THE SYNTHESIS OF VITAMIN B_6 and RELATED SUBSTANCES

BY MICRO-ORGANISMS

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CONTENTS

POSSIBLE EXPERIMENTAL APPROACHES

THE USE OF MICROORGANISMS IN THE STUDY OF VITAMIN BIOSYNTHESIS

GENERAL TECHNIQUES

EXPERIMENTAL

SECTION A

SECTION B

INTRODUCTION

Chapter 1

Vitamin B_6

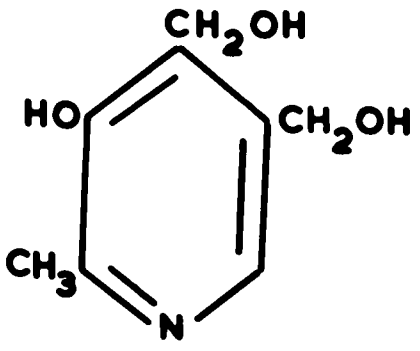
Existence in a number of forms

In the years immediately following its discovery, vitamin B_6 was identified with pyridoxine (2-methyl-3-hydroxy-4:5-bis(hydroxymethyl)pyridine). Then in 1942, the natural occurrence of more than one chemical compound with vitamin B_6 activity was deduced from a discrepancy between the values for the vitamin B_6 contents of various natural materials as assayed by the growth response of Streptococcus faecalis and by the chemical or rat and yeast biological methods of pyridoxine assay (Snell, Guirard and Williams, 1942). The incredibly high figures obtained with Strep. faecalis could only be explained by assuming the presence in natural materials of a 'pseudopyridoxine' more active than pyridoxine itself in supporting the growth of this organism. 'Pseudopyridoxine' proved to be not a single substance, but two., viz., pyridoxal and pyridoxamine, readily interconvertible both biologically and chemically and differing from pyridoxine only in the nature of their 4-substituent groups (Fig. 1) (Snell, 1944).

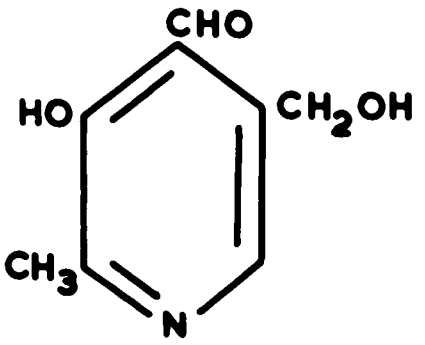
None of these compounds is the functional form of the vitamin in cell metabolism. In all instances except one, vitamin B_6-dependent enzymes have been found to utilise

FIG. 1.

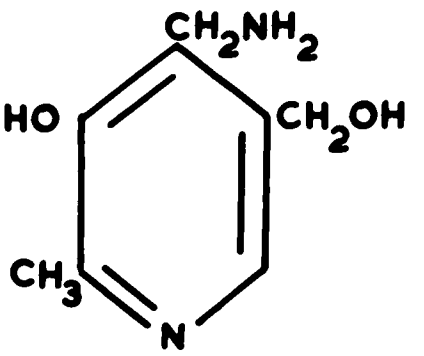
(a) Naturally occurring forms of vitamin B_6



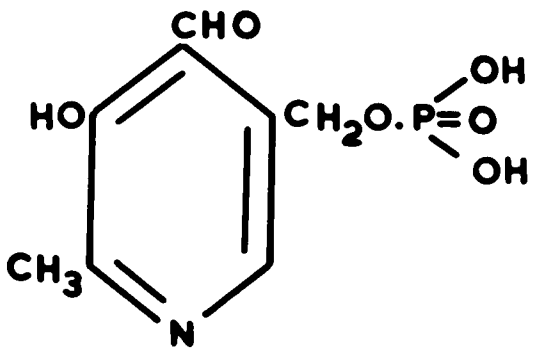
Pyridoxine



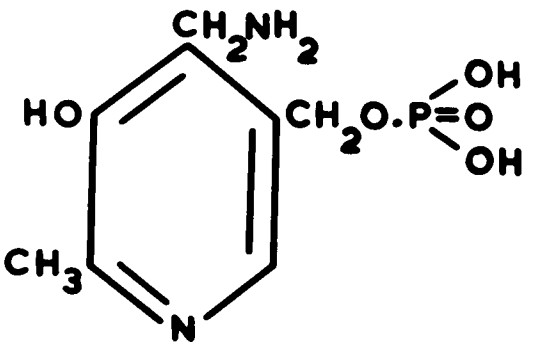
Pyridoxal



Pyridoxamine

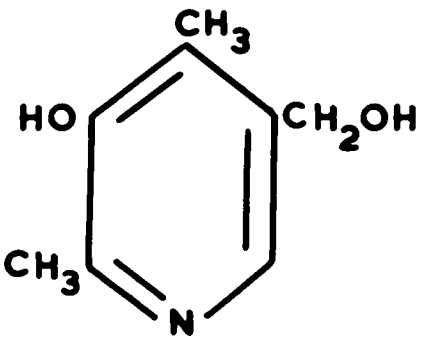


Pyridoxal-5'-phosphate

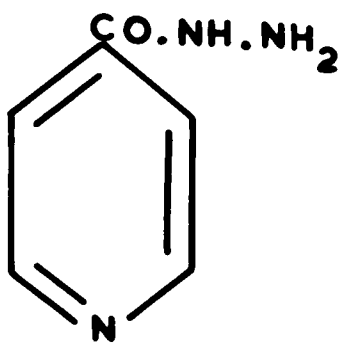


Pyridoxamine-5'-phosphate

(b) Vitamin B_6 antimetabolites



4-Deoxypyridoxine



Isoniazid

only pyridoxal-5'-phosphate as coenzyme (Heyl et al., 1951), e.g., amino acid decarboxylases (Baddiley and Gale, 1945; Lichstein and Cohen, 1945), and transaminase preparations (Lichstein, Gunsalus and Umbreit, 1945; Green, Leloir and Nocito, 1945; Kritzmann and Samarina, 1946; O'Kane and Gunsalus, 1947). Only in the case of an animal transaminase has pyridoxamine-5'-phosphate been shown to replace the pyridoxal ester (Meister, Sober and Peterson, 1952). Pyridoxamine-5'-phosphate may be the biological 'storage' form of the vitamin (Rabinowitz and Snell, 1947).

Any natural material might therefore contain three unesterified compounds with vitamin B_6 activity (the degree of activity depending on the test organism), as well as the 5'-phosphate esters of pyridoxal and pyridoxamine (Fig. 1), and perhaps other tissue-bound forms (Scudi, 1942; Scudi, Buhs and Hood, 1942; Jones and Morris, 1950). This multiplicity of naturally occurring forms of the vitamin presents a major problem when total vitamin B_6 is to be assayed. The various derivatives must be converted either to a single form, or to a mixture of forms which elicit equal response in the assay procedure.

Extraction

As the vitamin B_6 content of natural materials is often predominantly present in the form of 'protein bound' phosphates which are unreactive in several methods

of vitamin B_6 assay, the free forms must be 'liberated' by preliminary hydrolysis. This is usually carried out at an acid pH since all these compounds are at their most stable at low pH. Not unexpectedly, it appears that the conditions necessary for optimal extraction vary with the nature of the material treated. A large variety of methods has been suggested employing either elevated temperature or suitable enzymes, e.g., pepsin or papain (for review, see Sebrell and Harris, 1954). The most reliable of these procedures use a pH of 1.6 to 2.0, quite large volumes of extractant, and prolonged autoclaving (15 to 20 lb.sq.in. for 1 to 5 hr.) (Rabinowitz and Snell, 1947; Rubin, Scheiner and Hirschberg, 1947).

Assay

To estimate the total vitamin B_6 content of a digested sample a method must be used in which pyridoxine, pyridoxal and pyridoxamine show equal activity.

The possible physical methods, e.g., u.v. absorption or polarography, are relatively insensitive and, unless vitamin B_6-free extract is available as a blank, are applicable only to pure solutions.

Available chemical methods consist of colorimetric reactions which were devised when pyridoxine was the only compound known to possess vitamin B_6 activity. Different

TABLE II-1

Organisms used for the microbiological assay of vitamin B6

Organism	Disadvantages	References
Neurospora sitophila 299	1. 5-day incubation period. 2. Growth response measured gravimetrically. 3. Occasional failure to give consistent results.	Stokes, Larsen, Foster & Woodward (1943). Carpenter & Strong (1944).
Saccharomyces cerivisiae Ck (Gebruder-Bayer)	Pyridoxal and pyridoxamine only 40-46% as active as pyridoxine.	Williams, Makin & McMahn (1941). Siegel, Melnick & Oser (1943). Snell & Rannefeld (1945)
Saccharomyces oviformis 782	Response to pyridoxal and pyridoxamine dependent on cultural conditions and time of incubation; always only between 20-80% activity of pyridoxine.	Burkholder (1943). Snell & Rannefeld (1945)
Kloeckera brevis	Results frequently at variance with those given by Neurospora sitophila 299.	Emery, Meleod and Robinson (1946).
Schizosaccharomyces ludwigii	Basal medium contains u.v.-irradiated yeast autolysate.	Meisel & Pomoshchnikova (1952)
Saccharomyces carlsbergensis 4228	1. Rate assay. 2. Pyridoxamine is sometimes less active than other forms.	Atkin et al. (1943) Hopkins & Pennington (1947) Jones & Morris (1950) Snell (1950)

degrees of reactivity and differently coloured products are obtained with pyridoxal and pyridoxamine. Although the methods are sensitive and quantitative when applied to pure solutions of the individual compounds, they are so unspecific that very tedious preliminary purification procedures must be carried out to remove interfering substances from natural extracts.

Biological assay depends on the production of a vitamin B_6-deficiency state in higher animals; both prophylactic and curative methods have been adopted. Although no preliminary extraction procedures are here necessary to 'liberate' assayable vitamin, the methods are very tedious and relatively insensitive. Similar objection can be taken to the use of the growth response of the rice moth larva (Sarma, 1943; 1944).

Microbiological assay is the method of choice. It is sensitive, rapid and accurate, though few organisms respond equally well to each of the free forms of the vitamin (Table II-1). Of these, Saccharomyces carlsbergensis 4228 has given the most reliable results.

Biochemical functions

Acquaintance with the functions of vitamin B_6 and the consequences of its lack, is necessary in the present context only as an aid to the preparation of growth

media designed either (a) to enhance the vitamin B_6 requirement of an organism, or (b) so to diminish their vitamin requirement as to support the growth of organisms deficient in the vitamin.

Pyridoxal-5'-phosphate is the coenzyme of a great variety of enzymes acting upon amino acids. The reactions include decarboxylation, transamination, racemisation, dehydration and desulphydration besides a variety of miscellaneous transformations. So numerous are the possible reactions in fact, that vitamin B_6 can be considered as being obligatory for the non-oxidative metabolism of amino acids (Snell, 1952; 1955). The vitamin is thus also concerned with the biosynthesis of other essential cell constituents such as purines and pyrimidines (via glycine, aspartic acid, etc.), or other vitamins, e.g. kynureninase in mammalian nicotinic acid synthesis and aspartic decarboxylase in pantothenic acid formation.

Nutritional evidence points to a function for vitamin B_6 in fat metabolism (Sinclair, 1952), in particular in the synthesis of unsaturated fatty acids. It may have even more functions: pyridoxal-5'-phosphate was recently reported as an integral part of the enzyme muscle phosphorylase (Baranowski, Illingworth, Brown and Cori, 1957).

In all organisms showing a vitamin B_6 deficiency,

the enzymic reactions whose malfunction is primarily responsible for the deficiency symptoms vary according to the nutritional state of the organism. The manifestations of vitamin B_6 deficiency are determined as much by the manner in which this is produced as by its assayable extent (see Woods, 1953, concerning preferential utilisation of metabolites).

Antimetabolites

There is no truly catholic vitamin B_6 antimetabolite. Accurate assessment of the worth and mode of action of any one antimetabolite is hampered by the fact that its efficacy varies with the test enzyme preparation or organism employed. This is well illustrated by the action of various vitamin B_6 analogues on the growth of Strep. faecalis R (Olivard and Snell, 1955). The effects of ω-methylpyridoxal ranged from efficient substitution for pyridoxal to good competitive antimetabolite activity depending on the nature of the limiting amino acid in the growth medium (and hence on the nature of the limiting vitamin B_6-requiring enzyme).

In the course of the present studies, it was necessary to use,

(a) a vitamin B_6 antimetabolite capable of inhibiting the growth of micro-organisms able to synthesise their

normal requirements of the vitamin;

(b) a vitamin B_6 antimetabolite capable of selectively inhibiting the growth of those organisms unable to synthesise their requirements of the vitamin, being thus dependent on an exogenous supply of pyridoxine.

Isoniazid was chosen as a compound fulfilling the criteria of (a), and 4-deoxypyridoxine, those of (b).

Isoniazid - isonicotinoyl hydrazide (INH)

High concentrations of isoniazid inhibit the growth of many micro-organisms, the inhibition being relieved competitively by vitamin B_6 (Boone and Woodward, 1953). Administration of isoniazid to humans caused the excretion of excessive amounts of vitamin B_6 in the urine (Biehl and Vilter, 1952) as well as a peripheral neuritis which was counteracted by vitamin B_6 administration.

Again in comparatively high concentrations, isoniazid interferes with the functioning of many pyridoxal phosphate-dependent enzymes (Meister and Downey, 1956; Yoneda, Kato and Okajima, 1952; Yoneda and Asano, 1953) and prevents reactivation of certain apoenzymes by pyridoxal-5'-phosphate (Fried and Lardy, 1955; Lichstein, 1955).

Although it is certain that isoniazid can act as a vitamin B_6 antimetabolite, its mode of action is uncertain. Studying the kinetics of INH inhibition of cysteine

sulphinic acid decarboxylase, Davison (1956) showed that the reaction possesses a high energy of activation, of the same order as that found for the chemical reaction of isoniazid with pyridoxal phosphate. He concluded that chemical interaction with hydrazone formation was the cause of the inhibition. Lichstein (1955), on the other hand, believed that isoniazid competes with pyridoxal phosphate for the apoenzyme.

Whilst hydrazone formation with pyridoxal probably accounts for many of the anti-vitamin B_6 actions of isoniazid, it cannot explain all (Umbreit, 1955), and certainly does not account for its specific activity against mycobacteria.

4-Deoxypyridoxine

This inhibits the growth of some micro-organisms which are strictly dependent upon an exogenous supply of vitamin B_6 (Saccharomyces carlsbergensis 4228 and Neurospora sitophila 299) whilst it has no action on others equally incapable of synthesising the vitamin (Lactobacillus casei and Strep. faecalis). It might be incorrect, therefore, to assume that vitamin B_6-synthesising organisms are immune to its action merely because they are independent of an external vitamin supply. The growth inhibition is overcome by all free forms of vitamin B_6, and it might in fact be

significant that 4-deoxypyridoxine affects only those organisms that can utilise pyridoxine (Rabinowitz and Snell, 1953).

4-Deoxypyridoxine might inhibit the uptake of vitamin B_6 by susceptible organisms. If its inhibitory action is exerted subsequently, then the reactions accomplishing the utilisation of externally supplied vitamin must differ significantly from those concerned in the normal biosynthesis of pyridoxal-5'-phosphate.

While 4-deoxypyridoxine-5'-phosphate will not displace pyridoxal phosphate from holoenzymes when the latter has been firmly attached, it may compete with the natural coenzyme, as for example, for tyrosine apodecarboxylase (Umbreit and Waddell, 1949). Phosphorylation of the analogue and adsorption onto the apoenzyme only occur when the pyridoxal or pyridoxal phosphate concentrations are very low.

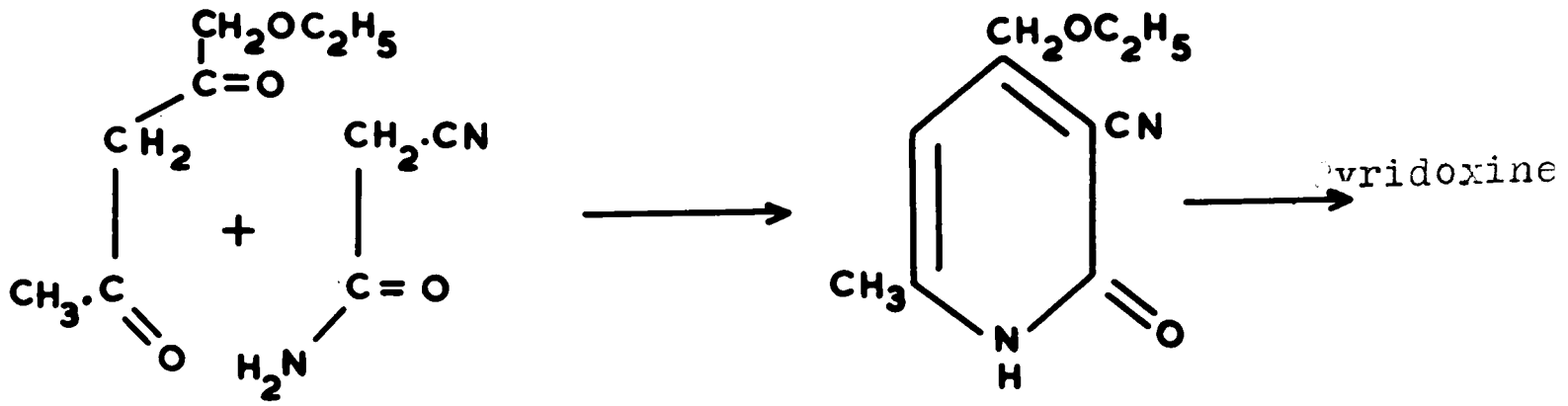
Again the mechanism of action of this antimetabolite is uncertain, perhaps changing with changed conditions of test.

Chemical synthesis of pyridoxine

Two major synthetic and one degradative procedure are now used for the chemical preparation of pyridoxine.

In the present context, interest in these chemical

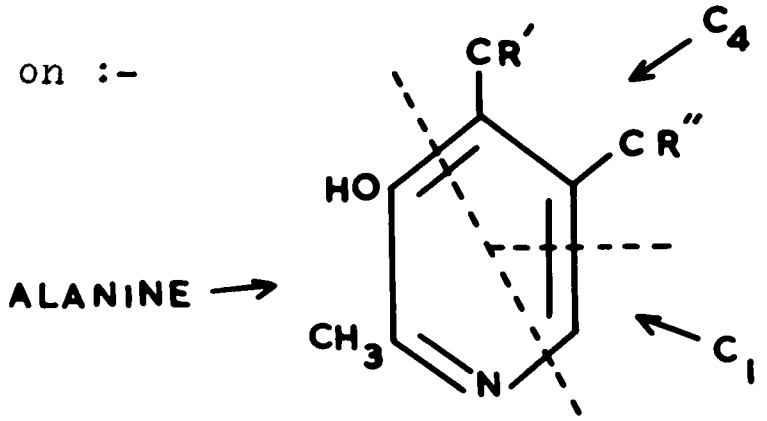
(a) Harris and Folkers (1939)

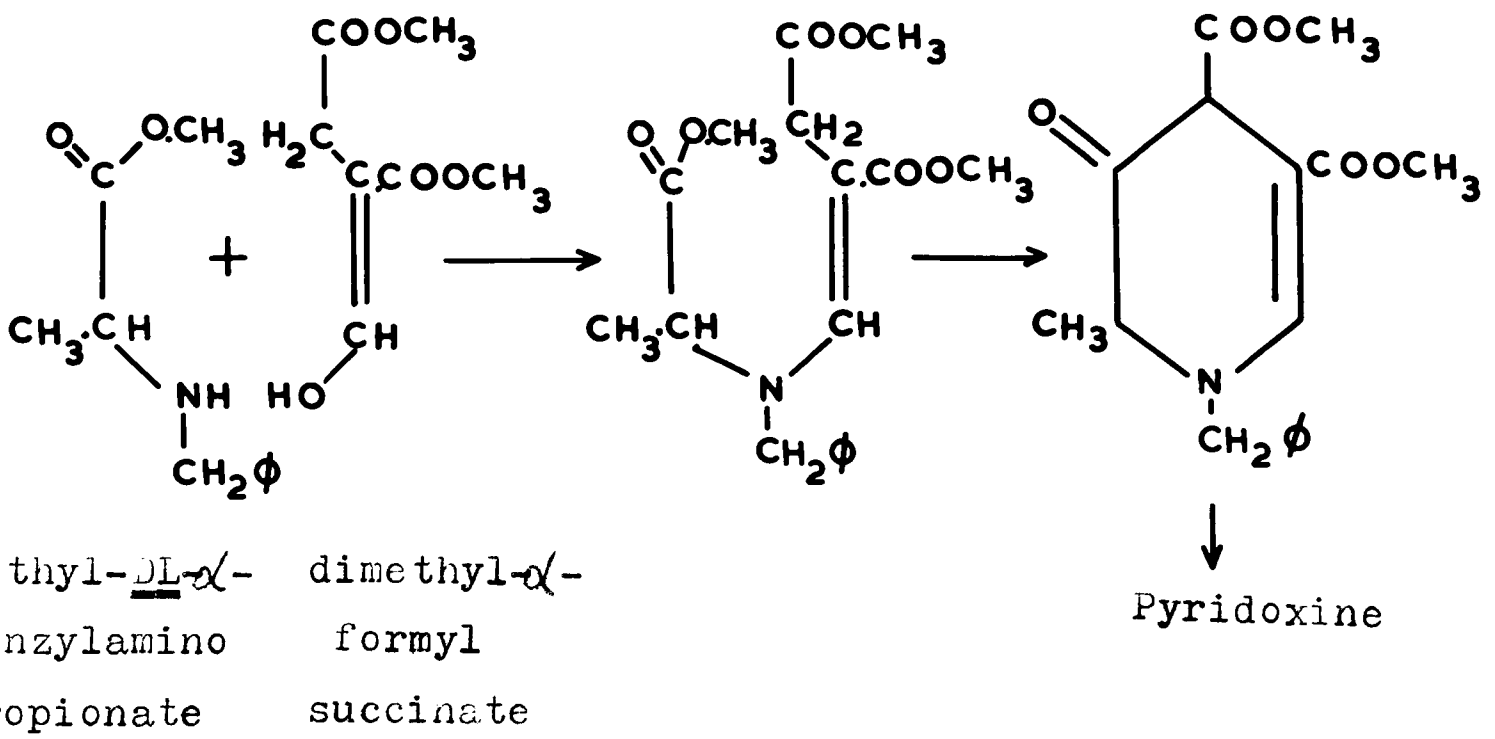


ethoxyacetyl-acetone + cyanoacetamide → 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone → Pyridoxine

(b) Cohen, Haworth and Hughes (1952)

Based on :-





methyl-DL-α-benzylamino propionate + dimethyl-α-formyl succinate → Pyridoxine

FIG 2. Ring closure in the chemical synthesis of pyridoxine

procedures stems from the possibility that they could suggest likely biological precursors. The degradative method, therefore, (Kuhn, Westphal, Wendt and Westphal, 1939; Ichiba and Michi, 1939) which subjects a suitably substituted isoquinoline derivative to controlled oxidation, is of little interest, as the starting material already contains the pyridine ring.

