ASPECTS OF THE METABOLISM OF MICRO-ORGANISMS,

being a

Dissertation

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LIST OF CONTENTS

	Page
List of figures	iv
List of tables	vi
Glossary	x
SECTION I	
Sulphonamide resistance in *Acetobacter suboxydans* 621	
Part I. Introduction:	
Preface	1
Chapter 1: p-Aminobenzoic acid as a growth factor and as an antagonist of sulphonamide action	2
Chapter 2: The biological function of p-aminobenzoic acid	8
Chapter 3: The mechanism of sulphonamide action	19
Chapter 4: Drug resistance and variation in micro-organisms	23
Chapter 5: Sulphonamide resistance in bacteria	33
Chapter 6: The present work	40
Chapter 7: *Acetobacter suboxydans*	42
Part II. Experimental methods:	
Chapter 8: Chemical and bacteriological methods	45

Page

Part III. Results:

Preliminary note on the experimental results. 57

Chapter 9: The cultivation of *A. suboxydans* 621 58

Chapter 10: On the nutrition of *A. suboxydans* 621 62

Chapter 11: The sulphathiazole-trained strain 65

Chapter 12: The non-exacting strain 69

Chapter 13: The synthesis of p-aminobenzoic acid by *A. suboxydans* 621 76

Chapter 14: The synthesis of "folic acid" by by *A. suboxydans* 621 80

Chapter 15: The synthesis of adenine by *A. suboxydans* 621 89

Chapter 16: Other trained strains of *A. suboxydans* 621 93

Chapter 17: Some experiments with *Escherichia coli* 273-384 96

Part IV. Discussion:

Chapter 18: p-aminobenzoic acid in the metabolism of *A. suboxydans* 621 98

Chapter 19: A summary of the properties of the trained strains of *A. suboxydans* 621 106

Chapter 20: The mechanism of the variation to dispense with p-aminobenzoic acid 110

Chapter 21: The mechanism of the variation to sulphonamide resistance 114

Page

Chapter 22: The variations of Esch. coli 273-384 119

Chapter 23: Some theoretical implications of the expanded enzyme hypothesis of sulphonamide resistance 120

Chapter 24: The conclusions reached in Chapters 20 to 23 129

SECTION II

The interaction of some organisms requiring p-aminobenzoic acid for growth

Chapter 25: Introduction: The accumulation of intermediates by biochemical mutants 131

Chapter 26: Experimental methods 135

Chapter 27: Results and discussion 142

APPENDICES

Appendix 1: Tests with some analogues of p-aminobenzoic acid and related compounds 149

Appendix 2: List of media 153

Appendix 3: Abstract 163

Appendix 4: Bibliography 172

LIST OF FIGURES

Figure		facing page
1.	Standard dry weight curves for bacterial suspensions	54
2.	Standard curve for sulphathiazole estimations	56
3.	Growth of A. suboxydans in cultures of various depths	58
4.	Growth and pH in cultures of A. suboxydans	60
5.	Growth and pH in cultures of A. suboxydans containing glucose	61
6.	Growth curves of various strains of A. suboxydans in Medium 2	66
7.	Growth of strain C with sulphathiazole at three levels of p-AB	67
8.	Growth of strain A with purified casein hydrolysate	73
9.	Growth curves of various strains of A. suboxydans in "Improved" Medium 2	74
10.	Folic acid content of once-washed cells of A. suboxydans	82
11.	Folic acid content of twice-washed cells of A. suboxydans	82
12.	Folic acid content of cells of A. suboxydans of various ages	82

Figure		facing page
13.	Folic acid content of *A. suboxydans* grown with glycerol and with glucose	83
14.	Folic acid content of cells of trained strains of *A. suboxydans*	84
15.	Folic acid content of cells of strain C grown with and without sulphathiazole	85
16.	Folic acid content of cultures of normal *A. suboxydans* and of strain C	85
17.	Folic acid content of cultures of normal *A. suboxydans* and of strain A	85
18.	Folic acid content of cultures of strain C grown with and without sulphathiazole	86
19.	Response of normal *A. suboxydans* and of strain C to p-AB	87
20.	Growth curves of strains A and A_2 in "improved" Medium 2	95
21.	Plate tests for syntrophism among mutant strains of *Oph. multiannulatum*	140

LIST OF TABLES

Table		facing page
1.	Growth of *A. suboxydans* with p-AB	62
2.	Growth of *A. suboxydans* with pteroylglutamic acid	62
3.	Growth of *A. suboxydans* with adenine	62
4.	Growth of *A. suboxydans* with adenine-ribose and adenine-pteroylglutamic acid mixtures	63
5.	Competitive anti-sulphanilamide activity of p-AB and pteroylglutamic acid for *A. suboxydans*	64
6.	Non-competitive anti-sulphathiazole activity of adenine for *A. suboxydans*	64
7.	Formation of the sulphathiazole-resistant strain (strain C)	65
8.	Comparison of the requirements of strain C and normal *A. suboxydans* for p-AB	67
9.	Failure of strain C to destroy sulphathiazole	68
10.	Stimulation of the growth of strain A by p-AB, pteroylglutamic acid or glucose	69
11.	Growth and pH of cultures of various strains of *A. suboxydans*	70
12.	Stimulation of the growth of strain A by additional "vitamin-free" casein hydrolysate	71
13.	Tests of aspartic acid and various other supplements as growth stimulants for strain A	72

Table		facing page
14.	Sulphathiazole resistance of various strains of A. suboxydans	74
15.	Competitive sulphathiazole antagonism by p-AB for strain A	74
16.	Requirement of strain A for pantothenic and nicotinic acids	75
17.	Growth of strain H on its first and third passages with extracts of A. suboxydans	77
18.	Assay of extracts of strains A and C for p-AB	78
19.	Assay of extracts of strains A and C for p-AB	79
20.	Growth of Lb. casei in the presence of a killed culture of A. suboxydans	80
21.	Effect of p-AB concentration during growth on folic acid content of cells of A. suboxydans	83
22.	Effect of glucose on folic acid synthesis by A. suboxydans	84
23.	Non-competitive sulphathiazole antagonism by extracts of A. suboxydans	89
24.	Growth of "adenine-less" N. crassa in the presence of a killed culture of A. suboxydans	90
25.	Comparison of the growth factor effects of extracts of A. suboxydans (various strains) for N. crassa ("adenine-less") with their anti-sulphathiazole effect for normal A. suboxydans	90
26.	Growth of strain CD with p-AB	94

Table facing page

27. Sulphathiazole resistance and p-AB requirement of "p-AB-less" Esch. coli; comparison with non-exacting strain 96

28. Training of "p-AB-less" Esch. coli to resist sulphathiazole 97

29. Enhanced p-AB requirement of the sulphathiazole-resistant strain of "p-AB-less" Esch. coli when grown with sulphathiazole 97

30. Tests for syntrophism among ten p-AB-requiring mutants 142

31. Stimulation of the growth of Esch. coli mutants by a filtrate of an Oph. multiannulatum mutant 142

32. Stimulation of the growth of A. suboxydans 621 by a filtrate of a N. crassa mutant 142

33. Relationship of the activity of extracts of N. crassa for A. suboxydans to their glucose contents 146

34. Effect of sucrose concentration on the formation of material active for A. suboxydans by N. crassa 147

35. Tests of p-aminobenzoylmethionine as a growth factor for A. suboxydans and as a growth stimulant and anti-sulphonamide for Esch. coli 149

Table facing page

36. Tests of ammonium N-8-benziminazolemethyl-p-aminobenzoate as an inhibitor of Esch. coli and as a growth factor for A. suboxydans 150

37. Test of p-hydroxybenzoic acid as a growth factor for A. suboxydans 151

GLOSSARY

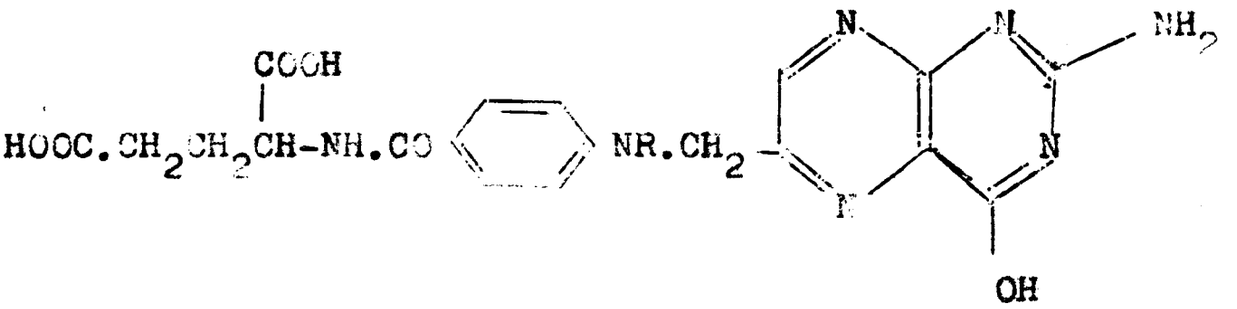
List of Terms used in this Dissertation

Chemicals:

p-Aminobenzoic acid. Abbreviated throughout to "p-AB". A member of the "B" group of vitamins; an antagonist of the sulphonamide drugs and a growth factor for certain micro-organisms. It has the structure:



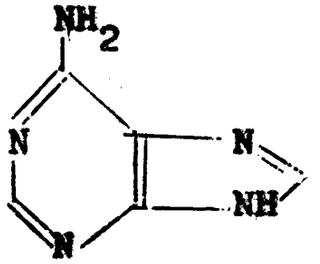
Pteroylglutamic acid. A synthetic product having the full biological activity of "Liver L.casei factor", one of the folic acid group of factors. It has the structure below, where R = H:



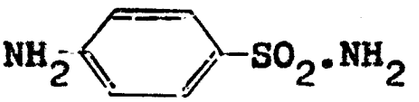
Rhizopterin. A synthetic product, formylpteroylglutamic acid, active for Streptococcus Faecalis R. It has the structure given above except that R = CHO.

Folic acid. A group of substances active as growth factors for various lactic acid bacteria and other organisms. Used in this dissertation to denote a substance capable of replacing

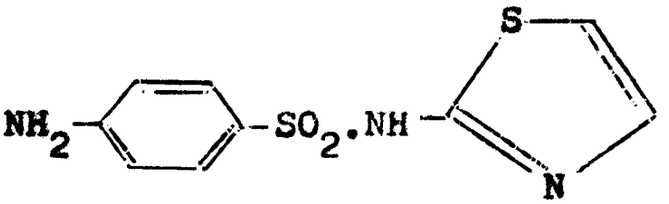
Adenine. 6-Aminopurine; a component of nucleoproteins and certain nucleotide-like co-enzymes. It is sometimes a growth factor for micro-organisms and there is little doubt that it is a common constituent of protoplasm. It has the structure:



Sulphanilamide. The simplest typical sulphonamide drug: p-aminobenzenesulphonamide.



Sulphathiazole. One of the more active members of the sulphonamide class of drugs. It is p-aminobenzene-N'-2-thiazolyl-sulphonamide, having the structure:



Sub-strains of Acetobacter suboxydans 621

"Strain A". A sub-strain obtained by training A. suboxydans to grow in the absence of p-AB, which is normally required by this organism.

"Strain A_2". A second sub-strain of the same type as strain A.

"Strain C". A sub-strain obtained after training by sub-cultivation in the presence of sulphathiazole. It resisted the action of more than two hundred times as much drug as the parent strain in the same conditions.

"Strain CD". A sub-strain derived from strain C with a decreased requirement for p-AB in the presence of large amounts of sulphathiazole.

"Strain H". During attempts to detect p-AB in extracts of certain variant strains (Chapter 13) it was necessary to assay such extracts with a strain having a normal requirement for p-AB. Since the extracts were slightly inhibitory towards the normal organism, a strain was trained to resist this inhibition by sub-cultivation in the presence of an inhibitory extract. It was called strain H.

Other terms

Certain terms common in the discussion of biological variation have been used in different senses by different authorities, and it is therefore desirable to define these terms carefully before using them. The definitions below give the sense in which certain words will be used in this dissertation.

Training. A term used to describe a laboratory procedure by which strains of micro-organisms may be obtained which grow more readily than the parent organism in specific environmental conditions. For example, organisms can sometimes be trained to

resist the action of a drug by repeated subcultivation in sub-inhibitory concentrations of drug. In this work the word is intended to imply nothing about the mechanism by which the variation occurs.

Mutation. A process by which a trained organism may arise: a homogeneous strain of organisms is regarded as producing a proportion of mutants spontaneously during multiplication. Some of these will be able to grow more readily in the training medium, and on prolonged subculture these mutants will supersede the parent type entirely, giving rise to a trained strain. A mutation is a single qualitative change of a genetical character and is independent of the nature of the training medium.

Adaptation. An alternative mechanism by which a trained organism may arise: the changed environment is regarded as altering the enzymic constitution of all the cells present so as to enable them to grow more readily in the training medium. Adaptation implies a continuous change which is not primarily genetical and which is engendered by the training medium. The process has also been referred to as enzyme adaptation, though other authorities restrict this phrase to an adaptive change which takes place without cell multiplication.

SECTION I

SULPHONAMIDE RESISTANCE IN ACETOBACTER SUBOXYDANS 621

P A R T I

Introduction

Preface This thesis is largely concerned with a study of the variations undergone by a micro-organism enabling it to resist the inhibitory action of the sulphonamide drugs. It is therefore necessary as a preliminary to discuss what is known about the mode of action of these drugs and the mechanism of resistance to them. So many hundreds of papers have been published on these matters that it would not be practicable to survey them in detail here. An outline of present knowledge is given in Chapters 1 to 7.

Chapter 1

p-Aminobenzoic Acid as a Growth Factor and as an Antagonist of Sulphonamide Action

p-Aminobenzoic acid, referred to in this thesis as "p-AB", is one of the more recent members of the "B" group of vitamins to be investigated in detail. Stamp (1939) showed that certain cell extracts could overcome the growth-inhibitory action of the sulphonamides, and Woods (1940) showed that p-AB duplicated the action of yeast extracts in this respect, and resembled them in chemical properties. The relationship between p-AB and sulphanilamide was competitive, ie: the number of molecules of p-AB required to antagonise a given number of sulphanilamide molecules was in constant ratio for a given organism and medium. For this reason, and because of their resemblance in chemical structure, he put forward the hypothesis that sulphanilamide acted by inhibiting the utilisation of p-AB by competition at an enzyme surface. It was necessary to emphasise that it was the utilisation of p-AB that was involved, since, if the drug interfered with its formation in the cell; one would not expect the relationship between drug and antagonist to be competitive. A small amount of antagonist added from outside the cell would be sufficient to satisfy the needs of the organism in the presence of drug and this amount would be independant of the amount of drug present; the relationship would therefore not be competitive.

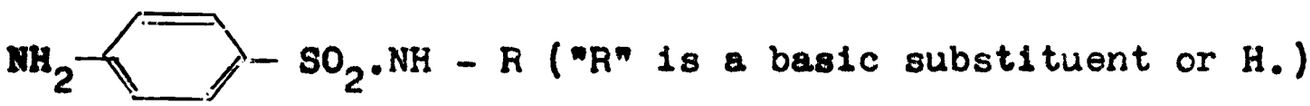
This type of competitive interaction may be illustrated schematically:-



This scheme indicates the use of p-AB by an enzyme to form other substances directly, but utilisation of p-AB as a co-enzyme in the formation of other products would also give rise to a competitive relationship (Woods, 1949).

Such interaction is not unusual in biological systems: the action of malonic acid in inhibiting the dehydrogenase of the structurally related succinic acid (Quastel & Wooldridge, 1928; Hopkins, Morgan & Lutwak-Mann, 1938) and the anti-vitamin effect of many vitamin analogues are well-known examples. Competitive inhibition is regarded as being due to a similarity in geometry and charge distribution in the molecules of the metabolite and those of inhibitor, as a result of which the inhibitor occupies some of the enzyme surface which would normally be involved with the metabolite. Thus the rate of utilisation of the metabolite is reduced, sometimes to zero, but can be restored by increasing the metabolite concentration in

accordance with the law of mass action. The effect of the inhibitor depends on its concentration in a precisely similar manner and the effect of a mixture of inhibitor and metabolite will therefore depend on their relative molar concentrations. In the case of p-AB and the sulphonamides the geometrical similarity lies in the p-aminbenzenesulphonamido- residue common to all sulphonamides as shown in the general formula below:



This view of the interaction has received wide support, and has stimulated more detailed investigation of the phenomenon.

Wyss (1941) investigated the competitive antagonism of sulphonamides by p-AB with Escherichia coli and showed that the competitive relationship could be accounted for in simple kinetic terms assuming adsorption of the two substances at an enzyme surface with differing equilibrium constants for the two adsorptions.

Klotz (1944) and Klotz & Gutman (1945) preferred to regard the competition as due to a series of simple equilibria between p-AB, drug and their dissociated forms, together with p-AB/enzyme and drug/enzyme complexes. To these the law of mass action may be applied, and Klotz has given a quantitative development of

this point of view which is as satisfactory as the approach of Wyss.

There is little difference between these two types of approach from the experimental point of view, though, as with the thermodynamic and kinetic treatments of chemical reactions, they are formally distinct and capable of development along different lines. In the present state of knowledge, however, they have the value of indicating that a physico-chemical approach to this type of problem can usefully be made.

The physico-chemical approach to the problem was taken in rather a different sense by Bell & Roblin (1942) who correlated the activity of various sulphonamides with their acid dissociation constants, and showed that the most active members of this group were those that had a pK_a in the region of that of p-AB. It should be noted that the agreement was not entirely satisfactory since the most active sulphonamides had a pK_a in the region of 6.5, whereas that of p-AB is about 4.8. The difference does not seem large until one remembers that the pK_a scale is logarithmic. pK_a is defined in the usual notation as:

$$pK_a = \log. \frac{1}{\frac{(A^-)(H^+)}{(AH)}}$$

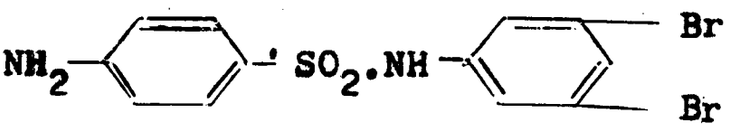
from which:

$$pK_a = pH + \log. \frac{(AH)}{(A^-)}.$$

This second relation indicates that at a given pH a small difference in pK_a represents a large difference in the ratio of ionised and non-ionised forms.

Bell & Roblin also confirmed the steric relationship between the sulphonamides and p-AB, in that the distance between the p-amino- and N-substituted sulphonamido- groups in sulphonamides was very similar to the distance between the amino- and carboxyl in p-AB. Given this geometrical similarity they gave a qualitative interpretation of the correlation of activity and pK_a in terms of the effect of the N-substituents on the electron distribution in the p-aminobenzenesulphonamido- system.

The antagonistic relationship has been shown (see Henry, 1943) to be true for all drugs containing the p-aminobenzene-sulphonamido- moiety, as well as for some other isosteres of p-AB. An exception to this rule occurs in a series of compounds called the sulphanilyl-anilides, which have structures of the form:



Although they contain a sulphonamide residue in the molecule, the action of these compounds is not antagonised by p-AB (Goetchus & Lawrence, 1945). The antagonistic relationship is true for all bacteria except Bact. tularense (Tamura, 1944) and

has been demonstrated in protozoa, fungi and higher organisms (Lwoff, Nitti & Trefouel, 1941; Lwoff, Nitti, Trefouel & Hamon, 1941; Bonner, 1942). The exception of Bact. tularense is possibly due to the extreme frailty of this organism (Eigelsbach, Chambers & Coriell, 1946).

Several other substances having effects antagonistic to the sulphonamide drugs are known; for example, methionine, certain purines and mixtures of purines, pteroylglutamic acid and complex materials such as "peptone" have activity of this kind in certain circumstances but few substances are as active as p-AB. Sulphonamide antagonists other than p-AB will be discussed in detail in a later section (page 11).

A direct implication of Woods's view of the anti-sulphonamide activity of p-AB was that this substance must be a metabolite of normal cells. This was soon confirmed by Rubbo & Gillespie (1940) (see also Rubbo, Maxwell, Fairbridge & Gillespie, 1941) who found that it was an essential growth factor for Clostridium acetobutylicum. Since then it has been shown (see Woods, 1947) to be a growth factor for a variety of micro-organisms including at least ten artificially induced mutants and has been claimed to have various effects on higher organisms, such as the stimulation of the growth of chicks and the prevention of grey hair in men and rats (see Ansbacher, 1944).

Chapter 2

The Biological Function of p-Aminobenzoic Acid

For some years after Woods's discovery, there was little evidence on the precise function of p-AB as a growth factor until, after 1945, a team of workers of the American Cyanamid Company published their work on folic acid (see Hutchings & Mowat, 1948). From a study of the degradation products of a folic acid ("fermentation L. casei factor") isolated from the fermentation residue of a diphtheroid organism, they were able to synthesise a substance which had, for Lactobacillus casei and other organisms, the full biological activity of a factor occurring naturally in liver. The synthetic material contained a p-AB residue in its molecule and was given the trivial name of pteroylglutamic acid. Its structure has been given in the glossary (page x). These workers also showed that pteroic acid, the same molecule without a glutamic acid residue, was active as a growth factor for Streptococcus faecalis (R), which accounted for the observation of Stokes & Larsen (1945b) that resting suspensions of enterococci would convert this factor to folic acid. "Vitamin B_c", present in yeast and liver, was also shown to be pteroylglutamic acid, existing naturally as conjugates containing more than one glutamic acid residue in the molecule. "Vitamin B_c conjugate" was pteroylheptaglutamic acid. "Fermentation L. casei factor" was a similar factor with

only three glutamic acid residues. Of interest in this connection is a report by Ratner, Blanchard & Green (1946) of a peptide isolated from yeast which appeared to contain ten or eleven glutamic acid residues, p-AB and another unidentified residue. It had, however, no biological activity.

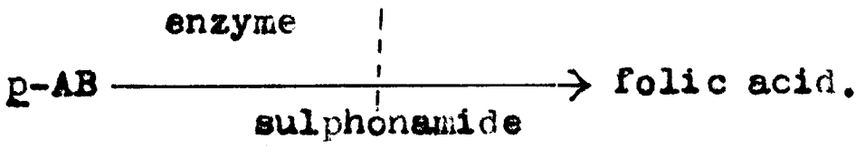
In 1947 a group of workers from the laboratories of Merck and Co., Inc., reported their work on a naturally occurring factor for the growth of Strept. faecalis (R) (see Hutchings & Mowat, 1948). They showed that a formylated derivative of pteroic acid, rhizopterin, was more active than pteroic acid itself. It had the structure given in the glossary (page x).

In view of the presence of a p-AB residue in the various compounds of the folic acid group it seemed likely that one function of p-AB in the cell was its use in the synthesis of such compounds. Folic acid is an essential metabolite as well as a growth factor for certain organisms; it is required by mammals and birds (see Jukes & Stokstad, 1948) and some organisms that do not require it have been shown to synthesise it. Consequently, much work was done on the biological relationship of p-AB and folic acid both as growth factors and as anti-sulphonamide substances. Lampen & Jones (1946) showed that the growth of some strains of enterococci was stimulated by either p-AB or folic acid. With the former the usual competitive inhibition by sulphonamides occurred, but with the latter the organisms were almost insensitive to sulphonamide inhibition. With Strept.

faecalis (Ralston) (which is not the Strept. faecalis (R) referred to above) and with Strept. zymogenes 26 C 1 they found that pteroylglutamic acid and pteroyltriglutamic acid were non-competitive antagonists of sulphadiazine at concentrations at which the closely related organisms Strept. faecalis (R) and Strept. zymogenes 5 C 1 respectively required them for growth. The same authors (Lampen & Jones, 1947) showed that pteroyl-glutamic acid, pteroic acid or p-aminobenzoylglutamic acid would replace the p-AB required for growth by Lb. arabinosus, though greater molar concentrations of these compounds were needed. At high concentrations thymine was also active, and this substance or pteroylglutamic acid acted as a non-competitive anti-sulphonamide for Streptobacterium plantarum. They also showed that thymine was able to replace folic acid for Strept. faecalis (R) at levels at which it was a non-competitive anti-sulphonamide for Strept. faecalis (Ralston). The activity of thymine was interesting in view of previous work (Stokstad, 1941) which showed that thymine, in the presence of purines, was capable of replacing folic acid for Lb. casei.

On the hypothesis of Woods, the discovery of a case of non-competitive anti-sulphonamide activity suggested that the antagonist was, or readily became, one of the products of the competitively inhibited reaction, and the work of Lampen & Jones suggested that one reaction of this kind was the building up of p-AB into pteroylglutamic acid or a similar substance. Nimmo-

Smith & Woods (1947, 1948) investigated the matter with a number of organisms for which p-AB was a growth factor and found that, for Cl. acetobutylicum and Sbm. plantarum, pteroylglutamic acid acted as a non-competitive anti-sulphonamide at concentrations at which it was effective in replacing p-AB for growth. On the other hand with A. suboxydans and a p-AB-requiring mutant of Neurospora crassa it acted as an inefficient competitive antagonist. For Sbm. plantarum 10 S, Nimmo-Smith, Lascelles & Woods (1948) showed that suspensions of the organism, in conditions in which no growth occurred, converted p-AB to folic acid (assayed with Lb. casei), in proportion to the amount supplied, and that this synthesis was competitively inhibited by sulphanilamide. This was in very satisfactory accord with the view that the point of primary attack by the sulphonamides in this organism was at a stage which one can write as:



Several compounds were known which would act in place of p-AB both as growth factors and anti-sulphonamide substances. These effects were often only partial in the sense that in the presence of these compounds the amount of p-AB needed to produce an effect was lessened. This suggested that in most cells there were reactions other than the formation of folic acid in which p-AB was involved.

Harris & Kohn (1941a) observed that methionine acted as an antagonist of sulphonamides for Esch. coli, but only over a limited range of sulphonamide concentration. Bliss & Long (1941) obtained a similar effect, and showed that arginine and lysine had a small and irregular action of this kind with the same organism. Strauss, Dingle & Finland (1949a,b) confirmed the activity of methionine for Esch. coli, but showed that, unlike p-AB, methionine did not prevent the acquirement of sulphonamide resistance by the organism. Strehler (1950) observed that methionine acted as a growth factor for a p-AB - requiring mutant of Neurospora, but he also showed that the mutant was able to synthesise small amounts of p-AB. In connection with the activity of methionine it is interesting to note that vitamin B_{12} has an action resembling methionine in certain circumstances. B_{12} - requiring mutants of Esch. coli were shown by Davis & Mingioli (1950) to use methionine in place of B_{12} for growth, and either methionine or B_{12} decreased the amount of p-AB required to antagonise sulphathiozole for those organisms. They suggested that B_{12} had this action indirectly, by being concerned in the synthesis of methionine from homocysteine.

Harris & Kohn (1941b,) further demonstrated that purines, while sometimes inhibitory for normal Esch. coli, increased the anti-sulphonamide activity of methionine, and Snell & Mitchell (1942) reported that purines could increase the activity of p-AB as an anti-sulphonamide in lactic acid bacteria; in some or-

ganisms they found that purines were active alone. Similarly, purines reduced the p-AB requirements of such organisms, sometimes to zero, and high concentrations of methionine could replace p-AB altogether. Landy & Streightoff (1943) observed that adenine would reduce the p-AB requirement of A. suboxydans 621 by a factor of about ten. Zalokar (1950), during his work on the sulphanilamide-requiring mutant of N. crassa (page 37), showed that the inhibition of the wild type of N. crassa by sulphanilamide was partially counter-acted by methionine, and that adenine or guanine enhanced this effect. Similarly, methionine decreased the quantitative p-AB requirement of a p-AB-requiring mutant, and adenine and guanine acted synergistically. Thymine was inactive.

Complex materials such as "peptone" (Kohn & Harris, 1941) also have anti-sulphonamide activity and Mirick (1941) showed that this action was not due to p-AB. Much of the earlier work mentioned above was done with media containing materials later found to have anti-sulphonamide or growth factor-sparing action themselves; thus the media in which lactic acid bacteria were grown always contained amino-acids which could well be acting synergistically with the substances tested.

The rather confusing picture that emerges from this work has been to some extent clarified by several more recent papers. In the first of these, Lampen, Roepke & Jones (1946) studied an induced mutant of Esch. coli requiring p-AB for growth. This

requirement could be abolished by a mixture of thymine, purines (adenine, guanine and xanthine) and a number of amino-acids of which methionine was the most important. Almost optimal growth in the absence of p-AB was supported by a mixture of these substances, and persisted for ten subcultures. Growth with this mixture was highly resistant to sulphadiazine. Pteroylglutamic acid did not replace p-AB for the mutant, and in another paper (Lampen & Jones, 1947) lysine is referred to as being one of the important amino-acids in the mixture.

Winkler & de Haan (1948) tested a series of compounds for their ability to reduce the amount of p-AB required for sulphonamide antagonism in Esch. coli and Salmonella typhimurium. As inhibitor they used sulphanilamide and showed that (a) methionine, (b) methionine and xanthine, and (c) methionine, xanthine and serine mixtures had successively greater activity in reducing the amount of p-AB needed. In the presence of the last mixture, pteroylglutamic acid or thymine gave complete non-competitive anti-sulphanilamide activity in the absence of p-AB.(1) In a footnote these authors mentioned that valine and, to a less extent, lysine or iso-leucine, had some activity in accelerating growth in these conditions, and a later paper (Winkler, de Haan &

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(1) Recent experiments in this laboratory have not confirmed the total replacement of p-AB by mixture (c) + pteroylglutamic acid reported by Winkler & de Haan.

van de Langerijt, 1949) included valine as one of the active amino-acids for Esch. coli, and reported that these amino-acids antagonised several other sulphonamides. These workers extended their studies to Staph. aureus cultured in a synthetic medium (de Haan, Winkler & Haljst, 1949) and showed that a mixture of methionine, xanthine, thymine and valine would antagonise the action of sulphanilamide, and that pteroylglutamic acid would not replace thymine in this mixture. Cutts & Rainbow (1950) described a strain of Saccharomyces cerevisiae which required p-AB for growth, and whose requirement for this could be abolished by a mixture of adenine and casein hydrolysate. A study of the ability of known amino-acids to replace casein hydrolysate revealed that adenine, methionine and histidine as a mixture would replace p-AB, but only limited growth was obtained. The addition of any of the amino-acids leucine, iso-leucine, nor-leucine, valine, nor-valine, tyrosine or phenylalanine improved growth, but no mixture supported growth as well as p-AB. The presence of active mixtures of amino-acids decreased the amount of p-AB needed to permit optimal growth.

Recently Lascelles & Woods (1951) showed that the requirement of Leuconostoc mesanteroides P-60 for serine could be abolished by a mixture of glycine, pyridoxal, CO_2 and p-AB, and that the synthesis of serine from glycine by Strept. faecalis (R) required the presence of pteroylglutamic acid and pyridoxal among other compounds. p-AB was inactive. In contrast, cell suspension

of a p-AB-requiring strain of S. cerevisiae showed increased synthesis of serine with p-AB, though pteroylglutamic acid was inactive. They suggested that folic acid or derivatives thereof, formed from p-AB, were concerned in the transfer of single carbon units in cell syntheses, and that the reaction:

$$CH_2NH_2COOH \xrightarrow[H.COOH]{CO_2 \text{ or}} HO.CH_2CH_2NH_2.COOH$$

was an example of this. These findings were consistent with those of Elwyn & Sprinson (1950), who observed that the reverse reaction, serine ⟶ glycine was much diminished in folic acid-deficient rats, and also with those of Plaut, Bethiel & Lardy (1950) who found that the incorporation of C^{14} from formate into serine was diminished in such rats.

Thus the overall picture of p-AB in the economy of the cell has been seen to be very complicated. The discovery of substances which have a p-AB-sparing effect, either as growth factors or anti-sulphonamides, suggests that these substances are among the normal products of utilisation of p-AB: that the inhibitor blocks the synthesis of the substances by a route involving p-AB. An interesting demonstration of the validity of this hypothesis was carried out by Wang, King, Cheldelin & Christiansen (1951), who showed that C^{14}-labelled methionine was incorporated into cells of Esch. coli without dilution if the

culture contained 2-chloro-p-aminobenzoic acid (an inhibitor of the sulphonamide type whose action is antagonised by p-AB or methionine (Strandskov, 1947)). Cells grown without inhibitor showed roughly 50% dilution of the labelled methionine by methionine synthesised by the cells. Thus methionine synthesis was prevented by the inhibitor even when growth occurred.

If this view of the mechanism of p-AB replacement is accepted, then there is evidence from the work outlined in this chapter that p-AB is involved in the synthesis of the substances listed below:

1) Folic acid
2) Adenine; probably xanthine and other purines
3) Thymine
4) Methionine
5) Serine, lysine, valine, histidine; possibly other amino-acids.

Folic acid may well be involved in the synthesis of all the compounds listed after it, so it probably represents the first product of the utilisation of p-AB. It is highly likely that these products will assume varying degrees of importance in various organisms, thus accounting for the conflicting results that have been found in studies of the biological function of p-AB.

Note added during revision of this dissertation: A growth factor for Leuconostoc citrovorum ("Citrovorum factor") has recently been reported (see Krehl & Liao, 1951). It resembled folic acid and acted as a competitive antagonist of the pteroylglutamic acid

analogue aminopterin (4-amino-10-methyl-pteroylglutamic acid). In this respect it was more active than pteroylglutamic acid with Lb. casei and it may be closer to the functional form of folic acid in the cell metabolism. Its structure has recently been shown to be N^5-formyl derivative of tetrahydropteroylglutamic acid.

Chapter 3

The Mechanism of Sulphonamide Action

Since the observations of Woods this subject has been the centre of much discussion. It is likely that one of the primary actions of the sulphonamides is to interfere with the metabolism of p-AB or a closely related substance in the cell, but it is undoubtedly the case that there are secondary actions, some common to all sulphonamides and others depending on the particular drug. A series of papers between 1938 and 1940 presented evidence suggesting that these drugs acted by destroying or inhibiting catalase in the cell, thus causing an accumulation of toxic H_2O_2 - the "anti-catalase" theory (Main, Shinn & Mellon, 1938, 1939, 1940; Shinn, Main & Mellon, 1938, 1939, 1940; Locke, Main & Mellon, 1939;). This view is no longer regarded as satisfactory, as sulphonamides act on organisms that contain no catalase, and on those that form no H_2O_2. Moreover, H_2O_2 is bactericidal, whereas in general the sulphonamides are bacteriostatic, and, in addition, Mann & Keilin (1940) have shown that sulphanilamide has no action on purified catalase.

Another school, whose outlook is summarised in reviews by Henry (1943) and Sevag (1946), is that of Sevag and his co-workers, who regard the primary action of sulphonamides as being on the respiratory enzymes of bacteria. This hypothesis has probably little validity in view of the work of Wyss, Strandskov & Schmelkes (1942) who studied the effect of various sulphona-

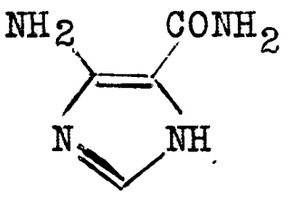
mides on the respiration of Esch. coli, Staphylococcus aureus and Streptococcus pyogenes. They showed (1) that therapeutically inactive isosteres of sulphanilamide affected the respiration of these organisms, sometimes more than sulphanilamide itself, (2) that sulphathiazole, a highly active sulphonamide drug, had no action whatever on the respiratory enzymes, and (3) that training Esch. coli to resist sulphonamides caused no change at all in the susceptibility of its respiratory enzymes to sulphonamides. It is therefore likely that Sevag and his school were concerning themselves with a secondary action of a few of the sulphonamides.

Such secondary actions are well known. Dorfman & Koser (1942) showed that the stimulation of respiration which occurs when nicotinamide is added to cells of Shigella grown under conditions of nicotinamide deficiency was antagonised by sulphapyridine and sulphathiazole, but not by sulphanilamide. Mann & Keilin (1940) demonstrated that sulphanilamide affected the enzyme carbonic anhydrase, though other sulphonamides were inactive, and van Goor (1943-5) found a similar effect. The effect of sulphanilamide on carbonic anhydrase was not due to the complete sulphonamide molecule, as removal of the p-amino-group gave compounds which still inhibited the enzyme though they were no longer sulphonamides (Mann & Keilin, 1940). Gale (1945) observed that, while p-AB alone was sufficient to overcome the inhibition of Group D streptococci by sulphapyridine and sulphathiazole, both p-AB and CO_2 were needed in the case of sul-

phanilamide; arginine would replace CO_2 through the action of the enzyme arginase. There is evidence (Dr. G. Wolf, personal communication) that sulphamezathine has a gonadotropic effect on rats which is due to the N-substituent rather than to the sulphonamido-residue.

While some of these secondary effects are due to special characteristics of individual sulphonamides, it is nevertheless likely that general secondary effects will occur. For example, sulphonamides have been shown to interfere with the metabolism of purines, and since these are known to be respiratory co-enzymes, it is understandable that sulphonamides may in general affect cell respiration indirectly.

Some interesting work on sulphonamide action was performed by Stetten & Fox and later by Shive and his colleagues using Esch. coli. Fox (1942) and Stetten & Fox (1945) showed that in certain conditions of sulphadiazine bacteriostasis this organism produceda diazotisable, non-acetylable amine. This material was shown by Shive, Ackermann, Gordon, Getzendaner & Eakin (1947) to be 2-aminoiminazole-3-carboxamide, which, from a consideration of its structure, they regarded as a precursor of purines in biological systems. It had the structure given below:



Ravel, Eakin & Shive (1948) later demonstrated that the output of this substance depended on the amount of glycine in the medium, and was assisted by glutamic acid. Their results are interesting in view of the known synergistic effects of purines with p-AB, and Shive regarded the effect as indicating that p-AB acted as a co-enzyme in purine synthesis in the cell, the purines being formed from this amine by the incorporation of one carbon atom.

It is more likely that folic acid or a derivative thereof is the co-enzyme for the incorporation of a single carbon atom into 2-aminoiminazole-3-carboxamide, since, not only is this view consistent with the proposal that folic acid is generally involved in the transfer of one-carbon groups in cells, but Woolley & Pringle (1950) showed that 2-aminoiminazole-3-carboxamide accumulated in cultures of Esch. coli grown with sub-inhibitory amounts of the folic acid antagonist aminopterin. This was hardly likely to occur if p-AB were involved directly as a co-enzyme for the utilisation of 2-aminoiminazole-3-carboxamide, and suggests that a preliminary conversion of p-AB to pteroylglutamic acid had occurred, and that the further utilisation of the latter was prevented by aminopterin.

Chapter 4

Drug Resistance and Variation in Micro-organisms

The phenomenon of acquired resistance to drugs is part of the wider subject of variation in micro-organisms. It is therefore necessary to survey this subject briefly before proceeding to the detailed topic of sulphonamide resistance.

The variations undergone by bacteria can be broadly divided for convenience into four types, though it is extremely likely that they are inter-related.

1) Variations involving a change in growth factor requirements. Organisms that are normally exacting in certain respects can often be trained to become less exacting. Thus Fildes, Gladstone & Knight (1933) found that freshly isolated Bact. typhosum which usually required tryptophan for growth, lost this requirement after three passages in tryptophan-deficient media; Postgate & Hinshelwood (1946) observed that Bact. coli mutabile was readily trained to dispense with asparagine. It is usually assumed that the organisms have, in these circumstances, developed the power to synthesise the metabolite they originally required, and Fildes & Knight (1933) demonstrated that this was the case with tryptophan in Bact. typhosum. The hypothesis is in any case intrinsically reasonable, as many of the requirements which bacteria can be trained to lose are for substances which are known constituents of protoplasm.

