in individual assays.

Expression of results

The L-methionine content of reaction mixture supernatants is the average of the values determined for the samples by comparing growth responses with those produced by known concentrations of methionine. The L-methionine formed is given throughout in terms of $\mu\text{m-moles/mg. protein}$ of the enzymic extract in the time incubated; results are not given per unit of time since in some cases there was a lag in methionine formation during the first hour of incubation.

A control, in which a reaction mixture containing all components was heated at zero time, was included in each experiment; the value obtained (about 4 $\mu\text{m-moles L-methionine/mg. protein}$) was deducted from the experimental and other control values.

Chemicals

Details of the more important chemicals used will be described in the first chapter of each Experimental Section. Details of other chemicals can be found in Appendix 1.

Tetrahydropteroylglutamic acid (PtH₄G)

This was prepared by the catalytic hydrogenation of commercial pteroylglutamic acid (folic acid) in glacial acetic acid with platinic oxide as catalyst; the method of O'Dell, Vandenbelt, Bloom & Pfiffner, (1947). The platinic oxide was prepared by the method of Adams, Voorhees & Shriner (1941). Pteroylglutamic acid (PtG, 1g.) was reduced in 100ml. glacial acetic acid using 400mg. of catalyst. To prevent the reduction of the p-aminobenzoyl residue the reaction was stopped after 80% of the theoretical amount of H₂ had been taken up; the catalyst and unreacted PtG were removed by filtration under hydrogen with a sintered disc. The PtH₄G was precipitated in an excess of ether (peroxide-free) and washed several times by centrifuging and resuspension in ether. The solvents were removed by standing over anhydrous CaCl₂ and paraffin wax in the presence of solid KOH in vacuo; after flushing the dessicator with N₂. PtH₄G was stored under H₂ in a dessicator at 0° in small corked tubes fitted with syringe needles to reduce contact with air when opened. It was dispensed from one sample tube into vials fitted with rubber seals to enable the introduction of H₂ and solvents so that solutions could be prepared under anaerobic conditions. When added to the reaction mixture for methionine synthesis, PtH₄G was prepared as a 0.01 M solution in 1.0 M-phosphate

buffer pH 7.8 under H_2 and dispensed with a calibrated syringe. PtH_4G (solid) was stored up to 8 weeks with no loss of activity although it slowly darkened to a pale brown colour. The purity of the product was assayed by three methods.

- 1) Titration with iodine. The reaction of PtH_4G with excess iodine for 5 min. (in 0.04 M-phosphate buffer pH7.5, under petroleum ether) and back-titration of the residual iodine with standard sodium thiosulphate gave an estimated purity of 103% with respect to the reducing power of the labile H atoms.
- 2) Binding of formaldehyde. The method of Kisliuk (1957) was used; PtH_4G was treated with an excess^{of} formaldehyde (standardised with dimedone) and the unreacted formaldehyde was collected by passing through a column of Dowex-1 resin (chloride form, X8, 100-200 mesh). Estimation of the formaldehyde with Nash (1953) reagent showed that 70% of the PtH_4G was capable of binding one molecular equivalent of formaldehyde.
- 3) Spectrophotometry. Solutions of PtH_4G are unstable in air and rapidly lose cofactor activity for C_1 -transfer (Blakley, 1957, b). The first product of oxidation is mainly dihydropteroylglutamic acid (PtH_2G) which is further degraded to a number of pterin and pteridine compounds (only a small proportion being PtG). Measurement of the absorption

spectrum of a solution of PtH_4G in 50mM-phosphate buffer pH7.5 with a Cary Recording Spectrophotometer (Model 14M-50) after a 30 sec. exposure to air shows a single absorption maximum $\lambda_{\text{max}}=299\text{m}\mu$ and a molecular extinction coefficient, $\epsilon=28,000$ cm^2/mole . Values for ϵ of 26,000-30,000 have however been obtained with different specimens. The spectrum was not greatly altered by the presence of 2-mercaptoethanol (20mM) or by pH changes from 7 to 11 with 2-mercaptoethanol present. These values agree with those recently published for PtH_4G using similar conditions; $\lambda_{\text{max}}=298\text{m}\mu$, $\epsilon=22,000$, Osborn, Vercamer, Talbert & Huennekens (1957); $\lambda_{\text{max}}=298\text{m}\mu$, $\epsilon=28,400$ in the presence of 2,3-dimercaptopropan-1-ol (BAL) 0.5mM, Blakley (1958).

Solutions of PtH_4G (0.05mM, in 0.05M-phosphate buffer pH7.5) underwent rapid spectrophotometric changes in air; in a period of 20 min., λ_{max} became 280m μ and the extinction coefficient fell to 88% of the original value. Similar changes have been reported by Osborn & Huennekens (1958), the final spectrum being similar to that of PtH_2G as reported by O'Dell *et al.* (1947) and Wright, Anderson & Herman (1958).

It would appear that although there is no satisfactory criterion of purity for this compound the material used here is similar to that prepared in other laboratories.

Spectrophotometric observations showed that extensive degradation of Pth_4G (0.04mM) could be prevented by addition of 2-mercaptoethanol (0.02M) or DL-homocysteine (0.4mM). Pth_4G was normally added last to the reaction mixture for testing methionine formation, immediately after DL-homocysteine to reduce oxidative decomposition occurring before anaerobic conditions were established. Whenever possible, the solution of Pth_4G was prepared with phosphate buffer in which the DL-homocysteine had been dissolved. In some experiments mercaptoethanol was used for stabilising the Pth_4G .

The formaldehyde derivative of tetrahydropteroylglutamic acid ($\text{CH}_2\text{O-Pth}_4\text{G}$)

This was prepared under H_2 by mixing equimolar quantities of formaldehyde and Pth_4G to give a 0.033M solution of both in 0.5M -phosphate buffer $\text{pH}7.8$ (Kisliuk, 1957). The resulting compound which is formed spontaneously is believed to be $\text{N}^5, \text{N}^{10}$ -methylene Pth_4G (Kisliuk, 1957; Blakley, 1959, 1960). This compound had a single absorption maximum at $292\text{ m}\mu$ and an extinction coefficient of 31,000 which is similar to that prepared by Blakley (1958) and to the same compound formed from formaldehyde and Pth_4G by an enzyme isolated from pigeon liver (Osborn *et al.* 1957).

Judged by changes in the absorption spectrum, $\text{CH}_2\text{O-Pth}_4\text{G}$ ($5 \times 10^{-5}\text{M}$ in 0.05M -phosphate buffer) was unstable in air (but

more stable than PtH_4G). In the presence of formaldehyde (0.01M) however the spectrum was unchanged by exposure to air. These observations suggest that formaldehyde and PtH_4G are combined in a dissociable complex. Similar observations have now been made and extended by Blakley (1960).

Cobalamin

Crystalline vitamin B_{12} (BDH) was dissolved in water and estimated spectrophotometrically using the specific extinction coefficients at the three wavelengths of maximal absorption (278, 361, 550 m μ) quoted by Brink *et al.* (1949). Solutions were stored at -15° and precautions were taken to shield them from strong light. The values given for cobalamin concentrations have been corrected for the estimated purity of 83%.

Factor B

The cobalamin analogue having no nucleotide residue was provided by Dr. E. Lester Smith. Aqueous solutions were assayed spectrophotometrically assuming that the molecular extinction coefficient in 0.01% (w/v) KCN at the absorption maximum (367 m μ) is the same as for cobalamin under the same conditions. The estimated purity was 70%.

5,6-Dimethylbenzimidazolylcobamide coenzyme

A solution of this coenzyme was kindly given by Dr. H.A. Barker and it was also assayed spectrophotometrically

(Weissbach, Toohey & Barker, 1959). Dilutions of this compound were stored at -15° and strict precautions were taken at all stages of its use to exclude light.

Other cobalamin derivatives

Aqueous solutions of pseudovitamin B₁₂, Factors A, D, F, III and the 5-methylbenzimidazole, 5,6-dichlorobenzimidazole and 2,6-diaminopurine analogues of cobalamin were provided by Dr. J.E. Ford. The chemical nature and biological properties of these members of the B₁₂ group are summarised by Kon & Pawelkiewicz (1960).

CHAPTER 2

SURVEY OF STRAINS OF *ESCHERICHIA COLI* AND CONDITIONS FOR METHIONINE SYNTHESIS

Survey of Strains of *Escherichia coli*

Several strains of *E. coli* were examined of their ability to synthesise methionine as ultrasonic extracts. Strain PA15, a serine or glycine requiring auxotroph was chosen because it had proved to be the most active for methionine synthesis in suspensions, particularly after growth in the presence of glycine (Gibson & Woods, 1960); and this strain has been much studied as acetone-dried powder extracts (by Cross, Szulmajster, Kisliuk & Woods). Two auxotrophic strains requiring methionine or cobalamin for growth were used, 121/176 and 113/3 the former was previously studied by Helleiner & Woods. In order to obtain enzymic preparations of these organisms (121/176, 113/3) which could be expected to be relatively deficient in cobalamin they were grown with methionine as supplement; concentrations only just optimal for growth were used since higher ones were likely to repress the formation of the enzyme systems under investigation (Wijesundera & Woods, 1960). Another strain (3/62), examined during the course of the work, had a growth requirement for methionine for which there was no known alternative. However it was not expected that ultrasonic extracts of this strain

would synthesise methionine. Finally two prototrophic strains (W and 518) were studied.

The synthesis of methionine was examined under three sets of conditions with regard to the nature of the C_1 -donor and the source of the folic acid cofactor; (a) serine with extract of heated E.coli (EHC), (b) serine with tetrahydropteroylglutamic acid (PtH_4G) and (c) the formaldehyde derivative of PtH_4G (CH_2O-PtH_4G) as both donor and cofactor source. Conditions (a) and (c) had been studied with ultrasonic extracts of E.coli 121/176 (Helleiner, Kisliuk & Woods, 1958) and condition (b) was used for extracts of acetone-dried powders of E.coli PA15 (Kisliuk & Woods, 1958). It was not possible to use free formaldehyde as a C_1 -donor when EHC supplied the folic acid cofactor; the formaldehyde is inactivated by spontaneous reaction with homocysteine to form 1,3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957) which is biologically inert.

As expected, extracts of strain 3/62 could not synthesise methionine under any of the three conditions (Table I.2). The results obtained with other strains are also shown in Table I.2. It was later found that there was quantitative variation in the activity of extracts prepared from the same strain, however this did not affect the validity of the results.

TABLE I.2

The synthesis of methionine by ultrasonic
extracts of strains of *Escherichia coli*

One-carbon unit donor: Source of folic acid cofactor: Cobalamin:		L-Methionine formed (μ -moles/mg. protein)					
		Serine		Serine		CH ₂ O-Pth ₄ C	
		EHC		Pth ₄ C		-	+
Strain of <i>E. coli</i>	W	26	27	4	20	5	8
	518	16	16	-	-	2	6
	PA15	100	103	5	80	4	40
	121/176	8	40	3	62	6	38
	113/3	20	30	5	14	5	8
	3/62	3	3	2	3	3	3

Reaction mixture I plus ultrasonic extracts, L-serine omitted if
 CH₂O-Pth₄C present. Cobalamin (13 μ moles,) Pth₄C (1 μ mole) and
 CH₂O-Pth₄C (3.3 μ moles) when present, EHC of the corresponding strain.
 Incubated 4hr.

In consequence, the strain chosen for further study was
 strain PA15 which possessed similar properties to the proto-
 trophic strains but gave extracts of superior activity. Of
 the cobalamin-requiring auxotrophs, strain 113/3 was chosen
 because it was more active than 113/2 and showed more charac-
 teristic responses to added cobalamin under the conditions of these
 tests. Strain 113/3 has however recently been used with

a) Serine plus extract of heated *E. coli* (EHC)

EHC served as a source of the cofactor for C_1 -transfer for all strains, but only in the case of ultrasonic extracts of strains having an alternative growth requirement for cobalamin or methionine (121/176; 113/3) was methionine formation increased by added cobalamin.

b) Serine plus tetrahydropteroylglutamic acid

PtH_4G would not replace EHC with extracts of strains W and PA15. However in the presence of cobalamin good methionine synthesis was obtained with extracts of these strains, as well as with the cobalamin-deficient extracts of strains 121/176 and 113/3.

c) Formaldehyde derivative of PtH_4G

When CH_2O-PtH_4G was used as the source of both the C_1 -units and the folic acid cofactor the synthesis of methionine by ultrasonic extracts of all strains, albeit low in some cases, required cobalamin.

In consequence, one strain chosen for further study was strain PA15 which possessed similar properties to the prototrophic strains but gave extracts of greatest activity. Of the cobalamin-requiring auxotrophs, strain 121/176 was chosen because it was more active than 113/3 and showed more clear-cut responses to added cobalamin under the conditions of these tests. Strain 113/3 has however recently been used with

success by Hatch, Takeyama & Buchanan (1959) for studying the role of cobalamin in methionine formation.

An overall variation of activity of about 3-fold in different preparations of the same extract was found. This may reflect the participation of a number of enzymes with varying stability in the overall reaction; the work of Emerson (1950) with mutants of Neurospora suggests that the reaction may be complex. Ultrasonic extracts of E.coli PA15 were examined in greatest detail because (a) the activity was as a rule subject to the least variation, (b) the stability on storing was greater than for extracts of strain 121/176 and (c) comparison was possible with parallel studies using extracts of acetone-dried powders of strain PA15 being made at the same time by Dr. R.L. Kisliuk and later by Dr. K.M. Jones and Mr. M.A. Foster (extracts of acetone-dried powders of strain 121/176 were only weakly active for methionine formation; Helleiner unpublished observations).

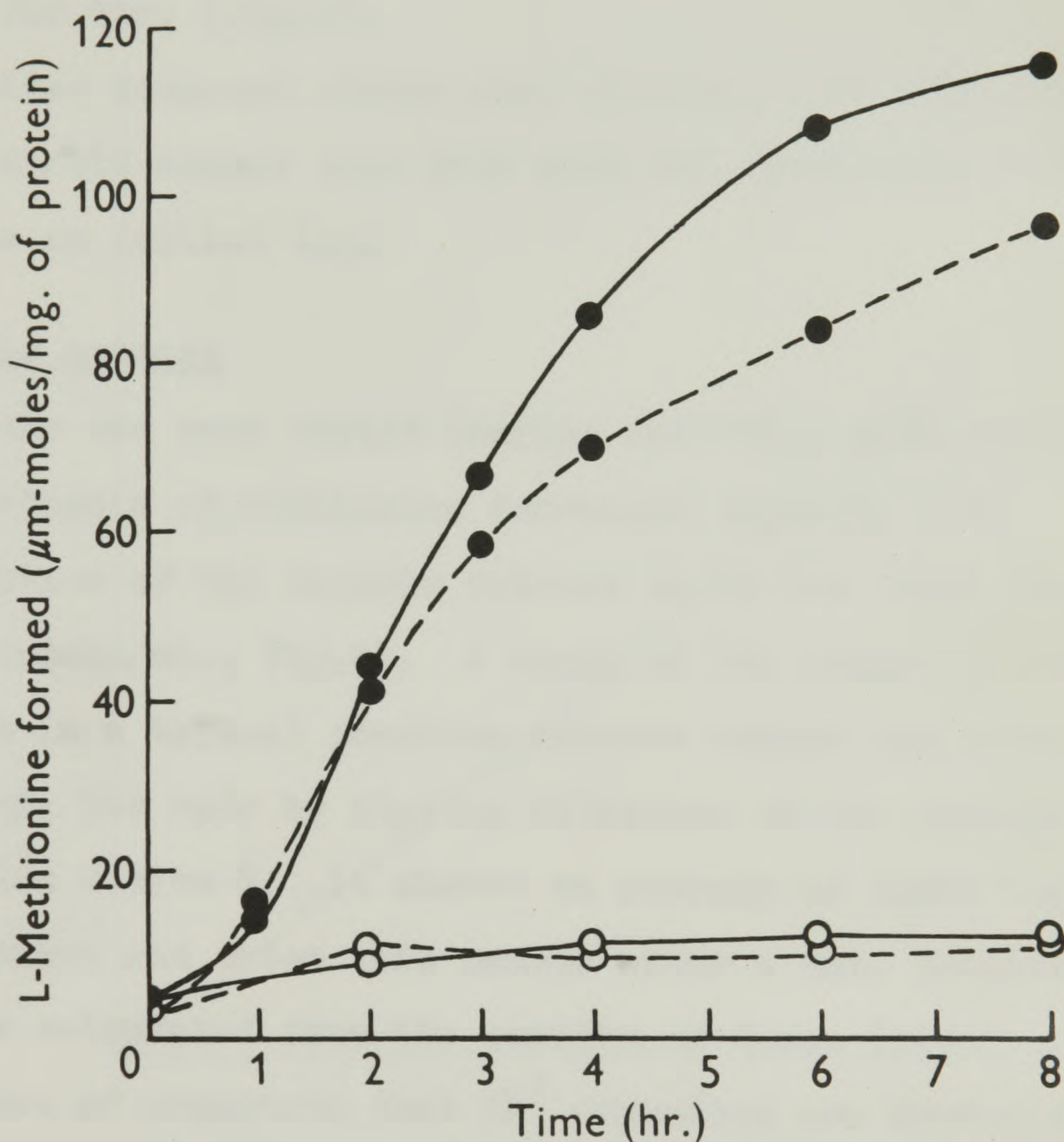
Conditions for Methionine Synthesis

Progress of methionine formation

The rate of methionine synthesis with strain PA15 and Pth₄G as the folic acid cofactor showed an initial lag of about 1hr. before it became almost linear up to 4hr. (Fig.7). The lag period was usually reduced if a much larger quantity

FIG. 7

The progress of methionine formation by ultrasonic extracts of *Escherichia coli* PA15



- , Serine C₁-donor. Reaction mixture I with enzymic extract (strain PA15) and PtH₄G (1μmole).
- - -, Formaldehyde (as CH₂O-PtH₄G, 3.3μmoles) as C₁-donor. Reaction mixture I (without L-serine and pyridoxal phosphate and with DL-homocysteine reduced to 5μmoles) plus enzymic extract. Cobalamin (65μmoles) present (●) or absent (○).

FIG. 8
 of cobalamin (about 1 μ m-mole) was used although the maximum rate of methionine formation was not necessarily increased. When EHC supplied the folic acid cofactor (no cobalamin required) there was no lag period and synthesis was proportional to time for 2hr. (Fig.9).

Similar progress curves were obtained with extracts of strain 121/176 except that even with EHC (cobalamin required) there was no initial lag.

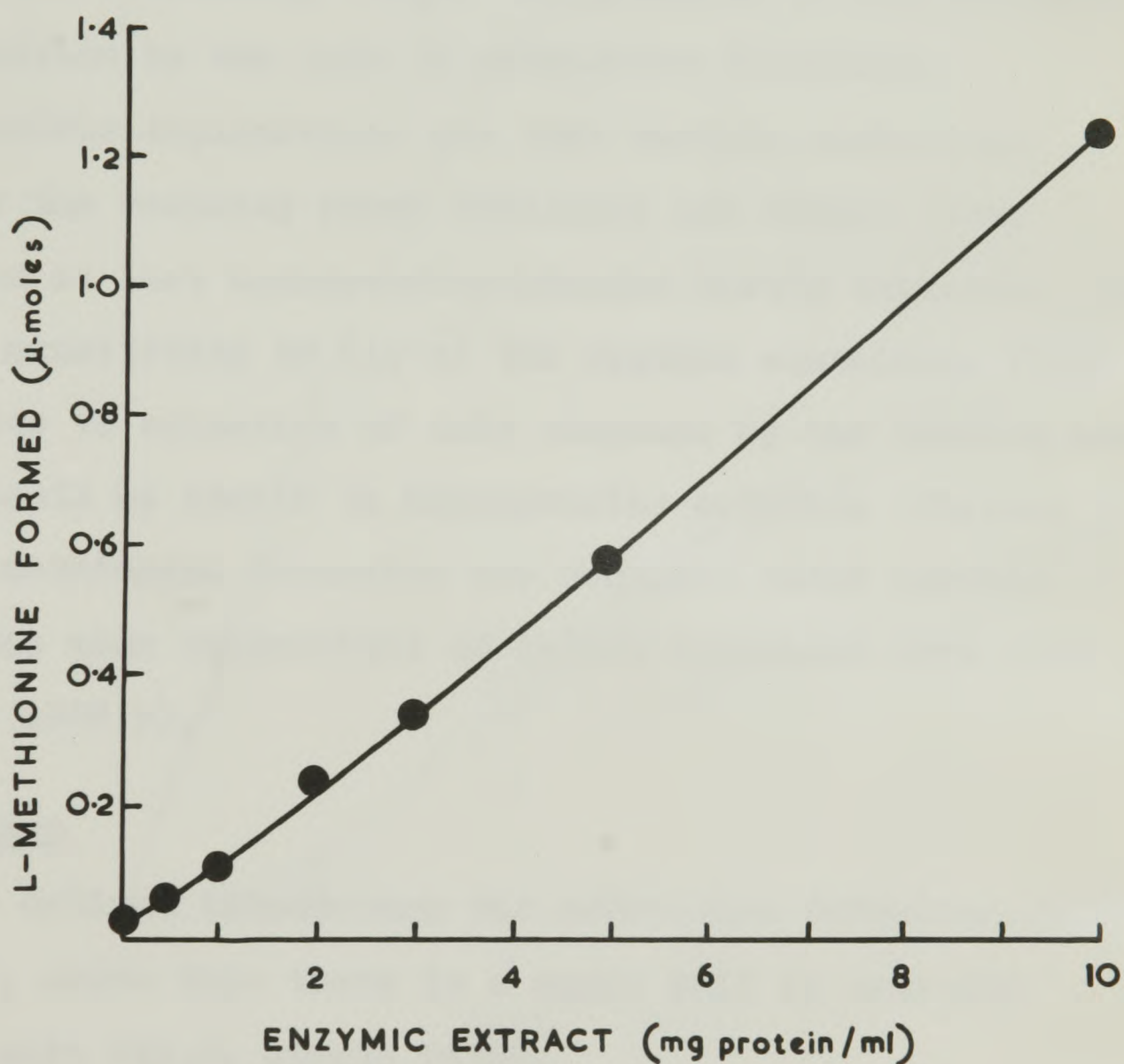
Ultrasonic extract

In the one case tested (serine and Pth₄G with strain PA15) synthesis of methionine increased linearly with concentration of the enzymic extract up to the limit tested (10mg. protein/ml., Fig.8). A count of the number of viable organisms in a typical reaction mixture before and after incubation, was made by plating dilutions of the mixtures on the complex Medium S. It showed an average of 5,500 organisms to be present and twice this number after a 4hr. incubation. It can be calculated from the results of Cross (1956) using suspensions of organisms, that 10⁷ organisms are needed for the formation of 1 μ m-mole L-methionine under similar circumstances; so there is no possibility of the synthesis being due to viable organisms.

The supernatant fluid from centrifugation (105,000xg for 60 min. at 0°) of the extract showed all the original activity for methionine synthesis, and an increase in activity per mg. protein of 7%.

FIG. 8

Effect of concentration of enzymic
extract on methionine synthesis



Reaction mixture I plus ultrasonic extract (strain PA15),
PtH₄G (1μmole) and cobalamin (65μμmoles). Incubated 4hr.

Aerobic incubation

With EHC as the source of folic acid cofactor (strain PA15) the initial rate of methionine synthesis was not affected by aerobic incubation, but the maximum synthesis was reduced to 70% of that obtained under anaerobic conditions (Fig.9). For systems containing Pth_4G , the presence of air caused a 40% reduction in the rate of methionine formation.

Possible explanations are that aerobic conditions decrease the reducing power available for methyl group formation or that homocysteine becomes partly oxidised. The greater sensitivity to air of the systems containing Pth_4G may be due to oxidation of this compound by the extract, because Pth_4G should be stable in homocysteine solution (Chapter 1). Optimal methionine formation was obtained under aerobic conditions when suspensions of intact organisms were used (Gibson, 1952 b).

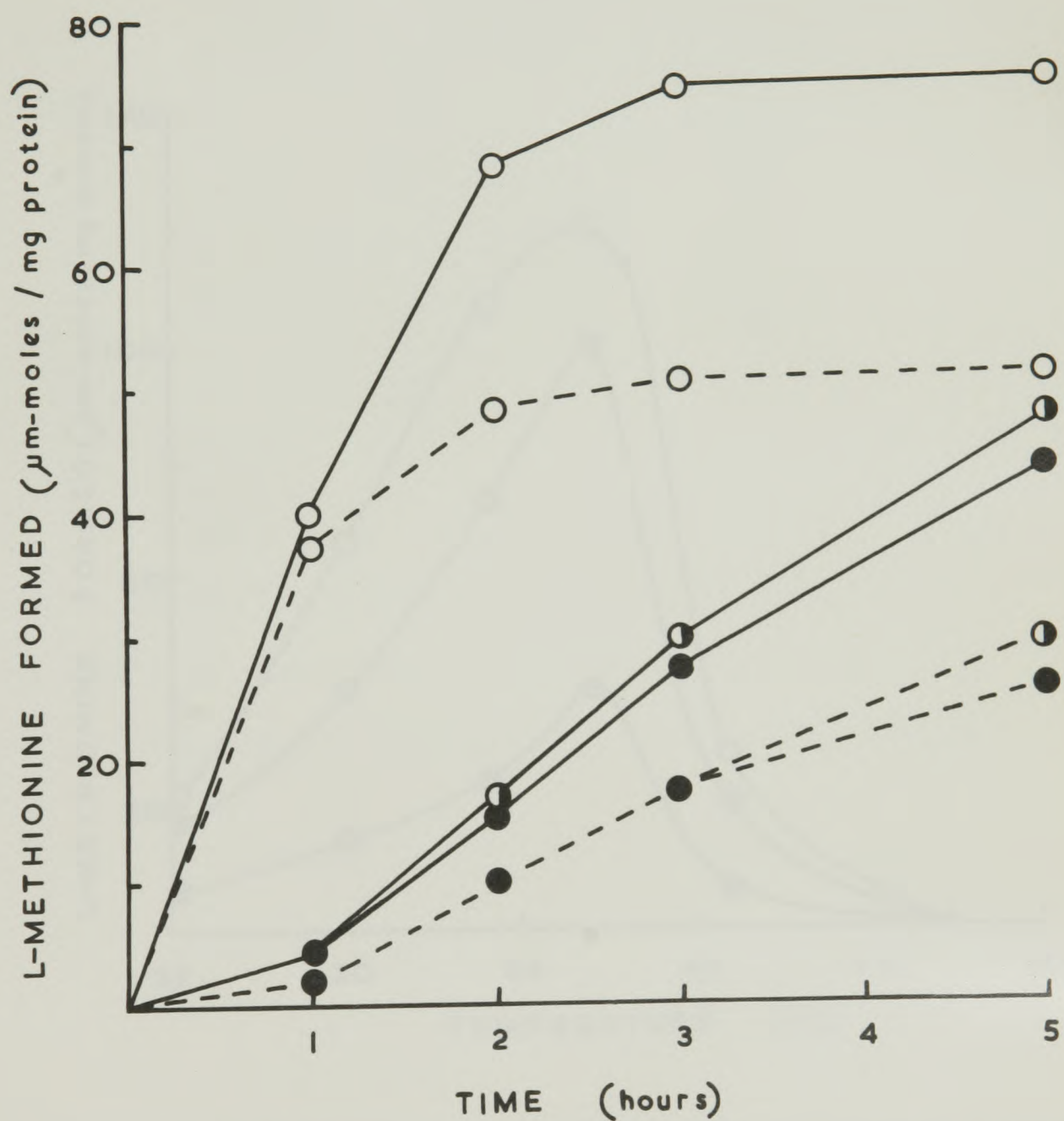
Temperature

The optimum temperature for methionine formation is 37° (Fig.10); above this there is a rapid fall in activity (serine with Pth_4G , strain PA15).

Hydrogen ion concentration

Using a range of phosphate buffers having different pH values it was found that methionine formation increases with increasing pH to pH7.6 (Table I.3). The initial pH of a

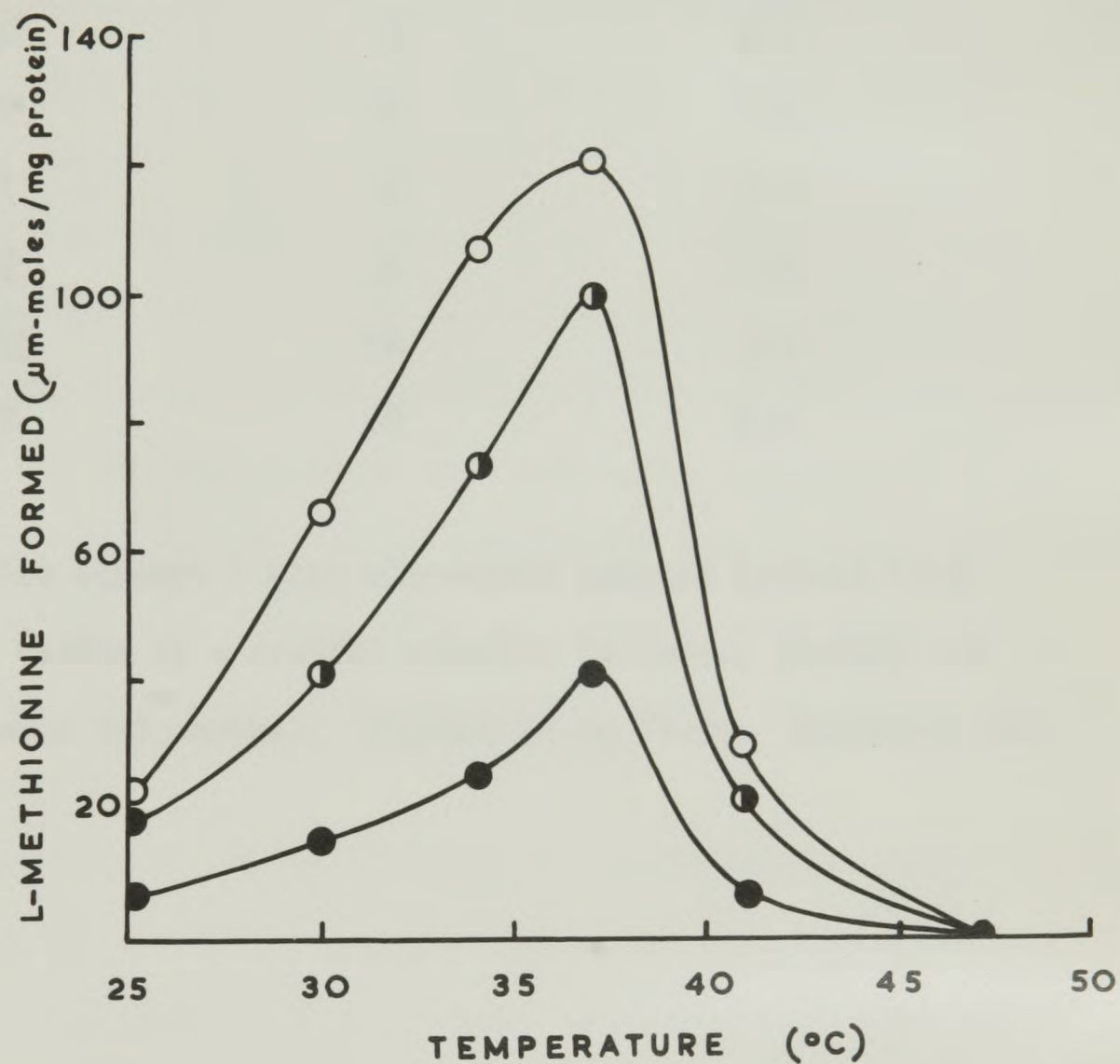
FIG. 9

Effect of aerobic incubation on methionine synthesis

Reaction mixture I plus Dowex-1-treated extract (strain PA15) with (O), EHC; (◐), PtH₄G (1μmole), cobalamin (65μμmoles) and, (●), CH₂O-PtH₄G (3.3μmoles, serine and pyridoxal phosphate omitted). Incubated in air (---), and under H₂ (——).

FIG. 10

Effect of temperature on methionine synthesis



Reaction mixture I plus ultrasonic extract (strain PA15)
 PtH_4G ($1\mu\text{mole}$), cobalamin ($65\mu\mu\text{moles}$). Incubated
 2 (●), 4 (◐) and 6hr. (○).

TABLE I.3

The effect of pH on methionine synthesis
by ultrasonic extracts of *Escherichia coli* PA15

<u>Initial pH</u>	<u>L-Methionine formed</u> <u>(μ-moles/mg.protein)</u>	<u>Final pH</u>
6.2	2	5.9
7.0	16	6.8
7.3	24	7.1
7.5	35	7.3
7.65	50	7.2
7.8	52	7.2

Reaction mixture I plus ultrasonic extract (strain PA15)

Pth₄C (added as a neutral solution in water, 1 μ mole) and

cobalamin (65 μ moles). Initial pH as shown. Incubated 4hr.

5) The synthesis of methionine by ultrasonic extracts of strain PA15 is directly proportional to the amount of extract protein added, and optimal synthesis occurs at 37° when the initial pH is 7.6-7.8 and in an atmosphere of H₂.

completed reaction mixture using phosphate buffer pH7.8 was pH7.65.

Summary

- 1) Ultrasonic extracts of strains of Escherichia coli not requiring methionine for growth (PA15, W,518) can synthesise methionine from homocysteine and serine, under certain conditions, when a heated extract of the organism supplies the folic acid coenzyme. When PtH_4G is the folic acid cofactor or when $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ is both donor of the C_1 -unit and the cofactor, cobalamin is essential for methionine synthesis.
- 2) Extracts of methionine or cobalamin requiring strains of E.coli (121/176, 113/3) need cobalamin for optimal methionine synthesis under all three sets of conditions, when the extracts are prepared from organisms grown on media devoid of the vitamin.
- 3) The synthesis of methionine by ultrasonic extracts of strain PA15 is directly proportional to the amount of extract protein added, and optimal synthesis occurs at 37° when the initial pH is 7.6-7.8 and in an atmosphere of H_2 .

CHAPTER 3

COBALAMIN IN METHIONINE SYNTHESIS

The catalytic effect of cobalamin in the synthesis of methionine by ultrasonic extracts of E. coli was first observed with strain 121/176 using EHC as the source of folic acid cofactor (Helleiner & Woods, 1956). Later, cobalamin was also found to promote methionine synthesis in extracts of the same strain when serine and EHC were replaced by $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ (Helleiner, Kisliuk & Woods, 1958). These observations have now been extended to include several other strains and also a third condition of synthesis where EHC is replaced simply by PtH_4G (Chapter 2).

Cobalamin Concentration

In Chapter 2 it was shown that cobalamin is essential for the synthesis of methionine by ultrasonic extracts, with PtH_4G as the folic acid cofactor (strains PA15 and 121/176) and with EHC as the source of folic acid cofactor (strain 121/176, but not strain PA15). A typical example of the response of extracts (strain 121/176) to increasing cobalamin concentration is given in Table I.4; similar concentrations ($14\mu\text{M}$) caused half-maximal activation with either EHC or PtH_4G supplying the folic acid cofactor. The concentration

TABLE I.4

Effect of the concentration of cobalamin on methionine synthesis by extracts of *Escherichia coli* 121/176

Cobalamin added (μ moles)	L-Methionine formed (μ -moles per mg. protein) when source of CoF is	
	EHG	Pth ₄ G
0	6	3
13	18	25
27	25	40
65	40	62
130	43	80
325	44	88

Reaction mixture I plus ultrasonic extract and either EHG or Pth₄G (1 μ mole). Incubated 4hr. using very high concentrations of ultrasonic extract and incubation for only 4hr.

Analogues and Derivatives of Cobalamin

A wide variety of forms of vitamin B₁₂ differing in the presence or absence of and the nature of the corrinoid residue have been isolated from natural sources and many more have been produced by guided biosynthesis (see e.g. Derivatives, 1960). The ability of these compounds to replace cobalamin as a growth factor depends upon the organism in question. It

for half-maximal activation of methionine synthesis with PtH_4G or $\text{CH}_2\text{O-PtH}_4\text{G}$ in extracts of strain PA15 (about $8\mu\text{M}$) was the same for each condition (Fig.11), and was also of the same order as that required for strain 121/176.

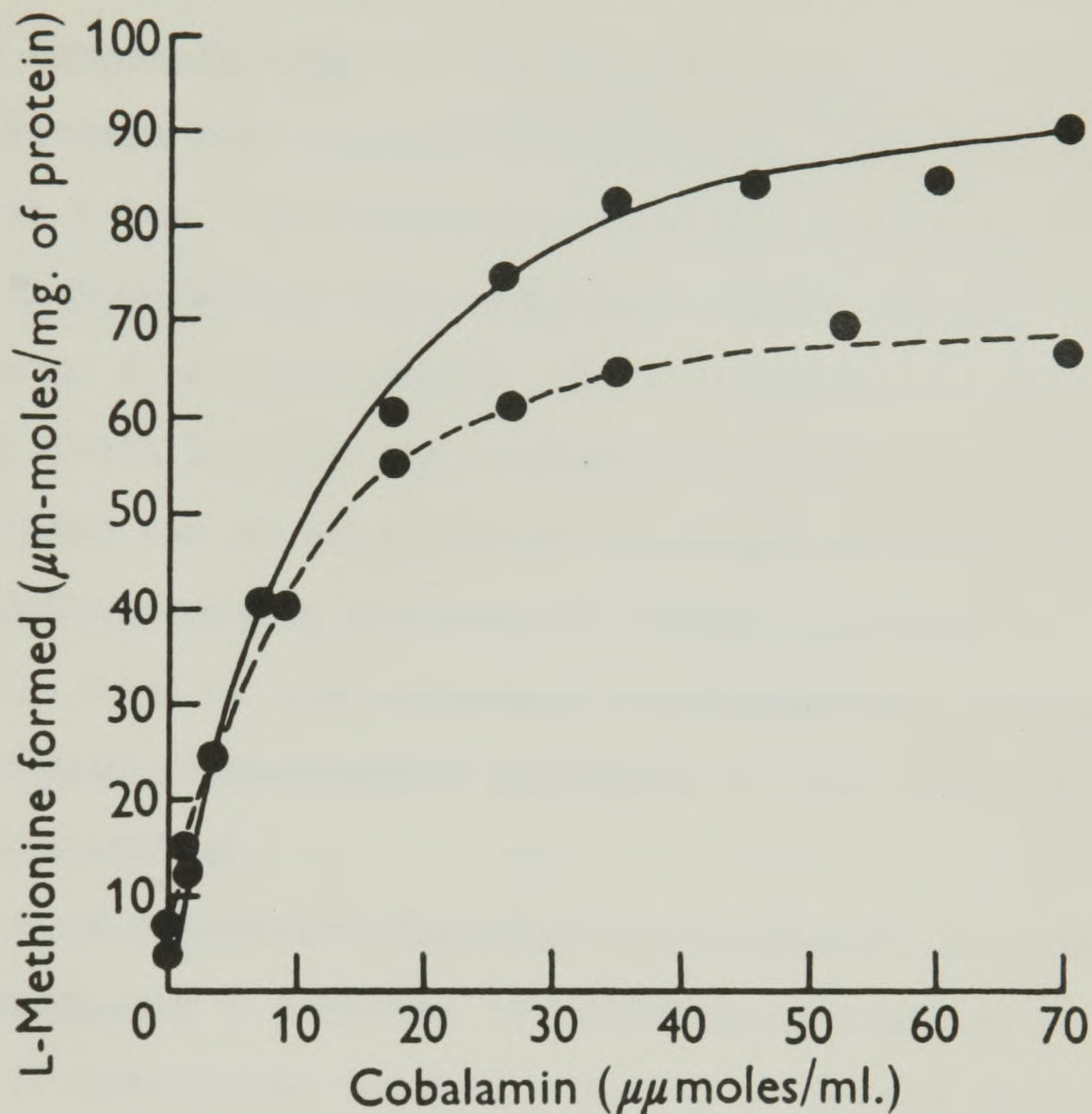
The effect of cobalamin was variable with different preparations of extracts of both strains in the sense that the higher concentrations of cobalamin did not always give further increases in methionine synthesis. The cobalamin concentration for half-maximal activation varied between $8\mu\text{M}$ and $35\mu\text{M}$. It appeared that this variability was caused by uncontrollable factors in the preparation of the extract, probably on the efficiency of ultrasonic disintegration because extracts produced over periods of several months would have similar relative activities. In some later experiments it was possible to get good methionine synthesis using very high concentrations of cobalamin ($0.65\mu\text{M}$ -moles) and incubation for only 2hr.

Analogues and Derivatives of Cobalamin

A wide variety of forms of vitamin B_{12} differing in the presence or absence of and the nature of the nucleotide residue have been isolated from natural sources and many more have been produced by guided biosynthesis (Kon & Pawelkiewicz, 1960). The ability of these compounds to replace cobalamin as a growth factor depends upon the organism in question. It

FIG. 11

Effect of the concentration of cobalamin on the synthesis of methionine by ultrasonic extracts of *Escherichia coli* PA15



—, Serine as C₁-donor. Reaction mixture I with ultrasonic extract, PtH₄G (1μmole). Incubated 4hr.

- - -, CH₂O-PtH₄G (3.3μmoles) as C₁-donor. Reaction mixture I without L-serine and modified as in Chapter 1, plus ultrasonic extract. Incubated 4hr.

is generally agreed that cobalamin or methionine auxotrophs of Escherichia coli are the least specific with regard to the type of cobalamin which satisfies their growth requirement. Most of the compounds replaced cobalamin under conditions in which that vitamin was required (Table I.5). This suggests that there is a low specificity for cobalamin; none were significantly more active than cobalamin at the concentrations tested. Factor D, a biologically inactive cobalamin pigment and Factor B (both lacking the nucleotide group) were almost inactive. There was no quantitative correlation with the relative growth-promoting activity of these compounds for E. coli 113/3. None of the compounds (0.65 μ m-moles) had any inhibitory effect on methionine synthesis in the presence of cobalamin (65 μ m-moles).

5.6- Addition of 5,6-dimethylbenzimidazole (itself inactive) together with factor B resulted in activity equivalent to 85% of that of cobalamin; further addition of ribose had no effect (Table I.6).

5,6-Dimethylbenzimidazolylcobamide coenzyme

Coenzyme forms of vitamin B₁₂ active in the metabolism of glutamate by Clostridium tetanomorphum have recently been described (Barker, Weissbach & Smyth, 1958; Weissbach, Toohey & Barker, 1959). The 5,6-dimethylbenzimidazolylcobamide coenzyme was 2-3 times as active as cobalamin (serine with

TABLE I.5

Ability of analogues to replace cobalamin

Nature of the base of the nucleotide residue	% Activity compared with cobalamin for		Growth of E. coli 113/3 (tube tests) (Kon & Pawelkiewicz, 1960)
	Methionine formation with analogue (μ moles)		
	65	650	
Absent (Factor D)	1	3	0
Absent (Factor B)	7	7	20
5-Methylbenzimidazole	5	45	70
Adenine (pseudovitamin B ₁₂)	30	100	50
2,6-Diaminopurine	55	80	20
5-Hydroxybenzimidazole (Factor III)	85	90	50
2-Methyladenine (Factor A)	100	95	50
5,6-Dichlorobenzimidazole	100	100	50
2-Methylmercaptoadenine	125	100	60

Reaction mixture I plus ultrasonic extract (strain PA15)
 and Pth₄G (1 μ mole). Incubated 4hr. (Factor D) etc.
 indicates trivial name of the complete cobalamin.

TABLE I.6

Replacement of cobalamin by factor B

<u>Additions</u>	<u>L-methionine formed</u> <u>(μm-moles/mg. protein)</u>
Cobalamin (0.65μ m-moles)	75
Factor B (0.65μ m-moles)	5
5,6-Dimethylbenzimidazole (DMB, 10μ m-moles)	1
Factor B (0.65μ m-moles) + DMB (10μ m-moles)	18
Factor B (0.65μ m-moles) + DMB (10μ m-moles) + ribose (1μ mole)	16

Reaction mixture I plus ultrasonic extract (strain PA15)

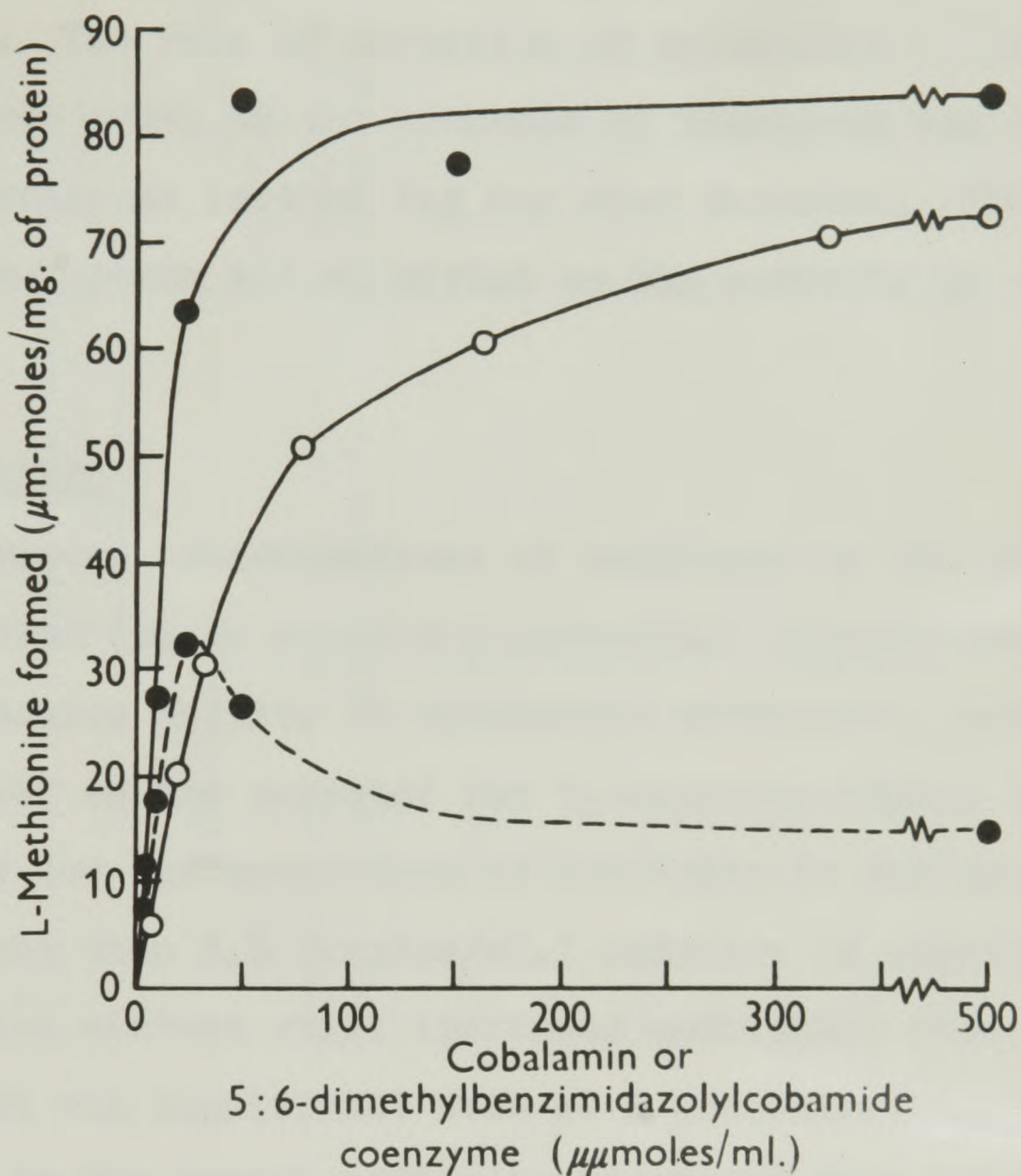
and PTH₄G (1μ mole). Incubated 4hr.