The first successful synthesis of pyridoxine from aliphatic precursors (Harris and Folkers, 1939; Morii and Makino, 1939) was based on an initial pyridone condensation of ethoxyacetylacetone with cyanoacetamide. The product, 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone, was convertible by usual pyridine ring reactions to pyridoxine (Fig. 2a).

Cohen, Haworth and Hughes (1952) based their method on a hypothetical derivation of pyridoxine from units of alanine, formaldehyde and a C_4 compound (Fig. 2b). For convenience the actual condensation was carried out between an alanine derivative (methyl-DL-α-benzylamino propionate) and a C_4 derivative already carrying the formyl group (dimethyl-α-formyl succinate). The product is easily converted to pyridoxine, and the method has been much used for the introduction of substituents in the 2-position.

Biogenesis

Strep. faecalis R, which normally requires vitamin B_6 for growth, grows in the absence of added vitamin in an otherwise complete amino acid medium when DL-alanine is provided in sufficiently high concentration (Snell and Guirard, 1943; Snell, 1945). Snell at first suggested that DL-alanine might be a precursor of vitamin B_6, perhaps being directly incorporated into the pyridine ring, but then found that when the organism was grown on a full replacement mixture including DL-alanine, only a very small amount of vitamin B_6 was synthesised (Holden, Furman and Snell, 1949). D-alanine alone was effective, the L-isomer being without activity, and it now seemed probable that D-alanine was essential for the growth of the organism, its synthesis proceeding normally via the L-isomer by the action of a vitamin B_6-dependent racemase. According to this theory, in the presence of an otherwise complete vitamin B_6-replacement medium, the omission of D-alanine would make provision of vitamin B_6 essential for growth. Conversely, growth could occur in the presence of D-alanine even though the organism remains unable to synthesise the vitamin. Confirmation came from the demonstration of the possession by the organism of a pyridoxal phosphate-requiring alanine racemase (Wood and Gunsalus, 1951) and from the assignment to D-alanine of an essential

function - in particular as a structural component of the cell wall (Snell, Radin and Ikawa, 1955). Growth of Strep. faecalis R could be made dependent upon the provision of other single amino acids. In the presence of the otherwise complete replacement mixture (now containing excess D-alanine), the organism when provided with α-oxo isocaproic acid would grow only if either leucine or pyridoxal were supplied. This was again not indicative of a vitamin B_6-precursor function of leucine, but was an expression of the pyridoxal phosphate requirement of the ketoleucine-leucine apotransaminase (Olivard and Snell, 1955).

A strain of brewer's yeast required both thiamine and vitamin B_6 for optimal growth, and it was suggested that these vitamins might be interconvertible via a common pyrimidine precursor which is but poorly synthesised by this organism (Moses and Joslyn, 1953). ... There undoubtedly exists a relationship between these vitamins in the metabolism of many micro-organisms, but the situation is very complex. The pyrimidine portion of the thiamine molecule, i.e., 'toxopyrimidine', can act as a vitamin B_6 antimetabolite (Abderhalden, 1954; Makino, Kinoshita, Sasaki and Shioi, 1954; Makino, /Kinoshita, Aramaki and Shintani, 1954; Makino and Koike, 1954), while thiamine itself causes an inhibition of growth of a strain of Saccharomyces carlsbergensis 4228 which is overcome noncompetitively by

pyridoxine (Schultz et al., 1947; Rabinowitz and Snell, 1951). There is evidence that vitamin B_6 may play an important role in thiamine biosynthesis in Neurospora sitophila 299 (Stokes, Foster and Woodward, 1943; Stokes, Larsen, Woodward and Foster, 1943), though with other pyridoxine-less Neurospora mutants Harris (1952) suggested that pyridoxine inhibited thiamine biosynthesis, while thiamine prevented degradation of vitamin B_6.

These findings suggest that the observations of Moses and Joslyn could be interpreted without assuming the common origin of thiamine and pyridoxine. Vitamin B_6 biosynthesis by their yeast was actually increased during thiamine deficiency, whilst pyridoxine deficiency caused a decreased thiamine synthesis. Thus, whilst vitamin B_6 could be concerned in thiamine synthesis, thiamine might exert a sparing effect on vitamin B_6 formation.

No further theories as to the route of vitamin B_6 biosynthesis have been forthcoming.

Chapter 2

The Biogenesis of the Pyridine Ring

The biosynthesis of vitamin B_6 may be only a facet of the more comprehensive problem of pyridine ring biogenesis. A common precursor of pyridine derivatives may exist in nature, by analogy with the origin of the aromatic amino acids from shikimic acid.

It is possible that most if not all of the ring substituents of the vitamin B_6 molecule form part of the immediate precursor(s) which undergo cyclisation. The ring closure reaction thus assumes particular importance, as the structure of plausible precursors could conceivably be extrapolated from the nature of the side chains and the type of reactive groupings probably required for cyclisation.

RING CLOSURE

This might be the result of (a) an intramolecular reaction, or (b) a hetero-molecular interaction.

Given suitable end groups, there is a tendency for aliphatic compounds of certain chain lengths to undergo intramolecular cyclisation. This tendency is particularly marked when 5- or 6-membered cyclic compounds would result, as these are particularly stable structures.

When the ring compound is a product of the fusion

of two or more molecules, at least two reactions must participate. Were the reactions to fall into a definite sequence, the last would necessarily represent an intramolecular cyclisation of an intermediate. In many instances however, it is not possible to decide which reaction takes precedence, e.g., in porphobilinogen biosynthesis from δ-amino-laevulic acid (Fig. 3b). Group (b) provides for just such cases as these.

With the N-heterocyclic compounds, one of the reactions always concerns the introduction of the nitrogen atom.

Chemical synthesis

The C-N link can be produced by an addition reaction, but this is comparatively rare, e.g., the Döbner-von Miller and the Skraup quinoline syntheses.

$$-NH_2 + H_2C{=}CH- \longrightarrow -NH-CH_2-CH_2-$$

The condensation of a ketone, or potentially ketonic group with a primary amino group is, however, by far the simplest and most frequently used method of synthesis of the C-N bond.

$$>C{=}O + H_2N- \longrightarrow >C{=}N-$$

or,
$$>C(OH)- + H_2N- \longrightarrow >C(NH)-$$

Use is made of such a condensation reaction in the Knorr pyrrole synthesis, the Hantzsch pyridine synthesis, and the Friedlander quinoline synthesis. It also occurs in

both main methods of pyridoxine synthesis (Harris and Folkers, 1939; Cohen, Haworth and Hughes, 1952). By an extension of the method, a $>C=N-C<$ link is obtained by the condensative insinuation of NH_3 between two ketonic (or enolic) groups, e.g., in the Tschitschibabin pyridine synthesis.

Apparent condensation between two primary amine groups, with the liberation of ammonia, can be utilised, though deamination might precede cyclisation with the formation of a ketonic intermediate. A simple example of such a reaction is the cyclisation of glutamine to pyrrolidone carboxylic acid. As this occurs on heating in aqueous solution, prior deamination is very likely. Condensation of amine with imine, could be an alternative mechanism,

$$R\text{-}NH_2 + HN{=}CH.R' \longrightarrow R.N{=}CH.R' + NH_3.$$

The secondary amine which is a co-product with primary amine of the catalytic reduction of a nitrile, is said to arise by such a reaction proceeding between the primary amine and the intermediate aldimine (Rodd, 1951).

Biochemical mechanisms

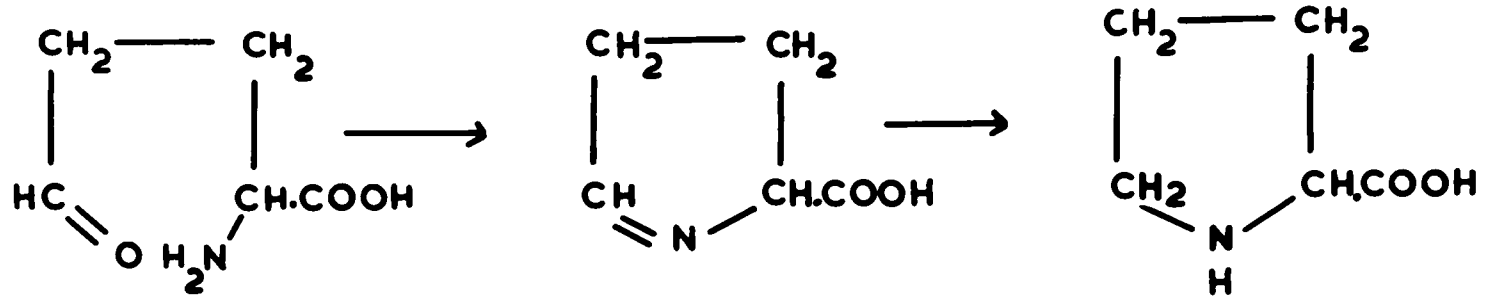
Formation of the C-N bond is a very common metabolic reaction, e.g., amidation as in glutamine formation, formylation of an amino group as in formylglycinamide

ribotide synthesis. Most commonly of all, it occurs in the synthesis of amino acids from their ketonic precursors and in peptide formation; incidentally, the formation of a cyclic polypeptide by carbonyl-amine condensation, might thus be considered as an example of N-heterocyclic biosynthesis. Most of these, and related reactions, e.g., dihydro-orotic acid formation by ring closure of carbamyl-aspartate, are endergonic and proceed only at the expense of ATP.

It seems though that the C-N ring closing bonds formed in the biosynthesis of pyrrole and pyridine derivatives might be the result of spontaneous condensations between ketonic and primary amino groupings. Thus in proline biosynthesis (see Fig. 3a), glutamic semialdehyde undergoes spontaneous ring closure to give the pyrroline carboxylic acid. In confirmation of the spontaneity of the cyclisation, the product obtained by treatment of α-keto-δN-benzyloxycarbonyl valeric acid with HBr, gave elemental analyses consistent with its being Δ'-pyrroline-2-carboxylic acid, yielded proline on hydrogenation, and yet possessed properties characteristic of an α-keto acid (Meister, 1954), suggesting that the compound was in fact a mixture of tautomeric forms including the enol form of the normal aliphatic compound, and the Δ'-cyclic form. The same compound was isolated from the culture filtrate

FIG. 3

(a) Ring closure in the biosynthesis of proline

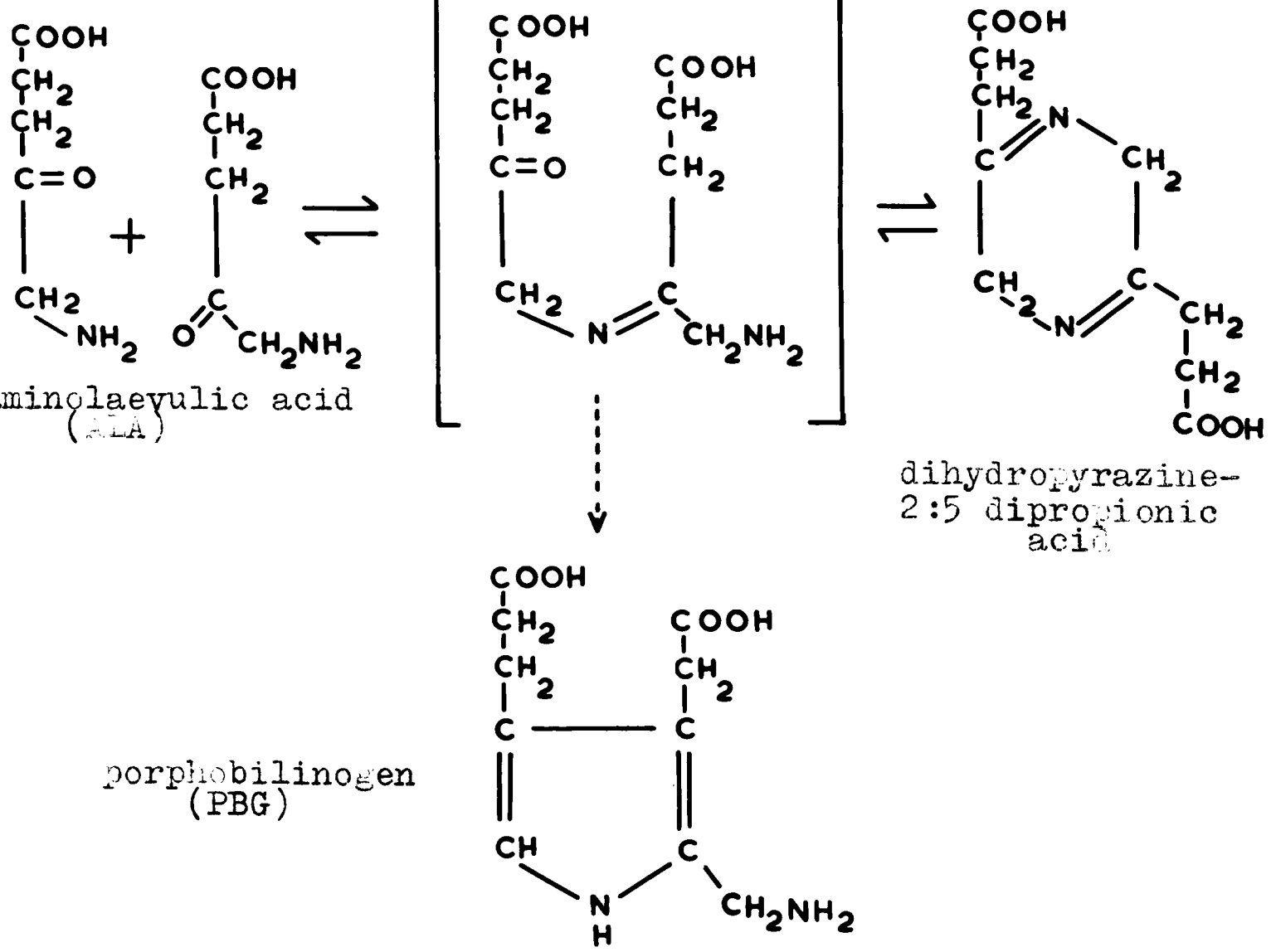


glutamic-γ-semialdehyde

Δ'-pyrroline carboxylic acid

proline

(b) Ring closure in porphobilinogen biosynthesis



δ-aminolaevulic acid (ALA)

dihydropyrazine-2:5 dipropionic acid

porphobilinogen (PBG)

of a proline auxotroph of E. coli by Vogel and Davis (1952). These authors too supposed the ring closure to be spontaneous though followed by an enzymically catalysed reduction. They stated that "it also seems possible that the biosynthesis of other heterocyclic compounds may involve similar reactions between amino and aldehyde groups".

The most thoroughly investigated reaction of this nature, is that responsible for the biosynthesis of porphobilinogen (PBG) from δ-aminolaevulic acid (ALA). The δ-aminolaevulic dehydrase (ALAD) which brings about this synthesis, has been studied (Gibson, 1955). The enzyme catalyses an intermolecular cyclisation though it is not known whether only one or both of the reactions concerned in ring closure are in fact enzymically catalysed. Under suitable conditions ALA will condense with itself to form some PBG without enzymic intervention, but at neutral or alkaline pH's and room temperature, dihydropyrazine-2:5-dipropionic acid is predominantly and spontaneously formed. Some evidence was obtained for the participation at neutral pH of an intermediate which is probably a linear dimer formed from two ALA molecules (Gibson, 1955) (see Fig. 3b). Only C-N bonds are formed in the predominant spontaneous reaction, and it is possible that only the new C-C bond in PBG is the result of enzymic action.

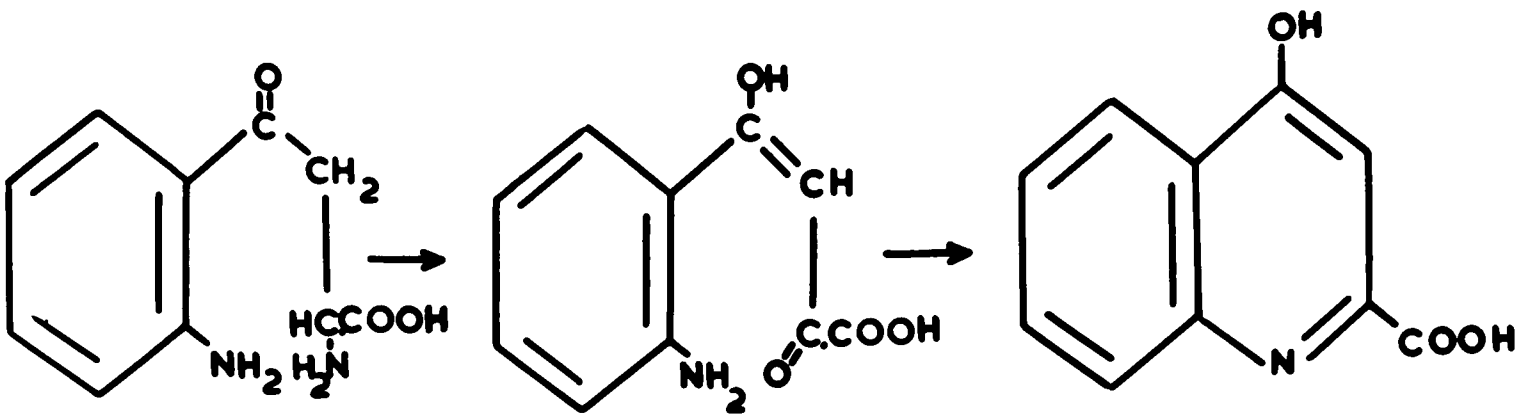
APPARENT AMINE-AMINE CONDENSATION

When all examples are closely examined, it seems that prior deamination of one of the amino groups precedes ring closure so that this is again a spontaneous keto-amine condensation, e.g., pyrrolidine ring formation from ornithine (Leete, 1955; Dewey, Byerrum and Ball, 1955).

The enzyme isolated from Pseudomonas sp. Tr. 7 forming kynurenic acid from kynurenine, was found to be a kynurenine transaminase, the formation of 2-aminobenzoyl-pyruvic acid from kynurenine being followed by spontaneous ring closure (Fig. 4a). A similar situation exists in the biosynthesis of pipecolic acid from lysine. Thus, pipecolic acid is formed by the hydrogenation of the product formed by the action on L-lysine, of either L-diamino acid oxidase of turkey liver (Boulanger and Osteaux, 1952), or the L-amino acid oxidase of Neurospora (Schweet, Holden and Lowy, 1954). Meister (1954) proposed that in the biosynthesis of the acid, oxidation or transamination of lysine, giving rise to α-oxo ε-amino-caproic acid, might be the first step. Spontaneous cyclisation to Δ'-dehydro-pipecolic acid would be followed by optically specific enzymic reduction to L(-)pipecolic acid. When α-oxo εN-benzyloxycarbonyl caproic acid was treated with HBr, the product gave the elemental analyses for either α-oxo

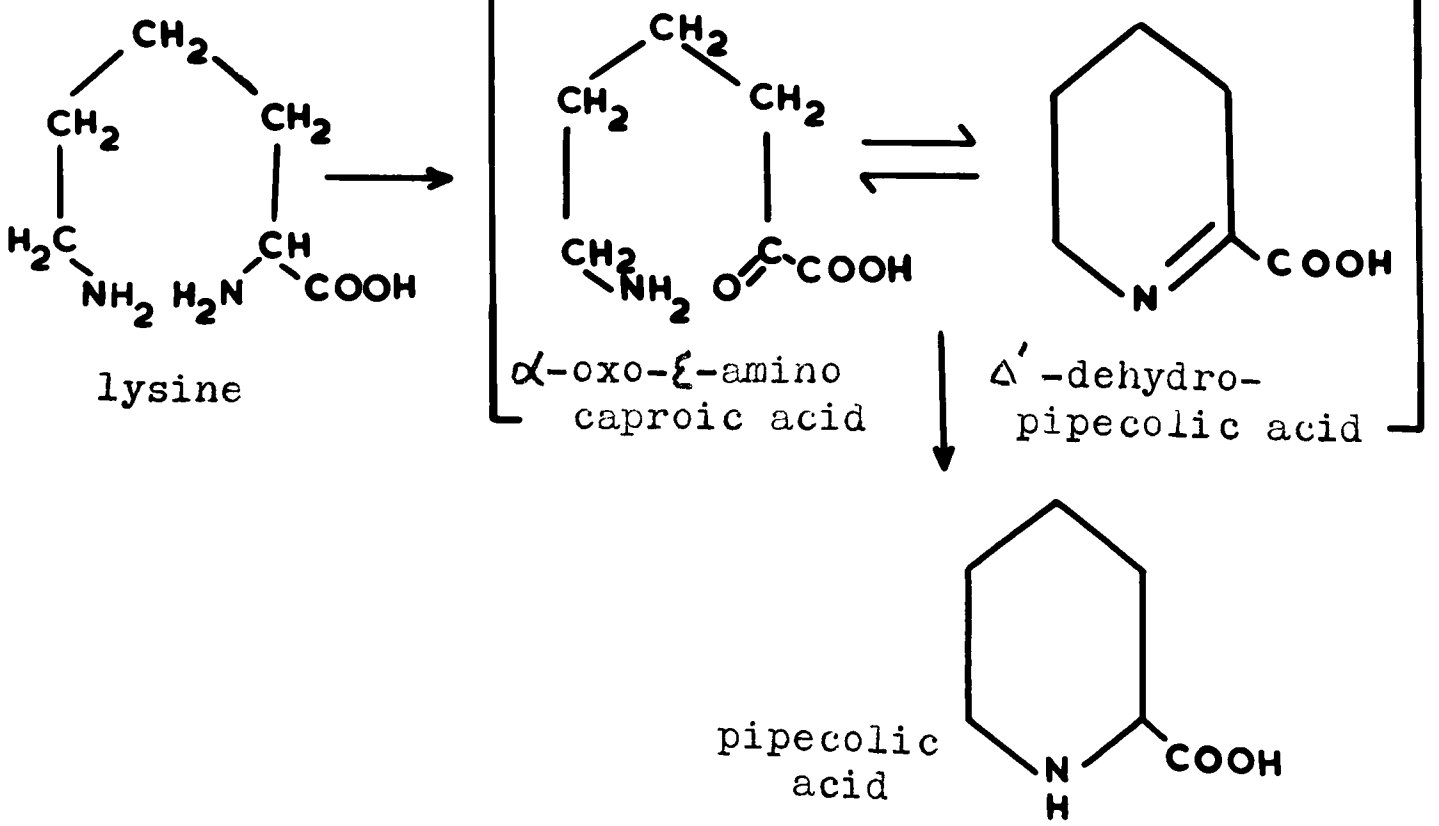
FIG. 4

(a) Ring closure in kynurenic acid biosynthesis



kynurenine — 2-aminobenzoyl-pyruvic acid — kynurenic acid

(b) Ring closure in pipecolic acid biosynthesis



lysine — α-oxo-ε-amino caproic acid ⇌ Δ′-dehydro-pipecolic acid → pipecolic acid

ε-aminocaproic acid, or Δ'-piperidene-2-carboxylic acid monohydrate, and otherwise reacted as a mixture of open chain and cyclic compounds. This oxo-analogue of lysine was identified with the product of action of Neurospora L-amino acid oxidase on L-lysine (Schweet et al., 1954) and was stated to exist mainly in the cyclised form. The ^{14}C-labelled oxo acid was converted by Neurospora both to pipecolic acid and back to lysine (Schweet, Holden and Lowy, 1954). By ^{15}N-labelling, Rothstein and Miller (1954) obtained incontrovertible evidence of the initial loss of the α-amino group when rats converted lysine to urinary pipecolic acid (Fig. 4b).