Non-exacting organisms sometimes develop a requirement for a growth factor, either spontaneously or after treatment in the laboratory. The artificial variants of the mould Neurospora produced by X-rays, ultra-violet light, mustard gas, etc. (see Beadle, 1945) are examples of this kind, and similar exacting variants of bacteria have been obtained artificially.

2) Variations involving a change in morphology. The training of an organism to grow in unfamiliar conditions is often accompanied by morphological variations whose mechanism is obscure. Perhaps the best known case of this kind is the classical one of Bact. typhosum, whose normally smooth colonies assume a rough appearance after passage for a little time on laboratory media. Lodge & Hinshelwood (1943) reported variations in the size of Bact. lactis aerogenes during training to m-cresol, and Hinshelwood (1946) mentioned a similar phenomenon during training the organism to 2-aminoacridine, "proflavine". Gardner (1940) has noted morphologically enlarged forms of Cl. welchii and other organisms during training to resist penicillin, and Gale & Rodwell (1949) have obtained a strain of staphylococcus trained to high concentrations of penicillin which was totally unrecognisable; it had a rod-like appearance and had become Gram-negative.

3) Variations involving a change of substrate. Many of the classical diagnostic tests used in bacteriology are based on the ability of a species to ferment some carbohydrates or similar carbon sources but not others. If, however, a certain organism

is normally incapable of utilising a certain substrate, or utilises it very inefficiently, it can often be trained to do so by prolonged passage in or exposure to the substrate. The best known case of this kind is that of Bact. coli mutabile, which Massini (1907) showed to be normally unable to ferment lactose, but which gave rise to "mutants" capable of doing so. Many similar cases are known, and led Karström (1937) to distinguish between constitutive enzymes which are normally present in the cell, and adaptive enzymes which appear only after training. The mechanism of adaptive enzyme formation and the validity of Karström's distinction have given rise to much discussion. Yudkin (1938) put forward a theory based on simple mass action effects by the substrate on enzyme precursors in the cell, which was modified and developed by Spiegelman (1945) and his colleagues (Spiegelman, Reiner & Cohnberg, 1947; Spiegelman & Reiner, 1947, a, b; Spiegelman & Dunn, 1947) in a series of papers on the ability of yeast suspensions to ferment various sugars. This type of variation in yeasts is one of the few cases in which it was shown (Stephenson & Yudkin, 1936) that the change could take place without cell multiplication.

Hinshelwood has applied his picture of an "auto-catalytic" self-duplicating system to the training of Bact. lactis aerogenes to grow on glycerol and lactose, regarding the new substrate as capable of inducing stereochemical distortion in a constitutive enzyme so as to form an adaptive enzyme (Lodge & Hinshelwood,

1944; Postgate & Hinshelwood, 1946). His general picture of adaptation is discussed further below, but it is interesting to note here that Spiegelman, in common with Hinshelwood, was caused to introduce a concept of "autocatalysis" into his interpretation of these phenomena.

Variations of this type have a number of experimental applications. Dubos (1939) trained a sporogenous bacillus to hydrolyse the capsular polysaccharides of various types of pneumococcus, and used a cell-free preparation of the enzyme to protect mice from infection by these organisms. Gale's amino-acid decarboxylases (Gale, 1946), which have been used for the estimation of specific amino-acids, are adaptive enzymes, and Mirick (1943) used a soil organism trained to oxidise p-AB as a means of estimating this substance. Stanier (1947) has used the method of "simultaneous adaptation" to demonstrate the metabolic pathways by which certain substances are broken down. Thus, if the oxidation of a substance "A" takes place by way of intermediates B, C, and D, etc. and not by way of B', C', and D', etc., then an organism trained to oxidise A will be found to have developed simultaneously the power to oxidise B, C, D, etc. but not B', C', D', etc.

This type of variation will not be discussed further as the present work is concerned entirely with the first and fourth types.

4) Variation to resist inhibitors. Organisms can often be trained to grow in the presence of substances which normally delay or prevent growth, and this fact is sometimes important in medicine. The example of training to resist penicillin, during which some organisms develop giant forms, has already been mentioned, and Todd, Turner & Drew (1945) showed that this particular type of adaptation could be reversible in Staphylococcus: the organism lost its resistance again in the absence of penicillin. Spink & Ferris (1945) demonstrated that a naturally penicillin-resistant Staphylococcus formed a powerful penicillin inactivator, but that trained organisms did not. They later showed (Spink & Ferris, 1947) that the penicillin inactivator of naturally resistant organisms was penicillinase. Gale & Rodwell (1949) observed that Staphylococcus aureus trained to penicillin had an increased ability to synthesise its amino-acid requirements, and a decreased ability to absorb pre-formed amino-acid from the medium. Correspondingly, cells trained to dispense with certain amino-acids developed a greater penicillin resistance. Organisms train very readily to the antibiotic streptomycin, and various workers reported (Kushnick, Randles, Gray & Birkland, 1947; Paine & Finland, 1948) that strains of both Gram-positive and Gram-negative organisms can not only become resistant to it but can actually develop a requirement for it: they fail to grow in its absence.

Organisms trained to resist the sulphonamide drugs have a

general resistance to all substances of this class, as would be expected from what is known of the mode of action of these drugs. Resistance is often acquired only slowly (Davies & Hinshelwood, 1943) but the resistance is usually permanent. James & Hinshelwood (1947) however, were able to cause Bact. lactis aerogenes to lose an acquired resistance to sulphanilamide by training it to resist 2-aminoacridine.

The subject of sulphonamide resistance, since it is closely related to the present work, is discussed in some detail later.

Variation enabling an organism to resists drugs is often regarded as being due to the artificial selection of resistant mutants which are held to occur spontaneously during growth. While this process can account for many cases, there are a few in which it is not satisfactory, and in which the evidence seems to favour a gradual change which takes place in all the organisms present. Hinshelwood and his colleagues (Hinshelwood, 1946), during studies of the adaptation of Bact. lactis aerogenes to resist drugs, including 2-aminoacridine, methylene blue, crystal violet and sulphanilamide, showed that reversion, or loss of resistance, took place readily in the early stages of training if the drug was removed, but rarely at later stages. Moreover they demonstrated that the trained strain grew at the same rate as the normal in the absence of drug. Consequently there was no disadvantage inherent in the trained strain which would allow selection of back-mutants to occur. Hinshelwood's school has

inclined to reject natural selection as an interpretation of acquired drug resistance, preferring to regard it as a simple physico-chemical process following the Law of mass action. They regard the growth and multiplication of cells as implying general reactions of an autocatalytic nature, which, since they are probably enzymic in nature, can be written:

Enzyme + substrate $\longrightarrow$ more enzyme + products.

The products of such a process are considered to be the substrates for enzymes which would operate further reactions of this kind.

Perhaps it should be emphasised now, as the point is often misunderstood, that this concept does not imply that there is any single reaction of this kind that could be observed in the cell, but merely that the overall processes leading to cell growth and multiplication can be represented in this manner. In the growing cell enzymes reacting in this way are linked by a series of equilibria resulting in the synthesis of more cell material, which may be written:

Enzyme 1 $\rightleftharpoons$ Enzyme 2 $\rightleftharpoons$ Enzyme 3 $\rightleftharpoons$ ... <u>etc</u>.

If a drug is regarded as interfering at one stage in such a series, say between enzymes 2 and 3, then the equilibria will be disturbed and the rate of formation of enzyme 3 from the products of the reaction of enzyme 2 will be lessened. Enzyme 2 and its products will therefore accumulate. Since the rate of a reaction above zero order is a function of the concentration of the reacting species, this accumulation will tend to bring the rate of the inhibited reaction back to normal again - the system will automatically become adapted to the inhibitor.

On the basis of ideas such as this Hinshelwood and his colleagues were able to suggest a mathematical account of the training of Bact. lactis aerogenes to resist the drugs mentioned above, and also to give some qualitative account of its training to utilise new carbon sources.

The ideas of Hinshelwood and his colleagues have not been widely accepted. Luria & Delbrueck (1943) regarded the appearance of 'phage-resistant strains of Esch. coli as due to random mutation, and Demerec (1945 a,b) took a similar view of penicillin resistance in Staph. aureus. Demerec showed that, if a number of cultures of Staph. aureus were grown from an inoculum free of resistant cells, and the whole cultures plated out in the presence of penicillin, then the number of resistant colonies on the separate plates differed widely. In contrast, a similar number of samples plated out from a single culture grown from the same inoculum showed considerable uniformity in the number of resistant

colonies on penicillin plates. He argued that, if penicillin resistance was not due to random mutation, but was conditioned by the presence of penicillin in the plates, then the uniform distribution of colonies found on plating out the samples from a single culture should also have been found in plating out the separate cultures. Eriksen (1949) criticised the technique used by Demerec and observed that penicillin-resistant colonies of Staph. aureus were only found on plates containing concentrations of penicillin sufficiently low to permit limited growth of the non-resistant cells. He also obtained a greater uniformity than Demerec by plating out larger cultures, and maintained that his findings were incompatible with a mutational mechanism for acquired penicillin resistance. Fildes & Whitaker (1948) recently published evidence that the variation of Bact. typhosum to tryptophan independence (page 23) was a mutational change.

Thus the interpretation of variation in bacteria is a subject which is still highly controversial, and in which the evidence one way or another seems to depend to a large extent on the organism used and the particular change in question. The American workers on the mould Neurospora have induced biochemical variants of the mould (see Beadle, 1945) which are undoubtedly mutants in the classical sense, and mutant bacteria have also been prepared by similar methods (Lederberg & Tatum, 1946; Tatum & Lederberg, 1947; Lampen, Roepke & Jones, 1946). On

the other hand, variation without cell growth, which has been shown to occur in yeasts (Stephenson & Yudkin, 1936; Spiegelman, 1945) is difficult to interpret in terms of mutations. Similarly cases of reversible training such as that of Bact. lactis aerogenes to glycerol (Lodge & Hinshelwood, 1944) are even more difficult to explain in these terms, and Postgate & Hinshelwood (1946) found a complete gradation of behaviours between the classical "mutational" case of Bact. coli mutabile in lactose and the typically adaptive and reversible behaviour of Bact. lactis aerogenes to this sugar.

It is therefore likely that there is no generally applicable interpretation of bacterial variation. The type of change undergone by the organism during training is often obscure, and in at least one case (penicillin resistance in staphylococci, (Spink & Ferris, 1945, 1947)) there is evidence that it can be different from the changes resulting in natural resistance.

Chapter 5

Sulphonamide Resistance in Bacteria

The view that variation to resist sulphonamides is due to the acquirement or development of an ability to synthesise sulphonamide antagonists, such as p-AB, has received support from several workers. Macleod (1943) found an increase in the production of such antagonists by the pneumococcus after training to resist sulphanilamide. With A. suboxydans 621 as an assay organism, Landy, Larkum, Oswald & Streightoff (1943) demonstrated an increased synthesis of p-AB-like substances in sulphonamide-fast staphylococci, though not in several other organisms. Landy & Gerstung (1944, 1945) have surveyed a large number of gonococci of varying natural *in vivo* resistance to sulphonamides, and showed a correlation between this resistance, their resistance *in vitro*, and their output of p-AB-like substances *in vitro*. As with all methods of microbiological assay, this work is open to the criticism that there are many substances having the action of p-AB to a greater or less extent, and it is difficult to be sure which is being assayed at any given time. Such criticism cannot be levelled at the ingenious work of Mirick (1943) who used a soil organism specifically trained to oxidise p-AB. With this he was able to demonstrate an increased formation of substrate for this organism by sulphapyridine-trained pneumococci. Lemberg, Tandy & Goldsworthy

(1946) and Lemberg, Callaghan, Tandy & Goldsworthy (1948) used a modification of the Bratton-Marshall test for aromatic amines, coupled with quantitative chromatography, to estimate p-AB in biological fluids and to distinguish it from its o-isomer, anthranilic acid. (The latter substance was not unexpectedly found in such fluids, it being an intermediate in tryptophan metabolism (Tatum & Bonner, 1944)). Though the quantitative p-AB recovery of Lemberg's method was only about 30%, he was able to show that there was no increase in p-AB synthesis by Esch. coli trained to resist sulphathiazole.

Strandskov (1947) gave an elegant demonstration that sulphonamide fastness in Esch. coli was not due to methionine synthesis. Using the inhibitor 2-chloro-p-aminobenzoic acid he showed that this substance was antagonised by both p-AB and methionine. On training the organism to resist this substance it was found not to be resistant to sulphonamide, whereas a sulphonamide-trained strain did resist 2-chloro-p-AB. Were resistance to either drug to have been acquired by reason of methionine synthesis, complete cross resistance would have been expected.

On the other hand it is likely that methionine is involved in some way in sulphonamide resistance. Kohn & Harris (1942) showed that a strain of Esch. coli trained to resist sulphanilamide in the presence of amino-acids (including methionine) and purines, developed an absolute requirement for added methionine.

A control strain cultured in the absence of sulphanilamide did not develop this requirement. They were able, however, to train a strain to resist sulphanilamide in the absence of methionine. They regarded this as showing that sulphonamide training involved certain metabolic re-adjustments among which was the protection of methionine synthesis in the cell. In the strain trained in the presence of added methionine this re-adjustment was unnecessary, but a requirement for added methionine was developed.

The relationship of p-AB to folic acid and the high sulphonamide resistance of folic acid-requiring organisms makes it clear that folic acid is involved in sulphonamide resistance, but whether this is true for acquired resistance is still uncertain. Even before the publication of the structure of pteroylglutamic acid, Miller (1944) had shown that the output of folic acid by Esch. coli, estimated with Lb. casei, was the same whether or not the organism was trained to resist sulphathiazole. The presence of active concentrations of inhibitor depressed the output in both cases (also, Miller, Bruno & Berglund, 1947).

Spink & Vivino (1943) reported the formation of a pigment by Staphylococci trained to high concentrations of sulphathiazole, which may be related to a pigment reported by Mayer (1944) which was formed by Mycobacterium tuberculosis hominis var. in the

presence of p-AB or procaine.[1] A different pigment formed by Neurospora in the presence of high and inhibitory concentrations of p-AB was reported by Cushing & Reid (1948). The importance of these pigments is at present unknown, but Spink & Vivino suggested that their pigment was formed by the organism from excess p-AB produced in response to the sulphonamide present.

Emerson & Cushing (1946) and Emerson (1947) obtained some peculiar variants of the mould Neurospora by training it to resist sulphonamides. One strain, when adapted to sulphanilamide, developed an enhanced sensitivity to inhibition by both sulphathiazole and high concentrations of p-AB. In the presence of these compounds it reverted, losing its resistance to sulphanilamide and behaving like the wild type organism. By crossing the resistant strain with a wild type organism Emerson obtained a strain with a partial requirement for sulphathiazole or sulphapyridine - its growth was stimulated by either of these substances at high temperatures, though at low temperatures growth was unaffected. It was inhibited by p-AB at all concentrations tested (10^{-2} to 10^{-5}M); though he did not test this substance at growth factor levels (ca. 10^{-7}M). Finally, by crossing this last variant with an artificial mutant requiring p-AB for growth he was able to obtain a temperature-sensitive variant requiring

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(1) Mayer's pigment is probably an oxidation product of p-AB as recent studies have shown that Mycobacterium smegmatis oxidises p-AB via aniline to p-aminophenol (Sloane, Crane & Mayer, 1951).

both p-AB and sulphanilamide for growth. He interpreted these phenomena in terms of an over-production of p-AB by the mould to such an extent that it became inhibitory and was antagonised by sulphonamides. Zalokar (1948) studied these particular mutants in some detail, and observed that pteroylglutamic acid and pteroyltriglutamic acid were inactive for the sulphanilamide-requiring strain, though they acted as weak competitive anti-sulphonamides for the p-AB-requiring mutant; p-AB was also inactive. In the absence of sulphanilamide the strain requiring both this and p-AB would grow only if the p-AB concentration were very low (5×10^{-8} M). He suggested that in the normal organism p-AB was converted to various products, and that in the mutant one of these was toxic. It was only formed at relatively high concentrations of p-AB, and its formation was antagonised by sulphonamides. Thus, at high p-AB concentrations, a sulphonamide-requirement appeared. In a second paper (Zalokar, 1950) he studied the behaviour of hybrids between the sulphanilamide-requiring mutant and certain methionine-requiring mutant strains, and showed that a strain requiring both methionine and sulphanilamide was so produced, and that high methionine concentrations were then inhibitory, though the inhibition could be antagonised by threonine. He suggested that the deleterious reaction which resulted in sulphonamide requirement, interfered with the normal utilisation of threonine, and that it required some precursor of methionine as a substrate.

An early observation by Macleod (1939) that a pneumococcus changed its fermentative properties and produced more H_2O_2 on training to resist sulphapyridine was of interest in view of the then current "anti-catalase" theory of sulphonamide action (page 19), but did not suggest any obvious interpretation of sulphonamide resistance. Cole & Hinshelwood (1947) noted recently an increase in the catalase activity of Bact. lactis aerogenes trained to resist sulphonamides, though the earlier papers of this school (eg: Davies & Hinshelwood, 1943) favoured an alternative proteosynthetic growth mechanism as an interpretation of sulphanilamide resistance in this organism.

There remains the formal possibility that sulphonamide-resistant organisms destroy sulphonamides, a point which seems to have been little investigated. However, Link (1943) found that a sulphonamide-resistant Staphylococcus could convert sulphonamides to substances stimulatory to itself. A strain trained to resist sulphathiazole still needed addition of p-AB to antagonise yet more sulphathiazole, but its growth in the presence of sulphathiazole could be stimulated by weakly active sulphonamides such as sulphacetamide, though this inhibited the normal strain. Thus these weak sulphonamides seemed to act as anti-sulphonamides in the resistant strain. When cell suspensions of this resistant strain were incubated with sulphonamides there was an increased formation of sulphonamide antagonists This process appeared to be enzymatically controlled as it was stopped when the organisms were killed by heat.

Thus the mechanism of development of sulphonamide-resistance remains obscure, and it seems likely that there may be different mechanisms for different organisms. While there is, in some cases, fairly strong evidence that the variation is due to increased synthesis of p-AB, it seems unlikely that this is a general mechanism. In many cases, such as Esch. coli, there is distinct evidence to the contrary. However, a failure to detect increased p-AB synthesis may mean merely that all the p-AB formed is at once converted into some higher product which would not be detected by the method used, so even with Esch. coli the case must be considered not proven.

Chapter 6

The Present Work

So far no reports have appeared in the literature of studies of the development of resistance to sulphonamides by an organism which has an absolute requirement for p-AB, and for this reason the work reported here was undertaken. It was thought possible that such studies might yield useful information on the mechanism of sulphonamide-resistance since the amount of p-AB present would always be under strict control as the organism itself would presumably be unable to synthesise it.

Since sulphanilamide itself is known to have such secondary effects on CO_2 enzymes as might necessitate growing the organism in CO_2 (page 20), sulphathiazole was chosen as the more convenient drug to which to train the organism.

With such a system at least three types of variation are theoretically possible:

1) Development of resistance to sulphathiazole in the presence of p-AB. In this case the organism would be grown in the presence of optimal amount of p-AB and would be trained to resist sulphathiazole without further increase in the p-AB concentration.

2) Ability to grow without p-AB. A p-AB-requiring organism trained to dispense with this growth factor might undergo interesting changes in its sulphonamide resistance and related properties.

3) Development of resistance to sulphathiazole in the absence of p-AB. This sort of variation depends in the first instance on the realisation of one of the two types already mentioned. In the first case the strain might be trained to dispense with p-AB while retaining its resistance to sulphathiazole, and in the second case the non-exacting strain might be trained to resist sulphathiazole in the same way as a natural non-exacting strain.

The first two types of "sub-strain" have been obtained, and variations of their properties are reported. The third type has not so far been realised, but the attempts to obtain it are also recorded. The properties selected for investigation are those related to the metabolism of p-AB and are listed below:-

a) The requirement of the strains for p-AB and similar substances.

b) Their resistance to sulphathiazole in the presence and absence of p-AB, and the nature of the inter-relation between p-AB and sulphathiazole in these strains.

c) The synthesis, if any, of adenine, folic acid and p-AB by these strains.

The amino-acid requirements of the various strains were not studied because, as is reported on page 49 it was not possible to obtain satisfactory growth on a medium in which casein hydrolysate was replaced by an amino-acid mixture.

Some of the results presented in this thesis have been reported briefly elsewhere (Marshall & Postgate, 1949).

Chapter 7

Acetobacter Suboxydans

The experimental work reported in this dissertation is chiefly concerned with variants of a particular strain (621) of A. suboxydans. The general properties of the organism will therefore be summarised.

Acetobacter suboxydans, earlier known as Bact. suboxydans, is classified in Bergey's "Manual of Determinative Bacteriology" (Breed, Murray & Hitchens, 1948) in the order Eubacteriales, family Pseudomonadaceae, genus Acetobacter. It is recorded as consisting of Gram-variable rods, ovoid and non-motile, occurring singly or in chains. It grows optimally at 30°C, and is an obligate aerobe showing a tendency to pellicular surface growth. It is normally isolated from spoiled beer.

One importance of this species of Acetobacter lies in the fact that it brings about certain limited oxidations (from which character its specific name is derived) which are used commercially Thus it oxidises glucose only to gluconic acid, glycerol to dihydroxyacetone, sorbitol to sorbose and so on, and the main industrial methods at present used to prepare the last two compounds make use of this organism. Since this aspect of the metabolism of the organism does not concern us here the literature will not be summarised; a guide to it is given in the introduction to the paper of Underkofler, Bantz & Petersen (1943).

During a survey of the growth factor requirements of various bacteria Lampen, Underkofler & Petersen (1942) showed A. suboxydans (A.T.C.C.621) to be among those requiring the newly discovered factor p-AB. This was the same strain as was used by the author in the present work. In a later paper Underkofler, Bantz & Petersen (1943) found that it also required nicotinic and pantothenic acids. They used a medium based on glycerol and acid-hydrolysed casein freed of vitamins, and also reported that the organism was able to "grow more slowly on a mixture of known amino-acids". They did not specify the amino-acids concerned. These workers also showed that the lactone moeity of pantothenic acid (L-2:2'-dimethyl-1:3-dihydroxy-1:3-lactone) would replace pantothenic acid as growth factor. Sarrett & Cheldelin (1945) confirmed this fact and developed a method for the microbiological assay of pantoyl-lactone. There was a stimulation of growth by both glucose and casein hydrolysate in conditions where pantoyl-lactone was being assayed. Underkofler et al. reported the need of A. suboxydans for nicotinic acid only in a footnote, and the fact was confirmed by Karabinos & Dicken (1944) who developed a method for the assay of nicotinic acid using this organism.

A. suboxydans has also been used for the microbiological assay of p-AB; conditions for assay were first described by Landy & Dicken (1942) and improved by Landy & Streightoff (1943) and Cheldelin & Bennett (1945). The media used were again based on casein hydrolysate, glycerol and vitamins, and a similar

medium was used for the present work.

Stokes & Larsen (1945, a) described a medium in which casein hydrolysate was replaced by a mixture of amino-acids of which valine, iso-leucine, alanine and cystine were essential, and proline, serine, histidine and phenylalanine were stimulatory. As is reported later, we have been unable to confirm these findings in our laboratory.

Since the work reported in this dissertation was carried out, further studies on the nutrition of A. suboxydans 621 have been published. King, Locker & Cheldelin (1948) showed that a conjugate of pantothenate found in heart muscle was specifically more active for the organism than free pantothenic acid, and Novelli, Flynn & Lipmann (1949) observed that the recently discovered pantothenic acid derivative, Co-enzyme A, enabled the organism to grow at a considerably greater rate than in any medium containing simple pantothenate. They suggested that the pantothenic acid conjugate of King, Locker & Cheldelin was a partial degradation product of Co-enzyme A. A related compound, the "Lactobacillus bulgaricus factor", was shown to be the disulphide form of N-(pantothenyl)-β-aminoethane thiol pantethine, (see Craig & Snell, 1951), but tests of this compound with A. suboxydans have not been reported.

PART II

Experimental Methods

Chapter 8

Chemical and Bacteriological Methods

The methods used by bacteriologists for the sterile manipulation of cultures and materials are so well known that they will not be described here except where there was a departure from the usual technique. Details of experimental procedure will be described under the heading of the organism in connection with which they arose. Details of media are collected in Appendix 2 (page 153)

Reagents and Apparatus. Since metabolites such as p-AB are active at very high dilution it was necessary to take elaborate precautions to ensure the purity of the reagents and the cleanliness of the apparatus used. Roblin (1946) quotes an example in which, in a certain laboratory, it was impossible to prepare a medium free of p-AB because previously p-AB derivatives had been made there. In another room, with the same apparatus and materials, there was no such difficulty.

As a matter of laboratory routine all glassware was cleaned by standing it in a solution of sodium dichromate in strong H_2SO_4 overnight, or by passage through a bath of this solution heated to about 70°C. Such glassware was then washed 10 times in tap water and twice in distilled water before sterilisation. Pipettes were wrapped individually in paper before being steri-

lised, and, to prevent contamination of materials by substances from cotton wool, all plugs, except those of pipettes, were covered with a layer of washed muslin. This precaution is especially important in work on folic acid-like compounds, since cotton seed, contained in crude cotton wool, has been shown to be a source of folic acid (Sherwood & Singer, 1944).

All solutions were made up in glass-distilled water and reagents of "analytical reagent" grade were recrystallised at least once using acid-cleaned glassware throughout. Where possible, amino-acids, purines and other naturally occurring substances were of synthetic origin, so that the possibility of contamination by substances of biological activity should be reduced to a minimum; where this was not possible the substance was recrystallised many times and tested microbiologically.

Where a filtration was necessary, Whatman's No. 50 acid-extracted paper was used.

Liquid reagents, such as acetic acid and glycerol of "analytical reagent" grade were re-distilled, more than once if necessary. Substances were weighed on a circle of No. 50 paper using a platinum spatula which had been washed and burned clean in a Bunsen burner flame immediately before use.

Sterilisation of Solutions. Solutions to be added to sterile media were usually autoclaved for 10 min. at 10 lb./in.^{2} before use, but in cases where the compound might be unstable to heat the solution was sterilised by Seitz filtration. The "all glass" apparatus described by Knight & Fildes (1936) was used,

but had two inevitable disadvantages. Firstly, there were two parts of the apparatus which could not be guaranteed free of growth factors since they could not be acid-cleaned, namely the asbestos filter and its stainless steel support. Secondly, in preparing standard sterile solutions there was a risk of adsorption of material in the filter pad which could sometimes be seen with pigmented compounds such as riboflavine. The error was minimised by using the strongest possible solution for the actual filtration and diluting it afterwards.

Acetobacter suboxydans American Type Culture Collection No. 621. This organism was obtained from the National Collection of Type Cultures and was kept on agar slopes containing glycerol and yeast extract (Medium 1). The stock strain was subcultured monthly and incubated at 30°C. for 20 hr. before storage in a refrigerator at 4°C. In order to minimise the number of subcultures undergone by the parent stock, and the consequent possibility of variation, four parallel slopes were prepared for routine use during each month.

Quantitative work was carried out using a medium of partly defined composition after Landy & Dicken (1942; Medium 2). It was based on vitamin-free casein hydrolysate, glycerol as carbon source, nicotinic and pantothenic acids and salts. As is recorded in the next chapter (page 57), it was later found necessary, not only further to purify Ashe's vitamin-free casein

hydrolysate, but also to increase its concentration considerably.

The medium was made up in a more concentrated form than that described in Appendix 2 so as to allow for the dilution consequent upon the addition of growth factors, inoculum, etc. The organisms were grown at 30°C. in 25 ml. Erlenmeyer flasks containing 5 ml. of the complete medium which had been sterilised in an autoclave for 10 min. at 10 lb./in.^{2}

The inoculum was prepared by harvesting in distilled water the growth after 24 hr. on agar slope. The suspension was centrifuged, resuspended in distilled water, and its opacity adjusted to 1 on the Brown scale, corresponding to about 3.4×10^7 viable cells per ml. It was added to experimental media to a final dilution of $1/10^3$. This was a large inoculum for work of this kind, but some early experiments in this laboratory had shown that the growth rate of the organism was sometimes irregular if a smaller inoculum was used. The large inoculum gave perfectly satisfactory experimental controls.

For serial subculture in liquid media the organism was treated in a similar way: the resuspended growth of a liquid culture was used as inoculum after adjustment of the opacity. In these conditions the organism tended to grow as a pellicle or in small clusters of cells, but usually the growth was readily dispersed by shaking the culture.

To measure the growth rate of this organism, 20 or 25 ml. amounts of the complete inoculated medium in 6 x $\frac{3}{4}$ in. tubes were

aerated continuously with damp air through a Pasteur pipette in a thermostat at 30°C. This not only served to maintain diffuse growth by stirring the culture, but also ensured adequate aeration. Samples (1 ml.) were taken at intervals and their optical density measured in a Hilger "Spekker" absorptiometer. In a few cases the pH of samples was also estimated.

To measure the drug resistance or growth factor requirement of a strain, cultures containing various concentrations of the drug or growth factor were incubated, and that concentration at which visible growth did not occur was recorded.

In determinations of the growth factor requirements, however, growth sometimes occurred, after prolonged incubation, in the control cultures containing no added growth factor. In these cases those concentrations of growth factor at which growth did not occur more rapidly than the "blanks" were recorded. All such tests were carried out in duplicate.

Stokes & Larsen (1945,a) have described a synthetic medium for A. suboxydans 621 based on glycerol, salts, vitamins, and the amino-acids valine, iso-leucine, alanine and cysteine (Medium 3). This mixture permitted limited growth, and optimal growth was obtained when it was supplemented with histidine, proline, phenylalanine and serine. It did not prove possible to obtain optimal growth in such a medium in this laboratory, nor yet in a similar medium supplemented with aspartic acid, glutamic acid, leucine, lysine, tyrosine, hydroxyproline, arginine, glycine and

tryptophan. The reasons for this failure were not clear, but the amino-acids used in this laboratory differed from those of Stokes & Larsen in that all, except a specimen of histidine (four times recrystallised), were synthetic, whereas several of those used in the original work were of natural origin. It is therefore possible that the materials of Stokes & Larsen contained impurities such as would not occur in synthetic products, and which were capable of stimulating the growth of the organism.

Marshall (1948) working in this laboratory improved the growth in a synthetic medium by addition of the non-toxic detergent "Tween-80", but growth was still inferior to that in Medium 2.

Viable Counts of A. suboxydans 621. The inoculum size in terms of viable organisms which was quoted above was measured by the method of Miles & Misra (1938). Plates of glycerol yeast agar were dried at 60°C. for 3 hr. and drops of a diluted suspension were added from a Pasteur pipette. The volume of the drops was subsequently determined by weighing. Miles & Misra recommended drying the plates at 37°C., but, possibly owing to their content of glycerol, these plates required a higher temperature. Moreover, it was necessary to perform the higher dilutions, those above 1/100, in a solution containing glycerol and yeast extract (Medium 1 without agar), as the organism was very sensitive to further dilution in distilled water and the count fell off abruptly.

Escherichia coli 273-384. This was an X-ray induced mutant of Esch. coli isolated by Roepke, Libby & Small (1944) and required p-AB for growth; it was obtained by courtesy of Dr. R. O. Roblin. It was stored at 4°C. after growth for 16 hr. on agar slopes containing a tryptic digest of meat (37°C.), and a reserve of dried organisms was kept in sealed tubes at 4°C. The minimal medium used was that of Roepke et al. (Medium 6) consisting of glucose, asparagine and salts, except that synthetic DL-aspartic acid was used instead of asparagine. The synthetic substance was equally effective and more easily obtained in a pure state.

The minimal medium was distributed in 5 ml. amounts in 6 x $\frac{5}{8}$ in. tubes and autoclaved for 10 min. at 10 lb./in.^{2}. The inoculum was a $1/10^5$ dilution of the culture obtained after 16 hr. incubation in the minimal medium with 10^{-7} M p-AB.

Dried Cultures. The dried stock of Esch. coli 273-384 was prepared from a single colony grown from Dr. Roblin's original culture. This was passed once through the minimal medium to confirm its requirement for p-AB and was grown for 16 hr. on tryptic meat agar. The growth was harvested in "Lemco" gelatin and ascorbic acid, and dried in vacuo at room temperature using the method described by Stamp (1947). The dried tubes were sealed off at normal pressure and stored.

Neurospora crassa 28610-1899-1a. This was an artificial mutant of N. crassa, requiring adenine or related compounds for

growth, which has been used for the assay of these substances (Mitchell & Houlahan, 1946; McElroy & Mitchell, 1946). It was received from Dr. H. K. Mitchell and was grown at 25°C. on agar slopes containing sucrose, malt extract and "Difco" yeast extract. It was stored on agar slopes in the refrigerator at 4°C. Tests were done using a minimal medium based on that described by Mitchell & Houlahan (1946), which consisted mainly of sucrose, salts and biotin (Medium 7). Mitchell advised that a mixture of trace elements should be added to the medium, but this proved unnecessary in the present case.

The mould was grown at 25°C. in 5 ml. aliquots of the culture fluid in 6 x § in. tubes, all of which had been autoclaved for 10 min. at 10 lb./in.^{2}. The inoculum was a barely opalescent suspension of conidiospores taken from an agar slope.

Lactobacillus casei ∈, A.T.C.C.7469. This organism was obtained through Dr. Barton-Wright in 1946, and was kept on agar stabs containing "Bacto-tryptose", liver extract and glucose. It was subcultured every fortnight and incubated for 16 hr. at 37°C. before storage at 4°C. As with A. suboxydans, parallel stabs from the parent stock were made for routine use.

The organism is one normally used for the assay of the folic acid group of growth factors, and the medium used in this work was that of Roberts & Snell (1946) based on a partial tryptic digest of casein. This was purified by treatment with Norite charcoal and then made up for assay purposes with glucose,

purines, vitamins and salts (Medium 4). The digestion with trypsin was carried out for only 24 hr., as against the 48 hr. of Roberts & Snell, and a larger amount of "Difco" trypsin was used: 1 g. per 100 g. of Glaxo alcohol-extracted casein. These modifications were recommended by Dr. Woolley (personal communication) as likely to conserve the growth factor strepogenin which is required by Lb. casei. In the complete medium DL-cysteine was used in place of the L-cystine of Roberts & Snell.

The inoculum was taken from a fresh stab incubated for 16 hr., which was subcultured into a broth of "Bacto-tryptose" (Difco), liver extract and glucose. After incubation for 24 hr. a loop of this was inoculated into the assay medium supplemented with 10^{-9} M pteroylglutamic acid, and the 24 hr. growth of this culture was used as inoculum for an assay at a final dilution of $1/10^{3}$. The media containing the material to be assayed were previously autoclaved for 8 min. at 10 lb./in.^{2}.

This procedure has been modified to some extent by other workers in this laboratory, but was used throughout most the work recorded here. Other modifications to allow for the assay of bacterial extracts are reported in Chapter 13.

It was not possible to obtain complete absence of growth in the cultures containing no added folic acid; in fact a complete "blank" of this kind has not been recorded in the literature on this method of assay. The method nevertheless gave a satisfactory graded response to pteroylglutamic acid if growth

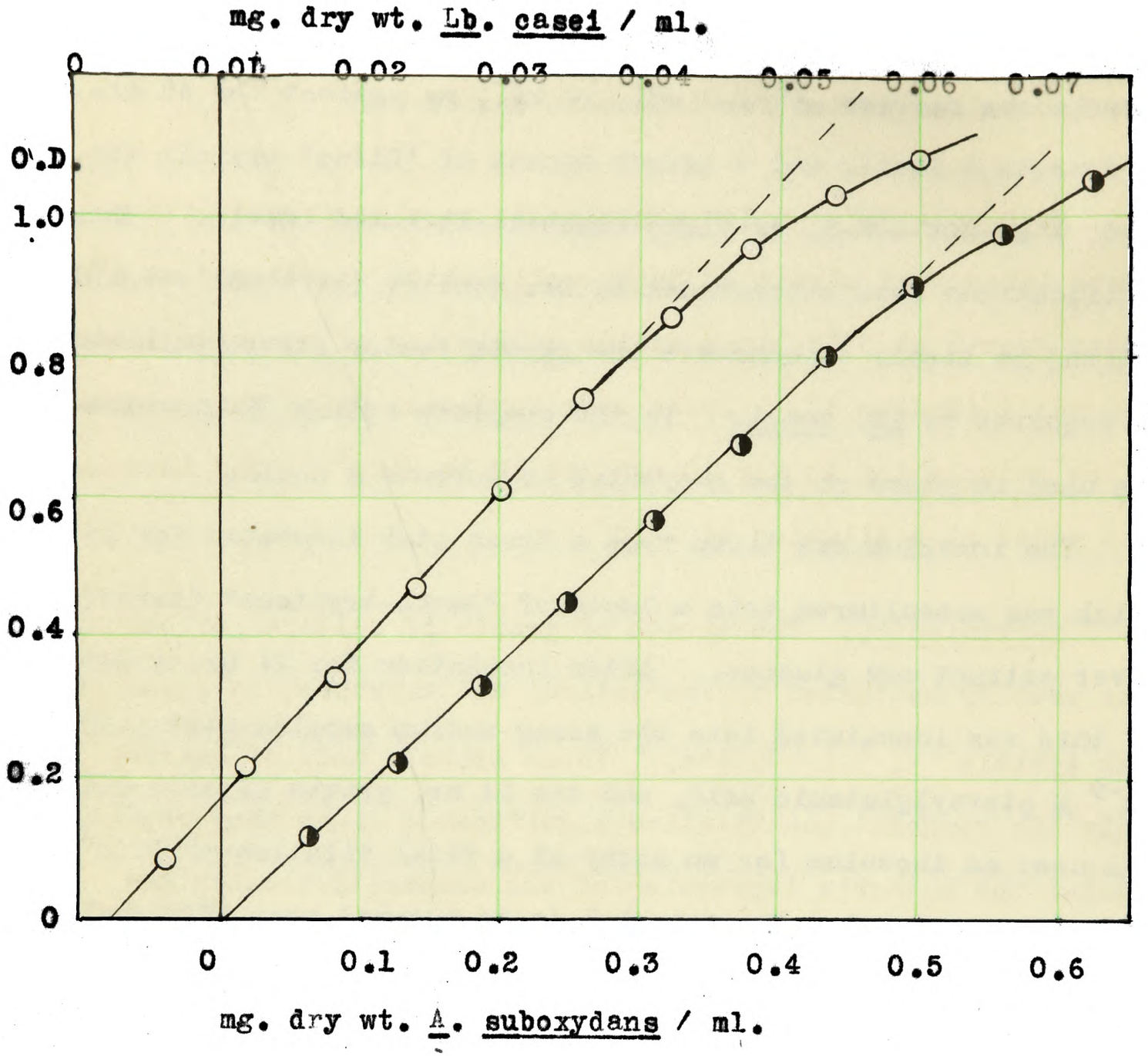


Fig. 1 Standard dry weight curves for bacterial suspensions.

The optical density (O.D.) of cell suspensions of known cell concentration was determined in the "Spekker" absorptiometer.

after incubation was measured in the "Spekker" absorptiometer. The incubation period for this work was 48 hr.

Esch. coli "F". A few experiments were performed with a stock laboratory strain of Esch. coli, which was stored at 4°C. after growth at 37°C. in a medium consisting of ammonium lactate and salts (Medium 5). Tests were conducted using this medium after autoclaving it for 10 min. at 10 lb./in.^{2}. The inoculum was added at a dilution of $1/10^5$.