PtH₄G and strain PA15, Fig.12). Irradiated specimens of cobalamin and the coenzyme were prepared by exposing 0.3ml. of a 10 μ M solution in a silica cell for 50 min. at 5°, 6ft. distant from an 80 watt white fluorescent light. This was sufficient to cause the changes in the absorption spectrum of the coenzyme reported by Barker, Weissbach & Smyth, (1958). Irradiation had no effect on the activity of cobalamin, but greatly reduced that of the coenzyme (Fig.12). With the latter, the effect of irradiation was apparently more marked at higher concentrations than at lower suggesting the possibility that an inhibitor is formed.

In Clostridium tetanomorphum the coenzyme is needed for the interconversion of glutamate and β -methyiaspartate. The effect of L-threo- β -methyiaspartate (0.05, 0.5 and 5mM) on methionine synthesis was examined with extracts of strain PA15. It had no ability to replace cobalamin with PtH₄G as the cofactor for C₁-transfer. It did not overcome (as cobalamin does) the inhibition of the activity of EHC by PtH₄G (see Chapter 4). In addition, β -methyiaspartate had no significant effect on methionine formation when present in any of the three test conditions for strain PA15; it would not replace serine as a C₁-donor and methionine formation was not affected by growing the organism in the presence of β -methyiaspartate (1mM).

FIG. 12

Comparative activity of cobalamin and cobamide coenzyme



○, Cobalamin (with or without irradiation).
 ●, 5,6-Dimethylbenzimidazolylcobamide coenzyme,
 (—, not irradiated; ---, irradiated).

Reaction mixture I plus PtH_4G (1μmole) and Dowex-1-treated ultrasonic extract of strain PA15. Incubated 3hr.

Growth of the Organisms in the presence of Cobalamin

In all the experiments so far described, enzyme preparations were made from organisms grown in the absence of cobalamin. The rate of formation of methionine by extracts of organisms grown in the presence of cobalamin was linear for 2hr. only; no initial lag was ever observed. Treatment with Dowex-1 resin had no effect on the activity of such extracts.

Strain 121/176

Increasing concentrations of cobalamin in the original growth medium (up to about 5 $\mu\text{moles/ml.}$) yielded extracts with increasing ability to synthesise methionine, with either Pth_4G or EHC as the cofactor for C_1 -transfer (Table I.7). At relatively low concentrations of cobalamin in the growth medium (less than 3.3 $\mu\text{moles/ml.}$) addition of cobalamin to the reaction mixture still increased methionine formation. This effect was significant even at a concentration of cobalamin in the growth medium in excess of that required for optimal growth of the organism (0.65 $\mu\text{moles/ml.}$).

Strain PA15

Although this organism does not require cobalamin for growth, the effect of cobalamin in the growth medium on the activity of the subsequent extracts (Pth_4G as folic acid

TABLE 1.7

Effect of growth in the presence of cobalamin on its subsequent activity with ultrasonic extracts

Cobalamin in growth medium (μ moles/ml.)	L-Methionine formed (μ -moles per mg. protein) with strain			
	121/176 and		PA15 and	
	PtH ₄ C	ERC	PtH ₄ C	ERC
	with cobalamin Absent Present	with cobalamin Absent Present	with cobalamin Absent Present	with & without cobalamin
0	(6) (67)	(43) (43)	2 44	103
0.05	18 69	23 50	- -	-
0.26	35 75	33 62	8 48	95
1.30	71 107	57 73	26 58	92
3.25	102 124	72 80	66 72	85
6.50	125 126	81 84	85 86	72
19.50	125 129	82 82	107 107	77

() indicates methionine was added to the growth medium. Methionine was omitted from the growth medium for strain 121/176 when cobalamin was added.

Reaction mixture I with ultrasonic extract of either strain 121/176 or PA15 and PtH₄C (1 μ mole). Cobalamin 0.65 (μ -moles) when present. Incubated 2hr.

cofactor) was similar to that observed for extracts of strain 121/176 (Table I.7). However, with EHC as the source of folic acid cofactor the presence of cobalamin during growth depressed the activity of the extracts to a small extent. A similar effect was observed with extracts of acetone-dried powders (Kisliuk & Woods, 1960) and was presented as evidence in the support of there being an alternative cobalamin-dependent pathway for methionine formation.

Growth on factor B (strain PA15 only)

Factor B was unable to replace cobalamin for methionine synthesis by extracts. Extracts prepared after growth in the presence of factor B (30 μ moles/ml.) had 35% of the activity of organisms grown with the same concentration of cobalamin (serine with PtH_4G) although normal activity was obtained with EHC as the cofactor. Addition of cobalamin to the reaction mixture had no stimulatory effect.

Summary

- 1) The concentration of cobalamin producing half-maximal methionine formation in ultrasonic extracts of E.coli varies between 8 and 35 μM and is of the same order for all cobalamin-dependent conditions viz. (a) Serine plus PtH_4G , and $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ alone, for strains 121/176 and PA15; (b) Serine plus EHC for strain 121/176.

- 2) Cobalamin can be replaced by several of its nucleotide substituted analogues but not by derivatives lacking the nucleotide residue.
 - 3) 5,6-Dimethylbenzimidazolylcobamide coenzyme in 2-3 times more active than cobalamin itself; L- β -methylaspartate has no function in methionine synthesis.
 - 4) Growth of either strain in the presence of cobalamin enhances the ability of ultrasonic extracts of the organisms to methylate homocysteine, except for strain PA15 when the folic acid cofactor is provided by EHC.
- been grown in the presence of cobalamin (Kislink & Woods, 1958, 1960). PnH_3G would serve as the cofactor with ultrasonic extracts of strain 121/176; here, cobalamin was an essential component of the reaction mixture (Holloiner, Kislink & Woods, 1960). Details of the nature of the folic acid requirement are examined more closely in this chapter and in Section III.

Requirements for EHC

The formation of methionine by dialyzed ultrasonic extracts of *E. coli* was enhanced considerably by the addition of EHC (Table I.6). Treatment of the aqueous extract with Dowex-1 resin almost completely abolished the original activity; presumably by removing the traces of the natural

CHAPTER 4

FOLIC ACID IN METHIONINE SYNTHESIS

Evidence that the extract of heated E.coli PA15 (EHC) satisfied the folic acid cofactor requirement for methionine formation by dialysed enzymic extracts of the same organism was obtained by Szulmajster & Woods (1955; 1960). Tetrahydropteroylglutamic acid (Pth_4G) would not however replace the EHC when extracts of acetone-dried strain PA15 were used as the source of enzymes, unless the organisms had first been grown in the presence of cobalamin (Kisliuk & Woods, 1958, 1960). Pth_4G would serve as the cofactor with ultrasonic extracts of strain 121/176; here, cobalamin was an essential component of the reaction mixture (Helleiner, Kisliuk & Woods, 1958). Details of the nature of the folic acid requirement are examined more closely in this chapter and in Section III.

Requirement for EHC

The formation of methionine by dialysed ultrasonic extracts of E.coli was enhanced considerably by the addition of EHC (Table I.8). Treatment of the enzymic extract with Dowex-1 resin almost completely abolished the original activity; presumably by removing the traces of the natural

TABLE I.8Effect of Dowex-1 treatment and EHC
on methionine synthesis

<u>Ultrasonic</u> <u>extract</u>	<u>Strain of</u> <u>E.coli</u>	<u>L-methionine formed</u> <u>(μ-moles/mg. protein)</u>	
		<u>EHC absent</u>	<u>EHC present</u>
Dialysed	(PA15	35	80
	(121/176	15	35
Dialysed and Dowex-1-treated	(PA15	4	76
	(121/176	4	35

Reaction mixture I with ultrasonic extract,

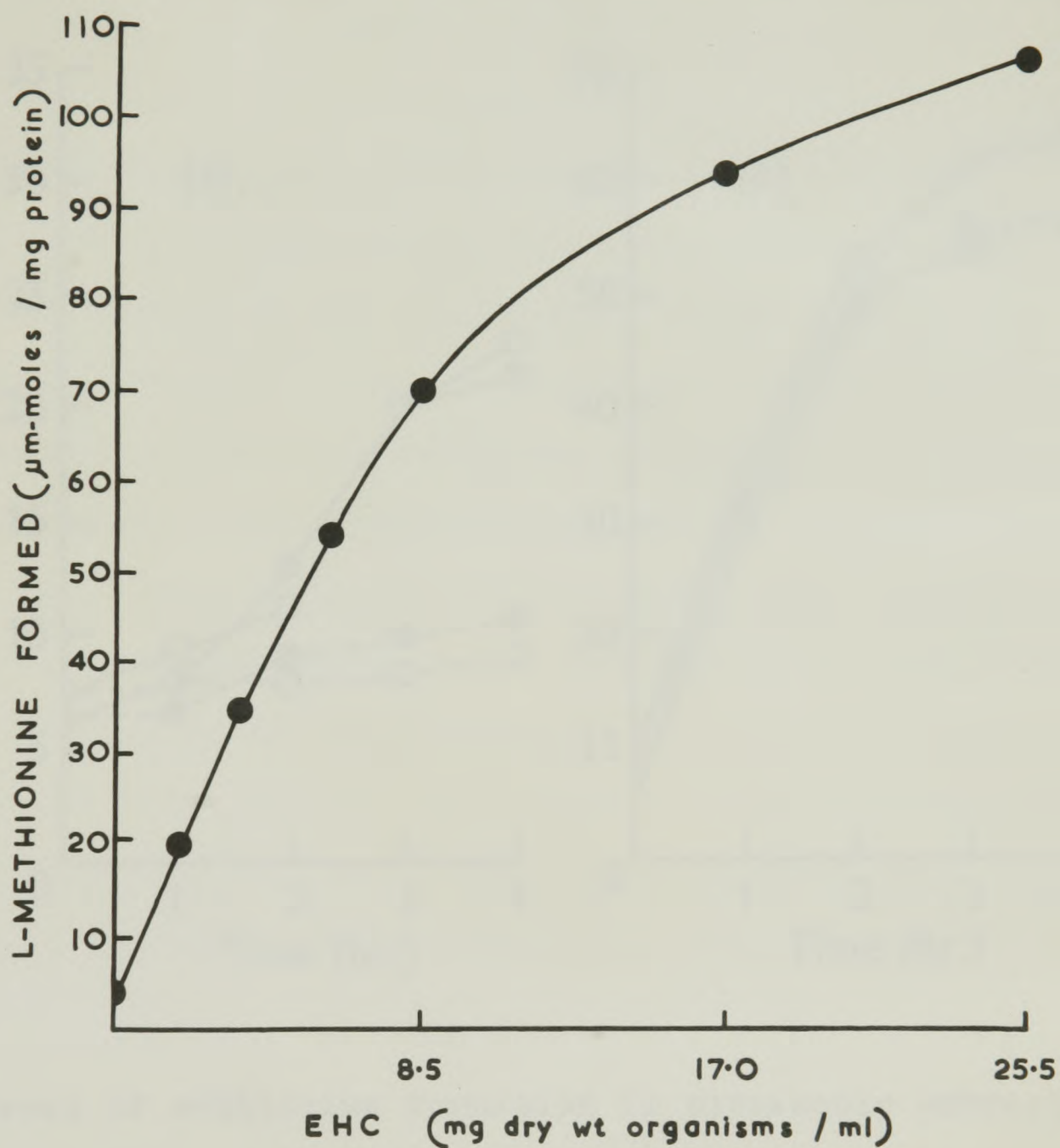
EHC as shown and cobalamin (65μ moles).

Incubated 4hr.

follic acid cofactor which remained in the extract after dialysis. Dowex-treated extracts were used routinely in subsequent investigations into the nature and function of the follic acid cofactor. The response to increasing concentrations of EHC was as shown in Figure 13. Interchanging the enzymic and heated extracts of the two strains (PA15 and 121/176) showed that dialysed and Dowex-treated ultrasonic extracts of strain 121/176 required cobalamin for methionine synthesis even if the EHC was derived from strain PA15, whereas similar enzymic extracts of strain PA15 showed no requirement for cobalamin if the source of EHC was either strain PA15 or strain 121/176 (Fig.14). This suggests that the follic acid components of the EHC of both strains are similar and that the EHC of strain PA15 does not contain cobalamin functional in methionine synthesis. Furthermore the synthesis of methionine by enzymic extracts of strain 121/176 with EHC prepared from the same strain, grown with a limiting concentration of cobalamin, was still dependent upon the addition of cobalamin. This sample of EHC contained less than 0.1 μ moles of cobalamin (assayed with Euglena gracilis). Cross (1956) also found that EHC of strain PA15 was unable to replace cobalamin in increasing the methionine formation by suspensions of whole organisms.

FIG. 13

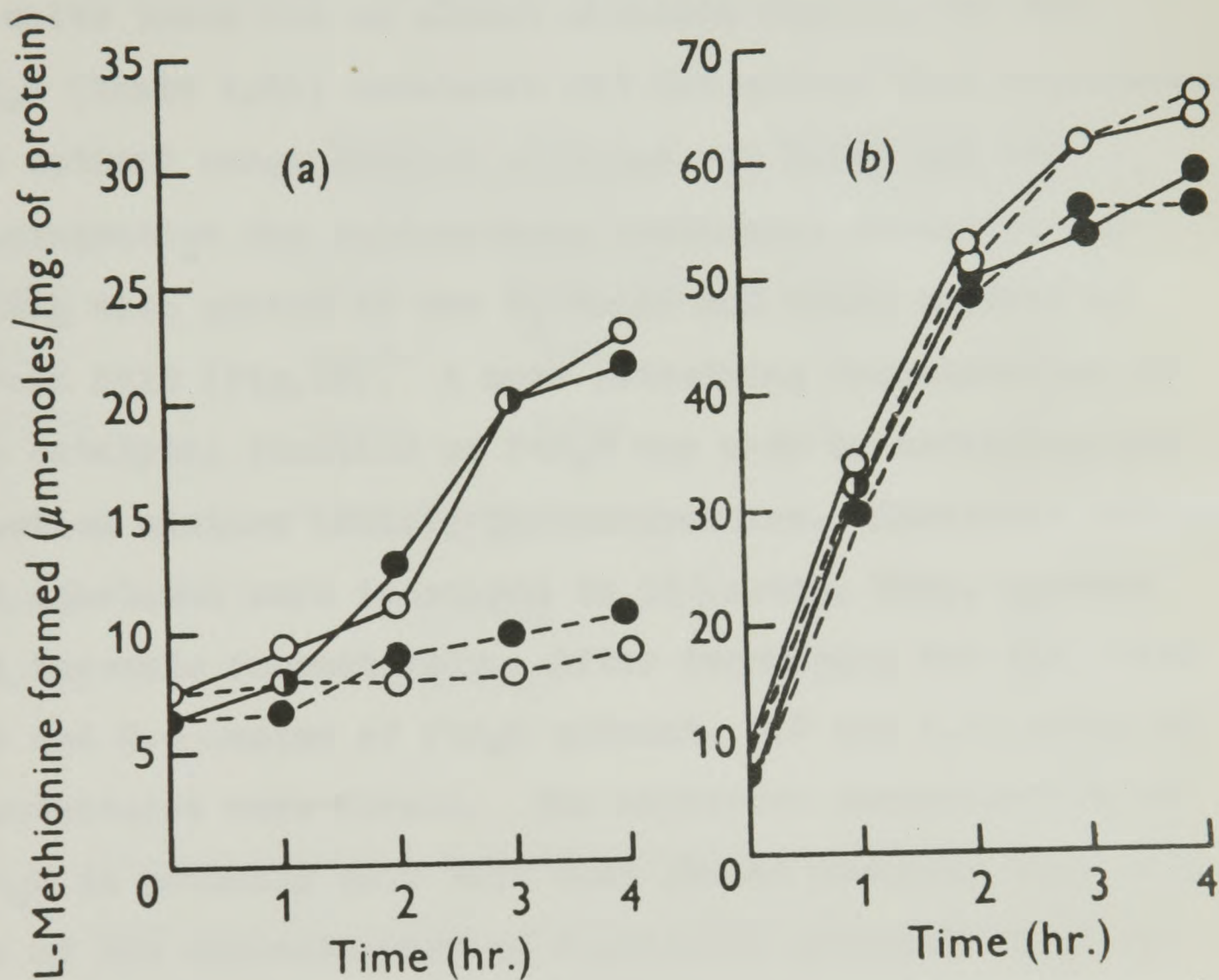
Effect of extract of heated *Escherichia coli* PA15



Reaction mixture I plus Dowex-treated extract
(strain PA15) and EHC as shown (0.1 ml. $\equiv$ 17 mg. dry wt.
organisms). Incubated 2hr.

FIG. 14

Activity of heated extracts of strains of *Escherichia coli*



Progress of methionine formation by ultrasonic extracts of (a) strain 121/176 and (b) strain PA15. Reaction mixture I plus Dowex-treated enzymic extract with: (O), EHC of strain 121/176; (●), EHC of strain PA15, and cobalamin (65μ m-moles) present (—); absent (---).

Requirement for Tetrahydropteroylglutamic acid

With either serine or formaldehyde as the source of C_1 -units there was an almost absolute requirement for PtH_4G (Table I.9); cobalamin did not affect this requirement. The optimal concentration of PtH_4G was $0.5mM$ and the concentration for half-maximum methionine formation was $0.15mM$ with serine as the C_1 -donor and using extract of strain PA15 (Fig.15). A more satisfying demonstration of the catalytic function of PtH_4G was made by modifying the reaction mixture (2ml.); DL-homocysteine, ultrasonic extract and cobalamin were increased to $15 \mu moles$, 20mg. protein and $1 \mu m$ -mole respectively. After incubating for 4hr. with 0.2 and $0.4 \mu moles$ of PtH_4G present, 1.0 and $1.4 \mu moles$ of L-methionine were formed. The effective concentration of PtH_4G is probably only half that stated assuming that only one of the stereoisomers of chemically prepared PtH_4G is active.

Inhibition of Methionine Synthesis by Tetrahydropteroylglutamic acid

The ability of EHC to promote synthesis of methionine in the absence of cobalamin was almost completely inhibited by PtH_4G at concentrations as low as $0.1 \mu mole/ml$. (Fig.15). PtH_4G also inhibits the residual activity of dialysed ultra-

TABLE I.9

Effect of tetrahydropteroylglutamic acid
on methionine synthesis by ultrasonic
extract of two strains of Escherichia coli

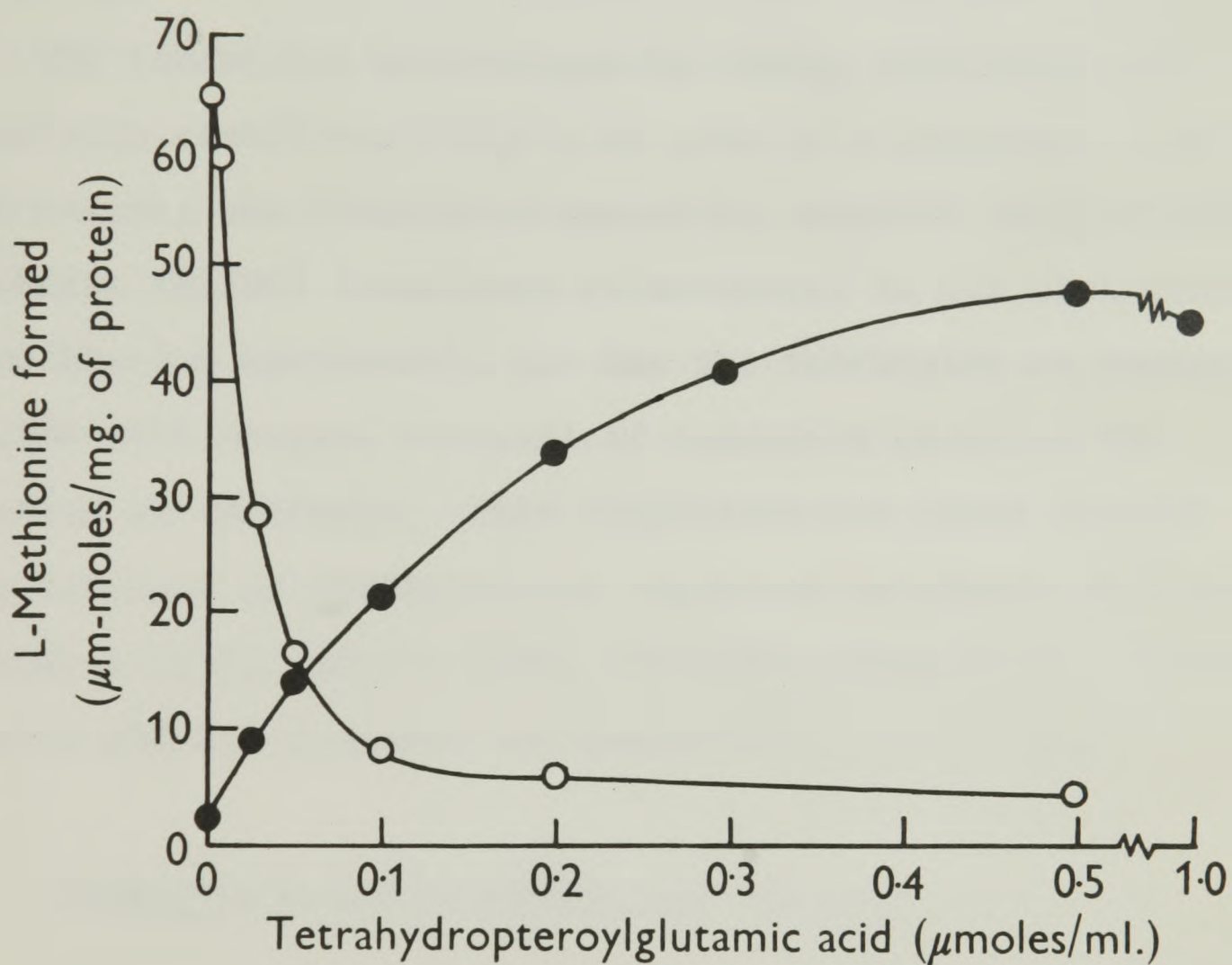
<u>Strain of</u> <u>E. coli</u>	<u>Conditions for</u> <u>methionine synthesis</u>		<u>L-Methionine formed</u> <u>(μ-moles per mg. protein)</u>	
	<u>One-carbon</u> <u>donor</u>	<u>Source of</u> <u>folic acid</u>	<u>Pth₄G</u> <u>absent</u>	<u>Pth₄G</u> <u>present</u>
PA15	Serine	Pth ₄ G	7	70
121/176	Serine	Pth ₄ G	5	30
				
PA15	CH ₂ O-Pth ₄ G		4*	68
121/176	CH ₂ O-Pth ₄ G		4*	28

Reaction mixture I with Dower-treated extract and cobalamin (13 μ moles, strain 121/176; 65 μ moles, strain PA15). Pth₄G (1 μ mole) when present and CH₂O-Pth₄G (3.3 μ moles, L-serine and pyridoxal phosphate omitted and DL-homocysteine, 5 μ moles). Incubated 4hr.

* CH₂O-Pth₄G omitted and formaldehyde (3.3 μ moles) added.

FIG. 15

Effect of the concentration of tetrahydropteroylglutamic acid on the synthesis of methionine by ultrasonic extracts of *E.coli* PA15 in the presence of either cobalamin or extract of heated *E.coli*



Reaction mixture I plus Dowex-treated ultrasonic extract supplemented with either 65 μmoles cobalamin and incubated 4hr., (●) or with EHC and incubated 2hr., (○).

sonic extracts. Experiments to determine the type of inhibition did not give conclusive evidence for competitive or non-competitive inhibition. This is probably a consequence of the crude nature of the EHC, which may contain other compounds which enhance or inhibit methionine formation.

The inhibition is overcome by adding cobalamin, and under such conditions PtH_4G also acts as a cofactor. For this reason, the inhibition cannot be observed with extracts of strain 121/176 (cobalamin is essential in all conditions of methionine synthesis), nor can the inhibition be demonstrated with enzymic extracts of organisms grown in the presence of cobalamin. This inhibition was first studied with extracts of acetone-dried organisms of strain PA15 by Kisliuk & Woods (1960); here, cobalamin added to the reaction mixture did not overcome the inhibition.

Interconversion of Serine and Glycine

1) The liberation of C_1 -units from serine is presumably due to the action of serine transhydroxymethylase. This enzyme has been studied with extracts of acetone-dried powders of strain PA15. (Kisliuk & Woods, 1960) by following the degradation and the synthesis of serine. Using the same techniques as these authors, the synthesis of serine by

ultrasonic extracts of strain 121/176 from $\text{CH}_2\text{O-PtH}_4\text{G}$ and added glycine was examined under conditions similar to those for methionine synthesis except that H_2 replaced air as the gas-phase. There was an active serine transhydroxymethylase present; equilibrium was reached in less than 10 min. so it is not possible for this enzyme to limit the relatively slow rate of methionine formation. PtH_4G and pyridoxal phosphate were essential for optimal activity, although the absence of pyridoxal phosphate only produced a 55% reduction in activity; cobalamin had no effect.

From a consideration of the requirements for methionine synthesis with serine or formaldehyde as the source of C_1 -units it is evident that PtH_4G has a dual role with the former: as cofactor for serine transhydroxymethylase and in the subsequent methylation of homocysteine. Cobalamin would appear to function at the second stage only.

Summary

- 1) Either heated extract of Escherichia coli or tetrahydropteroylglutamic acid plus cobalamin can supply the folic acid cofactor required for methionine synthesis by ultrasonic extracts of E.coli.
- 2) With extracts of strain 121/176 cobalamin is always required, even when the EHC is prepared from strain PA15.

3) Tetrahydropteroylglutamic acid inhibits methionine synthesis when EHC is the cofactor (strain PA15). The inhibition is overcome by the presence of cobalamin which also allows Pth_4G to act as a cofactor.

4) Ultrasonic extracts contain an active serine trans-hydroxymethylase which requires Pth_4G and pyridoxal phosphate. The components of the reaction mixture normally used were based on the results of Cross & Woods (1964), Hellewell & Woods (1965) and Hellewell & Woods (1966). Detailed investigations of the requirements for the various constituents have been made using Dows-treated ultrasonic extracts of strain PA15. That all components are necessary for best synthesis is shown in Table I.10. Similar extracts of organisms grown in the presence of cobalamin had the same requirements when tested in the serine plus Pth_4G system.

Source of One-carbon Units

Omission of serine or $\text{CH}_3\text{O-Pth}_4\text{G}$ reduced the synthesis of methionine to low values under all conditions (Table I.10). The concentration of serine which gave half-maximal activity (Pth_4G system) was 1.5mM (Fig. 16). With $\text{CH}_3\text{O-Pth}_4\text{G}$ half-maximal synthesis occurred on addition of 1.2 moles to the reaction mixture, optimal synthesis was obtained with 3.5 moles but higher concentrations reduced the activity probably due to interaction between formaldehyde and homocysteine.

Several compounds were tested for C_1 -donor activity with both EHC and Pth_4G as the folate acid cofactor. Compared with

CHAPTER 5

OTHER FACTORS CONCERNED WITH METHIONINE FORMATION

The components of the reaction mixture normally used were based on the results of Cross & Woods (1954), Helleiner & Woods (1956) and Kisliuk & Woods (1960). Detailed investigations of the requirement for the various constituents have been made using Dowex-treated ultrasonic extracts of strain PA15. That all components are necessary for best synthesis is shown in Table I.10. Similar extracts of organisms grown in the presence of cobalamin had the same requirements when tested in the serine plus Pth_4G system.

Source of One-carbon Units

Omission of serine or $\text{CH}_2\text{O}-\text{Pth}_4\text{G}$ reduced the synthesis of methionine to low values under all conditions (Table I.10). The concentration of serine which gave half-maximal activity (Pth_4G system) was 1.5mM (Fig.16). With $\text{CH}_2\text{O}-\text{Pth}_4\text{G}$ half-maximal synthesis occurred on addition of 1.2 μmoles to the reaction mixture, optimal synthesis was obtained with 3.3 μmoles but higher concentrations reduced the activity probably due to interaction between formaldehyde and homocysteine.

Several compounds were tested for C_1 -donor activity with both EHC and Pth_4G as the folic acid cofactor. Compared with

TABLE I.10

Effect of other components of the reaction mixture on the synthesis of methionine by strain PA15 under various conditions

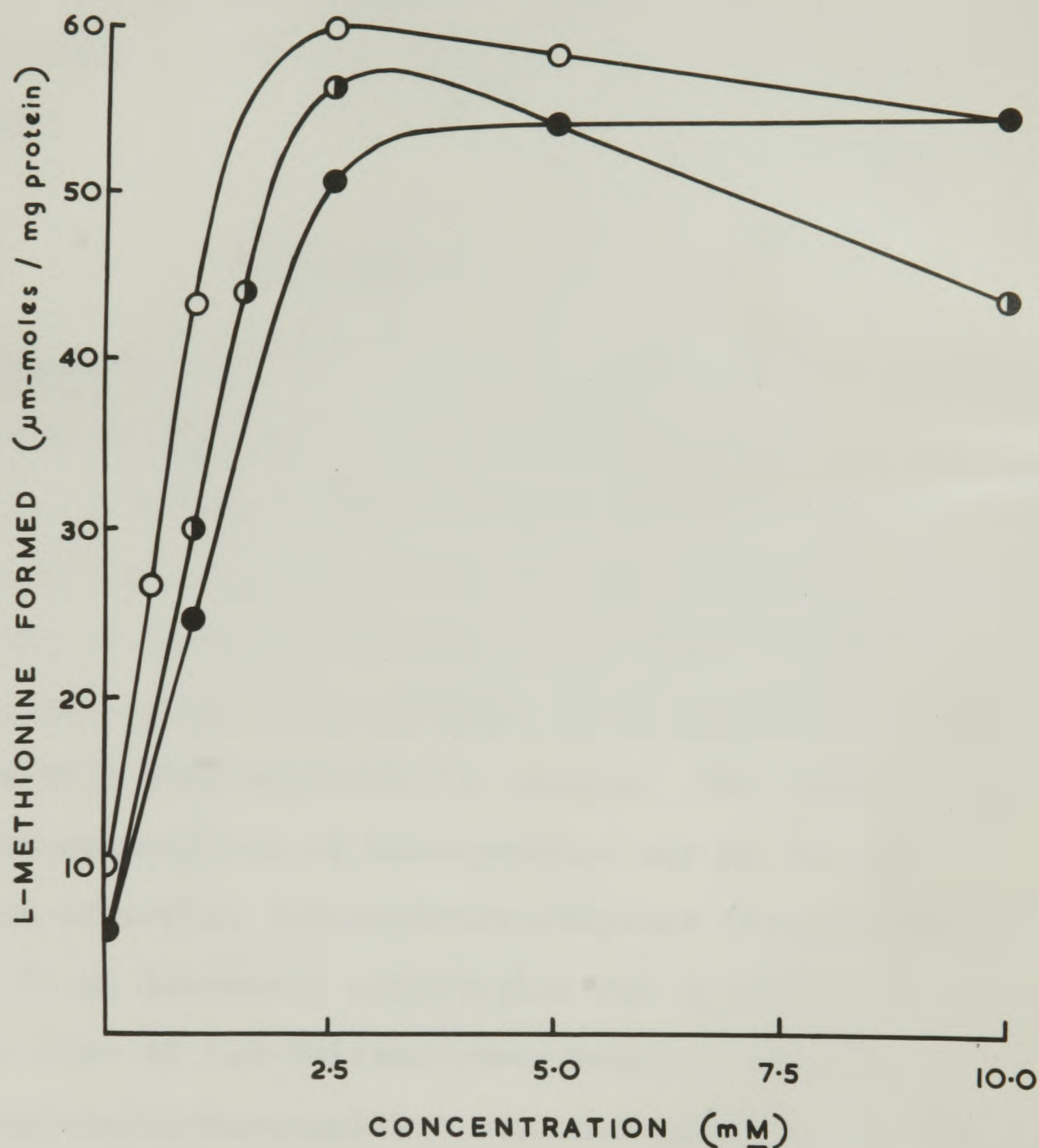
Nature of the one-carbon donor: Source of folic acid cofactor:	<u>L-Methionine formed (μm-moles/mg. protein)</u>		
	Serine EHC	Serine Pth ₄ G	CH ₂ O-Pth ₄ G
<u>Omission from reaction mixture</u>			
None	76	75	68
L-Serine	8	7	-
CH ₂ O-Pth ₄ G	-	-	7
DL-Homocysteine	4	5	8
ATP + Mg ²⁺	15	6	10
DPN	68	10	11
Glucose	39	10	15
Pyridoxal phosphate	78	62	47 [#]
DPN (TPN added)	25	11	17
None (TPN added)	61	75	68

Reaction mixture I with the appropriate modifications and Dowex-treated ultrasonic extract, EHC as in methods, Pth₄G (1 μ mole), CH₂O-Pth₄G (3.3 μ mole) and TPN (1 μ mole) when present. Cobalamin (65 μ mole) added in systems containing Pth₄G. Incubated 2hr. (EHC system); 4hr. (Pth₄G systems).

[#] pyridoxal phosphate (0.5 μ mole) added.

FIG. 16

Effect of glucose, ATP + Mg²⁺ and L-serine
on the synthesis of methionine



Reaction mixture I with appropriate omission plus Dowex-treated ultrasonic extract (strain PA15) with PtH₄G (1 μmole), cobalamin (65 μmoles) and glucose (0), ATP + Mg²⁺ (○), and L-serine (●) as shown. Incubated 4hr.

the same concentration of serine their activities as percentages were, glycine (12), formate (4), N-formylglycine(4), formimino-glycine (5), glycine + formate (16). None of the compounds were active with PtH_4G as cofactor unless cobalamin was added.

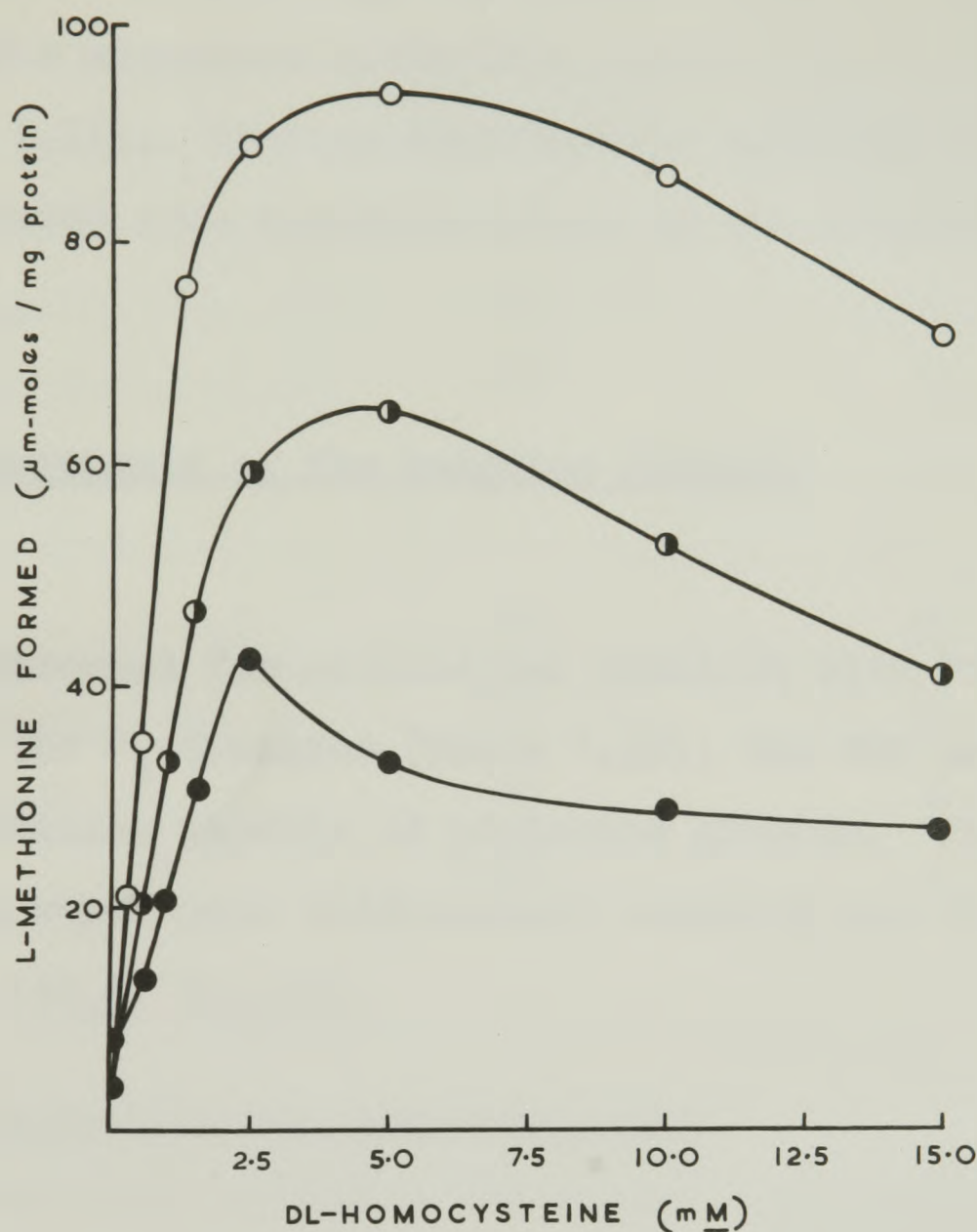
Homocysteine

Methionine formation was almost completely abolished by the omission of DL-homocysteine and half-maximal activity was obtained at a concentration of about 2mM (Table I.10, Fig.17). Optimal synthesis occurred over a restricted range of concentrations particularly when $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ was the source of C_1 -units; the optimal homocysteine concentration for $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ was half that required for serine. The lowered activity at high concentrations of homocysteine may be due to inhibition of serine transhydroxymethylase (Kisliuk & Woods, 1960) or to an increased competition for C_1 -units for the formation also of 1,3-thiazane-4-carboxylic acid.

S-adenosyl-L-homocysteine replaced DL-homocysteine using serine and either EHC or PtH_4G as the cofactor for C_1 -transfer (2-mercaptoethanol was added to give the same sulphhydryl group concentration in all test reactions). At low concentrations (less than 0.5mM of L-stereoisomer), S-adenosyl-L-homocysteine was 50% more active than homocysteine.

FIG. 17

Effect of DL-homocysteine on the synthesis of methionine by ultrasonic extracts of E.coli PA15



Reaction mixture I (DL-homocysteine as shown) with Dowex-treated enzymic extract and EHC, PtH_4G ($1\mu\text{mole}$), $\text{CH}_2\text{O-PtH}_4\text{G}$ ($3.3\mu\text{moles}$, L-serine and pyridoxal phosphate omitted) and cobalamin ($65\mu\text{moles}$) when added.

○, Serine, EHC. Incubated 2hr.

◐, Serine, PtH_4G and cobalamin. Incubated 4hr.

●, $\text{CH}_2\text{O-PtH}_4\text{G}$ and cobalamin. Incubated 4hr.

However, the maximum synthesis obtainable (at 1mM) was only about 80% of that for homocysteine (at 2.5mM L-stereoisomer). The presence of S-adenosyl-L-homocysteine does not alter the requirement for adenosine nucleotide derivatives (serine plus Pth₄G, Table I.11). Similar results were obtained with extracts prepared from organisms grown in the presence of cobalamin.

Other Components of the Reaction Mixture

Glucose

The requirement for glucose was greatest with Pth₄G as the cofactor for C₁-transfer (Table I.10); the EHC probably contains substances capable of replacing glucose. The concentration which gave half-maximal activity was 0.75mM (serine plus Pth₄G, Fig.16).

Adenosine triphosphate and magnesium ions

These were also necessary for optimal methionine synthesis; half-maximal activity was obtained with a concentration of 1mM (Table I.10, Fig.16). Adenosine diphosphate (ADP) was 85% as active as ATP but the monophosphate (AMP), adenosine and adenine were without activity under conditions using serine plus Pth₄G.

TABLE I.11

S-Adenosyl-L-homocysteine in methionine synthesis

<u>Substrate</u>	<u>Addition to reaction mixture (10 μmoles)</u>	<u>L-Methionine formed (μm-moles/mg. protein)</u>
<u>DL-Homocysteine</u> (10 μ moles)	ATP	70
	ADP	60
	AMP	3
	None	3
<u>S-Adenosyl-L-homocysteine</u> (5 μ moles)	ATP	58
	ADP	45
	AMP	4
	None	4

Reaction mixture I (homocysteine and ATP omitted), plus

Dowex-treated ultrasonic extract (strain PA15), Pth_4G (1 μ mole)

and cobalamin (0.65 μ m-moles). 2-mercaptoethanol (10 μ moles)

added when homocysteine omitted. Incubated 2hr.

Diphosphopyridine nucleotide

The requirement for DPN which was clear with the other test systems, could not be shown with EHC present; the EHC presumably contains sufficient for activity (Table I.10). Half-maximal activation was obtained with 0.25mM DPN.

Triphosphopyridine nucleotide (TPN) did not replace DPN and was somewhat inhibitory when EHC was the source of the folic acid coenzyme (Table I.10).

DPN was replaced by DPNH_2 and with higher concentrations of the latter both glucose and DPN could be replaced by the single addition of DPNH_2 ; furthermore, synthesis of methionine reached about half the maximum even when ATP was omitted as well (Table I.12).

Pyridoxal phosphate

Omission of pyridoxal phosphate resulted in a variable reduction (15-30%) with the serine plus Pth_4G system only (Table I.10). The absence of a clearly defined requirement may be due to the greater activity of serine transhydroxymethylase compared with methionine formation in the extracts: a trace of pyridoxal phosphate remaining in the extracts might be sufficient to permit the formation of C_1 -units from serine. EHC probably contains this coenzyme. In the $\text{CH}_2\text{O-Pth}_4\text{G}$ system there was no pyridoxal phosphate requirement, in fact its addition generally inhibited methionine formation

(Table 3.10), suggested **TABLE 1.12** *Strombolytic action in the**conversion* **Replacement of glucose and DPN by DPNH₂** *the conversion**to the methyl group of methionine.*

<u>Omissions</u>	<u>Additions (μ moles)</u>	<u>L-Methionine formed (μm-moles/mg. protein) with ATP (10 μmoles)</u>	
		<u>Present</u>	<u>Absent</u>
None	None	65	5
Glucose + DPN	None	8	5
DPN	DPNH ₂ (1)	69	-
Glucose + DPN	DPNH ₂ (4)	40	26
Glucose + DPN	DPNH ₂ (10)	63	39

Effect of added methionine

Reaction mixture I (ATP omitted) plus Dowex-treated
ultrasonic extract (strain PA15), Pth₄G (1 μ mole) and
cobalamin (0.65 μ m-moles). Incubated 2hr.

quantitatively recovered after incubation in a complete
reaction mixture minus serum.

Effect of methionine on methionine synthesis

Addition of up to 2 μ moles of DL-methionine to a complete
reaction mixture resulted in a 10% fall in methionine
formation; this is barely significant because of the
inaccuracy in assaying small differences in concentrated
samples. Stevens & Sakai (1959) however, have recently found
the presence of methionine in the reaction mixture increases
the methionine formation using extracts of pig liver.

(Table I.10), suggesting that the formaldehyde carbon is not converted to the β -carbon of serine before its conversion to the methyl group of methionine.

Phosphate

A requirement for phosphate was demonstrated by the fact that neither "TRIS" nor imidazole buffers could be substituted for phosphate. Addition of phosphate restored activity but in the presence of these other buffers maximum synthesis was never obtained.

Effect of added Methionine

Recovery of methionine

L-Methionine (0.5 μ moles added as DL-methionine) was quantitatively recovered after incubation in a complete reaction mixture minus serine.

Effect of methionine on methionine synthesis

Addition of up to 3 μ moles of DL-methionine to a complete reaction mixture resulted in a 10% fall in methionine formation; this is barely significant because of the inaccuracy in assaying small differences in concentrated samples. Stevens & Sakami (1959) however, have recently found the presence of methionine in the reaction mixture increases the methionine formation using extracts of pig liver.

Wijesundera & Woods (1953, 1960) observed that growth in the presence of the amino acid markedly decreased its subsequent synthesis from homocysteine and serine by suspensions of a number of strains of E.coli. Increasing concentrations of methionine in the growth medium also resulted in a similar reduction in the activity of the ultrasonic extracts of cobalamin-requiring strains of E.coli when the organisms were grown with methionine and when an extract of the heated organisms was the source of folio acid cofactor. (Table II.13, page 123).

Summary

- 1) A source of C_1 -units, homocysteine, glucose, DPN, ATP and Mg^{2+} are essential for optimal synthesis of methionine by ultrasonic extracts of E.coli PA15 in phosphate buffer.
- 2) Serine or CH_2O-Pth_4G can serve as C_1 -donors. Glycine is only 12% as active as serine and formate is almost inactive.
- 3) Homocysteine can be replaced by S-adenosyl-L-homocysteine.
- 4) The requirement for glucose and DPN can be replaced by high concentrations of $DPNH_2$.
- 5) A requirement for pyridoxal phosphate can only be shown when serine serves as source of C_1 -units.
- 6) Growth of the organism with methionine present represses the subsequent activity of extracts for its synthesis.

DISCUSSION OF EXPERIMENTAL SECTION I

The Relationship between Cobalamin and the Folic acid Cofactor

It is probable that treatment with acetone destroys some enzyme system, present in ultrasonic extracts, required for the further metabolism of cobalamin. A requirement for cobalamin in the synthesis of methionine from serine and homocysteine could be demonstrated with ultrasonic extracts of cobalamin-requiring strains of E.coli when the organisms were grown with methionine and when an extract of the heated organisms was the source of folic acid cofactor. Cobalamin had no effect on methionine formation by the other strains tested when EHC was the cofactor. This was not expected because the synthesis of methionine by suspensions of strains not requiring cobalamin for growth was increased two to six-fold by the addition of cobalamin (Gibson & Woods, 1960). Methionine synthesis by suspensions of cobalamin-requiring auxotrophs was completely dependent upon the presence of cobalamin. No significant effect of cobalamin was found using enzymic extracts prepared from acetone-dried powders of all the strains of E.coli.

By contrast, when Pth_4G replaced EHC as the source of folic acid cofactor or when its formaldehyde derivative replaced both serine and EHC, cobalamin was essential for the synthesis of methionine by ultrasonic extracts of all strains of E.coli. Enzymic extracts prepared from acetone-dried powders of strain PA15 were however incapable of using

PtH_4G as a folic acid cofactor, even with cobalamin added to the reaction mixture, unless the organisms were first grown in the presence of cobalamin (Kisliuk & Woods, 1958, 1960). It is probable that treatment with acetone destroys some enzyme system, present in ultrasonic extracts, required for the further metabolism of cobalamin.