The necessity for prior deamination of one of the two amine groups has led to a suspicion that amine oxidases might play some role in alkaloid biosynthesis, diamines and diamine oxidases coming in for greatest study (Späth and Berger, 1930; Tabor, 1951; Blaschko, 1952; Mann and Smithies, 1955). Whether ring compound formation will follow amine oxidase action upon aliphatic diamines, depends on the chain length of the substrate. Thus, the product of oxidation of 1:10-diaminodecane does not undergo ring closure (Mann and Smithies, 1955).

PIPERIDINE RING BIOSYNTHESIS

As a result of the keto-amine condensation reaction only one double bond is introduced into the ring.

Reduction to the fully saturated derivatives can occur. Pipecolic acid is formed from lysine in this way (see above, and Grobbelaar and Steward, 1953; Lowy, 1953); similarly L-lysine is the precursor of coniine-2-propyl-piperidine (Leete, 1955b).

Discussion of the piperidine derivatives merely as if they are reduced pyridine compounds might be very misleading, for the chemistry of these two groups differs widely; pyridine is related to piperidine as benzene is to cyclohexane. The chemistry of pyridine is dominated by its aromatic character, that of piperidine is essentially that of a secondary amine. Incidentally, there is a striking similarity between the substitution reactions of pyridine and p-nitrobenzene, a resemblance which assumes importance in the assessment of the mode of action of pyridoxal.

The possibility of pyrrolidine and piperidine ring formation by the cyclisation of aliphatic diamines (ornithine and lysine respectively), need not, therefore, suggest a similar precursor for pyridine ring formation, though both lysine and 4-hydroxylysine have been considered as plausible precursors of the pyridine rings of nicotine and anabasine. But 2-^{14}C-lysine was not incorporated into the nicotine of tobacco plants growing in hydroponic culture (Leete, 1955c), whilst 2-^{14}C-lysine and ε-^{14}C-hydroxylysine were only insignificantly converted to

anabasine (Aronoff, 1956). Aronoff (1956), however, interpreted his observation that photosynthesising excised leaves of Nicotiana glauca supplied with $^{14}CO_2$ synthesised uniformly labelled anabasine, as showing that both the pyridine and piperidine rings were of simultaneous origin.

PYRIDINE RING FORMATION

To obtain the aromatic pyridine ring, the product of spontaneous keto-amine condensation would have to be subjected to two further oxidation reactions unless the precursor was unsaturated or potentially unsaturated, e.g., capable of a keto-enol tautomerism, as in kynurenic acid synthesis. It is not known whether such oxidation of a tetrahydropyridine to a pyridine can be accomplished.

Virtually all knowledge concerning the biogenesis of the pyridine ring has come from the study of the route of formation of nicotinic acid.

Nicotinic acid (pyridine-2-carboxylic acid)

Nutritional studies with mammals (Krehl, Teply, Sarma and Elvehjem, 1945) exposed a relationship between L-tryptophan and nicotinic acid. Results obtained with mammalian tissues together with observations made with nicotinic acid-mutants of Neurospora, showed that in these organisms at least, nicotinic acid is a product of

3-hydroxyanthranilic acid metabolism. This in turn is derived from tryptophan via kynurenine and 3-hydroxy-kynurenine (Bonner and Yanofsky, 1951; Sebrell and Harris, 1954; Mehler, 1955; Yanofsky, 1955). The operation of the same pathway has been noted in other fungi, e.g. Trichophyton equinum (Georg, 1949), Phycomyces blakesleeanus (Schopfer and Boss, 1949) and in at least one bacterium, viz., Xanthomonas pruni (Davis, Henderson and Powell, 1951). However, alternative routes of nicotinic acid biosynthesis must exist. The possible absence of a tryptophan-nicotinic acid relationship in certain plant tissues (Terroine, 1948; Crane, 1954) and in the protozoon Tetrahymena (Kidder and Dewey, et al. 1949) has been remarked upon. Pontecorvo (1953) concluded that two pathways of nicotinic acid biosynthesis must operate in Aspergillus nidulans. Both proceed via 3-hydroxyanthranilic acid so that the duplicity is in the origin of this compound. One biosynthetic route seems identical with the tryptophan pathway described in Neurospora, in the other, anthranilic acid appears to be converted to its 3-hydroxy derivative either directly or via unknown intermediates.

Marnay (1951) concluded on the basis of growth inhibition studies with indole-3-acrylic acid that the path of nicotinic acid biosynthesis in Escherichia coli is the same as that in Neurospora. The method and conclusions

were criticised by Yanofsky, who, feeding overall ^{14}C-labelled indole and tryptophan to suitable tryptophan auxotrophs of E. coli and B. subtilis, demonstrated that the nicotinic acid then formed was unlabelled (Yanofsky, 1954a & b). These results confirmed earlier observations with E. coli (Ellinger and Abdel-Kadar, 1949a & b; Volcani and Snell, 1948). A strain of E. coli (presumably wild-type), directly hydroxylated anthranilic acid in the 3-position by an enzymatic reaction requiring a p-amino-benzoic acid derivative as cofactor, and proceeding at a rate compatible with its being the major route of nicotinic acid synthesis by the organism (McCullough, 1957). The occurrence of this reaction could explain the observations of both Pontecorvo and Yanofsky. Yet a few questions would remain. If the direct anthranilic to 3-hydroxyanthranilic acid 'shunt' is the only way of forming nicotinic acid in these organisms, mutants might be obtainable requiring either anthranilic acid or tryptophan plus nicotinic acid. These have not been described. If there is an alternative route of synthesis available, this cannot proceed via indole and tryptophan else some ^{14}C would in all probability have been incorporated into the nicotinic acid synthesised during Yanofsky's experiments.

The suggestion was made that the first step in the transformation of 3-hydroxyanthranilic acid to nicotinic

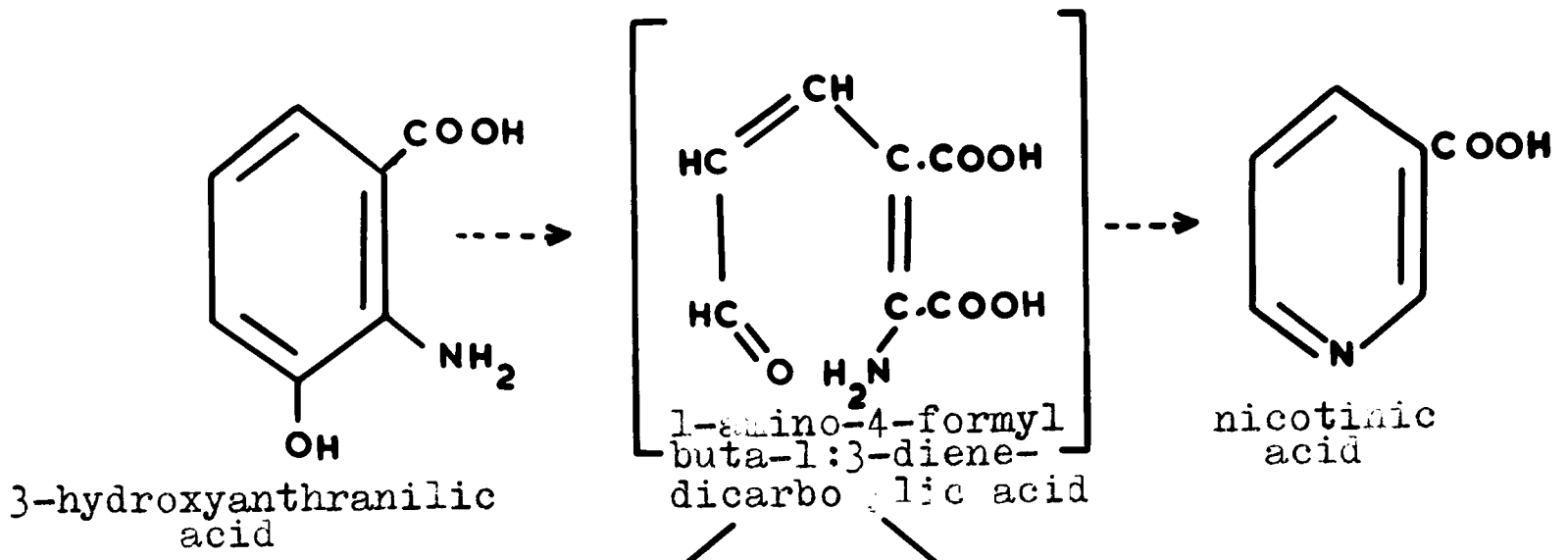
acid might be the oxidative cleavage of the benzene ring between C-3 and C-4, followed by spontaneous ring closure of the newly formed aldehyde group with the free amino group (Henderson and Hirsch, 1949). Evidence in support of this idea was obtained by the use of tryptophan labelled with ^{15}N in the pyrrolidine ring and with D in the benzene ring (Schayer and Henderson, 1952).

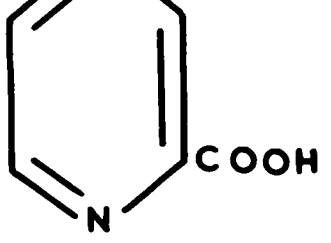
Only once has an enzymic synthesis of nicotinic acid from 3-hydroxyanthranilic acid been described (Makino et al., 1951; criticised by Hundley, 1954). 3:4-Dihydroxy-anthranilic acid (Fig. 5) was here reported to be as effective a substrate as the monohydroxy acid. No intermediary role for the dihydroxy compound could later be shown with Xanthomonas pruni, growing rats and rat liver slices (Henderson et al., 1951), or with a nicotinic acid auxotroph of Neurospora crassa (Hellmann and Wiss, 1952).

Nicotinic acid is not the only product of metabolism of 3-hydroxyanthranilic acid. Quinolinic and picolinic acids may also be formed. Thus mammalian tissue slices converted 3-hydroxyanthranilic acid to quinolinic acid (Schweigert, 1949; Schweigert,and Marquette, 1949), via an intermediate with a characteristic absorption maximum at 360 mμ (Bokman and Schweigert, 1951; Miyake, Bokman and Schweigert, 1954). Mehler (1956) showed that only the formation of this intermediate was enzyme catalysed,

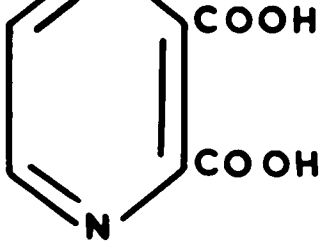
FIG. 5

(a) Ring closure in nicotinic acid biosynthesis

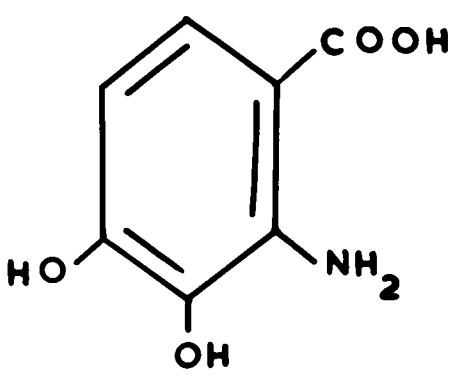




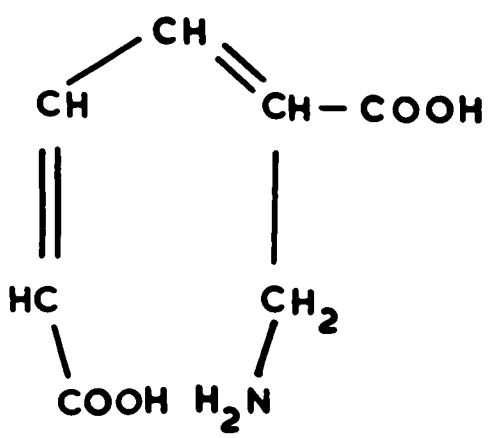
picolinic acid



quinolinic acid



3:4-dihydroxyanthranilic acid



α-aminomethylmuconic acid

the subsequent production of quinolinic acid being a spontaneous reaction (Fig. 5). The intermediate might be either the orthoquinone of 3:4-dihydroxyanthranilic acid or the open chain dicarboxylic acid amino aldehyde; its chemical properties were consistent with its being 1-amino-4-formyl-buta-1:3-diene dicarboxylic acid (Hiss, Simmer and Peters, 1956). A competing enzymic reaction is capable of converting this same intermediate to picolinic acid (Mehler, 1956a ~~& b~~). With ^{14}C-labelled 3-hydroxyanthranilic acid, it was confirmed that this is the carboxyl group cleaved by picolinic carboxylase (it is of course retained in the synthesis of nicotinic acid). Most endogenously produced 3-hydroxyanthranilic acid (in rats) seems to be oxidised and decarboxylated in this fashion. Quinolinic acid is not decarboxylated, and its formation *in vivo* is perhaps a reflection of the spontaneous cyclisation of the intermediate in the presence of a non-physiological excess of substrate.

Recently, some evidence has been obtained for the possible utilisation of α-aminomethyl-*trans*:*cis*-muconic acid for nicotinic acid biosynthesis by *Xanthomonas pruni* (Harris and Binns, 1957) (Fig. 5).

In the formation of nicotinic acid, it is possible that the placing of the amino group in a position suitable for subsequent condensation might be favoured or compelled

during decarboxylation, so that ring formation and decarboxylation might appear as simultaneous reactions effectively catalysed by the one enzyme. No such enzyme has as yet been isolated. Perhaps free nicotinic acid is not first formed and amidation and/or ribotidation actually precede ring closure.

Besides forming quinolinic and picolinic acids (together with the latter's derivatives, i.e., its methyl betaine homarine found in marine organisms, and the 3-hydroxypicolinic acid of etamycin), 3-hydroxyanthranilic acid must be considered as the precursor of products of the further utilisation of nicotinic acid in which the pyridine ring is retained. These include the detoxication products excreted by higher animals, e.g., N'methylnicotinamide, N'-methyl-6-pyridone-3-carboxamide and nicotinuric acid, and the pyridine ring in the nicotine alkaloids (Dawson et al., 1956). But while a very rapid conversion of nicotinic acid to the alkaloid trigonelline was carried out by excised pea stems, no label was incorporated from ^{14}C-labelled 3-hydroxyanthranilic acid (Aronoff, 1956).

DIPICOLINIC ACID (pyridine-2:6-dicarboxylic acid)

Some case has been made out for the possible formation of this pyridine derivative by direct cyclisation of an aliphatic precursor. The study of its biosynthesis

was facilitated by the fact that its calcium salt is excreted in large amount by germinating spores of both Bacillus and Clostridium species (Powell, 1953; Powell and Strange, 1956).

Perry and Foster (1955) pointed out that, apart from the unsaturated nature of the product, a similar relationship could be envisaged between 2:6-diaminopimelic acid and dipicolinic acid as had been shown to exist between lysine and pipecolic acid.

Washed suspensions of endotrophically sporulating Bacillus cereus var mycoides were supplied with totally ^{14}C-labelled diaminopimelic acid (DAPM- of biological origin and containing meso and LL isomers). A relatively small consumption of the exogenous DAPM was obtained, and from the degree of ^{14}C incorporation it was calculated that only about 4% of the dipicolinic acid synthesised could have arisen from the exogenous DAPM. The authors very properly pointed out that the data do not discriminate between prior breakdown of the supplied DAPM with utilisation of all or part of the fragments, or direct ring closure without previous rupture. The ^{14}C was preferentially incorporated into dipicolinic acid relative to some dozen or so amino acids isolated (including lysine). This cannot be used as an indication of specificity of conversion, for dipicolinic acid is very likely the major product of

synthesis during sporogenesis.

Powell and Strange (1956) failed to show DAPM conversion to dipicolinic acid even with disintegrated spore preparations. No increase in NH_3 production or O_2 uptake accompanied incubation of intact or disintegrated organisms and spores of B. cereus with synthetic DAPM.

The small conversion of exogenous DAPM to endogenously synthesised dipicolinate obtained in Perry and Foster's experiments, need not invalidate their hypothesis of direct conversion. It has been shown that exogenous DAPM is not incorporated into the endogenous DAPM of wild-type E. coli due presumably to the existence of permeability barriers (Roberts et al., 1955). The behaviour of DAPM auxotrophs of E. coli which show a relative requirement for lysine in addition to their absolute requirement for DAPM has thus been explained by concluding that since "added DAPM does not get into the wild-type cell fast enough to compete effectively with endogenous DAPM, it is not surprising that added DAPM does not get into the mutant cell fast enough to support rapid growth" (Davis, 1955). A similar situation might exist in B. cereus, though the negative results obtained with disintegrated preparations would suggest that impermeability to the substrate might not be the sole cause of the small utilisation.

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Conclusions

1. Incorporation of the N atom into N-heterocyclic compounds is achieved by spontaneous condensation of a free or potential ketonic group with a free amino group, and any precursor of vitamin B_6 might carry these reactive groups.
2. The singly unsaturated ring compounds so formed, can be reduced enzymically to the fully saturated homologues but there is no known instance of their further oxidation.
3. Such evidence as is available concerning the possible cyclisation of DAPM followed by its subsequent oxidation to dipicolinic acid is inconclusive.
4. The only biosynthetic routes to pyridine ring formation known at present, have as starting material, the already aromatic benzene ring, e.g., nicotinic, quinolinic and picolinic acids from 3-hydroxyanthranilic acid.

Ring-opening by oxidation is followed by the expected keto-amine condensative ring closure. Since the aliphatic intermediate has retained its unsaturated character, no further oxidation of the product is necessary.

POSSIBLE EXPERIMENTAL APPROACHES

THE USE OF MICOOORGANISMS IN THE STUDY OF VITAMIN BIOSYNTHESIS

The B-group vitamins are as essential to simpler forms of life as to the more highly organised, and the employment of microorganisms in a study of their biosynthesis has several advantages. It is assumed that the formation of any one vitamin is likely to proceed from the same ultimate precursor and through the same intermediates in very different organisms. Happily, divergences from a common route of biosynthesis are rare, though they have been described; nicotinic acid synthesis by certain moulds and bacteria, for example, proceeds by a route differing from that common to other microorganisms and mammals (Yanofsky, 1954).

The several experimental approaches which are available are summarised below. Most of these have been employed in the present study, and a critical evaluation is left until their actual use is described (see 'Experimental Section' and 'Discussion').

COMPETENT MICROORGANISMS

A. USE OF GROWING ORGANISMS

1. For the biosynthesis of large quantities of vitamin

Microorganisms capable of synthesising a particular vitamin sometimes produce it in excess of their needs. The free vitamin may be liberated by subsequent autolysis, but often the excess is directly excreted into the culture medium (Thompson, 1942). The overproduction of vitamin is in most instances not great, but the occasional organism is found which by some quirk of its metabolism produces astonishingly large amounts.

Once a chemically-defined medium has been developed which will support growth of the selected organism, different substrates and suspected precursors may be provided, conditions of incubation varied, plausible metabolic inhibitors tested in an attempt to influence the vitamin production while maintaining normal rate and extent of growth.

Although to perform such experiments one is not dependent upon organisms synthesising large amounts of vitamin, their use has one important advantage. Vitamins are normally synthesised in such small amounts as compared with the major cell constituents (amino acids, purines and pyrimidines, etc.) that there is some reason to believe

that the provision of a precursor might not unduly enhance their small but adequate production, the compound merely being used for other cell functions of greater quantitative significance. When, however, the vitamin is produced in large amount, then its biosynthesis must represent a major metabolic pathway and is more likely to reflect the presence of supplied precursors.

2. Isotope-labelling

There are at least three methods available by which isotopes may be employed in biosynthetic studies.

a. An unlabelled main carbon source is used together with labelled suspected precursors, when specific incorporation of labelling into the isolated product may take place.

b. The decrease in the degree of labelling of the product is determined when unlabelled suspected precursors are added to a medium containing an indiscriminately labelled, main carbon source, i.e., the isotope dilution technique.

c. A specifically labelled main carbon source is used, the position of the isotope in the product determined and an attempt made to correlate this pattern of incorporation with the utilisation of intermediates produced by recognised metabolic routes.

The isolation of an assayable sample of chemically pure product even when radioactively labelled is frequently difficult when the compound in question is normally formed in only small amount. Here again it is, therefore, desirable to use an organism capable of synthesising large quantities of the desired substance.

3. Use of antimetabolites

(a) In the investigation of routes of biosynthesis

A compound X, capable of interfering specifically with step B ⟶ C in the route of conversion of substrate A to vitamin V, when added in sufficient concentration would inhibit the growth of the organism (unless there is available an alternative route of synthesis which does not proceed via B ⟶ C).

$$A \longrightarrow B \overset{X}{\dashrightarrow} C \longrightarrow D \longrightarrow V$$

The inhibition might be overcome by the addition of V, or of other compounds subsequent to the blocked reaction, i.e., C, D.

The most useful of antimetabolites (Woolley, 1952) are compounds so closely related structurally to the normal substrates as to be able to compete with them for enzymic sites and yet be sufficiently different as to be unable to undergo the required enzyme-catalysed

reactions. Theoretically the inhibition brought about by any concentration of antimetabolite can consequently be overcome by the addition of a proportionately increased concentration of normal substrate. Thus B should overcome the inhibitory action of X competitively, while Y and all members of the reaction sequence subsequent to the locus of inhibition would act noncompetitively.

(b) <u>To achieve increased vitamin synthesis</u>

The accumulation of the substrate of a blocked reaction is frequently accompanied by its excretion into the culture medium. Where the antimetabolite acts on an enzyme for which endogenous vitamin itself is substrate (responsible for its conversion into coenzyme, or its degradation), the vitamin might thus "pile up".

It is evident from the concept of antimetabolite action outlined above, that a sufficiently increased concentration of normal substrate (B) could compete with the inhibitor and so overcome its action. The organism now grows in an otherwise inhibitory concentration of antimetabolite, i.e., it has developed a drug resistance (although this is by no means the only mechanism by which a drug resistance may be developed). There is, of course, a limit to the increased synthesis and therefore to the degree of resistance attained, but there may exist a many-fold difference between the amounts of vitamin produced by

resistant and parent strains.

A compensatory enhanced vitamin synthesis leading to drug resistance is also possible when the drug acts not as an antimetabolite (in the sense described above), but by chemical combination with the vitamin or coenzyme, resulting in its effective loss to the organism.

B. USE OF WASHED SUSPENSIONS OF ORGANISMS

Incubation of washed suspensions of organisms in a chemically defined medium designed to evoke maximum synthesis of the desired compound has the advantage that a greater density of organisms may be used than is ever encountered during growth, with the result that a given amount of product is formed in a correspondingly shorter time. Usually little or no reproduction occurs and the overall conditions are much better defined than at any time during growth.

It may be necessary to use organisms with a subnormal vitamin content if its subsequent synthesis is to be observed (Lascelles and Woods, 1952).

C. USE OF CELL-FREE PREPARATIONS

There is a limit to the utility of intact organisms, set by their impermeability to certain substrates.

It is not to be expected, though it sometimes happens, that disorganised extracts will be able to carry

out syntheses requiring the proper functioning of a number of interdependent reactions. They may be used with profit, however, in the confirmation of suspected reaction sequences, the responsible enzymes being isolated and purified - so enabling the details of their mode of action and cofactor requirements to be better studied. It would be suspicious were it consistently impossible to demonstrate the presence in the organism of an enzyme postulated to be a member of the supposed synthetic pathway.