Turbidimetry. The extent of growth of cultures of bacteria was usually estimated by inspection, and expressed in terms of the symbols +, ±, -, etc. These symbols had, of course, only the roughest quantitative significance since they represented the author's impression of the comparative rates and extents of growth during a single experiment. They were not comparable as between one experiment and another except in certain limiting conditions, such as the point at which the concentration of a drug was just sufficient to prevent visible growth altogether.

In certain cases, however, greater accuracy was required, and the optical density of cultures was then estimated in the "Spekker" absorptiometer. In this apparatus the optical densities of cultures of A. suboxydans and of Lb. casei were linear functions of the weight of dried cells/ml. (Fig. 1), so samples to be estimated were diluted to the working range of the instru-

ment, i.e.: between readings of 0.1 and 0.8. The "turbidity" was calculated from the instrument reading and was expressed as an arbitrary unit, the reading multiplied by the dilution.

In a few cases, where the cultures were to various extents coloured, neither turbidimetry nor inspection was altogether satisfactory as a measure of growth. Such cultures were counted under the microscope in a shallow Thoma haemocytometer.

The Chemical Estimation of p-AB and Sulphonamides. Since both p-AB and the sulphonamides are aromatic amines they could be estimated by the same method, though this fact precluded the estimation of one in the presence of another. The method chosen was that of Bratton & Marshall (1939) with certain modifications.

N.HCl (2 ml.) was added to an aliquot, up to 2 ml., of the solution to be tested. The volume was brought to 4 ml. and 1 ml. of 0.1% (w/v) $NaNO_2$ added. The mixture was shaken and left to stand at room temperature for 5 min. Ammonium sulphamate (0.5% w/v; 1 ml.) was then added and the whole left a further 2 min. during which time nitrogen was evolved from the unchanged NO_2' ion. Sodium acetate (25% w/v; 3 ml.) was added, bringing the pH to about 4.6, and also 1 ml. N-dimethyl-α-naphthylamine (0.4% w/v) in 95% (v/v) alcohol. After 10 min. 0.3 ml. of concentrated HCl was added to dissolve the precipitate of coupling reagent which formed at this pH, and the violet colour (from p-AB) was measured in the "Spekker" absorptiometer with a filter transmitting maximally light of 5200 Å wavelength.

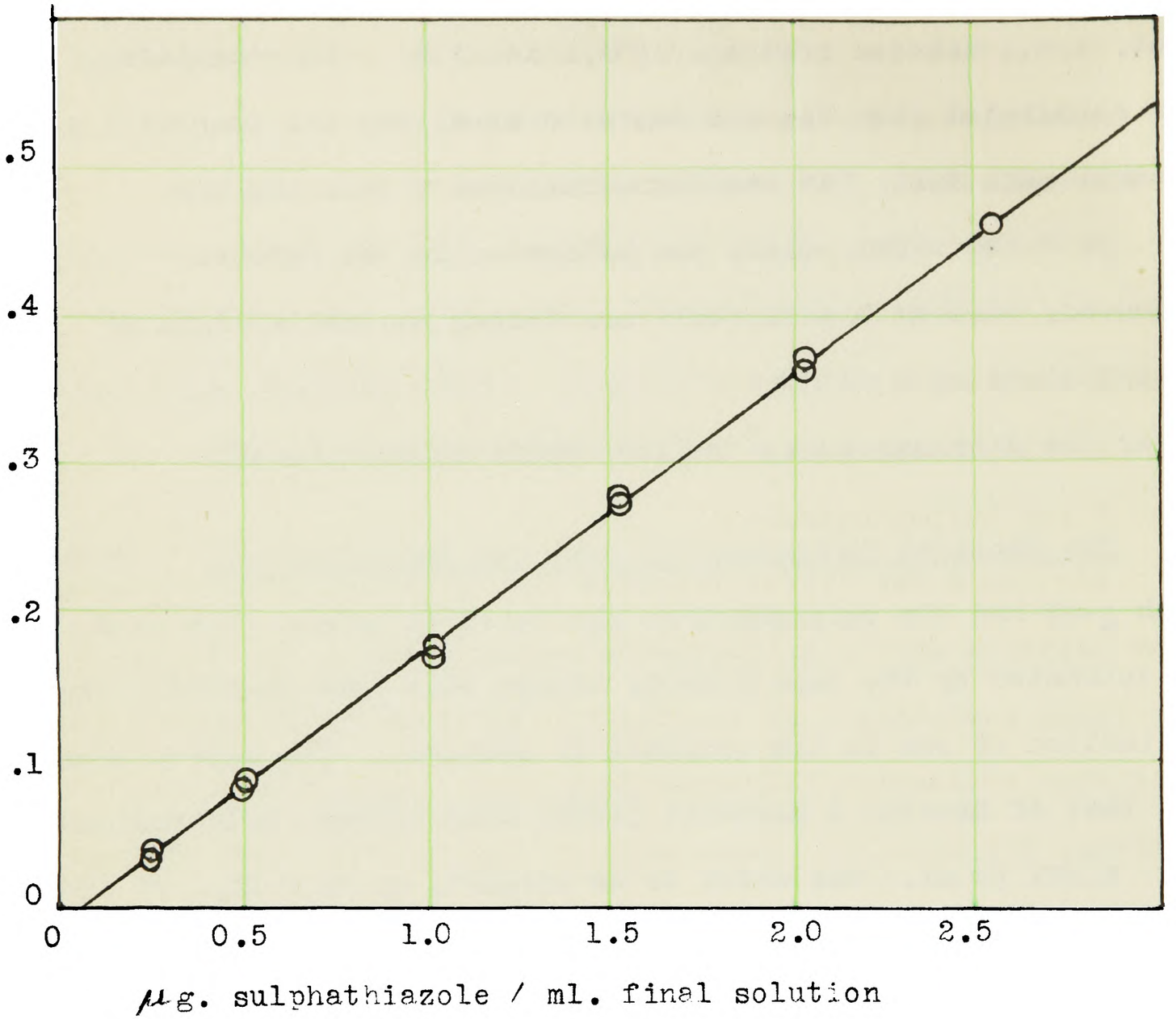


Fig. 2 Standard curve for sulphathiazole estimations.

Standard sulphathiazole solutions were diazotised and coupled with N-(1-naphthyl)ethylenediamine (for procedure see text). The intensity of the colour of the solutions so obtained was measured in the "Spekker" absorptiometer; the absorptiometer readings are given in the ordinate above.

For estimation of the sulphonamides the more sensitive N-∝-naphthylethylenediamine was used as a coupling reagent in aqueous solution. It was then unnecessary to bring the pH up with acetate. The colour was estimated in the "Spekker" absorptiometer with a filter transmitting maximally light of 5500 Å wavelength.

A calibration curve for the apparatus used is given in Fig. 2 for sulphathiazole.

Stetten & Fox (1945) reported the formation of an amine by some bacteria during sulphonamide bacteriostasis, which gave the diazo reaction. It was therefore necessary to correct for this when estimating sulphonamides, since its presence would increase the colour. These authors demonstrated that whereas sulphonamides were acetylated by standing with acetic anhydride at room temperature for 30 min., and consequently became no longer diazotisable, their amine remained unaffected. Hence, during sulphonamide estimations on bacterial extracts, control tubes acetylated with 1% acetic anhydride were also included. In all cases no amine was found.

The method has been confirmed as satisfactory, since some experiments not recorded in this thesis demonstrated the formation of this amine by Esch. coli "F" in Stetten & Fox's medium during sulphadiazine bacteriostasis.

PART III

Results

Preliminary Note on the Experimental Results

As a preliminary to the study of the variations of *A. suboxydans* related to sulphonamide resistance, it was necessary to establish the most satisfactory conditions for culture of the organisms. Investigations to this end are recorded in Chapter 9, and in Chapters 10 to 16 the results of studies following the plan outlined in Chapter 6 are recorded.

During this work certain properties of a non-exacting variant led to the discovery of deficiencies in the composition of Medium 2 which might otherwise have remained undetected. The medium not only contained traces of a substance which was most probably p-AB, but was also deficient in some constituent of Ashe's vitamin-free casein hydrolysate. To improve the medium large amounts of ether-extracted casein hydrolysate were required, so routine subcultures were maintained in ordinary Medium 2, and the use of the improved medium was restricted to occasions when quantitative measurements had to be made. Certain critical tests which had been performed before the discovery of the improved medium were repeated using it.

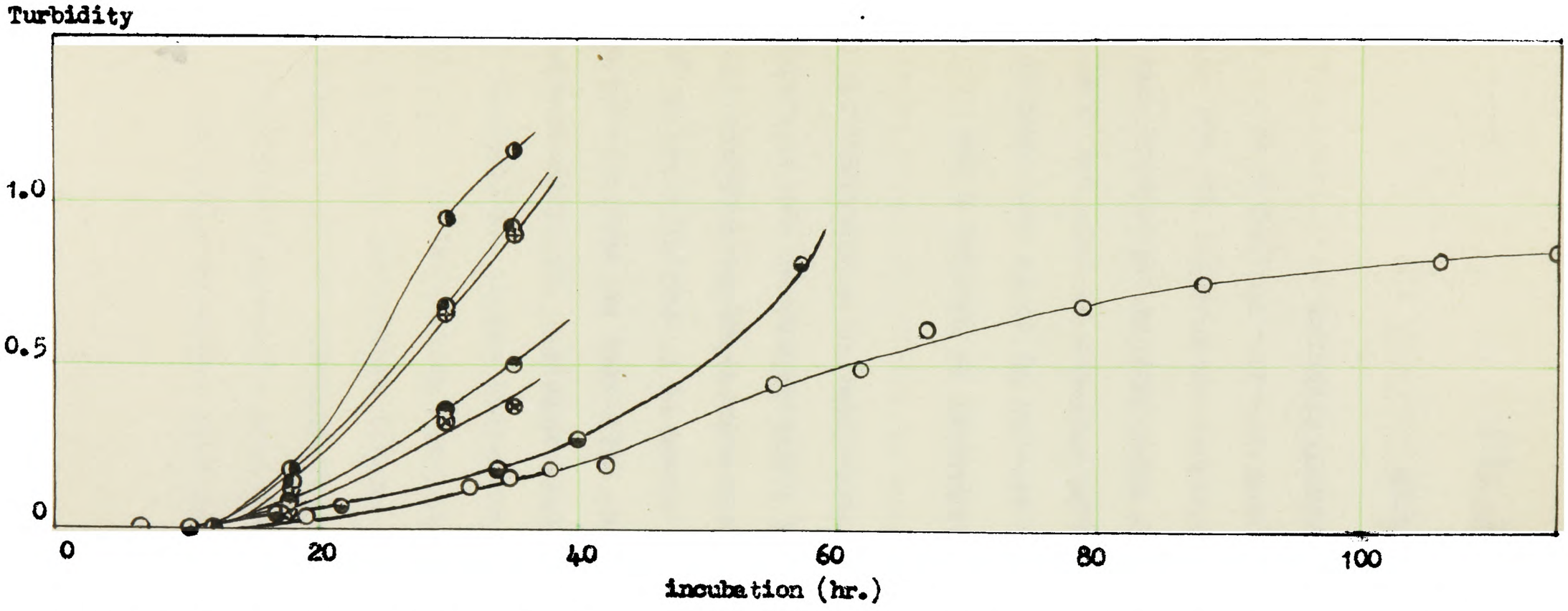


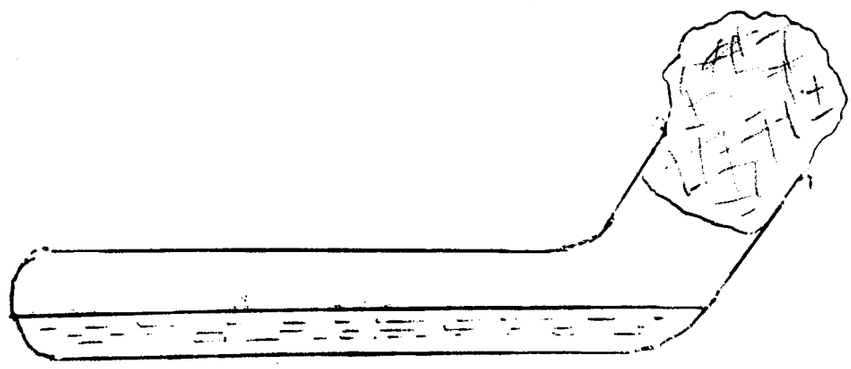
Fig. 3. Growth of A. suboxydans in cultures of various depths. Normal cells were grown in medium 2 with p-AB (10^{-7} M) contained in special test tubes (see text) or in 500 ml. flasks containing 50 ml. culture. ◑ 3 ml. culture ⊕◐ 4 ml. culture ⊗◓ 5 ml. culture ◒ 50 ml. culture, shaken frequently ○ 50 ml. culture, undisturbed

Chapter 9

The Cultivation of A. suboxydans 621

Effect of Aeration. The normal organism is aerobic and requires optimal conditions of aeration for optimal growth, as was well illustrated by the results shown in Fig. 3. It was inoculated into Medium 2 supplemented with 10^{-7}M p-AB in bent test tubes of the kind illustrated below, containing 3, 4 and 5 ml. portions of the medium. The total capacity of the tubes was 12 ml. and they were so designed as to fit into the Hilger "Spekker" absorptiometer, by means of which the growth rates were measured. Readings were not taken at high growth levels as the relation between turbidity and population was not linear above a reading of about 1.0 on the absorptiometer (page 54).

Simultaneously two 50 ml. cultures in 500 ml. conical flasks were sown, one being shaken frequently and the other being allowed to grow without disturbance except that consequent on the removal of samples.



Special test tube

In these conditions the cultures grew at the various rates shown in Fig. 3. While these results provided no quantitative data as to the amount of air available to the organisms, it was clear that the growth rate was conditioned by the availability of air. For this reason all the work reported here was carried out with the greatest aeration compatible with experimental convenience: using 5 ml. aliquots of culture in 25 ml. conical flasks. It is likely that even in these conditions the growth rate was not quite optimal, so where strictly optimal conditions were required the organism was continuously aerated during growth. This has been mentioned in Chapter 8 (page 48).

Amino-acid Medium. The failure of the laboratory strain of A. suboxydans 621 to grow satisfactorily in the synthetic medium of Stokes & Larsen (1945, a) has been mentioned in Chapter 8 (page 49) and will not be discussed further.

Effect of Glucose. Tosic (1946), during work on the oxidations performed by Acetobacter turbidans, used a medium containing ethyl alcohol and sodium lactate. The use of this mixture slowed down the decrease in pH that normally accompanied growth in alcohol, as the product of oxidation of the lactate was the bicarbonate ion, which reacted with the acetic acid formed from the alcohol. Some pilot experiments with A. suboxydans showed that it would not grow in a medium in which glycerol was replaced by either sodium lactate or a mixture of sodium lactate and ethyl

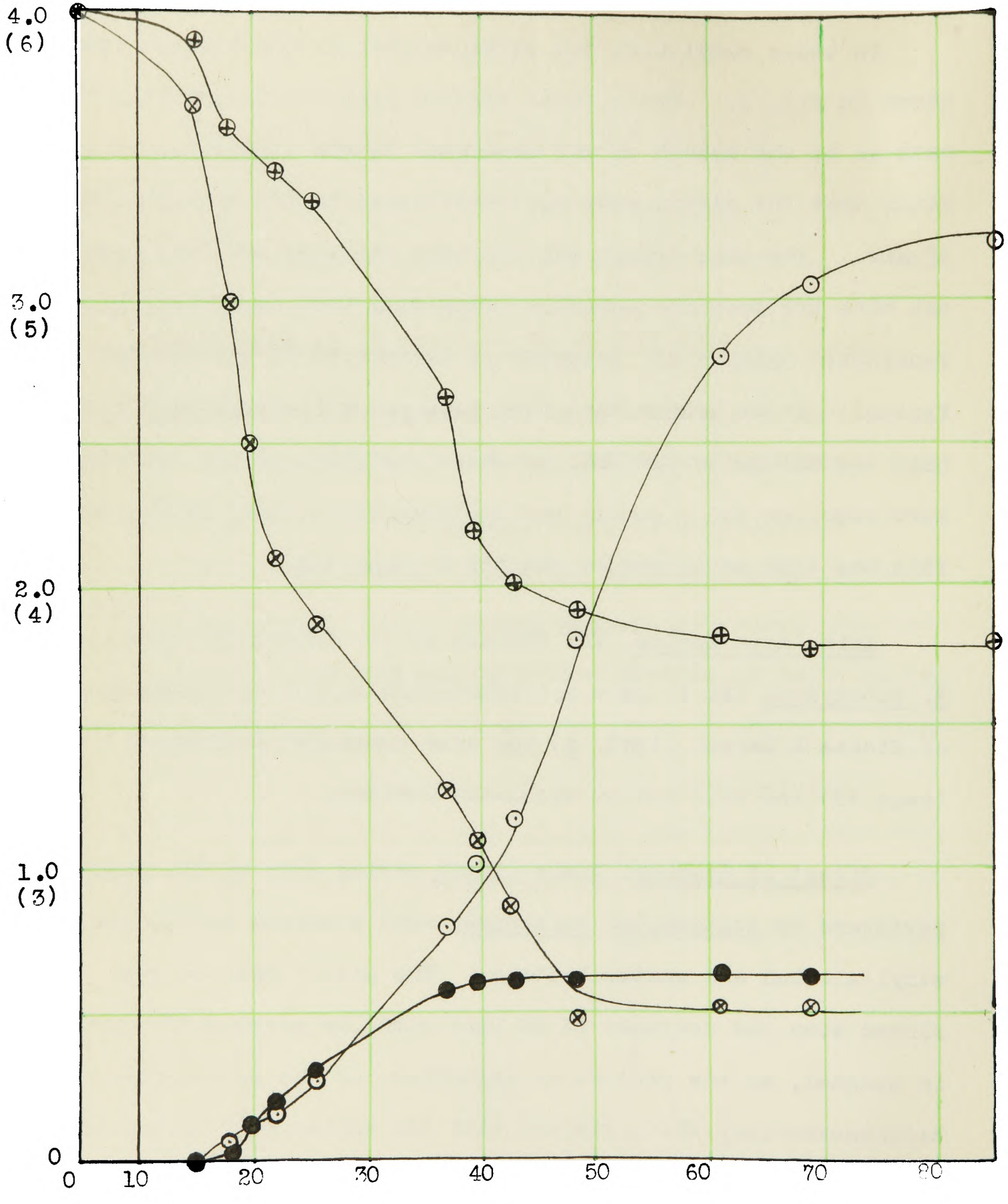


Fig. 4 for legend see opposite.

Fig. 4 **Growth and pH in cultures of *A. suboxydans*.**

Ordinate: turbidity of culture or pH (in brackets)

Abscissa: incubation (hr.)

○ growth in glycerol medium

⊕ pH of glycerol medium during growth

● growth in glucose medium

⊗ pH in glucose medium during growth

cultures of *A. suboxydans* were grown in media containing glucose or glycerol as carbon source and samples were removed during growth for estimation of pH and turbidity.

alcohol. If glucose or a mixture of glucose and lactate was used in place of glycerol the organism grew, but the final cell population was greatly diminished. It seemed likely that this was due to the extremely rapid decline in the pH which took place in the presence of glucose. This is illustrated in Fig. 4.

Although it is outside the scope of this thesis, it is interesting to speculate on the condition of the organism used in the present work, which reduced the pH of its medium to about 3.8 in the presence of glycerol, though it is reputed (page 42) only to be able to oxidise this to the neutral dihydroxyacetone. Even more surprising was the formation from 0.05 M glucose - from which only gluconic acid should be formed - of an acid capable of bringing the pH down to about 2.5. The pK values for gluconic and glucuronic acids (respectively formed as the first and third stages in the oxidation of glucose) are in the region of 4.1, and they are thus incapable of bringing the pH to a value much lower than this figure. It is therefore most likely that the organism had developed the power to oxidise glucose and glycerol far beyond the single stage originally reported.

During tests on the activity of the culture fluid of the mould Ophiostoma multiannulatum as growth factor for A. suboxydans (reported in Section II of this dissertation), it was noticed that the basal medium used for this mould (medium 9) stimulated growth when added to Medium 2 containing sub-

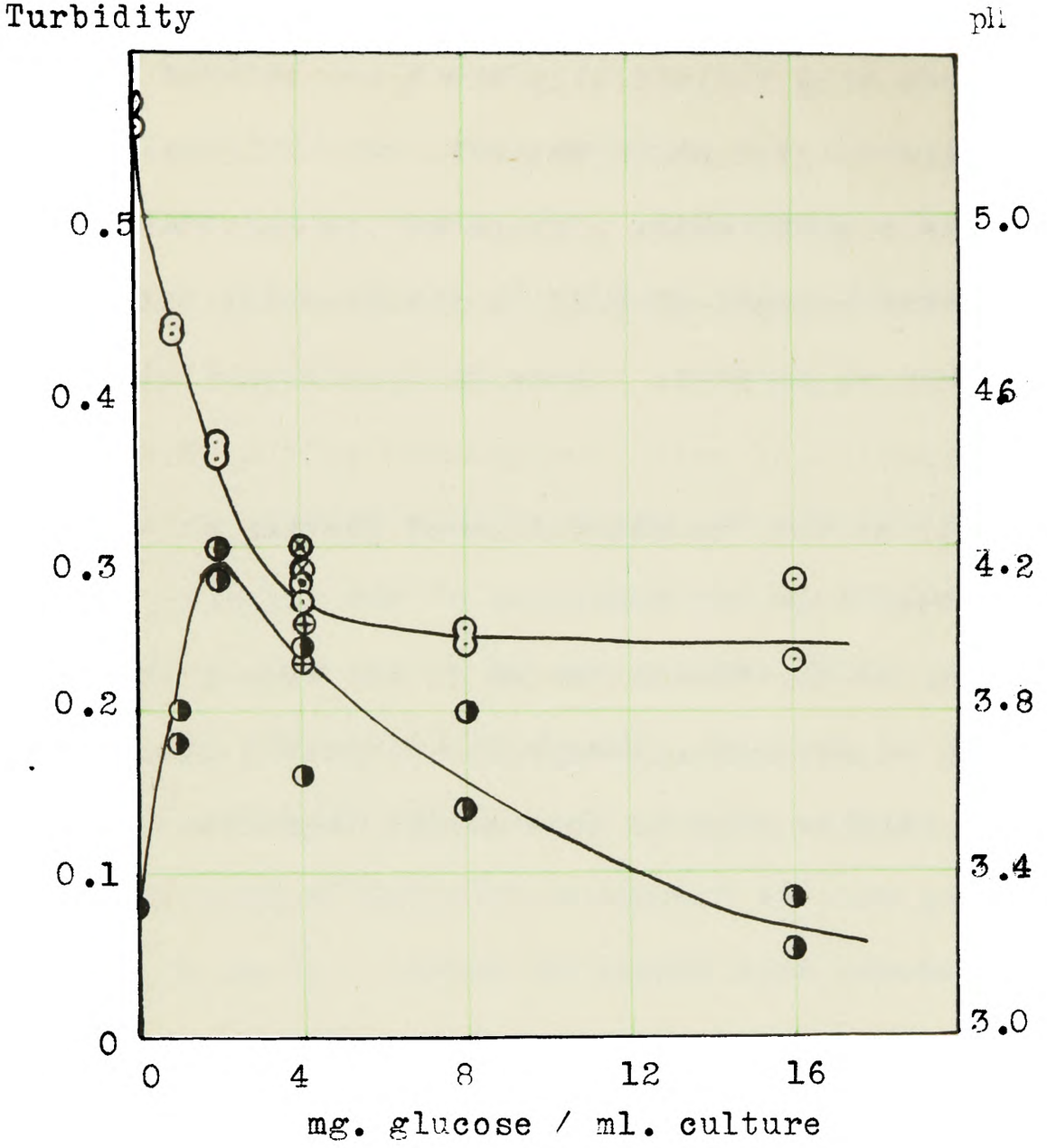


Fig. 5 Growth and pH in cultures of A. suboxydans containing glucose.

Cultures of A. suboxydans were prepared in medium 2 with suboptimal concentration of p-AB (5×10^{-9}M) and various amounts of glucose (added before autoclaving except where mentioned). Growth and pH were measured after 66 hr. incubation.

◑ growth; (⊕ Seitz-filtered glucose added after autoclaving)

○ pH; (⊗ Seitz-filtered glucose added after autoclaving)

optimal p-AB. By testing individually the components of Medium 9 the effect was traced to glucose. The effect is illustrated by the test recorded in Fig. 5, where glucose at low concentrations stimulated growth with suboptimal p-AB, but became inhibitory if added in greater amounts. It is likely that this inhibition was also due to the formation of acid from glucose as the pH reached a constant low level at that concentration of glucose at which inhibition began (Fig. 5).

The effect was not due to substances formed from glucose during autoclaving, since controls to which glucose sterilised by Seitz-filtration had been added gave similar results.

TABLE 1 Growth of *A. suboxydans* with *p*-AB

(a) in Medium 2 :

Added *p*-AB (M)	Incubation (hr.) 18	42	66	90	114
10^{-7}	-	++	+++++	+++++	+++++
4×10^{-8}	-	++	+++++	+++++	+++++
2×10^{-8}	-	++	+++++	++++	+++++
8×10^{-9}	-	+	++	+++	++++
4×10^{-9}	-	-	tr.	+	+++
1.6×10^{-9}	-	-	-	+	++
8×10^{-10}	-	-	-	tr.	+
3.2×10^{-10}	-	-	-	-	+
1.6×10^{-10}	-	-	-	-	tr.
no addition	-	-	-	-	tr.

(b) in the improved form of Medium 2 :

10^{-7}	-	+++	+++++	+++++
4×10^{-9}	-	-	tr.	+
1.6×10^{-9}	-	-	-	tr.
no addition	-	-	-	tr.

TABLE 2 Growth of A. suboxydans with pteroylglutamic acid

Pteroylglutamic acid represented as PtGA. Medium 2.

Added PtGA (M)	Incubation (hr.) 31	46	70	94
10^{-6}	tr.	+	+++	++++
2.5×10^{-7}	-	-	tr.	++
1.2×10^{-7}	-	-	-	+
no addition	-	-	-	+

TABLE 3 Growth of A. suboxydans with adenine

Medium 2, improved form; A control with p-AB is included for comparison.

Added adenine (M)	Incubation (hr.) 17	42	65	89
10^{-4}	-	tr.	tr.	+
10^{-5}	-	tr.	tr.	+
10^{-6}	-	?	tr.	tr.
no addition	-	-	-	tr.
From another experiment:				
2×10^{-4}	tr.	tr.	+	+
(10^{-7} M p-AB.)	tr.	+++	++++	+++++

Chapter 10

On the Nutrition of A. suboxydans 621

Growth factor requirements. Cultures prepared and inoculated as described in Chapter 8 grew slowly. Growth was perceptible after 24 hr. in Medium 2 with optimal added p-AB, but the logarithmic phase was not over until after some 72 hr.

A typical titration of growth response against added p-AB is given in Table 1. In the improved basal medium the limiting concentration of p-AB which would support growth lay between 4×10^{-9} and 1.6×10^{-9} M, as is also shown in Table 1.

p-Aminobenzoic acid was replaceable by synthetic pteroylglutamic acid, but the requirement for this was considerably greater (Table 2). It lay between 2.5×10^{-7} and 1.2×10^{-7} M.

Landy & Streightoff (1943) recorded that adenine would decrease the requirement of A. suboxydans for p-AB. The results given in Table 3, however, showed that visible growth occurred with adenine in the complete absence of p-AB. The experiment was performed with a synthetic specimen of adenine (kindly supplied by Prof. A. R. Todd) and with the improved form of Medium 2, so there is little likelihood that the effect was due to synergism between adenine and traces of p-AB present as an impurity. Since adenine is known to be a component of nucleoproteins it was thought worth while to see if D-ribose had any synergistic effect with adenine. The results shown in Table 4 gave no indication of such an effect.

TABLE 4 Growth of *A. suboxydans* with adenine - ribose and adenine - pteroylglutamic acid mixtures

Pteroylglutamic acid written as PtGA. Medium 2.

Added material (M)	Incubation (hr.) 18	42	62	90
Adenine, 10^{-4}	tr.	+	±+	++
Adenine, 10^{-4} + D-ribose, 10^{-3}	tr.	+	±+	++
From another experiment:	19	43	67	91
Adenine, 10^{-4} + PtGA, 2×10^{-7}	-	++	+++	++++
Adenine, 10^{-4} (synthetic) + PtGA, 2×10^{-7}	-	++	+++	++++
PtGA, 2×10^{-7}	-	+	+	++

Lampen, Roepke & Jones (1946) demonstrated that a mixture of thymine (3×10^{-4} M), methionine (2×10^{-4} M), guanine (4×10^{-4} M) and xanthine (5×10^{-4}M) could substantially replace p-AB as a growth factor for a mutant of Esch. coli requiring this substance. The mixture was not completely soluble in Landy & Dicken's medium at pH 6.0, as xanthine and guanine did not remain in solution, but a medium saturated with the mixture had no significant growth factor or anti-sulphonamide effect with A. suboxydans; that is to say its growth factor effect was no more than would be expected from the adenine present. No anti-sulphathiazole effect was noticed with the mixture in spite of its adenine content, but the presence of insoluble material was probably sufficient to mask any such effect. The mixture was found to act synergistically with pteroylglutamic acid as a growth factor, but again this effect was traced to adenine and was independent of the other components of the mixture. This is also indicated in Table 4.

It has been mentioned already that synthetic pteroylglutamic acid had roughly 2% of the activity of p-AB as a growth factor for A. suboxydans. The implication of this was either that it was acting as a poor source of p-AB for the organism, or that the specimen was contaminated with p-AB. Other work in this laboratory indicated that, while impurity such as p-AB might have been present, it did not exceed 0.03 moles % (Marshall, 1948). This was insufficient to account for the growth factor

TABLE 5 Competitive anti-sulphanilamide activity of p-AB and pteroylglutamic acid (PtGA) for A. suboxydans

Medium 2; cultures were incubated for 7 days.

Sulphanilamide (M)	p-AB (M)		PtGA (M)	
	(a)	(b)	(a)	(b)
10^{-3}	10^{-7}	4×10^{-8}	4×10^{-5}	2×10^{-5}
10^{-4}	2×10^{-8}	8×10^{-9}	10^{-5}	4×10^{-6}

(a) This concentration permitted visible growth

(b) This concentration prevented visible growth

TABLE 6 Non-competitive anti-sulphathiasole activity of adenine for *A. suboxydans*

Medium 2.

Adenine (M)	Sulphathiasole (M)	Incubation (hr.) 17	42	65	89
10^{-4}	2×10^{-3}	-	tr.	±	+
10^{-4}	10^{-3}	-	tr.	±	+
10^{-4}	2×10^{-4}	-	tr.	±	+
10^{-4}	10^{-4}	-	tr.	±	+
10^{-4}	2×10^{-5}	-	tr.	±	+
10^{-4}	10^{-5}	-	tr.	±	+
10^{-4}	-	-	tr.	±	+
no addition	-	-	-	-	tr.

activity of pteroylglutamic acid, so it is likely that this material itself acted as a growth factor.

Effect of growth factors as anti-sulphonamides. Measurements of the amounts of p-AB and pteroylglutamic acid respectively required to overcome the inhibitory action of a fixed amount of sulphonamide were made, with the results shown in Table 5. In both cases there was a tenfold decrease in the requirement for these substances following a tenfold decrease in drug concentration. Hence both substances were acting as competitive anti-sulphonamides. In order to fit in with other work in this laboratory, these particular experiments were performed using sulphanilamide in place of the customary sulphathiazole, but their general conclusions are doubtless valid for the latter substance.

In contrast, adenine could be shown to act as a non-competitive anti-sulphonamide substance, in spite of the weakness of its activity as a growth factor. The test recorded in Table 6 showed comparable, though weak, growth in a given concentration of adenine over a 200-fold range of sulphathiazole concentration.

Sulphathiazole resistance. For the purposes of comparison with variant strains of A. suboxydans, the resistance of the normal organism to sulphathiazole was measured. In the presence of 10^{-7} M added p-AB it grew with between 10^{-4} and 2×10^{-4} M

TABLE 7 Formation of the sulphathiazole-resistant strain (Strain C)

The normal organism was subcultured serially in Medium 2 with 10^{-7} M p-AB and sulphathiazole concentrations as below.

Serial numbers of subcultures	Sulphathiazole (M)
1, 2	10^{-5}
3, 4, 5	2×10^{-4}
6	4×10^{-4}
7, 8, 9	10^{-3}
10, 11, 12	2×10^{-3}
13	5×10^{-3}
14* et seq.	2×10^{-3}*

*Optimal growth occurred in this and subsequent subcultures.

Chapter 11

The Sulphathiazole-trained Strain

Origin and behaviour of the strain. A "sub-strain" of A. suboxydans (hereafter referred to as strain C) resistant to sulphathiazole was obtained by subculturing the normal organism every three or four days in Medium 2 with 10^{-7} M p-AB and increasing amounts of sulphathiazole. After 12 passages the organism grew optimally in 2×10^{-3} M sulphathiazole. This corresponded to a more than 200-fold increase in sulphathiazole resistance since some inhibition of the untrained strain occurred with 10^{-5} M sulphathiazole and 10^{-7} M p-AB. Owing to the insolubility of sulphathiazole in Medium 2 the resistance could not conveniently be increased further than this. During the training process the parent was inoculated into subcultures containing a range of sulphathiazole concentrations, and the culture that showed visible growth at the highest concentration was used as inoculum for the next subculture. Figures are given in Table 7 showing the progress of the training.

The strain was maintained by serial subculture in Medium 2 containing 10^{-7} M p-AB and 2×10^{-3} M sulphathiazole for more than one hundred and forty passages, and its growth rate and extent of growth were similar to those of normal cells grown in Medium 2 containing only 10^{-7} M p-AB. It was distinguished, however, from the normal organism in that it was now capable of

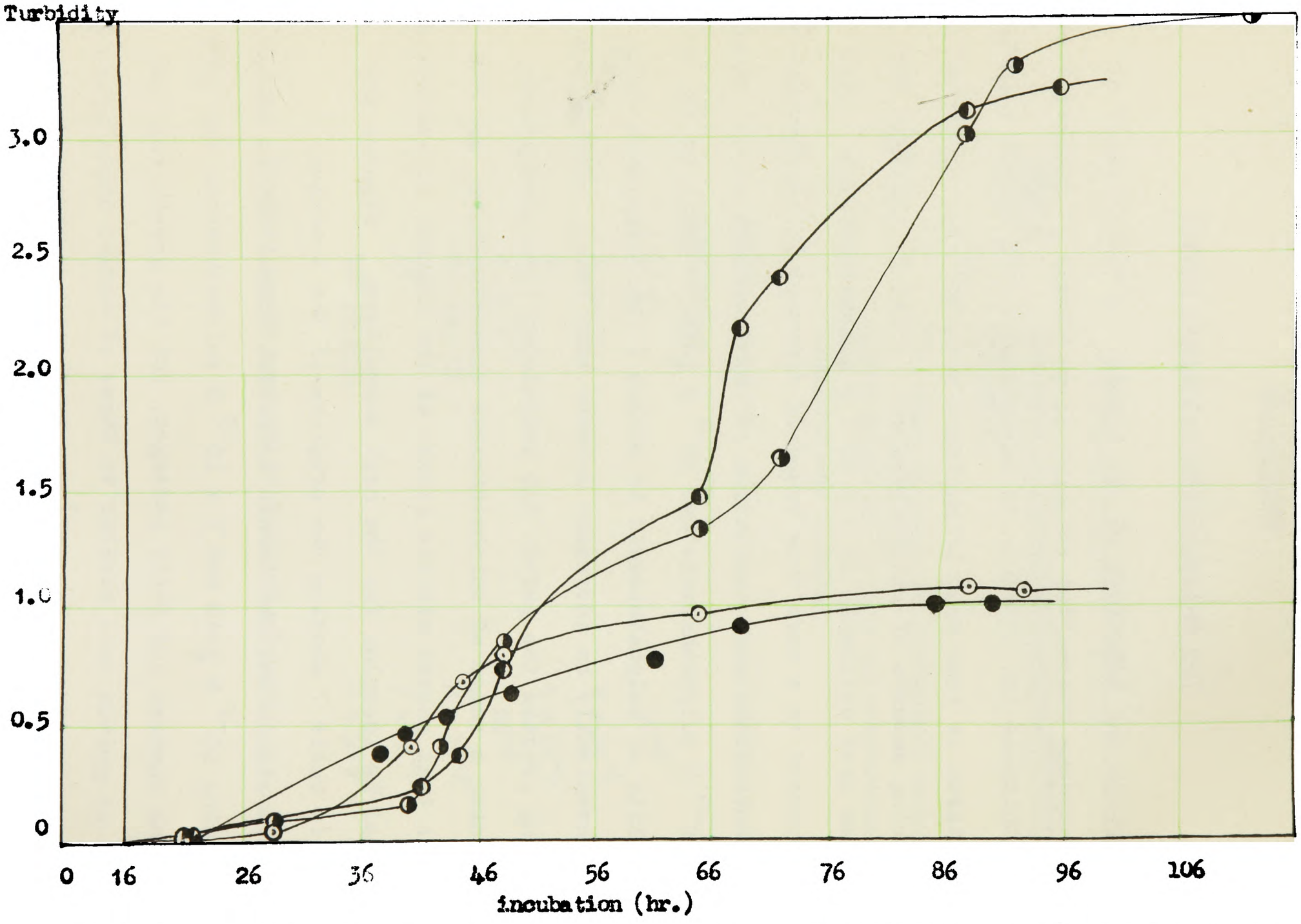


Fig. 6. Growth curves of various strains of *A. suboxydans* in medium 2. See legend opposite.

Fig. 6 Legend.

◑ normal A. suboxydans with p-AB (10^{-7}M)

◐ strain C with p-AB (10^{-7}M) and sulphathiazol (2×10^{-3}M)

○ strain A

● strain C with neither p-AB nor sulphathiazol

limited growth in Medium 2 without added p-AB if sulphathiazole was also omitted. These characteristics are illustrated by the growth curves in Fig. 6. These growth curves are rather irregular, like all growth curves obtained with A. suboxydans in Medium 2; growth curves obtained with the improved medium (page 73, also Fig. 9) were more regular. The limited final population obtained with strain C grown in Medium 2 with neither p-AB nor sulphathiazole was not due to self-inhibition by acid formation since growth ceased at a relatively high pH (c. 5.2) compared with cultures grown with p-AB (c. 3.6) (see also Table 11, page 70). Nor was it due to insufficient casein hydrolysate in Medium 2, as growth was also limited in the improved medium, which contained more casein hydrolysate (page 73, Fig. 9). Another difference between strain C and the normal strain is shown in Fig. 19 (facing page 87). The growth/concentration curve for p-AB was not linear with strain C, though it was for the untrained organism.

Stability. After more than 54 subcultures in Medium 2 with sulphathiazole, strain C was tested for any tendency to lose its resistance to sulphathiazole. It had its normal sulphathiazole resistance after (1) one passage in Medium 2 without sulphathiazole, (2) four passages in Medium 2 with neither p-AB nor sulphathiazole, or (3) thirty four passages on glycerol-yeast agar (Medium 1).