Comparison of the cobalamin requirements for methionine synthesis when the cobalamin is added to the reaction mixture or to the growth medium with enzymic extracts of strain 121/176 (EHC system) and strain PA15 (PtH_4G system) shows an essential similarity between the two systems. It might be suggested that cobalamin functions in the conversion of simpler reduced forms of folic acid to a coenzyme form or that the EHC of strain PA15 contains cobalamin. However, the heated extract of strain 121/176 must contain an effective form of folic acid because it was active for methionine formation by enzymic extracts of strain PA15 in the absence of added cobalamin. Also, heated extracts of strain PA15 were inactive for methionine formation by enzymic extracts of strain 121/176 unless cobalamin was added. It is also unlikely that cobalamin functions in the conversion of PtH_4G to a coenzyme form similar to that from natural sources unless the conversion were complete because the cofactor present in EHC is strongly inhibited by PtH_4G . Thus it is

concluded provisionally that the role of cobalamin (if there is one) in the formation of a folic acid coenzyme cannot be the sole function of the vitamin in the overall methylation of homocysteine. This and other theories concerning the function of cobalamin will be discussed in later sections.

Cobalamin and Cobalamin Derivatives

Several nucleotide substituted analogues of cobalamin had activity equal to or approaching that of cobalamin; it has not yet been determined whether they have intrinsic activity or whether they are first converted to one functional form by enzymes present in the extract. It can be inferred from the greater activity of extracts of cobalamin-grown organisms that some further metabolism of cobalamin does take place. Factor B replaced cobalamin to a significant extent only if 5,6-dimethylbenzimidazole was also present. This suggests that the ultrasonic extract possesses enzymes capable of synthesising a complete form of the vitamin. Enzymic extracts of strain PA15 grown in the presence of factor B also had characteristics similar to those of cobalamin-grown organisms.

The significantly higher activity (compared with cobalamin) of 5,6-dimethylbenzimidazolylcobamide coenzyme adds weight to the view that cobalamin must be transformed to

a more elaborate compound before it has functional activity in isolated enzyme systems. Of the three cobamide coenzymes isolated by Barker et al., the one tested here was the least active for the interconversion of glutamate and β -methylaspartate; benzimidazolylcobamide coenzyme was sixty times more active than the 5,6-dimethylbenzimidazole derivative.

β -Methylaspartate had no activity in the formation of methionine by E.coli.

Towards the end of this work the existence of a cobalamin-containing enzyme, forming part of the complex of enzymes catalysing the transfer of the β -carbon atom of serine to homocysteine by ultrasonic extracts of E.coli 113/3 (grown in the presence of cobalamin) was reported (Hatch, Takeyama & Buchanan, 1959). PtH_4G was used as cofactor, but with this strain, the particular relationship between cobalamin and PtH_4G would not be observed, nor the inhibitory activity of the latter.

Folic Acid

The use of Dowex-1-treated enzymic extracts enabled a more conclusive demonstration of the requirement for a folic acid cofactor for methionine formation by E.coli. PtH_4G is an essential cofactor for methionine synthesis with extracts of sheep and pig liver (Nakao & Greenberg, 1958; Stevens &

Sakami, 1959) and PtG enhances the incorporation of the β -carbon atom of serine and formaldehyde into methionine by chicken liver extracts (Doctor, Patton & Awapara, 1957). No cobalamin requirement like that with *E. coli* was described, though cobalamin deficient organisms were not used. PtH_4G can replace natural coenzymes for all the functions of folic acid studied except methionine synthesis by *E. coli*. Furthermore this system using strain PA15 is the only one in which PtH_4G can be attributed with the characteristics of an inhibitor. It is possible that PtH_4G acts as an inhibitory analogue of the folic acid coenzymes and that in the presence of cobalamin it can also act as a cofactor, possibly by a different mechanism. Alternatively the inhibition may be due to the unnatural isomer present in chemically prepared PtH_4G . In a system containing EHC and PtH_4G it was not possible to determine whether the addition of cobalamin overcame the inhibition or whether it simply enabled PtH_4G to be used as a cofactor. Other Factors

The exact function of ATP is not known; it may react with homocysteine forming S-adenosylhomocysteine or homocysteine adenylate; either could be the acceptor of the C_1 -unit in place of free homocysteine. S-adenosylhomocysteine

will replace homocysteine but it does not remove the requirement for ATP. Similar findings have been obtained with mammalian preparations and also some evidence for its function as an intermediate (Stevens & Sakami, 1959; Nakao & Greenberg, 1958). Enzymic exchange of P^{32} -labelled pyrophosphate with ATP in the presence of homocysteine has been reported in pig liver extracts (Stevens & Sakami, 1959) and more recently in extracts of E. coli 113/3 (Hatch, Cathou & Larrabee, 1960) although it has still to be correlated with methionine formation.

ATP may also be required for activation of PtH_4O or the other folic acid cofactors (Whiteley, Osborn & Huennekens, 1958). In this system with E. coli, ATP is almost certainly also required for the utilisation of glucose by glycolysis.

A requirement for DPN in the overall reaction showed up clearly in the test systems not containing extract of heated organisms; the latter presumably contained this factor. Both glucose and DPN could be replaced by $DPNH_2$ at substrate concentrations; the main function of glucose is probably the generation of $DPNH_2$. The reduced coenzyme was not however used as a routine since the enzymic extracts had a strong $DPNH_2$ oxidase activity.

TPN was more effective than DPN for the synthesis of methionine by enzyme systems derived from animal tissues

(Nakao & Greenberg, 1958; Stevens & Sakami, 1959); other requirements included ATP and Mg^{2+} . TPN was inactive with ultrasonic extracts of E.coli PA15 and was even inhibitory when EHC was the source of the folic acid. Similar results were obtained with extracts of acetone-dried organisms by Kisliuk and Woods (1960). Hatch, Takeyama and Buchanan (1959) report an absolute requirement for $DPNH_2$ and ATP, and in a later communication a requirement for flavin mono- or dinucleotide was also demonstrated using fractionated enzymic extracts of E.coli 113/3 (Hatch, Takeyama, Cathou, Larrabee & Buchanan, 1959). It was suggested that the flavin component of the system is the final hydrogen donor for methyl group biosynthesis and that $DPNH_2$ served to keep this reduced. No requirement for flavin has however been found in the system studied here, although flavin coenzymes are present in EHC and may be retained in sufficient quantities by the extracts even after treatment with Dowex-1 resin.

RESULTS

Details of the cobalamin requirements for the synthesis of methionine by extracts of *Escherichia coli* have been given in the first part of the experimental section. This aspect of methionine synthesis was more thoroughly studied with the aid of several other strains of *E. coli* using both

EXPERIMENTAL RESULTS

growing cultures and extracts of *E. coli*. Comparisons of ultrasonic extracts and extracts of acetone-dried *E. coli*

EXPERIMENTAL SECTION II

TABLE I revealed the effect of ultrasonic extracts (in the presence of cobalamin) on the synthesis of methionine by Factor (X),

THE FUNCTION OF COBALAMIN IN THE SYNTHESIS OF METHIONINE

which was essential for the synthesis of methionine by extracts of acetone-dried organisms where PNH₂S was the source of the necessary folie acid cofactor. The properties of this factor (X) and its formation were also investigated.

Table II shows the effect of cobalamin on the synthesis of methionine by Factor (X) and its formation were also investigated.

The ultrasonic extracts were found to be active in the synthesis of methionine by Factor (X) and its formation were also investigated. The ultrasonic extracts were found to be active in the synthesis of methionine by Factor (X) and its formation were also investigated.

PREFACE

Details of the cobalamin requirement for the synthesis of methionine by extracts of E. coli have been given in the first part of the experimental section. This aspect of methionine synthesis was more extensively studied with the aid of several inhibitory analogues of cobalamin using both growing cultures and enzymic extracts of E. coli. Comparison of ultrasonic extracts and extracts of acetone-dried E. coli PA15 revealed the ability of ultrasonic extracts (in the presence of cobalamin) to form a protein-like factor (X), which was essential for the synthesis of methionine by extracts of acetone-dried organisms when Pth_4G was the source of the necessary folic acid cofactor. The properties of this factor (X) and its formation were also investigated. In this case the vitamin (1250-1255) was added with the inoculum to the surface of each tray containing 500ml. of solidified medium B₂ (supplemented with 10mg. glycine).

The acetone-dried organisms were extracted by mixing with water (10ml./g. powder) in a Potter-Elvehjem homogeniser. After centrifuging (25,000xg. 45 min.) at 0° the supernatant fluid was dialysed for 18hr. at 3° against 0.05M-phosphate buffer pH 7.5 (750ml./g. of dried organism); the dialysis fluid was changed once. The product was the extract of acetone-

CHAPTER 1

METHODS

The techniques used were essentially similar to those described in Section I. Reference will only be made to methods and procedures used in this and later sections which have not already been described.

Extracts of acetone-dried *Escherichia coli* PA15

Large scale production of acetone-dried *E. coli* PA15 was carried out by the staff of the Microbiological Research Establishment, Ministry of Supply, Porton Down, Wilts, using the methods described by Szulmajster & Woods (1960). For a few experiments, the organism was grown in the presence of cobalamin; in this case the vitamin (13 μ m-moles) was added with the inoculum to the surface of each tray containing 500ml. of solidified medium B₂ (supplemented with 10mM glycine).

The acetone-dried organisms were extracted by mixing with water (10ml./g. powder) in a Potter-Elvehjem homogeniser. After centrifuging (25,000xg, 45 min.) at 0° the supernatant fluid was dialysed for 18hr. at 3° against 0.05M-phosphate buffer pH7.8 (750ml./g. of dried organism); the dialysis fluid was changed once. The product was the extract of acetone-

dried organisms normally used. In some experiments the procedure was modified by subjecting the homogenised material to ultrasonic vibration (2.5 min. at 600 watts as described for preparation of ultrasonic extracts) before centrifuging and dialysing. Such ultrasonic-treated extracts of acetone-dried organisms were only used with organisms grown without cobalamin. These acetone-extracts could be stored at -15° for up to 4 weeks with no appreciable loss of activity.

Formation of the Factor (X)

Ultrasonic extracts of organisms were incubated with cobalamin in Reaction Mixture II which was a modification of Reaction Mixture I and contained (per ml.):— glucose, 15 μ moles; ATP, 7.5 μ moles; DPN, 0.75 μ moles; $MgCl_2$, 7.5 μ moles; potassium phosphate buffer pH 7.8, 167 μ moles. The concentration of ultrasonic extract used was equivalent to 7.5 mg. protein/ml. and the reaction was carried out in a final volume of 2.7 or 6.0 ml. under an atmosphere of H_2 at 37° . In early experiments the concentration of cobalamin was 50 μ moles/ml. and the incubation was for 3-4hr. Later the concentration of the vitamin was increased to 0.5 μ m-moles/ml. and the time of incubation decreased to 45 min. The reaction was stopped by cooling the mixture to 0° .

Assay of the Factor (X)

Determination of the content of X in the material obtained from the previous reaction depended on its ability to promote methionine synthesis by extracts of acetone-dried organisms (cultured without cobalamin) in a system containing PtH_4G as source of the folic acid coenzyme. During the early work the method of assay used was based on that of Kisliuk & Woods (1960). A modified method was developed during the later work and will be described in a later chapter.

The samples of material containing X were generally equivalent to 6mg. of the protein of the ultrasonic extract used for X production. Reaction Mixture III, used to follow the synthesis of methionine contained (in 2.2ml. final volume):- DL-homocysteine, 10 μmoles ; L-serine, 10 μmoles ; fructose-1,6-diphosphate (HDP), 15 μmoles ; ATP, 10 μmoles ; MgSO_4 , 10 μmoles ; DPN, 1 μmole ; pyridoxal phosphate, 1 μmole ; PtH_4G , 1 μmole and potassium phosphate buffer pH 7.8, 250 μmoles . The enzyme system (dialysed extract of acetone-dried organisms) was added in an amount equivalent to 10mg. protein (normal extract) or 20mg. protein (ultrasonic-treated). Incubation was for 3hr. at 37° in an atmosphere of H_2 . After heating to 100° for 3 min. to stop the reaction, the precipitated protein was removed by centrifuging, and the supernatant fluid assayed for methionine using Leuconostoc

mesenteroides P60 (see Section I, Chapter 1). The same reaction mixture and conditions of incubation were used for the study of the direct synthesis of methionine by extracts of acetone-dried E.coli PA15.

In each assay there were controls in which the enzyme was incubated with and without cobalamin (65 μ moles) in reaction mixture III in the absence of a sample containing X; the values for methionine obtained were deducted from the experimental values. Controls in which the samples containing X were incubated in reaction mixture III without the extract of acetone-dried organisms were also carried out as a routine.

Expression of results

The activity of the enzymic extract used for assaying X varied slightly from batch to batch and the conditions for the assay were not completely reproducible; it was not possible therefore to define an absolute unit for X. Each complete experiment included a sample of X formed under standard conditions and this was titrated for its effect on methionine synthesis; the titration of other samples was compared with this. The arbitrary unit of activity of X chosen was that amount which caused the synthesis of 0.1 μ mole L-methionine in a particular assay. In some experiments the results are however recorded simply as the total amount of methionine formed (μ m-moles).

Experiments with Growing Cultures of
Escherichia coli

The method used was modified from that of Brown et al. (1949). Liquid culture technique was adopted for studying the growth of strains of *E. coli* in the presence of cobalamin and its analogues. Duplicate tests were carried out in 150x20mm. hard glass tubes containing medium B₁ (glucose, lactate and salts) with the appropriate supplements and inoculum; the final volume being 2ml. Solutions of cobalamin and its analogues were sterilised by Seitz-filtration and added to the medium which had been autoclaved at 115° for 10 min.

The inoculum was prepared from a tryptic meat agar slope culture of the organism and harvested after incubation for 16hr. at 37°. The organisms were washed once with 10ml. water and each tube of the test was inoculated with about 3.5µg. dry wt. organisms (in 0.1ml.). Incubation was for 24 or 48hr. at 37° with the tubes sloped 5° from horizontal to increase aeration.

The extent of growth was measured with an EEL photo-electric colorimeter (see Section I, Chapter 1). With very high concentrations of cobalamin analogues, the value for an uninoculated control was deducted from the corresponding experimental values. The results are expressed in terms of mg. dry wt. organisms/ml. of culture. The inhibition index for half-maximal growth and complete inhibition of growth is defined as the ratio: concentration of inhibitory analogue/ concentration of cobalamin.

Assay of Cobalamin

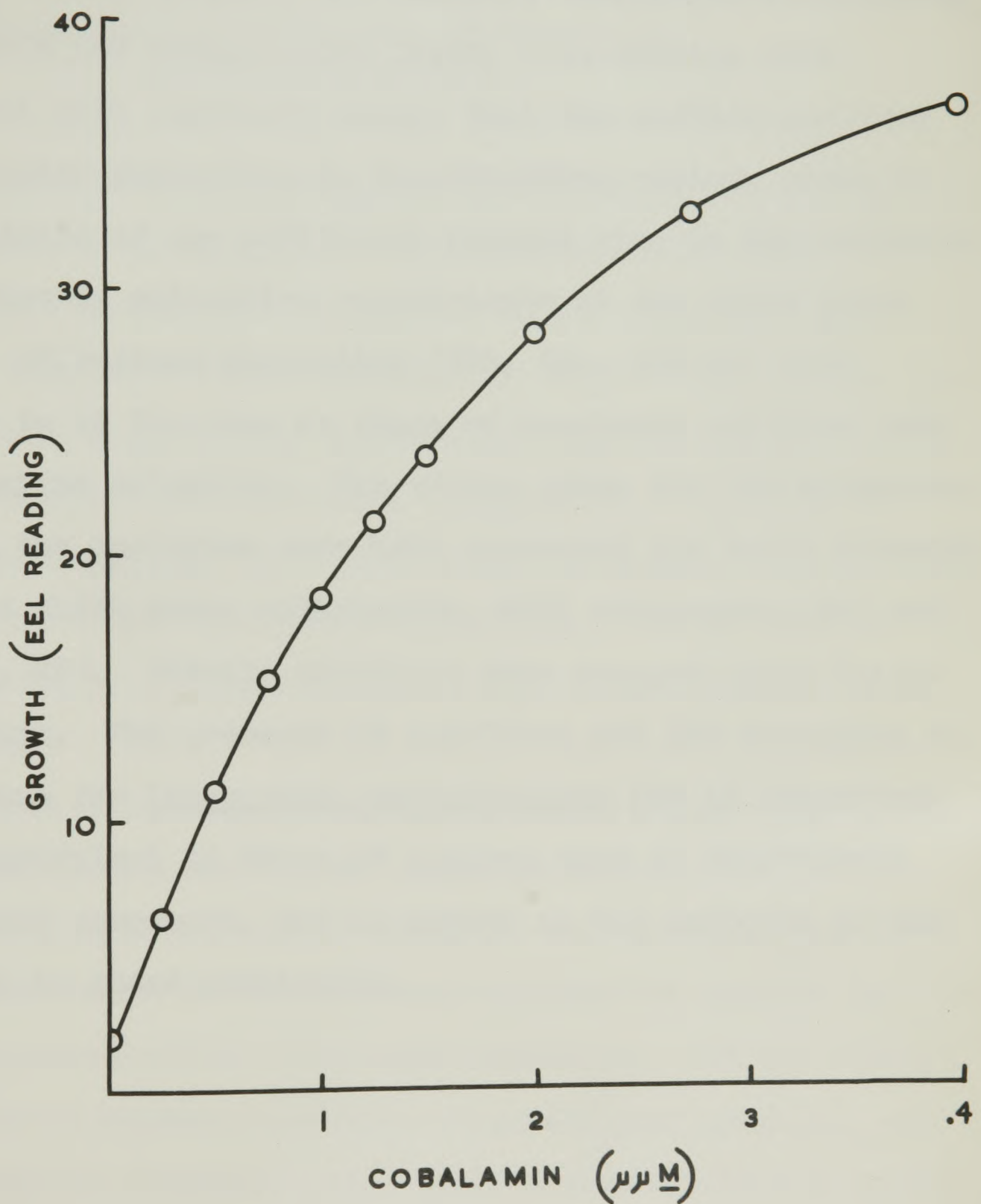
The method used was modified from that of Hutner et al. (1949); the assay organism was Euglena gracilis var. bacillaris. The malate, glutamate plus salts medium D (5ml. final volume in 125x16mm. tubes) was autoclaved 10 min. at 115° after addition of cobalamin or experimental samples. Standards covering the range 0 to 4 μ m-moles cobalamin/ml. were included in each assay. The inoculum was derived from a seven day culture on the same medium containing 0.8 μ m-moles cobalamin/ml. The organisms were washed twice with 5ml. of 0.9% (w/v) NaCl and finally resuspended in saline (5ml.); 0.05ml. of this suspension was added to each assay tube. Incubation was for 10 days at 30° and 36cm. distant from two 60 watt tungsten filament lamps. The growth response was assessed in an EEL photoelectric colorimeter using a neutral density filter (Fig.18). Test samples were also assayed in the presence of cobalamin to ensure the absence of growth inhibitors in the material.

Cobalamin-amide analogues

The methanamide, ethanamide and anilide of the monocarboxylic acids of cyanocobalamin (Smith, Parker & Gant, 1956) were provided by Dr. E. Lester Smith; they will be referred to as the methanamide, ethanamide and anilide

FIG. 18

Response of *Euglena gracilis* to cobalamin



analogues. They contained less than 0.02% of cobalamin itself (Smith, personal communication). Crystalline samples were dissolved in water and examined spectrophotometrically in visible and ultra-violet light. The spectra were identical with cobalamin except that the anilide analogue had a higher absorption in the 220-275m μ region, owing to the presence of one additional benzene ring in the molecule. The molecular extinction coefficients at the three wavelengths of maximum absorption (278, 361, 550 m μ) were assumed to be the same as those of cobalamin and were used for assaying solutions. The values given for the concentration of the analogues have been corrected for their probable purities which were: methylamide, 82%; ethylamide, 84% and anilide, 83%. Sterile solutions were assayed after Seitz-filtration. The presence of cobalamin and its analogues in the medium for Leuconostoc mesenteroides P60 at concentrations equivalent to those of samples used in experiments with these compounds, had no effect on the response of the organism to added methionine.

action of cobalamin in methionine synthesis. Factor B which was unable to replace cobalamin in methionine synthesis was also tested for inhibitory activity at concentrations higher than tested previously (Section I, Chapter 3).

CHAPTER 2

THE INHIBITION OF METHIONINE SYNTHESIS BY ANALOGUES OF COBALAMIN

Inhibition of Methionine Synthesis in Ultrasonic extracts

The synthesis of methionine from homocysteine by ultrasonic extracts of *E. coli* has been studied under three conditions (Section I): (a) serine as the C_1 -donor and PtH_4G as the folic acid cofactor, (b) CH_2O-PtH_4G as both the C_1 -donor and cofactor for C_1 -transfer, and (c) serine as the C_1 -donor and extract of heated *E. coli* (EHC) as source of folic acid cofactor. With ultrasonic extracts of strain 121/176, cobalamin is required in every case, but for strain PA15, cobalamin is only essential when PtH_4G is the cofactor for C_1 -transfer. Several analogues of cobalamin, the methylamide, ethylamide and anilide of the monocarboxylic acids of cobalamin designed as antimetabolites of cobalamin (Smith *et al.* 1956) were tested for their ability to inhibit the action of cobalamin in methionine synthesis. Factor B which was unable to replace cobalamin in methionine synthesis was also tested for inhibitory activity at concentrations higher than tested previously (Section I, Chapter 3).

In order to determine the type of inhibition, the rate of methionine formation was measured over a range of cobalamin

Serine plus tetrahydropteroylglutamic acid

The analogues of cobalamin replaced the requirement for cobalamin to a limited extent, but only at relatively high concentrations (Table II.1). The analogues however, were also inhibitors of the action of cobalamin and reduced the synthesis of methionine to the value obtained with the analogue alone. The order of increasing activity in this respect was methylamide < factor B < ethylamide < anilide (Table II.1). The two extreme cases of the analogue activities are illustrated in Figure 19. The effect of the anilide analogue on methionine synthesis with strains PA15 and 121/176 was very similar. Factor D, an inactive cobalamin compound of unknown structure but lacking the nucleotide residue, was also tested here and found to inhibit in the same manner as factor B. Because the analogues have some ability to replace cobalamin, and because this is different for each analogue, it is not possible to express their capacities for inhibition in precise quantitative terms. The anilide analogue was chosen for more detailed studies because it combines high inhibitory activity with a weak ability to replace the vitamin (Fig.19).

Treatment of the enzymic extract with Dowex-1 resin had no effect on the inhibitory action of the anilide analogue.

In order to determine the type of inhibition, the rate of methionine formation was measured over a range of cobalamin

TABLE II.1

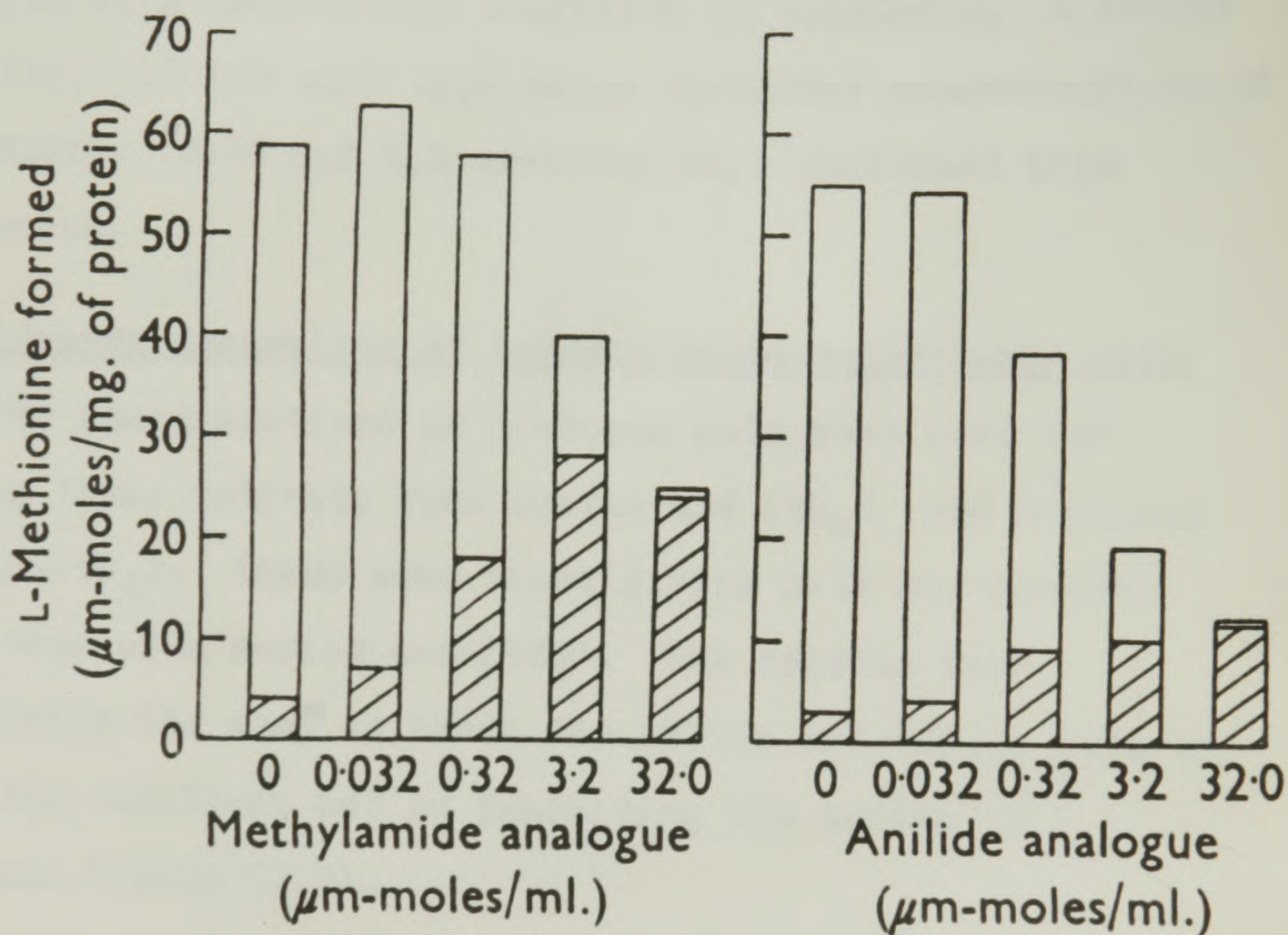
The effect of analogues of cobalamin on methionine synthesis in a system containing serine and tetrahydropteroylglutamic acid

Strain of <u>E. coli</u>	Analogue	<u>(μm-moles/mg. of protein)</u>									
		<u>L-Methionine formed (μm-moles/mg. protein)</u>					<u>Cobalamin present with analogue (μm-moles/ml.)</u>				
		0	0.032	0.32	3.2	32.0	0	0.032	0.32	3.2	32.0
PA15	Methylamide	4	7	18	28	24	59	63	58	40	25
	Ethylamide	5	7	13	20	21	73	72	63	33	22
	Anilide	3	4	9	10	12	55	54	38	19	12
	Factor B	3	5	7	8	9	62	61	59	24	11
	Anilide	2	3	5	7	8	68	68	34	10	8

Reaction mixture I plus ultrasonic extract and PTHG (1 μ mole).
Cobalamin (0.032 μ m-moles/ml.) when present. Incubated 4hr.

FIG. 19

Effect of the methylamide and anilide analogues
of cobalamin on methionine synthesis



Reaction mixture I plus ultrasonic extract of strain PA15 and PtH_4G ($1\mu\text{mole}$). Cobalamin ($0.032\mu\text{m-moles/ml.}$)

□ , present; ▨ , absent. Incubated 4hr.

concentrations (0-0.32 $\mu\text{m-moles/ml.}$) in the presence of two concentrations of the anilide analogue (0.73 and 1.45 $\mu\text{m-moles/ml.}$). The results were plotted by the method of Lineweaver & Burk (1934) and show (Fig. 20) that the anilide analogue is a competitive inhibitor of cobalamin. A second experiment of the same type using different concentrations of the anilide (0.29 and 2.9 $\mu\text{m-moles/ml.}$) confirmed this conclusion.

Formaldehyde derivative of tetrahydropteroylglutamic acid

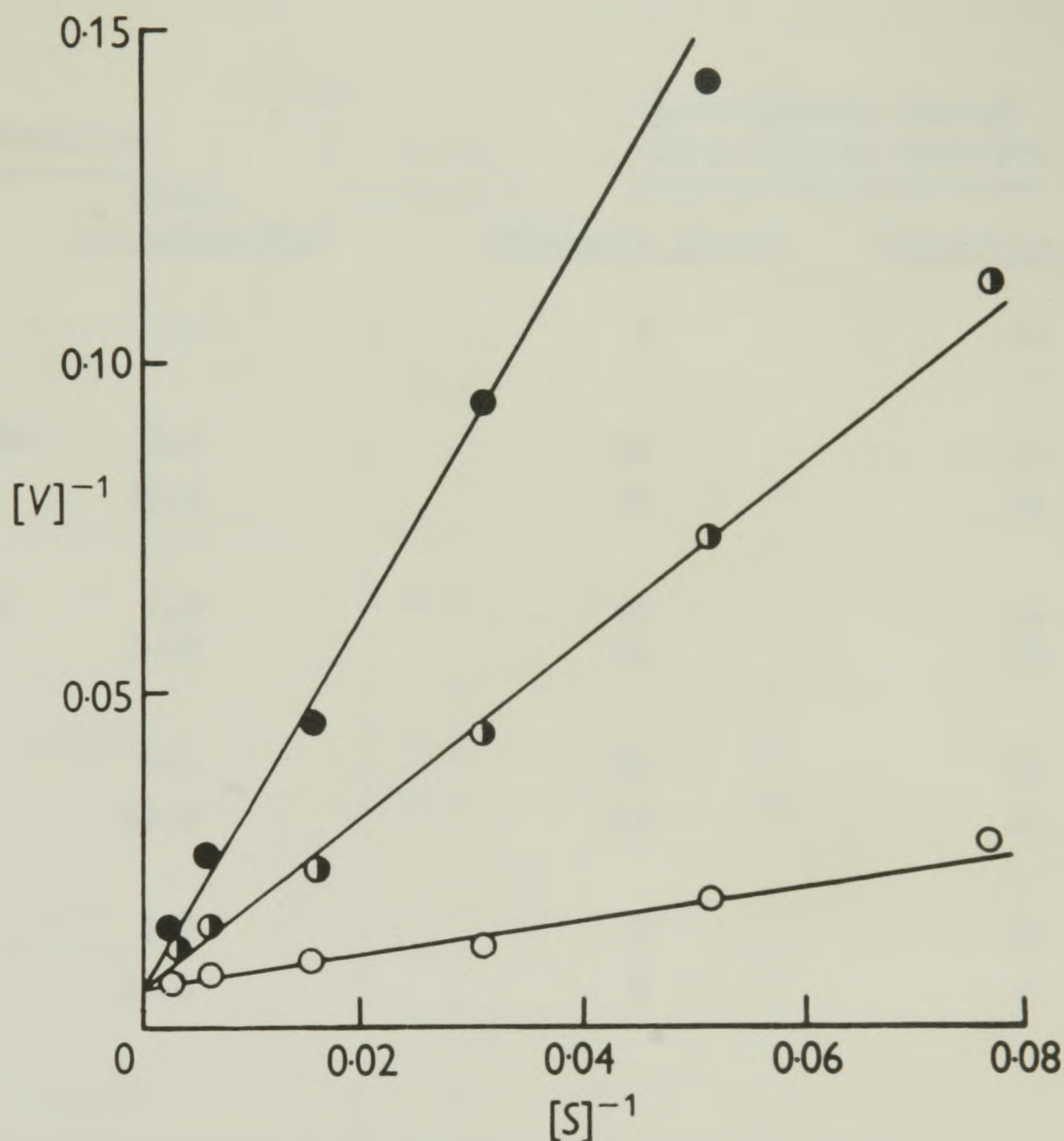
Two concentrations of analogue only were used for testing their activity when serine and Pth_4G were replaced by $\text{CH}_2\text{O-Pth}_4\text{G}$. These were those giving half and maximum inhibition with serine and Pth_4G . The results were essentially the same as those for serine and Pth_4G , both in replacing cobalamin and in inhibiting the action of cobalamin (Table II.2).

Serine plus extract of heated *E. coli*

The synthesis of methionine by ultrasonic extracts of strain PA15 with EHC as source of folic acid coenzyme was not inhibited by the analogues whether or not cobalamin was added (Table II.3). On the other hand with enzymic extracts of strain 121/176, methionine synthesis occurred only if cobalamin was added and here the anilide analogue was inhibitory: the degree of inhibition was the same whether the

FIG. 20

Effect of the anilide analogue on the rate of formation of methionine by ultrasonic extracts of strain PA15



Reaction mixture I plus PtH₄G (1μmole). S, cobalamin concentration (μμmoles/ml.); V, rate of methionine formation (μm-moles L-methionine/mg. protein/4hr.). Anilide analogue O, nil; ◐, 0.73μm-moles/ml; ●, 1.45μm-moles/ml.

TABLE II.2

The effect of analogues of cobalamin on methionine synthesis in a system containing the formaldehyde derivative of tetrahydropteroylglutamic acid

<u>Nature</u>	<u>Analogue</u>		<u>L-Methionine formed</u> <u>(μ-moles/mg. protein)</u>	
	<u>Concn.</u> <u>(μ-moles/ml.)</u>	<u>Cobalamin absent</u>	<u>Cobalamin present</u>	
	-	6	62	
	3.0	34	44	
	30.0	28	32	
Methylamide	1.0	21	51	
	30.0	24	25	
Ethylamide	0.5	12	53	
	29.0	19	24	
Anilide	1.7	7	36	
	34.5	8	10	
Factor B				

Reaction mixture I (modified as in Section I, Chapter 1)
plus ultrasonic extract of strain PA15 and $\text{CH}_2\text{O-PtH}_4\text{G}$
(3.3 μ moles). Cobalamin (0.032 μ -moles/ml.) when present.
Incubated 4hr.

TABLE II.3

The effect of analogues of cobalamin on synthesis of methionine in a system containing serine and heated extract of *Escherichia coli*

Strain of <i>E. coli</i>	Analogue Nature	Concn. (μ m-moles/ml.)	L-methionine formed (μ m-moles/mg. protein)	
			Cobalamin absent	Cobalamin present
PA15	Methylamide	0	54	-
		3.0	59	-
		30.0	57	-
	Ethylamide	0	54	-
		1.0	57	-
		30.0	60	-
	Anilide	0	67	70
		0.5	71	71
		29.0	70	74
	Factor B	0	54	60
		1.7	58	58
		34.5	56	56
121/176	Anilide	0	5	37
		0.5	7	18
		29.0	10	10

Reaction mixture I plus Dowex-treated ultrasonic extract

and EHC of the corresponding strain. Cobalamin (0.032 μ m-moles/ml.)

when present. Incubated 2hr. (strain PA15); 4hr. (strain 121/176).

source of folic acid was EHC or PtH_4G . Furthermore, the synthesis of methionine by enzymic extracts of strain PA15 with EHC derived from strain 121/176 was not affected (either in the presence or absence of cobalamin) by concentrations of the anilide analogue which were inhibitory in systems in which cobalamin is required. The anilide analogue did however inhibit methionine formation by ultrasonic extracts of strain 121/176 when the heated extract was derived from strain PA15. This evidence combined with that concerning the interchangeability of the heated extracts of strain PA15 and 121/176 (Section I, Chapter 4) supports the view that cobalamin has no function in the synthesis of methionine by extracts of strain PA15 when natural folic acid coenzyme is used for the transfer of C_1 -units.

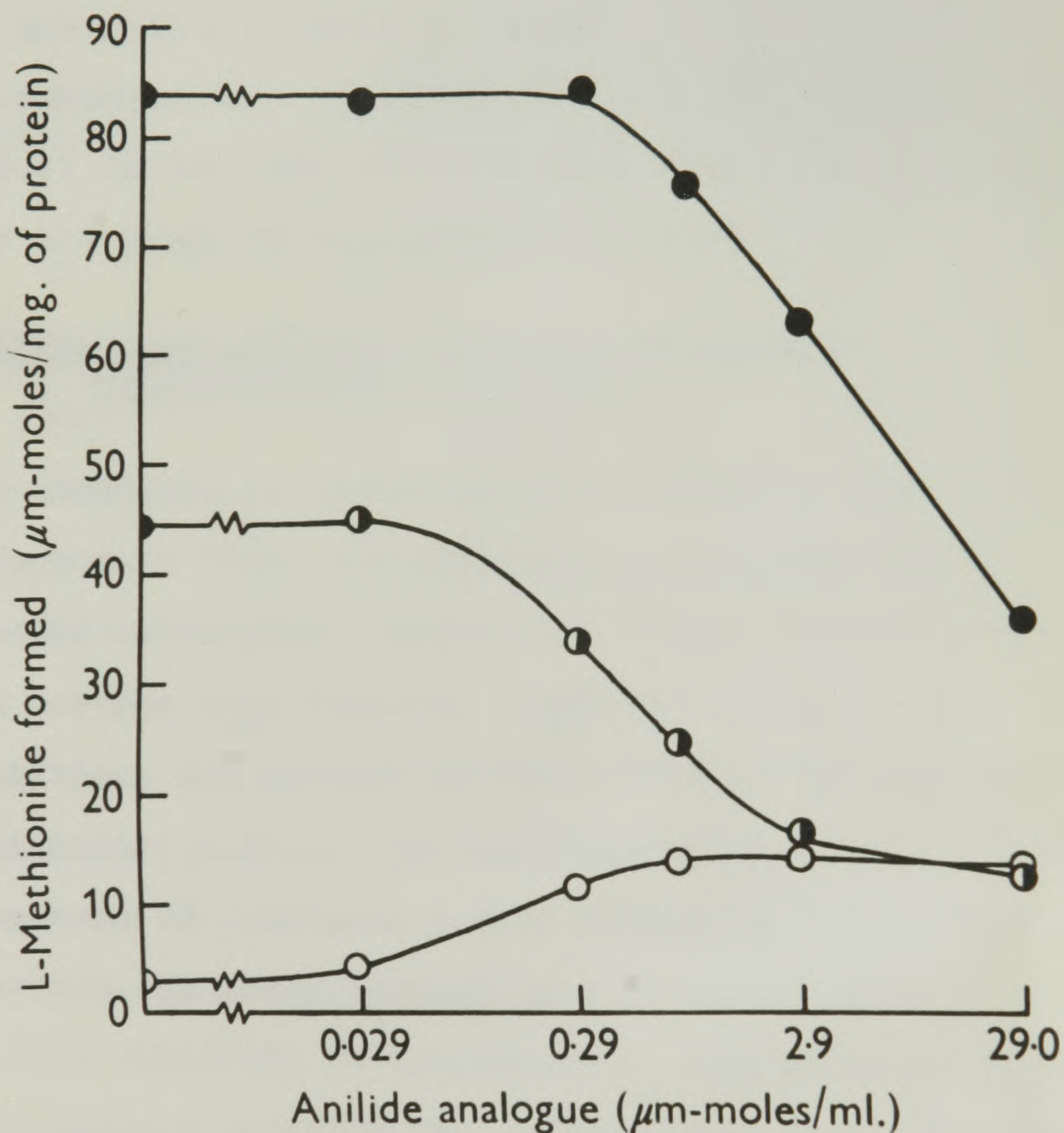
Inhibition in the presence of cobamide coenzyme

Under the first condition (serine plus PtH_4G) 5,6-dimethylbenzimidazolylcobamide coenzyme was 2-3 times more active than cobalamin in promoting the synthesis of methionine (Section I, Chapter 3). Much higher concentrations of the anilide analogue were required to inhibit the action of the coenzyme than those effective with an equimolar concentration of cobalamin (Fig. 21). In order to determine the type of inhibition, two concentrations of coenzyme, one three times

3. 5,6-Dimethylbenzimidazolylcobamide coenzyme
(52 $\mu\text{moles/l}$).

FIG. 21

Inhibition by the anilide analogue of the action
of cobalamin and 5,6-dimethylbenzimidazolylcobamide
coenzyme



Reaction mixture I plus Dowex-treated ultrasonic extract of strain PA15 and PtH_4G ($1\mu\text{mole}$).
 Incubated 4hr. in the dark.

○, No addition.

○, Cobalamin ($32\mu\mu\text{moles/ml.}$).

●, 5,6-Dimethylbenzimidazolylcobamide coenzyme ($32\mu\mu\text{moles/ml.}$).

higher than the other, were chosen, and the effect of a series of concentrations of the anilide analogue was studied. The percentage inhibition of the action of the cobamide coenzyme was constant for a constant ratio of anilide to coenzyme concentration (Table II.4). The inhibition is therefore as in the case of cobalamin, of the competitive type over the range of concentrations tested.

The Anilide Analogue and Ultrasonic extracts of
Cobalamin-grown Organisms

When cobalamin (4 μ moles/ml.) is added to the growth medium for strain PA15, the ability of ultrasonic extracts to synthesise methionine (serine with Pth_4O) without added cobalamin becomes just limiting (Section I, Chapter 3). Under these conditions the anilide analogue even at 29 μ m-moles/ml., had no inhibitory action. Dowex-1 treatment of such extracts or the presence of cobalamin had no effect on this result. If the anilide was added instead to the growth medium together with cobalamin (4 μ moles/ml.), the activity of the ultrasonic extract obtained was reduced (Fig.22). Considerable activity however was present in extracts of organisms harvested after growth in the presence of anilide only. The synthesis of methionine by extracts of organisms grown in the presence of cobalamin was not affected by addition of cobalamin (0.7 μ m-moles) to the reaction mixture. However, for extracts

TABLE II.4

Inhibition by the anilide analogue of the
synthesis of methionine promoted by
5,6-dimethylbenzimidazolylcobamide coenzyme

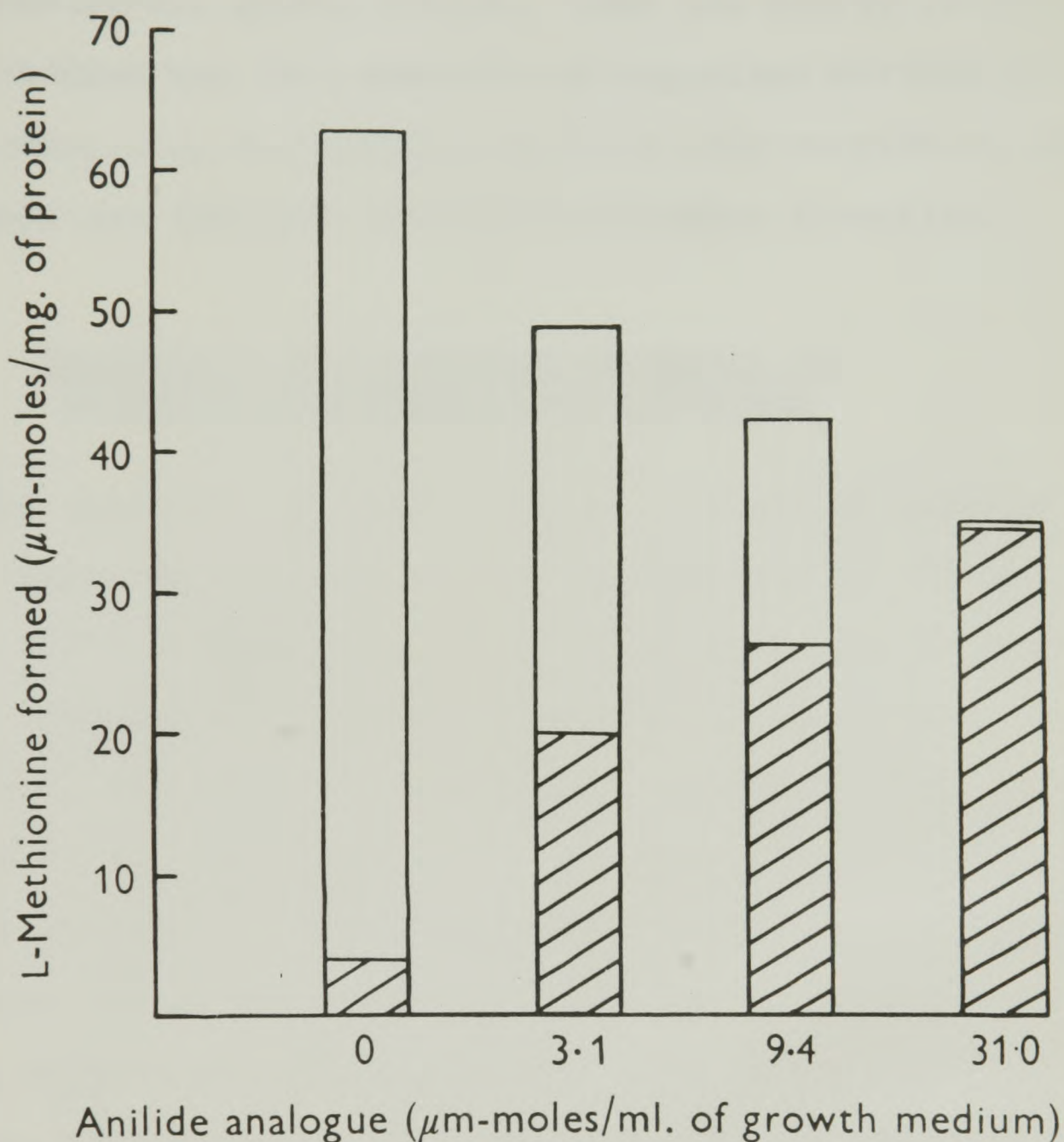
Anilide analogue (μ m-moles/ml.)	Inhibition (%) of the action of cobamide coenzyme in methionine synthesis with coenzyme (μ m-moles/ml.)	
	<u>0.0107</u>	<u>0.032</u>
0	0	0
0.29	20 (27)	0
0.87	40 (82)	23 (27)
2.61	54 (245)	38 (82)
7.83	70 (734)	56 (245)
23.49	-	73 (734)

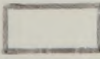
Reaction mixture I plus ultrasonic extract of strain PA15 and
Pth₄C (1 μ mole). Incubated 4hr. in the dark. Figures in
parentheses are the ratios of the concentrations of
anilide analogue and cobamide coenzyme.

Anilide analogues (μ m-moles/ml. of growth medium)

FIG. 22

The effect of growing *Escherichia coli* PA15 in the presence of the anilide analogue on the ability of ultrasonic extracts to synthesise methionine



Reaction mixture I plus PtH_4G ($1\mu\text{mole}$) and ultrasonic extract of organisms cultured with cobalamin ($4\mu\text{moles/ml. growth medium}$), , absent; , present. Incubated 1hr. 40min.

of organisms grown in the presence of the anilide analogue only, added cobalamin increased synthesis of methionine but not when the anilide analogue concentration was greater than $3.1 \mu\text{m-moles/ml.}$ growth medium. When the source of folic acid coenzyme was EHC, extracts of organisms derived from media containing the anilide analogue with or without added cobalamin had the same activityⁱⁿ methionine formation.