VITAMIN-DEPENDENT MICROORGANISMS

A. USE OF GROWING ORGANISMS

(1) With natural auxotrophs

Some naturally occurring microorganisms showing a growth requirement for a particular vitamin are alternatively able to utilise one or more of its precursors, and examples of natural symbiosis are sometimes encountered amongst organisms so differing in their ability to synthesise one vitamin. If a number of such organisms is available, it is occasionally possible to arrange their requirements in a sequence (usually of increasing complexity), from which emerges a possible route of vitamin biosynthesis.

(2) With induced auxotrophs

It is preferable to obtain all results where

possible with a single species, and mutant strains of Neurospora early proved of great value. The number of genetically dissimilar mutants requiring the same factor indicated the minimum number of steps in its synthesis, and the frequent accumulation of substrates of blocked reactions facilitated the identification of intermediates.

Auxotrophs of E. coli are now readily isolated by the penicillin-penicillinase technique (Lederberg and Zinder, 1948; Davis, 1948; Adelberg and Myers, 1953) and although genetic analysis of most strains is as yet impossible there are many instances of their great value in biosynthetic studies when they are subjected to nutrilite-replacement and cross-feeding analysis.

The relative concentrations of suspected precursor and product needed to support optimal growth of the mutant organism are of importance. If the precursor is utilised solely for the one biosynthesis, then (questions of permeability and degradation aside), its required concentration should be of the same order of magnitude as the active concentration of the product. The comparison often helps in distinguishing precursors of vitamins and products of their function.

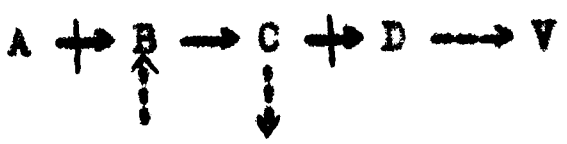
B. USE OF WASHED SUSPENSIONS OF AUXOTROPHS

When fully competent prototrophs are used in suspension to investigate the whole or part of a synthetic

sequence, the effect of adding a suspected precursor is often complicated by major alternative utilisation, degradation, or dilution by endogenously formed precursor. These difficulties can often be circumvented by the judicious use of mutant strains.

Organisms deficient in various factors are readily obtained by growth of suitable mutants on media containing suboptimal concentrations of the factor, and the effect determined of these deficiencies on the synthesis under examination.

Selected portions of a synthetic sequence may be 'isolated' and studied with suspensions of whole organisms by using a mutant blocked immediately before the precursor and immediately after the product of the reaction.



C. USE OF CELL-FREE EXTRACTS

Confirmation of an otherwise diagnosed metabolic lesion is obtained when it can be shown that the suspect enzymic activity, though detectable in cell-free extracts of the parent organism, is absent from similar preparations of the mutant strain.

Caution necessary in the interpretation of results obtained with mutant organisms

Since artificially-produced mutant strains figure large in the work to be reported, it must be stressed at the outset that the behaviour of such organisms may be extremely complex and the results obtained by their use consequently very difficult to interpret.

The assumption is commonly made that, as a consequence of a single gene mutation, the complete failure of a single enzymic reaction always results, evidenced by the development of a nutritional requirement for a single compound addition of which to the culture medium restores wild-type characteristics. In fact, so many and various are the possible effects of a single mutation that it is very probable that no mutant is ever completely restored to the parental biochemical state simply by supplying a growth requirement. There may exist a variety of differences between parent wild-type organism and a mutant formed therefrom as a result of a single irradiation of which the nutritional requirement may appear to be the most significant merely because it is the most obvious (Mitchell, 1953).

GENERAL TECHNIQUES

The usual precautions necessary in microbiological studies were observed throughout, especially with regard to the purity of the chemicals and the cleanliness of the glassware used. Hot chromic acid was the main cleaning agent employed, the glassware being thereafter thoroughly washed with resin-deionised water followed by glass distilled water. Chemicals were of the best quality available. Any special details concerning individual chemicals are given in 'Appendix 1'.

Organisms

Details regarding the origins and chief characteristics of these are given in 'Appendix 3'. Stock cultures were subcultured monthly and stored at 2°. No subculture was employed for longer than one week.

Media

Full details of the composition of all media used are given in 'Appendix 2'. They were usually stored and dispensed at twice the final concentration, being diluted to the desired volume with water and test solutions. To most, a little carbon tetrachloride-toluene mixture (50% of each, v/v) was added as preservative, storage being at 2°.

Conditions of incubation

1. Routine growth tests

(a) Static culture

i. Semi-aerobic:- 2 ml. volumes in 5 x 0.625 in. hard glass tubes, or 5 ml. volumes in 6 x 0.75 in. standing tubes.

ii. Aerobic:- same tubes held in wire baskets sloped at 5° to the horizontal.

(b) Shaken culture

Five ml. volumes of medium contained in optically-matched 6 x 6 x 0.625 in. L-shaped tubes (Monod, Cohen-Bazire and Cohn, 1951) were clamped to a rocking device (similar to that of van Heyningen and Gladstone, 1953) and shaken in a thermostatically-controlled water bath at 36 oscillations per minute, the excursion being 4 in.

2. Growth of larger quantities of organisms

(a) Semi-aerobic

Four hundred ml. volumes incubated in static culture in 1 litre Ehrlenmeyer flasks.

(b) Aerobic

i. Shaken. (a) Fifty ml. culture incubated in large L-shaped tube (horizontal limb dimensions 7 x 1.25 in.). (b) Five hundred ml. volume incubated in 1 litre Ehrlenmeyer flasks shaken with a circular motion at 220

rotations per min. of radius 0.75 in.

ii. Static. One hundred ml. volume incubated in shallow layer in a Roux bottle.

Inocula

Where at all unusual, the procedure for the preparation of the inoculum is described in full. With the majority of organisms used, a suspension was made in sterile water of organisms taken from a 24 hr. culture on solid medium. The density was determined photometrically and serial dilutions in sterile water made until the desired density was achieved. Small inocula were used routinely to minimise the danger of contamination by associated nutrients. In the case of all the E. coli mutants, the inoculum size was adjusted to give approximately 5×10^5 organisms per ml. of final medium.

Assessment of growth

The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Co., Harlow, Essex), using a neutral density filter; the uninoculated medium was used to give the zero setting. The cross arms of the 0.625 in. 1-tubes were constructed to fit the instrument; samples of other cultures were assessed in the 0.25 in. tubes supplied. A calibration

curve relating the readings in the two types of tubes was constructed, and the growth achieved by the E. coli mutants under all conditions of incubation expressed in terms of the EEL readings which would have been obtained in 1-tubes. The relation between 'EEL reading' and dry weight was determined for each organism used and was found to be linear up to a reading of 35 to 40.

Washed suspensions

Organisms were harvested after optimal growth on defined media (unless otherwise stated) and washed twice with 0.1 M phosphate buffer containing 4×10^{-4} M $MgSO_4.7H_2O$ before final suspension in the same buffer supplemented with test substrates. Incubation was with rocking for not more than 8 hr. in 1-tubes of 0.75 in. diameter holding 10 ml. of suspension, at 37°.

When prior 'starvation' of the organisms was desirable, after harvesting and washing, a 5 mg. per ml. suspension in 50 ml. of 0.1 M phosphate and Mg^{++} buffer was rocked in a large 1-tube at 37° for 2 to 3 hr. before re-harvesting and washing.

Sterilisation by filtration

Seitz-filtration under reduced pressure was used for volumes above 5 ml. Smaller volumes were sterilised by filtration through Hemmins filters centrifuged

at 1500 r.p.m. for 30 min.

Ultrasonic disintegration

Washed organisms were suspended in 0.5 M phosphate buffer (pH 7 to 7.4) to a density of approx. 25 mg. per ml. Samples (10 ml.) were subjected to ultrasonic vibration in a 50 ml. stainless steel conical flask. The vibrations (25 kc./sec.) were generated by a magneto-strictor brazed to the base of the flask. The exterior of the flask was cooled with running water so that its contents remained at <20° throughout.

Preparation of boiled extract of organisms

One g. dry wt. of freshly harvested and well-washed organisms were suspended in 6 ml. of water and placed in a boiling water bath for 3 min. After cooling, the residue was spun off at 24,000 g. for 10 min. at 0°. The opalescent supernatant was stored at -15°.

Ultra-violet absorption spectra

These were determined using 1 cm. silica cuvettes in the Unicam Model SP 500 spectrophotometer (Unicam Instruments, Cambridge).

pH measurements

The PYE Universal pH Meter and Millivoltmeter

was used (PYE & Co., Ltd., Cambridge).

Ion-exchange resins

Amberlite IR.50 - This weak cationic (carboxylic) resin was used in rough mesh H^+-form prepared by prior treatment with N-HCl and washing with glass distilled water until the eluate was neutral. This resin is a particularly effective acceptor of divalent cations, and was used to solubilise barium and calcium salts of phosphate esters.

Dowex 50 - A strong cationic (sulphonic) resin used in H^+-form. The resin was dispersed in water and lightly packed into a 10 x 0.75 cm. column. After settling, 2 N-NaCl was passed through until free salt could be detected emerging. Washing was carried out with two column volumes of distilled water and 2 N-HCl passed through until the effluent was of pH 1 to 2. Washing was now continued with glass distilled water until the effluent tested with indicator paper was neutral. The column was kept under water until required.

Dowex 1 - A strong anionic (quaternary ammonium) resin. This was obtained as its hydrochloride of 200-400 mesh. Each batch was prepared as close to use as possible. A thick slurry was made in 10 ml. water, 20 to 30 ml. of 2 N-NaOH added, and the mixture allowed to stand for 10

to 15 min. with occasional shaking. It was exhaustively washed with glass distilled water by alternate suspension and centrifugation until the supernate was of neutral pH.

Paper chromatography

(1) Carbohydrate derivatives

Descending chromatography with n-butanol:glacial acetic acid:water 4:1:5 mixture (organic phase), or with n-butanol containing 1% NH_3.

Spray reagents

(a) Ammoniacal silver nitrate

After drying, papers were sprayed with a 0.1 N solution of $AgNO_3$ in 5 N-NH_4OH and heated at 100° for 5 to 30 min. Addition of 2 N-NaOH to the mixture was made when greatest sensitivity (to sugar alcohols, etc.) was required. Alternatively, papers were dipped in an acetone solution of $AgNO_3$ (0.1 ml. satd. $AgNO_3$ aq. in 40 ml. acetone), allowed to dry, and dipped in a 0.5 N solution of NaOH in ethanol. Background colour was removed with 6 N-NH_4OH and the paper well washed in distilled water before final drying.

Brown-black spots are given with most reducing compounds and the reagent is amongst the most sensitive for these. Although unspecific, it is valuable because

almost every compound on the chromatogram either produces a stain or inhibits background colour.

(b) 3:4-dinitrobenzoic acid

Used as a 1% solution in ethanolic 2 N-Na_2CO_3. This reagent reacts more quickly with ketoses than with aldoses to produce a blue colour indicative of reduction to 2-nitro-4-carboxylic-phenylhydroxamine.

(c) Alkaline triphenyltetrazolium chloride (TTC)

Papers were sprayed with a freshly-made solution of 2% TTC (w/v) in N-NaOH and exposed for 20 min. to a water-saturated atmosphere at 40°. The remaining excess TTC was carefully washed out with water.

Red spots are obtained by reduction to triphenyl-formazan.

(d) Aniline phthalate

Used in 2% (w/v) solution in moist n-butanol. Brownish-red spots obtained with aldoses.

(2) Amino acids

Two main solvent mixtures were used for descending development:- phenol:NH_3:water - 80 g. phenol plus 0.3 ml. of 0.88 NH_3 dissolved in 20 ml. water; n-butanol:acetic acid:water - the organic phase from 4:1:5 mixt. In two-dimensional chromatography the phenol/NH_3 solvent was used first.

Spray reagent - A 0.25% (w/v) solution of

ninhydrin in n-butanol. After spraying, th4 papers were heated 5 min. at 80 to 100°, and the purple spots preserved by dipping in a solution containing 2 ml. satd. $Cu(NO_3)_2$ aq., 0.4 ml. 10% (v/v) nitric acid, and acetone to 100 ml.

(3) Carboxylic acids

The method of Denison and Phares (1952) was used with the ether:acetic acid:water mixture (13:3:1). Before spraying with the alcoholic solution of bromocresol green, the papers were steamed for 10 min. and warm air blown over them from a hair dryer for another 10 min. (temp. kept. <40°) to remove swamping acetic acid.

(4) Dinitrophenyl hydrazones

(a) of oxo-carboxylic acids - ascending development using the solvent mixture of Dagley and Fewster (1952) n-butanol:ethanol:$(NH_4)_2CO_3$ buffer (40:11:19).

(b) of neutral aldehydes and ketones - descending development using the two-phase solvent system of Meigh (1952) in which heptane was the mobile, and methanol the stationary phase.

The yellow spots were easily located visually, but were further characterised by spraying with 2 N-NaOH when colours ranging from brown to blue were obtainable.

Bioautography of vitamin B_6

The method of Winstein and Eigen (1948) was employed. Mixtures containing the three free forms of the vitamin were developed in the dark (descending) with n-butanol/water or n-butanol/acetic acid/water, and well dried. Petri dishes containing Medium A solidified with 3% washed agar (see 'Appendix 2') and thickly seeded (to EEL 0.5) with a washed suspension of Saccharomyces carlsbergensis 4228c were prepared and dried at 37° for 3 hr. Strips of the chromatogram (0.2 in. width) taken from the line of flow of the sample were laid on the surface of the medium and the plates incubated overnight at 30°.

The sensitivity of the method could be reduced by incorporation of 4-deoxypyridoxine into the basal medium (see 'Chapter 31 ').

Assay of tyrosine decarboxylase activity

This was carried manometrically by the method of Gunsalus, Bellamy and Umbreit (1944), using washed suspensions and acetone-dried preparations of Streptococcus faecalis R deficient in vitamin B_6.

Method of performing viable counts

Serial dilutions of the culture in sterile water

were prepared and drops of standard size delivered onto the surface of plates of nutrient medium. The plates were incubated overnight at 30° to limit the size of the colonies and so facilitate their subsequent counting. Four drops of each dilution giving between 5 and 35 colonies per drop were counted and the mean computed. Pasteur pipettes delivering a standard 0.03 ml. drop were used, and sterilised by immersion in boiling water before and after use. The nutrient plates were dried at 37 to 45° before use, so that drops placed on their surface would be absorbed within 30 min.

Production of mutants of Escherichia coli by ultra-violet irradiation

The method used was based on the Adelberg and Myers (1953) modification of the original Davis (1948) and Lederberg and Zinder (1948) penicillin selection technique.

At least three selection plates were prepared on each occasion. Selection of the mutants took 9 days, their characterisation another 5 or 6.

To produce 'log phase' organisms for irradiation, a suspension (EEL 7) was made in sterile water of E. coli 518 taken from a 24 hr. slope culture on Medium S_1. Medium G_1 (20 ml.) contained in a 500 ml. conical flask was inoculated with 0.4 ml. of this suspension and incubated

(standing) at 37° for 14 hr. At the end of this time, the culture (now EEL 12) was removed from the incubator and 5.5 ml. of a 1:500 dilution transferred to a sterile 7 x 1 in. quartz tube. The quartz tube was fitted into a shaking device run at a speed sufficient so to agitate the tube as to spread its contents around the walls in a thin, uniform film. The tube and its contents was exposed to the standard beam of u.v. light - 14 in. from the source for 3.5 min. (conditions found to result in the killing of 99.95% of the organisms). The post-irradiated suspension was immediately placed in ice-water in the dark. Subsequent operations were carried out in dim light to minimise photo-reactivation. The final 'selective' layer consisted of minimal agar fortified with that compound provision of which was to be obligatory for the growth of the required mutant organisms. Thus, when vitamin B_6 auxotrophs were required 6×10^{-6} M pyridoxine was incorporated into this layer; when glycolaldehyde auxotrophs were desired, 3×10^{-3} M was used.

Potential auxotrophs were isolated and screened on plates of defined media followed by tests in liquid media. When large numbers of isolates were to be tested, inocula were prepared in drops of sterile water arranged on the surface of sterile paraffin wax plates. Each plate accommodated 30 to 40 drops.

In the preparation of the tubes of minimal agar

used throughout the above procedure, washed agar was used ('Appendix 2') - best sterilised by separate autoclaving in 0.01 M phosphate buffer pH 7. The glucose was autoclaved separately in solution in glass distilled water and aseptically added after the separate sterilisation of the remaining components of the medium.

Chemical estimations

(a) Glycolaldehyde - The colorimetric method of Dische and Borenfreund (1947) was used. Trichloracetic and glacial acetic acids (AR grade) were employed and the diphenylamine recrystallised twice from 70% (v/v) ethanol before immediate use. The colour developed was accurately measured by reading optical densities at 660 and 580 mμ.

According to the original authors, this method allows of the determination of glycolaldehyde in the presence of a fifty-fold excess of trioses and other aldehydes and sugars. In the present work, no aldehyde or other chemically related compound tested at concentrations within this range was found to interfere appreciably with the assay of glycolaldehyde.

A standard curve relating $D_{660}-D_{580}$ to glycolaldehyde concentration over the range 2×10^{-4} to 10^{-3} M was constructed in each assay. Excellent straight line response was always obtained (Fig. 21b).

(b) Phosphate - The method of Fiske and SubbaRow (1925) was used.

Microbiological assay of vitamin B_6

The studies leading to the eventual adoption of this method are reported in 'Chapter A1'. The final details are given here.

Organism - This was a sub-strain of Saccharomyces carlsbergensis 4228 designated 4228c. It responded approximately equally well to the three free forms of the vitamin produced by previous hydrolytic extraction.

Extraction - The material containing usually 1 to 10 mμ moles vitamin B_6 was diluted to 40 ml. in a 7 x 1.25 in. hard glass tube, acidified with 5 ml. of 0.5 N-H_2SO_4 and autoclaved 3 hr. at 20 lb. per sq.in. Alternatively, 10 ml. samples plus 1.25 ml. 0.5 N-H_2SO_4 could be autoclaved in 6 x 0.75 in. tubes with no loss of accuracy.

After extraction, a sample was brought to pH 5 with a measured volume of N-NaOH (bromocresol green as external indicator). If necessary this extract was clarified by centrifugation.

When using heavily buffered samples (e.g., of growth media), it was necessary to add somewhat more acid to bring the pH down to the required range of 1.6 to 2.0. With most of the growth media used, 40 ml. samples required

the addition of approx. 8 ml. of 0.5 $\frac{N}{m}$-H_2SO_4.

Basal medium - Medium A ('Appendix 2') was modified from that of Atkin, Schultz, Williams and Frey (1943) in that it contained nicotinic acid (2.5 mg./litre) and a reduced concentration of acid-hydrolysed casein (the equivalent of 2 g. casein/litre).

Assay procedure - The samples or appropriate dilutions, at four different concentrations within the assay range, were added in 0.1 to 0.8 ml. quantities to 6 x 0.75 in. tubes containing 1 ml. double strength Medium A. Standards containing 5, 10, 20, 30, 40 and 50 μμmoles pyridoxine were included in each complete assay. After dilution to 1.9 ml. and autoclaving, the tubes were inoculated with 0.1 ml. of a suspension containing about 4 μg. dry wt./ml. of the yeast. This was prepared by suspending organisms harvested from a 24 hr. culture on Medium S_5 in 0.2% saline, centrifuging and resuspending to the required density. Incubation was for 24 hr. at 37° with the tubes sloped at 5° to the horizontal. Growth was assessed immediately the cultures were taken from the incubator.

The useful range of the assay was 5 to 40 μμmoles pyridoxine corresponding to growth of from 0.2 to 1.5 mg. dry weight of yeast per ml. All tubes were set up in duplicate. When pyridoxine was added to samples of the type assayed the recovery was 100% with an overall accuracy of ±10%.

EXPERIMENTAL

SECTION A

THE ASSAY OF VITAMIN B_6

WITH *Saccharomyces carlsbergensis* 4228

All vitamin B_6 assay methods using this organism are based on the original findings of Atkin, Schultz, Williams and Frey (1943). These authors described a thiamine-containing, chemically defined medium (pH 5) on which their strain of *S. carlsbergensis* 4228 would grow only slowly in the absence of added vitamin B_6. When incubated in shaken culture at 30° for 18 hr., excellent growth proportional to vitamin B_6 concentrations below 2×10^{-8} M was obtained. Growth of the organism was still proceeding rapidly at the end of incubation, i.e., the rate of growth and not its final extent, was dependent upon the vitamin concentration. The vitamin B_6 requirement of the organism was examined (Rabinowitz and Snell, 1951) and found to be induced by the presence of thiamine. Not all strains of *S. carlsbergensis* 4228 are as vitamin B_6-dependent as that used by Atkin et al. (1943); growth of the Frohberg strain was only slightly stimulated by pyridoxine (Hopkins and Pennington, 1947).

Although *S. carlsbergensis* 4228 responds

approximately equally well to each of the free forms of vitamin B_6, it cannot utilize the phosphorylated forms. Hence the need for hydrolytic extraction of natural materials (Atkin et al., 1943). Occasionally, pyridoxamine shows less activity than the other two forms, e.g., 70 to 90% of pyridoxine (Rabinowitz and Snell, 1948). This was confirmed by Jones and Morris (1950), who found, however, that if the medium was supplemented with DL-tryptophan (0.1 mg./ml.), pyridoxamine became equivalent in activity to pyridoxine and pyridoxal.

Hopkins and Pennington (1947) found that addition of nicotinic acid accelerated the growth of the organism in the presence of pyridoxine. They also reported that addition of a rather high concentration of $(NH_4)_2HPO_4$ decreased the growth without vitamin B_6 while slightly increasing readings at high pyridoxine levels. Adequate, uniform and continuous agitation was found to be essential to the success of the assay. This view is seemingly shared by most users of the method, though Snell pointed out that the assay could be carried out without shaking with only a slight decrease in the uniformity of the results. To maintain good aerobic conditions he suggested the sloping of the standing culture tubes (Snell, 1950).

The organism has always been grown at 30°, and the need to maintain rigid uniformity of temperature of all assay tubes at the time of incubation and thereafter

has often been emphasised. Various devices have been employed to ensure that growth does not continue whilst its extent is being measured, e.g., steaming, refrigerating, adding chlorothymol or thoral.

Since the basal medium is of a slightly acid pH there is less danger of light destruction of vitamin B_6, though precautions are still recommended.

EXPERIMENTAL

Organism

Three cultures, all reputed to have been derived from the same original strain were obtained from Dr. Rainbow (a); Distillers Company, Ltd. (b); and the Brewing Industry Research Foundation (c). When tested on arrival, all three were found to possess different nutritional properties. Variant (c) was selected for use, as it was found (Hughes, 1954) to be absolutely dependent on vitamin B_6 for rapid growth under all conditions tested.

Possible use in stationary culture

As a first step towards simplifying the assay procedure, the effect was examined of reduction of the culture volume and of incubation in stationary, sloped tubes; 2 ml. amounts of the basal medium described by

TABLE A1-1

Agreement between triplicates when assay carried out in static culture

Pyridoxine final conc. (µg)	Growth in triplicate tubes (EEL reading)		
Nil	0	0	0
2×10^{-9}	4.5	4.5	5.0
5×10^{-9}	9.0	9.25	9.0
10^{-8}	14.0	14.5	14.5
1.5×10^{-8}	20.5	19.5	21.0
2×10^{-8}	25.0	26.0	26.0

2 ml. basal medium of Atkin et al. (1943) in 6 x 0.75 in. tubes supplemented with pyridoxine, autoclaved 10 lb.sq.in. /7 min., cooled, inoculated with 0.4 µg. (dry wt.) of *S. carlsbergensis* 4228c, and incubated at 30° for 40 hr. sloped at 5°.