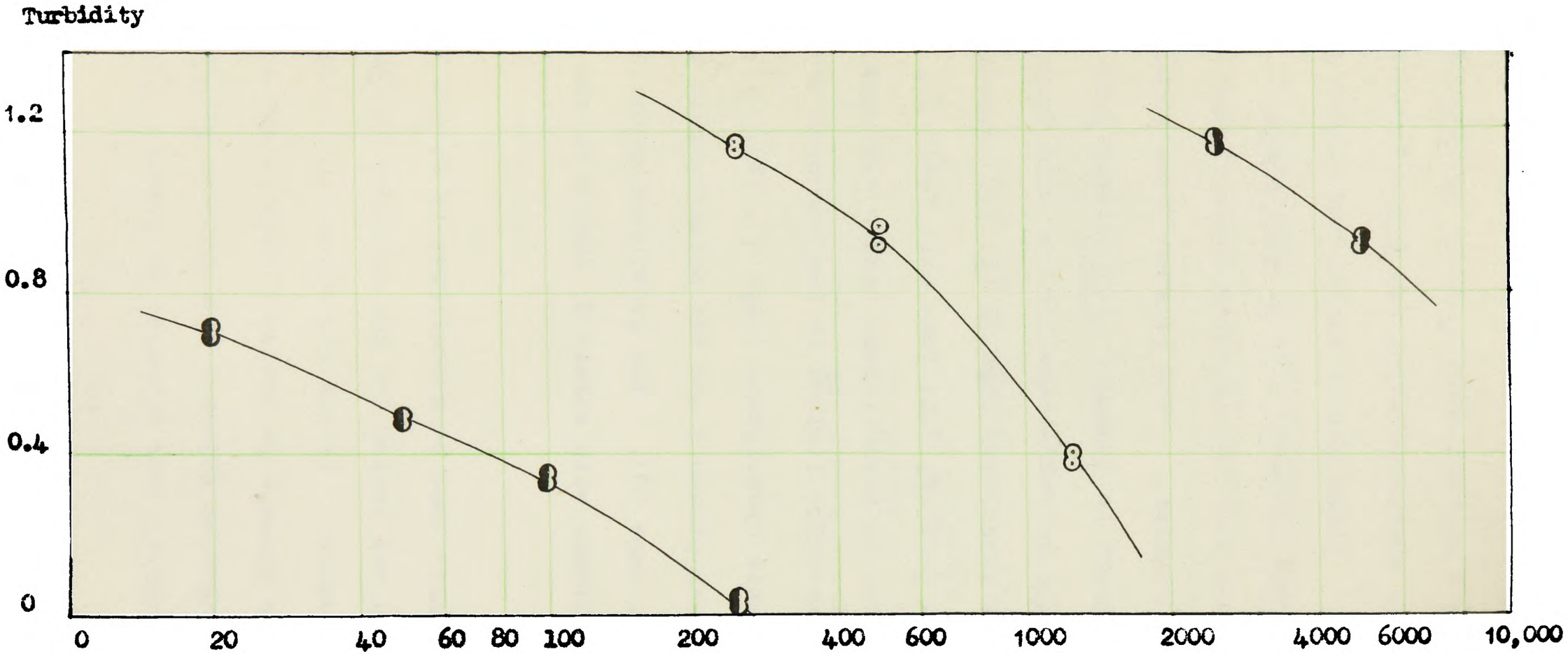


Fig. 7. Growth of strain C with sulphathiazole at various levels of p-AB. Cultures of strain C were grown in medium 2 and turbidities read after incubation for 95 hr.

◑ p-AB, 10^{-7} M ○ p-AB, 10^{-8} M ◐ p-AB, 10^{-9} M

TABLE 8 Comparison of the requirements of strain C and normal A. suboxydans for p-AB

Cultures were grown in Medium 2 containing various amounts of p-AB; the cultures of strain C all contained 2×10^{-3} M sulphathiazole.

Strain of bacteria	Incubation (hr.)	p-AB (M) (b)	(a)
Normal	90	4×10^{-9}	1.6×10^{-9}
C	90	4×10^{-9}	1.6×10^{-9}
C	162	1.6×10^{-9}	8×10^{-10}

(a) This concentration permitted visible growth.

(b) This concentration did not permit visible growth.

Sulphonamide resistance and p-AB requirement. The p-AB requirement of strain C in the presence of its normal amount of sulphathiazole was little different from that of normal cells growing in the absence of sulphathiazole. If measured at comparable times they were the same (Table 8). With normal cells there was a tendency for cultures containing no added p-AB to grow after about 100 hr. incubation, but this did not occur with strain C in the presence of sulphathiazole. Consequently it was possible to incubate the test cultures for a longer period, and when this was done growth occurred at p-AB concentrations slightly lower than the minimal requirement of the normal strain (Table 8). It is reasonable to conclude that the requirement of A. suboxydans for p-AB is unchanged by training to resist sulphathiazole if the two strains are tested in strictly comparable conditions.

The strain was susceptible to inhibition by sulphathiazole at still higher concentrations. Though the insolubility of this drug prevented the demonstration of complete inhibition in the presence of 10^{-7} M p-AB, sulphanilamide at 10^{-2} M concentration was shown to stop growth completely with this amount of p-AB. The resistance of the strain to sulphathiazole was still a function of the amount of added p-AB and the relationship was still competitive (Fig. 7). The curves have roughly the same form over a 100-fold range of concentration of both drug and inhibitor

Destruction of sulphathiazole. The possibility that the strain destroyed sulphathiazole was studied by estimating this

TABLE 9 Failure of strain C to destroy sulphathiazole

Cells were incubated with sulphathiazole in various media and sulphathiazole was subsequently estimated (for method see text) after removal of cells by centrifuging. Sulphathiazole was estimated similarly after growth of a culture in its presence.

A. Tests with a cell suspension:

Suspending medium	p-AB (M)	Incubation (hr.)	µg. sulphathiazole found/ml.
Medium 2	3×10^{-8}	6	1.67
Medium 2	-	6	1.67
Water	3×10^{-8}	6	1.67
Water	-	6	1.65
Medium 2 without cells	3×10^{-8}	6	1.81

B. Tests with a growing culture:

Medium 2 and an inoculum	10^{-7}	52*	3.58
Medium 2 and a boiled inoculum	10^{-7}	52*	3.62

*After 52 hr. these cultures showed respectively optimal growth and no growth at all.

substance after incubation with cells of strain C. This was investigated both with growing cultures and with heavy cell suspensions. The drug was estimated by Bratton & Marshall's (1939) method (page 55). In view of the possibility that the diazotisable non-acetylable amine of Stetten & Fox (1945, page 56) might be formed, and would then interfere with the sulphathiazole estimations, controls acetylated at room temperature for 30 minutes with 1% acetic anhydride were tested in all cases, but no such amine was found. The results, indicating that the cells had no action on sulphathiazole, are shown in Table 9.

TABLE 10 Stimulation of the growth of strain A with pteroyl-glutamic acid (PtGA), p-AB or glucose

Added to Medium 2	Incubation (hr.) 20	44	68
p-AB 10^{-7} M	tr.	+++	+++++
p-AB 10^{-8} M	tr.	+++	+++++
p-AB 10^{-9} M	tr.	++	+++
PtGA 10^{-5} M	tr.	+++	+++++
PtGA 10^{-6} M	tr.	++	+++++
PtGA 10^{-7} M	tr.	+	++++
no addition	tr.	+	+++
From another test:	17	41	65
Glucose 0.5 mg.	tr.	+++	++++
no addition	tr.	++	+++

Chapter 12

The Non-exacting Strain

Origin of the strain. The tendency for an occasional control culture to show growth in the absence of added p-AB after prolonged incubation has already been mentioned, and from one such culture a "sub-strain" capable of being serially subcultured in the absence of p-AB was obtained and was maintained for more than 150 passages in liquid medium. It will be referred to as "strain A".

Limited growth of strain A. In Medium 2 the extent of final growth of this strain was always less than that of the normal organism or of strain C growing optimally in their usual conditions, though the lag and growth rate were similar. The limited extent of growth is illustrated in Fig. 6 (page 66).

Stimulation of the growth of strain A. The final population of culture of strain A could be increased by several substances, sometimes so as to give growth comparable with the optimal growth of the other two strains. This is shown in Table 10, for the cases of p-AB and pteroylglutamic acid. As seems usually to be the case with A. suboxydans, pteroylglutamic acid had again only c. 1% of the activity of p-AB.

Table 10 also records a similar effect of glucose, though in this case stimulation of the final growth to optimal was not

TABLE 11 Growth and pH of cultures of various strains of A. suboxydans

Cultures were grown in Medium 2 ("glycerol") with and without various supplements as recorded. "Glucose-lactate" medium was Medium 2 in which glycerol was replaced by glucose (0.05 M) + sodium lactate (0.15 M).

Strain	Medium	growth after 72 hr.	pH after 72 hr.
Normal	Glycerol + p-AB, 10^{-7} M	+++++	3.60
A	Glycerol	+++	4.06
A	Glycerol + p-AB, 10^{-7} M	+++++	3.60
C	Glycerol + p-AB, 10^{-7} M + sulphathiazole, 2×10^{-3} M	+++++	3.60
C	Glycerol + p-AB, 10^{-7} M	+++++	3.68
C	Glycerol	+++	5.21
Normal	Glucose-lactate + p-AB, 10^{-7} M	++	3.95

obtained. The effect was not surprising in view of the known stimulatory effect of glucose on the growth of normal A. suboxydans, and its appearance with strain A made unlikely the possibility that the normal limited growth of strain A was due to a low pH being reached in the medium. It has been seen (page 59) that glucose could be inhibitory to A. suboxydans at higher concentrations, and that this inhibition was likely to be due to the low pH reached as a result of glucose fermentation. Were such a pH effect occurring with strain A, addition of glucose would be expected to enhance it; in other words, to repress growth further. That this was not so was confirmed by measuring the pH of various cultures after incubation for 72 hr. These measurements are recorded in Table 11, which also shows that a low pH did not account for the limitation of the growth of strain C in the absence of sulphathiazole and p-AB.

A number of vitamins, purines and similar compounds were tested for their effect on the growth of strain A. The substances listed below were shown to be without activity up to the concentrations mentioned:-

Methionine, Thymine, Uracil, Cytosine, Guanine, Adenine — Up to 10^{-4} M

Biotin — up to 0.1 µg/ml.

D-Ribose — up to 10^{-3} M

Histidine — up to 10^{-3} M

Riboflavin, Thiamine, Pyridoxin, Nicotinamide, m-Inositol, Glutamine, Choline-HCl — up to 1 µg/ml.

Pyridoxal — up to 5×10^{-6} M

TABLE 12 Stimulation of the growth of strain A by additional "vitamin-free" casein hydrolysate

Two batches of casein hydrolysate (Ashe) were tested in Medium 2 at 0.6%. Turbidities were read after incubation for 68 hr.

Batch of casein hydrolysate	mg. dry wt. cells/ml.
103	0.800
1147	0.785
-	0.225

The strain was found, however, to be stimulated by an increase in the amount of vitamin-free casein hydrolysate added to the medium, and it was this discovery which led to the use of the improved medium for quantitative work with A. suboxydans 621 (pages 57 & 73). The stimulation was shown by two different batches of Ashe's product when added to give a 3-fold increase in the amount of casein hydrolysate present. The effect is recorded in Table 12, and was also shown to occur with the normal strain. This latter result is not given here as the effect is better illustrated in the growth curves to be described later (Fig. 9, page 74).

A number of pure amino-acids were therefore tested for activity, excluding tryptophan and cystine which were already present in Medium 2 in high concentrations. The amino-acids were added in amounts based on the figures given by Jordan-Lloyd & Shore (1938) for the composition of caseinogen, so as to allow a two or three-fold increase in the amount of a given amino-acid present. Threonine, for which these authors did not give figures, was arbitrarily added to Medium 2 at 0.01%. The following amino-acids proved inactive:-

TABLE 13 **Tests of aspartic acid and various other substances as growth stimulants for strain A**

Three separate tests are recorded here.

	Added to Medium 2		Incubation (hr.) 19	42	66	
1.						
	Aspartate	3×10^{-3} M	-	++	+++	
	Aspartate	6×10^{-3} M	-	+++	++++	
	Aspartate	9×10^{-3} M	-	+++	++++	
	(10% CO_2)		-	+	+++	
	NH_4NO_3	0.01%	-	±	+++	
	-		-	±	+++	
			18	41	65	
2.						
	Malate	3×10^{-3} M	tr.	++	+++	
	-		tr.	++	+++	
			20	46	62	92
3.						
	Asparagine	6×10^{-3} M*	tr.	+	+++	+++
	-		tr.	++	+++	+++

*A norite-treated re-crystallised specimen of natural asparagine was used, as a synthetic specimen was not available.

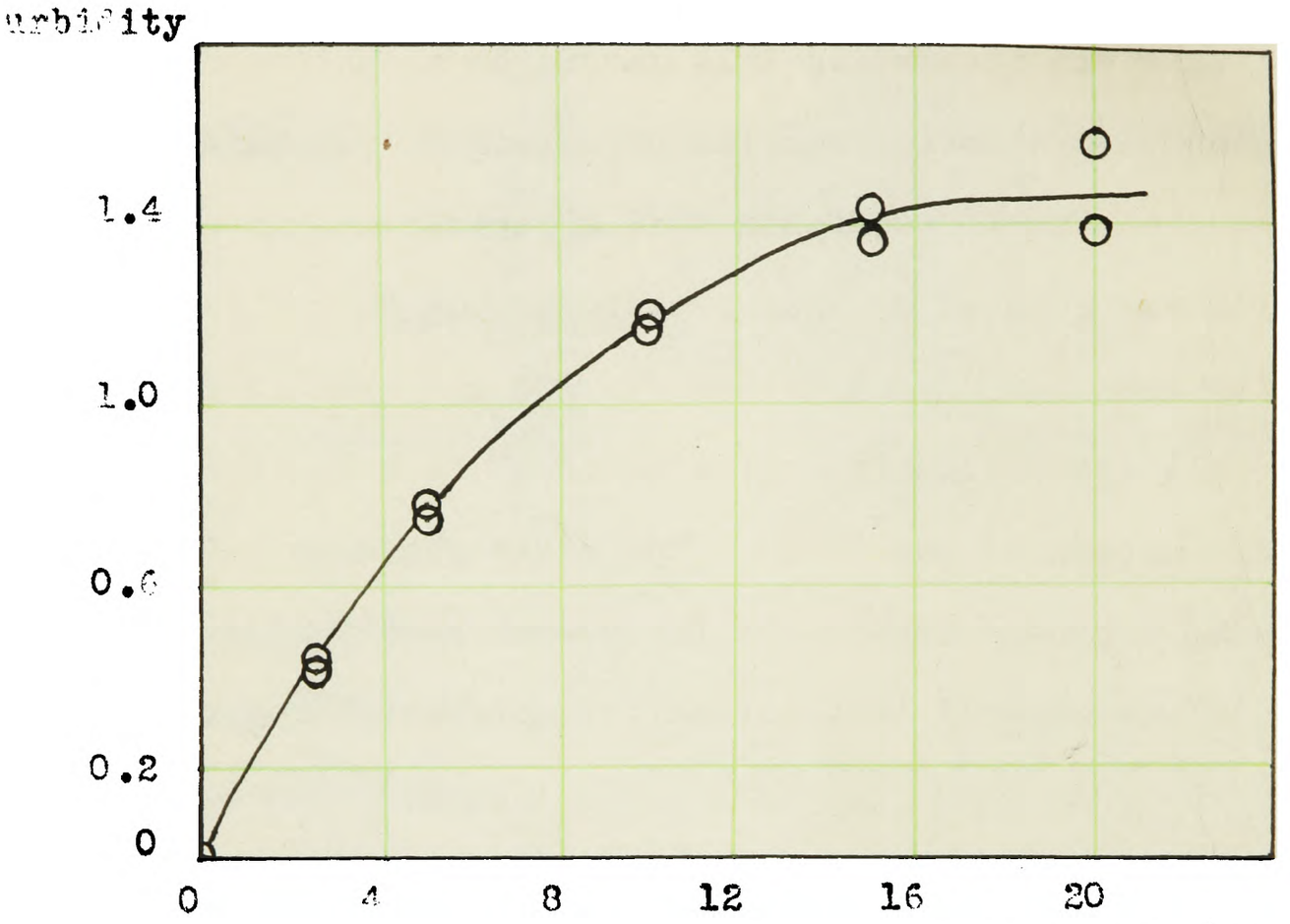
Amino-acid Concentration in Medium 2 (%)

Glycine	0.015
Alanine	0.03
Valine	0.1
Leucine	0.06
iso-Leucine	0.02
Serine	0.005
Phenylalanine	0.04
Tyrosine	saturated
Glutamic acid	0.3
Proline	0.04
Arginine	0.03
Lysine	0.03
Threonine	0.01

Aspartic acid added to 0.04% showed some stimulatory effect on the growth of strain A. Even at the highest active concentrations tested, however, this substance was not as effective as casein hydrolysate, and would not permit growth equivalent to that of the normal strain growing optimally. This is shown in Table 13, which also shows that asparagine and malic acid (both chemically related to aspartic acid) were inactive. In view of recent work relating aspartic acid to biotin and CO_2 metabolism a test was set up growing the strain in 10% CO_2 (biotin had been eliminated already). This showed no effect (Table 13). Ammonium nitrate was also tested and shown to be without activity (Table 13).

The enhanced growth of strain A in the presence of increased amounts of casein hydrolysate could, of course, be due to contamination of Ashe's product with p-AB, and it was shown that this was very likely. Woods (1940) demonstrated that p-AB

Fig. 3 Growth of strain A with purified casein hydrolysate.



Strain A was grown in medium 2 made up without casein hydrolysate to which were added various amounts of a specimen of vitamin-free casein hydrolysate (Ashe) which had been further purified by ether extraction (see text). Growth was estimated after incubation for 72 hr.

could be extracted from aqueous solution at pH 3.8 with ether, so 100 ml. of a 10% solution of Ashe's vitamin-free casein hydrolysate was treated in this manner in a continuous ether extractor. By testing samples of casein hydrolysate during ether-extraction it was found that a certain amount of active material was removed by this procedure, but that a residual activity remained. Extraction for 200 min. was sufficient to remove all ether-soluble active material from the specimen if the same apparatus was used. The ether phase contained material active as a growth factor for the normal strain of A. suboxydans, so it is most likely that the active material being removed was p-AB.

Since the ether-extracted casein hydrolysate still had a residual activity it was necessary to establish the optimal concentration of this material. This was done by adding purified casein hydrolysate to Medium 2 made up without it, and plotting the growth of strain A after a fixed time against the amount of purified material added. The curve obtained (Fig. 8) indicated that as much as 2% of the ether-extracted casein was required for optimal growth. This amount of purified vitamin-free casein hydrolysate was finally adopted for the "improved" basal medium, and used for all quantitative work with A. suboxydans.

The improved medium. Stimulation of the growth of normal A. suboxydans and strain C also occurred with increased amounts

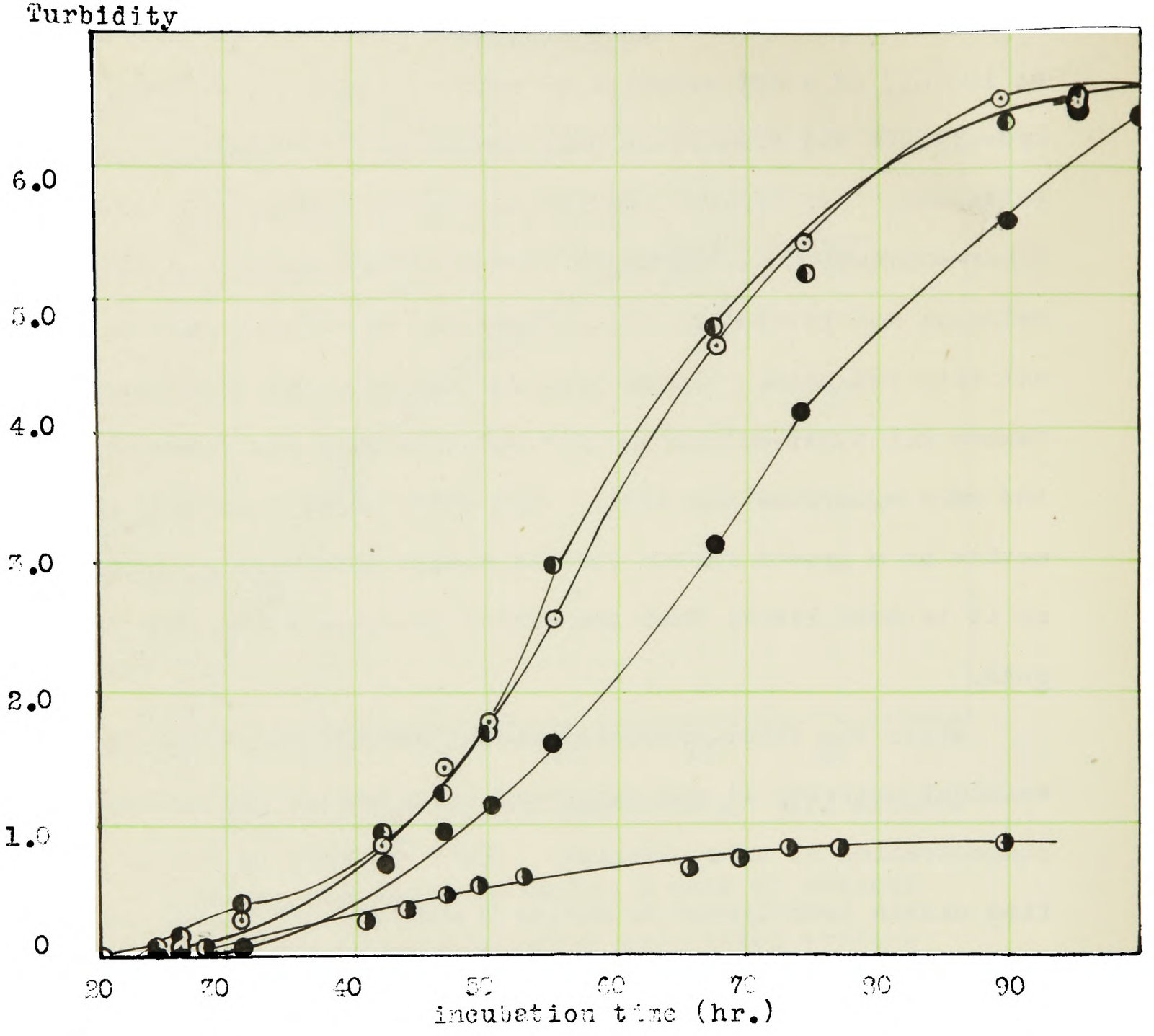


Fig. 9 Growth curves of various strains of A. suboxydans in "improved" medium 2.

- ○ normal A. suboxydans with p-AB (10^{-7}M)
- ● strain A
- ◐ strain C with p-AB (10^{-7}M) and sulphathiazole (2×10^{-3}M)
- ◑ strain C without p-AB or sulphathiazole

TABLE 14 Sulphathiazole resistance of various strains of A. suboxydans

Cultures were grown in Medium 2 with various supplements and after prolonged incubation the sulphathiazole concentrations limiting growth were recorded.

Strain	p-AB(M)	Incubation (hr.)	Sulphathiazole (M) (a)	(b)
Normal	10^{-7}	115	10^{-4}	2×10^{-4}
A	10^{-7}	115	10^{-3}	2×10^{-3}
A	-	115	10^{-5}	2×10^{-5}
C	10^{-7}	113	$>4 \times 10^{-3}$*	...
C	-	114	2×10^{-5}	2×10^{-4}

(a) This concentration permitted visible growth

(b) This concentration prevented visible growth

* Growth in this culture was about 2/5 optimal whereas only traces of growth were visible in the other tests recorded in this column.

TABLE 15 Competitive sulphathiazole antagonism by p-AB for strain A.

Strain A was grown in Medium 2 with various amounts of p-AB and sulphathiazole, and the sulphathiazole concentrations limiting growth were recorded.

p-AB (M)	Sulphathiazole (M) (a)	(b)
10^{-7}	10^{-3}	2×10^{-3}
10^{-8}	10^{-4}	2×10^{-4}
10^{-9}	10^{-5}	2×10^{-5}

(a) This concentration permitted visible growth.

(b) This concentration prevented visible growth.

of purified casein hydrolysate. This is shown in Fig. 9, which may be compared with Fig. 6 for growth in the usual medium. Not only were the growth rates and final populations of these two strains now greater than they were in Medium 2, but these properties for strain A were almost identical with those of the other strains: the limited final population of strain A in Medium 2 no longer occurred. The increased lag shown by strain A in this experiment was probably fortuitous; it was not usually observed. In contrast to strain A, the growth of strain C in the absence of both sulphathiazole and p-AB remained suboptimal even in this improved medium.

The lag phase of each strain in the improved medium was usually the same as in Medium 2.

Sulphathiazole resistance and growth factor requirements of strain A. The sulphathiazole resistance of strain A was considerably greater than that of the normal organism when measured under comparable conditions, though it was less than that of strain C (Table 14). In the total absence of p-AB the sulphathiazole resistance of strain A remained less than that of strain C. The relationship between p-AB and sulphathiazole was still competitive as the molar ratio of drug and inhibitor required to prevent visible growth was constant over a 100-fold range of p-AB concentration (Table 15).

As a matter of routine the requirement of this strain for the other two "B" vitamins- nicotinic and pantothenic acids -

TABLE 16 Requirement of strain A for pantothenic and nicotinic acids.

Strain A was grown in Medium 2 made up with p-AB (10^{-7} M) but with no nicotinic or pantothenic acids.

Added to medium	Incubation (hr.) 17	41	69	89	113
No addition	-	-	-	-	-
Nicotinic acid, 1 μg.	-	-	-	-	-
Ca pantothenate, 1 μg.	?	tr.	tr.	tr.	tr.
Ca pantothenate, 10 μg.	-	tr.	tr.	tr.	tr.
Ca pantothenate, 1 μg. + Nicotinic acid, 1 μg.	tr.	+++	+++++	+++++	+++++

was checked. The test recorded in Table 16 shows that they were still needed, and a trace of growth found in cultures containing only added pantothenate was probably due to the small amounts of nicotinic acid admitted by the manufacturers to be present in their preparation of vitamin-free casein hydrolysate. The improved basal medium made up without these vitamins but with 10^{-7}M p-AB was used for this test.

Stability of strain A. As with strain C, some tests on the stability of the variation undergone to give strain A were made. After its fourteenth subculture it did not revert when subcultured a further ten times with 10^{-7} M p-AB, and after its one hundred and fifteenth subculture it did not revert when subcultured thirty four times on glycerol-yeast agar (Medium 1).

Chapter 13

Synthesis of p-Aminobenzoic Acid by A. suboxydans 621

One of the most obvious interpretations of the variations undergone by this organism involves the assumption that the strains had developed the ability to synthesise p-AB, and it was thought desirable to test for the presence of this substance in fully grown cultures of strains A and C. The growth of both strains in Medium 2 in the absence of sulphathiazole and p-AB was limited, which suggested that any synthesis of p-AB would be small; certainly below the optimal concentration of 10^{-7} M. This was beyond the range of any chemical method for the detection of p-AB, so the investigation was carried out microbiologically using the normal strain of A. suboxydans as the assay organism.

Cultures of strain A and strain C, grown in the absence of both p-AB and sulphathiazole, were autoclaved at various pH values to release the cell contents. They were then brought to pH 3.8 and extracted with peroxide-free ether, in which conditions p-AB is known to go into the ether layer. The ether extracts, which were coloured, were evaporated and the residue taken up in sufficient water to give a 10-fold concentration of the extracted material; for example, the extract of a 50 ml. culture would be taken up in 5 ml. of water. The resulting solutions were assayed with A. suboxydans.

Such extracts had two characteristics which must be mentioned; (i) the higher the pH at which the culture was autoclaved, the

TABLE 17 Growth of strain H on its first and third passages with extracts of A. suboxydans

Extracts were added to Medium 2 in 0.5 ml. amounts.

A. Growth on first passage:

Added to medium	Incubation (hr.) 19	43	67	93	105
p-AB, 10^{-7} M	tr.	+++	+++++	+++++	+++++
p-AB, 10^{-7} M + extract of strain A	-	-	-	+	++++
no addition	-	-	-	-	-

B. Growth on third passage:

	19	43	75	91
p-AB, 10^{-7} M + extract of strain A	tr.	+++	+++++	+++++
p-AB, 10^{-7} M	tr.	+++	+++++	+++++
p-AB, 10^{-8} M	-	+	++++	++++
no addition	-	-	-	-

deeper was the brown colour of the culture and extract, and (ii) extracts were to a varying extent inhibitory to the assay organism.

The inhibitory nature of the extracts did not constitute a serious problem as adaptation to the relevant inhibitor appeared to be fairly rapid. A special strain of the normal organism - "strain H" - was therefore trained for assay work on these extracts by passing the normal organism three times through a medium containing 10^{-7} M p-AB and as much of a dark extract as was likely to be encountered in an assay. On its third passage strain H was shown to have undergone no change in its requirement for p-AB (Table 17) and consequently to be suitable for assay of these extracts. As a final check tests of such extracts were carried out both with the extract alone and with the extract and a known suboptimal amount of p-AB.

The cultures in which the assay was carried out were to various extents coloured, and, owing to the possibility that the bacteria might alter the colour of the medium, it was undesirable to estimate growth in the "Spekker" absorptiometer. Growth was therefore assessed by counting in a shallow Thoma haemocytometer. The haemocytometer used had a depth of 0.02 mm. and the counts recorded in Tables 18 and 19 are the average number of cells per unit square. If the count is n, the cell population was $1.25 \times 10^6 . n$ cells per ml. Loeffler's methylene blue (1 drop/5 ml.) was used to stain the cells, and clusters of cells were broken up before counting by boiling the suspension for a few seconds and

TABLE 10 Assay of extracts of strains A and C for p-AB

For preparation of extracts see text. Cultures contained 2 x 10^{-9}M p-AB in Medium 2 to ensure delayed growth and thus to detect inhibition by the extracts (0.5 ml.). Samples were counted haemocytometrically after inoculation with strain H and incubation for 32 hr. A control with optimal p-AB is included.

Extract of strain tested	pH at which culture under test was autoclaved prior to extraction	Count of strain H culture
-	-	35
A	3.0	29
A	6.0	39
A	9.0	48
C	3.0	(210)
C	6.0	49
C	9.0	34
(p-AB, 10^{-7}M)	...	1600

shaking it vigorously. The counting procedure for estimating growth was not very accurate with this organism: six cultures incubated 72 hr. with 10^{-8} M p-AB had a mean count of 20.5 with a standard deviation of 8.25, but the method was adequate for the detection of p-AB synthesis in amounts sufficient to account for sulphonamide resistance.

Cultures of strains A and C were brought to various pH values, autoclaved for 10 min. at 10 lb./in.^{2}, extracted, and the extracts assayed, with the results recorded in Table 18. Except in one case (C, pH 3.0) there was neither synergism with p-AB nor was any inhibition caused by the extracts. Inspection of strain H cultures containing no added p-AB gave a like result, except that the extract of strain C autoclaved at pH 3.0 showed no activity in this case.

The one positive result recorded in Table 18 was probably fortuitous, since a similar test with 68 hr.cultures of strains A and C autoclaved at pH 3.0 showed no such effect. In this case a normal strain of A. suboxydans was used as the assay organism, and, as is shown in Table 19, the extracts exhibited neither inhibition of nor synergism with 10^{-8} M added p-AB.

It therefore seems likely that these strains did not synthesise detectable amounts of p-AB, at least in excess of their utilisation of it. It is, however, possible that strain C synthesised p-AB only when grown in the presence of sulphathiazole and this theory ought to be tested as one of the formal possi-

TABLE 19 Assays of extracts of strains A and C for p-AB

0.5 ml. amounts of extract were assayed with normal *A. suboxydans* in Medium 2.

A. Test using extracts prepared from cultures autoclaved at pH 3.0 (a control with an extract of strain A is included).

Added to culture	incubation (hr.) 18	42	66	Count after 72 hr.
p-AB, 10^{-8} M	-	+	++	24 18
p-AB, 10^{-8} M + extract of A	-	+	++	31 13
p-AB, 10^{-8} M + extract of C	-	+	++	23 14
Extract of C	-	-	-	...
Extract of A	-	-	-	...

B. Test using an acid hydrolysate of strain C (see text).

	22	46	70	94
p-AB, 10^{-7} M	tr.	++++	+++++	+++++
p-AB, 10^{-8} M	?	+	+++	+++
p-AB, 10^{-8} M + hydrolysate of C	?	+	+++	+++
Hydrolysate of C	-	-	-	-
-	-	-	-	-

bilities. Unfortunately, it is not possible to design an experiment to settle this on the micro-scale required, as the sulphathiazole present would interfere with any method used to detect p-AB.

There remains the possibility that any p-AB synthesised was immediately incorporated into a larger molecule stable to autoclaving. This is not easily settled, but a fourteen day culture of strain C was tested after hydrolysis with c. 5N. HCl for 20 hr. This treatment should, according to Ratner, Blanchard & Green (1946), hydrolyse any p-AB-containing peptide present, but extracts made from the hydrolysed culture showed neither replacement or nor synergism with p-AB, nor yet any inhibition. This is also recorded in Table 19.

TABLE 20 Growth of Lb. casei in the presence of killed culture of A. suboxydans

0.5 ml. amounts of killed cultures of A. suboxydans were added to Medium 4 prior to sterilisation.

Added to culture.	Incubation (hr.) 19	43	68
Pteroylglutamic acid, 0.15 m g./ml.	...	...	++++
Extract of C	-	+++	++++
Extract of C diluted 1/100	-	-	-
Extract of A diluted 1/100	-	-	-
-	-	+	+

Chapter 14

The Synthesis of "Folic Acid" by A. suboxydans 621

In view of the direct connection in certain organisms between p-AB and both sulphonamide resistance and folic acid synthesis, it was of obvious interest to investigate that synthesis by the trained strains of A. suboxydans. Folic acid at the concentrations involved must be assayed microbiologically, and this was done with Lb. casei ∈ using the assay Medium 4. Preliminary assays of whole cultures of A. suboxydans showed definite synthesis of a substance active for Lb. casei, but also showed the presence of an inhibitor of it: for example, the slight growth obtained in the absence of added growth factor was suppressed by the material under assay (Table 20). The inhibition was not due to the acid formed in grown cultures of A. suboxydans since the pH of an assay material was always adjusted to that of the assay medium. Washing the cells of A. suboxydans once, however, appeared to remove the inhibitor and this was done in subsequent experiments. The results obtained therefore represented the folic acid content of the cells only, and did not in general make allowance for any extracellular material,

One cannot judge accurately the distribution of folic acid between cells of A. suboxydans and their medium, but the results of assays (recorded later) of whole cultures using a specially

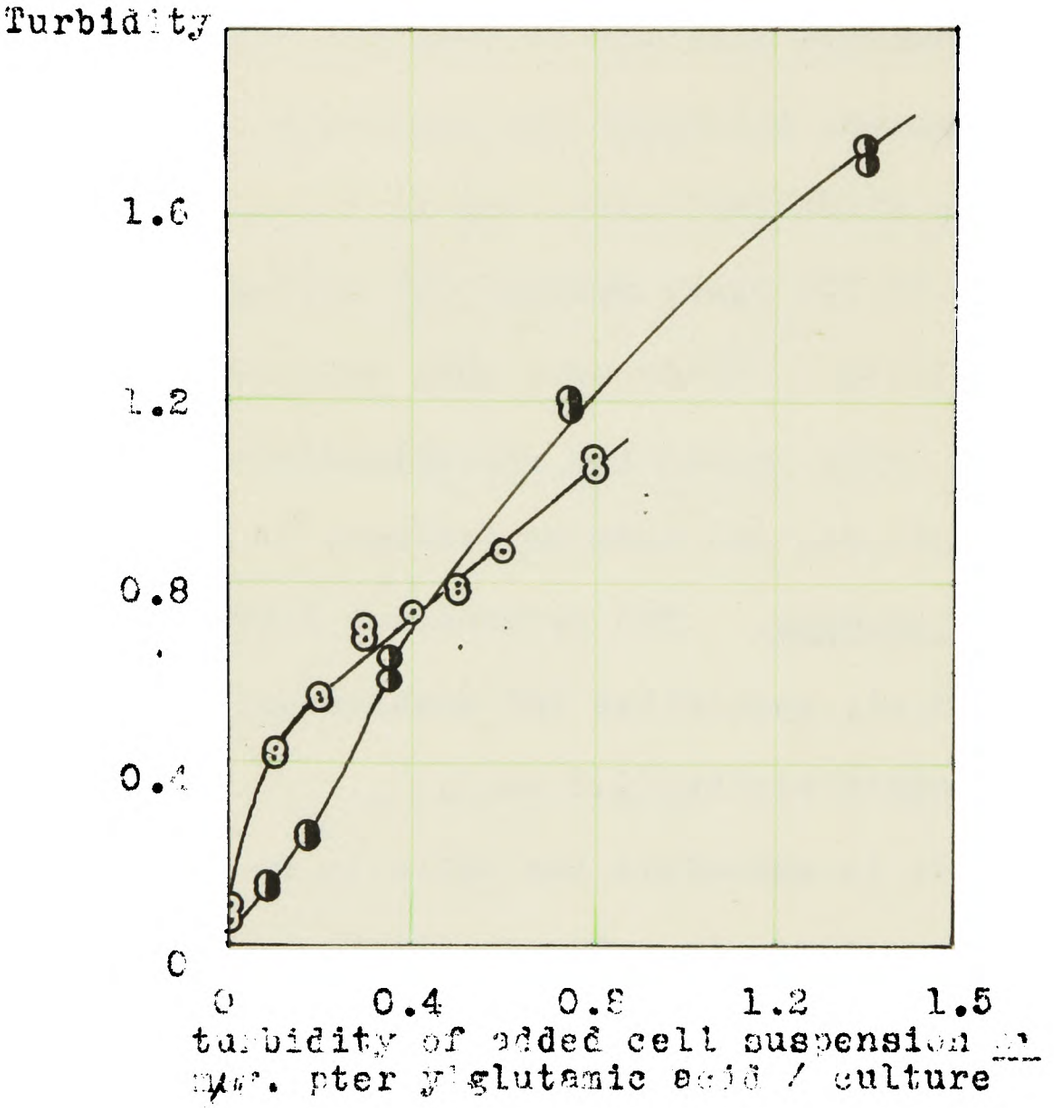
trained strain of Lb. casei showed the same order of response. Hence it was likely that the greater part of the folic acid formed was contained inside the cells.

The general technique used was to wash the cells to be tested by centrifugation, to adjust the suspension to a convenient opacity with distilled water and to add 1 ml. aliquots of serial dilutions to the assay medium (3 ml.) contained in plugged centrifuge tubes. These were then autoclaved, together with control tubes containing pteroylglutamic acid added at various concentrations, and were centrifuged to remove the dead cells of A. suboxydans. The supernatant liquid was divided into two 4.5 ml. quantities and assayed using the same inoculum as the standard series (0.5 ml.).

It was desirable to autoclave the cells to be assayed in the assay medium since there is evidence (Stokes & Larsen, 1945, b) that folic acid is to some extent destroyed by autoclaving, but that this destruction is minimal in the assay medium.

Since, as will be seen, the response curve given to bacterial extracts had a different shape from that given to pteroylglutamic acid, it was not possible to express this response in terms of equivalent amounts of pteroylglutamic acid. The turbidimetric readings were therefore plotted directly to obtain the curves given here. The difference in shape of the assay curves obtained with bacterial extracts compared with pteroylglutamic acid suggests that the bacterial folic acid was not pteroylglutamic acid. Lascelles & Woods (in press) have

Fig. 10 Folic acid content of once-washed cells of A. suboxydans.