Inhibition of Methionine Synthesis in Extracts of Acetone-dried Organisms

1) Under conditions where cobalamin is essential for the synthesis of methionine by ultrasonic extracts of strains of dried organisms has been studied extensively by Kisliuk & Woods, (1960). Its action was inhibited by the methylamide, ethylamide and anilide of the imino-carboxylic acids of cobalamin in the presence of cobalamin as described in Section II, Chapter 1, and tested for methionine synthesis with reaction mixture III in the presence and absence of both cobalamin and its analogues. The methylamide, ethylamide and anilide analogues had no effect on the synthesis of methionine even at the highest concentrations used with ultrasonic extracts. However, with factor B ($70 \mu\text{m-moles}$) added to the reaction mixture a 16% fall in activity was produced which could not be overcome by cobalamin ($6.5 \mu\text{m-moles}$).

2) The anilide analogue was not degraded by extracts of cobalamin-grown organisms. After incubation of the analogue the anilide analogue, and the ultrasonic extracts of organisms, inhibitory capacity after incubation with factor B.

with an extract of cobalamin-grown and acetone-dried strain PA15, in reaction mixture III (Pth_4G , homocysteine and pyridoxal phosphate omitted), the products were if anything slightly more inhibitory either than those of a reaction mixture to which the anilide analogue was added after incubation or than untreated analogue, when tested with ultrasonic extract (serine plus Pth_4G system).

Summary

- 1) Under conditions where cobalamin is essential for the synthesis of methionine by ultrasonic extracts of strains of Escherichia coli, its action was inhibited by the methylamide, ethylamide and anilide of the monocarboxylic acids of cobalamin and by factor B.
- 2) The anilide analogue inhibited competitively both the action of cobalamin and of 5,6-dimethylbenzimidazolylcobamide coenzyme, but was less active against the latter.
- 3) Extracts of organisms grown in the presence of cobalamin did not require added cobalamin for methionine synthesis and the anilide analogue was not inhibitory unless added with cobalamin to the growth medium.
- 4) Methionine formation by extracts of acetone-dried strain PA15 grown in the presence of cobalamin was not inhibited by the anilide analogue, and the analogue retained its full inhibitory capacity after incubation with these extracts.

CHAPTER 3

THE ANILIDE ANALOGUE AND GROWTH OF *Escherichia coli*

The mono-substituted cobalamin-amide analogues have been found to inhibit the growth of *Escherichia coli* 113/3 (Cuthbertson, Gregory, O'Sullivan & Pegler, 1956). Using the cup-plate method for growth of the organisms with graded levels of both cobalamin and the analogues they estimated the inhibition indices for the methylamide and anilide analogues as 150 and 2000-3000 respectively.

The effect of the anilide analogue on the growth of strains used for the study of methionine synthesis was examined in order to compare its effect on the growth of cobalamin-dependent and cobalamin-independent strains and, to compare its action on growth with its inhibitory action on the synthesis of methionine by cell-free preparations. Liquid culture technique was used throughout (see Chapter 1).

Growth in the absence of cobalamin

Over the range of concentrations tested, the anilide analogue did not support the growth of strains requiring cobalamin or methionine, nor did it inhibit growth when an optimal concentration of methionine was present (Table II.5). The anilide analogue had little effect on the growth of strain PA15 and Strain W.

TABLE II.5
Effect of the anilide analogue of cobalamin on
the growth of *Escherichia coli*

Strain of <i>E. coli</i>	Additions to the basal medium	Time of incubation (hr.)	Growth (mg. dry wt. organisms/ml.) with anilide analogue (µm-moles/ml.)					
			0	0.022	0.22	2.2	22.0	
W	-	24	0.7	0.7	0.7	0.8	0.9	
	-	48	2.3	2.4	2.5	2.6	2.8	
PM5	Glycine	24	0.4	0.4	0.4	0.4	0.4	
	Glycine	48	1.7	1.8	1.8	1.8	1.8	
121/176	-	48	0.0	0.0	0.0	0.0	0.0	
	Methionine	24	0.6	0.6	0.6	0.6	0.6	
113/3	-	48	0.0	0.0	0.0	0.0	0.0	
	Methionine	24	1.9	2.0	1.9	1.8	2.0	

Medium B₁ (glucose, lactate and salts) supplemented with glycine (5mM)
or DL-methionine (0.5mM) where stated.

Growth in the presence of cobalamin

The concentrations of cobalamin which are just optimal for the growth of E.coli 121/176 and 113/3 are about 0.26 and 1.3 μM moles/ml. respectively. When cobalamin (0.65 μM moles/ml.) was added to the basal medium in the absence of methionine, the anilide analogue inhibited the growth of strains 121/176 and 113/3 (Table II.6). The inhibition indices for 50% and 100% inhibition of growth are similar for the two strains over the range of cobalamin concentrations tested (Table II.7).

For strains PA15 and W, which do not depend on added cobalamin for growth, addition of the vitamin increased the early growth (compare Tables 5,6). With strain PA15 this effect was more pronounced after 16hr. but was still apparent after 24hr. The anilide analogue (at concentrations which inhibited the growth of strains 121/176 and 113/3) also inhibited this increase in early growth, but it had no effect on the extent of growth at 48hr.

L- β -methylaspartate (10^{-5} - 10^{-3}M) was unable to replace cobalamin or methionine as a growth factor for E.coli 121/176, nor would it increase the growth obtained with limiting concentrations of cobalamin or methionine. It has been reported recently however that the cobamide coenzymes are equally as active as cobalamin for the growth of E.coli 113/3 (Barker et al. 1960a,b).

TABLE II.6

Effect of anilide analogue on the growth of
Escherichia coli with cobalamin present

Strain of <i>E. coli</i>	Additions to the basal medium	Time of incubation (hr.)	Growth (mg. dry wt. organisms/ml.) with anilide analogue (μ m-moles/ml.)				
			0	0.022	0.22	2.2	22.0
W	Cobalamin	24	1.2	1.1	0.7	0.7	0.7
	Cobalamin	48	2.5	2.5	2.6	2.6	2.8
PM5	Glycine, cobalamin	24	0.5	0.5	0.4	0.3	0.3
	Glycine, cobalamin	48	1.7	1.7	1.6	1.7	1.7
124/176	Cobalamin	24	0.5	0.5	0.3	0.0	0.0
113/3	Cobalamin	24	0.6	0.6	0.4	0.0	0.0

Medium B₁ (glucose, lactate and salts) supplemented with glycine (5mM)
and/or cobalamin (0.65 μ m-moles/ml.) where stated.

TABLE II.7

Inhibition indices for the anilide analogue
for the growth of *Escherichia coli*
strains 121/176 and 113/3 in presence of cobalamin

Cobalamin concn. in basal medium (μ moles/ml.)	Inhibition index for a growth inhibition of			
	50%		100%	
	with strain		with strain	
	121/176	113/3	121/176	113/3
0.13	550	550	900	900
0.65	570	580	880	910
6.50	370	390	840	910

Medium B₁ (glucose, lactate and salts) supplemented with
 cobalamin and anilide analogue. Incubated 24hr.

Summary

The anilide analogue of cobalamin inhibits the growth of strains requiring cobalamin or methionine for growth when cobalamin is the growth factor, but not when methionine is supplied. It does not inhibit the growth of strains which do not require cobalamin for growth.

PTK_2O to be used as the collector for S_1 -transfer in methionine synthesis by extracts of acetone powders of the same organism grown in the absence of cobalamin (Woods, 1960; Kislick & Woods, 1960). It was immediately realized that the ultrasonic extracts were probably capable of forming this factor (X) in the presence of cobalamin under the reaction conditions. Treatment of the organism with acetone apparently caused the destruction of an enzyme or enzymes required for the formation of X. Indirect evidence for the formation of X by ultrasonic extracts was also obtained using the inhibitory analogue of cobalamin (Chapter 3).

Indirect Evidence for the Synthesis of X

Trial experiments showed that if ultrasonic extracts (strain PA15) were first incubated (60-100 min.) with cobalamin in the presence of glucose, ATP, Mg^{2+} , DVT and phosphate, the initial rate of synthesis of methionine was increased.

The effect of X on the synthesis of methionine was studied.

CHAPTER 4

COBALAMIN AND THE ENZYMATIC FORMATION OF A FACTOR (X) CONCERNED IN THE SYNTHESIS OF METHIONINE

A heat-labile, non-diffusible factor (X) was partially-purified from acetone-dried powders of *E. coli* PA15 cultured in the presence of cobalamin. This material (X) enabled PtH_4G to be used as the cofactor for C_1 -transfer in methionine synthesis by extracts of acetone powders of the same organism grown in the absence of cobalamin (Woods, 1960; Kisluk & Woods, 1960). It was immediately realised that the ultrasonic extracts were probably capable of forming this factor (X) in the presence of cobalamin under the reaction conditions. Treatment of the organism with acetone apparently caused the destruction of an enzyme or enzymes required for the formation of X. Indirect evidence for the formation of X by ultrasonic extracts was also obtained using the inhibitory analogues of cobalamin (Chapter 2).

Indirect Evidence for the Synthesis of X

Trial experiments showed that if ultrasonic extracts (strain PA15) were first incubated (60-100 min.) with cobalamin in the presence of glucose, ATP, Mg^{2+} , DPN and phosphate, then the initial rate of synthesis of methionine on the subsequent

addition of homocysteine, serine and Pth_4G was double that attained when cobalamin was omitted from the first incubation but was present during the second. This suggested that metabolism of cobalamin led directly or indirectly to the production of material which was more immediately active than cobalamin itself, in promoting the utilisation of tetrahydropteroylglutamate as cofactor. However, the maximum activity for methionine synthesis obtained after pre-incubation of the ultrasonic extract was never greater than that obtained without any pre-incubation, suggesting that partial inactivation of some enzymes concerned in methionine synthesis had occurred under these conditions. Consequently this approach to the function of cobalamin was discarded as being not entirely satisfactory.

The addition of small amounts of ultrasonic extract and cobalamin to complete reaction mixtures containing extracts of acetone-dried strain PA15 and Pth_4G enabled methionine to be synthesised. This synthesis was often 6-8 times greater than that with the ultrasonic extract as sole source of enzymes. The synergism suggested that X was being produced by the ultrasonic extract and then used in the synthesis of methionine by the acetone-powder extract.

Synthesis of X by Ultrasonic Extracts

The assay of X depends on its ability to promote synthesis

of methionine by extracts of acetone-dried organisms when PtH_4G is the folic acid cofactor; free cobalamin is not active (see Chapter 1). In order to obviate the possibility of X being formed during the assay procedure it was necessary to separate the X formed by the ultrasonic extract, as far as possible, from both the enzymes concerned in its formation and from free cobalamin; it was also desirable to remove other enzymes which participate in the conversion of serine and homocysteine to methionine. Acetone treatment of the incubated reaction mixture I (complete reaction for methionine synthesis) was not very satisfactory; the apparent recovery of X activity was low and considerable activity for formation of X remained in acetone-treated reaction mixtures from which cobalamin was omitted. A good separation was however achieved by treatment of the reaction mixture with protamine sulphate. Reaction mixture II (which contains no serine, homocysteine, pyridoxal phosphate or PtH_4G) was developed for studying the synthesis of X and the procedure used most frequently for separating X was as follows. Protamine sulphate (2%; 0.6ml. per 6.0ml. of reaction mixture II) was stirred in after incubation and the mixture stood for 20 min. at 0° before centrifuging at 25,000g for 5 min. The residue (first protamine precipitate, P1) was normally discarded and the supernatant fluid dialysed against 0.05M phosphate buffer pH 7.8 for 18hr. at 3° (150ml. buffer/ml.

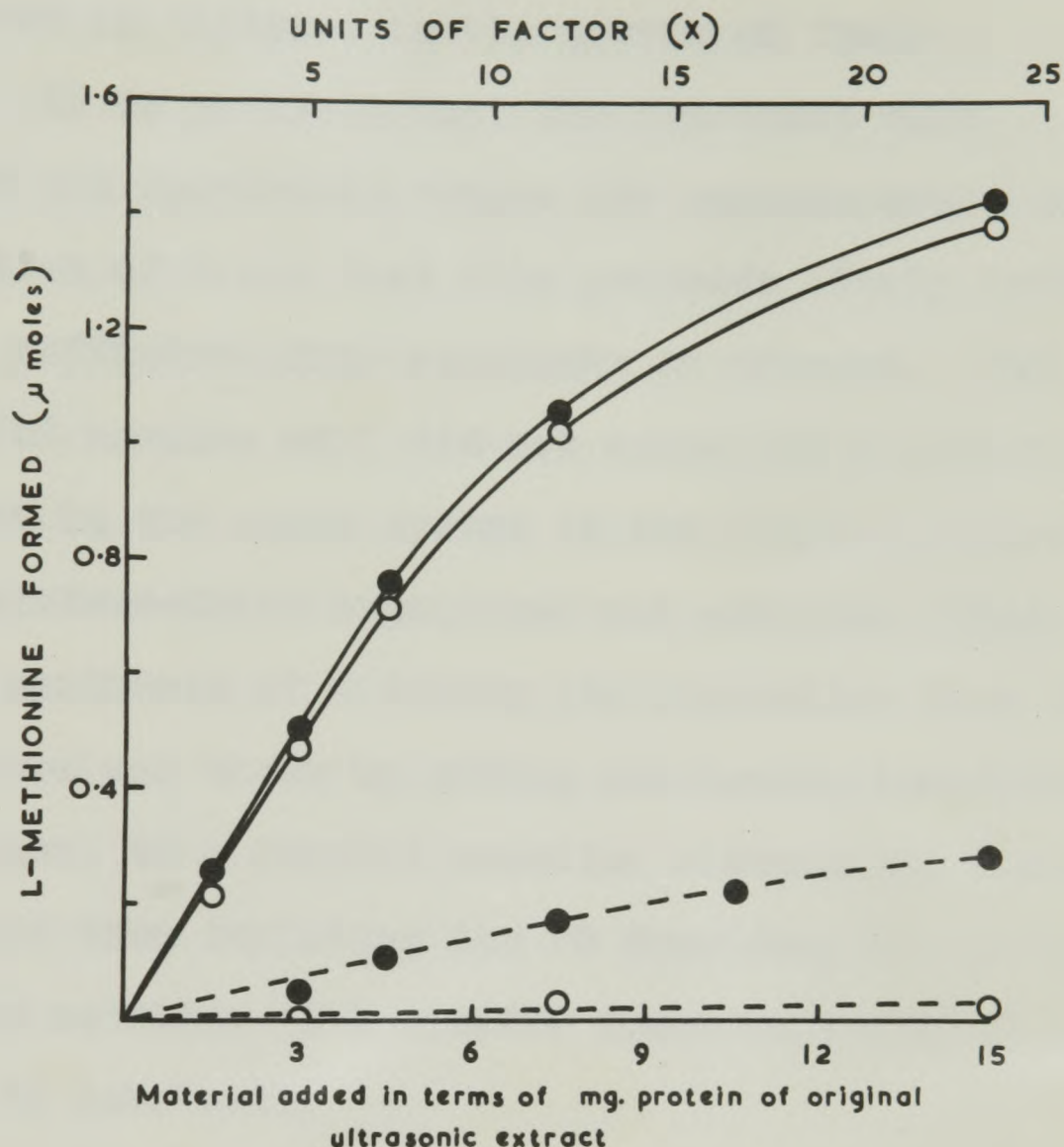
original reaction mixture); the dialysis fluid was changed twice. During the dialysis a fine white precipitate formed (second protamine precipitate, P2). After centrifuging (15000g, 10 min.) P2 was suspended in half the original reaction volume of 0.2M phosphate buffer, pH7.8 (3ml.) using a Potter-Elvehjem homogeniser.

With relatively small samples of the partly purified X (P2) synthesis of methionine in the assay system showed a linear relationship to the size of the sample (Fig.23), though with larger samples a proportionate increase was not obtained; possibly some other factor or enzyme becomes rate-limiting. A satisfactory assay of X was obtained using the range over which the response was linear. Samples used for the determination of the content of X in P2 were generally equivalent to 4.5 or 6.0mg. of the protein of the ultrasonic extract used to produce X; these by themselves caused the synthesis of less than 50 μ m-moles L-methionine.

The addition of free cobalamin to the assay system had no significant effect (Fig.23). There was a small but significant formation of methionine in the control in which cobalamin had been omitted from the system for synthesis of X but added to the assay system (Fig.23); this amounted to about 15% of the value obtained with cobalamin also added to the synthesis system, but was much smaller (2%) in the initial

FIG. 23

The function in methionine synthesis of the factor (X) formed by ultrasonic extracts of *Escherichia coli* PA15



Reaction mixture II with cobalamin ($50\mu\text{moles/ml.}$) present (—) or absent (---), incubated 4hr.

The reaction mixtures were then treated to obtain the second protamine precipitates (P2) and the latter assayed for X using reaction mixture III with normal extract of acetone-dried organisms equivalent to 10 mg. protein and with cobalamin ($65\mu\text{moles}$) present (●) or absent (○); incubated 3hr. (Units of factor activity were deduced from the curve 0—0).

experiments using a complete reaction mixture I for X synthesis. The validity of the assay was not affected by this since free cobalamin in the samples would have been largely removed by dialysis in the course of their preparation. It is probable that the treatment with protamine did not completely remove the enzymes concerned in the formation of X and that this proceeds slowly during the assay if sufficient free cobalamin is present. The partly-purified samples of X did not cause the synthesis of any methionine in the assay system if the enzyme preparation (extract of acetone-dried organisms) was omitted. That there was no synthesis of X during its separation from the reaction mixture was shown by adding cobalamin, immediately after incubation, to a control reaction mixture not containing the vitamin and then isolating the P2 fraction; it had less than 5% of the activity of a similar fraction isolated after incubation with cobalamin.

Recovery of X activity during isolation

The first protamine precipitate P1 and the supernatant fluid remaining after removing P2 had no X activity, nor did they affect the activity of P2. Furthermore, the activity of P2 was apparently the same as that of an equivalent quantity of the reaction mixture which had simply been dialysed. It is probable therefore that all of the X is

precipitated with the P2 fraction. When the suspension of P2 in 0.2M phosphate buffer pH7.8 was centrifuged, all the activity was recovered in the supernatant fluid; this final step was generally omitted. An overall purification of X of 4 to 5-fold was achieved during its separation; this was shown by dry weight determinations after exhaustive dialysis of the fractions against water.

It was concluded that incubation of the ultrasonic extract with cobalamin in a suitable reaction mixture leads to the production of material with similar biological activity to that extracted from acetone powders of organisms grown in the presence of cobalamin. It is clear that cobalamin functions in the intermediate formation of X which is necessary for the overall synthesis of methionine by ultrasonic extracts; and a method of assay for this property of the extract, independent of methionine formation is now available. 8 min. at 60°. The preparations of X were

unstable at pH values below 8 and above 8.5 (Table II.6).

The small: Properties of the Factor (X)

These tests were carried out with the partly-purified P2 fraction. The cobalamin content as measured with Euglena gracilis was 7% of the total cobalamin added to the original reaction mixture. For a sample of the size used for assay (equivalent to 6mg. original ultrasonic protein) this

corresponds to only about 2 $\mu\mu$ moles cobalamin compared with the 65 $\mu\mu$ moles added to the assay system in the controls of the experiments of Fig.23; it follows that methionine synthesis is not simply due to the presence of cobalamin. The cobalamin content of similar samples of P2 prepared from the reaction mixture for X synthesis in which the cobalamin was not added until after incubation and, in which no cobalamin was added were 0.27 and 0.02 $\mu\mu$ moles respectively. Considering that the fractions had been extensively dialysed it appears that cobalamin is bound during incubation. However it is not known whether there is any direct correlation between the bound cobalamin and X activity.

The activity of X was not reduced by dialysis for 48hr. against 0.05M-potassium phosphate buffer pH7.8, nor was it significantly altered by storing 6 weeks at -14° . It was heat labile; 70% activity was lost after 5 min. at 50° and 100% after 5 min. at 60° . The preparations of X were unstable at pH values below 5 and above 8.5 (Table II.8). The small residual activity for synthesis of X by P2 fractions prepared in the absence of cobalamin was no less stable to heat and pH so such treatments were of no value in the further isolation of uncontaminated X. The properties suggest that X is a protein or at least closely associated with a protein and is similar to the material

TABLE II.8

Effect of pH on the activity of factor (X)

<u>Buffer (0.1M)</u>	<u>pH</u>	<u>% activity remaining after treatment</u>
Acetic/acetate	{ 3.5	5
	{ 4.2	55
	{ 5.0	85
Phosphate	{ 6.1	92
	{ 7.0	96
	{ 7.8	100
Carbonate/bicarbonate	{ 9.0	75
	{ 9.9	50
	{ 10.7	35

Synthesis of factor. Reaction mixture II with ultrasonic extract of strain PA15 and cobalamin (50 μ moles/ml.); incubated 3hr. Samples of preparation P2 equivalent to 6 mg. original protein treated at the pH stated for 30 min. at 0°.

Assay of factor. Reaction mixture III with normal extract of acetone dried strain PA15 (10 mg. protein). Incubated 3hr.

separated from acetone-dried powders of cobalamin-grown organisms.

Summary

A heat-labile, non-diffusible material has been partly-purified from the products of the incubation of ultrasonic extracts of *E. coli* PA15 with a medium containing cobalamin. This factor (X) is essential for the synthesis of methionine by extracts of acetone-dried organisms of the same strain, when Pth_4G is the source of the necessary folic acid cofactor. The factor had properties similar to material with the same biological activity isolated from the same organism grown in the presence of cobalamin. The role of cobalamin in the overall process of methionine formation by ultrasonic extracts (serine with Pth_4G) is almost certainly in the formation of this functional factor; and an independent assay for this activity has been developed.

X can be formed in a cell-free system, independent of methionine synthesis, and since an assay for the formation of X has been developed, direct investigation of the site of action of these inhibitory analogues is possible. *Salmonella* strain PA15 was used throughout.

Effect on the activity of the Factor (X)

The amide of the carboxylic acid of cobalamin

CHAPTER 5

THE EFFECT OF ANALOGUES OF COBALAMIN ON THE FORMATION AND ACTIVITY OF FACTOR (X) AND THEIR USE IN THE ASSAY OF FACTOR FORMATION

Effect of Analogues of Cobalamin

Several analogues of cobalamin have been found to inhibit the action of cobalamin in the synthesis of methionine by ultrasonic extracts of *E. coli* PA15 (serine with Pth₄G). However growth of the organism on cobalamin rendered the subsequent extracts immune to inhibition by the anilide analogue; though methionine synthesis was reduced if the analogue was added with cobalamin to the growth medium (Chapter 3). From a consideration of the primary role of cobalamin in the synthesis of the factor (X) which functions in methionine synthesis, the results suggest that the analogues may interfere with the formation of X, but not with its activity once formed (viz: during growth). Now that X can be formed in a cell-free system, independent of methionine synthesis, and since an assay for the formation of X has been developed, direct investigation of the site of action of these inhibitory analogues is possible. *E. coli* strain PA15 was used throughout.

Effect on the activity of the factor (X)

The anilide of the monocarboxylic acids of cobalamin

had no effect on the activity of the partly-purified (P2) preparation of X derived from the incubation of cobalamin with ultrasonic extract (Table II.9). A more extended series of tests was carried out with a crude preparation of X derived from acetone-dried E. coli PA15 grown in the presence of cobalamin. The amount of this extract used catalysed the formation of negligible quantities of methionine and contained less than 15 μ moles of cobalamin. The anilide analogue again had no effect on the activity of X (Table II.9); this was also the case with the methylamide and ethylamide analogues. Factor B did however inhibit about 40% at relatively high concentrations; the inhibition (by 70 μ moles) was not relieved by cobalamin (up to 6.5 μ m-moles, cf. Chapter 2). High concentrations of cobalamin itself (6.5 and 65 μ m-moles) had no effect on the activity of X. In controls in which X was omitted the analogues and cobalamin did not themselves promote the synthesis of methionine in the assay system.

Effect on the formation of the factor (X)

All the analogues tested inhibited the formation of X completely if sufficiently high concentrations were used (Table II.10). The ratios of the concentration of cobalamin to analogue resulting in 50% inhibition were similar to those found for the inhibition of the activity of cobalamin in methionine synthesis (Chapter 2). The analogues themselves

TABLE II.9

Effect of analogues of cobalamin on the
activity of the factor (X)

<u>Source of factor (X)</u>	<u>Analogue (μm-moles)</u>	<u>L-methionine formed (μm-moles)</u>
A	None	1050
	Anilide (5.7)	1070
	(57.0)	1070
B	None	575
	Anilide (1.2)	580
	(57.0)	590
	Factor B (3.5)	465
	(70.0)	345

Source of factor. (A) Second protamine precipitate P2

from reaction mixture II incubated 4hr. with cobalamin

(50 μ m-moles/ml.); sample used was equivalent to 6 mg.

protein of the sonic extract. (B) Dialysed extract

(equivalent to 2 mg. protein) of acetone-dried organisms

grown in the presence of cobalamin.

Assay of factor. Reaction mixture III plus (A) normal

acetone-powder extract (10 mg. protein) or (B) ultrasonic-

treated acetone-powder extract (20 mg. protein) and

analogues as shown. Incubated 3hr.

TABLE II.10

Effect of cobalamin analogues on the
synthesis of the factor (X)

<u>Analogue</u> (μ m-moles/ml.)	<u>Units of factor (X) formed with</u>	
	<u>Cobalamin absent</u>	<u>Cobalamin present</u>
None	0.5	12.0
Methylamide (4.4)	2.4	3.0
(44.0)	2.2	2.1
Ethylamide (1.3)	-	5.7
(43.0)	1.3	1.8
Anilide (0.8)	-	3.6
(42.0)	1.3	1.3
Factor B (2.6)	-	4.4
(51.0)	0.7	0.8

Synthesis of factor. Reaction mixture II plus cobalamin (50 μ m-moles/ml.) when present and analogues as stated.

Incubated 3.5hr. Samples assayed were P2 fractions equivalent to 6 mg. original ultrasonic protein.

Assay of factor. Reaction mixture III plus normal extract of acetone-dried strain PA15 (10 mg. protein). Incubated 3hr.

when incubated with ultrasonic extracts did not give rise to significant X activity (Table II.10). Only in the case of Factor B was it possible that carry-over of the test substance into the final assay system could vitiate the results by inhibiting the function of X itself; it is unlikely however that inhibitory concentrations would remain after the treatment with protamine sulphate and dialysis used in preparing the samples for assay.

The conclusion that the anilide analogue inhibited the formation of X, though not its subsequent activity was supported by the experiment of Table II.11. Cobalamin was required for the synthesis of methionine by ultrasonic extracts with Pth_4G as the only sources of folic acid cofactor and such synthesis was completely inhibited by the anilide analogue. A preparation of X replaced cobalamin, but in this case the anilide analogue was not inhibitory. For this experiment a more purified preparation of X was used, derived from acetone-dried organisms grown with cobalamin (M.A. Foster & K.M. Jones, unpublished work in this laboratory); it had a higher ratio of specific activity to cobalamin content than the usual P2 preparation so that a negligible amount of free cobalamin was added with the sample of X.

No evidence could be obtained for the production of an inhibitory analogue of X when the anilide analogue was incubated with ultrasonic extract under the same conditions

TABLE II.11

Effect of the anilide analogue of cobalamin and factor (X) on the synthesis of methionine by ultrasonic extracts

<u>Additions</u>	<u>L-Methionine formed (μm-moles/mg. protein) with anilide analogue</u>	
	<u>Absent</u>	<u>Present</u>
None	4	10
Cobalamin	70	10
Factor (X)	38	40
Cobalamin + Factor (X)	110	42
Factor (X) (no ultrasonic extract)	2	0

The above results suggested a more concentrated reaction mixture I plus ultrasonic extract of strain PA15 (10 mg. protein) and PTH₄G (1 μ mole). Cobalamin (65 μ m-moles) and anilide analogue (30 μ m-moles) when present. Purified preparation of factor (X) equivalent to 0.1 mg. protein (see text). Incubated 4hr.

as led to the production of X from cobalamin. A second protamine precipitate was isolated from this reaction mixture (anilide analogue 12 μ m-moles/ml.); when added to the assay system at five times the concentration of a similar preparation of X (from a reaction mixture II containing cobalamin 50 μ m-moles/ml.) it did not inhibit the methionine production caused by the latter. Thus the inhibitory effect does not appear to be the result of a lethal synthesis type of mechanism (Markham, 1953).

Use of Analogues of Cobalamin in the Assay of Factor (X)

The above results suggested a more convenient method for the assay of X in which it would not be necessary to separate it (as the P2 fraction) from the reaction products of the first incubation. Addition of the anilide analogue to the reaction mixture (III) for the assay should prevent, during the assay, the formation of X from cobalamin carried over with the sample and, provided that the cobalamin concentration was not too great, dialysis alone should reduce the concentration sufficiently.

Ultrasonic extract was incubated with cobalamin in the usual reaction mixture (II). One portion of the mixture after incubation was simply dialysed against 0.05M potassium

phosphate buffer pH7.8 for 18hr. at 3° (150ml. buffer/ml. reaction mixture); the dialysis fluid was changed twice. The other portion was treated with protamine sulphate and a P2 fraction obtained as in Chapter 4. Both types of sample were assayed for X activity in the usual test system but with the anilide analogue either present or absent. Controls in which cobalamin was omitted from the reaction mixture for factor synthesis and others in which cobalamin (65 μ moles) was added to ^{the} reaction mixture for assay, were also done. The activity of the two types of sample were similar (Table II.12) whether the anilide analogue was present or not in the assay system. With samples of the control in which cobalamin was omitted from the synthesis system, but was present in the assay system, a higher value was obtained with the simply dialysed sample than with the protamine-treated sample. This apparent X activity was greatly reduced by the addition of the anilide analogue to the assay system (Table II.12).

The main disadvantage of this method for assay of X was that when the extract of acetone-dried organisms was omitted from the assay system there was a small synthesis of methionine due to the presence of a small quantity of all the enzymes of the ultrasonic extract (carried over with the sample). Under the conditions normally used this was equivalent to 2-3 units of X, and this value ^{was} not significantly lowered by the anilide analogue since X had been already formed in the

TABLE II.12

The anilide analogue of cobalamin and the assay of the factor

Assay of the factor

<u>Sample</u>	<u>Cobalamin (during factor synthesis)</u>	<u>Cobalamin (65 μmoles)</u>	<u>Units of factor activity in the sample in presence of anilide analogue (μm-moles)</u>		
			<u>0</u>	<u>5.7</u>	<u>57.0</u>
(A) (P2 fraction)	{ Present	(Absent	10.8	11.0	11.0
		(Present	11.2	-	-
	{ Absent	(Absent	0.2	-	-
		(Present	1.8	0.5	0.2
(B) (Dialysed only)	{ Present	(Absent	11.5	-	10.8
		(Present	11.3	-	-
	{ Absent	(Absent	0.4	-	0.8
		(Present	3.2	1.5	0.9

Synthesis of factor. Reaction mixture II, ultrasonic extract of strain PA15 and cobalamin (50 μ moles) when present. Incubated 4hr. Samples of factor tested (equivalent to 6 mg. protein of ultrasonic extract, (A) Second protamine precipitate P2, (B) dialysed reaction mixture.

Assay of factor. Reaction mixture III with cobalamin and anilide analogue as shown; normal extract of acetone-dried organisms (10 mg. protein). Incubated 3hr.

so that its concentration would not be rate-limiting.

first incubation. To overcome this difficulty several possibilities were investigated.

- 1) Growth of the E.coli strain PA15 in the presence of methionine is known to repress the activity of ultrasonic extracts for the synthesis of this amino acid (Section I, Chapter 5). However, the presence of methionine during growth was also found to reduce the synthesis of X (Table II.13).
- 2) Ultrasonic extracts of E.coli strain 3/62 are unable to synthesise methionine. However, under the same conditions, the activity of strain 3/62 for the synthesis of X was only 10-20% of that of strain PA15. This was too small for studying the synthesis of X. On the other hand, the synthesis of X during growth in the presence of cobalamin was almost 100% of that of strain PA15.
- 3) Several methods for the fractionation of the ultrasonic extract were examined in an attempt to separate the two activities, viz. synthesis of X and methionine formation. These included: heat treatment, iso-electric precipitation, ammonium sulphate, protamine sulphate and acetone treatment. However, no satisfactory separation could be found.
- 4) The problem was finally solved (a) by ensuring that all systems for assay of X contained the same amounts of protein of similar origin and (b) by increasing the amount of the main source of enzymes (extract of acetone-dried organisms) so that its concentration could not be rate-limiting.

TABLE II.13

Effect of growth with methionine on subsequent activity of ultrasonic extracts of *E. coli* PA15 for methionine synthesis and formation of factor (X)

Concentration of DL-methionine in growth medium (M)	% Activity of extracts compared with those prepared in the absence of methionine for	
	<u>Methionine synthesis</u>	<u>X formation</u>
0	100	100
10^{-5}	97	96
10^{-4}	93	72
10^{-3}	24	40
10^{-2}	15	33

Methionine synthesis. Reaction mixture I with ultrasonic extract of organisms grown with DL-methionine; Pth_4G (1 μmole) and cobalamin (65 μmoles). Incubated 4hr.

Formation of factor. Synthesis and assay of the factor were as in Table II.10.

1) Homosubstituted cobalamin-analogs inhibit the formation of the factor (X) by ultrasonic extracts but have

The final procedure for this alternative assay of X (which was adopted for most of the work still to be described) was to dialyse the products of the first reaction against 0.05M phosphate buffer pH7.8, and to assay samples in reaction mixture III in the presence of the anilide analogue (23 or 46 μ m-moles) and with the ultrasonic-treated extract of acetone-dried organisms increased to the equivalent of 20mg. protein. The curve relating size of sample to amount of methionine synthesised was similar to that of Fig.18 for the original method of assay except that the linear range was slightly reduced. Addition of cobalamin (0.1 μ m-moles) to the assay system did not increase the methionine produced by either experimental or control samples of the reaction mixture for the synthesis of X.

Apart from being more convenient this new assay for X enabled the formation of X to be studied when changes were made in the synthesis system, either by purification of the ultrasonic extract or by altering the composition of the reaction mixture II, which made protamine treatment no longer practicable.

Summary

- 1) Monosubstituted cobalamin-amide analogues inhibit the formation of the factor (X) by ultrasonic extracts but have

no effect upon its function in methionine synthesis. Factor B partially inhibits both the formation and the function of the factor.

2) A more convenient method of assaying the formation of X by ultrasonic extracts has been developed; it makes use of the inhibitory properties of the anilide analogue of cobalamin. The anilide analogue of cobalamin was used wherever possible.

Effect of the concentration of cobalamin

An optimal synthesis of X during the first hour of incubation was obtained with 0.5 μ -mole cobalamin/ml. (Fig. 2). Synthesis was proportional to time up to 45 min. With higher concentrations of cobalamin, although the initial rate was increased, synthesis of X stopped after a short period; the reason for this is not known. In subsequent experiments a cobalamin concentration of 0.5 μ -mole/ml. and 45 min. incubation was used as a routine. The cobalamin content of the resultant dialysed samples used for assay of X was estimated with *Escherichia coli* as 0.1 μ -mole, and this amount of cobalamin was added to the controls during the assay of X. The anilide analogue added to the assay system was increased to 10 μ -mole to cause complete inhibition of X formation (during the assay) promoted by cobalamin carried over.

CHAPTER 6

PROPERTIES OF THE SYSTEM FORMING THE FACTOR (X)

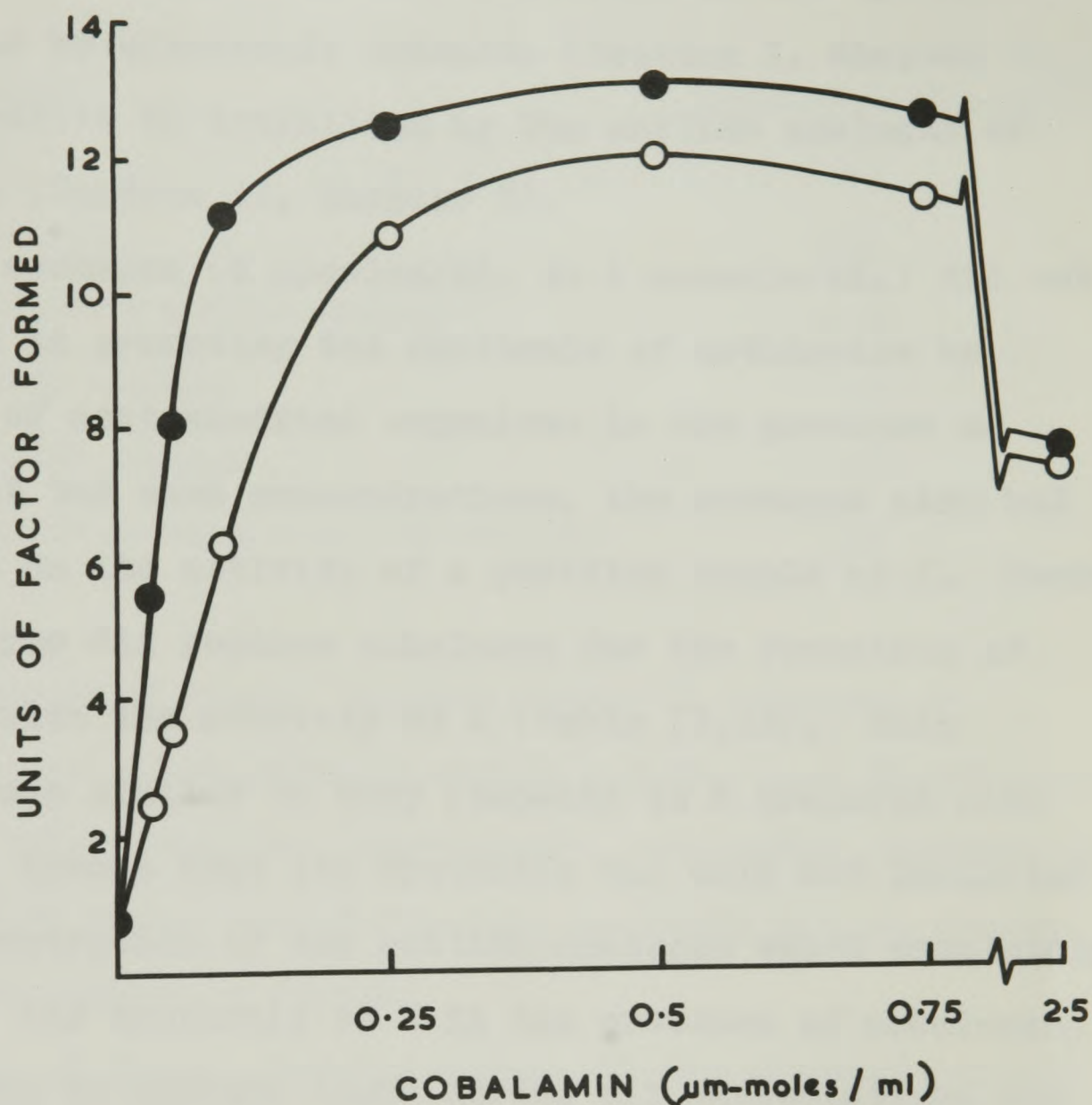
Details of the synthesis of X by ultrasonic extracts of E.coli strain PA15 were examined in an attempt to elucidate the nature of the factor. The modified assay system incorporating the anilide analogue of cobalamin was used wherever possible.

Effect of the concentration of cobalamin

An optimal synthesis of X during the first hour of incubation was obtained with 0.5 μm -moles cobalamin/ml. (Fig.24); synthesis was proportional to time up to 45 min. With higher concentrations of cobalamin, although the initial rate was increased, synthesis of X stopped after a short period; the reason for this is not known. In subsequent experiments a cobalamin concentration of 0.5 μm -moles/ml. and 45 min. incubation was used as a routine. The cobalamin content of the resultant dialysed samples used for assay of X was estimated with Euglena gracilis as 0.1 μm -moles, and this amount of cobalamin was added to the controls during the assay of X. The anilide analogue added to the assay system was increased to 46 μm -moles to ensure complete inhibition of X formation (during the assay) promoted by cobalamin carried over.

FIG. 24

The effect of cobalamin concentration on the synthesis of the factor (X) by ultrasonic extracts



Synthesis of factor. Reaction mixture II plus Dowex-treated ultrasonic extract and cobalamin; incubated 1hr. (O) or 3hr. (●). Dialysed samples equivalent to 6mg. protein of the original ultrasonic extract were used for the assay.

Assay of factor. Reaction mixture III plus extract of acetone-dried organisms (20 mg. protein) and anilide analogue (46μm-moles); incubated 3hr.

5,6-Dimethylbenzimidazolylcobamide coenzyme

This coenzyme form of cobalamin was previously found to be 2-3 times more active than cobalamin in the synthesis of methionine by ultrasonic extracts (Section I, Chapter 3), and less sensitive to inhibition by the anilide analogue of cobalamin (Section II, Chapter 3).

The coenzyme (5 μ moles/ml. to 1 μ m-mole/ml.) did not replace X in promoting the synthesis of methionine by extracts of acetone-dried organisms in the presence of PtH_4G . At the same concentrations, the coenzyme also had no effect on the activity of a purified sample of X. However the coenzyme did replace cobalamin for the formation of material with the activity of X (Table II.14). This material was similar in many respects to X prepared with cobalamin except that its synthesis was only 80% inhibited by a concentration of the anilide analogue which completely prevented the synthesis of X in the presence of cobalamin. Precautions to exclude light during all manipulations and incubations of the coenzyme and coenzyme-containing materials were taken; the X activity of a P2 fraction prepared from a reaction mixture containing the coenzyme was however completely stable to conditions of irradiation which inactivated aqueous solutions of the coenzyme (Section I, Chapter 3). The requirements for formation of X in the

TABLE II.14

5,6-Dimethylbenzimidazolylcobamide coenzyme
and the synthesis of the factor by ultrasonic
extracts of Escherichia coli PA15

<u>Additions</u>	<u>Units of factor formed</u>
None	0.7
Cobalamin	10.0
Cobalamin + anilide analogue	0.9
Anilide analogue	0.9
Cobamide coenzyme	11.2
Cobamide coenzyme + anilide analogue	3.0

Synthesis of factor. Reaction mixture II, cobalamin or 5,6-dimethylbenzimidazolylcobamide coenzyme (each 50 μ moles/ml.) and anilide analogue (95 μ moles/ml.) when added. Incubated 4hr. Samples assayed were P2 fractions equivalent to 6 mg. protein of the ultrasonic extract.

Assay of factor. Reaction mixture III plus ultrasonic-treated extract of acetone-dried organisms (20 mg. protein) and anilide analogue (25 μ moles). Incubated 3hr.

The synthesis of X increased linearly with the concentration of ultrasonic extract up to the limit tested (11mg. protein/ml.). Different batches of the extract varied

presence of the cobamide-coenzyme were similar to those for cobalamin (Table II.15). The product of the coenzyme function in Clostridium tetanomorphum, β -methylasspartate, had no activity for replacing X in methionine formation.

Requirements for the Synthesis of the Factor (X)

Components of the reaction mixture

Omission of any of the constituents of reaction mixture II reduced the rate of formation of X, the effect of ATP and of glucose being the most pronounced (Table II.15). The absence of DPN reduced synthesis by about 40%. Increasing concentrations of ATP would not replace the glucose requirement but reduced DPN replaced both DPN and glucose and at higher concentrations it also eliminated the requirement for ATP. Glucose could also be replaced by hexose-1,6-diphosphate and ATP by ADP but not by AMP. Addition of the other substrates and cofactors for the synthesis of methionine itself (DL-homocysteine, 7.5 μ moles/ml; L-serine, 7.5 μ moles/ml; Pth_4G , 0.75 μ moles/ml.) singly or in all possible combinations, had no significant effect on the synthesis of X.

Ultrasonic extract

The synthesis of X increased linearly with the concentration of ultrasonic extract up to the limit tested (11mg. protein/ml.). Different batches of the extract varied

TABLE II.15

Requirements for the formation of the factor by
ultrasonic extracts of *Escherichia coli* PA15

Omissions from reaction mixture II	Additions (μ moles)	Units of factor formed with	
		Cobalamin	Cobamide coenzyme
None		9.5	11.0
Cobalamin or cobamide coenzyme		0.6	0.9
Glucose		2.2	4.0
DPN		6.0	5.0
ATP		2.5	-
Mg ²⁺		3.4	-
ATP and Mg ²⁺		2.4	3.2
Glucose, ATP, DPN, Mg ²⁺		2.0	2.2
Glucose and DPN	DPNH ₂ (4)	9.5	-
Glucose, DPN and ATP	DPNH ₂ (16)	9.0	-

Synthesis of factor. Reaction mixture II plus Dowex-treated ultrasonic extract and cobalamin (0.5 μ -moles/ml.) or 5,6-dimethylbenzimidazolyl-cobamide coenzyme (0.4 μ -moles/ml.). Incubated 45 min. Samples for assay were equivalent to 4.5 mg. protein of the ultrasonic extract.

Assay of factor. Reaction mixture III plus ultrasonic-treated extract of acetone-dried organisms (20 mg. protein) and anilide analogue (4.6 μ -moles). Incubated 3hr.

in activity by an overall factor of two, but there was generally no loss of activity on storage for up to 6 weeks at -14° . In most experiments the extract was treated with Dowex-1 resin which produced a variable reduction in activity (usually by about 10-15%). The activity could be restored and sometimes enhanced by the addition of a heated extract of the organism (EHC).

In order to isolate and define more clearly the system causing the synthesis of X, fractionation of the ultrasonic extract by several methods was attempted (cf. Chapter 5). Of the methods tested only treatment with protamine sulphate and ammonium sulphate resulted in a significant purification. Under certain conditions a fraction containing 80% of the original activity with a 2-fold increase in specific activity was obtained. However, the specific activity for methionine synthesis was similarly increased. The extract lost 90% of its activity on heating to 45° for 3 min. and was completely inactivated by incubation at 50° for 3 min. The fractionations were however done before it was found that reduced DPN could replace glucose and DPN, and at higher concentrations, ATP also.

Ultrasonic extracts of E.coli 121/176 also catalysed the synthesis of X, and their activity was the same as those of strain PA15, using dialysed samples or P2 fractions in the

assay system containing extract of acetone-dried strain PA15. The synthesis of X by extracts of strain 3/62 was only 10-30% of that of strains PA15 and 121/176.

Other Properties of the System forming Factor (X)

The presence of substances possibly required for protein synthesis e.g. mixtures of amino acids (acid-hydrolysed casein supplemented with cysteine and tryptophan), mixtures of amino acids, purines, pyrimidines and B-group vitamins (Medium C), did not increase the synthesis of X. Also, the synthesis was not affected by analogues of a number of amino acids and purines: 4-methyltryptophan (1mM), p-fluorophenyl-alanine (1mM) and 8-azaguanine (200µM). Chloramphenicol (140µM) inhibited by 30% only, but could not be tested at higher concentrations because it would have inhibited the growth of the organism used in the assay of methionine.

Reducing agents, glutathione and ascorbic acid (each 5mM) caused a small and variable (ca10%) increase in X formation. Homocysteine often produced a similar increase.

Formation of X was decreased 20-30% under aerobic or semi-aerobic conditions compared with the anaerobic conditions normally used.