TABLE A1-2

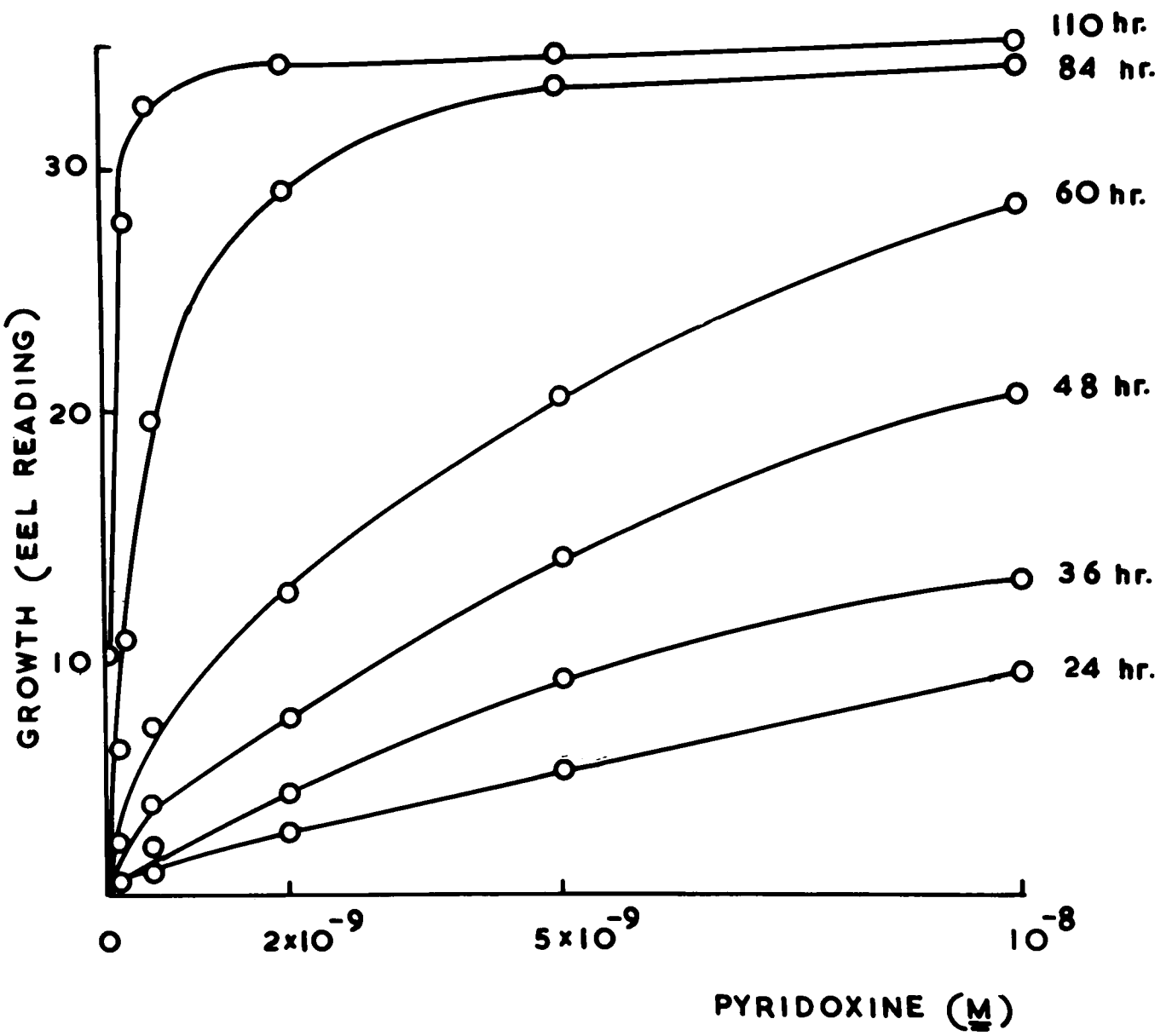
Rapid growth of *S. carlsbergensis* 4228c in shaken culture

Pyridoxine final conc. (mµg)	Growth (EEL readings) Duplicates		Mean
Nil	0	0	0
1	6.0	6.5	6.25
2	12.0	12.0	12.0
3	18.0	17.5	17.75
5	28.0	28.0	28.0
7	36.5	38.0	37.25

5 ml. basal medium of Atkin et al. (1943) in 0.625 in. diameter 1-tubes, supplemented with pyridoxine, autoclaved, inoculated with 1 µg. dry wt. *S. carlsbergensis* 4228c and rocked 16 hr. at 30°.

FIG. 6

Increasing time of incubation



1.9 ml. volumes of basal medium of Atkin et al. (1943), supplemented with pyridoxine as shown, were inoculated with 0.1 ml. of a 1:100 EEL 10 suspension of Saccharomyces carlsbergensis 4228c and incubated in sloped tubes at 30°.

Atkin et al. (1943) and incubation for the rather arbitrary time of 40 hr. at 30° were first used.

Under these conditions excellent agreement between triplicate tubes could be obtained (Table A-1). This was despite the fact that the organisms settled out completely during growth.

Time of incubation

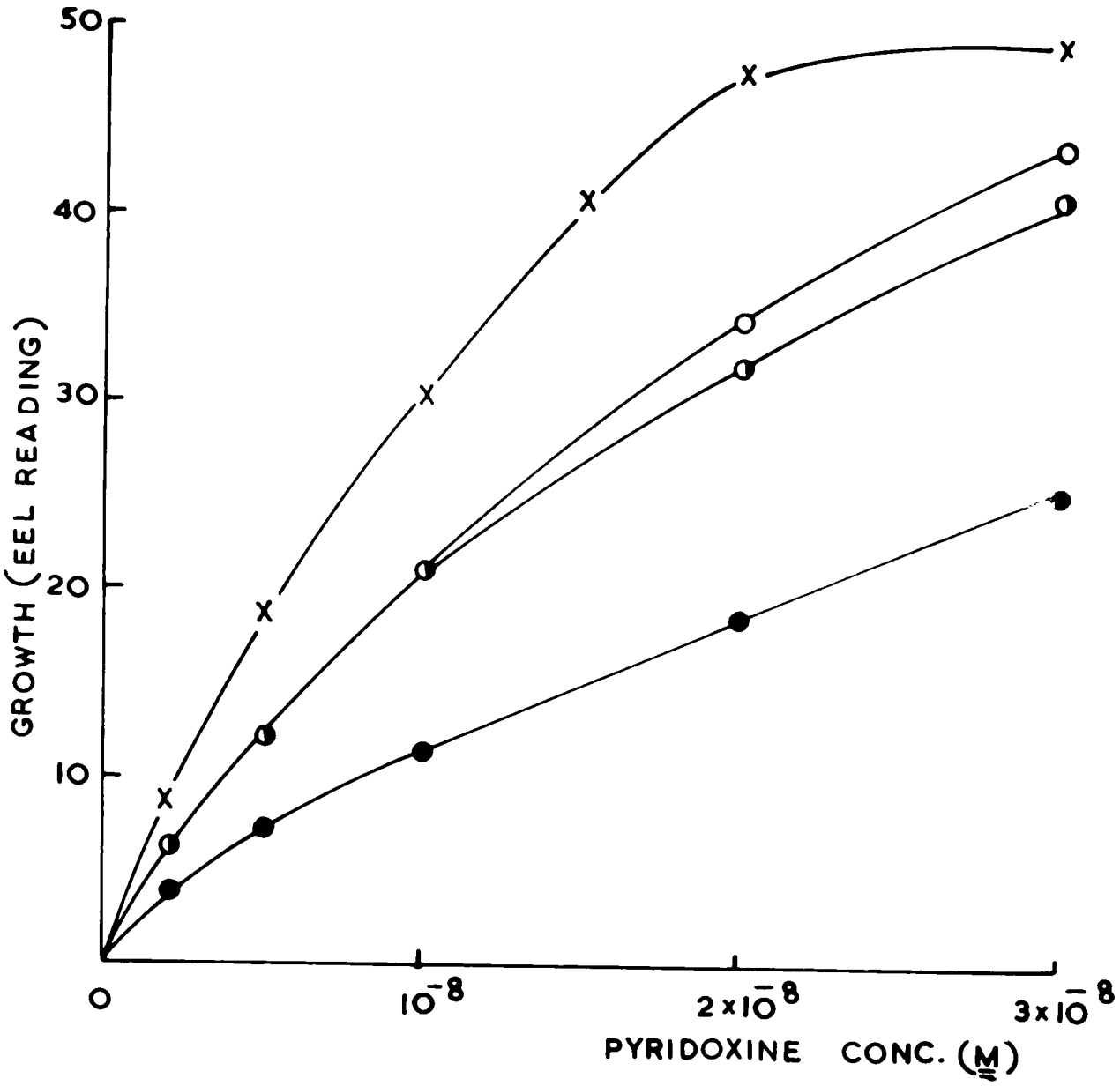
Photometric determination of the extent of growth was so rapid that no measures were necessary to prevent continued growth other than immediate reading on withdrawal from the incubator.

The effect of increasing times of incubation on the growth response of S. carlsbergensis 4228c in static culture, to concentrations of pyridoxine less than 10^{-8} M was determined (Fig. 6). The extent of growth obtained at any one concentration of vitamin increased with increasing time of incubation. Fairly good growth of the organism in the absence of added vitamin B_6 was obtained by incubation for 100 hr. or longer.

To obtain reproducible standard dose-response curves strict adherence to a selected time of incubation was essential. Even so, certain unavoidable fluctuations in the response curve were produced which made imperative the construction of a new standard curve in the course of each assay.

FIG. 7

Effect of increasing aeration



Key: ●—● = vertical tubes. o—o = 20% increased surface area.
◑—◑ = normal slope (5°). x—x = conical flasks.

1.9 ml. volumes of basal medium of Atkin et al. (1942), supplemented with pyridoxine as shown, were inoculated with 0.1 ml. of a 1:100 EET 10 suspension of S. carlsbergensis 4228c (once washed in 0.2% sterile saline), and incubated at 30° for 40 hr. in variously sloped tubes or 25 ml. conical flasks.

Degree of aeration

Good aeration is essential for the rapid growth of the organism (Atkin et al., 1943; Snell, 1950) and most rapid growth was indeed achieved by constant agitation of the culture (compare Tables A-1 and A-2).

If the increased rate of growth under these conditions was a function only of the increased aeration and was not due to the dispersal of the organisms, then it was to be expected that relatively small changes in the degree of aeration might be reflected in the growth response curve. When the assay was carried out in sloped tubes, such changes would result from alteration in the degree of sloping of the tubes (Fig. 7).

An identical degree of sloping on each occasion of assay was ensured by employing standard wire baskets sloped at 5° to the horizontal.

Temperature of incubation

S. carlsbergensis 4228c was found to grow much more rapidly at 37° than at 30°, and incubation for 24 hr. at the higher temperature gave a steeper and more linear response over the range of pyridoxine concentrations used (Fig. 8a).

Further work was carried out at 37° though

A. Effect of [illegible] temperature of incubation (24 hr.)

B. Increasing time of incubation at 37°

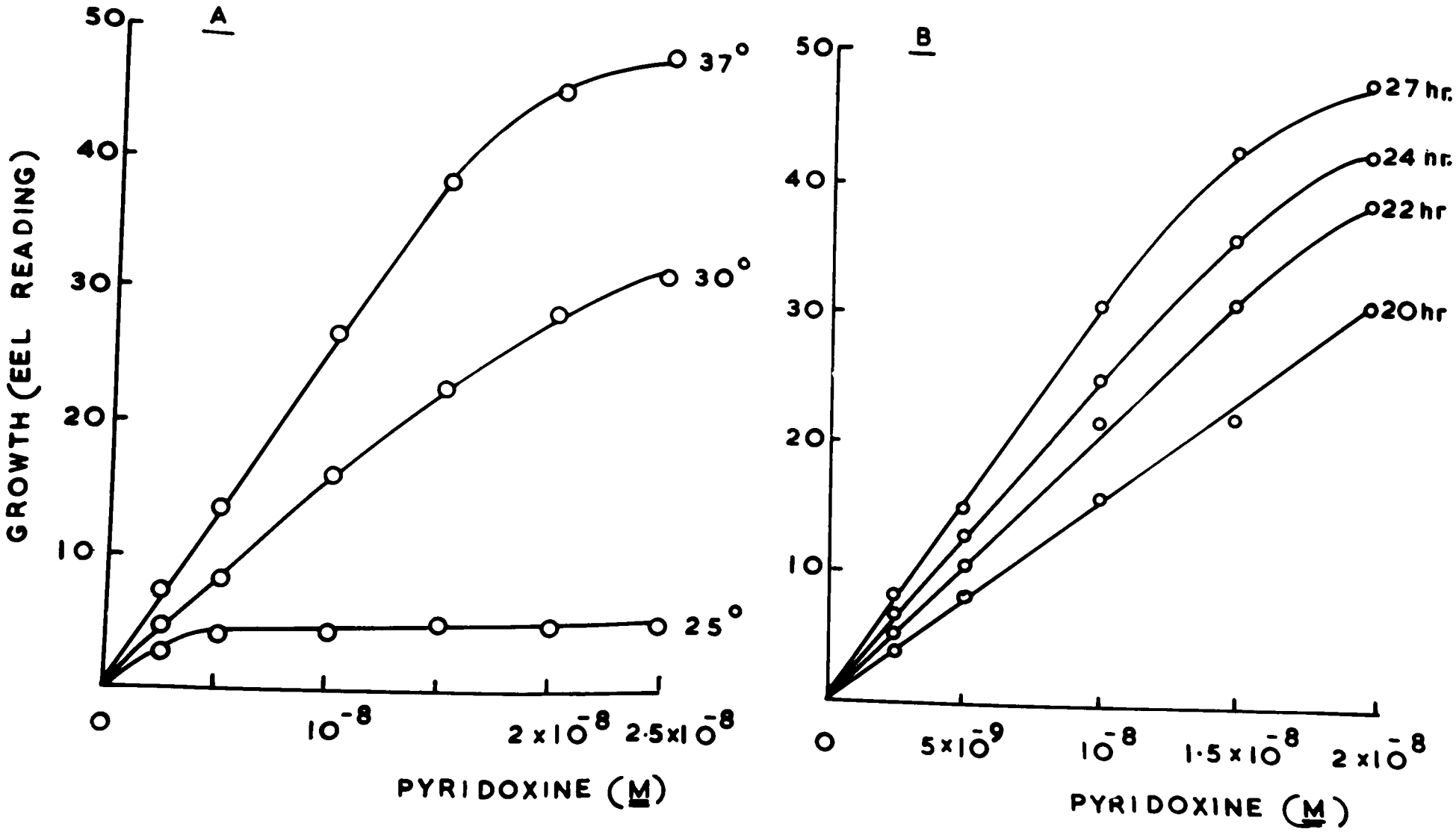


FIG. 8

1.9 ml. volumes - Medium A supplemented with pyridoxine as shown were inoculated with a washed suspension of E. carlsbergensis 4228c (0.4 mg. dry wt.) and incubated under the conditions shown in sloped tubes (50° to horizontal).

relatively small changes in incubation time now had a marked effect on the shape of the standard curve (Fig. 8b).

Maintenance of a uniform incubation temperature was particularly important when the assay was carried out at 37° for temperatures only slightly higher than this were markedly inhibitory to the growth of the organism (Mulder, 1958). The results here reported were obtained using an incubator whose temperature fluctuated between 36.4 and 37°.

Preparation of inoculum

From the outset, to diminish the transference of vitamin B_6 from the complex medium on which the inoculum was grown, a smaller inoculum size was used than had ever previously been recommended.

The organisms were taken from 24 hr. 30° slope cultures on Medium S_5. Such organisms behaved no differently during subsequent incubation in assay medium A, from those harvested after 24 hr. growth at 37° on Medium A supplemented with 10^{-7} M pyridoxine, i.e., inoculum development.

To reduce contamination still further, the harvested organisms were washed by centrifugation from 0.2% sterile saline (isotonic - Ingram, 1955), before suspension in fresh saline to a final concentration of

about 4 µg. dry wt./ml. This concentration was chosen as it represented a dilution of 1:100 of that giving a reading of 10 on a standardised EEL photoelectric colorimeter.

This suspension (0.1 ml.) was used to inoculate 1.9 ml. of medium plus supplements. With this reduced inoculum size, no growth was obtained in the absence of added vitamin B_6 under the conditions of incubation used.

Variation in inoculum size caused a variation in the growth level attained in the standard incubation period upon a particular concentration of pyridoxine - yet another consequence of its being a rate assay. The inoculum size was another factor, therefore, which was kept strictly standardised to ensure reproducible results.

Modifications of the basal medium

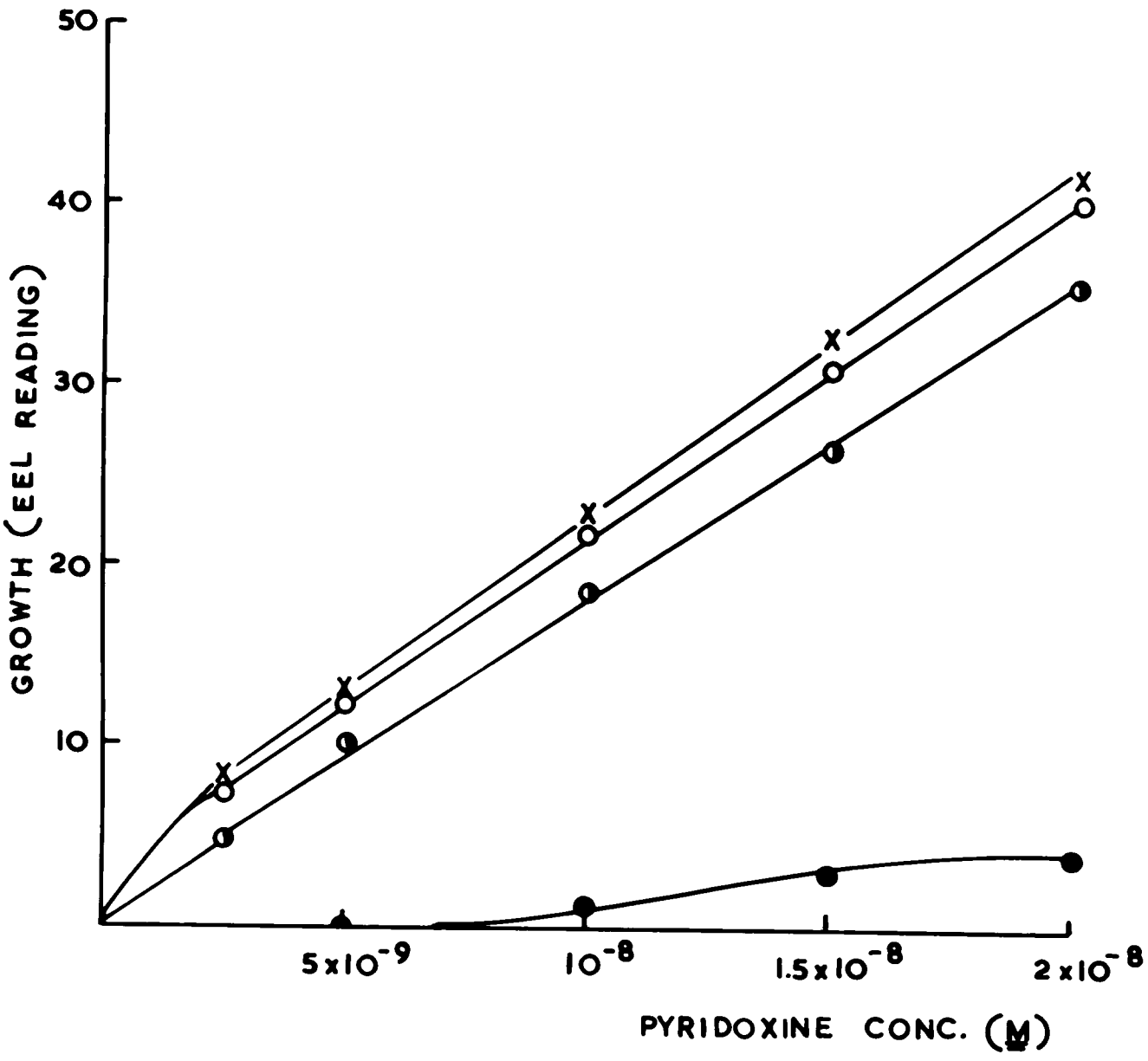
The better growth of strain 4228c in a medium containing less casein hydrolysate than recommended by Atkin et al. (1943) (Hughes, 1954) was confirmed, and thereafter an amount equivalent to 2 g. casein per litre of medium was used.

The addition of $(NH_4)_2HPO_4$ in the concentration (4 mg./ml.) recommended by Hopkins and Pennington (1947) upset the buffering of the medium, the pH rising from 5 to 6.7 with consequent caramelisation on autoclaving.

Nicotinic acid slightly stimulated the growth of the organism (Fig. 9) and was incorporated into the basal

FIG. 9

Effect of nicotinic acid and thiamine on the response of S. carlsbergensis 4228c to pyridoxine



Key: ●—● = minus thiamine
o—o = 7.5 x 10^{-7} M thiamine (usual Medium A)
x--x = 5.0 x 10^{-5} M thiamine
●--● = minus nicotinic acid

1.9 ml. volumes of Medium A minus thiamine and nicotinic acid were supplemented with these vitamins and pyridoxine as shown, and inoculated with 0.1 ml. of a washed suspension (4 µg.dry wt./ml.) of S. carlsbergensis 4228c. Incubation was at 37° for 24 hr. in sloped tubes.

medium in relatively high concentration (2×10^{-5} M).

Effect of thiamine

Rabinowitz and Snell (1948) reported that growth of their strain of S. carlsbergensis 4228 was not dependent upon added vitamin B_6 in the absence of added thiamine. In very small concentrations, thiamine caused a growth inhibition which was overcome non-competitively but in a strictly quantitative fashion, by pyridoxine. When the inoculum was grown in a thiamine-rich medium, sufficient was carried over endogenously to necessitate the provision of vitamin B_6 if growth were to be obtained even in a thiamine-free medium. Use of the organism for the assay of vitamin B_6 was therefore only possible in the concurrent presence of thiamine.

Strain 4228c showed an entirely different thiamine-vitamin B_6 relationship.