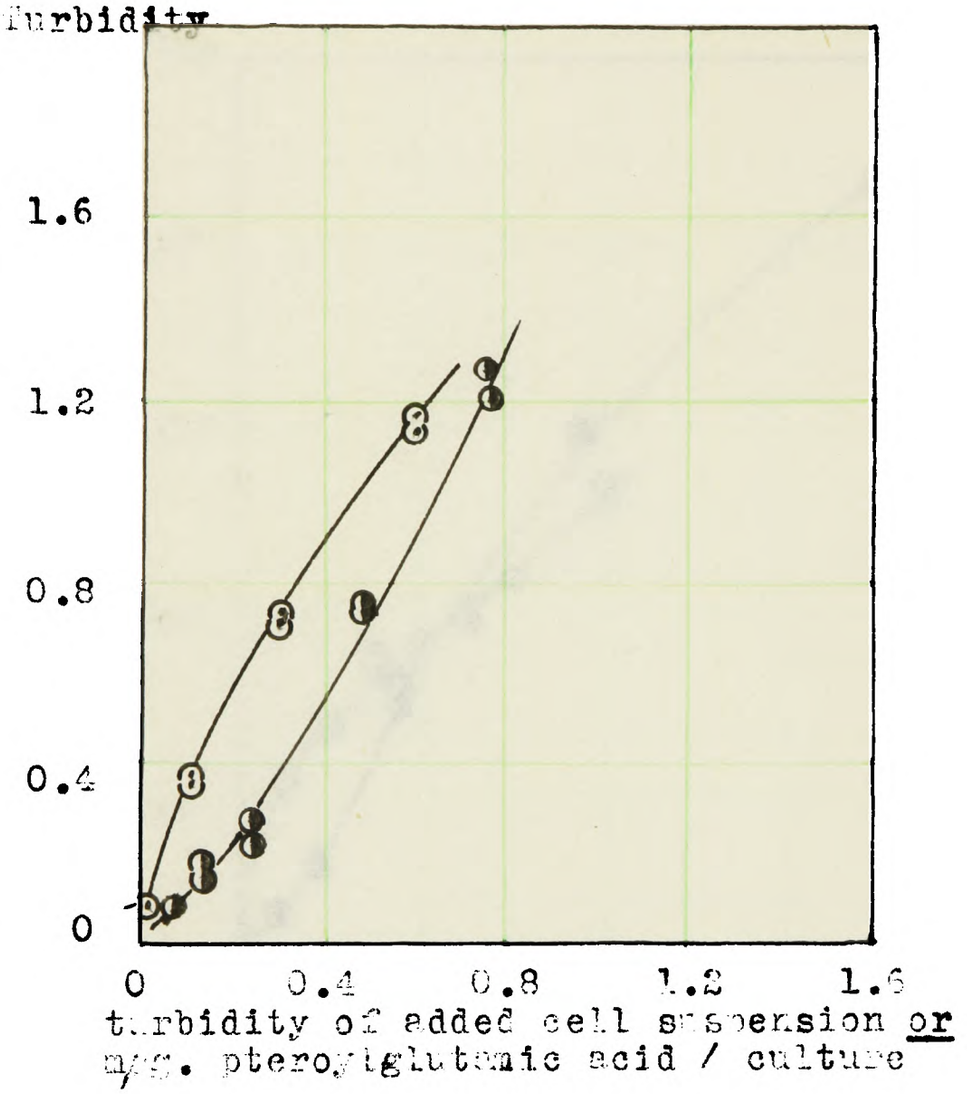


Cells of A. suboxydans were washed by centrifuging and resuspended in water. Samples (0.5 ml.) of suspension were added to medium 4 (5 ml. final vol.) and assayed with Lb. casei (for procedure see text).

● assay of cells

○ control assay with pteroylglutamic acid

Fig. 11 Folic acid content of twice-washed cells of A. suboxydans



Cells of A. suboxydans were washed by centrifuging and resuspending in water twice. Samples (0.5 ml.) of suspension were added to medium 4 (5 ml. final vol.) and assayed with Lb. casei (for procedure see text).

● assay of cells

○ control assay with pteroylglutamic acid

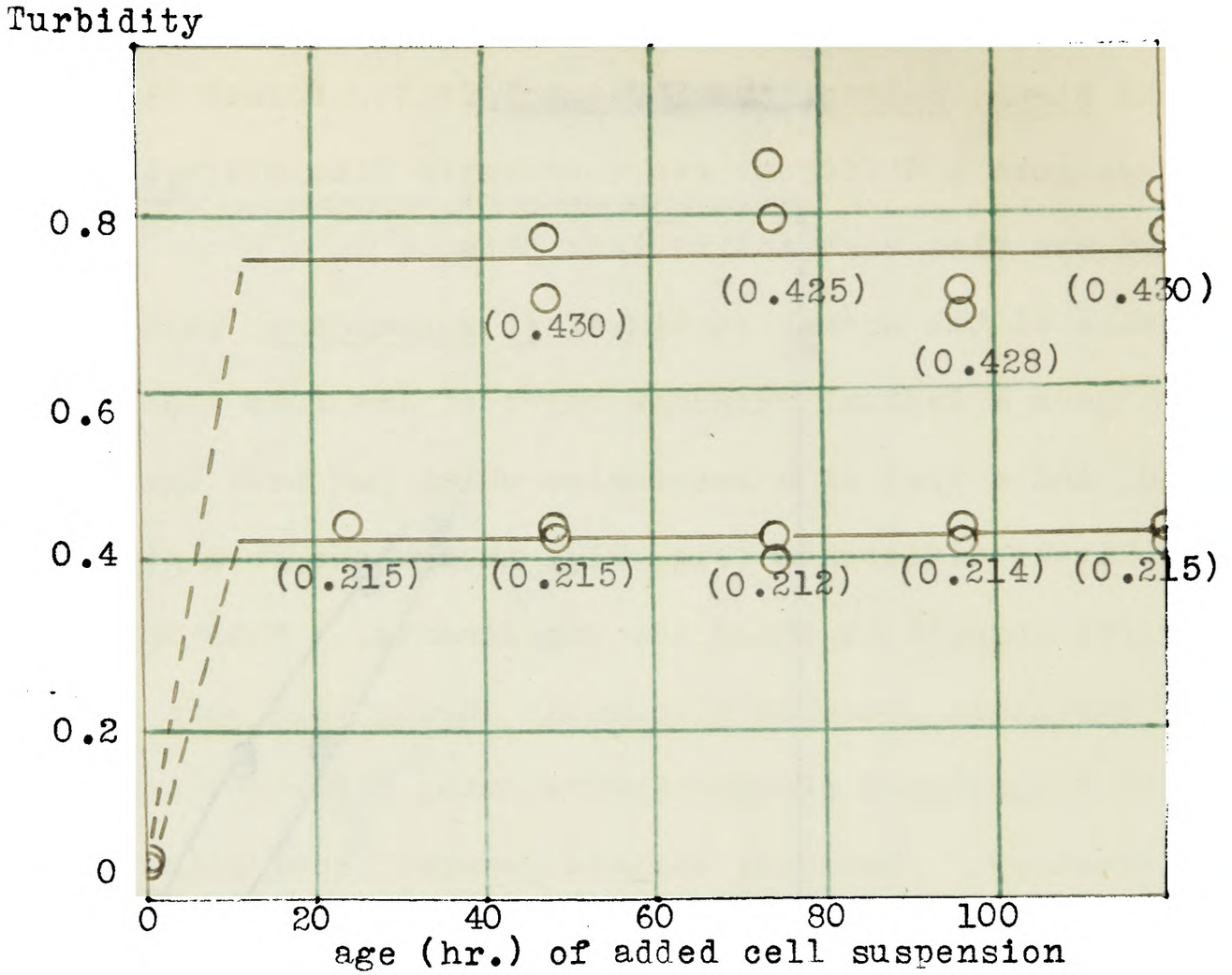


Fig. 12 Folic acid content of cells of A. suboxydans of various ages.

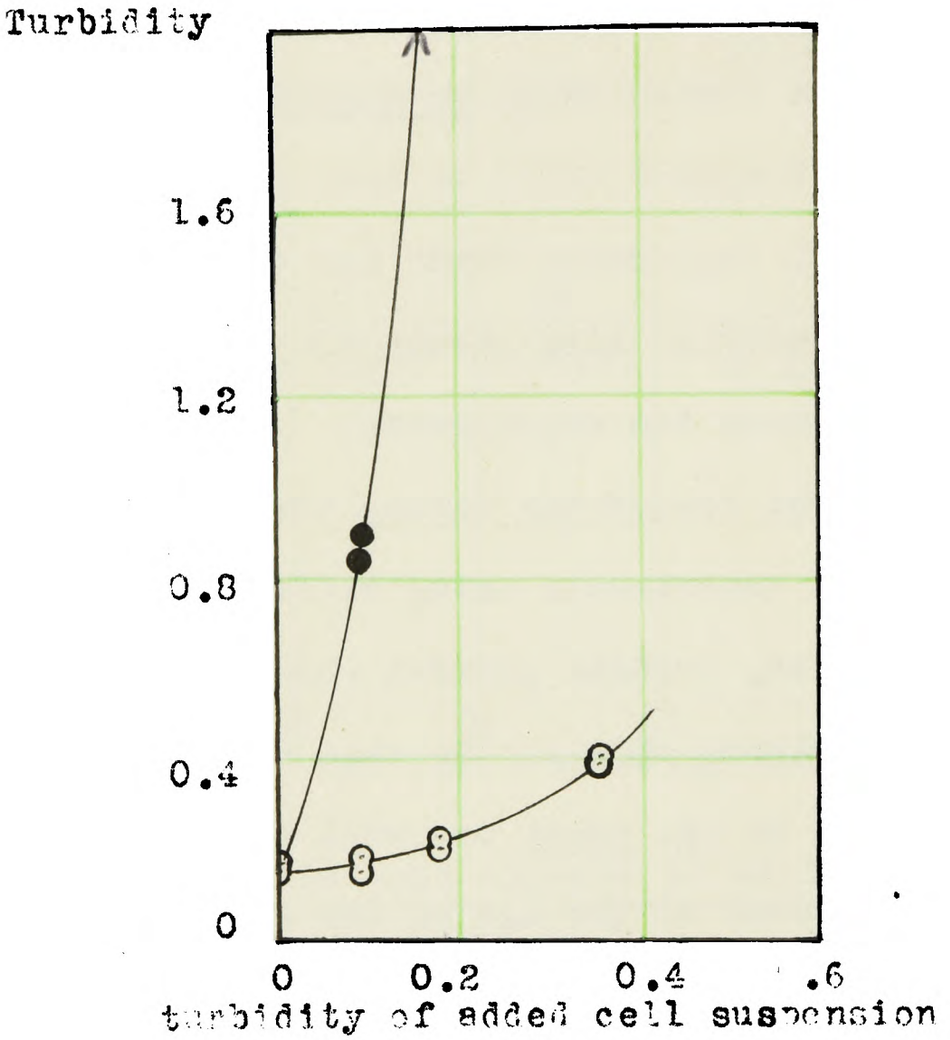
Samples (0.5 ml.) of cell suspensions of normal A. suboxydans of various ages were added to medium 4 (5 ml. final vol.) and assayed with Lb. casei (for procedure see text). The turbidities of the added cell suspensions were approximately equal and are written into the figure.

shown a similar effect in the synthesis of folic acid by Esch. coli and Staph. aureus: the Lb. casei factor formed by these organisms gave a different response curve from pteroylglutamic acid and was also more active than this.

Cells of the normal strain of A. suboxydans, assayed in this manner, gave a typical response curve of the form shown in Fig. 10, and a test of a suspension which had been washed twice showed little difference (Fig. 11), though the form of the standard curve changed in these two experiments. This type of day to day variation occurred frequently during this work and made detailed comparisons of experiments using different inocula unsatisfactory. However, certain general conclusions were established from comparisons made in the same assay.

The response given by Lb. casei per cell of A. suboxydans was found to be independent of the age of the culture of A. suboxydans. Cultures of this organism were sown at various times before the date of assay, were washed, brought to standard opacity, and assayed at two levels. The results are shown in Fig. 12. The reading plotted for the 24 hr. culture at the lower level (none was obtained for the higher level) is calculated from the response to cells added at an opacity of 0.135 (Spekker), as the culture had only shown slight growth. A seven-fold concentration of cells during washing was needed to achieve this figure. The calculation was performed assuming the slope of the assay curve for the 24 hr. culture to be the

Fig. 13 Folic acid content of *A. suboxydans* grown with glycerol and with glucose.



A. suboxydans was grown (1) in medium 2 made up with glucose in place of glycerol and (2) in medium 2. Samples (0.5 ml.) of the whole culture (1) and of washed cells from (2) were added to medium 4 (5 ml. final vol.) and assayed with *Lb. casei* (for procedure see text). For a test using washed cells grown in glucose see table 22.

● glucose culture ○ glycerol culture

TABLE 21 Effect of p-AB concentration during growth on folic acid content of cells of A. suboxydans

Cultures of A. suboxydans were grown with various concentrations of p-AB, washed and re-suspended in 0.8% saline. Samples (0.5 ml.) were added to Medium 4 before sterilisation and assayed with Lb. casei; incubation 48 hr.

p-AB (M) in A. suboxydans culture	µg. dry wt. cells/ml. in A. suboxydans suspension assayed	µg. dry wt. cells /ml. in Lb. casei assay culture
10^{-7}	140	25.0
10^{-8}	135	30.0
4×10^{-9}	144	24.5
2×10^{-9}	144	27.5
10^{-9}	140	23.5

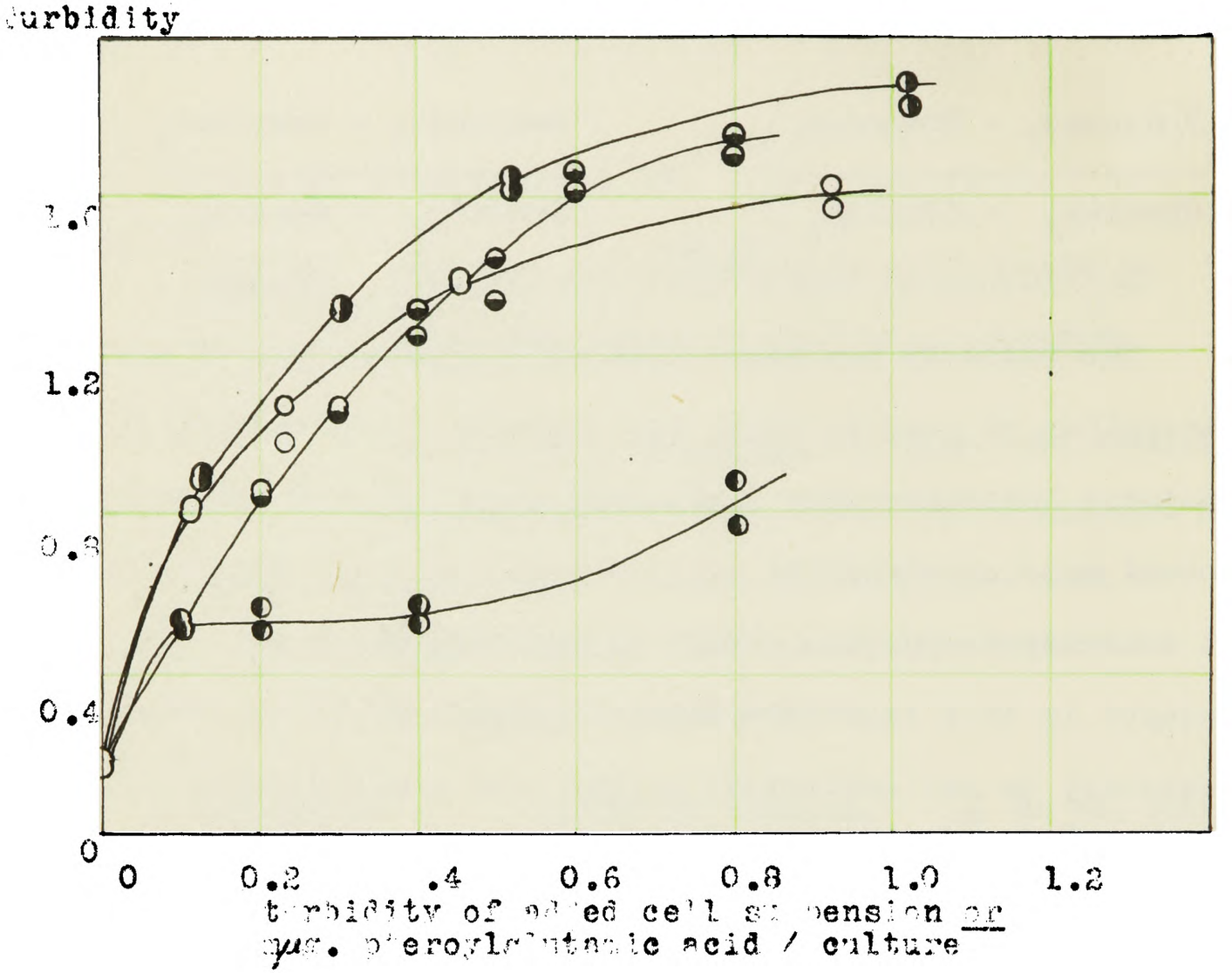
same as that for the 48 hr. one, and to be linear, which at this level it usually was. One could then apply the relation:-

$$\left(\frac{\text{Response}_2 - \text{Response}_1}{\text{Opacity}_2 - \text{Opacity}_1}\right)_{24\text{ hr.}} = \left(\frac{\text{Response}_2 - \text{Response}_1}{\text{Opacity}_2 - \text{Opacity}_1}\right)_{48\text{ hr.}}$$

The response was also shown to be independent of the concentration of p-AB in which the organism to be assayed was grown. Cultures incubated over a 100-fold range of p-AB concentrations showed approximately the same response with Lb. casei when assayed at a standard opacity. This is shown in Table 21. The cells assayed in this test were rather old (114 hr.), but this was necessary to get sufficient growth with small amounts of p-AB; even so a seven-fold concentration of the cells grown at the lowest level was needed.

It has been mentioned that the normal cells of A. suboxydans would grow in a medium in which glycerol was replaced by glucose and sodium lactate (page 59), though the extent of final growth was limited. It is probable that this repression of growth was due to the low pH reached in the presence of glucose. Whole cultures could be assayed directly as they were not inhibitory towards Lb. casei and there was a greatly enhanced production of folic acid by cells grown in the presence of glucose (Fig. 13). An experiment was therefore performed to compare the folic acid present in a whole culture grown in glucose-lactate medium with

Fig. 1. Folic acid content of cells of trained strains of *L. suboxydans*.



Samples (0.5 ml.) of washed cell suspensions were added to medium 4 (5 ml. final vol.) and assayed with *Lb. casei* (for procedure see text)

◑ normal *A. suboxydans* grown with p- 3 (10^{-7}M)

○ strain 3 grown with p- 3 (10^{-7}M) and sulphathiazole (2×10^{-3}M)

◐ strain A

◒ control assay with pteroylglutamic acid

TABLE 22 **Effect of glucose on folic acid synthesis by *A. suboxydans***

Cultures of *A. suboxydans* were grown in Medium 2 in which glucose (0.05 M) replaced glycerol. Samples (0.5 ml.) of culture and of washed cells were assayed with *Lb. casei* in Medium 4.

Material assayed	µg. dry wt. cells /ml. assay material (*A. suboxydans*)	µg. dry wt. cells /ml. assay culture (*Lb. casei*)
Killed cells in culture medium	180	132
Killed cells washed once	176	32.7

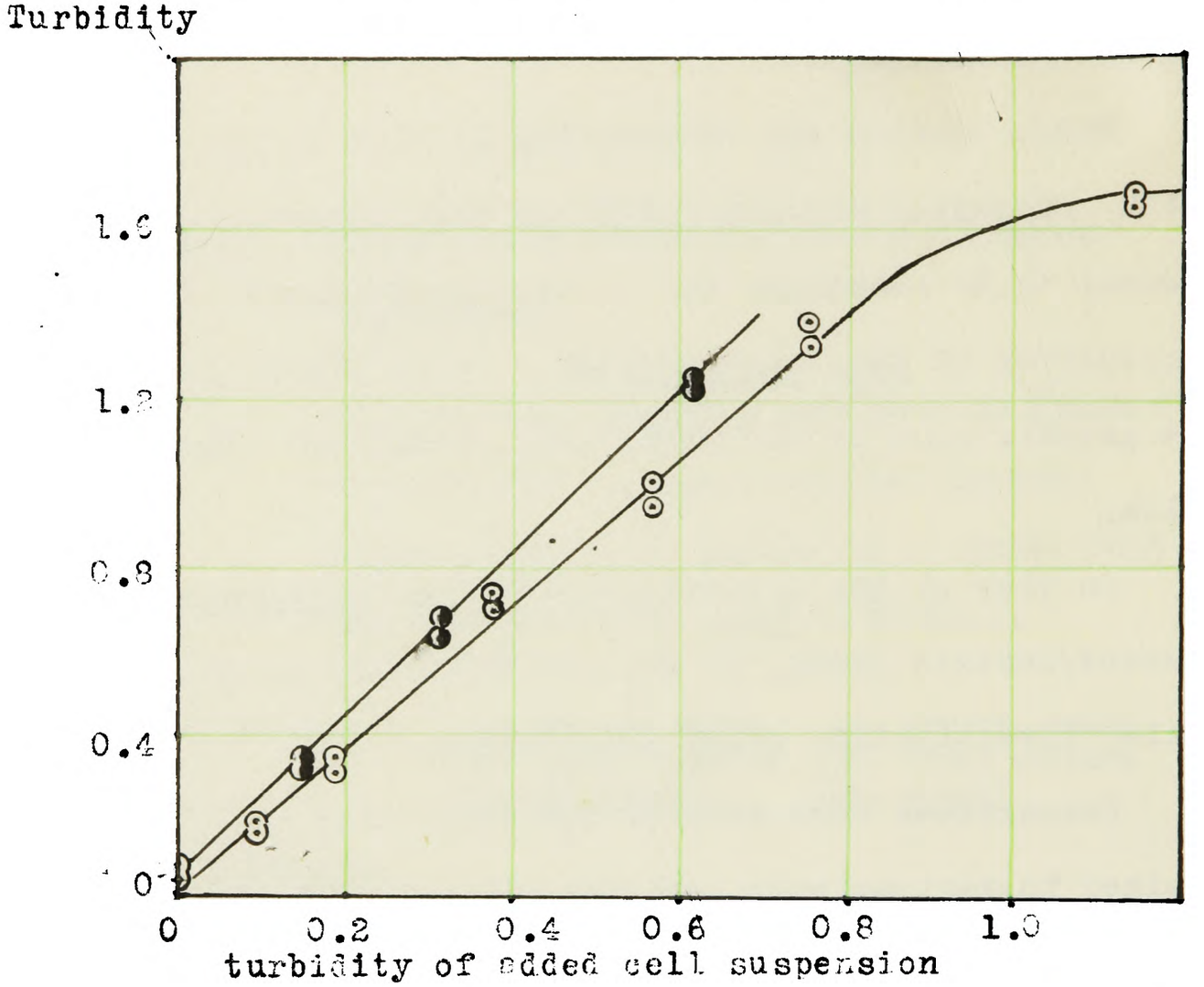
that in the cells separated from the same culture. The results, given in Table 22, showed that the greater part of the folic acid was in the medium.

These results are interesting in view of the work of Nimmo-Smith, Lascelles & Woods (1948) in this laboratory, who found glucose to be necessary for folic acid synthesis by non-growing suspensions of Sbm. plantarum10S. They found, however, that the greater part of the folic acid formed was contained in the cells.

In view of the limited growth of A. suboxydans in glucose or glucose/lactate media, it was not desirable to carry out a detailed investigation using these media.

Comparisons were made of the folic acid content of the cells trained in various ways, and are collected in Fig. 14. In general washed cells of the normal and sulphathiazole-trained strains showed little difference when grown in their normal media, but cells of strain A had a markedly lower folic acid content and gave a differently shaped response curve. These comparisons were repeated many times, and indicated that, while there was a definite stimulation of Lb. casei by strain A the effect was weak and variable. It was similar to effects obtained by other workers in this laboratory using high concentrations of thymine in place of pteroylglutamic acid, and may suggest that strain A was producing a weakly active substance very different from the folic acid of the other two strains.

Fig. 15 Folic acid content of cells of strain C grown with and without sulphathiazole.

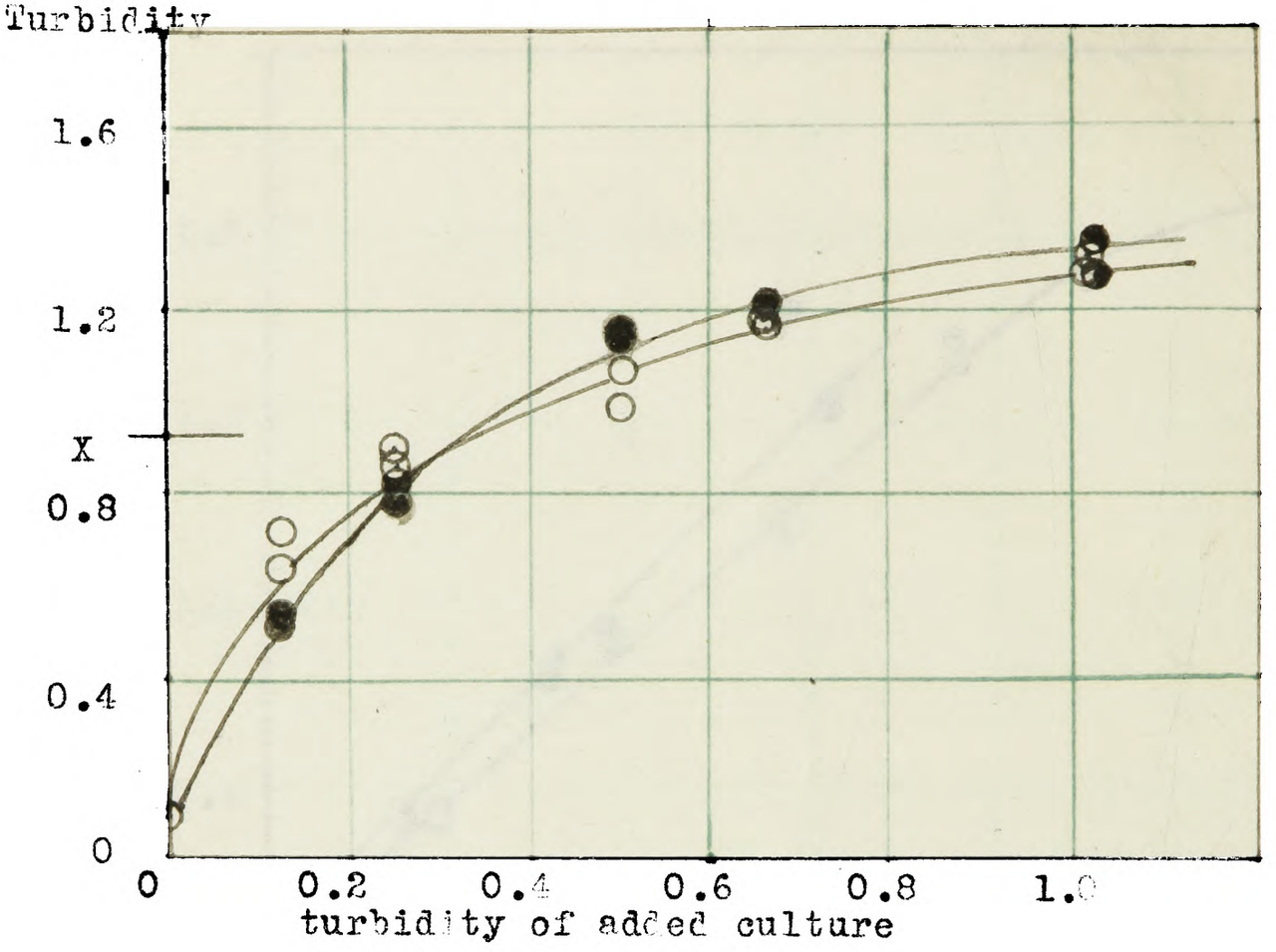


Samples (0.5 ml.) of washed cells of strain C were added to medium 1 (5 ml. final vol.) and assayed with Lb. casei (for procedure see text).

● cells grown with p- B (10^{-7}M) and sulphathiazole (2×10^{-3}M)

○ cells grown with p-AB (10^{-7}M)

Fig. 16 Folic acid content of cultures of normal A. suboxydans and of strain C.



Samples (0.5 ml.) of cultures of normal A. suboxydans and of strain C were added to medium 4 (5 ml. final vol.) and assayed with a specially trained strain of Lb. casei (for procedure see text).

● normal A. suboxydans

○ strain C

X highest growth level reached with pteroyl glutamic acid

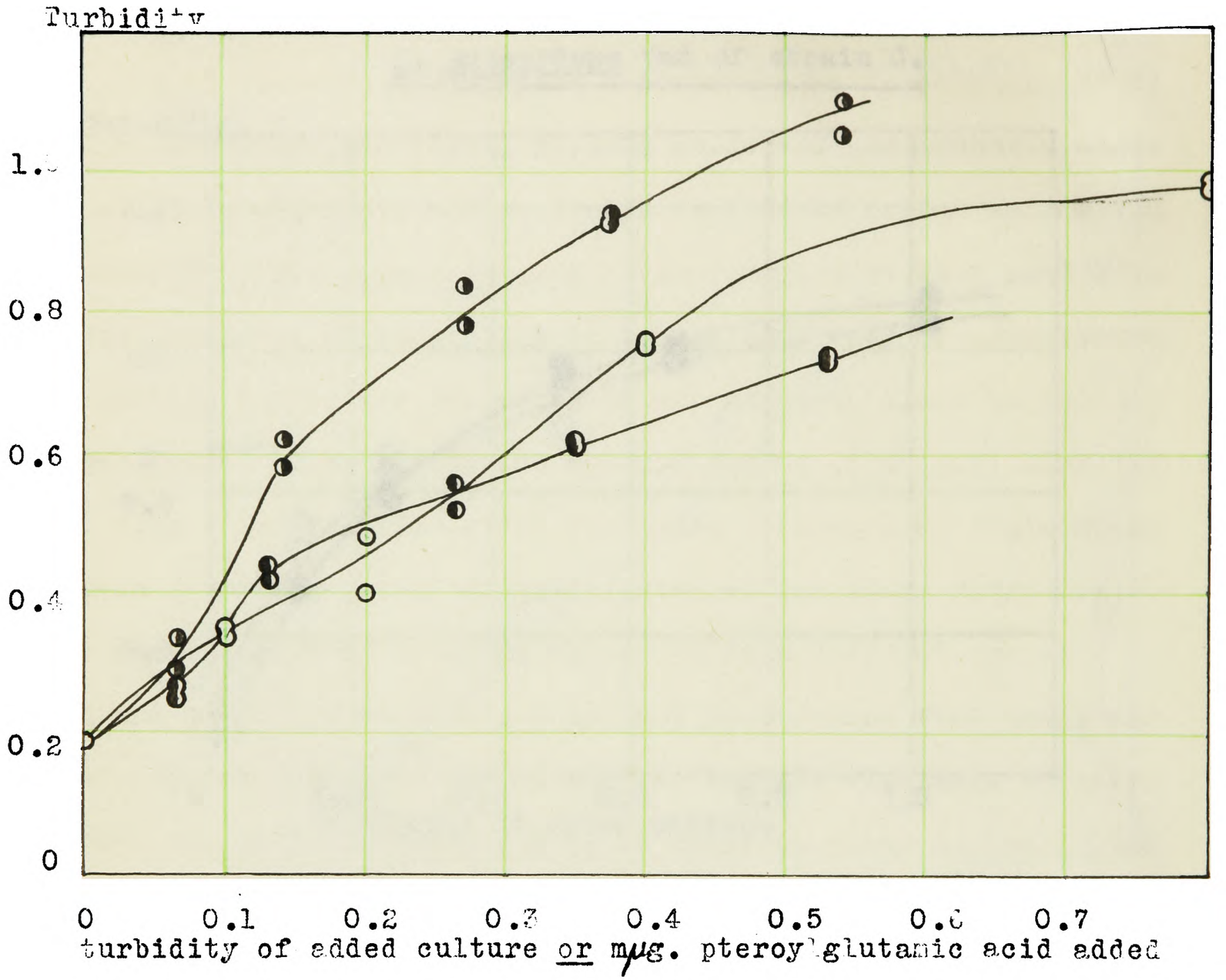


Fig. 17 Folic acid content of normal A. suboxydans and of strain A.

Samples (0.5 ml.) of the test cultures were added to medium 4 (5 ml. final vol.) and assayed with a specially trained strain of Lb. casei (for procedure see text).

● normal A. suboxydans

◐ strain A

○ pteroylglutamic acid

Strain C might have been expected to form more folic acid in the absence of sulphathiazole than in the presence of it, since Nimmo-Smith, Lascelles & Woods (1948) had shown that sulphonamides had an inhibitory effect on the synthesis of folic acid from p-AB by suspensions of Sbm. plantarum 10S. However, experiments to test this showed no difference in the folic acid content of cells grown in the presence and absence of sulphathiazole (Fig. 15), though perhaps one should again remark that there might be a greater amount of extra-cellular folic acid formed which would not be revealed by the assay procedure used.

It was felt that this criticism could be levelled at all the assay work reported so far, so a strain of Lb. casei was obtained specially adapted to grow in the presence of the inhibitors found in whole cultures of A. suboxydans. A normal strain of Lb. casei, which showed inhibition as reported in Table 20, was subcultured twice in the assay medium to which had been added 20% of a fully grown culture of normal A. suboxydans. The strain was used for assay work in the usual manner and showed no suppression of growth in the presence of assay material (Figs. 16, 17, 18).

The response curves obtained with this strain of Lb. casei had the same general characteristics as those found with the parent strain, but suggested that whole cultures contained rather more folic acid than washed cell suspensions of similar turbidity. It is not possible to give useful figures to illustrate this

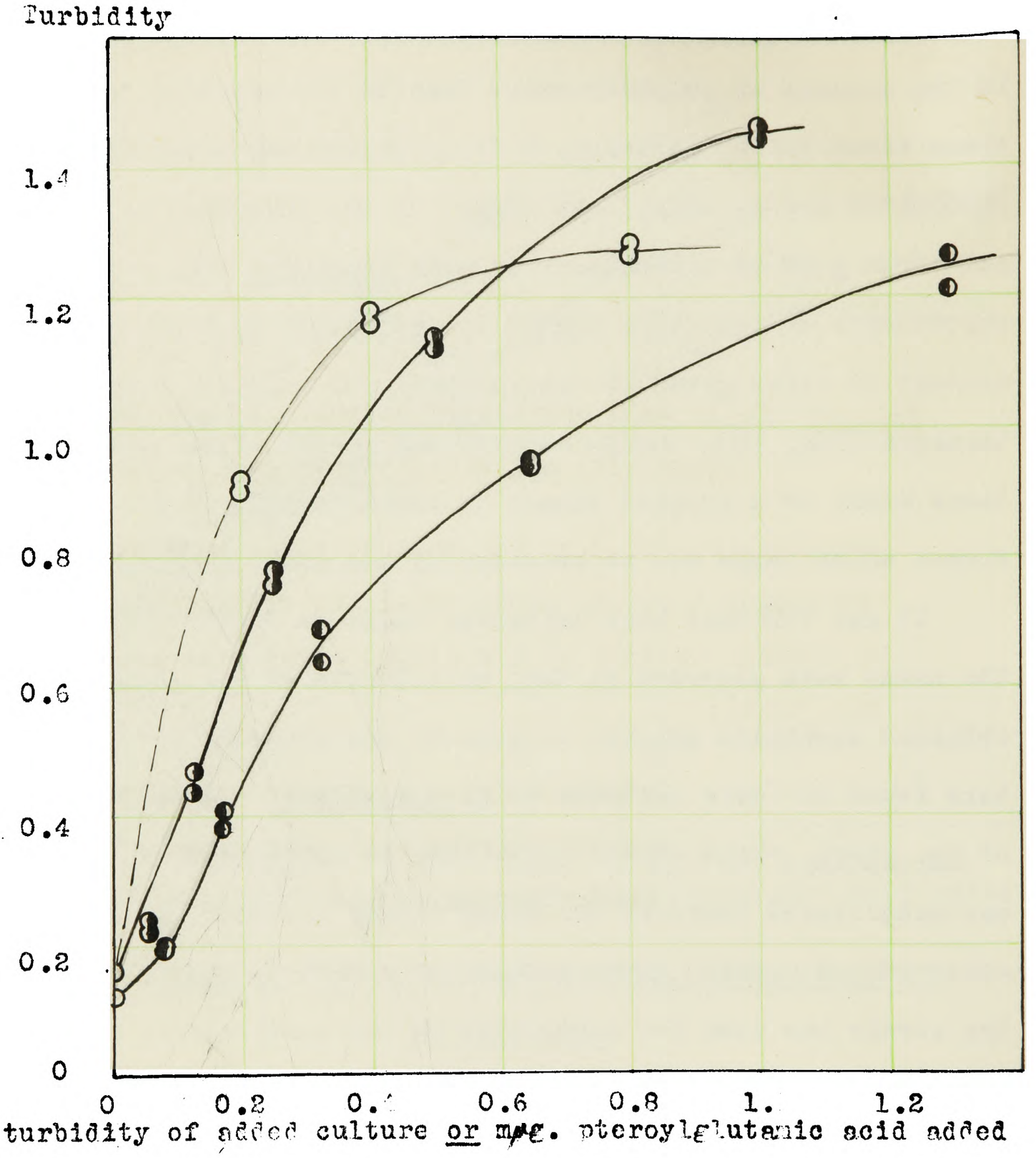


Fig. 18 for legend see opposite.

Fig. 18 Folic acid content of cultures of strain C grown with and without sulphathiazole.

Samples (0.5 ml.) of the test cultures were added to medium 4 (5 ml. final vol.) and assayed with a specially trained strain of Lb. casei (for procedure see text).