Cyanide (0.1M) almost completely inhibited the synthesis of X and the activity of X was destroyed by treatment with

cyanide (0.01M) at pH 7.8 and 16° for 15 min.

*The Formation of Factor (X) and its Role
in Methionine Synthesis*

Summary

1) Cobalamin, glucose, DPN, ATP and Mg^{2+} are necessary for optimal synthesis of factor (X) by ultrasonic extracts of *E. coli* PA15. $DPNH_2$ can replace glucose and DPN and, at higher concentrations, ATP also.

2) 5,6-Dimethylbenzimidazolylcobamide coenzyme has no activity as the factor but replaces cobalamin for its formation; the requirements for synthesis of X with the cobamide coenzyme are the same as with cobalamin.

the organism to form X, though acetone does not affect X once formed. Extracts of such acetone-dried organisms cannot synthesize methionine with $5H_4O$ as the source of folic acid cofactor, and do so only very feebly on the addition of free cobalamin. Extracts of acetone-dried organisms may therefore be used for the assay of the X which is formed either by growing the organism in the presence of cobalamin (Kisliuk & Woods, 1950) or, as in the present work, incubating cobalamin with the ultrasonic extract. The formation of the factor (X) and its assay are shown diagrammatically in Figure 25. The biological activities of X derived from both sources are identical; purified samples of X derived from acetone-dried organisms can also replace the cobalamin requirement for methionine synthesis by ultra-

DISCUSSION OF EXPERIMENTAL SECTION II

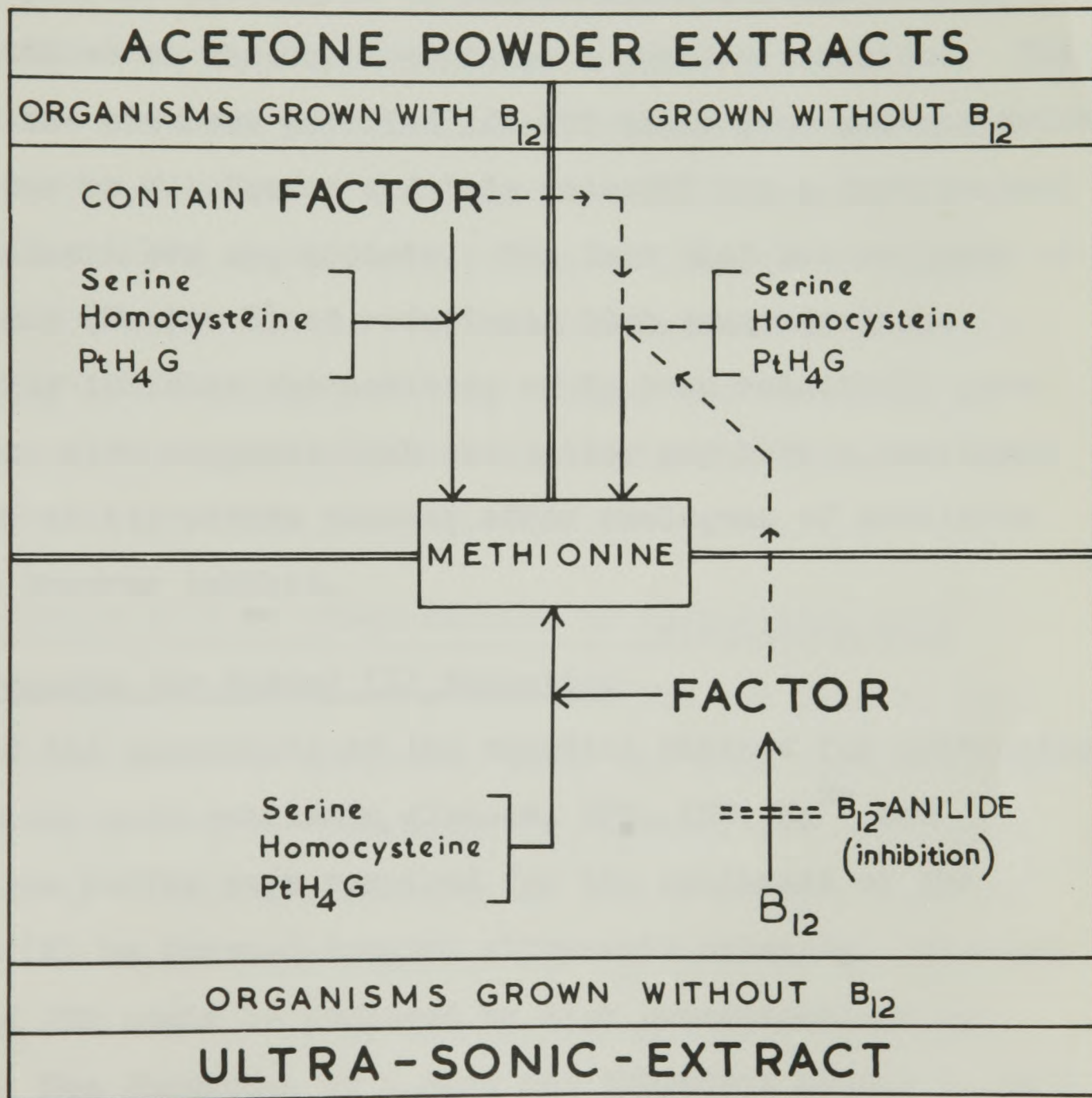
The Formation of Factor (X) and its Role in Methionine Synthesis

Biological activity and nature of the factor

The difference in behaviour of extracts of acetone-dried organisms and ultrasonic extracts appears to lie in the ability of the latter to utilise free cobalamin in the formation of factor (X) which is required for the formation of methionine by soluble extracts of E.coli PA15 when PtH_4G is provided as the cofactor for C_1 -transfer. Treatment with acetone appears to destroy almost completely the capacity of the organism to form X, though acetone does not affect X once formed. Extracts of such acetone-dried organisms cannot synthesise methionine with PtH_4G as the source of folic acid cofactor, and do so only very feebly on the addition of free cobalamin. Extracts of acetone-dried organisms may therefore be used for the assay of the X which is formed either by growing the organisms in the presence of cobalamin (Kisliuk & Woods, 1960) or, as in the present work, incubating cobalamin with the ultrasonic extract. The formation of the factor (X) and its assay are shown diagrammatically in Figure 25. The biological activities of X derived from both sources are identical; purified samples of X derived from acetone-dried organisms can also replace the cobalamin requirement for methionine synthesis by ultra-

FIG. 25

Cobalamin and the synthesis of methionine in
Escherichia coli PA15



sonic extracts. The physical and chemical properties of X from both sources are also similar and suggest that the factor is a protein or firmly associated with a protein. Although the material contains cobalamin it cannot be said at the present low degree of purification whether this is adventitious or whether X contains a cobalamin residue. The factor has now been purified 100-150 times from acetone-dried organisms by M.A. Foster and this material has a high content of cobalamin per mg. protein. The fact that one analogue of cobalamin (Factor B) at relatively high concentrations partially inhibits the activity of X, even relatively pure samples, also suggests that the latter may have a cobalamin residue at its active centre; other analogues of cobalamin do not however inhibit.

The synthesis of X by other strains of *Escherichia coli*

Requirements for factor (X) formation

Of the components of the reaction mixture for methionine formation, only cobalamin, glucose, DPN, ATP, Mg^{2+} and phosphate buffer were required for the synthesis of the factor (X) by Dowex-1-treated ultrasonic extracts. Glucose, DPN and ATP could be replaced by high concentrations of $DPNH_2$. The formation of X does not therefore appear to be a simple activation of a protein by cobalamin but a more complex process; no other evidence for the dissociation of X into a coenzyme and an apoenzyme was obtained during this work.

Although it was not proved that X is not a product of de novo protein synthesis, it is more likely to result from the modification of a pre-existing protein; the formation of X was not increased by mixtures of amino acids, purines and pyrimidines and it was not inhibited by analogues of these substances which would be expected to inhibit protein synthesis. Attempts to fractionate the extract were made before it was discovered that glucose and DPN could be replaced by DPNH_2 which would reduce the number of enzymes required for X formation. Further attempts to fractionate the extract in the light of this finding may therefore be more successful and information regarding the mechanism of the synthesis of X may be obtained.

The synthesis of X by other strains of *Escherichia coli*

Ultrasonic extracts of strain 121/176 are able to form X when incubated with cobalamin under the same conditions as used for strain PA15. Cobalamin is required for methionine formation by ultrasonic extracts of strain 121/176 when EHC supplies the folic acid cofactor for C_1 -transfer as well as when Pth_4G is used. It is therefore reasonable to suggest that the function of X in methionine synthesis is not confined to the utilisation of Pth_4G in this organism.

Ultrasonic extracts of strain 3/62 are unable to synthesise methionine but they are capable of forming X, albeit in smaller quantity than the other strains. With this strain, the mutation has probably blocked the conversion of homocysteine to methionine at some point before or after the functioning of X. This emphasises that concurrent methionine synthesis is not necessary or helpful to the formation of X. However the synthesis of X appears to be controlled by the same mechanism controlling methionine formation; the presence of methionine in the growth medium of strain PA15 represses simultaneously the formation of enzymes for the synthesis of methionine and of X. The formation of X from cobalamin during the growth of strain PA15 has also been found to be inhibited by the presence of methionine (R.J. Rowbury, private communication).

Organisms grown in the absence of cobalamin do not contain any detectable X; this has been confirmed by Kisliuk (1960). Now, cobalamin increases the early growth of strains PA15 and W, so this may be the result of an increase in methionine formation due to the synthesis of X.

Towards the end of this work, the existence of an enzyme containing cyano- or hydroxocobalamin which is concerned in the synthesis of methionine from homocysteine and serine by ultrasonic extracts of E.coli 113/3 (cobalamin grown) was

reported by Hatch, Takeyama and Buchanan (1959). More recently cobalamin was defined as the prosthetic group of this enzyme and the apo-enzyme was purified 30-fold from extracts of this organism after growth with methionine (Takeyama & Buchanan, 1960). The holo-enzyme was prepared by incubating the apo-enzyme with cobalamin, PtH_4G , flavin adenine dinucleotide and a sulphhydryl compound. This material may be the same as X although the requirements for the formation of X by crude ultrasonic extracts of E. coli PA15 are very different.

Theories concerning the possible functions of cobalamin and X in methionine synthesis will be discussed in a later section.

Inhibitory Analogues of Cobalamin

The use of factor B and the methylamide, ethylamide and anilide derivatives of the monocarboxylic acids of cobalamin has made a significant contribution to the study of the role of cobalamin in the synthesis of methionine.

Inhibition of methionine synthesis and factor (X) formation

The synthesis of methionine was inhibited by the analogues of cobalamin wherever cobalamin was required for such synthesis and, the sensitivity to the anilide analogue was the same

whatever strain of E. coli or source of folic acid cofactor was used. The anilide analogue shows a competitive relationship with cobalamin and the inhibitory activity is thus related specifically to the function of cobalamin. The ability of the analogues both to replace cobalamin and inhibit its action makes it difficult to interpret the results in a quantitative manner. There are several possible explanations for this phenomenon. First, the analogues may have sufficient structural similarity to cobalamin to function as cobalamin at low efficiency. Second, the enzymic extracts may convert a proportion of the analogue to cobalamin. The contamination of the analogues with cobalamin is less than 0.02% (E.L. Smith, private communication) and this would not account for their ability to replace cobalamin. ~~as with the high concentration~~

The mono-substituted cobalamin amide analogues had no effect on the activity of X but they inhibited the formation of X when added with cobalamin to the ultrasonic extract. The relative concentrations of analogue and cobalamin were similar for the inhibition of the formation of X and for inhibition of methionine synthesis. The formation of methionine by extracts of organisms, grown in the presence of cobalamin, was resistant to inhibition by the analogues, presumably because they contained preformed X. A direct demonstration of the inhibition of the formation of X during growth was not possible, due to the large quantities of

analogue which would have been required. However, the presence of the anilide analogue in the growth medium of strain PA15 in addition to a limiting concentration of cobalamin, inhibited the activity for methionine synthesis of the ultrasonic extracts subsequently prepared. Here, the relative concentrations of the anilide analogue and cobalamin which produced this inhibition were of a similar order to those which inhibited the growth of cobalamin-requiring strains. The inhibition was not absolute because the extracts of organisms grown in the presence of the anilide (with no added cobalamin) had themselves considerable activity. On the data available concerning the upper limit of possible contamination of the analogue with cobalamin; such contamination is of possible significance with the high concentrations of analogue relative to cobalamin used in these experiments. Also, any tendency to convert the analogue to cobalamin could be fully exploited during growth.

These observations confirmed the presence of a specific system for the formation of X in E.coli PA15 and they also indicate the similarity between X formed during growth and by incubation of the ultrasonic extract with cobalamin.

The action of the analogues was localised at the synthesis of X although this could be interpreted as (a) the inhibition of the conversion of cobalamin to a coenzyme form, (b) inhibition of the incorporation of a cobalamin residue

into X or (c) as the inhibition of a catalytic function of cobalamin in the formation of X.

The properties of the anilide analogue were important in the development of a convenient assay of the formation of X by ultrasonic extracts. This assay enabled the examination of the requirements for X formation and also permitted the study of many other properties of the system which would not have been possible using protamine sulphate precipitation, because the precipitation reaction was itself frequently influenced by the test conditions.

Factor B in the presence of 5,6-dimethylbenzimidazole replaced cobalamin to a limited extent. However at higher concentrations it not only inhibited methionine synthesis and the formation of X but also the activity of X.

The synthesis of methionine by ultrasonic extracts of Cobalamin analogues and the growth of *Escherichia coli* strain PA15 was not affected by the analogues of cobalamin when EHC supplied the folic acid cofactor. Even with factor B no inhibition was observed suggesting that neither cobalamin nor the factor (X) function in methionine synthesis in this strain when natural folic acid is available. The folic acid cofactor activities of EHC prepared from strain 121/176 and strain PA15 when tested with ultrasonic extracts of either benzimidazole and some other heterocyclic compounds were identical. This confirms the similarity of the folic acid cofactors of both strains and also suggests

that the EHC of strain PA15 does not contain any cobalamin having functional activity.

The activity of cobalamin antimetabolites in other cell-free systems

The cobalamin-containing enzyme of Hatch, Takeyama and Buchanan (1959) was inhibited competitively by an analogue of cobalamin. It is also apparent that this analogue prevented the combination of free cobalamin with the apo-enzyme in their system (Takeyama & Buchanan, 1960).

The anilide analogue of cobalamin inhibited the possible function of cobalamin in amino acid activation and incorporation into proteins in cell-free preparations of the liver of cobalamin-deficient rats (Wagle, Mehta & Johnson, 1959a,b; see Introduction).

Cobalamin analogues and the growth of *Escherichia coli*

The inhibitory effect of the anilide analogue on the growth of cobalamin dependent strains is in agreement with the findings of Cuthbertson, et al. (1956).

Since this work was completed many other antimetabolites of cobalamin have been examined for inhibitory effects on the growth of cobalamin-requiring micro-organisms. Substituted benzimidazoles and some other heterocyclic compounds have been used by Scott, Rogers & Rose (1958), Funk & Nathan (1959),

Timmis & Epstein (1959) and Ford (1959). The mono-acids of cobalamin and their derivatives have also been used by Baker et al. (1959) and Ford (1959). The quantitative details of these results are however very inconsistent, presumably due to the different techniques used by the various authors. The monocarboxylic acid derivatives can replace cobalamin for some organisms (Euglena gracilis, Lactobacillus leichmannii) but apparently not for Ochromonas malhamensis and E. coli 113/3. Both Cuthbertson et al. (1956) and Ford (1959) find that the methylamide analogue is a more potent inhibitor of the growth of E. coli 113/3 in the presence of cobalamin than the anilide analogue. Baker et al. (1959) report that these compounds are without inhibitory activity for this organism although they may not have been tested at sufficiently high concentrations.

The inhibitory analogues of cobalamin have also been found to antagonise the action of cobalamin when injected at the same time into cobalamin-depleted chicks and chick embryos (Coates, Davies & Harrison, 1960). It is of interest that here the analogues on their own, particularly the methylamide analogue, also have some ability to replace cobalamin. The order of decreasing inhibitory activity of the analogues was anilide > ethylamide > methylamide analogue. These properties are similar to those for the replacement and inhibition of cobalamin in the synthesis of

methionine by extracts of E.coli strain PA15. The order of decreasing inhibitory activity in both these cases is however the reverse of their inhibitory action on the growth of E.coli 113/3. The analogues did not produce any signs of cobalamin-deficiency when injected into non-depleted chicks and embryos; furthermore they were ineffective when injected 24hr. after cobalamin-depleted chicks and embryos had been injected with cobalamin. This represents a situation similar to that found with extracts of E.coli PA15 where the analogues antagonise the action of free cobalamin in methionine synthesis but they are ineffective against the product of its metabolism (X).

The critical ratio, concentration of anilide analogue/ concentration of cobalamin is higher for 50% inhibition of growth than for 50% inhibition of methionine synthesis by ultrasonic extracts of E.coli 121/176 (about 550 compared with 12). This probably reflects differences in the permeability of cobalamin and the analogue for the whole organism, a higher external concentration of the analogue being needed to achieve an inhibitory concentration at the site of cobalamin action. Such a phenomenon would explain why concentrations of anilide analogue equivalent to those needed to inhibit the growth of strains 121/176 and 113/3 are required to inhibit the formation of X during growth of strain PA15 in the presence of cobalamin. Differences in permeability

might also explain why the order of inhibitory activity of the three analogues for methionine formation by extracts of E.coli is the reverse of the order of inhibitory activity for the growth of cobalamin-requiring strains of E.coli.

5,6-Dimethylbenzimidazolylcobamide Coenzyme

Much higher concentrations of the anilide analogue were required to inhibit the activity of the cobamide coenzyme than cobalamin in the formation of methionine by ultrasonic extracts of E.coli PA15. The coenzyme had no ability to replace X; this has also been confirmed by Kisliuk (1960). The coenzyme could however replace cobalamin for the synthesis of X and although it was not tested directly, it appeared that it was more active than cobalamin and that greater concentrations of the anilide analogue were required to inhibit its activity in the formation of X. The factor (X) formed with the cobamide coenzyme had similar properties to that formed with cobalamin and the requirements for its synthesis were also the same.

Because the cobamide coenzyme is more active than cobalamin in the synthesis of methionine, it is conceivable that cobalamin is first converted to the coenzyme before participating in the formation of X, the coenzyme may even be present in the factor (X). Alternatively it is still possible

for both compounds to be active in their own right, or for both to be converted to another distinct functional form. It will be recalled that this cobamide coenzyme is the least active of the three isolated by Barker et al. when tested in the system for β -methyiaspartate formation with Clostridium tetanomorphum. The factor (X) was sensitive to cyanide though resistant to irradiation, but the cyanide effect could have been non-specific and the presence of protein may have protected the coenzyme from destructive radiation. Consequently it was not possible to decide whether a cobamide-coenzyme was present in X.

Takeyama and Buchanan (1960) have reported that 5,6-dimethylbenzimidazolylcobamide coenzyme will not replace cobalamin for the activation of methionine synthesis in a partially purified system from E.coli 113/3, which suggests that the coenzyme does not have a direct function in methionine synthesis even though it is equally as active as cobalamin for the growth of this organism (Barker et al. 1960b).

EXPERIMENTAL RESULTS

PREFACE

The requirement for a folic acid cofactor in the synthesis of methionine by ultrasonic extracts of Escherichia coli PA15 can be supplied by a heated extract of the organism (EHC) or by Pth_4G if cobalamin or the factor (X) is also present (Sections I & II).

With EHC as the source of folic acid cofactor (cobalamin absent) the synthesis of methionine is inhibited by Pth_4G (Section I, Chapter 4). Furthermore, the two sources of folic acid cofactor (EHC and Pth_4G) have widely differing stabilities in the presence of air.

In this section the function of folic acid in the synthesis of methionine was examined chiefly by two methods. In the first several derivatives of folic acid were synthesised and tested for their ability to replace EHC or Pth_4G and also for their effect on the activity of EHC. Secondly, the isolation and identification of the active components of the EHC was attempted.

reduced compounds). No such precautions were taken here because the folic acids under investigation were stable. The growth responses of the micro-organisms with several forms of stable folic acids are shown in Table III.1 (compiled from Stokstad, 1954; Selman, 1950. For definition of the

CHAPTER 1

METHODS

Microbiological Assay of Folic Acids

The specificity of certain micro-organisms for members of the folic acid group has contributed significantly to their isolation and to concepts of the chemical nature of the vitamin. The use of these organisms for the assay of folic acids has been extensively reviewed (Stokstad, 1954; Jukes, 1955). The three organisms most commonly employed are:

Streptococcus faecalis R, Lactobacillus casei and Pediococcus cerevisiae (Leuconostoc citrovorum). They differ in response to the reduced and oxidised states of the pteridine ring and to the number of glutamic acid residues in the molecule.

P. cerevisiae is unique in its requirement for reduced forms of the vitamin, the other organisms respond to both oxidised and reduced folic acids. Unless special precautions are taken during the assay, the tetrahydropteroylglutamic acid derivatives are decomposed (except for the N⁵-formylated reduced compounds). No such precautions were taken here because the folic acids under investigation were stable. The growth responses of the micro-organisms with several forms of stable folic acids are shown in Table III.1 (compiled from Stokstad, 1954; Rabinowitz, 1960. For definition of the

TABLE III.1

Growth factor activity of folic acid derivatives for the micro-organisms used for vitamin assay

<u>Folic acid derivative</u>	<u><i>S.faecalis</i> R</u>	<u><i>L.casei</i></u>	<u><i>P.cerevisiae</i></u>	<u><i>T.golei</i> W</u>
Pt (pteroic acid)	+++	-	-	-
N ¹⁰ -formyl Pt	+++	-	-	-
N ⁵ -formyl Pth ₄	+++	-	-	-
PtG	+++	+++	-	+++
N ¹⁰ -formyl PtG	+++	+++	-	-
N ⁵ -formyl Pth ₄ G	+++	+++	+++	-
PtG ₂	++	++	-	-
N ⁵ -formyl Pth ₄ G ₂	++	++	++	-
PtG ₃	-	++	-	+++
N ⁵ -formyl Pth ₄ G ₃ *	{ + -	{ ++ +++	{ +++ +	{ +++ +
PtG ₇	-	-	-	+++

- denotes no growth factor activity

+, ++, +++ denote relative growth factor activities (approximately 10-20%, 40-60% and 100% of the most active compound respectively).

* Conflicting reports in the literature.

A simple procedure for measuring the growth response of the three bacteria was used; it was developed in collaboration with Dr. E.M. Stone and was based on the method of Tonnies, Frank & Collett (1958).

abbreviations see page 1). Poly- γ -glutamyl derivatives of folic acid have not been tested rigorously because pure compounds are not available. S. faecalis will respond to derivatives of pteric acid and to pteroyl mono- and diglutamic acids; PtG₃ and PtG₇ will not supply the growth requirement. There are however conflicting reports about the activity of N⁵-formyl Pth₄G₃ for this organism: Bolinder, Widoff & Ericson (1953) and Wacker, Ebert & Kolm (1953) reported that it was active, but Silverman & Wright (1956), Hakala & Welch (1957) and Usdin (1959) have found it to be inactive. For P. cerevisiae the growth factor activity of N⁵-formyl Pth₄G₃ was found to have less than 10% of the activity of N⁵-formyl Pth₄G (Silverman & Wright, 1956) but Wacker et al. (1953) have reported the triglutamate as the most active folic acid derivative for supporting the growth of this organism. The growth requirement for L. casei can be supplied by mono-, di- and triglutamyl derivatives of pteric acid. Only the protozoan Tetrahymena geleii W can use pteroylheptaglutamic acid (PtG₇) as growth factor (Kidder & Dewey, 1949).

Assay of folic acids with S. faecalis R, L. casei and P. cerevisiae

A single procedure for measuring the growth response of the three bacteria was used; it was developed in collaboration with Dr. K.M. Jones and was based on the method of Toennies, Frank & Gallant (1952).

The inoculum development medium (5ml.) consisted of medium E (3.5ml.) supplemented with pteroylglutamic acid ($0.3\mu\text{M}$) or leucovorin ($0.8\mu\text{M}$). Organisms were harvested from a tryptose glucose broth culture (medium S_3) after growth at 37° for 16hr. and washed once with 0.9% saline. An amount equivalent to 0.1mg. dry wt. organisms was incubated in the inoculum development medium for 5 or 6hr at 37° . The inoculum for the assay was prepared from this log phase culture by washing once with saline and diluting ten times.

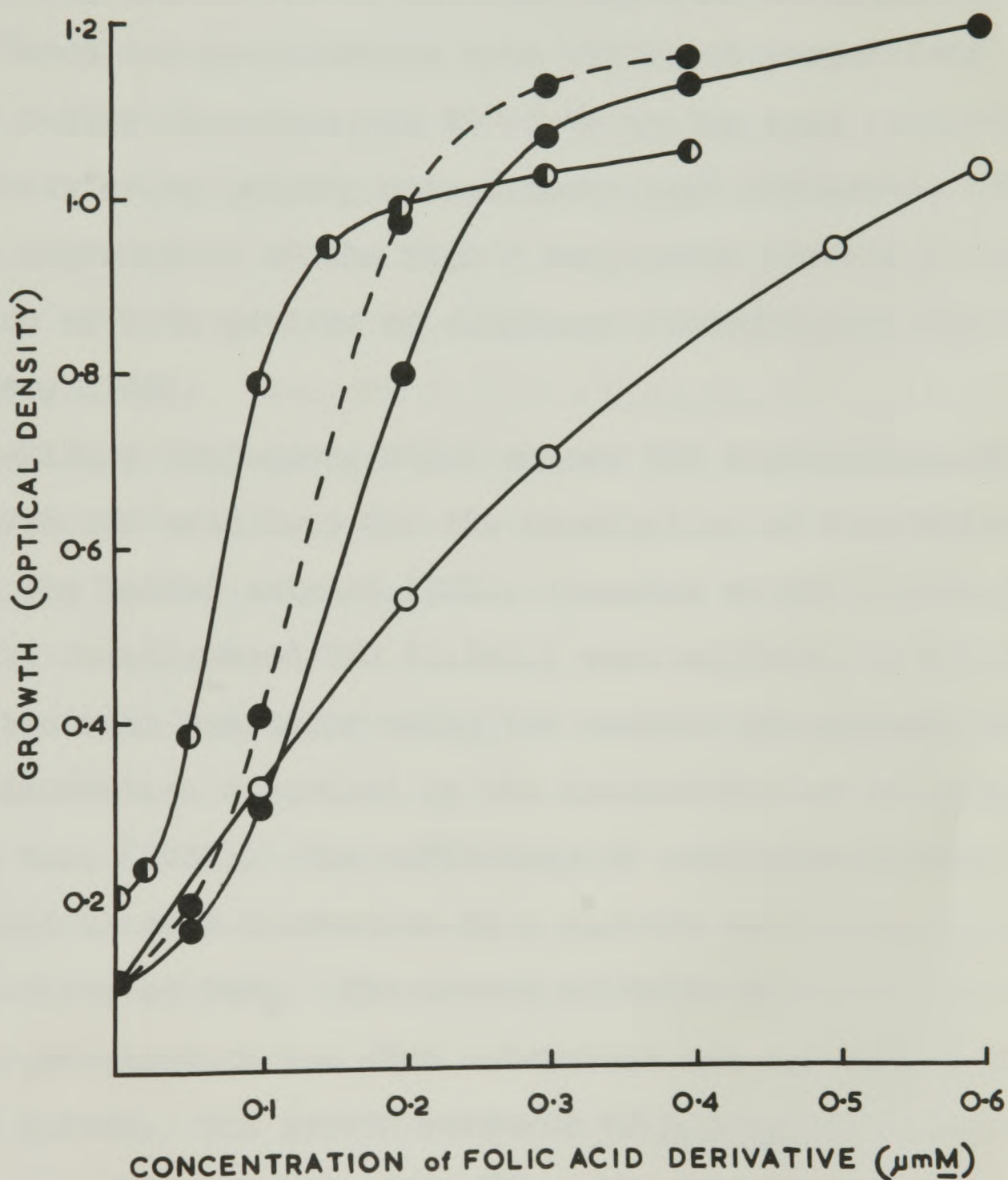
Several samples of each test solution with medium E (3.5ml.) and water added to give a final volume of 4.9ml. were sterilised by heating to 120° and allowing to cool (total time above 100° was 8min.) in 125mm.x 16mm. tubes. Standards containing PtG or PtG₃ (0.05 to $0.4\mu\text{M}$) or N⁵-formyl Pth₄G (0.1 to $1.0\mu\text{M}$) were included in each assay. The inoculum (0.1ml.) was added and incubation was for 22hr. at 37° , upright in air. Growth was assessed in 16mm. tubes with a Spekker Photoelectric Absorptiometer fitted with a neutral filter (H.508) and using the uninoculated medium for comparison with test cultures. Typical standard responses are shown in Figure 26.

Conjugase treatment

Enzymes which hydrolyse conjugated forms of folic acid to

FIG. 26

Response of three micro-organisms to various forms
of folic acid



- , S. faecalis R, pteroylglutamic acid.
 ●—●, L. casei, pteroylglutamic acid.
 ●—●, L. casei, pteroyltriglutamic acid.
 ○—○, P. cerevisiae, N⁵-formyltetrahydropteroylglutamic acid.

monoglutamyl and the lower polyglutamyl derivatives are present in many tissues. Preparations of the enzyme are made chiefly from chicken pancreas (Laskowski, Mims & Day, 1945) and hog kidney (Bird, Robbins, Vandenbelt & Pfiffner, 1946). These two preparations have different properties; with the latter digestion was found to be the most complete, the products being largely monoglutamic acid (Stokstad, 1954). Complete degradation of the higher conjugates however requires the action of both sources of conjugase (Sreenivasan, Harper & Elvehjem, 1949).

Hog-kidney conjugase, which causes 75% degradation of PtG_7 to PtG was used here for the examination of the folic acids in the heated extract (EHC). Samples of EHC (0.1ml.) and of the fractionated EHC (1.0ml.) were adjusted to pH4.5 and treated with conjugase using the methods for preparation and for incubation described by the Association of Vitamin Chemists Inc. (1951). The efficiency of conjugase action was checked in each incubation by a control containing a known quantity of PtG_3 . The amount of folic acid in the conjugase preparation was also determined and deducted from the test values. The growth response of L.casei was slightly different for PtG and PtG_3 (Fig.26). The response to PtG_3 was normally used as the standard for the assay of natural folic acids, but after conjugase treatment the growth response to PtG was used.

Tetrahymena vorax

This organism was used to confirm the presence of conjugated forms of folic acid in the fractions of EHC having cofactor activity but having no growth factor activity when tested with the three folic acid requiring bacteria. The method was based on that used for Tetrahymena geleii W by Kidder & Dewey (1949). Test samples and samples of PtG (final concentration 0.2 to 2.0 μ M) were added to medium F with water to 1.9ml. in 150mm.x20mm. tubes and sterilised (115° for 10 min.). For the inoculum T.vorax was harvested from a slope culture on medium S₄ which had been incubated for 72hr. at 30°. The organisms were depleted of folic acid by passaging twice in unsupplemented medium F for 72hr. at 30° and then washed with 0.9% saline and finally suspended in sterile pyridoxal phosphate (8 μ M) to an EEL value of 2. A sample of this suspension (0.1ml.) was used as the inoculum. Incubation was for 48hr. at 30° with the tubes sloped at 5° to the horizontal to increase aeration. Growth was assessed with an EEL colorimeter and although the growth obtained with no added folic acid was 30-40% of the maximum growth it was possible to assay the concentration of the vitamin in the test samples. Additional glutamic acid residues increased the values under the conditions used.

Chromatographic Methods

Chromatography coupled with bioautography and the use of conjugase have played an important part in the isolation and identification of natural folic acids.

Paper chromatography

The two most useful solvent systems proved to be: (1) Solvent A 0.1M Sodium phosphate buffer pH7.0, saturated with iso-amyl alcohol at 25° (Sakami & Knowles, 1958) and, (2) Solvent B 5% Citric acid and ammonia to pH9.0, saturated with iso-amyl alcohol at 25° (Wieland, Hutchings & Williams, 1952). Whatman No.1 and No.3 papers and descending chromatography were employed. The solutions of folic acid were neutralised with a dilute solution of ammonia, and 40-100µg of the material was spotted on the paper. After development of the chromatograms the folic acids were identified by examination in ultraviolet light or by bioautography. In the latter case much smaller amounts of folic acid were chromatographed. The chromatographic behaviour of several folic acid derivatives is shown in Table III.2; with Whatman No.1 paper the R_f values were slightly lower. The presence of a formyl group or additional glutamic acid residues increased the R_f values under the conditions used.

TABLE III.2

Chromatographic behaviour of folic acid derivatives

<u>Folic acid derivative</u>	<u>Rf value with solvent system</u>	
	<u>A</u>	<u>B</u>
PtG ²	0.48	0.30
N ¹⁰ -formyl PtG	0.82	0.65
N ⁵ -formyl PtH ₄ G	0.80	0.62
PtG ₃ ²	0.87	0.55
N ¹⁰ -formyl PtG ₃	0.95	0.85
N ⁵ -formyl PtH ₄ G ₃	-	0.80

The compounds were chromatographed on Whatman No.3 paper using descending technique and they were identified biologically or by their fluorescence in or quenching of (a) ultraviolet light. See text for details of solvent systems.

Anion-exchange cellulose chromatography

The method used was based on the work of Rabinowitz (1959) and Usdin (1959). Commercial diethylaminoethyl (DEAE-) cellulose and triethylaminoethyl (TEAE-) cellulose were graded by sedimentation; the fine particles were discarded. The exchanger was washed with 1% NaOH and then with water to neutrality. Columns were always 1cm.x17cm., packed under moderate hydrostatic pressure, with an initial flow-rate of 10ml./hr. The final flow-rate was 20-25ml./hr. at 3° with a 35-40cm. pressure head provided by the device for gradient elution. Samples containing 2-4 μ moles of folic acid derivatives or the equivalent of 5 or 6ml. of acetone-treated EHC were placed on the column which was then washed with water to displace unadsorbed material. The folic acids were eluted by a gradient of phosphate buffer pH6.1, 0 to 0.5M in 350ml. of effluent collected in 5ml. fractions. Gradient elution was by a method based on those described by Alm, Williams & Tiselius (1952) and Sober, Gutter, Wyckoff & Peterson (1956) using a closed system in flow equilibrium. The concentration of phosphate was measured by direct estimation of the eluent by the method of Fiske & Subbarow (1925). The observed gradient was almost always identical with the theoretical gradient calculated by using the equation of Cherkin, Martinez & Dunn (1953). 2-Mercaptoethanol (0.2M) was added to the developing fluids when the reduced

forms (PtH_4G and PtH_4G_3) were chromatographed. Spectrophotometric (280m μ) and biological investigation of the fractions enabled the % recovery of the chromatographed material to be determined. The behaviour of several folic acid compounds is shown in Table III.16, page 187; these compounds were identified spectrophotometrically and biologically by their cofactor or growth factor activities.

Preparation and Properties of Folic Acid Derivatives

Several derivatives of folic acid were prepared and a number of others were obtained as gifts (see Appendix 3).

The pteroylpolyglutamic acids used here were derived from (a) a sample of synthetic pteroyl- α -glutamylglutamic acid (PtG_2 , Mowat et al. 1948) which contains an α -glutamyl link not found in natural compounds and (b) two samples of pteroyl- γ -glutamyl- γ -glutamylglutamic acid (PtG_3), one being synthetic (Semb et al. 1949) and the other, fermentation Lactobacillus casei factor which was isolated from the culture fluid of an unidentified Corynebacterium by Hutchings et al. (1948). Pteroylheptaglutamic acid was not available; the material isolated from yeast by Pfiffner et al. (1946) has since been reported to have decomposed (Pfiffner, personal communication).

Paper chromatography of the synthetic PtG_3 revealed four fluorescent impurities. Purified specimens containing only minute traces of fluorescent impurity were obtained by cellulose column chromatography and paper chromatography (solvent A). The eluted material was precipitated several times from acid solution and recovered by lyophilisation. Samples were assayed spectrophotometrically in 0.1M phosphate buffer pH 7.0 by assuming the extinction coefficients at the absorption maximum (280m μ) to be the same as for PtG (Rabinowitz, 1960); the high purity of the PtG_3 was confirmed. Purified PtG was prepared similarly, details are described in Section III, Chapter 2.

The folic acids used throughout contain the L-Stereoisomer of glutamic acid in all cases.

Formylation of folic acids

Samples (100mg.) of purified PtG and PtG_3 were dissolved in 98% formic acid (4ml.) and stood in the dark for 48hr. (Blakley, 1959). The excess formic acid was removed by lyophilisation. In the case of PtG, the product, N¹⁰-formyl PtG, was identified spectrophotometrically and chromatographically; only traces of PtG remained. N¹⁰-formyl PtG_3 was extracted from the freeze-dried material by reprecipitation from acid solution and dried once more. It was spectro-

photometrically identical with N^{10} -formyl PtG and paper chromatography (solvent B) showed the presence of a little fluorescent impurity but only a trace of PtG_3 suggesting that a high yield of the formylated derivative had been achieved.

Dihydropteroylglutamic acid (PtH_2G)

This compound was prepared by the method of Futterman (1957). The dried product was stored and dispensed in the same way as PtH_4G (Section I, Chapter 1). The absorption spectrum in 0.05M phosphate buffer pH 7.8 showed a maximum at 283m μ with a molecular extinction coefficient 24,500cm.²mole⁻¹ which agrees with the data of O'Dell et al. (1947) and Wright, Anderson & Herman, (1958). This material is either 5,8-dihydro, or 7,8-dihydropteroylglutamic acid and most probably the latter (Rabinowitz, 1960). It has no asymmetric centre; a fact which is confirmed by its biological activity (O'Dell et al. 1947; Osborn & Huennekens, 1958 and Peters & Greenberg, 1958).

Tetrahydropteridine derivatives

Tetrahydropteroylglutamic acid and the other 5,6,7,8-tetrahydropteridine derivatives are racemates by virtue of the presence of an asymmetric carbon atom at position 6 of the pteridine nucleus. Thus chemically prepared PtH_4G and N^5 -formyl PtH_4G have been shown to react to the extent of 50% in

various enzymatic systems (Hatefi, Osborn, Kay & Huennekens, 1957; Peters & Greenberg, 1957; Blakley, 1958). Presumably reduced folic acids isolated from natural sources contain only one stereoisomer.

Hydrogenation of folic acids on a small scale

A modification of the method of Cross (1956) was used for the preparation of the tetrahydropteridine derivatives of PtG, N¹⁰-formyl PtG, N¹⁰-methyl PtG, PtG₂, PtG₃ and N¹⁰-formyl PtG₃.

A neutral solution of the folic acid (1ml.; 16 μ moles) in 25mM phosphate buffer pH 7.0 was placed in the side arm of a Warburg flask. The platinic oxide catalyst (6mg.) and 2.2ml. of 25mM phosphate buffer pH 7.0 were placed in the main vessel. The apparatus was flushed with H₂ after which the hydrogenation of the catalyst at 37° was followed manometrically. The folic acid was then hydrogenated, the flask being covered by a black cloth to exclude light. The theoretical H₂ uptake (two molecular equivalents) was generally obtained in 30-40 min. The solution of reduced folic acid was quickly freed from the catalyst using a plugged Pasteur pipette and it was either used immediately or stored at -14° under H₂ in a Thunberg tube.

N⁵-formyltetrahydropteroyltriglutamic acid (N⁵-formyl Pth₄G₃)

This compound was prepared by a modification of the method used for the synthesis of leucovorin (N⁵-formyl Pth₄G) by Shive et al. (1950) and Ericson (1953). Immediately after preparation, 2-mercaptoethanol (final concentration 0.4M) was added to N¹⁰-formyl Pth₄G₃. The solution was freed from the catalyst, adjusted to pH 8.2 and autoclaved at 120° for 1hr. After cooling and neutralisation this solution contained N⁵-formyl Pth₄G₃ with an expected yield of 20-40%.

Purified N⁵-formyl Pth₄G₃ was obtained by chromatography using TEAE-cellulose and development with a gradient of phosphate buffer pH 6.1. Spectrophotometric analysis of the eluate at 280mμ revealed the presence of two main components. Most of the folic acid cofactor activity was in the largest of these (eluted at 0.26M phosphate buffer). This procedure should separate N⁵-formyl Pth₄G₃ from all other possible contaminants except N¹⁰-formyl Pth₄G₃ which is eluted at 0.25M phosphate buffer; however this latter compound had no folic acid cofactor activity. The fraction containing the most N⁵-formyl Pth₄G₃ had a spectrum similar to that of N⁵-formyl Pth₄G a slow increase in absorption at 350mμ was observed when the solution was adjusted to pH 1.0 characteristic of the formation of an N⁵,N¹⁰-methenyl derivative from the N⁵-formylated tetrahydropteroyltriglutamate (Rabinowitz, 1960). The effect

of acid was analogous to that demonstrated by Wright (1956) using CoC type I isolated from Clostridium cylindrosporum and found to be an N^5 -formyl PtH_4G_3 containing glycine. The increase in absorption at 350m μ , produced by imidazolinium ring formation between the N^5 and N^{10} positions involving the formyl group, was used as an assay for the N^5 -formyl PtH_4G_3 content of the purified material by assuming an extinction coefficient of 22,000 cm.²mole⁻¹ as for N^5, N^{10} -methenyl PtH_4G (Greenberg, Jaenicke & Silverman, 1955). The absorption at 350m μ before acidification and the total absorption at 280m μ indicated the presence of N^{10} -formyl PtG_3 to the extent of 40% of the total folic acid in the purified material.

Monoglutamyl derivatives of pteric acid listed in Table III.3 were all inactive as cofactors for the transfer of the β -carbon of serine to the methyl group of methionine by ultrasonic extracts of *Escherichia coli* in the absence of cobalamin. However, if cobalamin was added to the reaction mixture, dihydropteroylglycine acid (PtH_2G) and the three formylated derivatives of PtH_2G , were active like PtH_2G itself (Table III.3, column 1). None of the compounds containing an unsaturated pteridine residue were active. It is probable that PtH_2G was first converted to PtH_4G (see Chapter 3). The formylated derivatives may be active in their own right although, liberation of PtH_2G may also account for their activity.

CHAPTER 2

THE ACTIVITY OF SEVERAL DERIVATIVES OF FOLIC ACID IN METHIONINE SYNTHESIS

One-carbon Unit Donor and Cofactor Activities

Early attempts to replace the requirement for the extract of heated E. coli in the synthesis of methionine by cell-free preparations of this organism by leucovorin, pteroylglutamic acid and anhydroleucovorin as well as by Pth_4G were unsuccessful (Cross & Woods, 1954; Helleiner, unpublished). This was before the role of cobalamin in the utilisation of Pth_4G had been discovered. By way of confirmation, the monoglutamyl derivatives of pteric acid listed in Table III.3 were all inactive as cofactors for the transfer of the β -carbon of serine to the methyl group of methionine by ultrasonic extracts of E. coli strain PA15 in the absence of cobalamin. However, if cobalamin was added to the reaction mixture, dihydropteroylglutamic acid (Pth_2G) and the three formylated derivatives of Pth_4G , were active like Pth_4G itself (Table III.3, column 1). None of the compounds containing an unreduced pteridine residue were active. It is probable that Pth_2G was first converted to Pth_4G (see Chapter 3). The formylated derivatives may be active in their own right although, liberation of Pth_4G may also account for their

* Degraded Pth_4G was prepared by slow exposure of solution to light.
phosphate buffer pH 7.6.

* Denotes not tested.

TABLE III.3

Cofactor activity of several folic acid derivatives in methionine synthesis and their effect upon the function of the heated extract

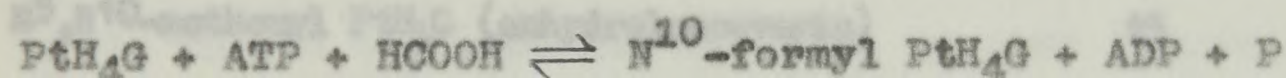
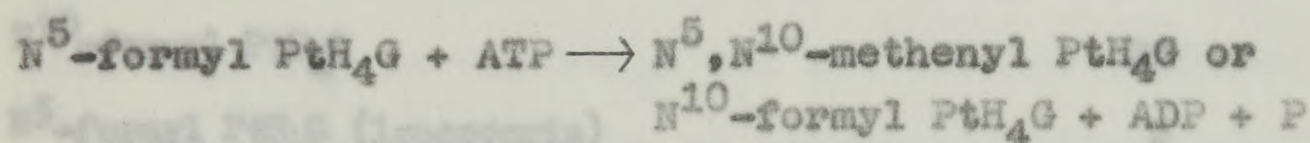
Folic acid derivative (1μmole)	L-Methionine formed (μm-moles/mg. protein)		
	1 Cobalamin	2 EHC	3 EHC + cobalamin
None	1	64	65
PtH ₄ G	60	3	63
PtH ₂ G	58	9	68
N ¹⁰ -formyl PtH ₄ G	52	4	60
N ⁵ -formyl PtH ₄ G (leucovorin)	13	19	60
N ⁵ ,N ¹⁰ -methenyl PtH ₄ G (anhydroleucovorin)	21	12	67
PtG	0	60	62
N ¹⁰ -methyl PtG	-	61	64
N ¹⁰ -methyl PtH ₄ G	1	42	52
N ¹⁰ -formyl PtG	0	58	62
N ¹⁰ -formyl Pt (rhizopterin)	-	63	62
2-Amino, 4-hydroxy, 6-methylpteridine	-	62	63
2-Amino, 4-hydroxypteridine- 6-aldehyde	-	63	66
2-Amino, 4,6-dihydroxypteridine (xanthopterin)	-	61	60
PtH ₄ G (degraded)*	4	49	-

Reaction mixture I plus Dowex-treated ultrasonic extract (strain PA15) with cobalamin (0.65 μm-moles) and folic acid derivative (1 μmole) when added. EHC equivalent to 17 mg. dry wt. organisms. Incubated 2hr.

* Degraded PtH₄G was prepared by 24hr. exposure of solution in 1M phosphate buffer pH 7.8.

- Denotes not tested.

activity. If this is the case, a small fraction of the methionine formed may be due to the use of formyl groups for methyl synthesis. In particular, the formyl groups of N^{10} -formyl PtH_4G and anhydroleucovorin were used for methyl group formation when tested at substrate levels in the absence of serine (Table III.4). Leucovorin and formate itself were almost inactive as donors of the C_1 -unit probably because the ultrasonic extracts are deficient in the enzymes N^5 -formyl PtH_4G isomerase and PtH_4G formylase which catalyse respectively the reactions



N^{10} -methyl PtH_4G was not active as a folic acid cofactor or as a donor of the one-carbon unit and is therefore not an intermediate in methionine formation; this observation also suggests that the reduction of C_1 -units to the methyl level of oxidation occurs after reaction with homocysteine. The formaldehyde derivative of PtH_2G (CH_2O - PtH_2G) prepared by mixing equimolar quantities of formaldehyde and PtH_2G at pH7.8 was only a poor C_1 -donor. However the dissociation constant for the interaction of these two compounds is about 100 times larger than that for formaldehyde and PtH_4G

TABLE III.4

The combined one-carbon unit donor and cofactor activity of certain folic acid compounds in the synthesis of methionine

<u>Folic acid compound</u> <u>(3.5 μmoles)</u>	<u>L-Methionine formed</u> <u>(μm-moles/mg. protein)</u>
PtH ₄ G with serine present*	60
CH ₂ O-PtH ₄ G	51
CH ₂ O-PtH ₂ G	6
N ¹⁰ -methyl PtH ₄ G	1
N ¹⁰ -formyl PtG	0
N ¹⁰ -formyl PtH ₄ G	22
N ⁵ -formyl PtH ₄ G (leucovorin)	2
N ⁵ ,N ¹⁰ -methenyl PtH ₄ G (anhydroleucovorin)	15

Reaction mixture I with L-serine and pyridoxal phosphate omitted and DL-homocysteine reduced to 5 μ moles, Dowex-treated ultrasonic extract and cobalamin (0.65 μ m-moles). Incubated 2hr.