In the absence of both vitamins, or of vitamin B_6 only, no growth of the yeast was obtained in 24 hr. incubation at 37° (Fig. 9). In the absence of added thiamine, ten times the concentration of pyridoxine was required to support maximal growth than when the normal basal medium recommended by Atkin et al. (1943) was used (containing 7.5×10^{-7} M thiamine). Increasing the concentration much above this value had little stimulatory

TABLE A1-3

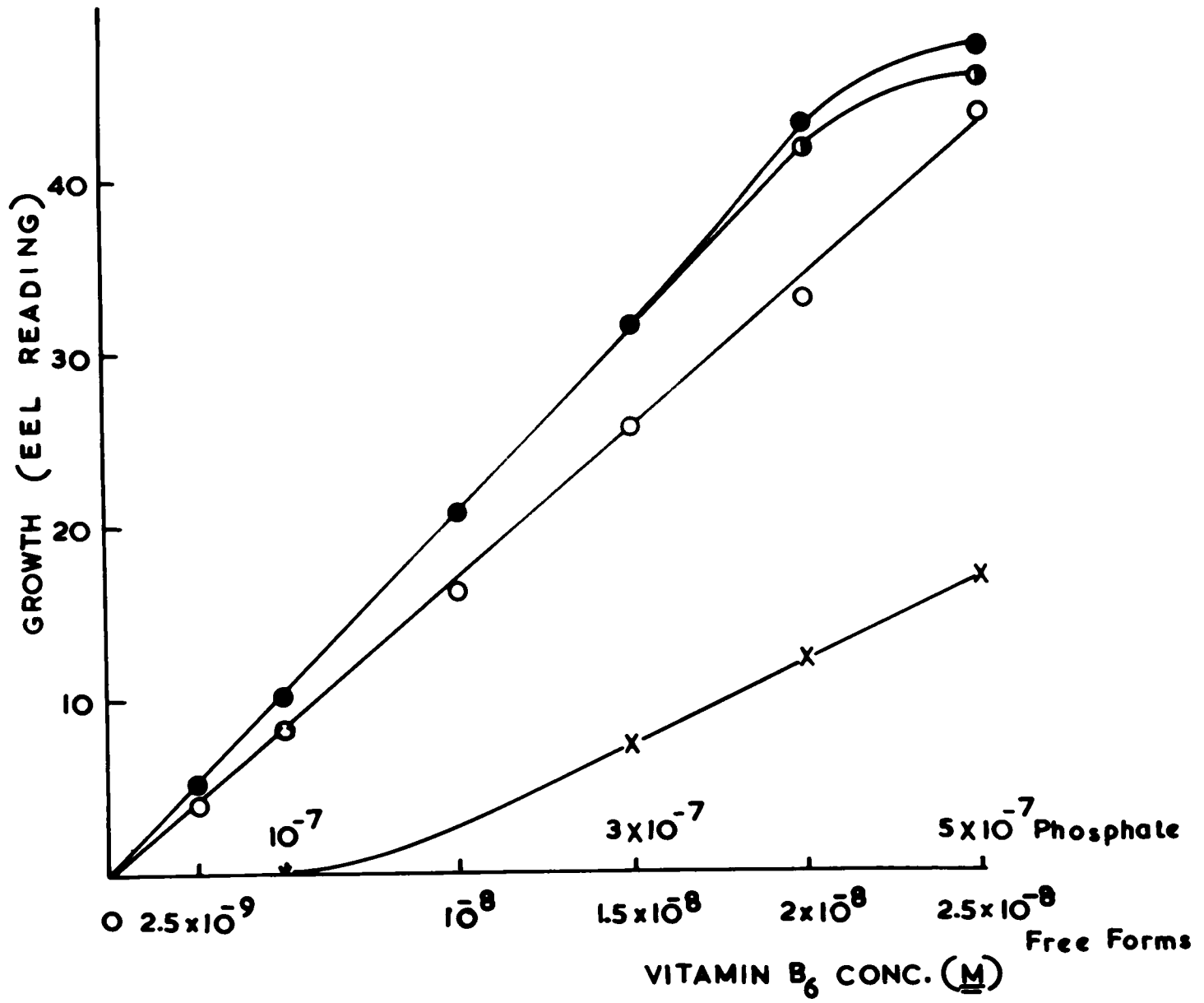
Growth response of S. carlsbergensis 4228c to various forms of vitamin B_6

Form of vitamin B_6	% growth promoting ability
Pyridoxine	100
Pyridoxal	100
Pyridoxamine	80 to 90
Pyridoxal phosphate	2
Pyridoxamine phosphate	<0.4

Determined by their ability to support growth of the yeast on medium A in static culture incubated 24 hr. at 37°.

FIG. 10

Response of S. carlsbergensis 4228c to various forms of vitamin B_6



Key: ●—● = pyridoxine

◑—◑ = pyridoxal

o—o = pyridoxamine

x--x = pyridoxal-5'-phosphate

1.9 ml. volumes of Medium A supplemented after autoclaving with Seitz-filtered solutions of the compounds shown, were inoculated with 0.1 ml. of a washed suspension (4 µg. dry wt./ml.) of S. carlsbergensis 4228c and incubated in sloped tubes at 37° for 24 hr.

effect (Fig. 9), so that the presence of even considerable concentrations of thiamine in natural extracts will not invalidate the assay. Similar results were obtained when the yeast was incubated at 30° as in the experiments of Rabinowitz and Snell (1948).

Response to various forms of vitamin B_6

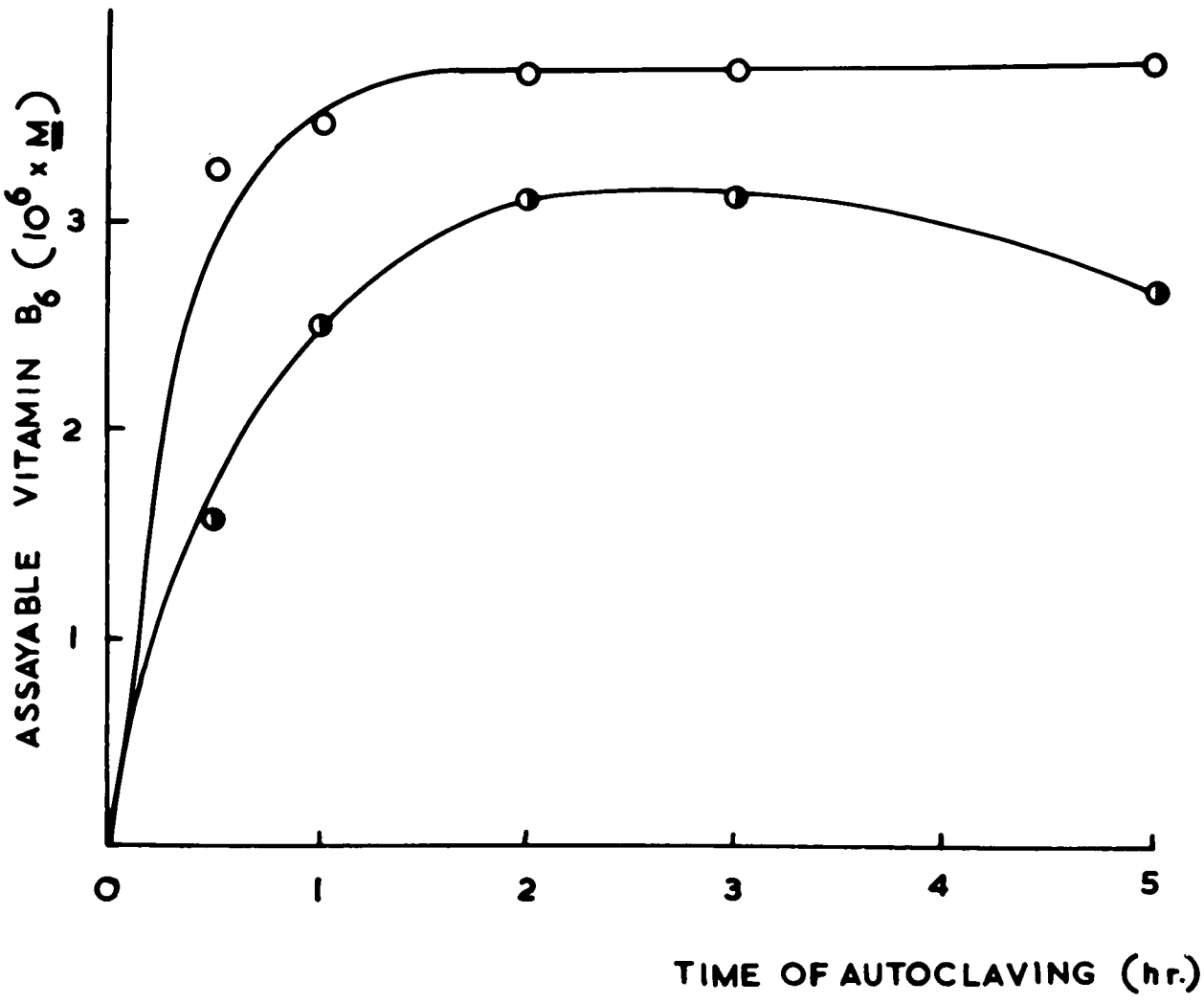
Pyridoxal was just as effective as pyridoxine in promoting the growth of strain 4228c on Medium A. Pyridoxamine appeared to be somewhat less active but it is uncertain to what extent this was merely due to decomposition of the available sample of pyridoxamine (Fig. 10 and Table A1-j). The addition of DL-tryptophan at a final concentration of 0.1 mg. per ml. had no effect on the response of the organism to pyridoxamine itself or to an extract of natural material (*E. coli* 518) known from bioautography to contain pyridoxamine.

Extraction procedure

When sufficient material was available, 45 ml. of extractant was used, as recommended by Hughes (1954). When necessary, 12 ml. volumes were similarly extracted with apparently no loss of accuracy. After autoclaving, the extract was brought to pH 5 with a measured volume of N-NaOH, centrifuged to clarify and usually assayed

FIG. 11

Hydrolysis of phosphates of pyridoxal and pyridoxamine by autoclaving 20 lb.sq.in. in 0.05 N-H_2SO_4



Key: o—o = pyridoxal phosphate

●—● = pyridoxamine phosphate

The compounds were dissolved in 40 ml. water and 5 ml. of 0.5 $\underline{N}$-H_2SO_4 added (final conc. of phosphates about 4 x 10^{-6} $\underline{M}$). After autoclaving for the times shown, the extracts were cooled, brought to pH 5 and immediately assayed with S. carlsbergensis 4228c. The sample of pyridoxamine phosphate used was obviously impure.

immediately. If kept at 2^{o} for any length of time before assay, the extracts were exposed as little as possible to light.

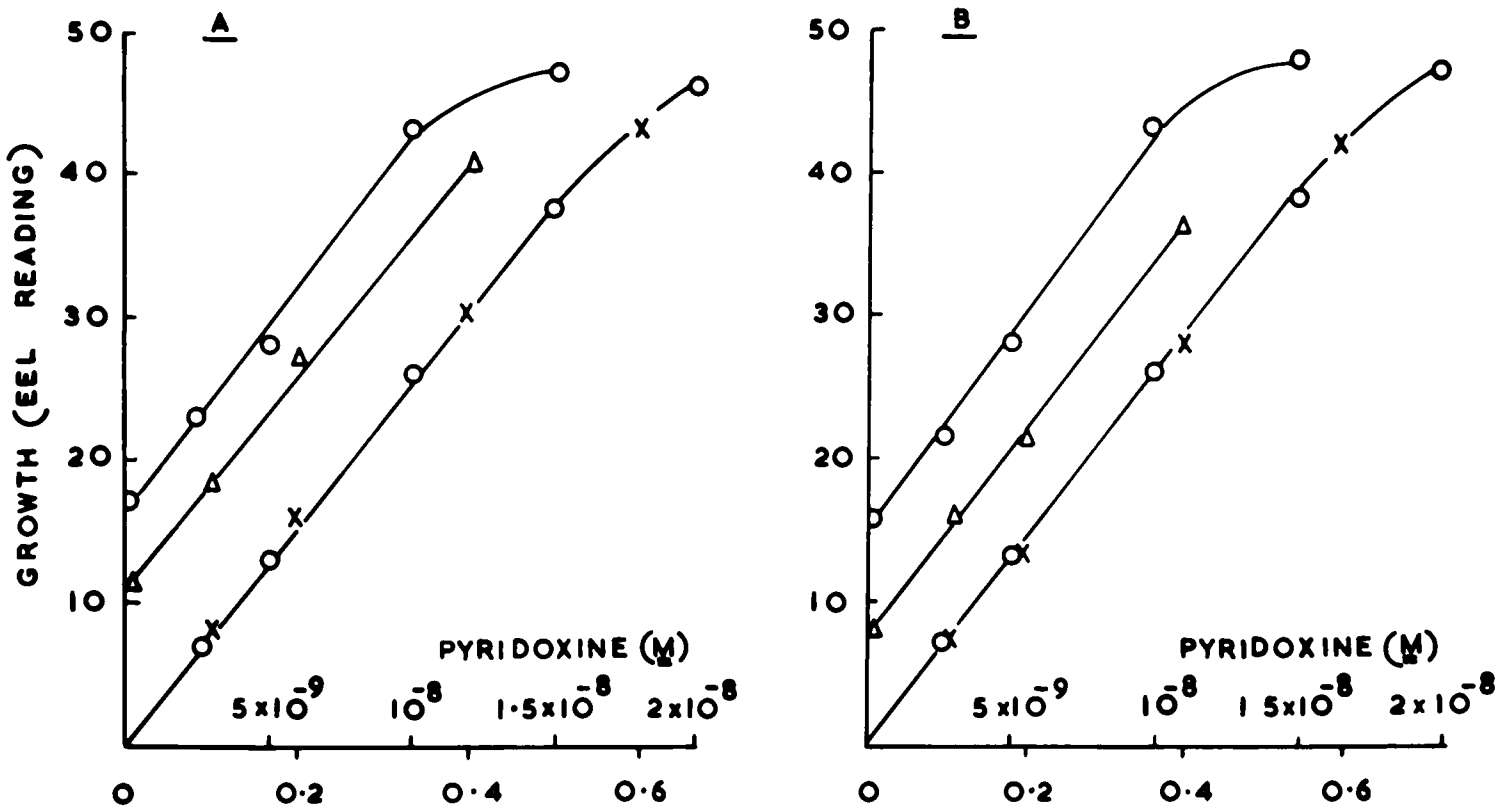
Hydrolysis of samples of pyridoxal phosphate and pyridoxamine phosphate was complete after 2 hr. autoclaving in 45 ml. volumes of 0.055 $\underline{N}$-H_2SO_4 at 20 lb.sq.in. (Fig. 11). With all samples of bacteria and growth media used, maximal extraction was always achieved by 2 to 3 hr. autoclaving, and 3 hr. at 20 lb.sq.in. was used routinely.

Assay of bacterial extracts

To discover whether any constituent of prepared extracts of various bacterial cultures would interfere with the assay of vitamin B_6 by stimulating or inhibiting growth of strain 4228c, experiments were performed to see whether the dose-response curves relating concentration of extract and resultant growth of the yeast were superimposable upon the standard curve obtained with pure solutions of pyridoxine. Similarly, the response curves obtained when a known concentration of pyridoxine was added to various concentrations of extract, and conversely when a small amount of extract was added to each of the standard tubes containing known concentrations of pyridoxine, were determined, and percentage recovery of the added material (in terms of pyridoxine content), calculated. Optimally,

FIG. 12

Assay of vitamin B_6 in extracts of A. Proteus morganii 231
B. E. coli B166



Key: ●—● = standard curve
x—x = extract
Δ—Δ = extract plus 4 x 10^{-9} M pyridoxine
o—o = pyridoxine plus aliquot of extract

Usual assay procedure followed with S. carlsbergensis 4228c, i.e., with Medium A and incubation for 24 hr. at 37°.

such control experiments should be carried out with each extract to be assayed.

Fig. 12 shows the results obtained with extracts of (a) a culture of Proteus morganii 231, and (b) a culture of E. coli B166. On no occasion when such a recovery experiment was performed were erroneous results obtained. The extract assay curves were always approximately superimposable upon the standard curve.

Accuracy

The assay procedure developed as a result of all these observations is described in 'General Techniques'.

The recovery of pyridoxine added to extracts was 100%, with an overall accuracy of ±10%.

When the results obtained by this method, but in another laboratory, were subjected to statistical analysis, the S.D. was found to be 8.2% (Powell, 1958).

Conclusions

1. The strain of Saccharomyces carlsbergensis 4228 used, differed from that of Rabinowitz and Snell (1948) in requiring thiamine for rapid growth in the presence of low concentrations of vitamin B_6, i.e., thiamine seemed to have a sparing effect on vitamin B_6 requirement; it was therefore added in excess to the basal medium.

2. A very reproducible growth response to vitamin B_6 concentrations in the convenient range of 2×10^{-9} to 2×10^{-8} M was obtainable in static culture.
3. S. carlsbergensis 4228c grew faster at 37 than at 30°.
4. The method finally used was a 'rate assay', and great care was exercised in maintaining standard time of incubation, temperature, degree of aeration and inoculum size.
5. As a corollary to (4), within limits, the sensitivity of the assay could be adjusted by varying the time of incubation.
6. 'Bound' vitamin B_6 was best released (from bacteria) by autoclaving in 45 ml. 0.055 N-H_2SO_4 at 20 lb.sq.in. for 3 hr.
7. No evidence was obtained of interference by any tested extract with the response of the assay organism to vitamin B_6.

SECTION B

THE SYNTHESIS OF VITAMIN B_6 BY COMPETENT ORGANISMS

Chapter 1

Search for microorganisms capable of synthesising large amounts of vitamin B_6

Routine examination of identified organisms

There have been made surprisingly few quantitative determinations of vitamin B_6 biosynthesis by microorganisms and many of those employed methods whose validity would be questioned now that the existence of more than one form of the vitamin has been established. All published figures for vitamin B_6 synthesis by moulds, yeasts and bacteria, are disappointingly small (e.g., Table B1-1).

In conjunction with other studies, the amount of vitamin B_6 synthesised by certain bacteria was estimated. To a large extent, choice of bacteria to be examined was made at random. Two criteria were used, viz., capability of growth, (a) in the absence of added vitamin B_6, and (b) on a chemically-defined medium. The media used were made as simple as was compatible with good growth, on the assumption that the presence of unnecessary supplements such as amino acids might possibly spare vitamin synthesis.

TABLE B1-1

Amount of vitamin B_6 synthesised by various bacteria - from Thompson (1942)

Organism	Vitamin B6 synthesised (as mμmoles/g. dry wt. cells)		
	Cells	Medium	Total
Aerobacter aerogenes (aerobic)	40	118	158
Aerobacter aerogenes (anaerobic)	106	<30	106-146
Serratia marcescens	65	135	200
Pseudomonas fluorescens	33	410	443
Proteus vulgaris	40	56	96
Clostridium butylicum	36	100	136

All organisms were grown on a glucose-casein-salts medium incubated for 24 hr. at 33°.

Vitamin B_6 was extracted by takadiastase and papain treatment at pH 4.7 (24 hr. at 37°), then assayed microbiologically with *Saccharomyces cerevisiae* GM.

Special attention was given to certain soil bacteria of the genera Agrobacterium, Erwinia, Azotobacter, Pseudomonas, as these had been implicated in the synthesis of vitamin B_6 in the rhizosphere (Shavlovskii, 1954). Also examined were the aerobic spore-forming Bacilli noted for their ability to synthesise dipicolinic acid. The other bacteria listed were examined as they became available (Table B1-2).

The organisms were grown on simple chemically-defined media under approximately optimal conditions of temperature, pH, aeration, until maximal growth was obtained. The extent of growth was measured, the organisms harvested and washed, and a sample, together with a quantity of the residual culture medium, extracted with acid and assayed for vitamin B_6. Although it was not necessary that organisms and residual medium be assayed separately, it was of some interest to do so, since Thompson (1942) had postulated that the vitamin B_6 contents of all organisms are very nearly identical. Thus, any gross variation in vitamin B_6 synthesis should be manifested by an increased excretion of the vitamin into the growth medium.

Table B1-2 lists the results obtained with some of the organisms examined. To facilitate comparison, the amount of vitamin is expressed in terms of mμmoles of

TABLE [illegible]

Amount of vitamin B_6 synthesised by various bacteria when grown on simple, chemically-defined media

Organism	Strain	Vitamin B_6 synthesis (mμmoles/g. dry wt.) Cells	Medium	Total	Basal medium	Growth temp.
Aerobacter aerogenes	64	[illegible]	13	292	G_2	37°
	71	178	80	258	G_2	37°
Acetobacter suboxydans	621	44	200	244		
Agrobacterium radiobacter	36	53	200	253	G_2	25°
Agrobacterium tumefaciens	A_1	73	150	223	G_2	25°
Alcaligenes faecalis	16	128	506	634	L plus asparagine	37°
Azotobacter sp.	RT	160	270	430	C + NH_4	30°
Azotobacter vinelandii		44	130	174	C + NH_4	30°
				165	C	
Bacillus megatherium		8	877	885	F	37°
Bacillus subtilis	RT	440	480	920	F	37°
	Pope	232	375	607	F	37°
	Porton	54	570	624	F	37°
	6346	85	390	475	F	37°
Corynebacterium erythrogenes	A1062	169	38	207	J	25°
Erwinia carotovora		180	174	354	G_2 plus asparagine	25°
Mycobacterium phlei	Grotta.	72	135	207	G_2 plus asparagine	25°
Proteus mirabilis				75	E	37°
Proteus morganii	231	277	1700	1977	E	37°
Proteus vulgaris	R	345	164	509	L plus nicotinate	37°
	X19	275	370	645	L plus nicotinate	37°
Pseudomonas sp.	Sc/30	114	303	417	G_2 plus asparagine	30°
Pseudomonas fluorescens	6251	289	28	317	L plus asparagine	30°
	950	25	290	315	D	30°
		125	226	351	L plus asparagine	30°
Rhodopseudomonas capsulatus	Oxford	404	700	1104	K	Anaerobic in light 30°
		355	462	817	K plus casein	Anaerobic in light 30°
Sarcina lutea	PCI.1001	150	65	215	I	30°
Vibrio sp.	O_1	105	170	275	L	30°
Chlorella pyrenoidosa		48	40	88	H	Aerobic in light 25°

pyridoxine per g. dry wt. of organism.

The amount of vitamin B_6 synthesised by all these bacteria was uniformly small. Very few bettered the synthesis obtained during growth of wild-type E. coli on Medium G_2. Of these, Proteus morganii was outstanding, but even this gave only a 4- to 5-fold better synthesis than E. coli. The ratio of vitamin B_6 present in the organism and in its culture medium varied with the organism and with the age of the culture, though there seemed to be a tendency towards excretion as growth entered upon its stationary phase.

Comparatively low values have been obtained for vitamin B_6 synthesis by all yeast strains tested in this laboratory (Hughes, 1954; Mulder, 1958). A variety of fungi both identified and unidentified have also only synthesised the vitamin in disappointingly low yield when grown on chemically-defined media (Mulder, 1958).

Isolation from soil of organisms synthesising vitamin B_6

A cross-feeding technique was developed to facilitate the screening of microorganisms with respect to their vitamin B_6 synthetic abilities, and the possible isolation of an organism capable of synthesising the vitamin in large amount. Three basal minimal media and two vitamin B_6-requiring organisms (which would respond

TABLE B1-3

Indicator organisms and basal media used for plate 'screening'

Organism	Basal medium
Saccharomyces carlsbergensis 4228c	A (pH 5.0)
Escherichia coli M_2	L (pH 7.4)
	or, (L plus acid-hydrolysed casein and mixture V)
	C_2 (pH 7.0)
	or, (C_2 plus acid-hydrolysed casein plus mixture V)

to the vitamin in any of its free forms) were selected for this purpose (Table B1-3).