● strain C grown with p-AB and sulphathiazole

◑ strain C grown with p-AB alone

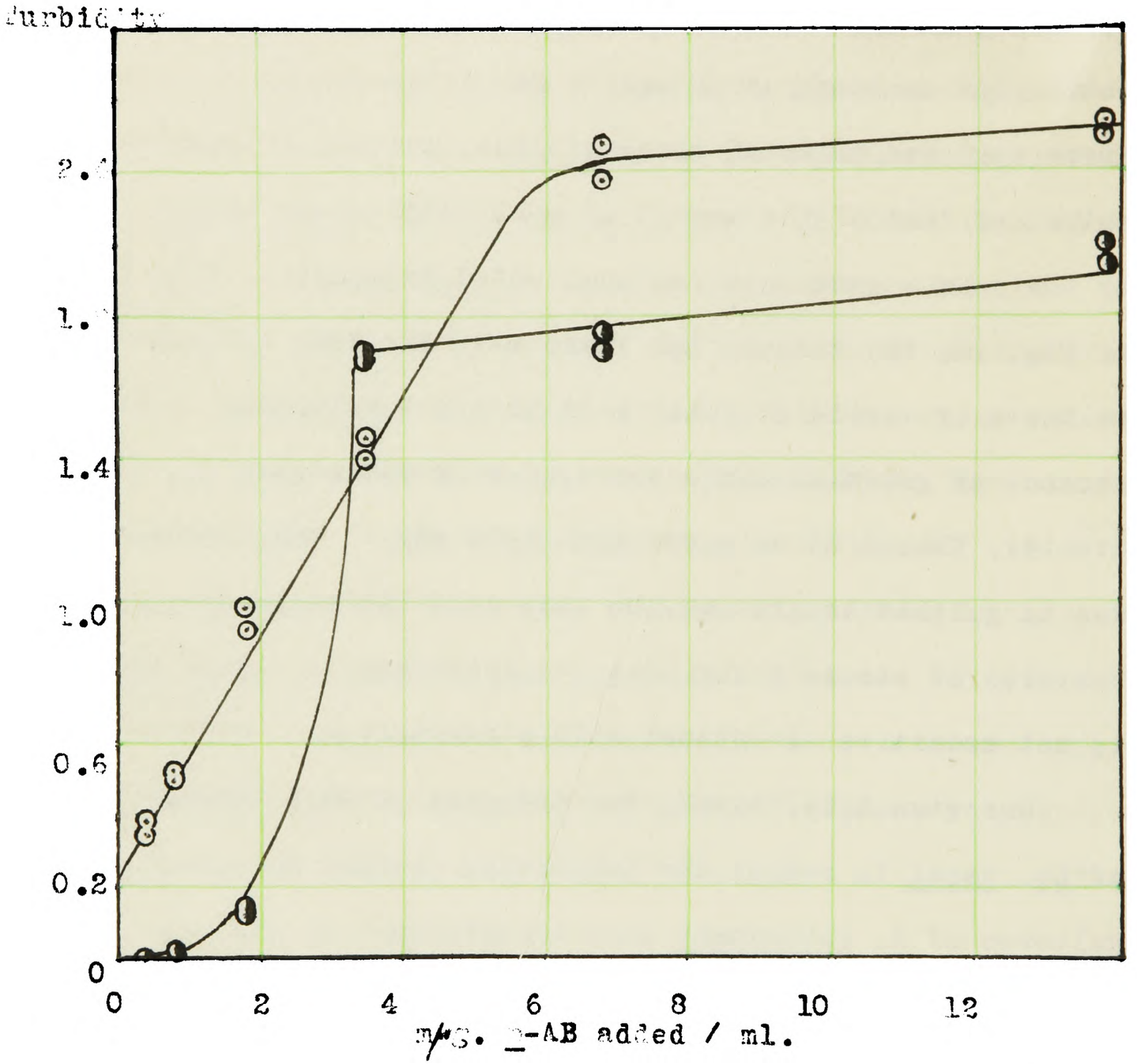
○ pteroylglutamic acid

because of the large day to day variation in these assays. The reduced synthesis of active material by strain A was still apparent, as was the lack of significant difference between the folic acid contents of strain C and of the normal organism. There appeared, however, to be a small difference between the output of factor by strain C grown in its normal medium and that by the same strain grown without sulphathiazole. This is shown in Fig. 18, which shows the results of an assay of whole 72 hr. cultures of strain C grown in these two conditions. In the absence of sulphathiazole the output of folic acid was definitely greater, though by no more than about 40%. This effect was not due to sulphathiazole carried over into the assay medium from cultures of strain C containing sulphathiazole, since Lb. casei is not sensitive to sulphathiazole inhibition.

Unfortunately, during the training of this special strain of Lb. casei to resist the inhibitors present in autoclaved whole cultures of A. suboxydans some interference in its response to synthetic pteroylglutamic acid took place. The agreement between duplicate cultures containing added pteroylglutamic acid was often poor, and in one case the response curve was so bad that only the optimal level achieved with pteroylglutamic acid is given (Fig. 16). Hence these curves are also without significance in terms of quantities of pteroylglutamic acid.

Summary. The results outlined above were unsatisfactory from a quantitative point of view, but they indicate one or two

Fig. 19 Response of normal _A. suboxydans_ and of strain _ to _-AB



Normal _A. suboxydans_ was grown in medium 2 with various concentrations of _- B, strain 3 in similar media with sulphathiazole (2×10^{-3}M) in addition. Turbidities were measured after incubation for 38 hr.

○ normal _A. suboxydans_

◑ strain 3

general points about the synthesis of folic acid by A. suboxydans, which are summarised below:

1) The synthesis of folic acid-like material by the various strains grown in glycerol medium was reflected in the folic acid content of the cells of these strains, and was independent of their age, and of the amount of p-AB supplied to them.

2) This synthesis was unaffected by adaptation to resist sulphathiazole, except that there may have been a slight increase in the extra-cellular folic acid when the sulphonamide-trained strain was grown in the absence of sulphathiazole.

3) This synthesis was much reduced, if not abolished, by training cells to grow in the absence of added p-AB.

4) It was unlikely that the folic acid synthesised by A. suboxydans was identical with pteroylglutamic acid as cell extracts gave a different response curve from pteroylglutamic acid.

Agreement with theory. In spite of the lack of quantitative significance of these results it is possible to give an interesting if rough calculation concerning them. Fig. 19 compares the p-AB assay curves shown by two strain of A. suboxydans, and from it one can see that about 10 mμg. (0.073 mμmoles) of p-AB was required to grow a 5 ml. culture of normal cells to a turbidity of 1.0 (approximately 0.56 mg. dry wt. cells/ml; page 54). From several assays of washed normal cells it can be said that

the folic acid content of this amount of cells was equivalent to about 8 mg. (0.018 m moles) of pteroylglutamic acid.

Assuming that the folic acid produced by A. suboxydans contains, like pteroylglutamic acid, one p-AB residue per molecule, and that its molecular weight is of a similar order, 444, then the yield of folic acid found in these assays was about 25% of theoretical.

TABLE 23 **Non-competitive sulphathiazole antagonism by extracts of A. suboxydans**

Extracts of A. suboxydans (for preparation see text) were tested as 0.5 ml. samples added to Medium 2. Growth of A. suboxydans after incubation is recorded.

Added to culture	Sulphathiazole (M)	Incubation (hr.) 17	41	69	89	113
p-AB, 10^{-7} M	-	-	++	+++++	+++++	+++++
-	-	-	-	-	tr.	+
Extract	2×10^{-3}	-	tr.	±	+	+
Extract	10^{-3}	-	tr.	±	+	+
Extract	2×10^{-4}	-	tr.	±	+	+
Extract	10^{-4}	-	tr.	±	+	+
Extract	2×10^{-5}	-	tr.	±	+	+
Extract	10^{-5}	-	tr.	±	+	+

Chapter 15

The Synthesis of Adenine by A. suboxydans 621

In view of the ability of adenine to support limited growth of A. suboxydans in the absence of p-AB, and also to act as a non-competitive anti-sulphonamide substance (pages 62 & 64) the synthesis of this substance by the various strains was investigated.

As a preliminary, extracts of the normal organism were shown to contain a non-competitive anti-sulphonamide substance. Cells of the normal organism grown for 72 hr. with 10^{-7} M added p-AB were washed twice to remove p-AB and suspended in the p-AB-free basal medium. The suspension was autoclaved for 10 min. at 10 lb./in.^{2} and brought to standard opacity; the supernatant liquid was tested and it was found that it supported the growth of A. suboxydans in the absence of p-AB and contained no inhibitor.

The solution was then extracted continuously with peroxide-free ether at pH 3.8 until after 60 min. tests showed that the last traces of p-AB had been removed. The purified extract was then tested as an anti-sulphathiazole substance for the normal strain, and, as recorded in Table 23, acted non-competitively over a 100-fold range of sulphathiazole concentration. The growth obtained with the extracts was feeble in all cases, and resembled the sulphathiazole-resistant growth found with adenine (Table 6, page 64).

TABLE 24 Growth of "adenine-less" *N. crassa* in the presence of a killed culture of *A. suboxydans*

1 ml. of a killed culture of *A. suboxydans* in Medium 2 was included in a 5 ml. culture of *N. crassa* in Medium 7.

Added to culture	Incubation (hr.) 18	42	66	90	114
Adenine, 10^{-4} M	-	+	++	+++	+++
Adenine, 10^{-4} M + killed culture	-	tr.	tr.	++	++
Killed culture	-	-	-	-	-
-	-	-	-	-	-

TABLE 25 Comparison of the growth factor effects of extracts of A. suboxydans, various strains, for N. crassa ("adenine-less") with their anti-sulphathiazole effects for normal A. suboxydans

For extraction procedure see text; extracts tested in 0.5 ml. amounts. A. suboxydans cultured in Medium 2 and N. crassa 28610-1899-1a in Medium 7. Extracts of strains A, C, and normal cells indicated as A, C and N.

1. Test with N. crassa:

Added to culture	Incubation (hr.) 17	41	66	89	113
Adenine, 10^{-4} M	-	±	++	+++	+++
C	-	tr.	tr.	±	+
A	-	tr.	tr.	±	+
N	-	tr.	tr.	±	+
-	-	-	-	-	-
From another test:	24	48	72	96	120
Adenine, 4×10^{-6} M	?	tr.	tr.	±	+
2. Test with A. suboxydans:	24	46	70	94	118
p-AB, 10^{-7} M	tr.	+++	+++++	+++++	+++++
-	-	-	-	-	-
C + Sulphathiazole, 10^{-3} M	-	tr.	±	+	+
A + Sulphathiazole, 10^{-3} M	-	tr.	±	+	+
N + Sulphathiazole, 10^{-3} M	-	tr.	±	+	+
Adenine, 4×10^{-6} M + Sulphathiazole, 10^{-3} M	tr?	tr.	±	+	+

The substance responsible for this activity may have been either a compound such as adenine or a folic acid-like compound which was formed from p-AB. It could not have been pteroyglutamic acid as this was a competitive anti-sulphonamide substance (page 64). The extract was therefore assayed with both Lb. casei and with "adenine-less" N. crassa. It was found active for both organisms and therefore presumably contained compounds of both classes - though the possibility of a single substance active for both assay organisms cannot be completely excluded. The assays with N. crassa were performed by suspending the cells to be tested in Medium 7, autoclaving, and inoculating the resulting medium with N. crassa, mutant No. 28610-1899-1a. Washed cells only were assayed, since preliminary tests had shown that whole cultures of A. suboxydans contained a weak inhibitor of the mould (Table 24).

Parallel extracts were then made from cells of strains A, C and the normal organism, starting at opacities 1.95, 2.20 and 2.24 (Spekker) respectively. The results of an assay of these with N. crassa are shown in Table 25, where the adenine content of the assay fluid is seen to have been similar for the three strains, in the region of 4×10^{-6} M.

These extracts were also tested by Dr. Nimmo-Smith for activity towards Lb. casei, and showed the effects to be expected from the work recorded in Chapter 14: the extracts of strain C and the normal organism supported optimal growth of the assay organism, but that of strain A gave only about half growth.

The same extracts were then tested for anti-sulphathiazole effects with the normal strain of A. suboxydans, using a fixed but high drug concentration (10^{-3} M). As is also shown in Table 25, there was no detectable difference in the activities of the three extracts, though there was a slight difference between these and a control with 4×10^{-6} M adenine.

It should be mentioned that controls showing inactivity of adenine for Lb. casei and pteroylglutamic acid for "adenineless" N. crassa were not included in these experiments. These were thought unnecessary since the medium for Lb. casei contains adenine in excess of its requirement (Roberts & Snell, 1946) and any effect of folic acid on the strain of N. crassa would have been noted during its original isolation (Mitchell & Houlahan, 1946).

Summary. The results described above can be summarised as follows:-

1) A. suboxydans contained a substance active in place of adenine for a mutant of N. crassa requiring this, and the two variations undergone to produce strains A and C caused no alteration in the content per cell of this factor.

2) Normal and variant cells produced a substance active as a growth factor and a non-competitive anti-sulphonamide for A. suboxydans. There was little evidence as to whether this was an adenine or folic acid-like compound, but it was more likely to be adenine since there was no difference in the anti-sulpha-

thiazole activity and adenine content of extracts from the three strains, whereas their folic acid content varied.

Chapter 16

Other Trained Strains of A. suboxydans 621

The origin of strain H of this organism has been reported already (page 77) and will not be discussed further.

As has been mentioned (page 41.), it ought to be possible to obtain strains of a p-AB-requiring organism trained not only to dispense with p-AB, but also to resist sulphonamide inhibition in the absence of this metabolite. Attempts to obtain this type of organism were made, without success, along two lines.

In the first case strain A, after its eleventh passage in the absence of added p-AB, was passed through a medium containing 10^{-5} M sulphathiazole, i.e.: just below the concentration which prohibited visible growth (page 74). After ten subcultures in such a medium there was no improvement in the extent of growth, and the sulphathiazole resistance of the strain, measured at the end of this time, remained at between 10^{-5} and 2×10^{-5} M sulphathiazole. These experiments were therefore abandoned.

In the second case attempts were made to reduce the p-AB requirement of strain C to zero in the presence of its normal amount of sulphathiazole, 2×10^{-3} M. This was not successful, though the sub-strain so obtained was in some ways peculiar. It is referred to as strain "CD", and was maintained for 60 passages with 10^{-9} M added p-AB, when its growth was weak and

TABLE 26 **Growth of strain CD with p-AB**

Medium 2 with sulphathiazole (2×10^{-3} M)

Controls with normal *A. suboxydans* are included for comparison.

p-AB (M)	Incubation (hr.) 18	43	60	90	114	139	162
10^{-7}	-	-	±	+	+±	++	+++
10^{-8}	-	-	±	+	+±	++	+++
10^{-9}	-	-	-	tr.	±	±	++
none added	-	-	-	-	-	-	tr.

with normal *A. suboxydans* :

	18	42	66	90	114
10^{-7}	-	++	+++++	+++++	+++++
none added	-	-	-	-	tr.

slow. Its requirement for p-AB was reduced as compared with that of strain C, in fact, even with a washed inoculum the cultures having no added p-AB showed visible growth after 7 days. These cells, however, would not grow when subcultured in similar conditions, and the conclusion seems to be that there had been sufficient intracellular growth factor carried over in the inoculum to permit visible growth.

The growth in Medium 2 without p-AB is illustrated in Table 26, where another peculiarity of strain CD is also recorded. Growth was stimulated by an increased p-AB concentration, but even with a concentration optimal for the normal strain and for strain C growth was poor. Table 26 shows that the growth with 10^{-7} M p-AB was identical with that with 10^{-8} M p-AB and was poor in both.

The growth of strain A in Medium 2 was limited (Fig. 6, page 66), but could be stimulated by the increased amount of casein hydrolysate in the improved medium (Fig. 9, page 74). When strain C was grown in the absence of both p-AB and sulphathiazole, however, this stimulation did not occur; growth was limited in both forms of Medium 2 (cf. Fig. 6, page 66 and Fig. 9, page 74). This is illustrated diagrammatically below:

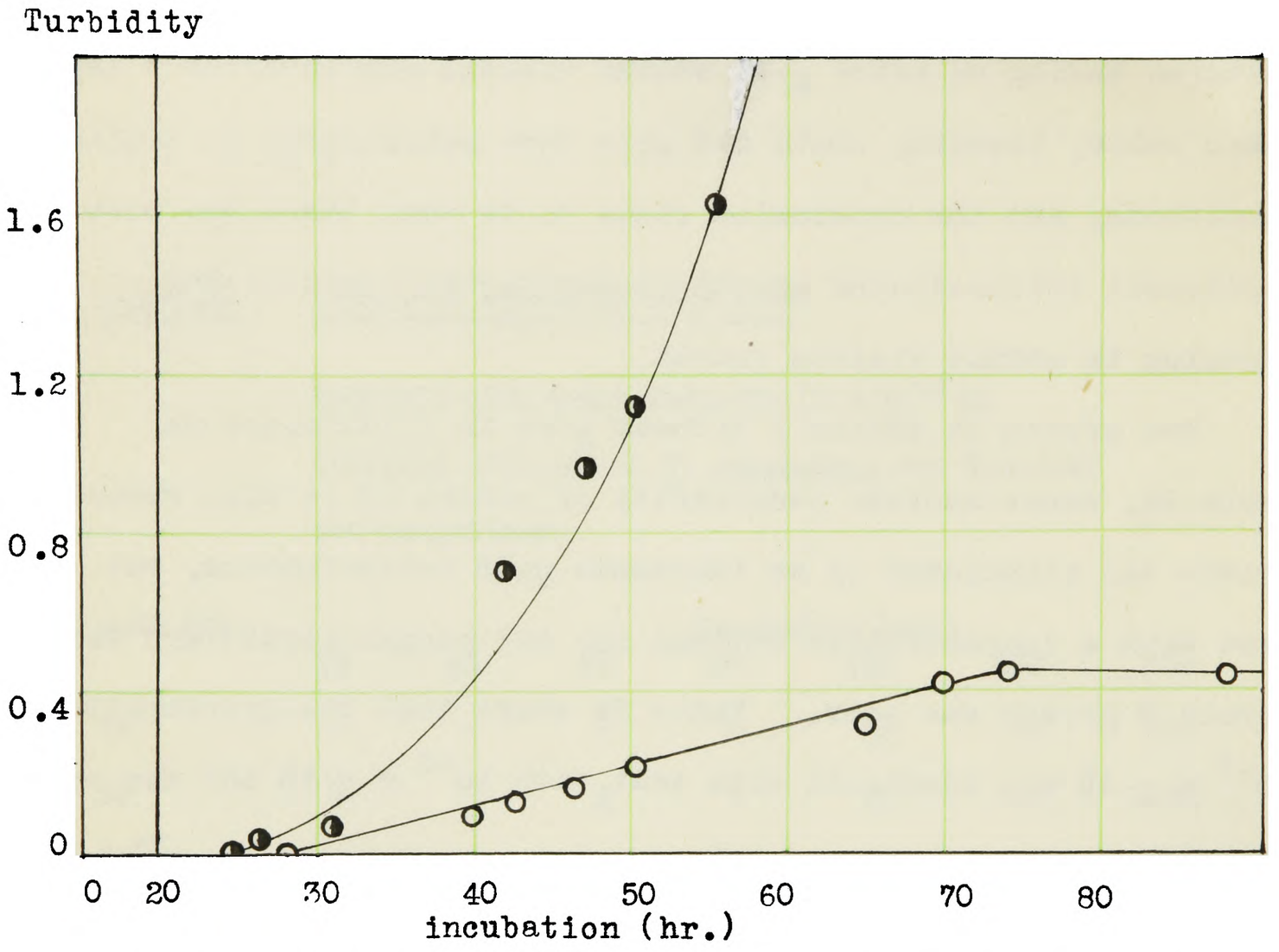
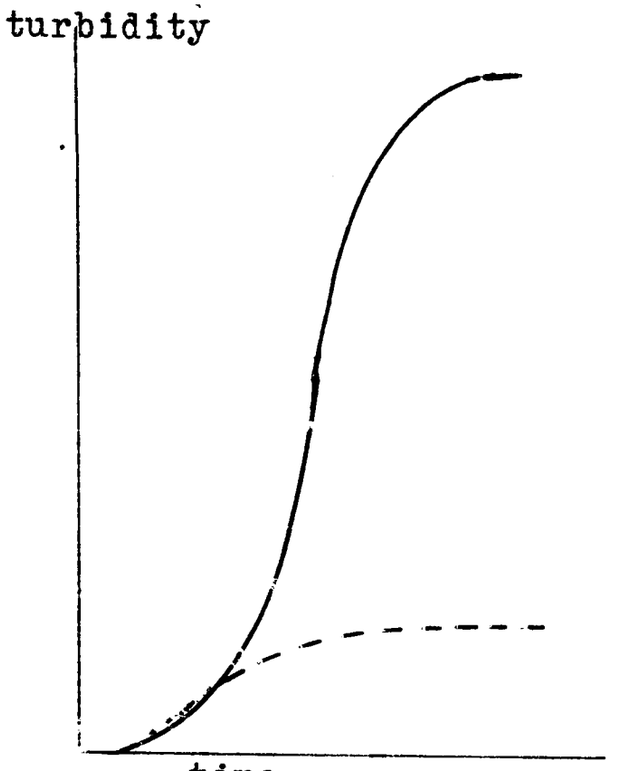


Fig. 20 Growth curves of strains A and A_2 in "improved" medium 2.

◑ strain A

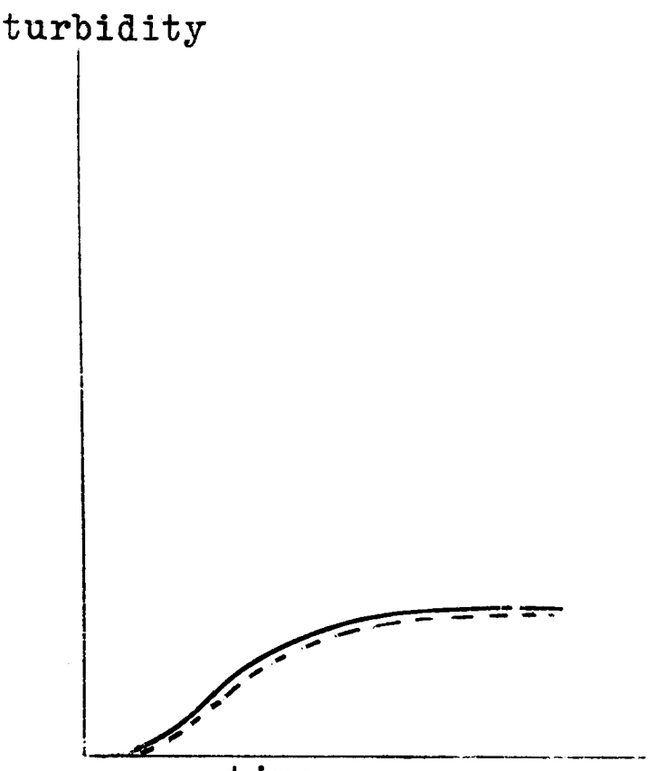
○ strain A_2



growth curves of strain A

—— in improved medium 2

- - - in medium 2



growth curves of strain C

—— in improved medium 2

- - - in medium 2

To see if the stimulation of growth by casein hydrolysate was a general property of non-exacting variants, a new strain of this kind, "strain A_2", was obtained from the 138 hr. growth of a control culture of the normal organism without p-AB. After one passage in Medium 2, in which it grew to the usual limited extent, its growth curve in the improved form of Medium 2 was measured (Fig. 20). The growth of strain A_2 was not stimulated in this medium; it was quite unlike that of strain A grown in similar conditions, and resembled strain C.

TABLE 27 Sulphathiazole resistance and p-AB requirement of "p-AB-less" Esch. coli ; comparison with non-exacting strain.

Medium 6.

A. Test with normal strain:

p-AB (M)	Sulphathiazole (M)	Incubation (hr.) 18	42	66	90	115
10^{-6}	-	+++	+++	+++	+++	+++
10^{-7}	-	+++	+++	+++	+++	+++
10^{-7}	4×10^{-6}	-	-	-	tr.	++
10^{-7}	10^{-5}	-	-	-	-	-
-	-	-	-	-	-	-
		17	43	65	89	113
2×10^{-10}	-	-	-	tr.?	tr.	tr.
8×10^{-11}	-	-	-	-	-	-

B. Test with strain trained to grow without p-AB:

		18	42	66	90	115
10^{-7}	-	+++	+++	+++	+++	+++
-	-	+++	+++	+++	+++	+++
10^{-7}	4×10^{-6}	-	-	±	++	++
10^{-7}	10^{-5}	-	-	-	-	-

Chapter 17

Some Experiments with Escherichia coli 273-384

Sulphonamide-resistant and non-exacting variants could also be obtained from a p-AB-requiring mutant of Esch. coli. In Medium 6 the mutant 273-384 gave optimal growth with 10^{-7} M p-AB, and required between 2×10^{-10} and 8×10^{-11} M p-AB for visible growth. With 10^{-7} M p-AB it would grow in the presence of between 4×10^{-6} and 10^{-5} M sulphathiazole (Table 27).

After two passages in the presence of suboptimal p-AB (10^{-8} M and then 2×10^{-10} M) the strain no longer required this substance, and its growth was the same as that of the original strain when grown with optimal p-AB. After five passages in the absence of added p-AB it was totally unaffected by addition of p-AB, and resisted between 4×10^{-6} and 10^{-5} M sulphathiazole in the presence of 10^{-7} M p-AB. Its sulphathiazole resistance was thus identical with that of the original mutant (Table 27). This behaviour was altogether different from A. suboxydans strain A, which was stimulated by added p-AB, and had an increased sulphathiazole resistance compared with its parent organism.

It is possible that the non-exacting strain of Esch. coli 273-384 was a back mutation to the parent Esch. coli from which Roepke, Libby & Small (1944) originally obtained this mutant, but this is not necessarily true since Raut (1950) observed that a pantothenic acid-requiring artificial mutant of yeast reverted

TABLE 28 Training of "p-AB-less" *Esch. coli.* to resist Sulphathiazole

The normal organism was subcultured serially in Medium 6 with 10^{-7} M p-AB and sulphathiazole concentrations as below.

Serial numbers of subcultures	Sulphathiazole (M)
1	2×10^{-6}
2	10^{-5}
3	2×10^{-5}
4, 5, 6	4×10^{-5}
7	8×10^{-5}
8	10^{-4}
9	2×10^{-4}
10	10^{-4}
11 to 16	4×10^{-4}
17 *et seq.*	10^{-4}

TABLE 29 **Enhanced p-AB requirement of the sulphathiazole-resistant strain of "p-AB-less" Esch. coli. when grown with sulphathiazole**

Medium 6; a test with the normal strain is included for comparison.

p-AB (M)	Sulphathiazole (M)	Incubation (hr.) 22	46	70	94	118	142
10^{-7}	10^{-4}	-	++	+++	+++	...	...
5×10^{-8}	10^{-4}	-	-	tr.	++	+++	+++
2×10^{-8}	10^{-4}	-	-	-	-	-	-
-	-	-	-	-	-	-	-
		18	43	66			
10^{-7}	10^{-4}	-	+++	+++			
10^{-7}	2×10^{-5}	-	+++	+++			
10^{-7}	4×10^{-6}	-	+++	+++			

Test with the normal strain:

		18	42	66
10^{-7}	-	+++	+++	+++
-	-	-	-	-

to pantothenate-independence by a different genetical mechanism from that which caused pantothenate-dependence. Thus the reverted strain was not a genetical back-mutation. The parent strain from which Esch. coli 273-384 was obtained was not available for testing so the point could not be investigated.

Esch. coli 273-384 was also trained to resist sulphathiazole by the same procedure as was used to isolate A. suboxydans, strain C, i.e.: by serial subculture with increasing amounts of sulphathiazole in the presence of optimal (10^{-7} M) p-AB. The progress of the training is recorded in Table 28. Resistance was increased by about 10-fold, but there was some difficulty in increasing it further. The strain still had an absolute requirement for p-AB in the absence of sulphathiazole, and when grown in the presence of its normal amount of sulphathiazole (10^{-4} M) its growth rate was considerably less than that of the parent strain growing with optimal p-AB. Thus, the parent strain would grow almost to its final extent within 16 hr., whereas the resistant strain required 48 hr. even after 30 passages with 10^{-4} M sulphathiazole. Moreover, there was no change in this growth rate over a 25-fold decrease of sulphathiazole concentration (Table 29).

The behaviour of these strains was markedly different from that of comparable strains of A. suboxydans and was not studied further.

PART IV

Discussion

Chapter 18

p-Aminobenzoic Acid in the Metabolism of A. suboxydans 621

The normal strain of A. suboxydans was shown to have the following properties.

1) It required p-AB for growth and this substance was replaceable by synthetic pteroylglutamic acid or by adenine. Pteroylglutamic acid was only effective at fifty to one hundred times the molar concentration of p-AB, but would, in sufficient amounts, support optimal growth of the organism. Adenine was only feebly active, in the sense that the growth of the organism was limited and little affected by the adenine concentration. While it has been reported that adenine will reduce the p-AB requirement of some organisms, including A. suboxydans (page 12), it is probable that in the work reported here adenine supported growth in the total absence of p-AB. It is unlikely that there was any free p-AB in the improved form of Medium 2 devised during this work, but the effect of adenine was nevertheless clear.

The situation may have been like that of the mutant of Esch. coli (Lampen, Roepke & Jones, 1946) whose requirement for

p-AB was abolished by a mixture of purines, thymine and amino-acids. The casein hydrolysate used throughout this work in Medium 2 may well have provided the components, other than adenine, of a similar mixture active for A. suboxydans. The resemblance was not complete, however, since the mixture used by Lampen, Roepke & Jones had no greater activity than adenine alone for A. suboxydans. Even here the reservation must be made that not all the components of Lampen, Roepke & Jones's mixture have been published, and certain minor constituents may be of major importance with A. suboxydans.

2) Both p-AB and pteroylglutamic acid acted as competitive anti-sulphonamide substances, though, as with its growth factor activity, pteroylglutamic acid had only about 1% of the activity of p-AB on a molar basis. These two facts taken together suggest that pteroylglutamic acid had no intrinsic activity, but merely acted as an inefficient source of p-AB. Woods (1940) showed that procaine (N-diethyl-p-aminobenzoic acid) could act as weak anti-sulphonamide for Esch. coli, probably for a similar reason. If pteroylglutamic acid had had intrinsic activity as a product of the utilisation of p-AB in normal conditions, it would have been expected to show non-competitive anti-sulphonamide activity. Nimmo-Smith & Woods (1947, 1948) showed that pteroylglutamic acid acted as a non-competitive anti-sulphonamide with Cl. acetobutylicum and with Sbm. plantarum spp., though it acted competitively with A. suboxydans 621 and a p-AB-requiring

mutant of N. crassa. As a growth factor for the former organisms it had in all cases between 1% and 10% of the activity of p-AB. With Cl. tetanomorphum, however, Sims & Woods (1950) later showed that pteroylglutamic acid was two to four times as active as p-AB as a growth factor, and was a non-competitive antagonist of sulphonamide inhibition. Lampen & Jones (1946) showed a similar non-competitive sulphonamide antagonism in enterococci stimulated by pteroylglutamic acid. In cases in which pteroylglutamic acid is a non-competitive sulphonamide antagonist it is likely that it is, or is readily converted to, a normal product of the utilisation of p-AB. Since it acts competitively with A. suboxydans, it is unlikely that the pteroylglutamic acid molecule is normally formed from p-AB by this organism. It is possible, however, that preformed pteroylglutamic acid is merely unable to enter the cell.

The fact that pteroylglutamic acid was probably not formed by A. suboxydans does not imply that no compounds of the folic acid group were formed by the organism, nor that these would not have shown non-competitive anti-sulphonamide activity if isolated. Indeed, there was evidence that A. suboxydans normally formed a substance, active in place of pteroylglutamic acid for Lb. casei, which was very probably a folic acid. This phenomenon will be discussed in more detail later.

Adenine acted as a non-competitive anti-sulphonamide for A. suboxydans, permitting limited growth both with and without

sulphathiazole. This suggests that adenine was one of the products of the reaction involving p-AB with which sulphonamides interfered competitively. In view of the limited growth obtained with adenine, however, it is likely that other important products were also normally formed from p-AB which adenine could not entirely replace. The activity of adenine both as a growth factor and as an anti-sulphonamide resembled its activity for the lactic bacteria studied by Snell & Mitchell (1942), though these workers used adenine as a component of a purine mixture. Since Medium 2 contained methionine in the casein hydrolysate, the behaviour of A. suboxydans with adenine may also have resembled that of Esch. coli studied by Harris & Kohn (1941) who were able to show anti-sulphonamide activity only in the presence of methionine. A. suboxydans differed from Esch. coli and Salmonella typhimurium (Winkler & de Haan, 1948) for which xanthine rather than adenine was involved in sulphonamide antagonism. An interesting difference in detail arises between the present work and the findings of Landy & Streightoff (1943). Using A. suboxydans 621 these authors found that adenine reduced the p-AB requirement by a factor of about ten, whereas it has been shown here that it replaced p-AB altogether, though growth was then limited in amount.

No experiments were performed with the "citrovorum factor" (page 17) as this was not available during the work reported here. Woods (unpublished) has shown that its behaviour with

A. suboxydans resembles that of pteroylglutamic acid.

3) A. suboxydans required various amino-acids for growth, though the details of this requirement were not studied fully. In this work amino-acids were provided in the form of vitamin-free casein hydrolysate, and the amount of this was shown to affect the response of the organism to p-AB: increased amounts of casein hydrolysate stimulated growth at fixed levels of p-AB.

Large amounts of casein hydrolysate were required to permit optimal growth, and the studies with strain A (Chapter 13) suggested that no single amino-acid was responsible for this effect, though aspartic acid had some activity. This kind of result suggests that there was some active factor present in very limited concentrations in casein hydrolysate, and that large amounts of casein hydrolysate had to be added before the factor reached an adequate concentration. The factor was not free p-AB as it was not removed at pH 3.8 by ether extraction, and it was not likely to have been bound p-AB released by the organism from a precursor, since growth of the organism was stimulated by casein hydrolysate in the presence of optimal added p-AB. It was also unlikely to be co-enzyme A or a derivative thereof (though these compounds are active for A. suboxydans (Novelli, Flynn & Lipmann, 1949)), since such compounds are very sensitive to acid hydrolysis (King, Locker & Cheldelin, 1948) and are unlikely to survive the preparation of an acid casein hydrolysate, even if present in the original casein. Nucleoproteins are possible

contaminants of casein, and partially hydrolysed derivatives of these might have survived acid hydrolysis and been responsible for the activity observed. No nucleosides or nucleotides were tested during this work.

An alternative possibility is that p-AB was concerned with the utilisation of amino-acids in the cell, and that the effect of casein hydrolysate at suboptimal p-AB levels was merely to increase the availability of these substances. Consistent with this possibility is the fact that casein hydrolysate would not replace p-AB altogether but only acted synergistically with it. Unfortunately the complete composition of vitamin-free casein hydrolysate is not known, so this possibility could not be tested with an artificial mixture.

It is possible that p-AB has some general function in the utilisation of amino-acids by micro-organisms. Amino-acids have been shown to be involved in the metabolism of p-AB in many organisms (Chapter 2, page 8), but in detail each case differs. Studies with Esch. coli indicated that methionine (Strauss, Dingle & Finland, 1941; Harris & Kohn, 1941 a,b; Davis & Mingioli, 1950), possibly arginine and lysine (Bliss & Long, 1941), methionine, valine, lysine, serine and isoleucine (Winkler & de Haan, 1948), and methionine, lysine and other unspecified amino-acids (Lampen, Roepke & Jones, 1946) were involved. Studies with other organisms implicated methionine, serine, valine, lysine and isoleucine for Salmonella typhimurium (Winkler

& de Haan, 1948), methionine and valine for Staph. aureus (de Haan, Winkler & Haljet, 1949) and methionine, histidine, leucine, isoleucine, norleucine, valine, norvaline, tyrosine and phenylalanine for Saccharomyces cerevisiae (Cutts & Rainbow, 1950). The findings with strain A of A. suboxydans reported in the present work suggest that asparagine may be involved in p-AB metabolism. While there are many points of agreement in the results of work in this field, the overall picture is obscured by differences in detail, which occur even with the same species of organism. These inconsistencies can be to some extent resolved if p-AB is regarded as being directly involved in the synthesis of only one or two amino-acids, but as conditioning the utilisation of other amino-acids through these. For example, the evidence is particularly strong that p-AB is directly involved in the synthesis of methionine and serine, and the synthesis of peptides and proteins by the cell would obviously depend on the presence of these amino-acids. The incorporation of other amino-acids into peptides may thus be determined by the availability of serine and methionine, which would in its turn be dependent on the availability of p-AB. The effects of amino-acids other than methionine and serine may simply be mass action effects: in conditions of methionine or serine deficiency increase in the concentration of the other amino-acids may merely assist the formation of peptides containing both groups of compounds.

4) A. suboxydans normally synthesised a factor active in place of folic acid for Lb. casei and one active for "adenine-less" N. crassa; they were probably folic acid and adenine respectively. The behaviour of adenine as a growth factor and as an anti-sulphonamide suggested that p-AB was involved in its synthesis, but there was no direct evidence from this work that p-AB was involved in the synthesis of folic acid. However, the fact that A. suboxydans trained to dispense with p-AB (strain A) produced very little, if any, folic acid suggested that p-AB played some part in folic acid synthesis.

It was unlikely that the folic acid formed by A. suboxydans was pteroylglutamic acid for two reasons. Firstly, as has been mentioned (page 99), pre-formed pteroylglutamic acid should have acted as a non-competitive anti-sulphonamide if it were a normal product of p-AB metabolism, and, secondly, the shapes of the response curves given by Lb. casei to bacterial extracts were quite different from those given to pteroylglutamic acid. In this respect the behaviour of A. suboxydans contrasted with that of Sbm. plantarum 10 S, which synthesised from p-AB a substance very like pteroyglutamic acid in its activity towards Lb. casei (Nimmo-Smith, Lascelles & Woods, 1948). Its behaviour was more like that of Staph. aureus and Esch. coli, which synthesised from p-AB a Lb. casei factor giving a response curve different from that of pteroylglutamic acid (Lascelles & Woods, in press).

Chapter 19

A Summary of the Properties of the Trained Strains of *A. suboxydans* 621

Strain A. The strain of *A. suboxydans* which could grow without added p-AB had the following properties which differed from those of the parent organism.

1) It had become more sensitive to the amount of vitamin-free casein hydrolysate provided, which had to be increased considerably to give optimal growth. Aspartic acid had some stimulatory effect in the presence of suboptimal casein hydrolysate but did not support optimal growth. Malic acid (the deaminated form of aspartic acid) and asparagine would not replace aspartic acid, nor would biotin or CO_2, both of which are biochemically related to aspartic acid (Landy, Potter & Elvehjem, 1947; Shive & Rogers, 1947). The only substances which would replace casein hydrolysate effectively were p-AB and pteroylglutamic acid, and we already have evidence (page 99) that the latter compound was only acting as an inefficient source of p-AB.

2) The strain had an enhanced sulphathiazole resistance compared with the normal organism, though it was not as resistant as a strain trained deliberately to resist sulphathiazole. The p-AB : sulphathiazole relationship remained competitive.

3) Assay with normal A. suboxydans did not indicate any synthesis of p-AB by this strain.

4) The folic acid synthesis of this strain was limited as compared with that of the normal organism of strain C, though substances weakly stimulatory to Lb. casei were produced.

5) The adenine synthesis of this strain was quantitatively the same as that of the normal strain and of strain C.

Strain C. Cells trained to grow in the presence of sulphathiazole had the following properties.

1) They retained their requirement for p-AB in these conditions and this requirement was the same as that of the normal organism. The p-AB : sulphathiazole ratio had decreased by some 100-fold, but the relationship was competitive. Growth in the absence of sulphathiazole and presence of p-AB was normal, but the strain had also developed the power to grow in the absence of p-AB provided that sulphathiazole was also absent. Growth in this case was weak in Medium 2, like that of strain A, but was not stimulated by increased casein hydrolysate. In this respect it resembled strain A_2 (page 95).

2) Assay, with normal A. suboxydans, of cells grown without either p-AB or sulphathiazole did not indicate synthesis of p-AB by this strain.

3) The synthesis of folic acid by this strain was unchanged as compared with that of the normal organism, and the folic acid content of cells of strain C was the same whether they were grown in the presence or in the absence of sulphathiazole. These findings resemble those of Miller (1944) and Miller, Brune

& Berglund (1947) who observed that sulphonamide-trained Esch. coli synthesised the same amount of folic acid as the untrained strain. Whole cultures of strain C grown in the absence of sulphathiazole, however, appeared to contain slightly more folic acid than those grown in the presence of the drug.

4) The synthesis of adenine by this strain was similar to that of the parent organism.

5) The strain did not destroy sulphathiazole.