* Reaction mixture I, PtH₄G (1 μ mole) and cobalamin (0.65 μ m-moles).

(Blakley, 1959). It is therefore highly probable that formaldehyde is removed by spontaneous reaction with homocysteine to form 1,3-thiazane-4-carboxylic acid.

Inhibition of Methionine Synthesis by
Tetrahydropteroylglutamic acid
and Derivatives

The synthesis of methionine by ultrasonic extracts of *E. coli* PA15 when extract of heated organisms supplies the folic acid cofactor for C_1 -transfer is inhibited by PtH_4G (Section I, Chapter 4). The possibility that this was due to some impurity in the PtH_4G was not however excluded.

Paper chromatography of commercial pteroylglutamic acid (PtG) showed the presence of three or four fluorescent impurities (solvent A). Purified PtG, containing very little fluorescent material was obtained by extraction with Darco-G60 at pH7.0 followed by recrystallisation from acid solution. Highly purified PtG was obtained by column chromatography on cellulose powder (Whatman Standard Grade, solvent A) followed by filtration through acid-washed Darco-G60 and recrystallisation (Sakami & Knowles, 1959). However, the PtH_4G prepared from these purified samples of PtG had exactly the same activity as the PtH_4G prepared from commercial PtG for both cofactor (cobalamin present) and inhibitor (serine with EHC and no cobalamin) activities in

methionine synthesis. The concentration of PtH_4G for half-maximal inhibition was $20\mu\text{M}$ in all cases (conditions as in Fig.15).

Several compounds known to be formed during the oxidative decomposition of PtH_4G (e.g. the 6-methyl and 6-aldehyde derivatives of 2-amino,4-hydroxypteridine) had no inhibitory effect on methionine synthesis (Table III.3, column 2). It was also found that the inhibitory and cofactor activities of a solution of PtH_4G disappeared simultaneously on exposure to air for up to 24hr. More recently, PtH_4G itself was purified by anionic-exchange chromatography on TEAE-cellulose under reducing conditions (see Chapter 1); freshly isolated material eluted as a single band, and identified by spectrophotometry still retained its inhibitory effect on methionine synthesis. Thus it was concluded that the inhibition was in fact caused by PtH_4G and not by an impurity or a decomposition product. Of the other folic acid derivatives tested, only those which were active as C_1 -donors or cofactors in the presence of cobalamin could act as inhibitors of methionine synthesis (Table III.3, column 2); the extent of inhibition was related directly to their efficiency as folic acid cofactors. However, as in the case of cofactor activity, the inhibitory activity could be explained by the conversion of these compounds to

PtH₄G. The formaldehyde derivative of PtH₄G also inhibited the synthesis of methionine when the folic acid cofactor for C₁-transfer was supplied by the EHC. Here the half-maximal inhibition was obtained with a concentration of 80μM which is four times greater than for PtH₄G alone, again suggesting that it is free PtH₄G or reduced pteridine derivatives with unsubstituted N⁵ and or N¹⁰ atoms which are responsible for the inhibition. In all these examples, the inhibition was relieved by addition of cobalamin to the reaction mixture (Table III.3, column 3).

Addition of N¹⁰-methyl PtH₄G caused a 30% inhibition of methionine synthesis which was only partly overcome by cobalamin. It would have been interesting to have found a compound, capable of completely inhibiting the activity of EHC in methionine synthesis but inactive as a cofactor in the presence of cobalamin. The effect of cobalamin on the inhibited system in such a case would probably have indicated whether cobalamin acts simply by relieving the inhibition of EHC function or whether normally its effect is to permit the use of PtH₄G as the folic acid cofactor, or whether both processes occur simultaneously. At the present state of this research no such decision can be made.

Summary

- 1) Several reduced pteroylmonoglutamic acid derivatives were active as C_1 -donors and cofactors for the synthesis of methionine but, like PtH_4G , only if cobalamin was also present.
- 2) In the absence of cobalamin the inhibition of methionine formation by PtH_4G (serine plus EHC system) was probably caused by this compound and not by an impurity or by any of its degradation products.
- 3) The same reduced pteroylmonoglutamic acid derivatives which served as C_1 -donors or cofactors in the presence of cobalamin also inhibited methionine formation in the absence of cobalamin when EHC supplied the folic acid cofactor.

mammalian systems. Reduced triphosphopyridine nucleotide ($TPNH_2$) was required by preparations of dihydropteroylglutamic acid reductase purified from chicken liver (Zakrzewski & Nichol, 1958; Osborn & Huennekens, 1959) although Futterman (1957) had found that $DPNH_2$ as well as $TPNH_2$ was active for this enzyme. The pyridine nucleotide requirement for PtH_2G reduction with an enzyme from sheep liver was dependent upon the pH used for the reaction (Peters & Greenberg, 1958). If this enzyme is present in the enzymic extracts of *E. coli* the activity of PtH_2G could be explained by its conversion to PtH_4G ; particularly in

CHAPTER 3

DIHYDROPTEROYLGLUTAMIC ACID AS A COFACTOR IN THE SYNTHESIS OF METHIONINE

Introduction

In the previous chapter dihydropteroylglutamic acid (Pth_2G) was found to replace tetrahydropteroylglutamic acid (Pth_4G) both as a cofactor for C_1 -transfer in the synthesis of methionine by extracts of Escherichia coli strain PA15 (cobalamin present) and as an inhibitor of methionine formation in the absence of cobalamin and with EHC as the source of folic acid.

The enzymatic reduction of Pth_2G by reduced pyridine nucleotides has been described in a number of avian and mammalian systems. Reduced triphosphopyridine nucleotide (TPNH_2) was required by preparations of dihydropteroylglutamic acid reductase purified from chicken liver (Zakrzewski & Nichol, 1958; Osborn & Huennekens, 1958) although Futterman (1957) had found that DPNH_2 as well as TPNH_2 was active for this enzyme. The pyridine nucleotide requirement for Pth_2G reduction with an enzyme from sheep liver was dependent upon the pH used for the reaction (Peters & Greenberg, 1958). If this enzyme is present in the enzymic extracts of E. coli the activity of Pth_2G could be explained by its conversion to Pth_4G ; particularly in

the reducing environment offered by the conditions used for methionine synthesis. It was hoped that this reaction could be demonstrated with the aid of aminopterin and amethopterin which are non-competitive inhibitors of chick liver dihydropteroylglutamic acid dehydrogenase (Osborn, Freeman & Huennekens, 1958; Futterman, 1957).

In the synthesis of thymidylic acid from deoxyuridylic acid and formaldehyde by extracts of rat thymus gland, it has been suggested that PtH_4G not only acts as a carrier for the C_1 -unit but also functions in the reduction of the hydroxymethyl group to the methyl level of oxidation (Humphreys & Greenberg, 1958). Evidence was obtained for the production of one methyl group during the oxidation of a molecule of PtH_4G to PtH_2G and that regeneration of PtH_4G can be mediated by DPNH_2 . Support for this mechanism of methyl synthesis has also been obtained by Friedkin (1959) from a study of the same reaction using tritiated tetrahydropteroylglutamic acid and an enzyme from E. coli; the thymidylic acid formed was labelled with tritium. Such a mechanism may also operate for the synthesis of methionine methyl groups from formaldehyde or the β -carbon of serine. The effect of reduced pyridine nucleotide (added as DPNH_2 or formed by glycolysis) would be mediated entirely by dihydropteroylglutamic acid reductase in a cyclical regeneration of PtH_4G from the PtH_2G which had been formed during the final

reduction of the C_1 -unit. Due to the active $DPNH_2$ -oxidase of the extracts, direct examination of the stoichiometry of methionine formation with respect to $DPNH_2$ and PtH_4G was not feasible; it was however hoped that the use of the inhibitors of dihydropteroylglutamic acid reductase would reveal whether or not this enzyme was of importance in methionine-methyl formation.

Dihydropteroylglutamic acid and the
Inhibition of Methionine Synthesis
by Antimetabolites of Folic Acid

The concentration of PtH_2G for half-maximal activation of methionine synthesis under normal reaction conditions ($75\mu M$) was exactly half the concentration required in the case of PtH_4G ($150\mu M$) presumably because the D-stereoisomer of chemically prepared PtH_4G is inactive. Half-maximal inhibition of methionine synthesis by PtH_2G (serine plus EHC system) occurred at a concentration ($15\mu M$) rather more than half the value for PtH_4G ($30\mu M$); furthermore, the maximum inhibition with PtH_2G was never as great as that obtained with PtH_4G . This would be expected if PtH_2G had first to be converted to PtH_4G because methionine formation would proceed normally until an inhibitory concentration of PtH_4G was formed.

Effect of the antimetabolites of folic acid

Neither aminopterin nor amethopterin had any effect on the synthesis of methionine by ultrasonic extracts of E. coli strain PA15 when either EHC or PtH_4G supplied the folic acid cofactor for C_1 -transfer (Table III.5). With PtH_2G as the cofactor, both folic acid antagonists inhibited methionine formation. Similar results were obtained with extracts of acetone-dried organisms where cobalamin was replaced by factor X (Table III.9, page 171). The use of higher concentrations of aminopterin and amethopterin was not possible because of their effect on the growth of Leuconostoc mesenteroides P60 in the assay of methionine.

In the synthesis of methionine there are two reactions which require a folic acid cofactor, serine transhydroxymethylase and the transfer of the hydroxymethyl group to homocysteine. One or both of these stages may require PtH_4G as the cofactor, PtH_2G being inactive. Blakley (1957c, 1959) found that PtH_2G would activate crude preparations of serine transhydroxymethylase but it was inactive for preparations of the enzyme after purification from rabbit liver.

It will be remembered that the formaldehyde derivative of PtH_2G was almost completely inactive in methionine synthesis, probably due to its low binding capacity for

formaldehyde which TABLE III.5 Effect of aminopterin and methopterin on the synthesis of methionine (Chapter 3).

Source of folic acid cofactor	L-Methionine formed (μ -moles/ng. protein) with							
	Aminopterin (μ M)				Methopterin (μ M)			
	0	0.05	0.5	5.0	0	0.005	0.05	0.5
(a) EHC	55	53	57	55	92	95	90	92
(b) Pth ₄ G	60	56	58	62	70	72	72	74
Pth ₂ G	60	34	8	4	66	56	33	9

Reaction mixture I plus Dowex-treated ultrasonic extract of E.coli strain PA15 and:

- (a) EHC equivalent to 17 ng. dry wt. organisms.
Incubated 2hr.
- (b) Cobalamin (65 μ moles) and Pth₄G or Pth₂G (1 μ mole).
Incubated 4hr.

Methionine formation using CH₃O-CH₂-S as source of the C₂-unit and cofactor for its transfer was not affected by aminopterin (Table III.7, Expt. a).

formaldehyde which allows spontaneous formation of 1,3-thiazane-4-carboxylic acid in the presence of homocysteine (Chapter 2). It was therefore not possible to study the effect of aminopterin or amethopterin on this part of the reaction. It would however seem unlikely that PtH_2G acts as a cofactor in the transfer of the C_1 -unit to homocysteine.

Indirect evidence for the function of dihydropteroylglutamic acid reductase in the utilisation of PtH_2G with formaldehyde was obtained in an experiment using Thunberg tubes fitted with two side-arms (Table III.6). Preliminary incubation of PtH_2G with the basal reaction mixture produced a compound (probably PtH_4G) which enabled formaldehyde to serve as the source of the C_1 -unit; the effect was prevented by the addition of aminopterin during the first incubation but not at a later stage.

Assuming that the folic acid antimetabolites are inhibiting dihydropteroylglutamic acid reductase it is apparent that PtH_2G must be converted to PtH_4G before it can exhibit cofactor activity. This being so, it is probable that the serine transhydroxymethylase and the subsequent transfer of the C_1 -unit to homocysteine are both inhibited.

Methionine formation using $\text{CH}_2\text{O}-\text{PtH}_4\text{G}$ as source of the C_1 -unit and cofactor for its transfer was not affected by aminopterin (Table III.7, Expt.a).

TABLE III.6

Dihydropteroylglutamic acid and the
utilisation of formaldehyde for the
synthesis of methionine

Additions to the basal reaction mixture during			<u>L-Methionine formed</u> <u>(μm-moles/ng. protein)</u>
<u>1st Incubation</u> <u>(60 min.)</u>	<u>2nd Incubation</u> <u>(2 min.)</u>	<u>3rd Incubation</u> <u>(120 min.)</u>	
-	{ PTH ₂ G Formaldehyde	Homocysteine	5
PTH ₂ G	Formaldehyde	Homocysteine	16
{ PTH ₂ G (Aminopterin	Formaldehyde	Homocysteine	4
PTH ₂ G	{ Aminopterin Formaldehyde	Homocysteine	15

Reaction mixture I (L-serine, pyridoxal phosphate and DL-homocysteine omitted) with Dowex-treated ultrasonic extract of strain PA15 (10 mg. protein) and cobalamin (0.65 μ m-moles). PTH₂G (3 μ m-moles), formaldehyde (2.5 μ m-moles), DL-homocysteine (4 μ m-moles) and aminopterin (10 μ m-moles) when added; final volume 2.0 ml.

TABLE III.7

Effect of aminopterin on methionine synthesis
with (a) the formaldehyde derivative of
tetrahydropteroylglutamic acid and (b) a
catalytic concentration of tetrahydropteroylglutamic acid

<u>Source of folic</u> <u>acid cofactor</u>	<u>Concentration</u> <u>(μmoles/2ml.)</u>	<u>L-Methionine formed (μmoles)</u> <u>with aminopterin (5μM)</u>	
		<u>Absent</u>	<u>Present</u>
(a) $\text{CH}_2\text{O-Pth}_4\text{G}$	1.0	0.14	0.16
	3.0	0.38	0.38
(b) Pth_4G	0.2	0.99	0.95
	0.4	1.42	1.37

(a) Reaction mixture I (L-serine and pyridoxal phosphate omitted and DL-homocysteine reduced to 5 μ moles) plus Dowex-treated ultrasonic extract of E. coli strain PA15 (10 mg. protein) and cobalamin (65 μ moles). Incubated 4hr.

(b) Reaction mixture I with Dowex-treated extract equivalent to 20 mg. protein and cobalamin (1 μ mole). Incubated 4hr.

The mechanism of methyl group formation

A more satisfactory demonstration of the purely catalytic role of PtH_4G in methionine synthesis was obtained by increasing the amount of ultrasonic extract and decreasing the amount of PtH_4G which were normally added to the reaction mixture (Table III.7, Expt.b). Under these conditions aminopterin still had virtually no inhibitory effect. This suggests that dihydropteroylglutamic acid reductase plays no part in the formation of methionine. Unlike the mechanism for the formation of thymidylic acid methyl groups, PtH_4G does not appear to serve as the final reductant of the hydroxymethyl group, unless there is an aminopterin insensitive pathway for the regeneration of PtH_4G . The results are sufficiently clear-cut to exclude the possibility of the resynthesis of PtH_4G from two molecules of PtH_2G even if such a dismutation reaction were to exist. Furthermore if reducing power for methyl group formation could be obtained from the further oxidation of PtH_2G to PtG , methionine synthesis would still be blocked by the need for PtH_4G in the serine transhydroxymethylase reaction. It is therefore concluded that the final reduction of hydroxymethyl groups for methionine formation is not mediated by PtH_4G but possibly by direct action of reduced pyridine nucleotides or by a reduced flavin compound as suggested recently by Hatch et al. (1959).

The Effect of Folic acid Antimetabolites
on the Inhibition of Methionine Synthesis
by reduced Pteroylglutamic acids

The inhibition by PtH_4G of the synthesis of methionine by ultrasonic extracts of E.coli PA15 with EHC as the cofactor was not affected by aminopterin or amethopterin (Table III.8); the inhibition caused by PtH_2G was however greatly reduced by both antimetabolites. Similar results were obtained with extracts of acetone-dried organisms (Table III.9). These results provide further evidence for believing that PtH_2G is inactive in this system until it is reduced to PtH_4G . If this is so, the results also support the argument that it is PtH_4G itself and not an impurity of the chemically prepared material which causes the inhibition of methionine formation.

Dihydropteroylglutamic acid Reductase

The existence of dihydropteroylglutamic acid reductase in the extracts of E.coli has been assumed from the results obtained with the folic acid antimetabolites. The presence of an extremely active DPNH_2 -oxidase in the enzymic extracts prevented the direct observation of a DPNH_2 oxidation coupled to the reduction of PtH_2G in a system similar to that used by Osborn & Huennekens (1958) for the assay of dihydropteroylglutamic acid reductase. Even with the aid of anaerobic

TABLE III.8

Effect of aminopterin and amethopterin on the inhibition of methionine synthesis by dihydro- and tetrahydropteroylglutamic acids

Additions to the basal reaction mixture	L-Methionine formed (μ m-moles/mg. protein)					
	with					
	Aminopterin (μ M)			Amethopterin (μ M)		
	0	0.5	5.0	0	0.05	0.5
EHC + Factor (X)	74	72	74	92	90	90
EHC + Pth ₂ G	3	4	3	6	6	6
EHC + Pth ₄ G	12	39	60	12	32	65

Reaction mixture I plus Dowex-treated ultrasonic extract of strain PA15, extract of heated organisms (EHC) equivalent to 17 mg. dry wt. organisms and Pth₂G or Pth₄G (1 μ mole) when added. Incubated 3hr.

Factor (X) was purified 7-fold from autoclaved products of organisms which had been grown in the presence of substances by R.A. Foster (1 mg. protein). Incubated 3hr.

TABLE III.9

The effect aminopterin on methionine formation
by extracts of acetone-dried *Escherichia coli* PA15

Additions to the basal reaction mixture	L-Methionine formed (μ -moles/mg. protein) with aminopterin (μ M)		
	0	0.5	5.0
EHC	47	46	45
Pth ₄ G + factor (X)	50	90	51
Pth ₂ G + factor (X)	47	19	8
EHC + Pth ₄ G	4	4	4
EHC + Pth ₂ G	8	29	42

Reaction mixture III plus Dowex-treated extract of acetone-dried strain PA15 (10 mg. protein), with Pth₄G or Pth₂G (1 μ mole) and EHC equivalent to 17 mg. dry wt. organisms when added. Factor (X) was purified 7-fold from acetone-dried powders of organisms which had been grown in the presence of cobalamin by H.A. Foster (1 mg. protein). Incubated 3hr.

1) Aminopterin and 6-aminocaproic acid inhibited the folate acid synthetase activity of Pth₄G in the synthesis of methionine, but not that of Pth₂G or EHC, presumably by inactivating dihydropteroylglutamate synthetase.

spectrophotometer cuvettes the endogenous oxidation of DPNH_2 was still too large to permit identification of the reductase. Examination in the Cary recording spectrophotometer also showed that incubation of Pth_2G in a system similar to that used for studying methionine synthesis produced only a small shift towards the spectrum of Pth_4G .

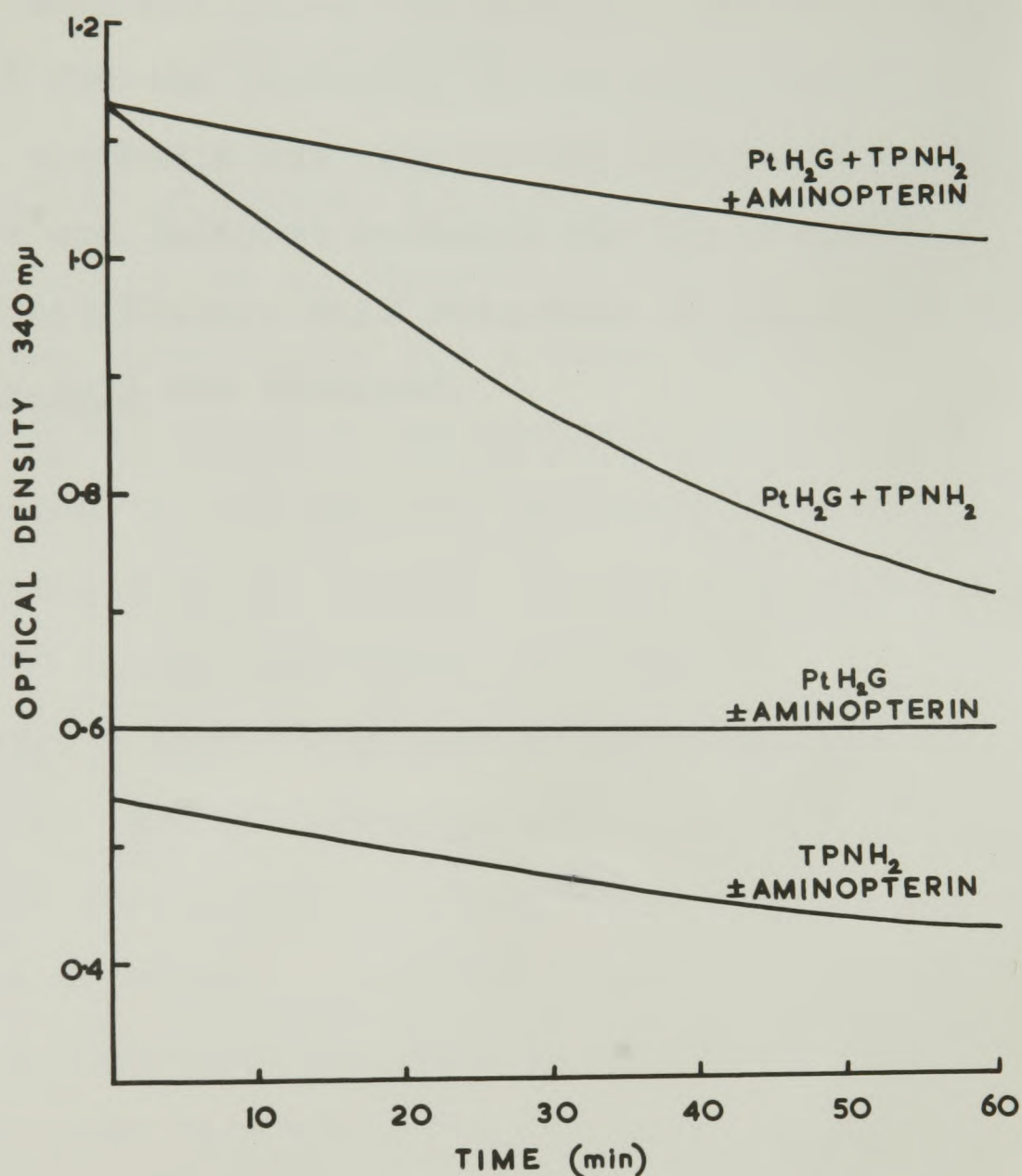
The existence of dihydropteroylglutamic acid reductase was finally proved by using TPNH_2 as the pyridine nucleotide and the spectrophotometric method of Osborn & Huennekens (1958). The endogenous oxidation of TPNH_2 was sufficiently low to permit a study of the enzyme in either aerobic or anaerobic conditions. Ultrasonic extracts of *E. coli* contained dihydropteroylglutamic acid reductase activity and this was inhibited by aminopterin at concentrations equivalent to those which inhibited the function of Pth_2G in the synthesis of methionine (Fig. 27). Because DPNH_2 could not be tested in the crude enzyme preparations, no conclusions can be drawn about the pyridine nucleotide specificity of the enzyme from this bacterial source.

Summary

- 1) Aminopterin and amethopterin inhibited the folic acid cofactor activity of Pth_2G in the synthesis of methionine, but not that of Pth_4G or EHC, presumably by inactivating dihydropteroylglutamic acid reductase.

FIG. 27

The effect of aminopterine on dihydropteroylglutamic acid reductase



Reaction mixture contained (in 3.8 ml. final volume): ultrasonic extract of strain PA15 (2 mg. protein); phosphate buffer pH 7.4 (150 μ moles) and 2-mercaptoethanol (40 μ moles) with PtH₂G (0.3 μ moles); TPNH₂ (0.4 μ moles) and aminopterine (0.05, 0.5 or 5 μ M) when added. The reaction was followed by change in optical density at 340 $m\mu$ in a Unicam SP500 spectrophotometer.

2) The inhibition by PtH_2G of methionine synthesis promoted by EHC was overcome by the folic acid antimetabolites but the inhibition caused by PtH_4G was unaffected.

3) PtH_4G does not appear to serve as the final reductant of the C_1 -unit for the formation of the methyl group in methionine synthesis but only as the cofactor for C_1 -transfer.

4) Direct and indirect evidence for the presence of dihydropteroylglutamic acid reductase in enzymic extracts of *Escherichia coli* was obtained.

In the first place it was apparent that the concentration of PtH_4G needed to activate the synthesis of methionine must be such in excess of the amount of folic acid added as a sample of EHC having equivalent activity.

Secondly, a high percentage of naturally occurring folic acid is in the form of pteroylpolylglutamic acid derivatives or conjugates which can have two, three, six or seven and even ten or eleven γ -linked glutamic acid residues per molecule (Introduction, Chapter 4). These conjugates sometimes possess high biological activity in cell-free systems. Wright (1960) has purified from heated *Clostridium* mylindromycum several pteroylpolylglutamates and these were 100-200 times more active than PtH_4G for the conversion of serine to glycine by enzymic extracts of *Clostridium* *alkalophilum*. A crude mixture of these was active in the synthesis of methionine by *E. coli* (Gordon & Woods, 1960). More recently an oxygen-labile folic acid enzyme has been isolated

CHAPTER 4

1959). CONJUGATED FORMS OF FOLIC ACID IN THE
SYNTHESIS OF METHIONINE BY EXTRACTS OF
Escherichia coli

Introduction

The ability of several conjugated forms of folic acid to serve as cofactors for C_1 -transfer in the synthesis of methionine was investigated for a number of reasons.

In the first place it was apparent that the concentration of PtH_4O needed to activate the synthesis of methionine must be much in excess of the amount of folic acid added as a sample of EHC having equivalent activity.

Secondly, a high percentage of naturally occurring folic acid is in the form of pteroylpolyglutamic acid derivatives or conjugated forms which can have two, three, six or seven and even ten or eleven γ -linked glutamic acid residues per molecule (Introduction, Chapter 4). These conjugates sometimes possess high biological activity in cell-free systems. Wright (1956) has purified from heated *Clostridium cylindrosporum* several pteroylpolyglutamates and these were 100-200 times more active than PtH_4O for the conversion of serine to glycine by enzymic extracts of *Clostridium sticklandii*. A crude mixture of these was active in the synthesis of methionine by *E. coli* (Szulmajster & Woods, 1960). More recently an oxygen-labile folic acid coenzyme has been isolated

from boiled extracts of Clostridium acidii-urici (Rabinowitz, 1959). It was tentatively identified as tetrahydropteroyl-glutamyl- γ -glutamylglutamate (PtH_4G_3) and was 5 times more active than PtH_4G for the tetrahydropteroylglutamic acid formylase reaction although the maximum reaction rate was unaltered. Both PtH_4G and PtH_4G_3 were however equally active for the formimino-tetrahydropteroylglutamic acid transferase and N^5 -formimino-tetrahydropteroylglutamic acid cyclodeaminase reactions (Rabinowitz, personal communication; see Introduction, Chapter 4). In two mammalian systems PtH_4G_3 also had greater activity than PtH_4G : serine transhydroxymethylase of rabbit liver (Blakley, 1957c) and formiminoglutamic acid formimino-transferase for histidine degradation in calf liver (Miller & Waelisch, 1957).

A third reason for continuing the search for a folic acid cofactor for the replacement of EHC was to enable a more detailed study of methionine synthesis using a completely defined reaction mixture, and at the same time to avoid the requirement for cobalamin or factor (X) which accompanies the use of PtH_4G .

The folic acid content of the heated extract was measured before and after treatment with pig kidney conjugase; average values are shown in Table III.10. The overall variation between different preparations of the extract in

TABLE III.10

The folic acid content of the extract
of heated *E. coli*

Concentration of folic acid (μM) in RHC

Assay organism	Untreated	Conjugase treated
<i>Lactobacillus casei</i>	23	40
<i>Streptococcus faecalis</i> R	1.2	70
<i>Leuconostoc citreorum</i> (<i>Pediococcus cerevisiae</i>)	0.9	22

Assays described in Chapter 1 using typical samples of an extract of heated *E. coli* strain PA15. Carried out in collaboration with Dr. K.M. Jones.

Conjugated form of folic acid as one-carbon unit donors and cofactors for the synthesis of methionine

Conjugated folic acids as cofactors for C_1 -transfer

Unlike the polyglutamic acid derivatives of folic acid several triglutamic acid compounds possessed C_1 -cofactor activity for the synthesis of methionine by ultrasonic extracts of *E. coli* H15 in the absence of added substrates (Table III.11). In this respect PnH_2G_2 , $\text{P}^{12}\text{-feroyl}$ PnH_2G_2 and $\text{P}^{12}\text{-feroyl}$ PnH_2G_2 exhibited directly the properties

different assays was approximately $\pm 20\%$. Examination of the growth responses of the different organisms show that many types of folic acid are present and that conjugated derivatives form a large percentage of the total. However using such an assay it is impossible to define the types of folic acid in quantitative terms because the growth response to different growth-promoting forms of folic acid varies greatly even for individual assay organisms. Taking the maximum and minimum values for the concentration of folic acids in EHC as 70 and $20\mu\text{M}$ then the concentration of folic acid causing half-maximal activation of methionine formation (serine plus EHC) is 0.6 to $2.4\mu\text{M}$. This proves that some form of folic acid possessing high cofactor activity compared with PtH_4G (concentration for half-maximal activation, $150\mu\text{M}$) is present in the heated extract.

Conjugated forms of folic acid as one-carbon unit donors and cofactors for the synthesis of methionine

Conjugated folic acids as cofactors for C_1 -transfer

Unlike the monoglutamic acid derivatives of folic acid several triglutamic acid compounds possessed C_1 -cofactor activity for the synthesis of methionine by ultrasonic extracts of E. coli PA15 in the absence of added cobalamin (Table III.11, column 1). In this respect PtH_4G_3 , N^{10} -formyl PtH_4G_3 and N^5 -formyl PtH_4G_3 exhibited directly the properties

TABLE III.11

Cofactor activity of several conjugated forms of folic acid and the effect of added cobalamin and HIC

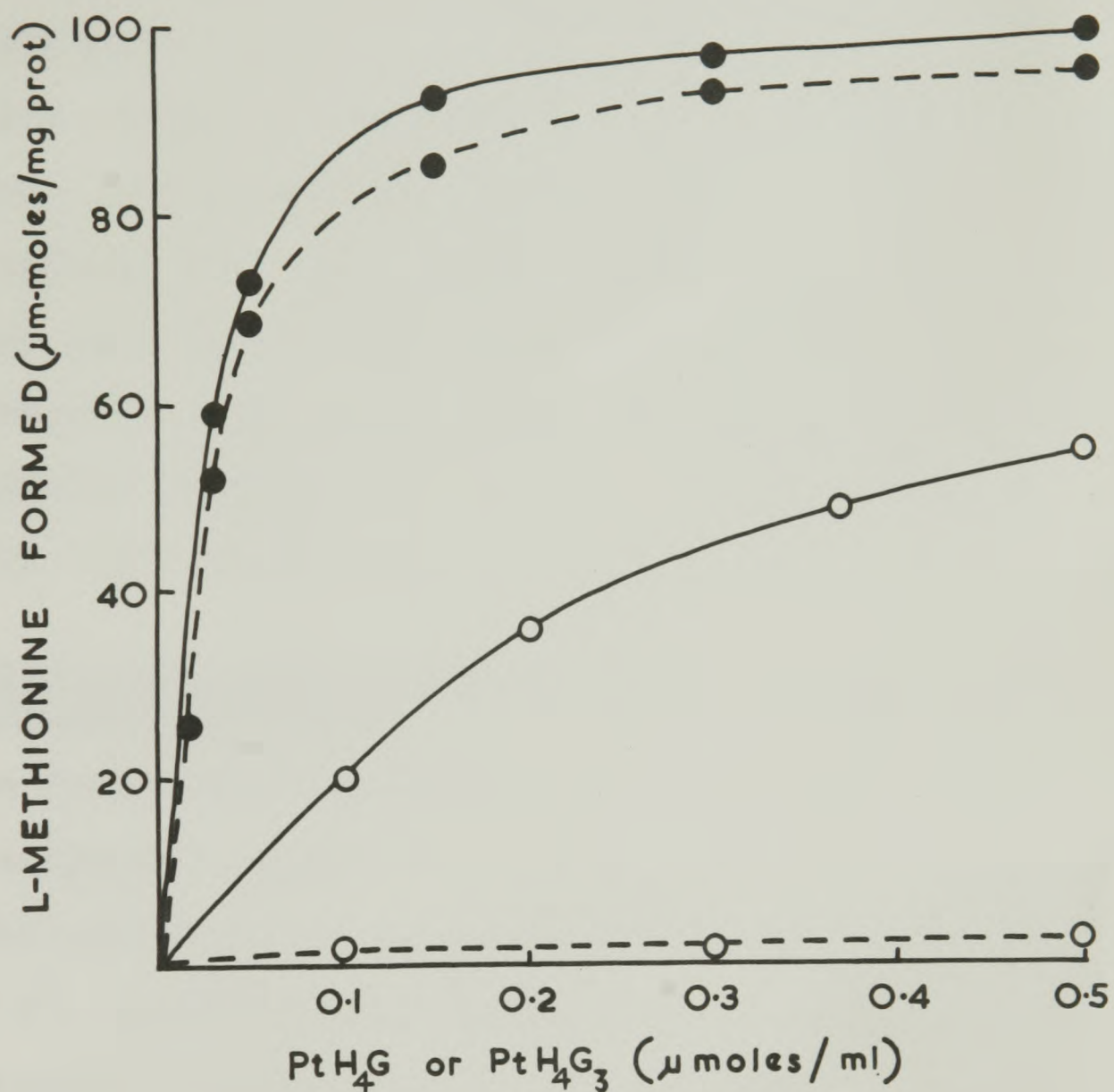
Source of folic acid (1μmole)	L-Methionine formed (μm-moles/mg. protein) with		
	1 <u>No additions</u>	2 <u>Cobalamin</u>	3 <u>HIC</u>
None	2	2	68
PtH ₄ G	2	58	4
PtG ₂	2	2	70
PtH ₄ G ₂	2	2	18
PtG ₃	3	2	70
PtH ₄ G ₃	96	100	105
N ¹⁰ -formyl PtG ₃	2	3	-
N ¹⁰ -formyl PtH ₄ G ₃	96	99	105
N ⁵ -formyl PtH ₄ G ₃ (crude)	99	110	106

Reaction mixture I plus Dowex-treated ultrasonic extract of E.coli PA15 with cobalamin (1μm-mole) and HIC equivalent to 17 mg. dry wt. organisms when added. Incubated 2hr.

of the folic acid coenzymes of the EHC. Addition of cobalamin had no appreciable effect (Column 2). The unreduced derivatives were completely devoid of cofactor activity even when cobalamin was added. PtG_2 and PtH_4G_2 were inactive but this was not surprising because they contain an α -linked glutamic acid residue which is not found in natural folic acid conjugates; pteroyl- α -glutamylglutamic acid has no folic acid activity for man or for Lactobacillus casei and Streptococcus faecalis R (Spies, Stone & Toca, 1947; Semb et al. 1949). The concentration of the triglutamic acid derivatives used for comparison with PtH_4G in the Table III.11 were much in excess of the requirement (Table III.15). The activities of PtH_4G_3 and PtH_4G are compared in detail in Figure 28; PtH_4G_3 was more than six times as active as PtH_4G and also produced a higher maximum rate of methionine formation. Tetrahydropteroyl-triglutamic (PtH_4G_3) acid was chosen as the cofactor for a more detailed investigation of methionine formation with the completely defined reaction mixture because PtH_4G_3 was most readily prepared. Samples of PtH_4G_3 prepared from natural PtG_3 or synthetic PtG_3 or from crude or chromatographically pure PtG_3 were all equally active within experimental error. PtH_4G_3 itself was chromatographed on TEAE-cellulose with a gradient of phosphate buffer pH6.1 containing 0.2M 2-mercaptoethanol. The reduced triglutamate was eluted as a single band

FIG. 28

The cofactor activity of tetrahydropteroyltriglutamic acid and tetrahydropteroylmonoglutamic acid in the synthesis of methionine



Reaction mixture I plus Dowex-treated ultrasonic extract strain PA15 with:

(○), PtH₄G; (●), PtH₄G₃ and cobalamin (0.65 μmoles), present (—); absent (---). Incubated 2hr.

by 0.29M phosphate buffer and identified spectrophotometrically; it still retained its cofactor activity.

With extracts of acetone-dried E.coli PA15 results analogous to those with ultrasonic extracts were obtained; PtH_4G_3 replaced the heated extract for the activation of methionine synthesis whether or not the organisms had first been grown in the presence of cobalamin.

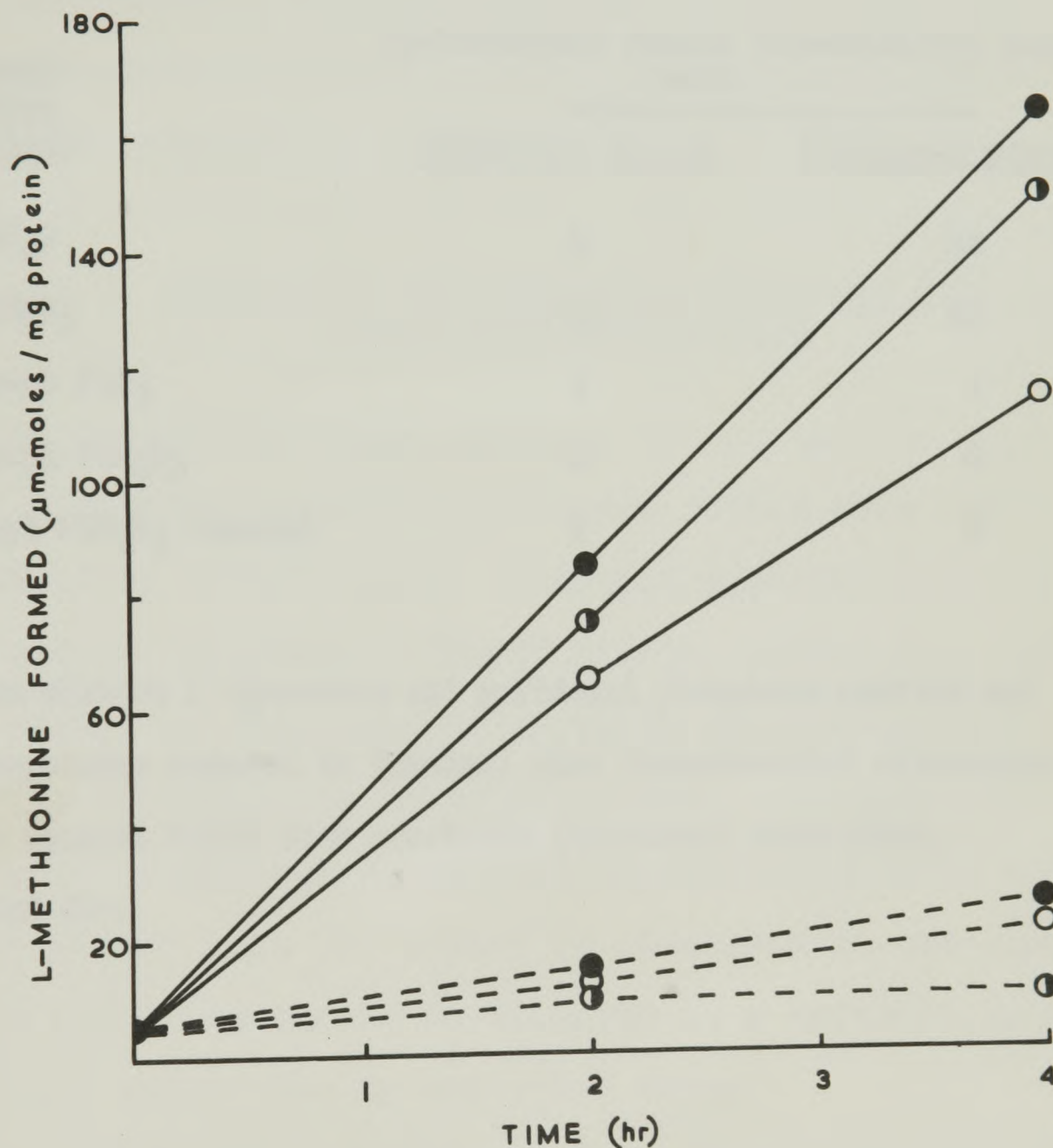
Cobalamin was still necessary for optimal methionine formation by ultrasonic extracts of E.coli strain 121/176 (a B_{12} -requiring auxotroph) even with PtH_4G_3 as the folic acid cofactor (Fig.29); the range of concentrations of cobalamin required was identical with that needed with PtH_4G .

Conjugated folic acids as donors of the one-carbon unit for methionine synthesis

The formaldehyde derivative of PtH_4G_3 was prepared by mixing equimolar quantities of PtH_4G_3 and formaldehyde in 25mM phosphate buffer pH 7.0 for 1hr. under hydrogen. This compound and the N^5 , and N^{10} -formyl derivatives of PtH_4G_3 were only weakly active as substrates for the synthesis of methionine (serine absent) and then only in the presence of cobalamin (Table III.12). The formaldehyde derivative of PtH_4G_3 was the most active.

Evidence for the combination of formaldehyde with PtH_4G_3 to form an N^5 or N^{10} -hydroxymethyl derivative or $\text{N}^5, \text{N}^{10}$ -methylene

Tetrahydropteroyltriglutamic acid as a cofactor for
the synthesis of methionine by ultrasonic extracts
of *Escherichia coli* 121/176



Reaction mixture I plus Dowex-treated ultrasonic extract (10 mg. protein, strain 121/176). Cobalamin (0.65 μm-moles) ———, present; — — —, absent.

(O), EHC equivalent to 17 mg. dry wt. organisms.

(◐), PtH₄G (1 μmole).

(⊗), PtH₄G₃ (0.2 μmoles).

TABLE III.12

Combined C₁-donor and cofactor activity
of conjugated derivatives of folic acid

Folic acid derivative (2.5 μmoles)	L-Methionine formed (μm-moles/mg. protein) with	
	Cobalamin absent	Cobalamin present
CH ₂ O-PtH ₄ G	4	42
CH ₂ O-PtH ₄ G ₃	4	10
N ¹⁰ -formyl PtG ₃	1	1
N ¹⁰ -formyl PtH ₄ G ₃	2	6
N ⁵ -formyl PtH ₄ G ₃ (crude)	2	6

as the source of cofactor (Table III.11, column 5). Cobalamin
Reaction mixture I (L-serine and pyridoxal phosphate omitted and
had no effect on this inhibition which may reflect the
DL-homocysteine reduced to 5 μmoles) plus Dowex-treated ultrasonic
extract (strain PA15) with cobalamin (1 μm-mole) when added.
The rate of inhibition is similar to that observed by PtH₄G is
Incubated 2hr.

might be argued that the effect of cobalamin in the case of
PtH₄G is simply to enable it to serve as a cofactor and not
relieve the inhibition by any other means.

The unreduced derivatives had no inhibitory effect on
methionine formation and the triglutamic acid derivatives
possessing cofactor activity even increased the rate of
synthesis to the maximum.

PtH_4G_3 was obtained by estimating the formaldehyde bound by PtH_4G_3 (Section I, Chapter 1); 85% of the PtH_4G_3 was capable of binding one molecular equivalent of formaldehyde. However the dissociation of this complex may still be too high to prevent spontaneous formation of 1,3-thiazane-4-carboxylic acid in the presence of homocysteine.

The EHC by PtH_4G_3 allowed the investigation of the nature of this inhibition in

Inhibition of methionine synthesis by folic acid derivatives

a system free of cofactor (Table III.10). The percentage

Effect of conjugated folic acids

Of the pteroylpolyglutamic acids tested only PtH_4G_2 had any inhibitory effect on the synthesis of methionine with EHC as the source of cofactor (Table III.11, column 3). Cobalamin had no effect on this inhibition which may reflect the inactivity of PtH_4G_2 to serve as a folic acid cofactor. If the mode of inhibition is similar to that imposed by PtH_4G it might be argued that the effect of cobalamin in the case of PtH_4G is simply to enable it to serve as a cofactor and not relieve the inhibition by any other means.

The unreduced derivatives had no inhibitory effect on methionine formation and the triglutamic acid derivatives possessing cofactor activity even increased the rate of methionine synthesis to the maximum. The concentrations required for half-maximal activation of methionine synthesis were much smaller than for PtH_4G (cobalamin present; Table III.10). PtH_4G_2 and N^{10} -formyl PtH_4G_2 were

Effect of tetrahydropteroylglutamic acid

PtH_4G inhibited methionine synthesis when PtH_4G_3 , N^{10} -formyl PtH_4G_3 or N^5 -formyl PtH_4G_3 were the source of the folic acid cofactor for C_1 -transfer just as it inhibited the activity of EHC (Table III.13); in all cases cobalamin restored the activity. Replacement of the EHC by PtH_4G_3 allowed the investigation of the nature of this inhibition in a system free from the complexities caused by the use of a crude source of cofactor (Table III.14). The percentage inhibition of the rate of methionine formation was constant for a constant ratio of PtH_4G_3 to PtH_4G suggesting that the inhibition is of the competitive type over the admittedly narrow range of concentrations tested.

Comparison of the folic acid cofactor activities of pteroyltriglutamic acid compounds and the heated extract

The conjugated forms of folic acid possessing C_1 -cofactor activity were similar to the active constituents of EHC both in their independence of any requirement for cobalamin (strain PA15) and in their sensitivity to inhibition by PtH_4G . The concentrations required for half-maximal activation of methionine synthesis were much smaller than for PtH_4G (cobalamin present; Table III.15). PtH_4G_3 and N^{10} -formyl PtH_4G_3 were

TABLE III.13

Effect of tetrahydropteroylglutamic acid on
the cofactor activity of several conjugated
forms of folic acid

Source of folic acid (1μmole)	L-Methionine formed (μm-moles/mg. protein) with		
	No additions	Pth ₄ G	Pth ₄ G and cobalamin
None	2	2	58
EHG	68	4	63
Pth ₄ G ₃	96	4	90
N ¹⁰ -formyl Pth ₄ G ₃	96	3	93
N ⁵ -formyl Pth ₄ G ₃ (crude)	99	3	94

Reaction mixture I plus Dowex-treated ultrasonic extract of E. coli
PA15 with cobalamin (1μm-mole), Pth₄G (1μmole) and EHG equivalent
to 17 mg. dry wt. organisms when added. Incubated 2hr.