Double strength basal media (10 ml. amounts) were diluted with equal volumes of water, 2.5% w/v washed agar added, and sterilised by autoclaving at 10 lb.sq.in./ 7 min. If basal media of pH 7 contained glucose, this was autoclaved separately in aqueous solution and added aseptically. The autoclaved media were cooled to 45° and a washed suspension of indicator organism added to give a final density of EEL 0.5 before plating and drying at 37° for 3 hr.

To test the vitamin B_6 synthetic ability of a particular organism X, it was streaked on the surface of such a plate which was then incubated at a suitable temperature. The appearance of opalescent haloes around colonies of X represented the utilisation by satellite colonies of the indicator organism of free forms of vitamin B_6 excreted during the growth of X. The radius of satellite growth was proportional to the amount of the vitamin excreted and hence approximately to the total quantity synthesised by X.

Zones of satellite growth also developed around surface colonies resulting from the incubation of similar plates streaked with suitable dilutions of soil washings. Incubation was for several days at 25, 30 or 37°. Many soil samples from very varied localities were examined

and a large number of organisms isolated which cross-fed the indicator organisms. The pH 5 basal medium A, especially at lower temperatures, supported the growth of yeasts and moulds in large numbers besides the bacteria which also developed in abundance on the other media. To isolate selected surface colonies, they were transferred to slopes of the basal medium lacking vitamin B_6 and incubated at their optimal growth temperature. Single colonies were then selected after streaking on plates of minimal medium solidified with washed agar.

All isolated organisms when subsequently grown in liquid culture in Roux bottles at their optimal growth temperatures synthesised less than 500 mμmoles vitamin B_6 per g. dry wt. of organism.

It was demonstrated by Rabinowitz and Snell (1953), and confirmed in the present study, that 4-deoxypyridoxine competitively inhibits the growth of some organisms which are dependent on added vitamin B_6, but does not affect those that can synthesise their own requirements of the vitamin. It was thought that judicious incorporation of 4-deoxypyridoxine into the basal media of the selection plates might decrease the sensitivity of the assay, i.e., a higher concentration of vitamin B_6 would now be required to support the same growth of the indicator organism. Within certain limits the degree of sensitivity

of the response could be adjusted by selecting the concentration of antimetabolite to be used. This was found to work well when pure solutions of pyridoxine were assayed by the cup-plate method. The background growth which normally appeared after incubation of the S. carlsbergensis 4228c plates for longer than 40 hr. was concurrently depressed. However, when 10^{-5} to 10^{-4} M 4-deoxypyridoxine was used, i.e., sufficient to inhibit the satellite growth around colonies of organisms synthesising about 500 mμmoles vitamin B_6/g. dry wt., no colony developing after streaking samples of soil washings, ever gave indication of significant vitamin B_6 excretion.

Thus, no organism was isolated from soil which was better able than Escherichia coli to synthesise vitamin B_6.

Attempts to enhance the vitamin B_6 biosynthesis of competent microorganisms

(1) Isoniazid

If high concentrations of isoniazid were bacteriostatic in consequence of its ability to combine with pyridoxal and so render it unavailable to the organism ('Introduction', Chapter 1), then one means by which isoniazid resistance could be developed would be by

TABLE B1-4

Isoniazid inhibition of growth of wild-type E. coli, and its reversal by pyridoxine

(a) Growth inhibition by isoniazid

Isoniazid conc. (M)	Growth (EEL readings)
0	26
10^{-3}	26
4×10^{-3}	24
6×10^{-3}	16
10^{-2}	Trace

(b) Reversal of inhibition by pyridoxine

10^{-2} M isoniazid used throughout.

Pyridoxine conc. (M)	Growth (EEL reading)
0	Trace
10^{-6}	Trace
10^{-5}	2
3×10^{-5}	11
10^{-4}	23
3×10^{-4}	26

Isoniazid was added aseptically as a Seitz-filtered solution.

5 ml. amounts of Medium G_2 supplemented as shown and inoculated with E. coli 518 incubated in sloped tubes 36 hr. at 37°.

enhanced synthesis of the vitamin.

In confirmation of the findings of Boone and Woodward (1953) growth of wild-type E. coli 518 in Medium G_2 was progressively inhibited by increasing isoniazid concentrations above 10^{-3} M. This inhibition was countered by the concurrent addition of relatively high concentrations of pyridoxine (Table B1-4).

On prolonged incubation of E. coli 518 in the presence of 10^{-2} M isoniazid and the absence of vitamin B_6 good growth was eventually obtained. The organism was passaged six times through Medium G_2 containing 10^{-2} M isoniazid. The delay in the onset of growth decreased on each subcultivation until eventually growth commenced after only 16 hr. incubation at 37^{o}. A single colony of this isoniazid-resistant strain was isolated by streaking on a plate of solidified vitamin B_6-free medium supplemented with 10^{-2} M isoniazid.

The amount of vitamin B_6 synthesised by this resistant strain when grown in the presence of 10^{-2} M isoniazid was double that of the parent non-resistant strain, and of the same strain when grown in the absence of the antimetabolite (Table B1-5).

The enhancement of vitamin B_6 biosynthesis so obtained was considered too small to justify the further use of this method, since objection could be taken to the

TABLE B1-5

Synthesis of vitamin B_6 by an isoniazid-resistant strain of Escherichia coli

Organism	Medium	Growth (EEL reading)	Vitamin B_6 synthesis (mμmoles/mg. dry wt.)		
			Cells	Medium	Total
Parent wild-type *E. coli* 518	G_2	40	165	290	455
Isoniazid-resistant substrain of *E. coli* 518	G_2	38	185	325	510
	G_2 plus 10^{-2} M INH	34	130	945	1075

Organisms grown in shaken culture 36 hr. at 37°.

Pyridoxine recovery from medium originally containing 10^{-2} M isoniazid was 100%.

use of an antimetabolite, on the grounds that it might otherwise interfere with the metabolism of the organism. Especially might objection be taken to isoniazid whose mode of action is after all unknown ('Introduction', Chapter 1).

Addition of 10^{-2} M isoniazid caused virtually no increase in the extent of vitamin B_6 synthesis from glucose plus NH_4^+ by aerobic, washed suspensions of Proteus morganii 231.

(2) EDTA (ethylenediaminetetra acetic acid, tetrasodium salt).

As pyridoxal phosphate probably chelates with a metal ion to form the active substrate-metal ion-coenzyme complex, it was thought that addition of a strong metal-chelating agent might effectively inactivate the pyridoxal phosphate complex by competing either with the pyridoxal phosphate for the metal ion, or with the substrate for the pyridoxal phosphate-metal ion complex. The decrease in available active coenzyme might then lead to compensatory enhanced vitamin B_6 synthesis by the organism.

EDTA was chosen as the chelating agent and the experiment conducted with washed suspensions of organisms, as growth would not occur in the presence of effective concentrations of the chelator. Washed suspensions of Proteus morganii 231 in phosphate buffer pH 7.4, synthesised

no more vitamin B_6 from glucose and NH_4^+, in the absence of added Mg^{++} and presence of 0.04% EDTA than when an excess of Mg^{++} was supplied.

Conclusions

1. No tested microorganism which was capable of growth in a vitamin B_6-free chemically defined medium, was found to synthesise large amounts of vitamin B_6. The best synthesis was given by Proteus morganii but even this was only 4 to 5 times that of E. coli.
2. No microorganism was isolated from soil capable of better vitamin B_6 synthesis in chemically-defined media than E. coli.
3. A cross-feeding technique was developed using S. carlsbergensis 4228c and E. coli M_2 as indicator organisms and adjusting the sensitivity of response by incorporating 4-deoxypyridoxine into the vitamin B_6-free basal media.
4. An isoniazid-resistant substrain of E. coli 518 synthesised twice as much vitamin B_6 when grown in the presence of 10^{-2} M isoniazid as it did when grown in the absence of this antimetabolite.
5. Isoniazid (10^{-2} M) and EDTA (0.04%) did not affect vitamin B_6 biosynthesis by aerobic washed suspensions of Proteus morganii 231.

Chapter B2

Biosynthesis of Vitamin B_6 by Competent Bacteria

1. Escherichia coli

This organism grows well on a simple glucose-ammonium - salts medium at 37°, concurrently synthesising vitamin B_6. The amount of the vitamin formed compares very favourably with the quantities similarly produced by other bacteria (Table B1-2).

The total amount of vitamin B_6 synthesised increased commensurately with growth of the organism and did not significantly decrease thereafter, though the relative distribution of vitamin between organism and its culture medium changed. The concentration of vitamin B_6 present in the organisms rose steeply during the logarithmic phase of growth and then fell away remarkably quickly at the onset of the stationary phase. The vitamin B_6 content of the medium increased steadily and more slowly (Fig. 13). The decrease in endogenous vitamin B_6 after the cessation of growth could be accounted for by its appearance in the culture medium.

It is very obvious that in a comparison of vitamin B_6 synthetic abilities, it is insufficient to assay only the cell content of vitamin, for this will vary

TABLE B2-1

Synthesis of vitamin B_6 by washed suspensions of E. coli B

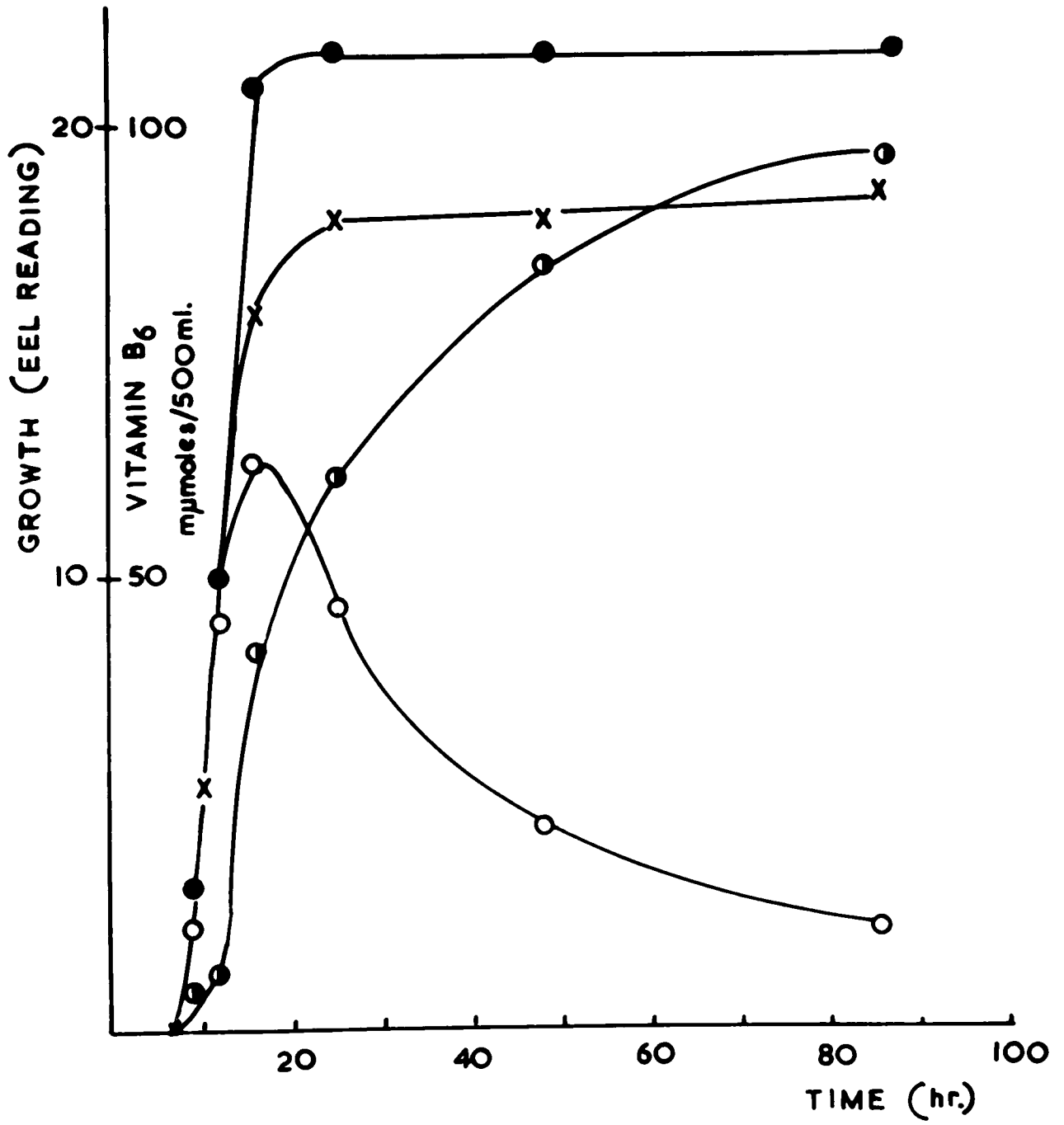
Supplement	Vitamin B_6 synthesis (mμmoles/g. dry wt.)
Nil	0
NH_4^+	0
Glucose	125
Glucose + NH_4^+ (aerobic)	310
Glucose + NH_4^+ (anaerobic)	125
Glutamine	310
Glucose + glutamine	400
Glucose + casein hydrolysate	200

Supplement conc: Glucose 1%; $(NH_4)_2SO_4$ 0.05%; L-glutamine 10^{-2} M; casein hydrolysate 0.05%.

The organisms were grown in shaken culture in Medium G_2 (21 hr. at 37°). Washed suspensions (1.5 mg./ml.) were incubated in 0.1 M phosphate buffer pH 7 supplemented with 0.01% $MgSO_4.7H_2O$ and other substrates as shown. Ten ml. volumes of suspension were rocked in 1-tubes for 6 hr. at 37°. For anaerobic incubation, the tube was evacuated several times and filled with a H_2:CO_2 mixture - 95:5% (v/v).

FIG. 13

The distribution of synthesised vitamin B_6 between organisms and culture medium during growth of *E. coli* 518 on Medium G_2



Key: x—x = growth
o—o = vitamin B_6 in organisms
◑—◑ = vitamin B_6 in growth medium
●—● = total vitamin B_6 synthesis

Incubation was at 37° in static culture (500 ml. in a 1 litre conical flask).

markedly with the age of the culture.

The aerobic growth of E. coli B at 37° was improved by the supplementation of Medium G_2 with casein hydrolysate, but the vitamin B_6 synthesis was virtually unchanged (if expressed in terms of molarity). Similarly little enhancement of vitamin B_6 synthesis was obtained by supplementation of Medium G_2 with purines and pyrimidines (Mixture PP) and vitamins (Mixture V).

Wild-type E. coli synthesised vitamin B_6 from glucose plus NH_4^+, when washed suspensions were incubated aerobically at 37° with these substrates in phosphate buffer pH 7.0 (Table B2-1). No endogenous synthesis of the vitamin was obtained when the suspensions were incubated in unsupplemented buffer. Glutamine could replace both glucose and NH_4^+ but synthesis was better with glucose also present. With casein hydrolysate replacing $(NH_4)_2SO_4$ as sole nitrogen source, vitamin B_6 synthesis was decreased as it was too when the suspensions were incubated anaerobically (Table B2-1). Synthesis of the vitamin was not enhanced by the provision of several compounds of interest, e.g., 3-hydroxyanthranilic acid.

Vitamin requirements

Suitably vitamin-deficient organisms were used to investigate the vitamin dependence of vitamin B_6

TABLE B2-2

Effect of vitamin deficiencies on vitamin B_6 biosynthesis from glucose and NH_4^+ by washed suspensions of *E. coli* mutants

(a) Nicotinic acid - with *E. coli* 9/8

	Vitamin B_6 content (10^8 conc. M)	
	Replete	Deficient
Initially	12	6
After incubation:-		
minus nicotinic acid	24	7.5
plus nicotinic acid (10^{-5} M)	48	48

(b) Thiamine - with *E. coli* 2/57

Initially	12	10
After incubation:-		
minus thiamine	40	14
plus thiamine (10^{-7} M)	60	60

Ten ml. washed suspension (1.5 mg. dry wt./ml.) in 0.1 M phosphate buffer pH 7, supplemented with 0.01% $MgSO_4.7H_2O$ 1% glucose and 0.5% $(NH_4)_2SO_4$ and as indicated.
Incubated with rocking in 1-tubes for 6 hr. at 37°.

biosynthesis from glucose and NH_4^+. For reasons which will later be apparent, nicotinic acid and thiamine were of especial interest.

Nicotinic acid

E. coli 9/8 required 10^{-6} M nicotinic acid for optimal growth on Medium G_2, 2×10^{-7} M allowing of somewhat less than half maximal growth after 15 hr. incubation at 37°. Two batches of cells were grown aerobically, differing in their final content of nicotinic acid and its products, (a) replete, grown on 2×10^{-6} M nicotinic acid, and (b) deficient, grown on 2×10^{-7} M nicotinic acid. When the synthesis of vitamin B_6 from glucose and NH_4^+ by washed suspensions of these organisms was examined, it was evident that maximum synthesis was dependent upon the provision of optimal amounts of nicotinic acid (Table B2-2).

Thiamine

E. coli 2/57 required 2×10^{-8} M thiamine for optimal growth; 2×10^{-9} M supported just over half-maximal growth in 15 hr. at 37°.

Thiamine replete and deficient cells were grown on Medium G_2 supplemented with these two concentrations of the vitamin and were examined as to their ability to

TABLE B2-3

Biosynthesis of vitamin B_6 during growth of various strains of Proteus morganii

Strain	Total vitamin B_6 synthesis (mμmole pyridoxine/g. dry wt.)
231	1900
232	2200
8168	2750
235	4000

All strains were grown in 100 ml. volumes of Medium E in Roux bottles incubated at 37° for 36 hr. Inoculum (per 100 ml. medium) was 0.2 ml. 1:10 EEL 10 suspension taken from a 24 hr. slope on Medium S_1.

synthesise vitamin B_6 from glucose and NH_4^+ in washed suspension in the presence or absence of added thiamine. Subnormal synthesis only of vitamin B_6 was obtainable in thiamine deficiency (Table B2-2).

2. Proteus morganii

Although strain 231 was used routinely, when they became available, three other strains were examined with respect to their synthesis of vitamin B_6 when growing on Medium E. Strain 235 was found to synthesise most vitamin B_6 under these conditions (Table B2-3), and thus demonstrates the best vitamin B_6 synthetic ability of any organism tested.

In preliminary experiments, it was found that best growth on Medium E was achieved by incubation for 24 hr. at 37° under highly aerobic conditions, i.e., either 100 ml. volumes in a Roux bottle or 500 ml. in a 1 litre conical flask kept constantly shaken. These were also the best conditions for vitamin B_6 biosynthesis.

Although Medium E is much more complex than the minimal Medium G_2 sufficing for E. coli, it was found that harvested organisms of Proteus morganii could again synthesise vitamin B_6 from glucose and NH_4^+. The synthesis, obtained with these washed suspensions proceeded best at pH 7.0 to 7.5 and was complete in 6 hr. Very little

TABLE B2-4

Synthesis of vitamin B_6 by washed suspensions of Proteus morganii 231

Supplement	Vitamin B_6 synthesis (mμmoles/mg. dry wt.)
Expt. 1	
Nil	0
NH_4^+	10
Glucose	156
Glucose + NH_4^+ (aerobic)	750
Glucose + NH_4^+ (anaerobic)	280
Casein hydrolysate	156
Glucose + casein hydrolysate	925
Glucose + NH_4^+ and Mixture PP	645
Expt. 2	
Nil	0
Glucose + NH_4^+	985
Glucose + glutamine	1370
Lactate + NH_4^+	520
Lactate + glutamine	520

Supplements (all final conc.):
Glucose 10^{-1} M; glutamine 10^{-2} M; lactate 2×10^{-1} M (neutral) $(NH_4)_2SO_4$ 0.05%; casein hydrolysate 0.05%; purine and pyrimidine mixture PP in 1:10 dilution; H_2/CO_2 - 95:5% (v/v). The organisms were grown aerobically on Medium E (15 hr. 37°). Harvested, washed and resuspended in 0.1 M phosphate buffer pH 7 supplemented with 0.01% $MgSO_4.7H_2O$ and other substrates as shown. Incubated with rocking in 1 tubes at 37° for 6 hr.

synthesis occurred when casein hydrolysate was provided as sole source of carbon and nitrogen, but when glucose was also given, quite good vitamin B_6 production resulted (Table B2-4). A comprehensive purine and pyrimidine mixture (PP) somewhat inhibited vitamin B_6 synthesis, as also did the addition of several compounds of possible relevance, e.g., pAB, anthranilic acid, DL-tryptophan and shikimic acid (all at 10^{-3} M). L-glutamine (10^{-2} M) was a better source of nitrogen than free NH_4^+ ions (Table B2-4). Little or no synthesis of vitamin B_6 was obtained when several compounds at equivalent concentrations were used in place of glucose, e.g., acetate, pyruvate, glyceraldehyde, xylose. With lactate as sole carbon source, vitamin B_6 synthesis was only about half that found with glucose. Anaerobic incubation also diminished the yield of vitamin.

As Proteus morganii shows an absolute growth requirement for both nicotinic and pantothenic acids, the effect of these vitamins on vitamin B_6 synthesis by this organism could be investigated.

Nicotinic acid

Organisms deficient in nicotinic acid were obtained by harvesting the limited growth developed on 5×10^{-8} M nicotinic acid. Vitamin B_6 biosynthesis by

TABLE B2-5

The effect of (a) pantothenic acid, and (b) nicotinic acid deficiencies on the synthesis of vitamin B_6 from glucose and NH_4^+ by washed suspensions of Proteus morganii 231

Organisms	Nil addition	Vitamin B_6 synthesis (m.moles/g. dry wt.) Pantothenate (10^{-5} M)	Nicotinate (10^{-5} M)
Normal	1030	1030	1030
Pantothenic acid deficient	722	832	—
Nicotinic acid deficient	105	—	735

The organisms were harvested after growth as described in the text.
The washed suspensions (0.75 mg./ml.) were incubated in 0.1 M phosphate buffer pH 7.4 containing 0.01% $MgSO_4.7H_2O$, 0.5% $(NH_4)_2SO_4$; 1% glucose, and the supplements shown.
Incubation was for 6 hr. with rocking in 1-tubes at 37°.

washed suspensions of these organisms was compared with that obtained by the use of organisms which had been grown in the presence of an excess (10^{-6} M) of nicotinic acid. The deficiency was reflected in poor synthesis of vitamin B_6, this being restored to near normal levels by the addition of 10^{-5} M nicotinic acid (Table B2-5).

Pantothenic acid

Vitamin B_6 biosynthesis from glucose plus NH_4^+ by washed suspensions of Proteus morganii 231 grown on Medium E, was compared with that obtained when pantothenic acid-deficient organisms were used (prepared by aerobic growth at 37° for 15 hr. in the presence of 10^{-8} M panto-thenate). The effect of supplementing the suspensions with 10^{-5} M pantothenate was examined. Vitamin B_6 synthesis was scarcely affected by pantothenic acid deficiency (Table B2-5).

CELL-FREE EXTRACTS

Thick suspensions of Proteus morganii 231 were easily disrupted by ultra-sonic treatment. Organisms grown overnight at 37° in 500 ml. shaken batches of Medium E, were harvested, washed and suspended to a density corresponding to EEL 500 in 0.5 M phosphate buffer pH 7.4. Samples (10 ml.) of this suspension were subjected to

ultra-sonic treatment for increasing lengths of time of up to 8 min., the cell debris spun off at 24,000 g. for 10 min. at 0°, and the supernatant (suitably diluted) assayed for its protein content.