Other trained strains. It did not prove possible to obtain a strain of cells trained to grow both without p-AB and in the presence of large amounts of sulphathiazole. Strain CD was the result of an attempt to do this, and while this strain had some unusual properties (page 93) it still required added p-AB for growth in the presence of high concentrations of sulphathiazole. The failure of the strain to give optimal growth with added p-AB even in the absence of sulphathiazole suggested that the intense training process which it had undergone had effected some permanent damage to the cell metabolism.

Strain A_2, the freshly isolated non-exacting strain, showed an interesting difference from strain A proper in that its growth was not stimulated by increased amounts of casein hydrolysate. Hinshelwood (1946) reported that the ease with which Bact. lactis aerogenes reverted when it had been trained to resist drugs depended on the number of times it had been subcultured through the training medium, and Postgate & Hinshelwood (1946) observed

a similar effect when this organism was trained to grow in various carbon sources; the ease with which the organism lost its ability to grow rapidly with lactose depended on the number of subcultures it had undergone in a medium containing this sugar. Such results suggest that in certain kinds of training the variation becomes more fully established the more the training process is protracted, and a similar phenomenon may have occurred with strains A and A_2. Strain A was the more completely trained in that it would, with excess casein hydrolysate, give optimal growth in the absence of p-AB, whereas strain A_2 was unable to do this.

Chapter 20

The Mechanism of the Variation to Dispense with p-Aminobenzoic Acid

The variation undergone to produce strain A can have taken place by one of two mechanisms, either the development of the ability to synthesise p-AB or the development of a different growth mechanism in which p-AB was no longer concerned.

Taking the first view, the limited growth of the organism in Medium 2 was presumably due to its inability to synthesise p-AB in sufficient amounts. Now, the normal growth of this strain in Medium 2 reached 0.42 mg. dry wt. cells/ml., and it will be seen from Fig. 19 (page 87) that the concentration of p-AB required to grow this amount of normal cells was about 10^{-8} M. We should therefore have to suppose that strain A could maintain an equilibrium concentration of this magnitude during growth.

This quantity of p-AB would have acted additively with respect to any p-AB coming from outside, and with 10^{-7} M added p-AB one would have expected effects equivalent to 1.1×10^{-7} M p-AB added to a culture of the normal organism. Such an effect would be, for instance, a 10% increase in the sulphathiazole resistance of the strain as compared with the normal organism grown in the presence of 10^{-7} M p-AB. In fact, the increase in sulphathiazole resistance shown by strain A in these conditions was some 1000% (page 74).

Again, with this strain the p-AB/sulphathiazole relationship was competitive down to 10^{-9} M p-AB (Table 15, page 74) which could hardly be the case if the organism were synthesising 10^{-8} M p-AB by itself.

One could imagine synthesis of p-AB by this strain occurring at the site of p-AB utilisation in such a manner that the active mass of p-AB was greater than that of an equivalent concentration of added p-AB. Such a theory would account for an increased p-AB : sulphathiazole ratio at low levels of added p-AB, but this altered ratio would not have been detectable at levels of added p-AB significantly greater than 10^{-8} M.

These considerations, together with the failure to detect p-AB by microbiological assay even after hydrolysis of the organism, suggest that it is unlikely that p-AB was synthesised. It is more likely that a variation by the second mechanism took place to form strain A: the strain was using a new mode of growth in which p-AB was no longer important. It is unlikely that this new mode of growth was by way of adenine synthesis: not only was the production of adenine by this strain normal, but adenine was shown to be a non-competitive sulphonamide antagonist for A. sub-oxydans at sulphathiazole concentrations considerably in excess of that required to prevent growth of strain A. On the other hand, it is likely that this mode of growth avoided folic acid as well as p-AB, since the production of Lb. casei factor by strain A was much depressed compared with the production by strains grown in the presence of p-AB. The sensitivity of the strain to

casein hydrolysate, however, suggests that this new mode of growth depended either on an unidentified factor present to a small extent in Ashe's vitamin-free casein hydrolysate, or on the presence of a mixture of some or all of the amino-acids to be found in casein hydrolysate which, except for aspartic acid, were inactive when tested separately. The action of aspartic acid, which was weak, could have been due to antagonism between it and the structurally related glutamic acid, which is present in large amounts in casein hydrolysate. This is not very likely, however, as glutamic acid ought then to have had the converse effect: increase in the glutamic acid concentration ought to have inhibited growth, whereas a doubling of the glutamic acid concentration had no effect in practice (page 72).

The two possible general mechanisms of the variation to give strain A which have been discussed in this chapter have so far been regarded as alternatives, but they are not incompatible. It is also possible that strain A, like the mutant of N. crassa described by Strehler (1950), had limited powers of p-AB synthesis, sufficient for certain key reactions in cell growth, but insufficient for amino-acid synthesis. Hence the presence of the amino-acids in casein hydrolysate, and possibly other compounds, was essential for growth and conditioned its extent. The facts obtained during this work are consistent with this hypothesis, but, if the strain had developed some ability to synthesise p-AB, it is difficult to understand why further adaptation or mutation did not occur, during the many passages

the strain underwent in p-AB free medium, enabling it to synthesise its complete p-AB requirements.

Chapter 21

The Mechanism of Variation to Sulphonamide Resistance

It is possible to apply similar reasoning in the case of strain C, but, since p-AB was normally present in the growth medium, there are now three theoretical possibilities.

i) Development of the ability to synthesise p-AB, and thus to antagonise greater amounts of sulphonamide for a given amount of added p-AB.

ii) Development of the power to grow by mechanisms not involving p-AB so that sulphonamide, while reducing the availability of p-AB, did not affect the growth of the cells.

iii) Development of a mechanism for the more efficient utilisation of p-AB, probably by an expansion of the amount of enzyme at the surface of which drug and factor competed. If the extent of growth of A. suboxydans were dependent on the amount of enzyme surface ("a") occupied by p-AB molecules at any given instant, then an increase in the amount of enzyme present would allow for adsorption of more molecules of inhibitor without reducing "a" to a suboptimal level; the p-AB:sulphonamide ratio would merely decrease.

As to the first possibility, it has been shown that the normal organism had less than 1% of the sulphonamide resistance of strain C for a given amount of added p-AB, so strain C must have maintained during growth an equilibrium concentration of 10^{-5} M

p-AB to account for its greater sulphonamide resistance when tested with 10^{-7} M added p-AB. If p-AB synthesis of this magnitude were in fact occurring, sufficient p-AB should have been present in cultures of strain C to antagonise all concentrations of sulphathiazole below the usual 2×10^{-3} M: below this drug concentration the p-AB/sulphathiazole relationship should not have appeared competitive. This was not so.

. Moreover, with the synthesis of this amount of p-AB, optimal growth of the strain would have been expected in the absence of both p-AB and sulphathiazole, but this was not found. Only limited growth occurred and no p-AB was detectable by assay of such cultures.

The theory cannot be entirely eliminated, however, since it is possible that an ability to synthesise p-AB was developed, which only operated in the presence of sulphathiazole, and the extent of which was conditioned by the amount of sulphathiazole present. It is difficult to envisage a mechanism by which this could have happened.

The second interpretation, that the organism had developed a new mode of growth in which p-AB was no longer important, implies that it had undergone a change of the type that yielded strain A. As was seen in Chapter 20, there are at least three types of change which may have given strain A, so the details of these will not be discusssed again in connection with strain C. The sulphathiazole resistance of strain C was, however, ten times

as great as that of strain A, so some further change must also be envisaged to account for this increase. Moreover, though strain C resembled strain A in giving limited growth in Medium 2 with neither p-AB nor sulphathiazole, its growth in similar conditions using the improved form of Medium 2 was not stimulated, though that of strain A was stimulated to optimal.

It is the opinion of the author that the most likely interpretation is the third: that there was a quantitative expansion of a p-AB-utilising enzyme, at the surface of which (or, more formally, with respect to which) sulphathiazole and p-AB competed. If optimal growth occurred when a portion "a" of the total enzyme surface "s" was occupied by p-AB molecules, then this amount "a" of enzyme would be available to the p-AB molecules in the presence of high concentrations of sulphathiazole only if there were a large total surface "s" to take up excess sulphathiazole molecules The variation to give this type of system would manifest itself solely in a decreased p-AB : sulphonamide ratio. Moreover, the quantitative p-AB requirement of the strain (the number of molecules required to saturate the surface "a") would be the same as that of the normal organism in comparable conditions of growth. This was found to be true. Further, if the utilisation of p-AB is regarded as having resulted in the formation of folic acid and adenine, among other products, a view for which there is some evidence, then the yield of these in normal growth conditions ought to have been unchanged as between normal and trained cells. This also proved to be the case.

There is, however, one reason why this interpretation, taken alone, is not entirely satisfactory: the strain had lost its absolute requirement for p-AB, in the sense that in the absence of sulphathiazole it could grow without any p-AB at all. The expanded enzyme picture does not account for this, but it is possible that the loss of p-AB requirement was a secondary variation not directly concerned with the sulphonamide resistance. Such a variation is unlikely to have been due merely to prolonged passage in liquid medium, since Marshall (1948) found that normal A. suboxydans could be subcultured at least 10 times in Medium 2 and that, provided the p-AB concentration was optimal, no change of this kind occurred. He also found that variations of this kind, giving non-exacting types like strain A which exhibited limited growth in Medium 2, occurred at suboptimal levels of p-AB as well as in its absence, the effect being observable with 10^{-8} M p-AB and less. In training A. suboxydans to give strain C it was subcultured for several passages in conditions where growth was limited by sulphathiazole present before it finally developed the ability to grow optimally with this drug. The expanded enzyme picture suggests that sulphathiazole reduced the availability of p-AB by competition, and thus produced conditions equivalent to growth in suboptimal p-AB. Since these conditions favour the appearance of non-exacting variants it is possible that the ability of strain C to grow without p-AB and sulphathiazole was a variation that occurred in its early history

and which was due to the nature of the training process but irrelevant to sulphonamide resistance.

There was a difference between the behaviours of strains A and C grown in the absence of drug and metabolite: the growth of strain C in these conditions was not stimulated by increased amounts of casein hydrolysate. This, however, is not necessarily a fundamental distinction, since, from the interpretation outlined in the previous paragraph, strain C grown in these conditions was more analagous to a freshly isolated non-exacting variant such as strain A_2 than to a fully trained variant such as strain A proper. In so far as the growth of neither strain A_2 nor strain C in Medium 2 was stimulated by increased casein hydrolysate in these conditions, their behaviours supported this view.

The sulphathiazole resistances of strains A and C in the absence of p-AB were, however, different, that of strain C being the greater. This is difficult to interpret on the hypothesis outlined above, though differences in resistance in the presence of p-AB are readily understood.

Since it is not easy to design experiments to investigate this the matter will not be discussed further, but it is useful to know that the variation which enabled strain C to grow in the absence of both p-AB and sulphathiazole is explicable in terms of what is already known of the behaviour of A. suboxydans.

Chapter 22

The Variations of Esch. coli 273-384

From the few experiments done with this p-AB-requiring mutant of Esch. coli it is clear that the situation was entirely different from that of A. suboxydans. The variation enabling the organism to grow in the absence of p-AB may or may not have been a "back mutation", but it resulted in the organism being entirely independent of p-AB for its growth. Its growth was not stimulated by p-AB, unlike the analogous strain A of A. suboxydans. It also underwent no change in its resistance to sulphathiazole as compared with that of the original mutant.

In training the mutant to resist sulphathiazole the concentration of drug required to prevent visible growth was increased, but the requirement for p-AB in the presence of the drug was very large. Moreover, the variation was not complete compared with that of strain C of A. suboxydans since, after as many as twenty-four passages with 10^{-4} M sulphathiazole, the organism still grew suboptimally. Finally, this increase in resistance to sulphathiazole did not alter the absolute need of the mutant for p-AB in the absence of drug.

In view of the few experiments which were done with this organism it would not be fruitful to discuss the mechanisms of the variations which took place. It is unlikely, however, that they occurred by the same mechanisms as yielded the analogous strain of A. suboxydans.

Chapter 23

Some Theoretical Implications of the Expanded Enzyme Picture of Sulphonamide Adaptation

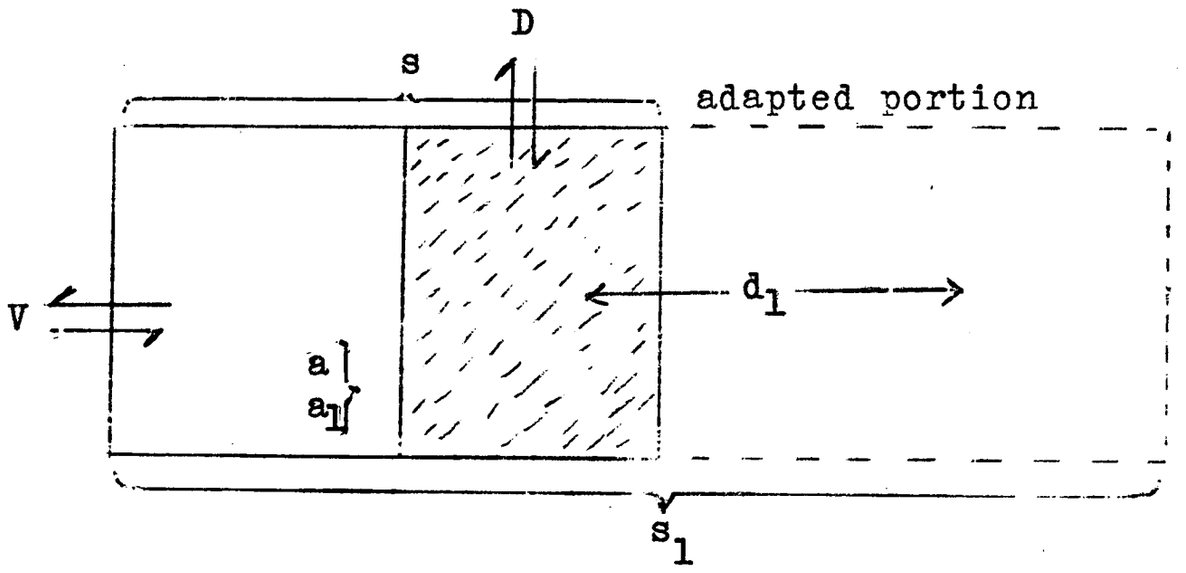
The concept of quantitative enzyme expansion has often been used as a basis for the interpretation of adaptive processes, particularly by Hinshelwood (1946) but at no time has any idea been given of the quantitative meaning of this picture in terms of the amount of enzyme present. The reason for this is probably that in all such interpretations the enzyme in question, the one whose function is interfered with by the inhibitor, is hypothetical. Nothing more is known about it than that, if it exists, it is inhibited. In the case of A. suboxydans, however, while the enzyme was still in a sense hypothetical, we can say that, if it existed, it was involved in the utilisation of p-AB, and that its function was interfered with by sulphonamides in a competitive manner. Hence the interaction between p-AB and the enzyme was of very much the same type as the interaction between sulphonamides and the enzyme.

If the primary interaction is regarded as having been an adsorption process, obeying the usual physico-chemical laws of adsorption, then the metabolite and inhibitor can be considered as being in an adsorption equilibrium with the enzyme surface. By examining the precise conditions for this both in a normal and in a trained organism it is possible to get some idea of the magnitude of the enzyme expansion needed to account for the

variation in question.

It should be emphasised that the expanded enzyme picture and the theoretical development of it which follows make no assumption whatever about the mechanism by which the variation takes place. It is of no importance to the theory whether the change is strictly an adaptation or a series of mutational steps, and there is no evidence from this work which would enable one to decide between these views.

If, for the normal organism, "s" is the total area at which adsorption takes place, "a" that occupied by the vitamin "V" and "d" that occupied by the drug "D", and if "s_1", "a_1" and "d_1" are the corresponding quantities for the adapted organism in which the expansion has occurred, then expressions can be written for the rates of adsorption and evaporation of drug in the usual notation as below:



For the normal strain, as soon as drug and vitamin are added:

$$\text{Rate of drug adsorption} = k[D](s - d - a)$$

and:

$$\text{Rate of drug evaporation} = k_1 d.$$

where k and k_1 are rate constants for the two processes. At equilibrium, for all levels of V and D, these processes will be balanced. Writing k/k_1 as K_d, we have:

$$d = K_d[D](s - d - a) \qquad (1)$$

Applying exactly similar considerations to the vitamin, we have:

$$a = K_v[V](s - d - a) \qquad (2)$$

Similarly, for the adapted strain we can write:

$$d_1 = K_d[D_1](s_1 - d_1 - a_1)$$

and:

$$a_1 = K_v[V_1](s_1 - d_1 - a_1).$$

Now, both from theory and by experiment, the vitamin requirement of the two strains is unchanged, the growth of the organism being conditioned by the area available for adsorption of the vitamin. When the strains are giving equivalent growth this surface will be the same in the two strains:

$$a = a_1 \quad \text{(at equivalent growth levels)}$$

Therefore:

$$d_1 = K_d[D_1](s_1 - d_1 - a) \qquad (3)$$

and

$$a = [K_v][V_1](s_1 - d_1 - a) \qquad (4)$$

When growth is 50% inhibited it can be assumed that half the available enzyme surface is occupied by vitamin and half by drug in the normal strain, i.e.:

$$a_{\frac{1}{2}} = d_{\frac{1}{2}}$$

from which, applying (1) and (2):

$$a_{\frac{1}{2}} = K_v[V_{\frac{1}{2}}](s - d_{\frac{1}{2}} - a_{\frac{1}{2}}) = d_{\frac{1}{2}} = K_d[D_{\frac{1}{2}}](s - d_{\frac{1}{2}} - a_{\frac{1}{2}}).$$

Therefore:

$$K_v[V_{\frac{1}{2}}] = K_d[D_{\frac{1}{2}}]$$

or:

$$K_v/K_d = [D_{\frac{1}{2}}]/[V_{\frac{1}{2}}]. \qquad (5)$$

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The sulphathiazole concentration causing 50% inhibition in A. suboxydans 621 grown with 10^{-7} M p-AB was not evaluated, but may be taken from Table 14 to be in the region of 10^{-5} M. Applying (5) to these figures, K_v/K_d becomes about 10^2 for this case.

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Considering the normal organism in the absence of sulphonamide, then, as before:

Rate of p-AB adsorption = $k_2[V](s - a)$

Rate of p-AB evaporation = $k_3 \cdot a$

whence:

$$a = K[V](s - a).$$

Again, if half growth is due to occupation by the vitamin of half of the available enzyme surface ($a = s - a$) then:

$$K_v = 1/[V_{\frac{1}{2}}]. \qquad (6)$$

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Fig. 19 (page 87) shows that the amount of p-AB giving half growth with normal A. suboxydans was in the region of 10^{-8} M, so from (6) K_v can be taken as about 10^8. From the previous result for the ratio of these constants, K_d becomes about 10^6.

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The quantitative expansion of enzyme surface required to account for the type of adaptation observed may now be calculated. This expansion can be represented algebraically as s_1/s, which will now be evaluated.

From (1) and (2):

$$s - d - a = \frac{d}{K_d[D]} = \frac{a}{K_v[V]}.$$

Hence:

$$\frac{s}{a} = \frac{K_d[D]}{K_v[V]} + 1 + \frac{1}{K_v[V]}.$$

Similarly, from (3) and (4):

$$\frac{s_1}{a} = \frac{K_d[D_1]}{K_v[V_1]} + 1 + \frac{1}{K_v[V_1]}.$$

Therefore:

$$\frac{s_1}{s} = \frac{K_d[D_1] + K_v[V_1] + 1}{[V_1]} \times \frac{[V]}{K_d[D] + K_v[V] + 1}.$$

If we now restrict ourselves to cases where comparisons are made at the same vitamin concentration, where $[V] = [V_1]$, we can simplify this expression to:

$$\frac{s_1}{s} = \frac{K_d [D_1] + K_v [V_1] + 1}{K_d [D] + K_v [V] + 1} \qquad (7)$$

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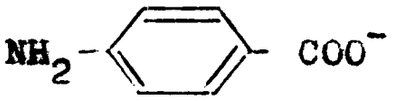
Table 14 (page 74) showed that equivalent suboptimal growth was given by normal A. suboxydans and strain C at respectively 4×10^{-5} M and 4×10^{-3} M sulphathiazole in the presence of 10^{-7} M p-AB. Since the growth was in both cases limited, the strains were strictly comparable and the enzyme surface cannot have been saturated with any one component of the system. Applying (7) we find:

$$\frac{s_1}{s} = \frac{(10^6)(4 \times 10^{-3}) + (10^8)(10^{-7}) + 1}{(10^6)(4 \times 10^{-5}) + (10^8)(10^{-7}) + 1}$$

$$\simeq 80.$$

In other words, an 80-fold increase in the area of the enzyme surface is sufficient to account for the approximately 200-fold increase in resistance observed; the ratio of increase in resistance to enzyme expansion for a variation of this kind should be 5:2.

It should be noted that this calculation makes no assumption concerning the active molecular species. At pH 6.0 p-AB is almost completely ionised in the form:



whereas only one fifteenth of the sulphathiazole is ionised at this pH, according to the figures of Bell & Roblin (1942). (In the usual notation,

$$\text{fraction ionised} = \frac{K_a}{K_a + [H^+]} = \frac{7.6 \times 10^{-8}}{7.6 \times 10^{-8} + 10^{-6}} = 1/15).$$

However, this correction will appear on both sides of relation (7), merely increasing K_d and not affecting the ratio significantly. A re-calculation, not recorded here, has been done, giving a similar result.

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It should perhaps be emphasised also that the treatment used in these calculations, picturing the process as a competitive adsorption of metabolite and antagonist at a surface, makes no real assumption as to the nature of the process. A precisely similar calculation could have been carried out in terms of mass action equilibria in solution between the enzyme, metabolite and inhibitor. Expressions for the concentrations of dissociable enzyme/drug and enzyme/vitamin complexes, and of free enzyme, would have replaced those representing areas of enzyme in the calculation above and would have given the same numerical result. With such a picture, an 80-fold increase in the active

mass of the enzyme species would have been required to account for the change in resistance observed. Similarly, an 80-fold increase in the affinity or free energy of interaction between the enzyme and p-AB as compared with that between the enzyme and sulphathiazole would account for the results found in this work. The treatment used was chosen as being perhaps rather easier to picture.

The figures obtained are of little more than theoretical interest, since we have little knowledge of the reaction in which p-AB is antagonised by sulphonamides. One can predict that there will be an 80-fold increase in the rate of formation of the products of this reaction when the trained and untrained strains are compared in the absence of inhibitor, and similarly an 80-fold increase in the rate of utilisation of p-AB can be expected. Work along the lines of that of Nimmo-Smith, Lascelles & Woods (1948) with active suspensions of p-AB-utilising cells would be of considerable interest in this connection. The 40% increase in absolute yield of folic acid found in the present work (page 86) is of no significance in this respect as there is no information as to how rapidly it was formed or disappeared.

Lascelles & Woods (unpublished) have recently studied the synthesis of Lb. casei factor by suspensions of Staph. aureus. They observed that, with 10^{-5} M p-AB, the synthesis of factor in the absence of sulphathiazole was ten times as great with a strain trained to resist sulphathiazole as with the normal

strain. The sulphathiazole resistance of the trained strain was fifty times that of the normal organism. The organism did not require p-AB for growth, so a calculation of the kind outlined above could not be applied. Their results are consistent with the theory above, however, since the increase in sulphathiazole resistance resulted in a corresponding but smaller expansion of p-AB-utilising enzyme as reflected in the increased formation of Lb. casei factor. The ratio of resistance to yield of factor (5 : 1) is of the order of the ratio of resistance to enzyme expansion predicted above (5 : 2). Though this agreement may be no more than coincidence, the change in the quantitative properties of Staph. aureus on training to resist sulphathiazole is clearly of the order to be expected on the expanded enzyme theory of sulphonamide resistance. The agreement between these results is the more surprising in view of the fact that the medium used by Lascelles & Woods contained no direct precursor of the pterin residue in the folic acid molecule. The rate of synthesis of this might easily have been rate-determining in the synthesis of Lb. casei factor by the trained strain, and might have masked any effect of an increased rate of p-AB utilisation.

Chapter 24

The Conclusions reached in Chapters 20 to 23

The formation of strains A and C. The changes that took place in A. suboxydans, firstly to enable it to grow in the absence of p-AB, and, secondly, to enable it to resist sulphathiazole, can be said to be different in kind. In both cases it is unlikely that the organism developed the power to synthesise p-AB since the quantitative properties of the strains were not in keeping with such a view, and it was shown that the sulphathiazole-resistant strain did not destroy sulphathiazole. The variation to grow without p-AB seems more likely to have been due to the development of an alternative mode of growth in which p-AB and folic acid were not important, but which may well have depended on the presence of an increased concentration of amino-acids. Alternatively, growth in the absence of p-AB may have depended on some unknown factor in vitamin-free casein hydrolysate.

On the other hand, while a variation of this kind may have taken place during the training to resist sulphonamides, it was not the primary change, and theories based on such a change do not account satisfactorily for the properties of the resistant strain. It seems likely that the primary effect was an expansion of the existing enzyme involved in the utilisation of p-AB, with respect to which p-AB and sulphonamides compete.

An 80-fold expansion of this kind has been shown theoretically to be sufficient to account for the more than 200-fold increase in drug resistance observed.

The variant strains of Esch. coli 273-384 behaved differently from the analogous strains of A. suboxydans and it is likely that growth without p-AB and sulphathiazole resistance were achieved by mechanisms different from those which operated in A. suboxydans. The precise processes which had occurred in Esch. coli 273-384 were not discussed in detail.

SECTION II

THE INTERACTION OF SOME ORGANISMS REQUIRING p-AMINOBENZOIC ACID FOR GROWTH

Chapter 25

Introduction : The Accumulation of Intermediates by Biochemical Mutants

Concurrently with the work described in the foregoing part of this dissertation an attempt was made to investigate the biosynthesis of p-AB itself, using the elegant approach of Beadle and his colleagues (see Beadle, 1945) with biochemical mutants. An organism synthesising a metabolite M presumably does so by a chain of synthetic reactions, and utilises it by a further sequence of reactions. Organisms requiring the metabolite M are presumably unable to conduct certain stages of the chain of reactions leading to M. In different organisms the chain of reactions may be interrupted at different stages before M, and the organisms will consequently differ in their ability to grow with preformed intermediates on the way to M. Many cases of this kind are known among organisms having a natural requirement for a given growth factor; several examples were discussed by Lwoff (1943). An example is the case of Strigomonas spp. and Mucor ramannianus which require aneurin for growth. Strigomonas spp. will only grow with the complete aneurin molecule, but M. ramannianus can grow with only part of the molecule, the thiazole residue. The situation can be represented schematically as below. If

A → B ——→ ..... → M →..... → X → Y → etc.

represents the natural biosynthesis and utilisation of a metabolite M from a simple compound A, then one organism requiring M may only be able to conduct the series of reactions from M itself, whereas another may be able to start from some intermediate between A and M. The artificial biochemical mutants obtained by treatment of N. crassa, Esch. coli and other organisms with X-rays, U.V. light, mustard gas, etc. (see Beadle, 1945) are derived from organisms able to synthesise most, if not all, of their growth factor requirements. Beadle (1945) and Mitchell (1950) have given reasons for believing that an artificial mutation resulting in a growth factor requirement is caused by the elimination of one step in a scheme of synthesis such as that given above. The gene responsible for synthesis of the enzyme conducting the missing stage has been damaged by the mutagen. If the reaction eliminated is, for example, that between I and J, then all the stages leading up to I will still operate in the mutant. If, now, the mutant is grown in the presence of M, then the intermediate I, or some stabilised by-product from it, may accumulate in the medium, assuming it is not further degraded by the organism. Also, any metabolite coming between J and M, and any after M, may be active in place of M if it can enter the cell.

If one discovers two genetically dissimilar mutants requiring the same metabolite M, then it is likely that the interruptions in their chains of syntheses will be at different stages

in the series A to M, for example, at D to E and I to J respectively. In both cases an intermediate may accumulate when the organism is grown, and if so, the intermediate I formed by that mutant interrupted between I and J will be found to be active for the mutant interrupted between D and E.

This type of accumulation of intermediates was shown by Tatum & Beadle (1945) to occur in several cases. It was found that a tryptophan-requiring mutant of N. crassa formed anthranilic acid, which was isolated (Tatum & Bonner, 1944) and mutants of this mould requiring thiamine and pantothenic acid respectively were shown to produce some of the component parts of these molecules. Bonner & Beadle (1946) found that one of five mutants of this organism requiring nicotinic acid or nicotinamide for growth produced a substance active for one of the others. This substance was converted to a pigment after prolonged incubation. From cultures of the first mutant they isolated a substance which proved to be 3-hydroxyanthranilic acid (Bonner, 1948; Mitchell & Nyc, 1948) and which was almost as active as nicotinamide for the second mutant. A second substance, which had only 0.1% activity, was thought to be a methylated derivative of the first. Liefer, Langham, Nyc & Mitchell (1950), using 3-hydroxyanthranilic acid labelled with N^{15}, confirmed that this compound was converted to nicotinic acid by a mutant of N. crassa able to utilise it, and showed that the amino-nitrogen of the anthranilic acid molecule became the pyridine nitrogen of nicotinic acid.

Unfortunately, the intermediates which accumulate in cultures of biochemical mutants are not always biologically active. Bonner & Yanofski (1949) found that a nicotinic acid-requiring mutant of N. crassa accumulated quinolinic acid, which was relatively inactive even for mutants able to utilise 3-hydroxyanthranilic acid. They suggested that it was a degradation product of a labile intermediate in the conversion of 3-hydroxyanthranilic acid to nicotinic acid.

For the present work it was thought to be of interest to extend this type of work to mutant organisms requiring p-AB for growth, in the hope of demonstrating interactions of the type found by Bonner & Beadle (1946). Ten different p-AB-requiring artificial mutants were available from other laboratories, derived from two species of mould and one bacterium, and the procedure used was in principle to test the culture fluid from a given mutant grown with suboptimal p-AB as a growth factor for the other mutants. Culture fluids were also tested for growth factor activity with A. suboxydans 621, which has a natural requirement for p-AB.

Chapter 26

Experimental Methods

The p-AB-requiring mutants

All the mutant organisms used in this work were obtained from other laboratories. Their sources will be mentioned in the description of the organism. All media were autoclaved for 10 min. at 10 lb./in^2.

Esch. coli 273-384. This organism has already been described (page 51). It was grown on Medium 6 which consisted mainly of glucose, salts and asparagine.

Esch. coli Y.44. This is an X-ray-induced double mutant requiring both p-AB and histidine for growth, and it was obtained from Dr. E. L. Tatum. It was stored at 4°C. after growth at 37°C. on agar slopes containing a tryptic digest of casein. As with Esch. coli 273-384 a dried stock of the strain was kept in sealed tubes. The minimal medium used consisted of glucose and salts (Medium 8) and was slightly modified from that of Gray & Tatum (1944) in that addition of trace elements (boron, manganese, etc.) was not necessary with the reagents used. Asparagine, which Gray & Tatum stated was optional, was not included, and since this work is only concerned with the organism's requirement for p-AB, a four times recrystallised specimen of L-histidine was regularly added to the minimal medium.

The inoculum for tests was the washed growth from a 16 hr. agar slope, diluted finally by $1/10^5$, and the organism was grown in 5 ml. portions of medium in 6 x $\frac{5}{8}$ in. tubes.

N. crassa 1633. This is a p-AB-requiring mutant which was obtained from Dr. H. K. Mitchell. It was treated in a similar way to the adenine-requiring mutant already described (page 51) except that it was grown at 30°C.

N. crassa Y.6516-1-19. This is a genetically dissimilar mutant also requiring p-AB for growth. It was obtained from Dr. E. L. Tatum and was treated in the same way as the previous mutant.

Both of these moulds, as has been mentioned, were grown on a medium containing sucrose, salts and biotin (Medium 7).

Ophiostoma multiannulatum. This is a heterothallic mould which has been used by Dr. Fries for work on artificially induced mutants. He kindly provided five mutant strains requiring p-AB for growth, which could be seen to be morphologically distinct. It was not known whether they were genetically distinct in their requirement for p-AB.

All strains were grown on a medium containing glucose, salts, pyridoxin and thiamine (Medium 9) (Fries, 1947, a, b) and stocks were maintained on sucrose-malt agar at 4°C. The strains were grown at 25°C. in 6 x $\frac{5}{8}$ in. tubes. The inoculum was a barely opalescent spore suspension added to give a dilution of 1/10.

The particular strains were:

617. The spores and mycelium of this mutant were white, and it grew within three days in optimal conditions on the minimal medium.

1109. This strain was similar to 617 except that it grew more readily in suboptimal amounts of p-AB.

899. This strain grew rather less readily than the previous organisms, particularly on the minimal medium. Its spores and mycelium were white.

872. This was similar to 899, but the spores and possibly the mycelium had a fawn colour on agar. On liquid culture the mycelium was white.

1758. This was a double mutant, requiring hypoxanthine or adenine for growth in addition to p-AB. When grown on agar the mycelium and spores appeared irregularly pigmented. It grew as rapidly as the first two strains in optimal conditions; it was regularly provided with 10^{-4} M adenine for work on its p-AB requirement.

For some purposes during this work a preparation of the minimal medium of Oph. multiannulatum set with purified agar was required and this was prepared as follows:

10 gm. of agar powder were put in a muslin bag, loosely packed so as to allow for an almost two-fold swelling in water, and washed under a running tap for 48 hr. The agar in the bag was then washed ten times in distilled water, the first two

washings lasting for about an hour each, and dried for several days in an acid-cleaned Petri dish at 37°C.

The purified agar did not permit growth of any of the Ophiostomata nor of either of the p-AB-requiring mutants of Neurospora when made up to 1.5% in the minimal medium.

Absidia glauca 1200. This was a p-AB-requiring mutant mould formed by treatment with U.V. light. It had been briefly reported in the literature (Giles, 1946) and was obtained from Dr. Giles. The parent stock was stored at 4°C. after growth on sucrose-malt agar. The minimal medium for this mould had not been published, but a personal communication from Dr. Giles recommended Burkholder's medium for yeasts (Burkholder, 1943) based on glucose, asparagine and salts (Medium 10). The organism was treated similarly to the Ophiostomata, and grown at 25°C.

Streptobacterium plantarum 10 S. This is not a mutant organism, but is a naturally occurring species with a requirement for p-AB. It was obtained from Prof. R. Kuhn and was stored at 4°C. after growth in agar stabs containing glucose and "Marmite" yeast extract. The basal medium consisted mainly of glucose, casein hydrolysate, salts and vitamins (Medium 11) and was developed by Dr. Nimmo-Smith in this laboratory.

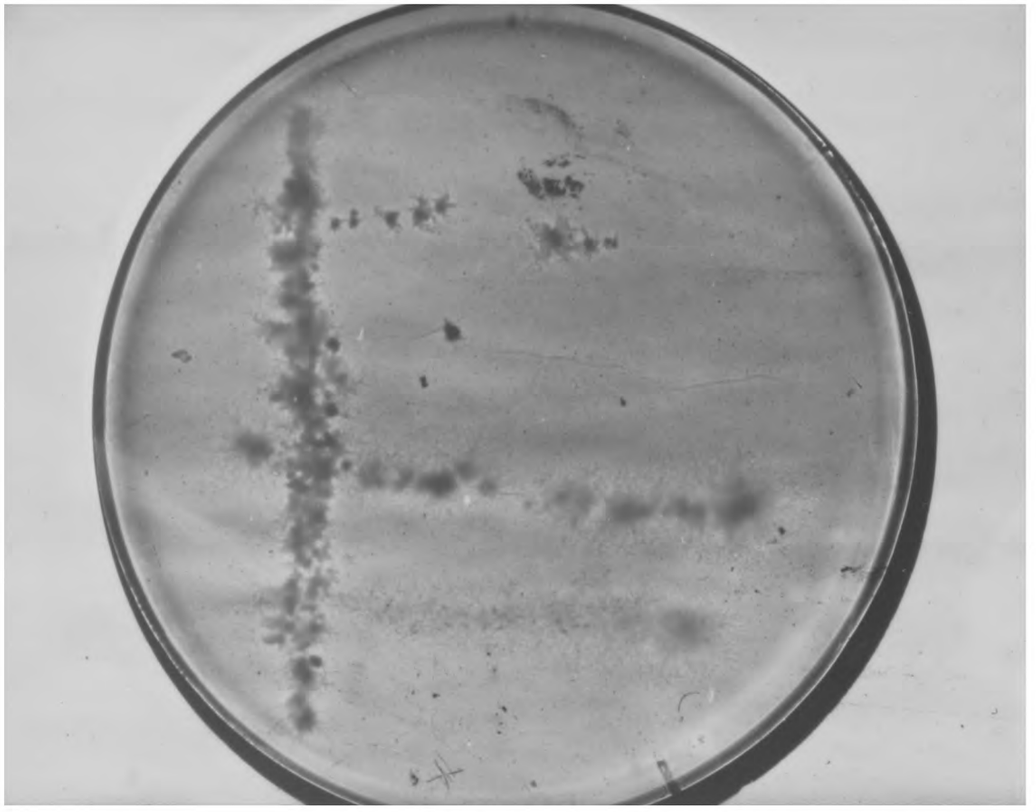
The organism was grown at 30°C. in 6 x ⅝" tubes containing 5 ml. of medium; the inoculum was a $1/10^5$ final dilution of a 16 hr. culture on the same medium grown with 10^{-7} M p-AB.

The organism had a particularly strong tendency to grow in the cultures containing no added p-AB, but if the growth after 30 hr. was inspected there was usually a satisfactorily graded response to added p-AB. After 46 hr. the "blank" cultures usually grew to the same extent as those containing optimal p-AB.

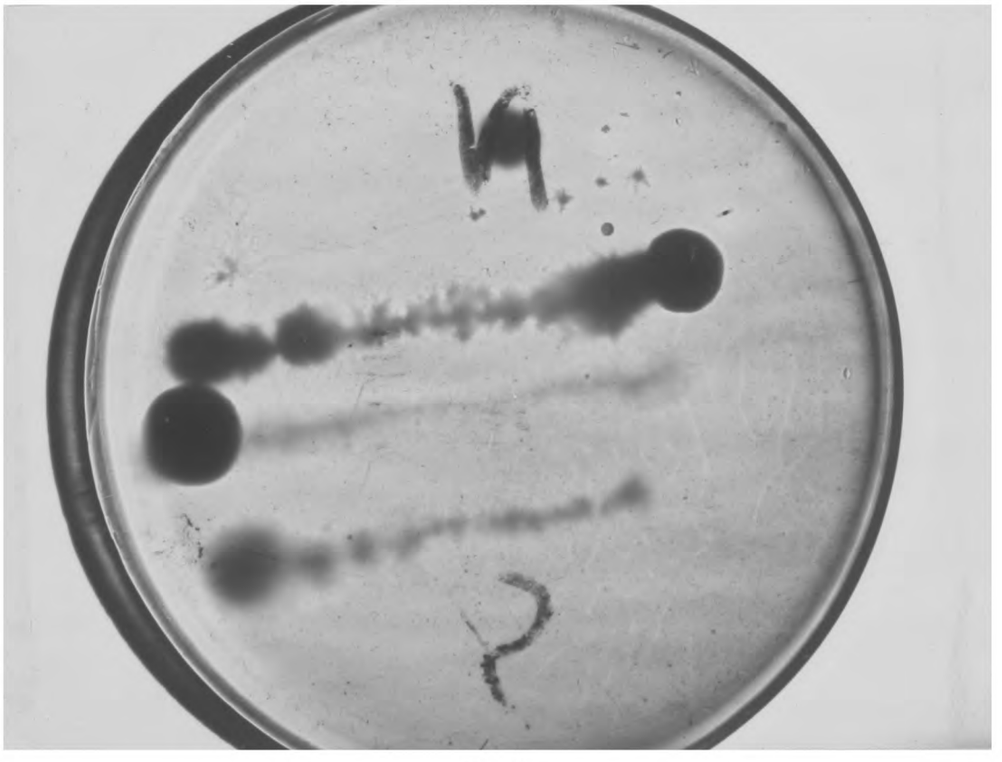
Testing of Mutants

To find out whether a certain p-AB-requiring mutant (A) (growing in p-AB) was nevertheless forming a precursor active for another such organism (B), the following general procedure was adopted. Organism A was grown in its medium (Medium A) with a known suboptimal concentration of p-AB (usually 10^{-5} M) and was removed by filtration (moulds) or centrifuging (bacteria). The fluid so obtained was added to the basal medium (Medium B) of organism B before sterilisation. The growth of organism B in these conditions was compared with its growth in Medium B with a similar amount of Medium A containing the same concentration of p-AB as that in which organism A was grown. Medium A and the fluid from the growth of organism A were usually tested at a final dilution of 1/10. If the response of organism B to the fluid from the growth of organism A was greater than its response to the control with Medium A, then it was possible that an active precursor of p-AB was being formed.