TABLE III.14.

Inhibition of the cofactor activity of
tetrahydropteroyltryptophanic acid by
tetrahydropteroylglutamic acid

<u>Pth₄G₃</u> <u>(μmoles/ml.)</u>	<u>Inhibition (%) of methionine formation with</u> <u>Pth₄G₃ as cofactor by Pth₄G (μmoles/ml.)</u>				
	<u>0</u>	<u>0.025</u>	<u>0.05</u>	<u>0.1</u>	<u>0.2</u>
0.075	0	16	30	44	69
0.150	0	-	19	29	42
0.30	0	-	-	15	22

Reaction mixture I plus Dowex-treated ultrasonic
 extract (strain PA15) and the folic acids as shown.

Incubated 110 min. plus Dowex-treated ultrasonic

extract of strain PA15. Incubated 110 min.

8. Methionine (0.05 μmoles) added.

TABLE III.15

Comparison of the one-carbon cofactor
activities of synthetic derivatives
of folic acid for the synthesis of methionine

<u>Source of folic acid cofactor</u>	<u>Concentration of folic acid required for half-maximal activation of methionine formation (μM)</u>
$\text{Pth}_4\text{G}^{\#}$	150
Pth_4G_3	24
N^{10} -formyl Pth_4G_3	24
N^5 -formyl Pth_4G_3 (purified)	1.8
EMC	0.6 - 2.4

Reaction mixture I plus Dowex-treated ultrasonic extract of strain PA15. Incubated 2hr.

* Cobalamin (0.65 μm -moles) added.

the EMC. On the other hand the cofactor activities of samples of the purified N^5 -formyl Pth_4G_3 were completely unaffected by heating at 100° in air for 3 min. at pH 7.0. Consequently with a combination of heat stability and a high folic acid cofactor activity, N^5 -formyl Pth_4G_3 qualifies as a possible component of the heated extract.

equally active and the N^5 -formyl PtH_4G_3 prepared and purified from the latter was thirteen times as active as these. If it is assumed that only one isomer of N^5 -formyl PtH_4G_3 is active then the concentration giving half-maximal activation of methionine synthesis ($0.9\mu M$) is well within the limits set by the folic acid concentration provided by the amount of heated extract which is required to produce a similar activation (and assuming that the folic acids of EHC possess cofactor activities with the same efficiencies). The intrinsic cofactor activity of N^5 -formyl PtH_4G_3 is almost 100 times greater than that of PtH_4G .

In addition to the difference in intrinsic cofactor activity of PtH_4G_3 or N^{10} -formyl PtH_4G_3 compared with the folic acids of EHC these two compounds were rapidly inactivated by contact with air or by heating in air. They cannot therefore be considered as possible constituents of the EHC. On the other hand the cofactor activities of samples of the purified N^5 -formyl PtH_4G_3 were completely unaffected by heating at 100° in air for 3 min. at pH 7.0. Consequently with a combination of heat stability and a high folic acid cofactor activity, N^5 -formyl PtH_4G_3 qualifies as a possible component of the heated extract.

Summary

- 1) Tetrahydropteroyltriglutamic acid (Pth_4G_3), N^{10} -formyl Pth_4G_3 and N^5 -formyl Pth_4G_3 served as folic acid cofactors for the synthesis of methionine by enzymic extracts of Escherichia coli PA15 and no cobalamin was required.
- 2) The cofactor activity of these conjugated folic acids was inhibited by Pth_4G and in the case of Pth_4G_3 the inhibition was of the competitive type.
- 3) With ultrasonic extracts of strain 121/176 cobalamin was still required when Pth_4G_3 was the source of the folic acid cofactor.
- 4) $\text{CH}_2\text{O-Pth}_4\text{G}_3$, N^{10} -formyl Pth_4G_3 and N^5 -formyl Pth_4G_3 were only weakly active as sources of the C_1 -unit for methionine formation and then only in the presence of added cobalamin.
- 5) From a comparison of the stabilities and the relative activities as cofactors only N^5 -formyl Pth_4G_3 qualified as a possible active component of the extract of heated Escherichia coli (EHC).

The heated extract could be stored up to 2 weeks at -14° in air with no apparent loss of activity.

CHAPTER 5

PROPERTIES AND PURIFICATION OF THE EXTRACT OF HEATED *Escherichia coli*

In conjunction with the search for synthetic folic acid derivatives possessing one-carbon cofactor activity similar to that of the active components of the heated extract (EHC), attempts were also made to isolate and identify the forms of folic acid present in the heated extract. In order to determine the stability and thus the range of techniques available for purification of the EHC, several of its physical and chemical properties were first examined. Of the methods which have been used for isolating folic acids from natural sources, paper chromatography and more recently anion-exchange cellulose chromatography have been the most successful. These techniques combined with bioautography have been used here in a preliminary examination of components of the heated extract which are folic acids.

Physical and Chemical Properties of the Heated Extract (EHC)

The heated extract could be stored up to 8 weeks at -14° in air with no apparent loss of activity.

Effect of hydrogen ion concentration

Treatment of the EHC at pH 1.0 or pH 10.0 for 1hr. at 0° destroyed 50% of the cofactor activity. By comparison, the crude mixture of folic acid coenzymes (CoC) of Wright (1955a) were destroyed by acid but stable to boiling in 0.05N alkali.

Effect of oxidising agents

In addition to being stable in air, the cofactor activity of the extract was unaffected by shaking in a stream of oxygen for 4hr. at 25°. To test whether extensive oxidative degradation might have taken place during the original preparation of the extract, samples were prepared in 2-mercapto-ethanol (1M) and in potassium ascorbate (25mM) and were found to be no more active than the normal EHC.

The cofactor activity was however reduced by 60% when the extract was treated with hydrogen peroxide (4 volumes final concentration) at 25° for 1hr.

Effect of visible and ultraviolet radiation

A sample of EHC was placed in a silica tube 25cm. distant from the combined radiation of a 50 watt tungsten lamp and a 7 watt ultraviolet lamp for 1hr. at 25°. No change in activity was observed, thus providing more evidence that a cobamide coenzyme is not a functional component of the

extract. Neither cobalamin nor 5,6-dimethylbenzimidazolyl-cobamide coenzyme had any effect on the EHC before or after irradiation.

Cobalamin content

The cobalamin concentration of a heated extract of *E. coli* strain PA15, grown in the absence of added cobalamin was $16\mu\text{M}$ when assayed with *Euglena gracilis*. For a heated extract of strain 121/176 grown with a limiting concentration of cobalamin the value was $10\mu\text{M}$. It is clear therefore that the EHC of strain PA15 does not supply cobalamin for the synthesis of methionine because the cobalamin requirement for methionine synthesis with strain 121/176 or for strain PA15 in the presence of Pth_4G is of the order of $50\mu\text{M}$. The heated extract of neither strain could supply this concentration under the conditions normally used.

Purification of the Heated Extract (EHC)

Folic acids represent only a small proportion of the total solid material in the EHC (approximately 5 μmoles folic acid per gram dry wt. of EHC). No increase in folic acid cofactor concentration was achieved by adding pteroyl-glutamic acid (10^{-3}M) to the growth medium from which the organisms were originally harvested. The use of methods which

did not give full recovery of the folic acids was prevented by the possibility that the relative concentrations of the components would be altered. For this reason, adsorption on charcoal was not used. Material suitable for chromatographic examination was obtained by treatment of the extract with an equal volume of acetone at 0°. After centrifuging the mixture (3,000xg for 15 min. at 0°) the precipitate was discarded and acetone removed from the supernatant fluid by lyophilisation. The folic acid cofactor activity was recovered without loss and a two-fold purification was obtained.

Anion-exchange cellulose chromatography

The chromatography of folic acid and its derivatives on DEAE-cellulose and TEAE-cellulose has recently been described by Usdin & Porath (1957), Usdin (1959) and Rabinowitz (1959). These adsorbents offer great advantages over other anion-exchange resins such as Dowex-1 because it is possible to elute the folic acids with very mild agents such as neutral phosphate buffers.

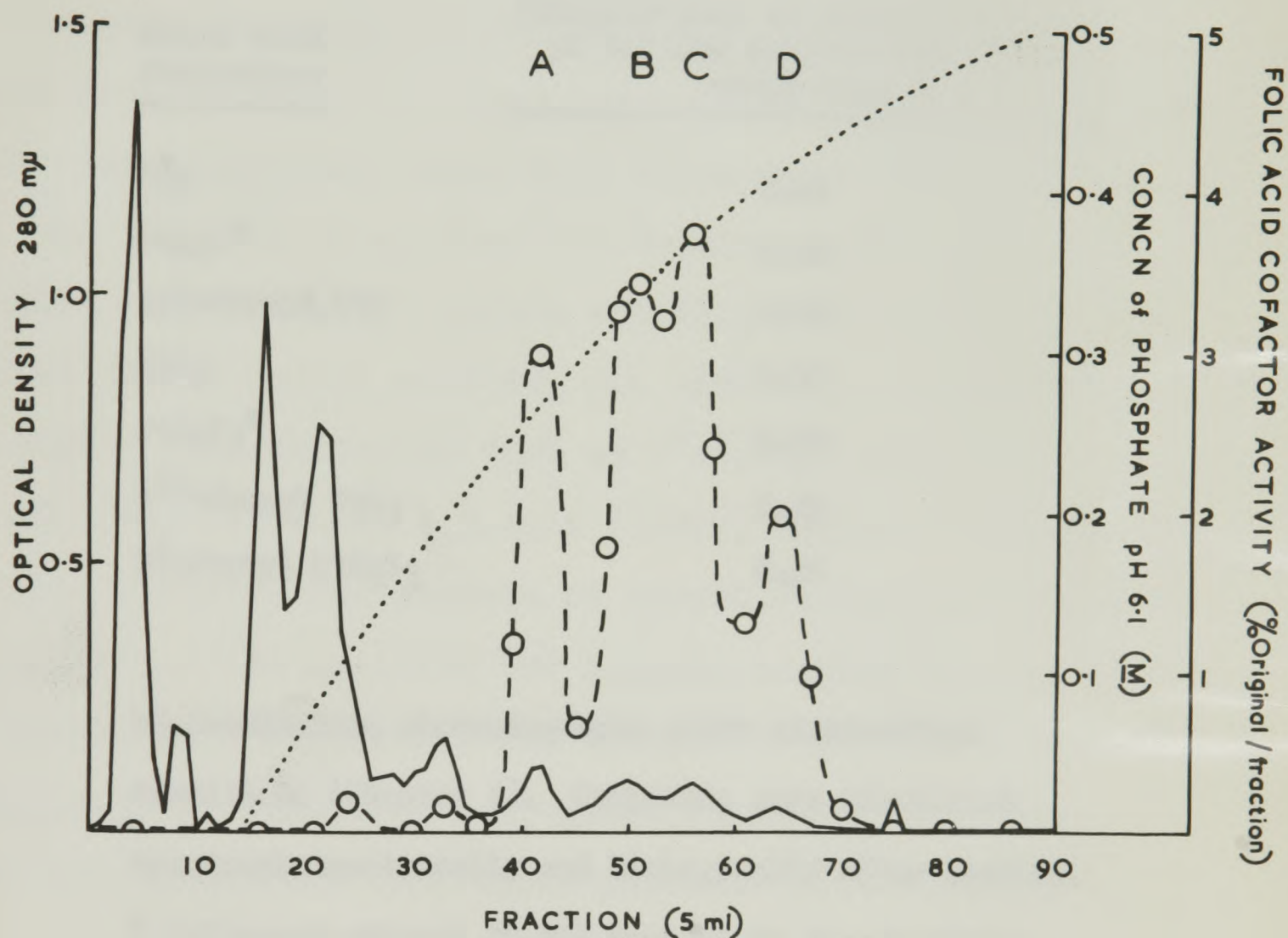
TEAE-cellulose was preferred to DEAE-cellulose because although higher concentrations of phosphate buffer were required for the elution of the folic acids, a greater resolution of the components was possible. A typical fractionation of a sample of EHC by gradient elution on

TEAE-cellulose is shown in Figure 30. Approximately 65% of the cofactor activity and 75% of the adsorbancy at 280m μ were recovered. The existence of several components having cofactor activity was confirmed. There may however be other folic acids having no activity in the synthesis of methionine but these were not investigated. The chief components having cofactor activity were eluted by A, 0.26M; B, 0.34M; C, 0.38M and D, 0.41M potassium phosphate buffer pH6.1. Component A was well separated from the other components and accounted for approximately 20-25% of the total eluted activity. The fractions retained their activity for 4 weeks at pH7.8 and 0° in air; the active components of EHC are therefore still resistant to oxidation when isolated from the crude extract.

Several synthetic folic acid derivatives were also chromatographed under exactly the same conditions; the concentrations of phosphate buffer required for their elution are shown in Table III.16. These values differ from those quoted by Usdin (1959); small variations in the technique and in the TEAE-cellulose employed probably account for this. Comparing the chromatography of synthetic and natural folic acids it is apparent that component A and N⁵-formyl Pth₄G₃ have similar properties. It will also be remembered that N⁵-formyl Pth₄G₃ was the only synthetic

FIG. 30

Chromatography of the heated extract of *Escherichia coli*
strain PA15 on TEAE-cellulose



Fractionation of the equivalent of 5.5 ml. EHC after treatment with acetone.

- , Optical density at 280mμ.
-, Phosphate buffer pH 6.1 (M).
- - -, Folic acid cofactor activity for methionine formation expressed as a percentage of the original activity in the fraction.

TABLE III.16

Chromatographic properties of several
folic acid derivatives

Folic acid derivative

Concentration of phosphate buffer
pH 6.1 for elution from TEAE-
-cellulose (M)

PtG	0.41
PtH ₄ G ²	0.20
N ¹⁰ -formyl PtG	0.12
PtG ₃	0.46
PtH ₄ G ₃ ²	0.29
N ¹⁰ -formyl PtG ₃	0.25
N ⁵ -formyl PtH ₄ G ₃	0.26

TEAE-cellulose chromatography under standardised conditions (Chapter 1). Compounds were identified spectrophotometrically and biologically after elution.

² 2-Mercaptoethanol (0.2M) present in the phosphate buffer.

derivative so far prepared which had both stability and cofactor activity comparable with that of the EHC (Chapter 4).

Microbiological activity of the components of the fractionated extract

The peak fractions of components A, B, C and D were diluted from 10 to 40000 times and assayed for folic acid growth factor activity using four micro-organisms (Table III. 17). The same fractions were also treated with pig kidney conjugase before assay. Before treating with conjugase the relative growth factor activity for different organisms of component A was similar to that of N^5 -formyl PtH_4G_3 as determined by Silverman & Wright (1956), Hakala & Welch (1957) and Uedin (1959). The absence of growth factor activity for S.faecalis and the activity for L.casei suggest that it must be a triglutamyl derivative and because it is also active for P.cerevisiae it must be a conjugated form of citrovorum factor having no more than three glutamic acid residues (Table III.1, page 146).

Of the other components B, C and D, C and D had growth promoting activity for Tetrahymena vorax but not for the other micro-organisms suggesting that they must be higher conjugates of folic acid. This was confirmed by conjugase treatment after which all the components (C and D and also B) exhibited growth factor activity for the three bacteria

(Table III.17). TABLE III.17

Microbiological activity of fractions of
the heated extract containing cofactor activity

<u>Assay micro-organism</u>	Folic acid concentration (μM) as determined with							
	Untreated fractions of components				Conjugase-treated fraction of components			
	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
<u>Streptococcus faecalis</u> R	0	0	0	0	3.0	1.2	1.5	0.6
<u>Lactobacillus casei</u>	2.5	0	0	0	3.7	1.2	1.6	0.7
<u>Pediococcus cerevisiae</u>	0.4	0	0	0	4.0	1.0	1.2	0.4
<u>Tetrahymena vorax</u>	-	-	2.0	1.0	-	-	-	-

Diluted peak fractions of components A, B, C & D from TEAE-cellulose chromatography (Fig.30) were assayed as described in Chapter 1. 0, denotes inactive and -, denotes that the fraction was not assayed.

Further samples were examined for their growth factor activity for *L. casei* and *P. cerevisiae* by direct incubation in the growth medium. With solvent A (page 181) all the cofactor activity was recovered as one band having R_f 0.25 - 1.0; most of the growth factor activity was located here. These are the properties expected of conjugated and/or formylated folic acids and possibly compounds containing additional polar groups (Table III.2, page 181).

(Table III.17). The relative concentrations of folic acid measured in each assay suggest that components B, C and D consist largely of conjugated forms of citrovorum factor (N^5 -formyl PtH_4G). Their chromatographic properties also suggest that they contain more than three glutamic acid residues.

The concentration of component A required for half-maximal activation of methionine synthesis (approximately $1.5\mu M$) was of the same order found for N^5 -formyl PtH_4G_3 ($0.9\mu M$, assuming only one isomer is active).

Paper chromatography and bioautography of the heated extract

Samples of the acetone-treated EHC were chromatographed on strips of Whatman No.3 paper; the strips were cut into sections and assayed both for folic acid cofactor activity by direct incubation in the complete reaction mixture for methionine formation. Smaller samples were examined for their growth factor activity for L.casei and P.cerevisiae by direct incubation in the growth medium. With solvent A (page 151) all the cofactor activity was recovered as one band having R_f 0.85 - 1.0; most of the growth factor activity was located here. These are the properties expected of conjugated and/or formylated folic acids and possibly compounds containing additional polar groups (Table III.2, page 151).

FIG. 31A

Solvent B partially resolved the cofactor activity of the EHC into two regions, $R_f=0.8$ and $R_f=0.95$ (Fig.31A). Only the former had growth factor activity for L.casei and P.cerevisiae suggesting that this contains the same material as the component A obtained from the anion-exchange cellulose fractionation. Presumably the other region ($R_f=0.95$) is composed of more highly conjugated forms of folic acid which also have cofactor activity.

The crude preparation of N^5 -formyl PtH_4O_3 (Chapter 4) was chromatographed in a similar manner (Fig.31B). Two bands with blue fluorescence were observed, $R_f=0.6$ and $R_f=0.8$ and these coincided with the regions of cofactor activity. Growth factor activity for L.casei was present in both regions whereas the activity for P.cerevisiae was largely at $R_f=0.8$. After purification, the N^5 -formyl PtH_4O_3 possessed growth factor activity for both organisms at $R_f=0.8$ only (Fig.31C). Because of the high concentration of phosphate buffer in the purified material it was not possible to chromatograph a large enough sample to identify the compound using its activity in methionine synthesis as the assay. However it is almost certain that most of the cofactor activity would now be found at $R_f=0.8$. Thus N^5 -formyl PtH_4O_3 has the same R_f as the microbiologically active component of the EHC which provides further evidence for its presence in the EHC.

FIG. 31A

Paper chromatography of the heated extract

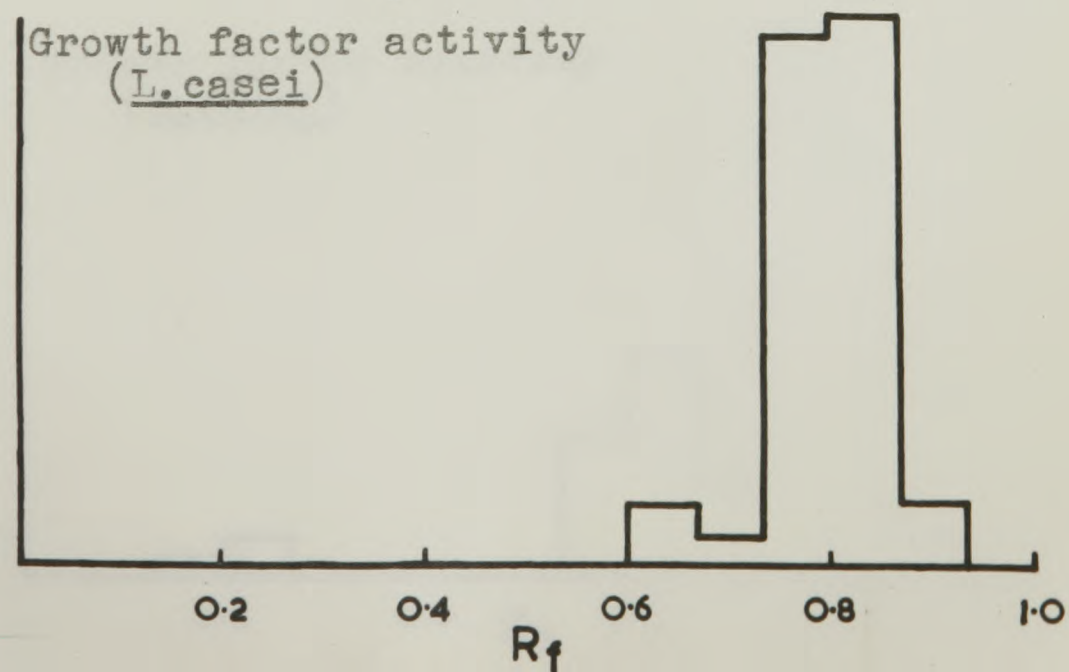
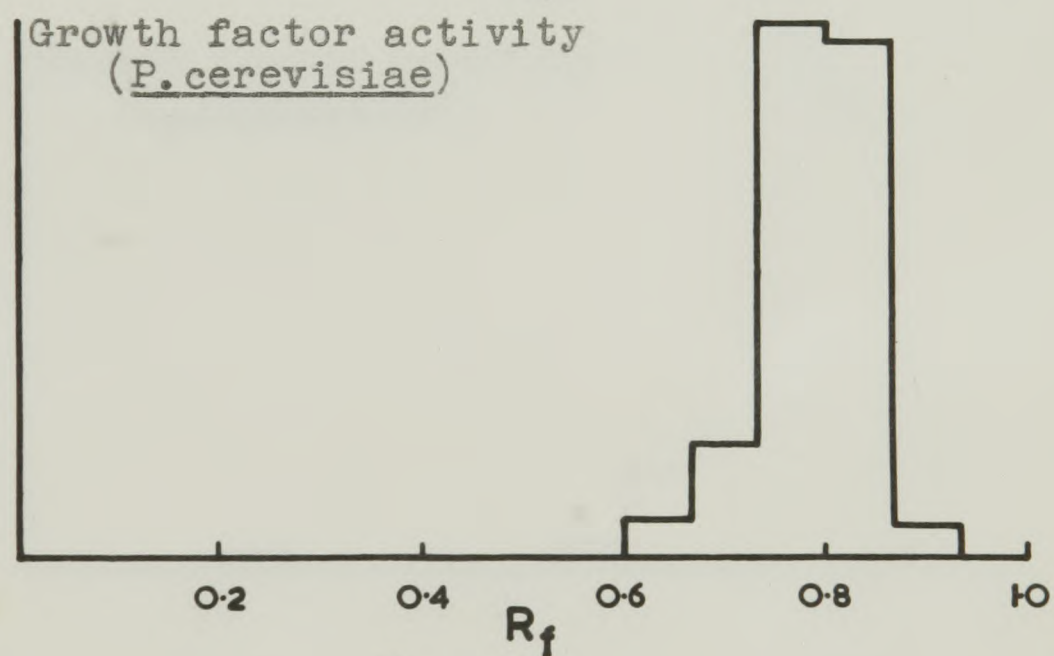
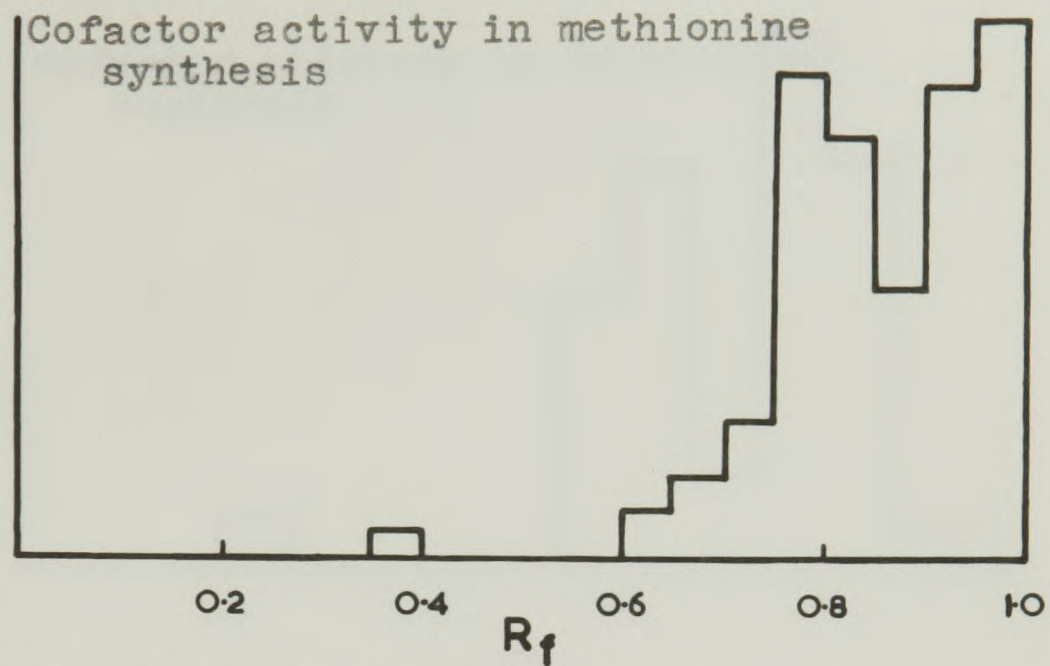


FIG. 31B

Paper chromatography of N^5 -formylPtH₄G₃ (crude)

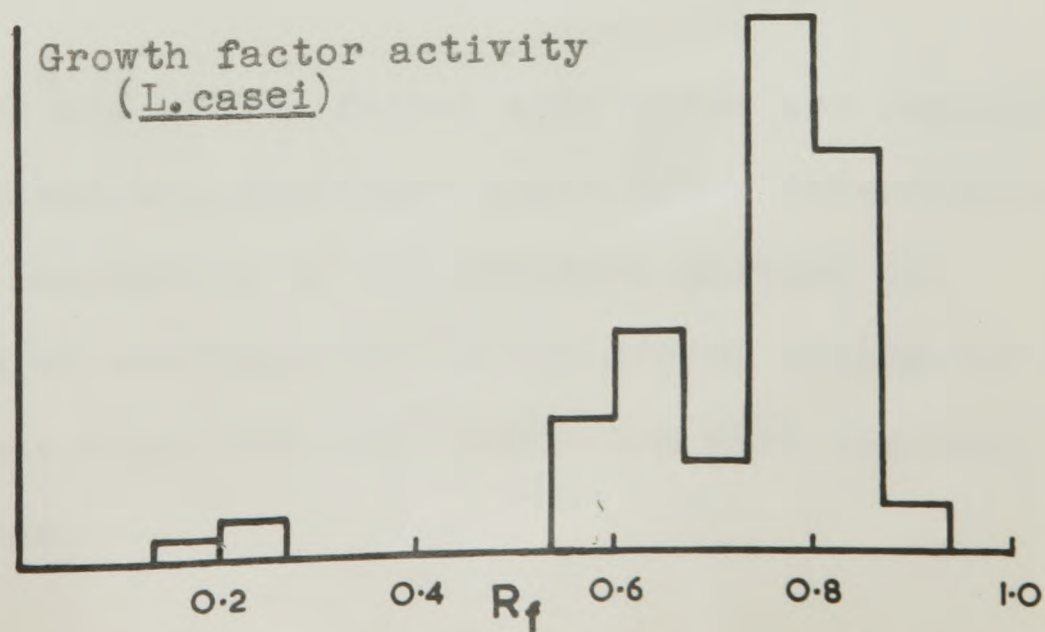
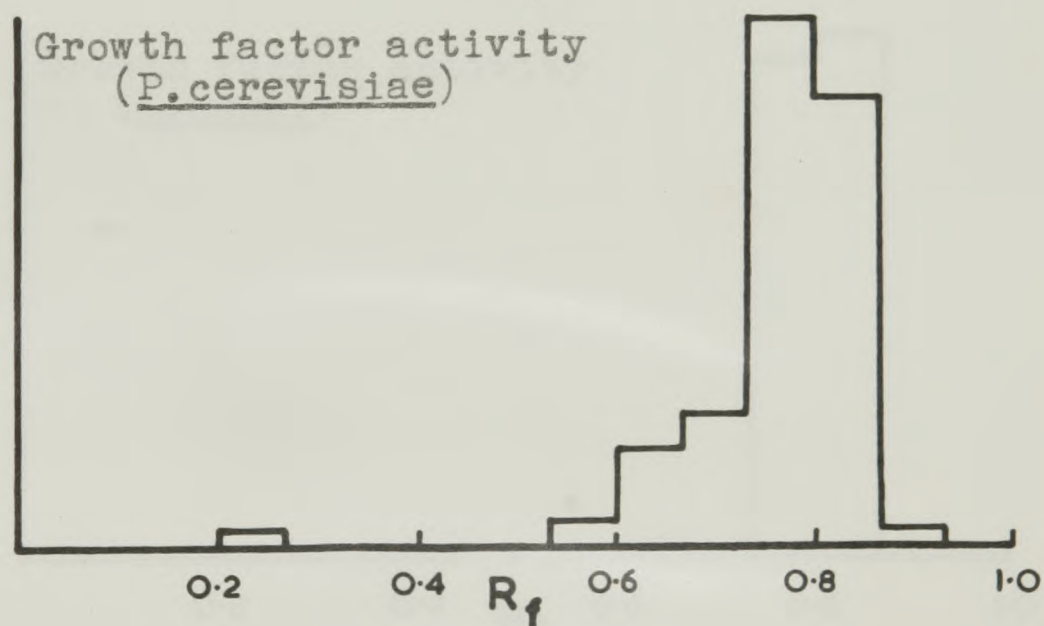
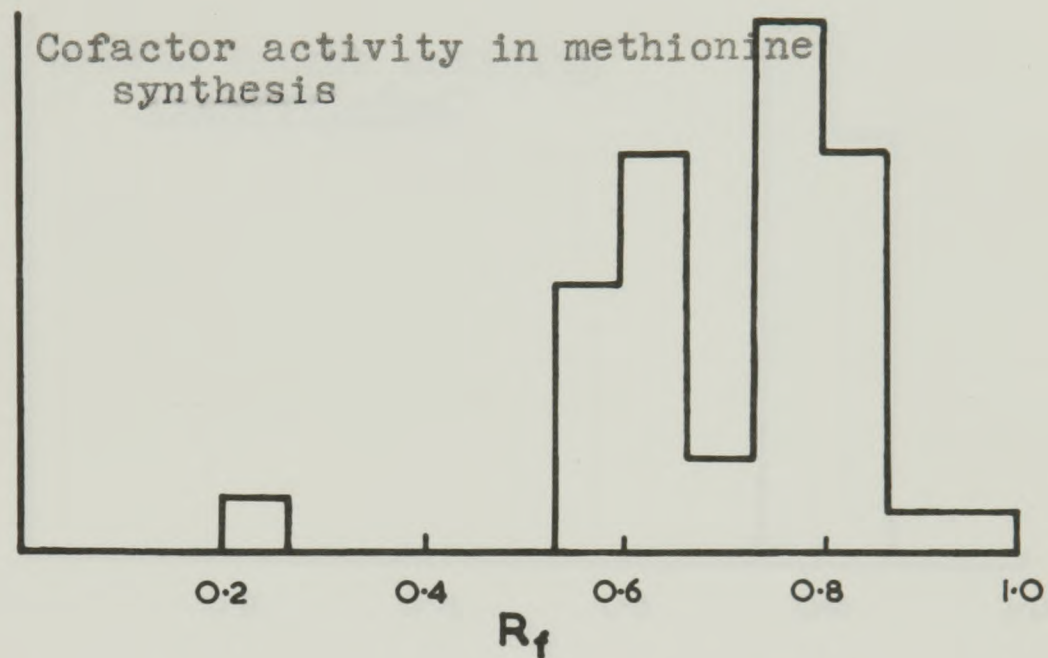
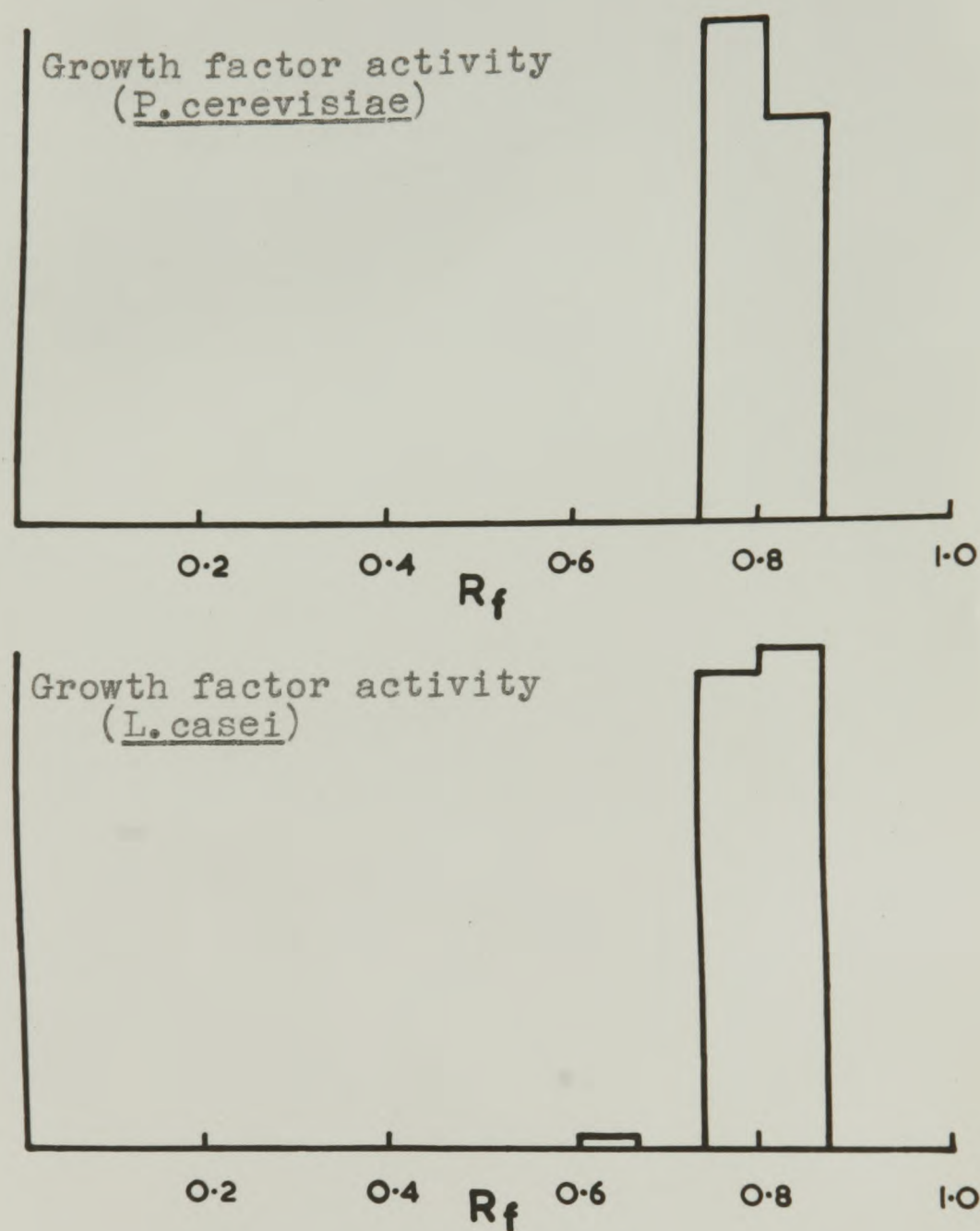


FIG. 31C

Paper chromatography of N⁵-formylPtH₄G₃ (purified)



Cofactor and growth factor activities are represented as a linear and arbitrary scale after determination by direct incubation in the reaction mixture for methionine synthesis or in the growth medium for the two assay organisms and comparison with standard responses.

In conclusion, there is a considerable amount of evidence which qualifies N^5 -formyl PtH_4G_3 or a close relative as a constituent of the EHC: (a) Its specific activity as a cofactor for methionine synthesis is similar to that of EHC and to that of the component A isolated from the EHC by TEAE-cellulose chromatography. (b) It is eluted from TEAE-cellulose by the same concentration of phosphate buffer as component A and its resistance to heating in air is the same as the EHC. (c) N^5 -formyl PtH_4G_3 has the same microbiological growth activities as the component A. (d) In paper chromatography it behaves similarly to the constituent of the EHC with these same growth factor activities. Final proof of the identity of the component A and N^5 -formyl PtH_4G must however await the isolation of sufficient of the purified compounds from both the EHC and the crude synthetic material to allow a rigorous comparison of their chemical, physical and biological properties.

SUMMARY

- 1) The effect of pH, oxidising agents and visible and ultraviolet radiation on the stability of the EHC were investigated.

2) A method for the fractionation of the active folic acid cofactors of EHC was developed and one component was tentatively identified as N^5 -formyl PtH_4G_3 by comparing its cofactor and microbiological activities and its behaviour in chromatography with the same properties of synthetic N^5 -formyl PtH_4G_3 .

acid and the reduced pteroylglutamic acids. In order to exert their cofactor functions, the former were dependent upon the presence of added cobalamin. The latter group, like the cofactor of the EHC, were active and no requirement for the simultaneous metabolism of cobalamin was observed if *E. coli* PA18 was the source of the ultrasonic extract used for examining the synthesis of methionine. With ultrasonic extracts of the cobalamin auxotroph (strain 181/176) cobalamin was essential for the utilisation of both groups of cofactors. This suggests that the metabolic block in strain 181/176 is at some stage in the direct pathway of methionine synthesis and not in the formation of a competent folic acid coenzyme.

Pteroylglutamic acids

Only reduced forms of folic acid served as cofactors for methionine synthesis. There was also strong evidence that PtH_2G_3 was activated by conversion to PtH_4G_3 . Tetrahydropteridine derivatives are in fact the only forms of folic acid which serve as coenzymes for all the purified enzymes of

DISCUSSION OF EXPERIMENTAL SECTION III

The Specificity of the Requirement for a Folic acid Cofactor in Methionine Synthesis

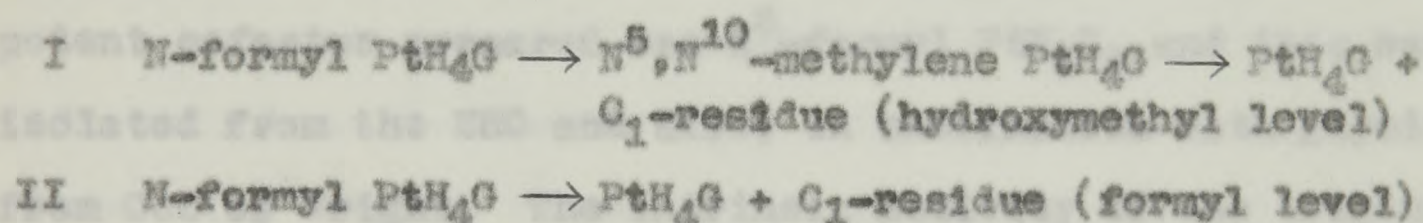
The compounds having folic acid cofactor activity fall into two distinct groups, the reduced pteroylmonoglutamic acids and the reduced pteroyltriglutamic acids. In order to exert their cofactor functions, the former were dependent upon the presence of added cobalamin. The latter group, like the cofactor of the EHC, were active and no requirement for the simultaneous metabolism of cobalamin was observed if E.coli PA15 was the source of the ultrasonic extract used for examining the synthesis of methionine. With ultrasonic extracts of the cobalamin auxotroph (strain 121/176) cobalamin was essential for the utilisation of both groups of cofactors. This suggests that the metabolic block in strain 121/176 is at some stage in the direct pathway of methionine synthesis and not in the formation of a competent folic acid coenzyme.

Pteroylmonoglutamic acids

Only reduced forms of folic acid served as cofactors for methionine synthesis. There was also strong evidence that PtH_2G was activated by conversion to PtH_4G . Tetrahydropteridine derivatives are in fact the only forms of folic acid which serve as coenzymes for all the purified enzymes of (see below). The work of Stollman (1957) and Blakley (1959, 1960)

C_1 -unit metabolism so far isolated (Rabinowitz, 1960). Enzymic reduction of PtG and PtH_2G to PtH_4G is probably responsible for the numerous observations with crude enzyme systems in which the more oxidised forms are active (e.g. Doctor, Patton & Awapara, 1957).

It is also possible that the formylated derivatives possessing cofactor activity were also converted to PtH_4G in methionine synthesis by extracts of E. coli and also in the animal system of Nakao & Greenberg (1958). Two pathways are available for such a conversion



Studies on the synthesis of methionine in the absence of serine suggest that the former mechanism (I) may occur with N^{10} -formyl PtH_4G and N^5, N^{10} -methenyl PtH_4G ; N^5 -formyl PtH_4G does not readily serve as a donor of C_1 -units possibly because it is at a lower energy level.

It has previously been assumed that folic acids with unsubstituted N^5 and N^{10} positions are required for hydroxymethyl group transfer. If however the N^5 , and N^{10} -formylated derivatives of PtH_4G have cofactor activity without prior conversion to PtH_4G this assumption will have to be modified (see below). The work of Kisliuk (1957) and Blakley (1959, 1960)

confirms that only pteridines possessing unsubstituted N^5 and N^{10} atoms readily form complexes with formaldehyde. However the conditions used in such experiments will certainly differ from those at the active centre of an enzyme.

Pteroyltriglutamic acids

Two of the seven components of CoC which served as cofactors for serine transhydroxymethylase in the system of Wright (1960) were oxidised derivatives (PtG_3 and N^{10} -formyl PtG_3). Only the reduced derivatives were active for methionine synthesis with extracts of *E. coli*. The most potent cofactor prepared was N^5 -formyl PtH_4G_3 and this was isolated from the EHC and also, in combination with glycine, from CoC by Wright. The intrinsic activity of the triglutamates was much greater than for the monoglutamates though it is surprising that the N^5 -formylated derivative of PtH_4G_3 had the greatest activity whereas the N^5 -formylated derivative of PtH_4G was the weakest cofactor form of PtH_4G . Even more unusual is the fact that the formaldehyde derivative of PtH_4G_3 would only serve as a donor of C_1 -units when cobalamin was present (and then only weakly). This suggests that the N^5, N^{10} -methylene derivative is not a normal intermediate with polyglutamyl forms of cofactor. It is possible that with the formaldehyde derivative of PtH_4G_3 as C_1 -donor a different mechanism of C_1 -transfer and reduction is adopted

and this may be similar to the mechanism used with mono-glutamyl cofactors and with enzymic extracts of strain 121/176, cobalamin being essential.

The extract of heated organisms

Analysis of the EHC showed that the folic acid cofactors present were mainly conjugated forms of Leuconostoc citrovorum factor (N^5 -formyl PtH_4G). The extract was very stable and its activity was not increased significantly by preparation under anaerobic conditions, which confirms the presence of a high percentage of relatively stable forms.

One component of the fractionated EHC was tentatively identified as N^5 -formyl PtH_4G_3 . The presence of conjugated forms of N^5 -formyl PtH_4G including the triglutamyl derivative has been demonstrated in a wide variety of biological materials e.g. plants (Iwai, Yoshida & Kondo, 1954); yeast and liver (Doctor & Couch, 1953; Winsten & Eigen, 1950), blood (Usdin, 1959), algae (Ericson, Widoff & Banhidi, 1953) and bacteria (Hakala & Welch, 1957). The enzymic conversion of PtG_3 to N^5 -formyl PtH_4G_3 has also been demonstrated with Bacillus subtilis and Streptococcus faecalis (Hakala & Welch, 1957; Zakrzewski & Nichol, 1955). Progress regarding these compounds has been limited by the methods available for their isolation; the use of anionic-exchange cellulose promises to accelerate research in this field.

The Inhibition of Methionine Formation
by Tetrahydropteroylglutamic acid

The function of natural folic acid cofactors and pteroyltriglutamic acid cofactors was inhibited by Pth_4G and those other monoglutamyl derivatives which were themselves potential cofactors in the presence of cobalamin. One possible reason is that Pth_4G , having a strong affinity for hydroxymethyl residues, may compete with the other cofactors and so remove the C_1 -units derived from serine or divert them into other metabolic channels. In the case of Pth_4G_3 , the inhibition by Pth_4G is of the competitive type.

In the presence of cobalamin or factor (X), Pth_4G is utilised as a cofactor although relatively high concentrations are required. The possible conversion of Pth_4G into a coenzyme form mediated by X has been mentioned previously. Following incubation, typical reaction mixtures containing Pth_4G and cobalamin or X have in fact been tested for the presence of a folic acid coenzyme before and after chromatographic removal of residual Pth_4G ; no evidence for such a conversion was obtained. Spectrophotometric investigations and experiments in which various combinations of the requirements for methionine synthesis were preincubated before completion of the reaction mixture also failed to yield conclusive evidence regarding the interrelationship between cobalamin and Pth_4G . In addition, the cobalamin

requirement for methionine synthesis using ultrasonic extracts of strain PA15 and PtH_4G as the cofactor for C_1 -transfer is similar to that for extracts of strain 121/176 with all sources of folic acid cofactor. Also, the extract of heated E. coli 121/176, obtained after growing the organism on a limiting concentration of cobalamin, contained over 600 times more cobalamin than a similar extract of strain PA15 (grown in the absence of the vitamin). It is therefore probable that cobalamin acts by providing an alternative pathway for the transfer and reduction of the hydroxymethyl group. The metabolic lesion of the cobalamin or methionine mutants of E. coli would then be at a step in the normal pathway of methionine synthesis and not in the synthesis of cobalamin or a folic acid coenzyme.

The Mechanism of Methionine Methyl Group Formation

Reduction of the hydroxymethyl group

The effect of aminopterin and amethopterin on the synthesis of methionine with either PtH_4G or EHC as the cofactor for C_1 -transfer (cobalamin present or absent) suggests that reduction of the hydroxymethyl group is not coupled to the oxidation of the folic acid cofactor as it appears to be in de novo synthesis of the methyl groups of

FIG. 32

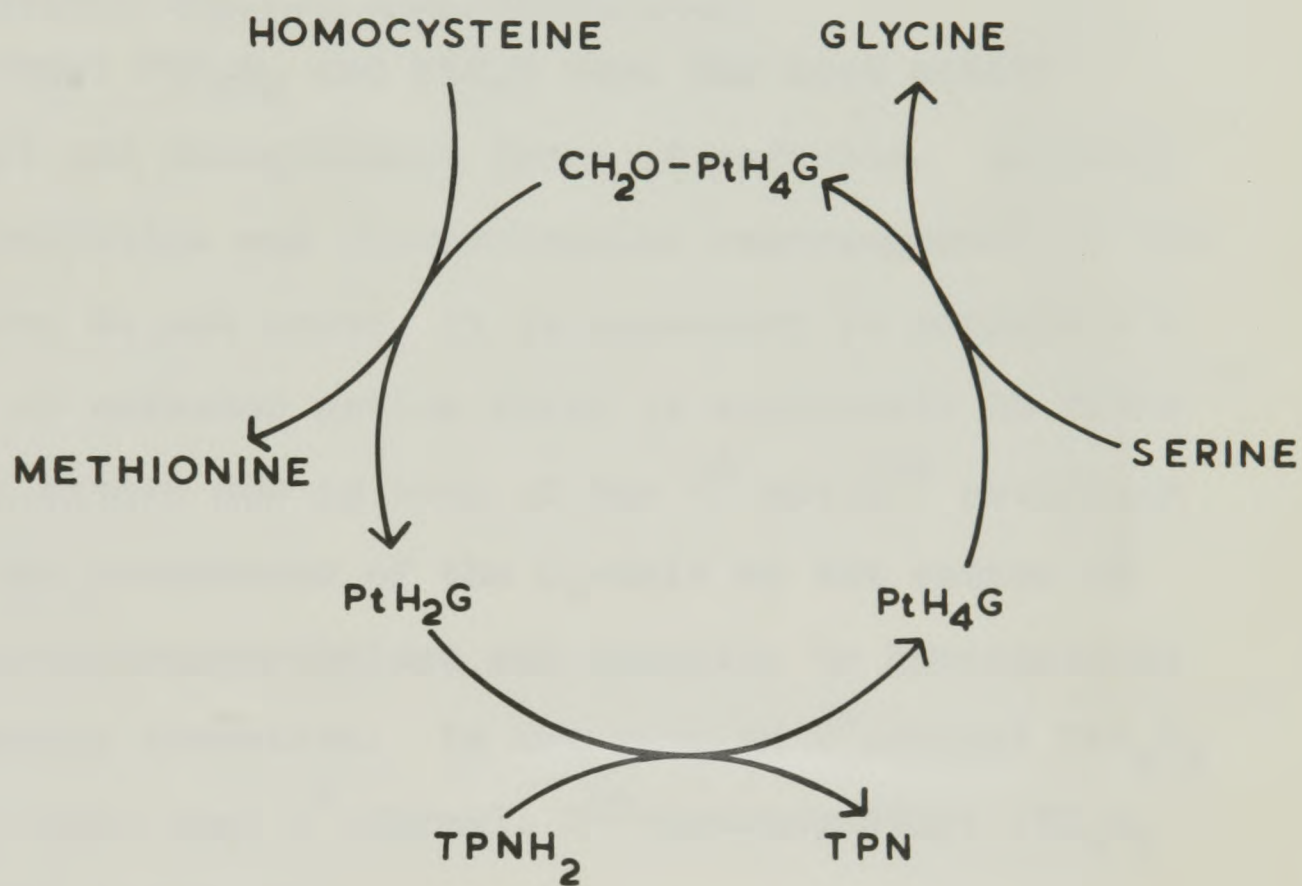
choline or thymidylic acid (Venkataraman & Greenberg, 1958; Humphreys & Greenberg, 1958). The mechanism of thymidylic acid synthesis has since been confirmed with enzymic extracts of S. faecalis; the reaction was inhibited by aminopterin when limiting concentrations of PtH_4G were used (McDougall & Blakley, 1960). The absence of a stoichiometric relationship between methionine formation and available reducing power prevented confirmation of the results obtained with the folic acid antagonists in methionine synthesis. However, the validity of the conclusion was supported by the finding that the dihydropteroylglutamic acid reductase of E. coli was pyridine nucleotide-linked and aminopterin sensitive, unlike the folic acid reductase of Clostridium sticklandii (Wright & Anderson, 1958). Mechanism B (Fig. 32) is therefore preferred to mechanism A, with DPNH_2 or reduced flavin nucleotides supplying the reducing power for methionine methyl group formation.

A difference in methyl group formation in the cases of methionine and thymidylic acid synthesis in E. coli PA15 is also indicated by the ability of PtH_4G to serve as cofactor for the incorporation the β -carbon atom of serine into thymidylic acid by extracts of this organism prepared by grinding with alumina (Birnie & Crosbie, 1958); no requirement for cobalamin or inhibition by PtH_4G was reported. Both the pathway of choline and thymidine methyl group biosynthesis

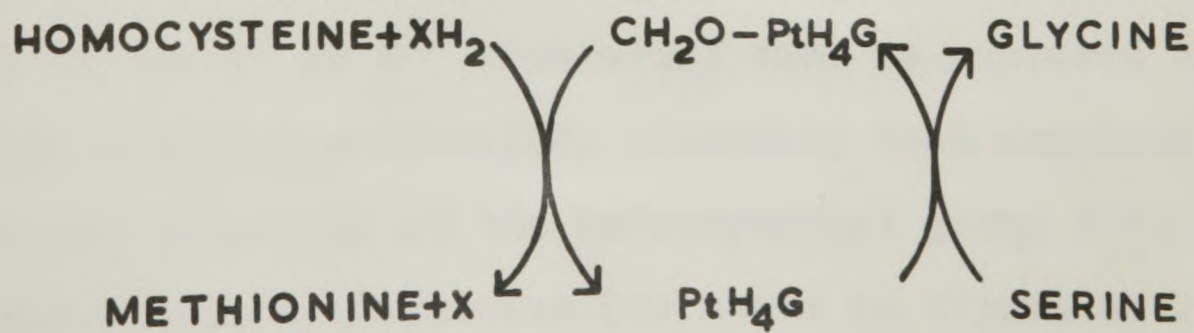
FIG. 32

Mechanism of methyl group formation

A



B



in animals and Lactobacilli (Venkataraman & Greenberg, 1958; Dinning & Young, 1959a,b) and the possible site of action of cobalamin therefore appear to differ from methionine methyl group synthesis in E.coli.

The folic acid cofactor and C₁-transfer

N⁵-formyl Pth₄G₃ and Pth₄G were the most active triglutamyl and monoglutamyl forms of cofactor. Assuming that deformylation and intramolecular rearrangement of the formyl group do not occur, it is necessary to postulate a mechanism of cofactor action which is applicable to folic acids with either one or both of the N⁵ and N¹⁰ positions free for the acceptance of the C₁-unit by the action of serine transhydroxymethylase and donation to homocysteine for methionine formation. In the case of N⁵-formyl Pth₄G₃ this would mean that N⁵-formyl, N¹⁰-hydroxymethyl Pth₄G₃ is the final C₁-donor.

For Pth₄G it has been proposed that the N⁵,N¹⁰-methylene bridge compound is the product of serine transhydroxymethylase activity and is also the final C₁-donor (Fig.5, page 32). The bond to the N⁵ or N¹⁰ atom would then be ruptured during the reaction with homocysteine. Assuming that complete transfer and reduction of the hydroxymethyl group follows the reaction with homocysteine (as shown in Figure 5) it now

would appear that the N^5 and N^{10} -hydroxymethyl derivatives and the N^5, N^{10} -bishydroxymethyl derivative may serve as the ultimate substrates. There is probably an equilibrium between the N^5, N^{10} -methylene, N^5 -hydroxymethyl, N^{10} -hydroxymethyl and N^5, N^{10} -bishydroxymethyl derivatives and between these and free formaldehyde and reduced folic acid, the relative proportions of each depending on physico-chemical factors. At the active centre of the enzyme concerned, interaction with the protein could stabilise any one of these structures for participation in methionine synthesis. All the three formylated derivatives of PtH_4G function in transformylation, and the same may be true at the hydroxymethyl level.

Cobalamin or factor (X), when required almost certainly function in the reductive transfer of the C_1 -unit but the nature of its interrelationship with the monoglutamyl derivatives of folic acid is unknown.

The Role of Cobalamin in the Biosynthesis of Methionine

The action of cobalamin in methionine biosynthesis is almost certainly mediated by the factor (X) which appears to be a protein complexed with cobalamin and may contain cobalamin or a derivative of cobalamin. The metabolic lesions of mutants of *E. coli* responding to either cobalamin or methionine has been assumed to be a block in the synthesis of cobalamin. The work presented in this thesis, however, suggests that the lesion is primarily due to a block in the biosynthesis of methionine.

CONCLUDING DISCUSSION

No positive evidence for the exact function of cobalamin or factor (X) has been obtained. During the course of this work, several working hypotheses have been formulated and it is proposed to discuss these and also to consider the role of cobalamin in methionine synthesis with respect to the other known functions of the vitamin.

Alternative pathways of methionine synthesis

The most plausible hypothesis, in agreement with all the experimental results, postulates the existence of two pathways for the transfer and reduction of the C₁-unit in the formation of methionine (Fig. 25). One of these is independent of cobalamin and presumably operates in pro-

The Role of Cobalamin in the Synthesis of Methionine

The action of cobalamin in methionine synthesis is almost certainly mediated by the factor (X) which appears to be a protein (possibly an enzyme) and may contain cobalamin or a derivative of cobalamin. The metabolic lesion of mutants of E.coli responding to either cobalamin or methionine has been assumed to be a block in the synthesis of cobalamin. The work presented in this thesis however, suggests that the lesion is primarily due to a block in the biosynthesis of methionine.

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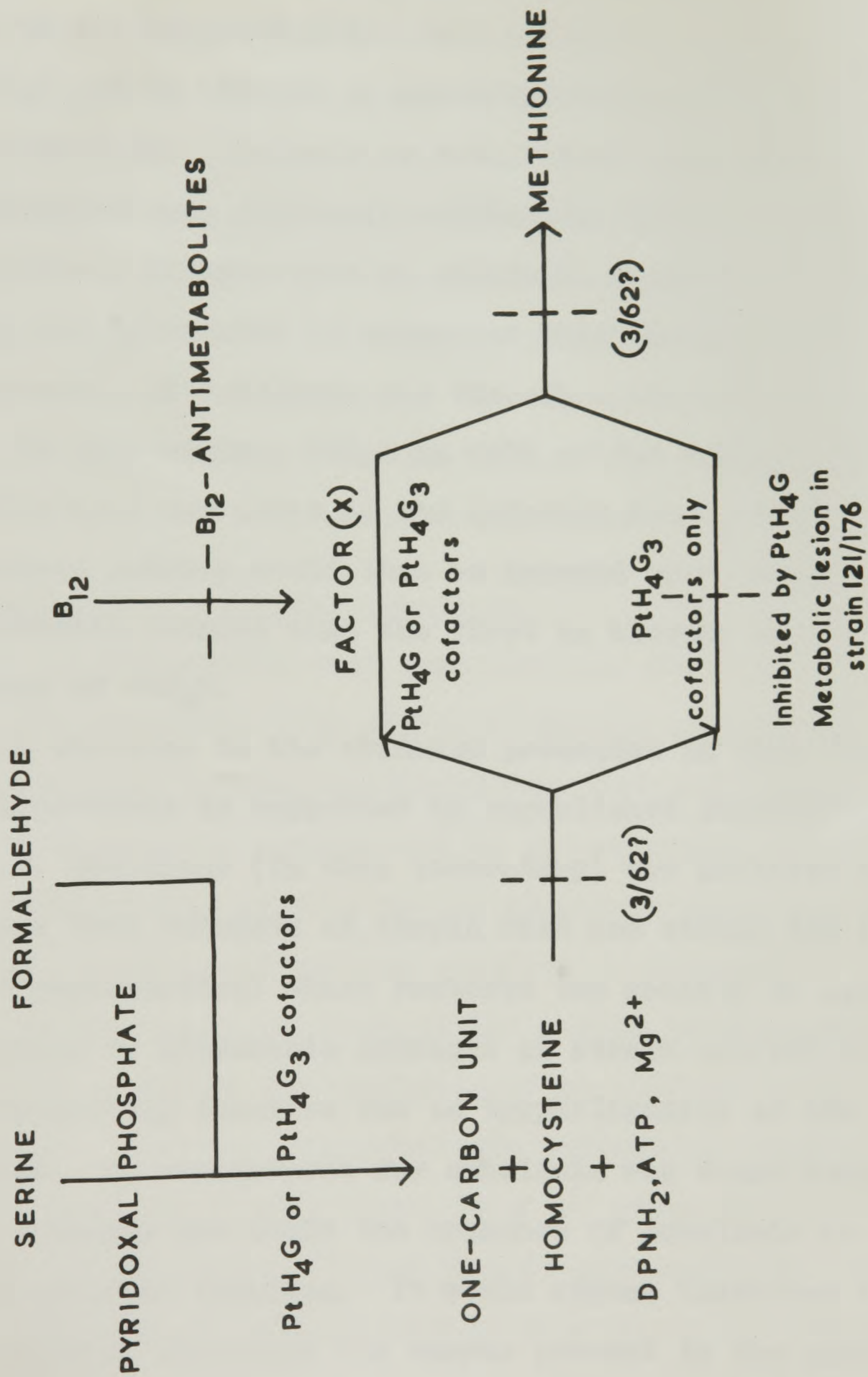


FIG. 33

Alternate pathways of homocysteine methylation in *Escherichia coli* PA15

trophic strains and possibly in higher plants. This pathway requires a conjugated folic acid cofactor, it is inhibited by PtH_4G and is blocked in auxotrophic strains having a requirement for cobalamin or methionine. The second pathway is dependent upon cobalamin metabolism (factor X formation). This pathway is operative in cobalamin-requiring strains of E.coli and presumably in organisms which have been grown in the presence of cobalamin and therefore contain the factor (X). In this system, PtH_4G as well as the conjugated forms of folic acid can serve as the cofactor for C_1 -transfer. The second pathway would then be imposed upon extracts of prototrophic strains when the first is blocked by the presence of PtH_4G .

In addition to the evidence presented in this thesis, this hypothesis is supported by unpublished observations of Dr. C.W. Helleiner (in this laboratory) who isolated a fraction from extracts of strain PA15 and strain 518 (with a 6-fold purification) which restored the ability to synthesise methionine to ultrasonic extracts of strain 121/176 which were relatively inactive due to lyophilisation of the organism. No requirement for cobalamin was found with these mixed extracts nor could the presence of cobalamin be detected in the purified fraction. It would appear therefore that this fraction contained the enzyme present in the prototrophs but missing from the cobalamin requiring auxotroph.

Strain 3/62, which responds only to methionine, formed factor (X) during growth (Dr. K.M. Jones, unpublished observations) or by incubation of the ultrasonic extract with cobalamin. It would seem that this organism is blocked at some other reaction in the conversion of homocysteine to methionine (Fig.33). Investigation of other mutants blocked at the methylation of homocysteine would probably yield further information concerning the sequences of reactions in the biosynthetic pathway and it may also provide^a useful assay for specific enzymes concerned therein.

Increased requirement for cobalamin

It is conceivable that cobalamin may function in methionine formation (via factor X) by the prototrophic strains as well as the auxotrophs and that sufficient of the vitamin is normally synthesised by the organism to satisfy this requirement. If this is so, an enzyme concerned in the function of cobalamin must be modified in the mutants so that more cobalamin and more of the enzyme protein are required to promote the synthesis of an adequate supply of methionine. However, the cobalamin content of strain PA15 was extremely small and, there is no evidence for the presence of the factor (X) in prototrophic strains. The chief disadvantage to this view is to explain how PtH_4O can

be an inhibitor and a cofactor in the same reaction mechanism; some other function of cobalamin in the metabolism of Pth_4G would have to be postulated.

The formation of a folic acid coenzyme

Some of the evidence obtained suggested that cobalamin (or X) functions in the formation of folic acid coenzymes in E.coli 121/176 and that it promotes the conversion of Pth_4G into a coenzyme form with extracts of strain PA15. However, the results of a considerable number of experiments suggest that this is not the case.

- a) Preparations of EHC from strains PA15 and 121/176 (cobalamin or methionine-grown) were equivalent with respect to their properties as folic acid cofactors for methionine synthesis when tested with the enzymic extracts from either strain. Furthermore, the addition of the anilide analogue of cobalamin to the test systems had no effect. It is concluded that the EHC of all strains (even cobalamin-deficient strains) contain competent folic acid coenzymes.
- b) Conjugated forms of folic acid isolated from the EHC or synthesised chemically, and therefore free from contamination by cobalamin, were active cofactors for the synthesis of methionine by extracts of strain PA15 and no cobalamin was required. The same compounds were inactive with extracts of

strain 121/176 unless cobalamin was also added.

c) The activity of a coenzyme form of folic acid synthesised from PtH_4G would be inhibited by residual PtH_4G unless the conversion were complete or unless the coenzyme, unlike the natural compounds, was resistant to the inhibition.

d) Direct attempts to demonstrate or isolate a coenzyme as the product of the interaction of cobalamin (or X) and PtH_4G were unsuccessful.

Comparison of the Function of Cobalamin in Methionine Synthesis with the other known Functions of Cobalamin

Protein Synthesis

It is possible that cobalamin promotes the synthesis or modification of a protein (factor X) which is specifically required for the utilisation of PtH_4G . This would probably mean that the factor (X) is not itself a cobalamin derivative. The conditions in which the factor (X) can be formed, though crude, would not appear to be optimal for protein synthesis.

Amino acid activation

Cobalamin could be concerned with the activation of homocysteine to form homocysteine adenylate which might be the actual acceptor of the C_1 -unit. However the cobalamin

requirement is particularly related to the utilisation of PtH_4G (with strain PA15) so homocysteine metabolism could not be the only function of cobalamin. The interrelationship between cobalamin and homocysteine could not be studied satisfactorily because homocysteine in the absence of PtH_4G (but not in its presence) inhibited the subsequent synthesis of methionine when it was preincubated with the enzymic extracts.

Cobamide-coenzyme functions

The molecular rearrangement of methylmalonyl CoA and formation of succinyl CoA (see Fig.4, page 24) occurs by transcarboxylation with the participation of a molecule of propionyl CoA. It has been suggested that glutamic acid could be converted to β -methylaspartic acid by transfer of a glycine residue to propionic acid in a similar manner, the latter being regenerated at each transfer (E.L.Smith, private communication). It was then suggested that the labile propionamide side chains of cobalamin (see Fig.1, page 2) or cobamide coenzymes could function as intermediate carriers in these reactions. That the most active antimetabolites of cobalamin have substituted propionamide side-chains also adds weight to such an hypothesis. Cobalamin derivatives may therefore function in the transfer of a one-carbon residue from a folic acid cofactor to homocysteine in some similar

manner; it is however difficult to envisage the precise mechanism by which this could be accomplished.

Alternatively the relationship between cobalamin and glutamic acid metabolism suggests that cobalamin may be concerned in the replacement of the glutamyl residues of folic acid cofactors by β -methylasspartyl residues. Experimentally the presence of β -methylasspartic acid during growth or in the reaction mixture for methionine synthesis had no effect on the requirement for cobalamin.

Oxidation-reduction

The structural similarity between cobalamin and the metalloporphyrins has suggested a role in oxidation-reduction reactions. The cobamide coenzymes probably contain a divalent cobalt atom compared with the trivalent cobalt of cobalamin (Smith, private communication; Pawelkiewicz, Bartosinski & Walerych, 1960). It may be significant that reducing power was essential for the formation of X from cobalamin. Alternatively a protein-bound derivative of cobalamin having a divalent cobalt atom (analagous to a reduced cytochrome) might transfer electrons from DPNH_2 or reduced flavin nucleotides to the hypothetical complex of homocysteine and hydroxymethyl- PtH_4G (Fig.5, page 32) and so bring about a reductive cleavage liberating methionine and PtH_4G . This mechanism might be used by strain 121/176 and by

strain PA15 when grown with cobalamin present, or by extracts of strain PA15 when PtH_4G was supplied as the cofactor for C_1 -transfer. An alternative mechanism would be required when conjugated forms of folic acid were used as cofactors with extracts of strain PA15.

The reduction of formate has also been suggested as the site of action of cobalamin in methyl group biosynthesis in Lactobacilli and bone marrow (Dinning & Young, 1959a,b; Dinning & Hogan, 1960). Cobalamin was required for the synthesis of methionine from homocysteine and formate or formylated derivatives of PtH_4G , but only when PtH_4G was the folic acid cofactor (strain PA15) and the conversion was very much less than obtained when formaldehyde or serine was the C_1 -donor. It seems unlikely that there is a formylated intermediate in methionine synthesis, though a general action of cobalamin in the interconversion of C_1 -units between different states of oxidation is quite possible.

The participation of cobalamin in the reduction of sulphhydryl groups supports a role in oxidation-reduction reactions. Dubnoff and Phillips (1959) have isolated factors from Propionibacterium freundenrichii which activate sulphhydryl compounds including homocysteine and these now appear to be analogous to the cobamide coenzymes.

Thus it can be seen there are many possible ways in which cobalamin could function and more may be discovered.

However at this stage of our knowledge no decision can be made regarding its precise function in methionine methyl group formation.

The Pathway of Methionine Biosynthesis

The methylation of homocysteine in Escherichia coli is apparently the result of a complex series of reactions. The work described with crude enzymic extracts has firmly established the requirement for a folic acid cofactor and in certain circumstances products of the metabolism of cobalamin. No intermediate derivatives of homocysteine have been found. Stekol (1955) has suggested that S-hydroxymethyl-homocysteine is an intermediate; the formation of the same compound from homocysteine and hydroxymethyl PtH_4O has also been postulated by Doctor, Patton and Awapara (1957). Many attempts to prepare this compound have been made in this laboratory but these were unsuccessful because at neutral pH it undergoes spontaneous cyclisation to form 1,3-thiazane-4-carboxylic acid and at slightly acid pH it condenses with another molecule of homocysteine to form homodjenkolic acid.

Evidence for S-adenosylmethionine (Nakao & Greenberg, 1958), S-adenosylhomocysteine and methylmethionine sulphonium (Stevens & Sakami, 1959) as intermediates have been presented. It is possible that the S-hydroxymethyl derivative of homo-

cysteine, S-adenosylhomocysteine or homocysteine adenyate could be an intermediate particularly if it was stabilised by interaction with the enzyme protein. The nature of the C_1 -donor, the reduction of the hydroxymethyl group and the role of cobalamin have been discussed previously. It would seem that further elucidation of this reaction will depend upon the fractionation of the enzymic extracts (possibly using several different strains of E.coli) and the isolation and characterisation of the steps involved. The requirements for methionine synthesis have now been defined in relatively simple terms; EHC can be replaced by synthetic polyglutamyl cofactors or by PtH_4G and purified factor (X), and the products of glucose metabolism can be replaced by $DPNH_2$ (and possibly ATP). A successful fractionation should therefore be possible in the future.

APPENDIX 1

Chemicals

The sources of some of the important special chemicals not given in the text were as follows.

Coenzymes and Substrates

DPN, DPNH₂, TPN and TPNH₂ - sodium tetrahydrate salts; 90-100% pure. (Sigma Chemical Corp.).

Pyridoxal-5-phosphate - (Roche Products Ltd.).

ATP, ADP, AMP - sodium trihydrate salts; 98-100% pure. (Sigma Chemical Corp.).

DL-Homocysteine - (Nutritional Biochemical Corp. and Mann Research Laboratories, Inc.). Solutions were prepared immediately before use.

S-adenosyl-L-homocysteine - gift from Dr. W. Sakami.

Fructose-1,6-diphosphate - dibarium salt (British Drug Houses, Ltd.). The sodium salt was prepared in solution by the addition of an equivalent amount of Na₂SO₄ to precipitate all the barium as BaSO₄.

6-Methylaspartic acid - gift from Dr. H.A. Barker.

5,6-Dimethylbenziminazole - gift from Dr. J. Lascelles.

N-formylglycine and formimino-glycine - were prepared by Mr. R.W. Wakelin.

Protamine sulphate - from herring roe. (L. Light & Co., Ltd.).

Derivatives of Folic Acid

Pteroylglutamic acid (PtG) - (British Drug Houses, Ltd.).

N¹⁰-methyl PtG - gift from Dr. E.L.R. Stokstad.

Leucovorin (N⁵-formyl PTH₄G) and anhydroleucovorin (N⁵,N¹⁰-

methenyl PTH₄G) - were gifts from Dr. T.H. Jukes

(Lederle laboratories).

Aminopterin, Amethopterin, Pteroyl- α -glutamylglutamic acid,

pteroyl- γ -glutamyl- γ -glutamylglutamic acid and

Fermentation L.casei factor - (Lederle Laboratories

Division of the American Cyanamid Co.).

N¹⁰-formyl Pt (rhizopterin) - (Merck and Co., Inc.).

2-Amino, 4,6-dihydroxypteridine (xanthopterin), 2-amino,

4-hydroxy, 6-methylpteridine and 2-amino, 4-hydroxypteridine-

6-aldehyde - gifts of Professor A. Albert.

Anion-exchange Resins

Dowex-1 resin - Analytical Grade 1-3X, 100-200 Mesh,

chloride form. (Bio-Rad Laboratories).

TEAE-cellulose - capacity 0.6 mequivalents/g. (Serva

Research Laboratories).

Medium S₂ - tryptone glucose broth

As medium S₂, with addition of CaCO₃ and urea, the pH being adjusted from pH 6.7 to 8.2.

Composite solutions, see page

APPENDIX 2

Medium S₁ - solidMEDIA AND COMPOSITE SOLUTIONSCrude MediaMedium S₁ - tryptic meat agar

Tryptic meat broth [#]	500ml.
Agar (BDH)	20g.
Water	500ml.
pH adjusted to	7.6

Procedure: agar melted by steaming with the water, the tryptic meat broth also heated and mixed with the agar. Distributed, autoclaved 15 lb.sq.in./10 min. and sloped.

Medium S₂ - tryptose glucose agar

Bacto tryptose (Difco)	10g.
Glucose	10g.
K ₂ HPO ₄	2g.
CaCO ₃	3g.
Salts solution (mixture B)	5ml.
16% liver extract (Oxoid)	10ml.
Agar (BDH)	15g.
Water to	1000ml.

Procedure: dissolved by steaming, mixed and distributed. Autoclaved 15 lb.sq.in./10 min. Solidified. Stabs.

Medium S₃ - tryptose glucose broth

As medium S₂, with omission of CaCO₃ and agar, the pH being adjusted from pH 6.7 to 6.9.

[#] Composite solutions, see page xi.

Medium S₄ - solid medium for slime-moulds and protozoa

(Raper, 1951)

Peptone (Evans)	1.0g.
Yeast extract (Difco)	1.0g.
Glucose	2.5g.
Water	500ml.
Agar (BDH)	7.5ml.

Procedure: dissolved by steaming, mixed, distributed and autoclaved 15 lb.sq.in./10 min. and sloped.

Medium B₁ - glucose and lactate maintenance medium for *Leishmania* of Cross (1966). pH 7.0 strength (pH 7.0) contains:

K ₂ HPO ₄	2.0g.
KH ₂ PO ₄	4.0g.
(NH ₄) ₂ SO ₄	1.0g.
NH ₄ Cl	2.0g.
Glucose	2.0g.
Lactic acid (80% soln. adjusted)	2.0ml.
Iron citrate (1.0000g)	1.0ml.
NaOH (40% soln.)	10.0ml.

Procedure: dissolved in water to 500ml. and pH adjusted.

Medium B₂ - modification of medium B₁ after C.V. Hollander contains all constituents at twice the concentration.

v

Synthetic and Semi-synthetic Media

Medium A - minimal medium for E.coli (Davis & Mingioli, 1950).

500ml. double strength (pH 7.0) contains,

K_2HPO_4	7.0g.
KH_2PO_4	3.0g.
$(NH_4)_2SO_4$	1.0g.
Sodium citrate. $2H_2O$	0.5g.
$MgSO_4 \cdot 7H_2O$	0.1g.
Glucose	2.0g.

Procedure: dissolved in 500ml. water and pH checked.

Medium B₁ - glucose and lactate containing medium for E.coli

of Cross (1956). 500ml. double strength (pH 7.0) contains,

K_2HPO_4	9.6g.
KH_2PO_4	4.4g.
$(NH_4)_2SO_4$	1.0g.
NH_4Cl	2.5g.
Glucose	2.0g.
Lactic acid (BDH. 88% reagent)	9.0ml.
Iron citrate (0.004M) ^W	1.0ml.
NaOH (40% soln.)	10.0ml.

Procedure: dissolved in water to 500ml. and pH adjusted.

Medium B₂ - modification of medium B₁ after C.W.Helleiner

contains all constituents at twice the concentration.

Salts	2.0g.
Riboflavin	0.5mg.
Nicotinic acid	1.0mg.
Calcium pantothenate	1.0mg.

Medium C - for the assay of L-methionine using Leuconostoc mesenteroides P60. modified from Steele et al. (1949).

500ml. of double strength medium contains,

<u>DL</u> -alanine	500mg.
<u>L</u> -arginine.HCl	200mg.
<u>DL</u> -aspartic acid	200mg.
<u>L</u> -asparagine	400mg.
<u>L</u> -cysteine.HCl	170mg.
<u>DL</u> -glutamic acid	600mg.
Glycine	200mg.
<u>L</u> -histidine.HCl	60mg.
<u>DL</u> -isoleucine	240mg.
<u>DL</u> -leucine	240mg.
<u>DL</u> -lysine.2HCl	120mg.
<u>DL</u> -phenylalanine	100mg.
<u>DL</u> -proline	50mg.
<u>DL</u> -serine	300mg.
<u>DL</u> -threonine	60mg.
<u>DL</u> -tryptophan	40mg.
<u>DL</u> -tyrosine	200mg.
<u>DL</u> -valine	240mg.
Glucose	20g.
Sodium acetate.3H ₂ O	20g.
Ammonium chloride	3g.
KH ₂ PO ₄	600mg.
Na ₂ HPO ₄ . 12H ₂ O	600mg.
Adenine	10mg.
Guanine	10mg.
Xanthine	12mg.
Uracil	12mg.
Salts B ^M	10ml.
Riboflavin	0.5mg.
Nicotinic acid	1.0mg.
Calcium pantothenate	1.0mg.

Thiamine.HCl	1.0mg.
Pyridoxine.HCl	1.0mg.
Biotin	1 µg.
p-Aminobenzoic acid	0.34mg.

Procedure: amino-acids dissolved in 80ml. 1N-NaOH and pH adjusted to 6-7 with 1N-HCl. Water added to 250ml. and other ingredients except vitamins dissolved by warming. Volume brought to about 500ml. with water and adjusted to pH 6.8. Medium brought to boil and simmered for 3 min. After cooling the medium was filtered through Whatman No.50 paper and the vitamins added.

Biotin	10 µg.
Iron citrate (0.004g) ¹⁰	1.5ml.
Acid hydrolyzed casein (10%) ¹⁰	5ml.

Procedure: dissolved in less than 500ml. water, adjusted to pH 4.5 and brought to 500ml.

Medium D -- for the assay of cobalamin using Euglena gracilis (Lacelles, quoted by Kisliuk & Woods, 1960). 500ml. double strength (pH 4.5) contains,

K_2HPO_4	0.5g.
KH_2PO_4	0.5g.
$(NH_4)_2PO_4$	0.8g.
$MgSO_4 \cdot 7H_2O$	0.2g.
$CaCl_2$	40mg.
L-glutamic acid (monosodium salt)	3.7g.
Malic acid	2.7g.
Thiamine.HCl	1.0mg.
Nicotinic acid	1.0mg.
Biotin	10 μ g.
Iron citrate (0.004M) ²	1.3ml.
Acid hydrolysed casein (10%) ²	5ml.

Procedure: dissolved in less than 500ml. water, adjusted to pH 4.5 and brought to 500ml.

Procedure: all ingredients except vitamins dissolved, adjusted to pH 4.5 and brought to boiling point, stirred 2-3 min. and cooled. Vitamins added and made to 700ml. with water, pH checked and solution filtered through No. 50 paper.

Medium E - for the assay of folic acids using Lactobacillus casei, Leuconostoc citrovorum and Streptococcus faecalis R.

Modified from Teennies, Frank & Gallant (1952). 700ml.

medium contains,

Tryptic digest of casein [®]	500ml.
DL-alanine	0.3g.
L-asparagine	0.1g.
L-cystine	0.2g.
Glucose	20g.
Adenine	10mg.
Guanine	10mg.
Xanthine	10mg.
Uracil	10mg.
Salts B [®]	5ml.
Ascorbic acid	250mg.
Riboflavin	0.5mg.
Nicotinic acid	0.5mg.
Calcium pantothenate	0.5mg.
Thiamine.HCl	0.5mg.
Pyridoxamine.HCl	0.2mg.
Biotin	10 µg.
p-Aminobenzoic acid	0.1mg.

Procedure: all ingredients except vitamins dissolved, adjusted to pH 6.5 and brought to boiling point, simmered 2-3 min. and cooled. Vitamins added and made to 700ml. with water, pH checked and medium filtered through Whatman No.50 paper.

Medium F - for the assay of folic acids with Tetrahymena
vorax, medium of Lascelles(1957) omitting pteroylglutamic
 acid and thiocetic acid, 500ml. double strength contains,

Acid Hydrolysed casein (10%) [*]	250ml.
L-cysteine.HCl	100mg.
DL-tryptophan	140mg.
Glucose	3.6g.
Na ₂ HPO ₄ .12H ₂ O	1.4g.
KH ₂ PO ₄	540mg.
MgSO ₄ .7H ₂ O	100mg.
MnSO ₄ .4H ₂ O	1.0mg.
Adenine	10mg.
Guanine	10mg.
Uracil	10mg.
Tween 80	0.4ml.
Riboflavin	0.5mg.
Nicotinic acid	1.0mg.
Calcium pantothenate	1.0mg.
Thiamine.HCl	1.0mg.
Biotin	1.0 µg.

Procedure: all ingredients except the vitamins dissolved
 with acid hydrolysed casein in 400ml. water by heating at
 pH 6.8. Boiled momentarily, cooled and filtered. Vitamins
 added and made to 500ml. with water.

^{*} Composite solutions, see page xi.

Composite Solutions

Tryptic meat broth. Bullocks' hearts were defatted, minced, weighed and 1.1 times their weight of hot tap water added. The mixture was brought to 80° and maintained at this temperature and at pH 8 for 30 min. After cooling to 45°, pancreatic extract was added (0.1 volume of water used) and the mixture stirred for 4 hr. at this temperature. The pH was then adjusted to 6 and temperature raised to 95°. Following 1hr. steaming in an autoclave, the mixture was filtered through muslin and Green's FP904¹ while still hot, then cooled and pH adjusted to 7.6. Storage was at room temperature in the dark over CHCl₃.

Acid hydrolyzed casein (10%). Vitamin free, prepared according to the method of Snell & Rannefeld (1945).

Tryptic digest of casein (Lascelles, 1951). 100g. Glaxo vitamin free casein was suspended evenly in 1400ml. 0.8% sodium bicarbonate solution, which had been warmed to 40°. A suspension of 1g. Difco trypsin in 20ml. water and 5ml. toluene were added. The mixture was shaken, and then incubated at 37° for 18-20hr. with occasional shaking.

The mixture was steamed in an autoclave for 40 min. to remove toluene, and cooled. The pH was brought to 6.6 with 4.4ml. glacial acetic acid and the undigested casein

removed by centrifuging. The supernatant was then brought to pH 3.8 with 54ml. of acetic acid and 40g. of "Norite" added with frequent shaking for 30 min. The suspension was filtered as quickly as possible through two layers of Whatman No.54 paper with suction. The residue on the paper was washed twice with 20ml. water. 20g. "Norite" was added to the filtrate and the mixture shaken frequently. Again after 30 min. the suspension was filtered as quickly as possible through a Ford's Sterimat S/GS/22 filter mat. 25g. KH_2PO_4 was added and 40g. NaOH. The pH was adjusted to 6.8, and the volume of the digest was made up to 2l. with water.

Salts mixture B

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	20g.
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	1g.
NaCl	1g.
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1g.

Dissolved in 200ml. water containing 0.5ml. conc. HCl aq. Volume made up to 500ml.

Iron citrate (0.004M)

$(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$	504mg.
Sodium citrate. $2\text{H}_2\text{O}$	708mg.
Water	500ml.

Tetrahymena strain VB, from Dr. V. Dreyer.

Maintained on mist slope of Sodium H_2 , inoculated 7th. at 20° and stored at room temperature.

APPENDIX 3ORGANISMSEscherichia coli

The growth factor requirements and sources of the strains used in this work are given in Table I.1, page 45. All strains were maintained on slope cultures of medium S₁, incubated 18hr. at 37°.

Organisms used in Microbiological Assays

Lactobacillus casei (helveticus). A.T.C.C. No.7469, obtained from Dr. B. Wright.

Leuconostoc mesenteroides (Streptococcus equinus) P60.

A.T.C.C. No.7881.

Leuconostoc citrovorum (Pediococcus cerevisiae). A.T.C.C. No.8081.

Streptococcus faecalis R. A.T.C.C. No.8043, the Rogers strain.

The above four strains were maintained as stab cultures in Medium S₂, incubated 18hr. at 37°.

Euglena gracilis var bacillaris. from Dr. J. Lascelles.

Maintained on a moist slope of Medium S₄, incubated 72hr. at 30°, 36cm. distant from two 60 W tungsten filament lamps. Stored at room temperature in daylight.

Tetrahymena vorax strain VE, from Dr. V. Dewey.

Maintained on moist slopes of Medium S₄, incubated 72hr. at 30° and stored at room temperature.

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INTRODUCTION

Nutritional and isotopic tracer studies with both animals and micro-organisms have shown that vitamin B₁₂ and folic acid are concerned in the biosynthesis of methionine from homocysteine and a source of C₁-units (e.g. serine or formaldehyde).

Using washed suspensions of E.coli serine has been found to be the most important C₁-donor and requirements for p-aminobenzoic acid and cobalamin were also demonstrated with the corresponding auxotrophic strains.

Soluble extracts of acetone-dried E.coli PA15 (a serine or glycine requiring strain) catalysed the formation of methionine from serine and homocysteine when an extract of the heated organism (EHC) served as the source of folic acid cofactor; no requirement for cobalamin was observed. However replacement of the EHC by tetrahydropteroylglutamic acid (PtH₄G) was not possible unless the enzymic extract was prepared from acetone-dried powders of organisms which had first been grown in the presence of added cobalamin. A heat-labile, non-diffusible factor (X) was partially-purified from the extract of cobalamin-grown organisms and this factor (X) was essential for the utilisation of PtH₄G as a cofactor for the synthesis of methionine by extracts of acetone-dried organisms grown without added cobalamin.

The synthesis of methionine by ultrasonic extracts of E.coli 121/176 (a cobalamin or methionine requiring strain, grown with methionine) was on the other hand dependent upon the presence of cobalamin in the reaction mixture with either EHC or Pth_4G as the cofactor for C_1 -transfer.

The object of this work was to investigate further the complexities of the methylation of homocysteine by ultrasonic extracts of Escherichia coli with particular regard to the functions of folic acid and cobalamin.

EXPERIMENTAL RESULTS

Section I The Synthesis of Methionine by Ultrasonic extracts of Escherichia coli

A preliminary survey of methionine formation by extracts of several strains of E.coli was made using three test systems: (I) serine as the C_1 -donor and EHC as the source of the folic acid cofactor, (II) serine as C_1 -donor and Pth_4G as the cofactor for C_1 -transfer and (III) the formaldehyde derivative of Pth_4G ($\text{CH}_2\text{O-Pth}_4\text{G}$) as both the donor and cofactor for the transfer of C_1 -units. Cobalamin was necessary for optimal synthesis of methionine under all conditions when extracts of cobalamin or methionine requiring strains (E.coli 121/176, 113/3) were used. With strains having no nutritional requirement for cobalamin or

methionine (E.coli PA15, W. 518) cobalamin was essential under conditions II and III i.e. only with Pth_4G or $\text{CH}_2\text{O-Pth}_4\text{G}$ present.

Quantitatively the requirement for cobalamin was similar for all cobalamin-dependent conditions with extracts of strain PA15 and 121/176. Cobalamin could be replaced by several of its nucleotide-substituted analogues but not by derivatives lacking the nucleotide residue. None of these compounds was significantly more active than cobalamin itself though 5,6-dimethylbenzimidazolylcobamide coenzyme was 2 to 3 times more active than cobalamin for promoting the synthesis of methionine. Growth in the presence of cobalamin enhanced the ability to form methionine of ultrasonic extracts of strain 121/176 and PA15 and the requirement for cobalamin in the reaction mixture (strain 121/176, system I and II; strain PA15, system II) was eliminated if sufficiently high concentrations were present in the growth medium.

In addition to the inability of Pth_4G to replace EHC in the absence of cobalamin (strain PA15), Pth_4G also inhibited methionine synthesis when EHC supplied the cofactor for C_1 -transfer. This inhibition was overcome by cobalamin which also permits the Pth_4G to function as a cofactor.

The synthesis of methionine by ultrasonic extracts of

E. coli PA15 was proportional to the amount of extract present. Optimal synthesis occurred at 37° in phosphate buffer with an initial pH of 7.6 to 7.8 under anaerobic conditions. Glucose, DPN, ATP and Mg^{2+} were also essential for optimal methionine formation although glucose and DPN could be replaced by $DPNH_2$. S-adenosyl-L-homocysteine replaced homocysteine but ATP was still required. Serine and CH_2O-Pth_4G were the most active C_1 -donors and with the former a requirement for pyridoxal phosphate was demonstrated. This was presumably for the intermediate functioning of serine transhydroxymethylase in methionine synthesis. More direct evidence for the presence of this enzyme in ultrasonic extracts was also obtained. Growth of organisms in the presence of increasing concentrations of methionine progressively repressed their ability to form methionine in cell-free extracts.

Section II The Function of Cobalamin in the Synthesis of Methionine

Inhibitory analogues of cobalamin

Under conditions where cobalamin was essential for the synthesis of methionine (strain 121/176, systems I, II & III; strain PA15, systems II & III) its action was inhibited by the methylamide, ethylamide and anilide of the monocarboxylic acids of cobalamin and by Factor B. Furthermore

the anilide analogue inhibited competitively both the action of cobalamin and the cobamide coenzyme but was less effective against the latter.

The anilide analogue did not inhibit the growth of prototrophic strains of E.coli and only inhibited the growth of cobalamin or methionine requiring auxotrophs when cobalamin was the growth factor. If extracts were prepared from organisms (strain PA15) which were first grown in the presence of cobalamin, the anilide analogue inhibited methionine formation only if it was added to the growth medium together with cobalamin.

The synthesis of factor (X) by ultrasonic extracts

A comparison of the different activities of ultrasonic extracts and extracts of acetone-dried E.coli PA15 suggested that in the presence of cobalamin the ultrasonic extract was capable of effecting the formation (in vitro) of the factor (X) which had previously been purified from cobalamin-grown organisms and was required for the utilisation of PtH_4O as a cofactor for the synthesis of methionine by extracts of acetone-dried organisms (Kisliuk & Woods). This system for the formation of X was presumably destroyed by treatment with acetone.

A method using protamine sulphate was devised for the isolation of factor (X) from the products of incubating

ultrasonic extracts of E.coli PA15 with cobalamin and the material was assayed by its ability to promote the synthesis of methionine by extracts of acetone-dried organisms incapable of utilising Pth_4G as a cofactor. The isolated factor (X) was heat-labile, non-diffusible, unstable in acidic and alkaline environments and resembled closely the material defined by Kisliuk and Woods.

The mono-substituted cobalamin-amide analogues inhibited the formation of X by ultrasonic extracts but had no effect on its function in methionine synthesis. Factor B however inhibited both the formation and function of X.

A more convenient method for assaying the formation of X was developed using the anilide analogue of cobalamin. Cobalamin, glucose, DPN, ATP and Mg^{2+} were necessary for the optimal formation of X though DPNH_2 would replace glucose and DPN; the requirements for X formation were unchanged if cobalamin was replaced by the cobamide coenzyme. The preparation of X formed with the cobamide coenzyme had the same properties as that formed with cobalamin by strain PA15. The material formed by incubating cobalamin with ultrasonic extracts of the cobalamin auxotroph E.coli 121/176 also had similar properties.

Section III The Function of Folic Acid in the Synthesis of Methionine

Pteroylmonoglutamic acids

Several reduced pteroylmonoglutamic acid derivatives were active as C_1 -donors and cofactors for the synthesis of methionine but as with PtH_4G cobalamin was an essential component of the reaction mixture. The same reduced pteroylmonoglutamic acids, also like PtH_4G , inhibited the synthesis of methionine in the absence of cobalamin when EHC supplied the folic acid cofactor (strain PA15).

The presence of an aminopterin-sensitive dihydropteroylglutamic acid reductase in ultrasonic extracts of E. coli PA15 was demonstrated. The cofactor activity of dihydropteroylglutamic acid (PtH_2G) in methionine synthesis was inhibited by aminopterin and amethopterin whereas the activity of PtH_4G and EHC were unaffected by these anti-metabolites. This suggested that the activity of PtH_2G depends on its conversion to PtH_4G . Furthermore, aminopterin and amethopterin prevented the inhibitory effect of PtH_2G , but not of PtH_4G , on methionine formation in the absence of added cobalamin (EHC present, strain PA15). Even in the presence of concentrations of aminopterin which completely inhibited the function of dihydropteroylglutamic

acid reductase, PtH_4G behaved as a catalyst for the formation of methionine. This suggests that PtH_4G does not serve as the final reductant for methionine methyl group formation (by its conversion to PtH_2G) a mechanism which has been postulated for the synthesis of the methyl groups of thymidylic acid.

Conjugated forms of folic acid

Several derivatives of tetrahydropteroyltriglutamic acid (PtH_4G_3) resembled EHC in their ability to serve as cofactors for the synthesis of methionine by enzymic extracts of E.coli PA15 in the absence of added cobalamin. With ultrasonic extracts of E.coli 121/176 cobalamin was however still required when PtH_4G_3 was used as the cofactor for C_1 -transfer. From a consideration of stability and the cofactor activity per molecule of folic acid, one of the conjugated folic acids, N^5 -formyl PtH_4G_3 , qualified as a possible active component of the EHC. PtH_4G inhibited the cofactor activity of the derivatives of PtH_4G_3 in the same manner as it inhibited the activity of EHC (strain PA15).

Finally the active folic acid components of the EHC were fractionated by chromatography on triethylaminoethyl-cellulose and one component was tentatively identified as N^5 -formyl PtH_4G_3 by comparing its cofactor activity, microbiological growth factor activity and its chromatographic behaviour with a sample of synthetic N^5 -formyl PtH_4G_3 .

DISCUSSION

The most satisfactory explanation of the results so far obtained is the postulation of two mechanisms for the synthesis of methionine in Escherichia coli, one being completely dependent upon the presence of cobalamin and the other being less dependent or independent of the metabolism of cobalamin. It is suggested that the second pathway is blocked in cobalamin-requiring auxotrophs whereas other strains can use both pathways. Unlike most other C_1 -transfer reactions, PtH_4G does not serve as a folic acid cofactor for the synthesis of methionine (unless cobalamin was present). Furthermore PtH_4G appeared to be an inhibitory analogue of the natural folic acid cofactors of the EHC and of PtH_4G_3 and its derivatives. Nevertheless PtH_4G was an active cofactor for methionine formation in the presence of cobalamin, presumably by the mechanism normally operating in the cobalamin auxotrophs.

It would seem that the mechanism of C_1 -transfer in the formation of methionine is more complex than other C_1 -transfer reactions which have been examined; no specific requirement for either a conjugated folic acid cofactor or the added participation of cobalamin has previously been described.

It seems certain that the function of cobalamin is mediated by a protein-like factor (X) which may contain cobalamin or a cobamide coenzyme. The exact function of cobalamin was not defined; it may however participate in either the transference of C_1 -units or their reduction. No other intermediates of methionine formation were isolated and it would appear that further elucidation of the mechanism of this reaction will depend on the fractionation of the enzymic extracts of several strains of Escherichia coli.