The method was by no means exhaustive; it had at least four defects:

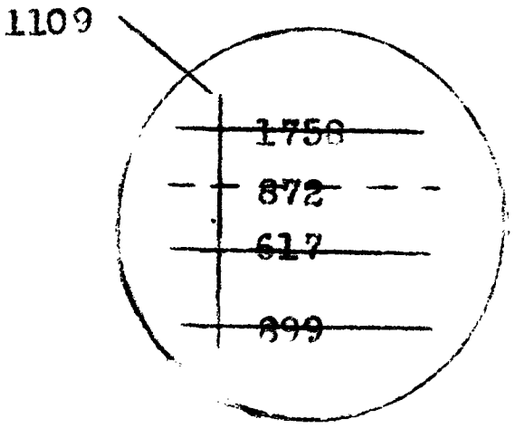


(a)



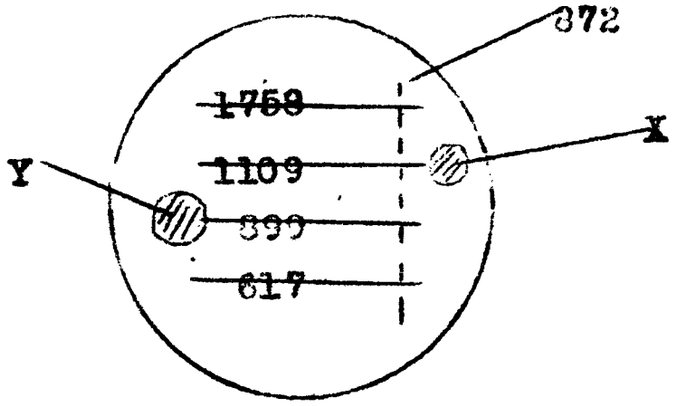
(b)

Fig. 21 Plate tests for syntrophism among mutants of Oph. multiannulatum. (for legend see opposite)



(a)

Sketch of plate "a" opposite showing position of streaks. Mutant 872 did not grow sufficiently to photograph.



(b)

Sketch of plate "b" opposite showing position of streaks and of contaminant moulds X and Y. Mutant 872 did not grow sufficiently to photograph.

1) If the biological activity of the metabolite sought was less than that of p-AB, then it would have remained undetected, as the control contained p-AB equivalent to that in which the organism A was grown. There is in principle no way of avoiding this difficulty without some knowledge of the properties of the metabolite concerned.

2) Only metabolites stable to heat sterilisation would be detected.

3) The possibility that one organism was converting p-AB into a substance more active than p-AB for another was not eliminated by this method, but could be investigated by further tests on the extracts.

4) The possibility that one organism was forming stimulants unrelated to p-AB for another also arises and could be investigated similarly.

In the case of the strains of Oph. multiannulatum it was useful to have a rapid preliminary test for syntrophism, and a plate test was devised using the minimal medium and p-AB-free agar. These plates were prepared with a suboptimal amount of p-AB and spore suspensions of the various strains were streaked across them so as to intersect. Enhanced growth at the point of intersection was taken as a sign of syntrophism between the intersecting strains.

A contact print is given (Fig. 21), taken from a plate which showed no syntrophism between the strains present. That the method worked is indicated by the second print showing stimulation of certain strains by a contaminant mould.

Estimation of Glucose

This was carried out by a modification of the Hagedørn-Jensen method due to Nimmo-Smith (1946), the principle of which was the degree of reduction of potassium ferrocyanide in a fixed time. The ferrocyanide formed was titrated against standard ceric sulphate in the presence of the redox indicator Lissamine green.

The method was originally worked out for blood glucose estimations, and involved a protein precipitation stage which removes most of the non-glucose reducing substances from blood. This stage was not used here, and the method is therefore open to the objection that it estimates most other reducing substances present. This was not important for the present work, because, as will be seen, the results obtained by this method were checked by biological tests on artificial mixtures containing pure glucose.

The method was also open to the general objection to the Hagedørn-Jensen method, that the reaction taking place between glucose and ferrocyanide is obscure. With careful control of the reduction time, however, the method yielded very satisfactory results.

TABLE 30 Tests for syntrophism among ten p-AB-requiring mutants.

For test procedure see text; tests with *A. subglaucus* were also included.

Extract prepared from:	Extract tested for activity towards: *N. crassa* 1633	*N. crassa* Y6516-1-19	*Esch. coli* 273-384	*Esch. coli* Y. 44	*Oph. multiannulatum* 1109	617	899	872	1758	*Abs. glauca* 1200	*A. suboxydans*
N. crassa 1633	...	-	-	-	-	-	-	-	-	-	+
N. crassa Y 6516-1-19	-	...	-	-	-	-	-	-	-	-	+
Esch. coli 273-384	-	-	...	-	-	-	-	-	-	-	-
Esch. coli Y 44	-	-	-	...	-	-	-	-	-	-	-
Oph. multiannulatum 1109	-	-	-	-	...	-	-	-	-	-	-
617	-	-	+	+	-	...	-	-	-	-	-
899	-	-	-	-	-	-	...	-	-	-	-
872	-	-	-	-	-	-	-	...	-	-	-
1758	-	-	-	-	-	-	-	-	...	-	-
Abs. glauca 1200	-	-	-	-	-	-	-	-	-	...	-

TABLE 31 Stimulation of the growth of Esch. coli mutants by a filtrate of an Oph. multiannulatum mutant

Esch. coli 273-384 was cultured in Medium 6, Esch. coli Y.44 in Medium 8 and Oph. multiannulatum 617 in Medium 9. Mould medium and filtrate contained 10^{-9} M p-AB and were added in 0.5 ml. aliquots to 5 ml. cultures of the Esch. coli mutants.

A. Test with Esch. coli 273-384:

Test material	Incubation (hr.) 17	41	65
10^{-7} M p-AB	++	+++	++++
-	-	-	-
mould filtrate	+	+++	+++
Medium 9 + 10^{-9} M p-AB	-	tr.	tr.

B. Test with Esch. coli Y.44:

10^{-7} M p-AB	+	+++	+++
-	-	-	-
mould filtrate	±	++	++
Medium 9 + 10^{-9} M p-AB	-	tr.	tr.

TABLE 32 Stimulation of the growth of A. suboxydans 621 by a filtrate of a N. crassa mutant

N. crassa Y 6516-1-19 cultured in Medium 7 with 10^{-9} M p-AB; medium and filtrate added in 0.5 ml. portions to 5 ml. cultures of A. suboxydans in Medium 2.

Test material	Incubation (hr.) 17	41	66	89	113
p-AB, 10^{-7} M	-	++	+++++	+++++	+++++
-	-	-	-	-	-
N. crassa filtrate	-	-	tr.	++	+++
Medium 7 + 10^{-9} M p-AB	-	-	tr.	±	++

Chapter 27

Results and Discussion

The ten mutant organisms described in the previous chapter were tested for synergism among themselves, and also for activity towards A. suboxydans 621, since this might also have been able to utilise simpler substances in place of p-AB. Since the number of tests to be performed was the factorial of the number of mutants available, interaction between several of the Ophiostomata was eliminated by the plate method already mentioned. These findings were later confirmed by testing liquid cultures.

The results of these tests are summarised in Table 30. There was only one case of interaction between mutants proper: extracts of Oph. multiannulatum 617 were active towards Esch. coli 273-384 and Esch. coli Y.44 (Table 31). The two p-AB-requiring mutants of Neurospora, however, produced substances active for A. suboxydans 621. A typical test indicating activity of this kind is given in Table 32.

Since glucose at low concentrations was known (page 60) to stimulate the growth of A. suboxydans 621, carry-over of glucose from the media in which the moulds Ophiostoma were grown might give false results in tests with A. suboxydans. It was found, however, that the moulds gave quite satisfactory growth with one tenth of their normal amount of glucose, at which concen-

tration the carry-over of glucose was insufficient to affect the response of A. suboxydans 621 to added p-AB. The same technique was used to test extracts of Abs. glauca 1200.

Similarly, adenine was known to affect the response of A. suboxydans 621 to p-AB (page 62), and since adenine was needed for the growth of Oph. multiannulatum 1758 the latter was not tested for activity towards A. suboxydans 621. The effect of adenine on the response of the laboratory strain to p-AB had not been sufficiently investigated for a useful test of such activity to be devised.

Some further work was carried out on the factor formed by the two mutants of N. crassa and which was active for A. suboxydans. Most of the work recorded below was carried out with extracts of N. crassa Y.6516-1-19, but the conclusions were shown to be true of N. crassa 1633 also.

Some Chemical tests on Neurospora Factor.

If this factor was in fact a biological precursor of p-AB it was not likely to be very plentiful in the extracts as normally obtained, and therefore the effect of chemicals on solutions of the factor had to be determined by microbiological assay. This limited the number of chemical tests which could be performed, but nevertheless several were possible and their results are given below. They are not recorded in detail since they led to little useful information.

1) No preparation of the factor was obtained which would support the optimal growth of A. suboxydans, though a few appeared to support limited and delayed growth in the absence of added p-AB.

2) The activity of the extracts was retained during diazotisation and boiling: conditions that destroyed p-AB in controls. The diazotised material was boiled at pH 6.0 rather than in more acid conditions, since this procedure favours formation of p-hydroxybenzoic acid from p-AB rather than poly-azo condensation products. p-Hydroxybenzoic acid was inactive for A. suboxydans 621 (Appendix I, page 150).

3) Treatment of the extracts with acetic anhydride at room temperature did not destroy the activity, though this treatment destroyed p-AB in controls, presumably by acetylation.

4) Hydrolysis for two hours with HCl at pH 1.0 destroyed the activity, and some charring was noted after this treatment.

5) The extracts in general contained 10^{-8} M p-AB, and tests for solubility in organic solvents were performed by trying to reduce the activity of extracts to that of 10^{-8} M p-AB alone by solvent extraction. The factor was insoluble in toluene, xylene, 60° - 80° ligroin, chloroform and ether, the latter being tested at pH values of 8.0, 6.0, and 3.8. Ether is known to remove p-AB from solutions at pH 3.8 (Woods, 1940), so ether extraction in these conditions, together with the diazotisation procedure, provided two methods of obtaining the factor free from p-AB.

At one time the factor appeared to be soluble in n-butyl alcohol, but the effect was not readily reproducible and activity was never successfully demonstrated in the solvent phase.

6) The yield of the factor was slightly improved by boiling the mould culture or by autoclaving it for 10 min. at 10 lb./in.^{2} before filtration, and a similar effect was noticed if the mycelium was broken up by shaking at 400 vibration/min. with Ballotini beads. A curious phenomenon was observed, however, that aqueous extracts freed of p-AB did not increase in activity after a two-or seven-fold concentration *in vacuo* at room temperature; in fact in some cases a reduction in activity was found.

7) The amount of the factor formed was independent of the age of the mould, of the amount of p-AB provided, and of the pH at which the mould was grown. pH values of 4.0 and 6.0 as against the customary pH of 5.4 were tested. Nor did the addition of sodium acetate, aspartic acid or vitamin-free casein hydrolysate to the mould medium affect the yield, though the latter substance improved the growth of the mould. A tryptic digest of casein freed of vitamins for folic acid assay appeared to improve the yield slightly.

8) The extracts were inactive for the adenine-requiring mutant of *N. crassa*, and had no effect on the growth of *Lb. casei*. Activity for the other p-AB-requiring mutants had been eliminated in the preliminary tests, and the extracts showed no activity towards *Sbm. plantarum* 10 S.

TABLE 33 Relationship of the activity of extracts of N. crassa for A. suboxydans to their glucose contents

A. suboxydans 621 cultured in Medium 2, N. crassa Y 6516-1-19 in medium 7; 0.5 ml. aliquots of test material added to cultures of A. suboxydans.

A. Tests with mould medium containing 10^{-8} M p-AB:

Test Material	Analytical glucose content	Incubation (hr.) 29	42	67	90	114
Extract of mould culture	5 mg.	+	+±	++	++	++++
Mould medium	...	tr.	±	+±	+±	+++
Mould medium + 5 mg. glucose	...	+	+±	++±	++±	++++

B. Tests with mould medium containing 5×10^{-9} M p-AB:

Extract of mould culture	5 mg.	tr.	±	+	++	+++
Mould medium	...	-	tr.	±	+	++
Mould medium + 5 mg. glucose	...	-	tr.	+±	++	+++

C. Tests with mould medium containing 10^{-9} M p-AB:

Extract of mould culture	5 mg.	tr.	tr.	tr.	tr.	++ / ±
Mould medium	...	-	-	-	tr.	tr.
Mould medium + 5 mg. glucose	...	-	tr.	tr.	tr.	+

D. Controls:

No addition	...	-	-	-	-	-
10^{-7} M p-AB	...	+	++	+++++	+++++	+++++

The purpose of testing the last organism was that, had the extracts proved active, one could have obtained some idea of the nature of the active components from its anti-sulphonamide activity. A precursor of p-AB should show competitive activity with this organism, whereas a product formed from p-AB might well exhibit non-competitive anti-sulphonamide activity.

These results gave no indication as to the chemical nature of the factor, but eliminated the possibility of its being any of the known stimulants of A. suboxydans 621 (adenine, folic acid or amino acids) except glucose. It was possible that the mould was hydrolysing the large amount of sucrose in its medium to glucose, which then stimulated the growth of the test organism. This explanation was shown to be most probably correct.

Cultures of the mould were grown with various amounts of p-AB, and extracts prepared from them, though that grown with the least p-AB showed only traces of growth. These extracts were then estimated for glucose by the cerimetric method already described (page 141), and artificial mixtures of the mould medium, p-AB and glucose were made up to correspond in composition to these extracts. Their activities for the test organism were then compared with those of the extracts proper.

The results, given in Table 33, showed that the glucose content of the extracts was more than sufficient to account for their activity towards A. suboxydans 621 in all cases, even when the glucose content and activity of the extract were low.

TABLE 34 Effect of sucrose concentration on the formation of material active for A. suboxydans by N. crassa

0.5 ml. amounts of test material added to A. suboxydans 621 cultures in Medium 2. N. crassa Y 6516-1-19 cultured in Medium 7 with various amounts of sucrose.

Material tested	Sucrose content of mould medium	Incubation (hr.) 22	46	70	94
Filtrate of N. crassa grown with 10^{-9} M p-AB	1%	-	tr.	+++	++++
Uninoculated mould medium with 10^{-9} M p-AB	1%	-	-	+	+++
Filtrate of N. crassa grown with 10^{-9} M p-AB	0.1%	-	-	+	+++
Uninoculated mould medium with 10^{-9} M p-AB	0.1%	-	-	±	+++

This finding was confirmed by another approach recorded in Table 34. Cultures of a mutant mould were sown in the minimal medium containing one tenth of the normal amount of sucrose, when a very slight depression of growth was noticeable. Extracts of these were made and compared in activity with extracts prepared in the usual manner. In both cases the extracts of the mould grown in decreased sucrose were without perceptible activity for A. suboxydans 621. An analogous test using extracts of N. crassa 1633 gave a result similar to that shown in Table 34 for N. crassa Y 6516-1-19.

Discussion

The results given in this section of the work require little discussion: the stimulation of A. suboxydans 621 by extracts of the two mutants of N. crassa was undoubtedly due to glucose formed in cultures of the latter. If the method of Bonner & Beadle (1946) is to be extended to unlike organisms cultured on unlike media, it can only be done with a complete knowledge of the behaviour of the organisms concerned. Though Sarrett & Cheldelin (1945) reported stimulation of A. suboxydans 621 by glucose in cultures with a deficiency of pantoyl-lactone, this is not an effect that one would expect a priori in this case; particularly as there is no evidence that p-AB is involved in fermentative or oxidative reactions of cells. It was fortunate that the effect was traced to glucose in time to avoid further

work on the factor.

No work has been done on the factor formed by Oph. multiannulatum 617 active for the two mutants of Esch. coli.

A P P E N D I C E S

TABLE 35 **Tests of p-aminobenzoylmethionine as a growth factor for A. suboxydans and as a growth stimulant and anti-sulphanilamide for Esch. coli.**

A. Test with Esch. coli "F" in Medium 5:

Sulphanilamide (M)	p-ABM (M)	Incubation (hr.) 24	46	70
10^{-3}	-	-	-	-
2×10^{-4}	-	-	+	+++
10^{-3}	10^{-5}	-	-	-
2×10^{-4}	10^{-5}	-	+	++++
-	-	++	++++	...
-	10^{-4}	++	++++	...

B. Test with A. suboxydans 621 in Medium 2:

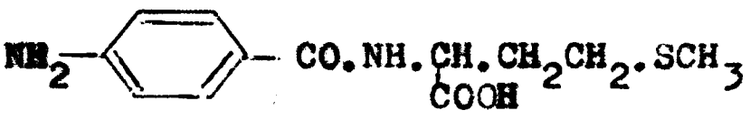
Added to culture medium	Incubation (hr.) 18	46	66	90
p-AB, 10^{-8} M	-	++	++	++++
p-ABM, 10^{-4} M	-	+	++	+++
-	-	-	-	-

APPENDIX 1

Tests on Some Analogues of p-Aminobenzoic Acid and Related Compounds

During this work certain routine tests on substances chemically related to p-AB were undertaken, with a view to detecting growth factor or anti-sulphonamide activity. The results of such tests are given in the tables below.

DL-p-aminobenzoylmethionine



This material was provided by Dr. King and his co-workers, and was tested for growth factor activity towards A. suboxydans 621 and for stimulatory and anti-sulphonamide activity for the laboratory strain of Esch. coli. In the latter case it showed no activity of either kind, and a slight activity for the former organism (0.01% of p-AB) was not regarded as significant. It could well have been due to slight hydrolysis of the peptide during sterilisation or growth. Tests with this compound are recorded in Table 35.

TABLE 36 Tests of ammonium N-8-benziminazolemethyl-p-aminobenzoate as an inhibitor of Esch. coli and as a growth factor for A. suboxydans.

A. Test with Esch. coli F in Medium 5:

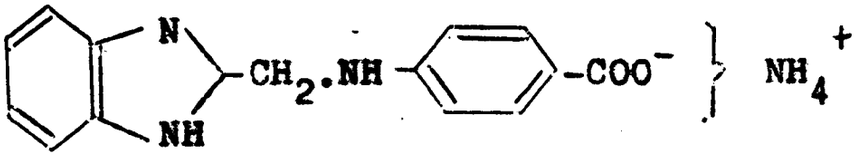
Test substance (M)	Incubation (hr.) 24	46	74
10^{-3}	-	+++	++++
-	-	+++	++++

B. Test with A. suboxydans 621 in Medium 2:

Added to culture medium	Incubation (hr.) 16	40	66	88	112
Test substance, 10^{-3} M	- *	++	++++	++++	++++
Supernatant after precipitation of test material; equivalent to test material at 10^{-3} M	tr.	+++	++++	++++	++++

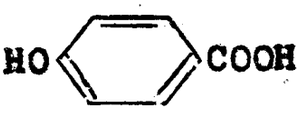
*Culture obscured by a slight precipitate of test substance.

Ammonium N-8-benziminazolemethyl-p-aminobenzoate



This material was provided by the same workers, and is a structural analogue of pteroic acid. It was shown to have no anti-bacterial activity for Esch. coli (Table 36). Some activity as a growth factor for A. suboxydans was observed, but as this seemed in principle unlikely it was studied further and shown to be due to impurity in the material. This follows from the data presented in Table 36, where the activity of the supernatant after precipitation of the test substance was the same as that of the original solution. Its inhibitory effect on Strept. faecalis R has been reported (King, Spensley & Nimmo-Smith, 1948).

p-Hydroxybenzoic acid



In view of the use of diazotisation and boiling as a method of removing p-AB from extracts of N. crassa, (page 144) it was thought desirable to show that p-hydroxybenzoic acid would not replace p-AB in the growth of A. suboxydans. The compound

TABLE 37 Test of p-hydroxybenzoic acid as a growth factor for A. suboxydans.

Medium 2.

Added p-HB (M)	Incubation (hr.) 24	46	70	94	118	142
10^{-7}	-	-	-	tr.	+	++
10^{-8}	-	-	-	-	-	tr.
-	-	-	-	-	-	tr.
From another test:	16	40	66	88	112	136
10^{-9} M p-HB	-	-	-	+	+	++

was prepared by diazotising 7.5 gm. of p-AB and heating on a steam bath until all evolution of nitrogen had ceased. The product was recrystallised three times from water, the second time after treatment of the solution with norite charcoal and the third being deliberately extravagant and using acid cleaned glassware. The yield was therefore small (500 mg.) but could be expected to be reasonably free of p-AB. It had the required melting point of 164°C, and before testing was recrystallised again from re-distilled alcohol.

The test in Table 37 shows that it still had about 1% of the activity of p-AB, but this could easily be due to about 1% of p-AB as impurity which was not detected in the melting point determination. The finding that p-hydroxybenzoic acid was probably inactive as a growth factor for A. suboxydans was consistent with Woods's (1940) report that it was inactive as an anti-sulphanilamide substance for Strept. haemolyticus and Bact. coli. Recently Davis (1950, 1951) observed that in certain conditions it acted as a growth factor for an induced mutant of Esch. coli. This mutant required tyrosine, phenylalanine, tryptophan and p-AB for growth, all compounds containing an aromatic ring, and p-hydroxybenzoic acid acted as a growth stimulant. All these compounds could be replaced by shikemic acid (a 3:4:5-trihydroxycyclohexene-1-carboxylic acid), and Davis suggested that shikemic acid was a natural precursor of several aromatic metabolites.

Benzoic acid While this substance is known to be inactive for most p-AB-requiring organisms, it was at one time thought advisable to confirm this for the strain of A. suboxydans used. An analytical grade specimen was found to be totally without activity up to 10^{-4} M, above which concentration it was not tested.

APPENDIX 2

List of Media used during this Work

Note on the preparation of media. Media 2 to 11 were made up by adding the materials listed to glass-distilled water. The volume of water added was chosen to give a more concentrated form of the medium which would allow for addition of various test solutions. The pH was adjusted and the medium was boiled gently for ten minutes, filtered, and stored at 4°C under toluene after checking the pH value. It was distributed and autoclaved immediately before use.

Medium 1 Agar for A. suboxydans 621

	g./litre
Glycerol	50 (40 ml.)
Agar	15
Yeast extract ("Difco")	5
NaOH	to pH 6.0 - 0.03

Medium 2 Medium for A. suboxydans 621 (Landy & Dicken, 1942)

	g./litre
Glycerol	50 (40 ml.)
Casein hydrolysate (Ashe, "vitamin free")	3
DL-Tryptophan	0.05
DL-Cysteine	0.075
KH_2PO_4	5
$MgSO_4.7\ H_2O$	0.2
$Fe(NH_4)_2(SO_4)_2.\ 6\ H_2O$	0.015
$MnSO_4.4\ H_2O$	0.010
Calcium pantothenate	0.001
Nicotinic acid	0.001
NaOH	to pH 6.00 - 0.03

In the "improved" form of Medium 2, Ashe's casein hydrolysate (3 g./l.) was replaced by a solution of this material which had been treated with ether to remove traces of p-AB (page 73). The final concentration of casein hydrolysate in the medium was then 20 g./l.

Medium 3 Medium for *A. suboxydans* 621 (Stokes & Larsen, 1945 *a*).

	g./litre
Glycerol	50 (40 ml.)
KH_2PO_4	4.5
$(NH_4)_2SO_4$	0.5
NH_4Cl	0.5
$MgSO_4.7\ H_2O$	0.2
$FeSO_4.(NH_4)_2SO_4.6\ H_2O$	0.015
$MnSO_4.4\ H_2O$	0.010
Calcium pantothenate	0.001
Nicotinic acid	0.001
DL-Valine	0.1
DL-*iso*Leucine	0.1
DL-Alanine	0.1
DL-Cysteine	0.1
NaOH	to pH 6.00 ± 0.03

Medium 4 Medium for assay of folic acid with Lb.casei

(after Roberts & Snell, 1946)

	g./litre
Tryptic casein digest	400 ml.
KH_2PO_4	2.5
K_2HPO_4	2.5
DL-Cysteine	0.1
DL-Tyrosine	0.1
Adenine hydrochloride	0.01
Guanine hydrochloride	0.01
p-AB	0.00015
Biotin	0.00004
Nicotinic acid, Calcium pantothenate Pyridoxin , Riboflavine & Aneurin hydrochloride	0.001
$MgSO_4.7\ H_2O$	0.8
$FeSO_4.7\ H_2O$	0.04
$MnSO_4.4\ H_2O$	0.016
Glucose	18
HCl (conc.)	0.08 ml.
NaOH	to pH 6.80 ± 0.03

Medium 5 Medium for Esch. coli "F"

	g./litre
KH_2PO_4	4.5
$(NH_4)_2SO_4$	0.5
NH_4Cl	0.5
Lactic acid	2.25
$MgSO_4.7\ H_2O$	0.01
$FeSO_4.(NH_4)_2SO_4.6\ H_2O$	0.015
NaOH	to pH 7.6 - 0.03

Medium 6 Medium for Esch. coli 273-384 (Roepke, Libby & Small, 1944)

	g./litre
NaCl	5
$(NH_4)_2SO_4$	4.72
KH_2PO_4	2.72
Glucose	2
Aspartic acid	0.2
$FeSO_4.(NH_4)_2SO_4.6\ H_2O$	0.003
$MgSO_4.7\ H_2O$	0.0015
$CaCl_2.6\ H_2O$	0.0016
NaOH	to pH 7.0 - 0.03

Medium 7 . **Minimal** medium for mutants of N. crassa (after Mitchell & Houlahan, 1946)

	g./litre
Sucrose	10
Ammonium tartrate	5
NH_4NO_3	1
KH_2PO_4	1
NaCl	0.5
$MgSO_4.7\ H_2O$	0.5
$CaCl_2.6\ H_2O$	0.1
Ferrous citrate (as Na citrate Ferrous ammonium sulphate)	0.0036
Biotin	0.00008

Medium 8 Minimal medium for *Esch. coli* Y.44 (Gray & Tatum, 1944)

	g./litre
NH_4Cl	3
$(NH_4)_2SO_4$	2
NaCl	2
NH_4NO_3	1
$MgSO_4.7\ H_2O$	1
$CaCl_2.6\ H_2O$	1 drop 10% soln.
KH_2PO_4	1
K_2HPO_4	3
Glucose	5
$FeSO_4.(NH_4)_2SO_4.6\ H_2O$	0.015
L-Histidine	0.1
NaOH	to pH 7.00 ± 0.03

Medium 9 Minimal medium for mutants of Oph. multiannulatum

(Fries 1947 a, b)

	g./litre
Glucose	20
Ammonium tartrate	5
KH_2PO_4	1
$MgSO_4.7\ H_2O$	0.5
NaCl	0.1
$CaCl_2.6\ H_2O$	0.1
$FeSO_4.(NH_4)_2SO_4.6\ H_2O$	0.015
$MnSO_4.4\ H_2O$	0.01
$ZnSO_4.7\ H_2O$	1 drop 10% soln.
Aneurin hydrochloride	0.00004
Pyridoxin hydrochloride	0.00004
(Sometimes) washed agar	15

Medium 10 Minimal medium for *Abs. Glauca* 1200 (Burkholder, 1943)

	g./litre
Glucose	20
Asparagine	2
$MgSO_4.7\ H_2O$	0.5
KH_2PO_4	1.5
$(NH_4)_2SO_4$	2
KI	0.0001
$Fe(NH_4)_2(SO_4)_2.6\ H_2O$	0.015
NaOH	to pH 5.00 - 0.03

Medium 11 Minimal medium for Sbm. plantarum 108

	g./litre
Casein hydrolysate ("Ashe", vitamin-free)	5
DL-Cysteine	0.1
DL-Tryptophan	0.1
$NH_4.COOCH_3$	5
KH_2PO_4	2
$MgSO_4.7\ H_2O$	0.4
Ferrous citrate (as Na citrate Ferrous ammonium sulphate)	0.0092
$MnSO_4$	0.01
Thiamine hydrochloride	0.0001
Nicotinic acid	0.0001
Biotin	0.000002
Calcium pantothenate	0.0004
Pyridoxin	0.001
Glucose	9
NaOH	to pH 6.50 - 0.03

APPENDIX 3

ABSTRACT OF DISSERTATION

Aspects of the Metabolism of Micro-organisms

Section 1. *Acetobacter suboxydans* (American Type Culture Collection No. 621) is an organism of the Pseudomonas family found in spoiled beer. Among the substances it requires for growth is p-aminobenzoic acid (p-AB), and since this substance is known to prevent the inhibition of bacterial growth by sulphonamides, the inter-relations of p-AB and a sulphonamide drug in the growth of *A. suboxydans* were investigated. The main approach was through a study of the variations which enabled the organism (1) to grow without p-AB and (2) to resist inhibition by sulphonamides. Such changes were likely to be associated with interesting variations in the metabolism of p-AB, since there is evidence, for instance, that in certain organisms sulphonamide resistance is due to an increased ability to synthesise p-AB.

For the majority of organisms p-AB overcomes sulphonamide inhibition in a competitive manner: for a given degree of inhibition the ratio of the concentrations of p-AB and sulphonamide is constant, though the absolute amounts of these may be varied considerably. Competitive relationships of this kind suggest that both metabolite and inhibitor react with the enzyme normally using the metabolite, and that the proportion of each

reacting is governed by the law of mass action. As a contrast, in non-competitive relationships, a small amount of metabolite will overcome inhibition over a wide range of concentrations of inhibitor. It is likely that a non-competitive antagonist of an inhibitor is, or readily becomes, a product of the cell's utilisation of a competitive antagonist.

As a preliminary to this study it was necessary to obtain certain information about the properties of the unchanged strain of A. suboxydans, and the following points were established:

1) The growth of the organism was very sensitive to the air supply: optimal aeration gave optimal growth.

2) The organism was normally cultured in a medium of glycerol, casein hydrolysate, vitamins and salts (Medium 2, Appendix 2), but it grew to a limited extent in a medium in which glucose replaced glycerol. The limited growth in glucose medium was probably due to rapid acid formation from this substrate Growth in glycerol medium was also affected by addition of glucose: at low concentrations glucose stimulated growth, but at higher levels it was inhibitory.

3) The strain used in this laboratory would not grow satisfactorily on a synthetic medium similar to that used by Stokes & Larsen (1945).

4) The organism required between 4×10^{-9} and 1.6×10^{-9} M p-AB to show visible growth in Medium 2, p-AB could be replaced by synthetic pteroylglutamic acid when added at about 100-fold greater molar concentration.

5) Adenine replaced p-AB entirely, but not only was it required at a greater concentration than 10^{-5} M, but only a small stationary population of cells was reached in adenine cultures.

6) The response to p-AB was affected by the amount of casein hydrolysate added to the medium, and certain amendments had to be made to the original medium as published by Landy & Dicken (1942). The effect was not due to p-AB contained as impurity in the casein hydrolysate.

7) Both p-AB and pteroylglutamic acid overcame sulphonamide inhibition of growth in a competitive manner. With 10^{-7} M p-AB the organism was able to resist between 10^{-4} and 2×10^{-4} M sulphathiazole. Adenine, however, acted non-competitively, permitting growth in all concentrations of drug up to 2×10^{-3} M.

8) A synergism between pteroylglutamic acid and a mixture due to Lampen, Roepke & Jones (1946) of purines, methionine and thymine was traced to the adenine in the mixture.

9) Extracts of the organism were prepared which replaced folic acid for Lactobacillus casei and adenine for an "adenine-less" mutant of Neurospora crassa. The folic acid content of cells of A. suboxydans was independent of their age and of the amount of p-AB in which they were grown.

Extracts of the kind mentioned above were used for comparison of the quantitative properties of the variant strains described below.

"Sub-strains" of A. suboxydans 621 were obtained trained to dispense with p-AB (strainA) and to resist some 200 times the normal amount of sulphathiazole for a given amount of p-AB (strain C). Throughout this work sulphathiazole was used as the sulphonamide type drug in preference to sulphanilamide as the latter is known to effect the CO_2 enzymes of cells in a manner not shown by its analogues.

The sulphathiazole-resistant strain, strain C, was shown to have undergone no change in its requirement for p-AB in its normal medium. That is to say that it required the same amount of p-AB to give visible growth after a fixed time as did the parent strain, though its medium contained 2×10^{-3} M sulphathiazole. Its growth curve in a medium containing this amount of sulphathiazole and 10^{-7} M p-AB was similar to that of the parent strain growing in the same conditions but without sulphathiazole. On the other hand, strain C could grow in the absence of added p-AB if sulphathiazole were also omitted; the extent of growth was then limited.

The relationship between p-AB and sulphathiazole in strain C was still competitive; there had merely been a change in the molar ratio of these two substances required to cause inhibition. The strain did not destroy sulphathiazole.

The non-exacting strain, strain A, grew without added p-AB, but still resembled the parent organism in that it required nicotinic and pantothenic acids for growth. It had an increased

resistance to sulphathiazole compared with the parent organism when both were tested with 10^{-7} M p-AB, though this resistance was not as great as that of strain C. The relationship between p-AB and sulphathiazole was also competitive in this strain.

The growth of strain A was affected by the amount of "vitamin-free" casein hydrolysate provided in its medium: growth in Medium 2 was limited compared with the other strains. In this medium its extent of growth was increased by:

1) p-AB.

2) Pteroylglutamic acid at some 100 times the molar concentration at which p-AB was active.

3) High concentrations of aspartic acid.

4) Glucose at some concentrations.

5) Increased amounts of vitamin-free casein hydrolysate.

This last effect was not shown, however, by a second strain of this type called strain A_2. The stimulation of growth by casein hydrolysate was not due to the presence of p-AB in this material.

Many pure vitamins, amino-acids and other compounds of importance in general cell metabolism were tested for ability to stimulate the growth of strain A; only those mentioned above were active.

Other strains. An unsuccessful attempt was made to train strain A to resist sulphathiazole further by serial subculture in partially inhibitory concentrations of drug. Similarly, an attempt was made to reduce to zero the p-AB requirement of

strain C in the presence of its normal concentration of sulphathiazole (2×10^{-3} M). This was also unsuccessful, though a peculiar strain (strain CD) was obtained after prolonged subculture with suboptimal amounts of p-AB.

The possible synthesis of p-AB, folic acid and adenine by the trained and normal strains of A. suboxydans was investigated.

Attempts to detect synthesis of p-AB by strains A and C were made by ether extraction of cultures grown without this growth factor. The extracts were concentrated and assayed with a strain of normal A. suboxydans trained to grow in the presence of the extracts. No synthesis of p-AB was detected.

Synthesis of folic acid did occur with strain C, and the folic acid content of cells of this strain (assayed with Lb. casei) was the same as the content of cells of the parent strain. This was true of cells grown both with and without sulphathiazole. The folic acid content of cells of strain A was very much diminished compared with the other two strains. It is unlikely that any of the strains of A. suboxydans synthesised the pteroylglutamic acid molecule as such, because the shapes of the assay curves with Lb. casei using bacterial extracts were different from the control curves obtained with a sample of pteroylglutamic acid.

Assays of whole cultures of A. suboxydans with Lb. casei were complicated by the presence of an inhibitor of Lb. casei in the assay material, and no conclusive results were obtained.

Synthesis of adenine occurred in strains A and C as well as in the parent strain; it was detected by assay with an "adenine-less" mutant of N. crassa. The adenine content of cells of all three strains was similar, and its presence probably accounted for the non-competitive anti-sulphathiazole activity of cell extracts for the normal strain.

Comparisons with Escherichia coli 273-384. This was a mutant of Esch. coli requiring p-AB for growth, and some variant strains analogous to A and C in A. suboxydans were isolated and investigated. The organism was readily trained to grow in the absence of vitamin and the resulting variant grew to the same extent as its parent growing optimally and its growth was not stimulated by p-AB. Its resistance to sulphathiazole was the same as that of its parent when both were tested with 10^{-7} M p-AB.

A strain was obtained having a sulphathiazole resistance increased by about 50-fold for a given amount of p-AB, but its resistance was not successfully increased further. In the absence of sulphathiazole it still required p-AB for growth (unlike strain C of A. suboxydans), and in the presence of drug its requirement for p-AB was considerable. The training was in a sense incomplete, since the growth rate of the resistant organism was less than that of the parent strain even after

many subcultures in the training medium.

Discussion. The results outlined above were discussed in an attempt to understand further the mechanisms of the variations that had taken place in A. suboxydans. It was considered to be unlikely that either strain had developed an ability to synthesise p-AB, though the possibility that extremely small amounts were synthesised could not be eliminated entirely. Strain A was regarded as having developed a mode of growth independent of this vitamin, and probably also of folic acid; its growth was probably dependent on the supply of amino-acids.

The view was taken that, while strain C may possibly have undergone a change of the same type as that undergone by strain A, this was not the change responsible for its high sulphathiazole resistance. The primary change was considered to be a quantitative expansion of p-AB-utilising enzyme in the cell, at the surface of which p-AB and sulphonamides can be regarded as competing. An increase in affinity of the enzyme for p-AB as compared with sulphathiazole would also account for the experimental data. The point was made that further work on the rates of utilisation of p-AB, or rates of formation of products from p-AB, would be of interest.

A brief mathematical consideration of this view was given to show that a roughly 80-fold expansion of p-AB-utilising enzyme would account for the 200-fold increase in sulphathiazole resistance observed.

Section 2. Concurrently with the work on the variations of A. suboxydans some experiments were undertaken on the interactions of artificially induced mutants requiring p-AB for growth. In view of the results of other workers on induced mutants it is likely that mutations resulting in a growth factor requirement are due to damage to one stage in a sequence of biochemical reactions leading normally to the synthesis of the factor required. This may be represented:

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow F..... \rightarrow \text{metabolite.}$$

In various organisms with a similar requirement these interruptions may well be at different stages in the sequence, and, as Beadle (1945) and his co-workers have shown with N. crassa, a mutant damaged, for example, between A and B will sometimes respond to extracts of one damaged between C and D, since C may accumulate in cultures of the latter.

Ten mutant organisms requiring p-AB for growth were obtained from other laboratories and tested with this possibility in view, and interactions between the mould Ophiostoma multiannulatum 617 and two mutants of Esch. coli were recorded. Interactions between two mutants of N. crassa and A. suboxydans were shown to be due to formation of glucose by N. crassa from sucrose provided in its medium. This was formed at concentrations which stimulated the growth of A. suboxydans when fluid from a grown mould culture was added to its medium.

APPENDIX 4

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