Ultra-sonic treatment for 3 min. gave maximum protein liberation. The extract so obtained (with u.v. absorption ratio 260/280 = 0.51) was rich in nucleic acid and had a protein content of about 17 mg./ml.

There was no 'endogenous' synthesis of vitamin B_6 when the cell-free extract was incubated in the presence of 0.01 M $MgSO_4$ at 37° for 6 hr. Slight vitamin B_6 synthesis (265 mμmoles pyridoxine/g. protein) took place when a boiled extract of Proteus morganii was added. This was not further enhanced by the addition singly, or in combination, of a number of plausible cofactors and substrates in relatively high concentrations, including:- ATP, DPN and nicotinamide, NH_4^+ and L-glutamine, DL-serine, glucose, glucose-6-phosphate, ribose-5-phosphate and 3-phosphoglyceric acid. Sodium fluoride (0.01 M) was without effect.

Not surprisingly, it appears that the profitable use of cell-free extracts must await the discovery of some intermediate(s) on the route of vitamin B_6 biosynthesis by these organisms.

Conclusions

1. Wild-type E. coli synthesised vitamin B_6 in fair yield when grown on Medium G_2. During later stages of growth, the vitamin was to be found predominantly in the growth medium. This synthesis was not enhanced by the addition of casein hydrolysate, mixtures of purines, pyrimidines and vitamins, or other substrates, e.g., 3-hydroxyanthranilic acid.
2. Washed suspensions of E. coli 518 synthesised vitamin B_6 when incubated at 37° in phosphate buffer pH 7 supplemented with Mg^{++}, glucose and NH_4^+. L-glutamine was a somewhat better nitrogen source than NH_4^+.
3. Use of nicotinic acid and thiamine auxotrophs of E. coli revealed a requirement for these vitamins in vitamin B_6 biosynthesis from glucose and NH_4^+.
4. Although Proteus morganii required for growth a more complex medium than did E. coli, it was again able to synthesise, in suspension, vitamin B_6 from glucose and NH_4^+. Maximum synthesis was obtained, with the light suspensions used (1 to 2 mg./ml.), in 6 hr. incubation at 37° and pH 7 to 7.5. Lactate was less good than glucose, glutamine again somewhat better than NH_4^+. There was very little synthesis when casein hydrolysate served as sole carbon and nitrogen source. No enhancement of synthesis was obtained by

the addition of any tested supplement.

5. Proteus morganii 235 gave the best yield of vitamin B_6 of any organism tested, both during growth and in washed suspension.

6. Nicotinic acid-deficient suspensions of Proteus morganii 231 gave poor synthesis of vitamin B_6 from glucose and NH_4^+. The synthesis was restored to normal levels by provision of excess nicotinic acid. On the other hand, vitamin B_6 synthesis from the same substrates was little affected by pantothenic acid deficiency.

7. An ultra-sonically produced extract of Proteus morganii 231 was capable of very slight vitamin B_6 synthesis, and then only when provided with a crude boiled cell extract. This could not be enhanced by the further addition of a number of cofactors and substrates.

SECTION C

Chapter 1

Properties of Vitamin B_6 Auxotrophs of Escherichia coli

Very few vitamin B_6-requiring mutant organisms have been described (Table C1-1). Mutants of Escherichia coli which show a vitamin B_6 requirement are rare, and all of those available at the outset of this study could under certain conditions grow in the absence of added vitamin. Of these mutants, three were reported to grow at 37° in the presence of vitamin B_6 or either DL-serine or glycine, i.e., mutants B166, B167 and 22/99. No vitamin B_6 requirement could be demonstrated with cultures of M154/59L (tested immediately on reception) and B170 (after subcultivation for two months), probably due to their having reverted to strains with prototroph-like characters. The remaining mutant, DW, whilst showing an absolute requirement for vitamin B_6 at 37°, was able to grow at 25° in the absence of added vitamin if a mixture of amino acids was provided (Table C1-1).

Attempted production of vitamin B_6 auxotrophs

Although interest was mainly focussed on those mutants which demonstrated an apparently alternative amino

TABLE 61-1

Described vitamin B_6 auxotrophs

Organism	Characteristics	References
Neurospora crassa 37803 A	Absolute requirement for vitamin B_6 even at an elevated temperature.	Strauss(1951)
44204	1. pH sensitive. 2. Grows in absence of vitamin B_6 if relatively high conc. of free NH_3 present. 3. When grown in absence of added pyridoxine, it apparently synthesises only 60% of that amount of vitamin B_6 made by the parent wild type. ? Greater destruction of B_6 by this mutant.	Houlahan et al.(1949) Strauss(1951)
44602	pH sensitive - as for 44204.	Strauss (1951)
Neurospora sitophila 299	1. Assay organism for B_6. 2. Growth stimulation by thiamine. 3. At pH 5.6 and in the presence of NH_4^+, vit.B_6 synthesis equals that of the parent wild-type organism. 4. No effect of DL-alanine.	Stokes, Foster & [illegible] (1943) Stokes, Larsen Woodward & Foster (1943)
Aspergillus nidulans pyro	8 mutants requiring only pyridoxine for full growth. Many multiple mutants including a vit.B_6 requirement.	Pontecorvo (1953)
bi.pyro	Pyro mutation superimposed on a biotin auxotroph.	
Escherichia coli M154-59L	1. Grows in absence of B_6 at 30°. Lichstein grew it on casein medium at 30°. Growth then obtained was 10-2% that found in presence of B_6. Stated that aerobic conditions essential for growth in absence of the vitamin. Resulting organisms were B_6-deficient. Metzler and Snell grew it on a complex medium containing amino acids, purines, pyrimidines and vitamins (without B_6 and without aeration). Organisms were then deficient in B_6 and had reduced D-serine dehydrase activity. 2. D-alanine did not replace B_6.	Lichstein(1955) Metzler & Snell (1952)
DW (a substrain of M154-59L)	1. Grows on casein media or media containing a comprehensive amino acid mixture, in absence of B_6 only at 25°. 2. Shows absolute requirement for B_6 at 37°. 3. Used to prepare B_6-deficient organisms.	B.D. Davis and E. Work Wijesundera (1954) Denman, Hoare & Work (1955)
B166, B167 and B179	All grow in absence of added B_6 at 37° if DL-serine or glycine present.	J. Gots
22/99	Grows in absence of vit. B_6 if DL-serine or glycine present.	B.D. Davis

acid/vitamin B_6 requirement, attempts were made to produce an auxotroph of E. coli showing an absolute requirement for the vitamin under all conditions of growth. It was thought that indirect evidence could concurrently be obtained as to the possible existence in this organism of a common route of pyridine ring biosynthesis, for, if a common precursor existed, it might prove possible to produce mutants requiring both nicotinic acid and vitamin B_6 for growth, or even some auxotroph showing a requirement for yet another pyridine derivative of as yet unknown metabolic function.

Wild-type E. coli 518 was subjected to u.v. irradiation and passed through the penicillin-penicillinase plate screening procedure of Adelberg and Myers (1953). Selective final layers were poured containing one of the following sterile mixtures: (a) pyridoxine, (b) pyridoxine plus nicotinic acid, (c) pyridoxine plus nicotinic acid plus yeast extract (giving 10^{-6} M final concn. of vitamins and 0.005% yeast extract). Many irradiations were carried out and a large number of mutants produced. Over 1,000 of these were isolated and their growth requirements studied. The majority were amino acid auxotrophs while a few, obtained from plates carrying supplement (c), showed requirements for adenine, guanine, thiamine and pantothenic acid.

Only one vitamin B_6 mutant, designated E. coli M_2,

was isolated, from a plate carrying a pyridoxine selective layer. Single colonies were isolated after twice streaking on plates of nutrient agar. One of these was maintained as the parent stock culture, and freeze dried. E. coli M_2 showed an absolute requirement for vitamin B_6 under all conditions of growth tested, and gave rapid and maximal growth on Medium G_2 in the presence of 10^{-6} M pyridoxine.

Over thirty nicotinic acid mutants were isolated but no auxotroph was found which required both nicotinic acid and vitamin B_6, or these two vitamins together with a suboptimal concentration of yeast extract.

No evidence has in fact been obtained in support of a common route of origin of nicotinic acid and vitamin B_6 in E. coli. If both these pyridine derivatives arose from a common precursor, a sparing effect of vitamin B_6 on the vitamin requirement of some nicotinic acid auxotroph, and vice versa might be found. In fact, there was no sparing effect of pyridoxine or pyridoxal (10^{-6} M) on the nicotinic acid requirements of Proteus vulgaris R growing on Medium L or of E. coli 9/8 on Medium G_1A. Similarly, nicotinic acid (10^{-7} to 10^{-4} M) had no effect on the pyridoxine requirements of E. coli B166 and E. coli M_2 growing on Medium G_2.

Conclusions

1. Four vitamin B_6 mutants of *E. coli* were available. Not one showed an absolute requirement for the vitamin under all conditions of growth. Three were said to grow in the absence of added vitamin B_6 if provided with DL-serine or glycine.
2. *E. coli* M_2 was produced by u.v. irradiation of *E. coli* 518. This mutant showed an absolute requirement for vitamin B_6.
3. Although many nicotinic acid auxotrophs were produced by u.v. irradiation of *E. coli* 518, no multiple mutants requiring both nicotinic acid and vitamin B_6 (with or without yeast extract) were obtained.
4. No evidence of mutual sparing actions of nicotinic acid and vitamin B_6 could be obtained with the nicotinic acid-requiring organisms *Proteus vulgaris* R and *E. coli* 9/8 and with the vitamin B_6 auxotrophs *E. coli* B166 and *E. coli* M_2.

Chapter C2

Growth of E. coli B166 on Minimal Media

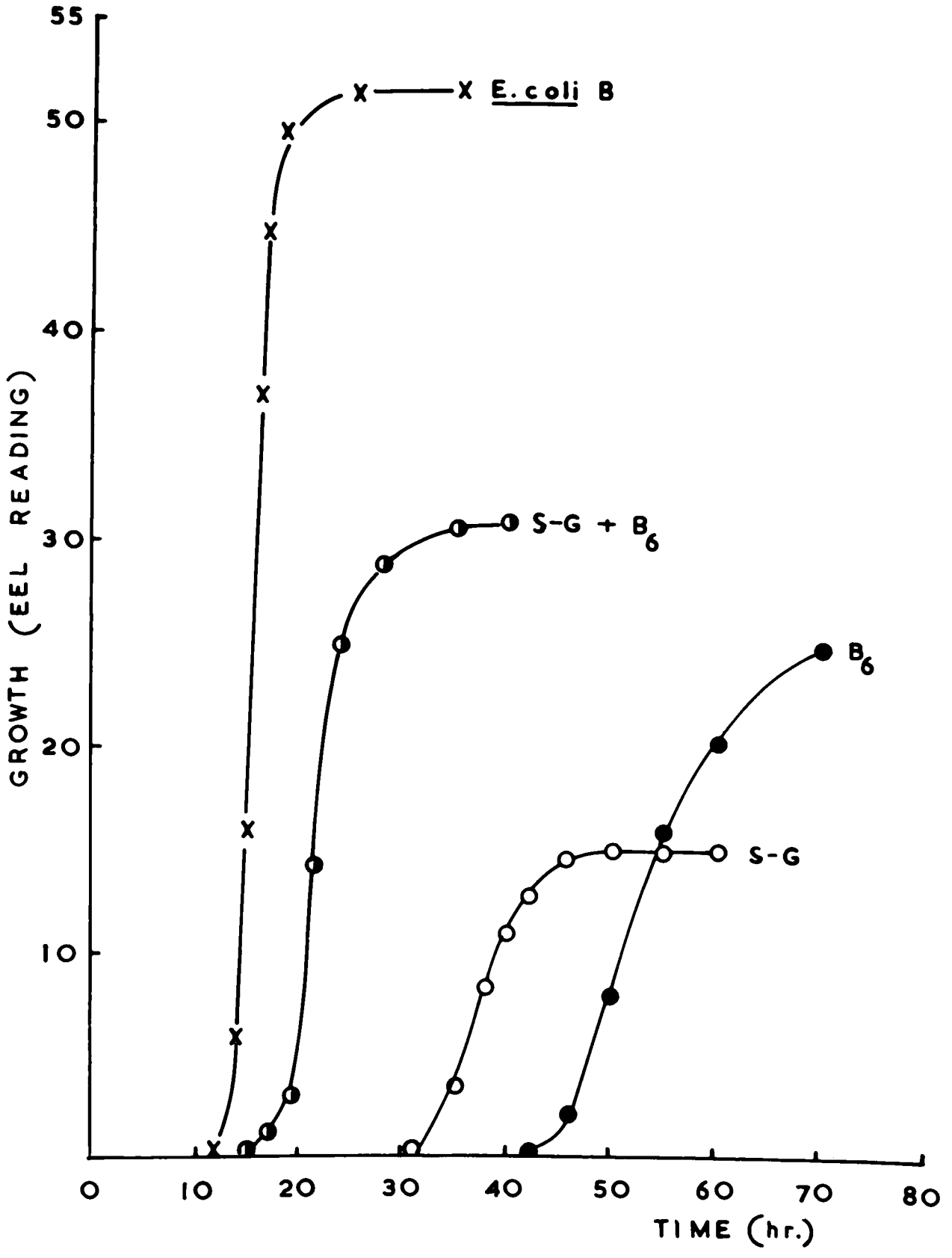
One explanation for the ability of many of the available vitamin B_6 mutants of Escherichia coli to grow alternatively on DL-serine or glycine, is that these amino acids are direct or indirect precursors of the vitamin and that the auxotrophs are capable only of suboptimal synthesis of these amino acids. It was of interest to attempt the identification of the metabolic lesion in these strains. For this purpose, it was necessary to examine in detail the growth characteristics of at least one of the mutant organisms. E. coli B166 was selected for this detailed study.

For reasons explained later, the amino acids were generally used as an equimolar mixture of DL-serine and glycine, the molar concentration being given in terms of the total amino-nitrogen. Serine and glycine are known to be metabolically very closely related in E. coli (Cutinelli et al., 1951; Abelson, 1954; Meinhart and Simmonds, 1955), and as they were essentially interchangeable in the present study, their use in admixture introduces no difficulties of interpretation.

Vitamin B_6 will be used hereafter in general

FIG. 14

Growth of E. coli B166 on Medium G_1A supplemented with serine-glycine and/or pyridoxine



Key: S-G - serine-glycine (10^{-3} M)

B_6 - pyridoxine (10^{-6} M)

E. coli B - prototrophic parent strain growing on unsupplemented medium. 5 ml. cultures in 1-tubes were rocked at 37°.

reference to the group of compounds whose individual members will be specifically named.

Comparison of glucose and lactate as main carbon source

The response of 3166 to pyridoxine (10^{-6} M), serine-glycine (10^{-3} M) and mixtures, was tested on the simple glucose containing medium G_1A, and the ammonium lactate medium L (Figs. 14 and 15). The main carbon source was added to each of the media before autoclaving.

A notable difference was that serine-glycine did not support growth on the lactate medium. In both media, however, most rapid and extensive growth occurred with pyridoxine plus serine-glycine, the rate then approaching that of the parent strain growing on unsupplemented medium. With pyridoxine alone growth was delayed in both media but the final mass was 80 to 90% of that obtained with the mixture. In all cases where growth occurred on lactate medium, it was slightly delayed, but finally 10 to 20% greater than with the same supplement in the glucose medium.

In glucose medium supplemented only with serine-glycine, growth was delayed less than with pyridoxine alone, but the final mass was only 50 to 60% of that found with both supplements.

TABLE C2-1

Response of E. coli B166 to

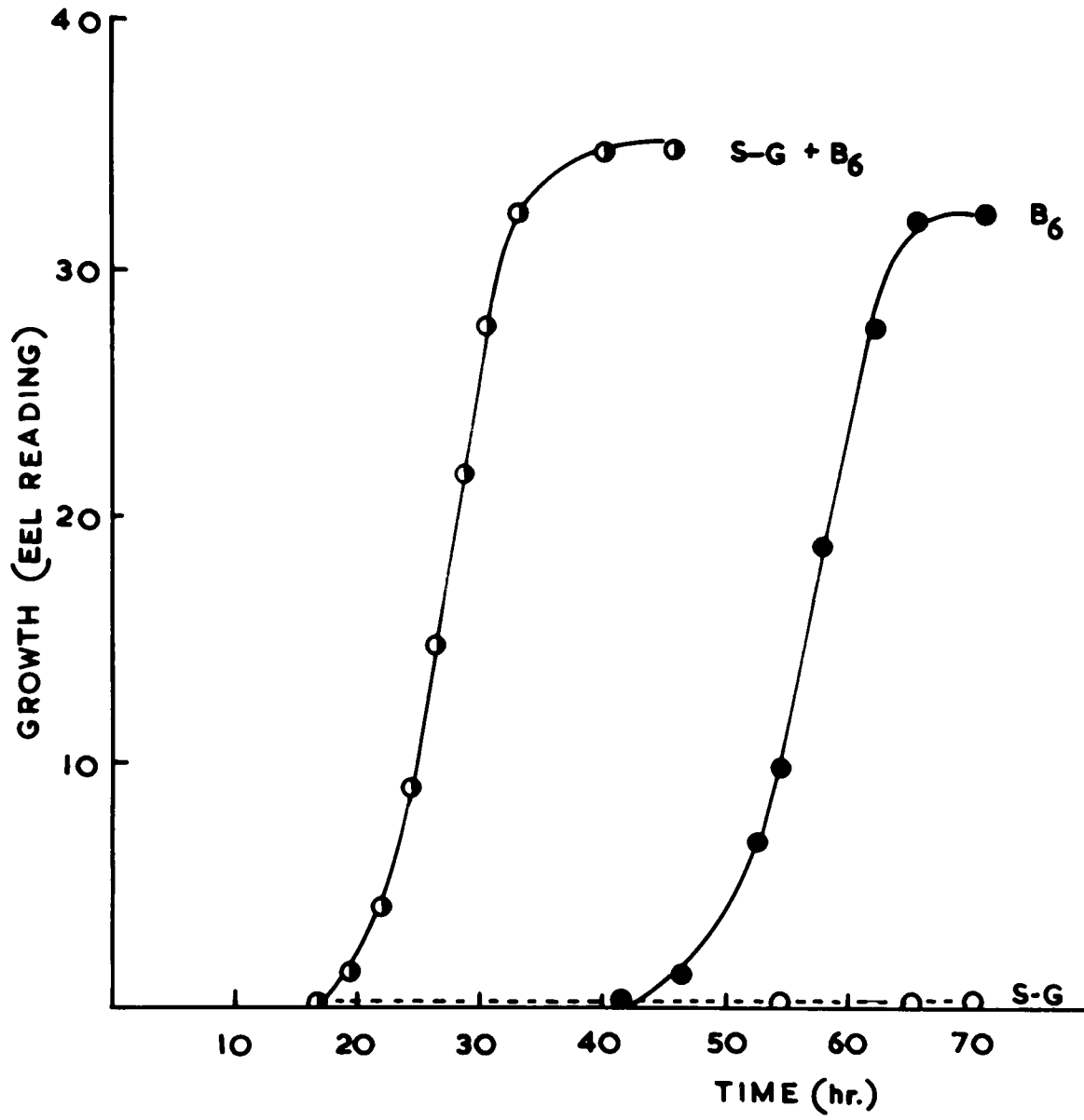
various forms of vitamin B6

Supplement	Conc. (M)	Growth (KEL reading) with Nil addition	Serine-glycine (10^{-3} M)
Pyridoxine	10^{-6}	14	18
	3×10^{-7}	10.5	18
	10^{-7}	4	10
	3×10^{-8}	0	6
	10^{-8}	0	0
Pyridoxal	10^{-6}	12	17
Pyridoxamine	10^{-6}	6	14
Pyridoxal -5'-phosphate	10^{-6}	5	-
Pyridoxamine -5'-phosphate	10^{-6}	0	-

Basal medium was O1A autoclaved at 10 lb.sq.in./7 min. Five ml. volumes, supplemented as above, were inoculated with E. coli B166 and incubated in sloped tubes at 37°. Those tubes containing serine-glycine were incubated for 30 hr. only, the remainder for 80 hr.

FIG. 15

Growth of E. coli B166 on ammonium lactate medium supplemented with serine-glycine and/or pyridoxine



Key: S-G = serine-glycine (10^{-3} M)
B_6 = pyridoxine (10^{-6} M)
Basal medium = Medium L. 5 ml. volumes in L-tubes rocked at 37°.

Response to vitamin B_6

With the five forms of vitamin B_6 tested on Medium G_1A (Table C2-1) growth was most rapid with pyridoxine; the concentration for half maximal growth was 2×10^{-7} M. Pyridoxal was intrinsically more active (10^{-7} M gave half-maximal response) but growth was delayed a further 12 hr. Similar results were obtained on Medium L. Pyridoxine was subsequently used routinely.

With serine-glycine also present (Table C2-1) the amount of pyridoxine required was reduced to about one-third (6×10^{-8} M for half maximal growth); in these experiments results were read at 30 hr. since on longer incubation there was growth in the absence of added pyridoxine.

Response to serine, glycine and related compounds

The concentration of serine-glycine required on Medium G_1A (no added pyridoxine) was 10^{-3} and 2.5×10^{-4} M for optimal and half-optimal growth respectively (Table C2-2). Similar concentrations were required for the other effect of serine-glycine, i.e., to promote earlier growth when pyridoxine was present.

Glycine and DL-serine were each individually active in both the above senses but the mixture gave somewhat more rapid and heavier growth. In addition,

TABLE C2-2

Concentrations of amino acids required to support growth of E. coli B166 in the absence of added vitamin B_6

Amino acid	Conc. (M)	Growth (KU reading)
Nil	-	0
DL-serine-glycine	4×10^{-3}	12
	10^{-3}	12
	5×10^{-4}	9
	2.5×10^{-4}	6
	1.25×10^{-4}	4
	6.25×10^{-5}	2.5
L-serine	5×10^{-4}	7
D-serine	5×10^{-4}	0
Glycine	5×10^{-4}	7.5
DL-homoserine	10^{-3}	0
DL-threonine	10^{-3}	*Trace

*Achieved KU 5 after 110 hr. incubation.

Basal medium was G_1A autoclaved at 10 lb.sq.in./7 min. Five ml. volumes, supplemented as above, were inoculated with E. coli B166 and incubated in sloped tubes at 37° for 70 hr.

results were erratic with suboptimal concentrations of the separate amino acids. This appeared to be due to partial reversion of the mutant to prototroph-like character. Random individual tubes grew heavily after 48 hr. incubation and sub-cultures from such tubes into basal medium gave growth (though less rapidly than the parent strain) without the addition of either pyridoxine or serine-glycine. The properties of these sub-strains have not been further pursued. These changes were less frequent with mixtures of glycine and serine at suboptimal concentration and did not occur when the concentration of the individual amino acids exceeded 4×10^{-4} M.

In experiments with serine alone (at optimal concentration) only the L-isomer was active, either in the presence or absence of pyridoxine.

There is evidence that E. coli can form glycine from both threonine and homoserine (Gibson, 1952; Abelson, 1954). DL-threonine at high concentration supported detectable growth of B166 in the absence of pyridoxine on Medium G_1A, but DL-homoserine was inactive (Table C2-2).

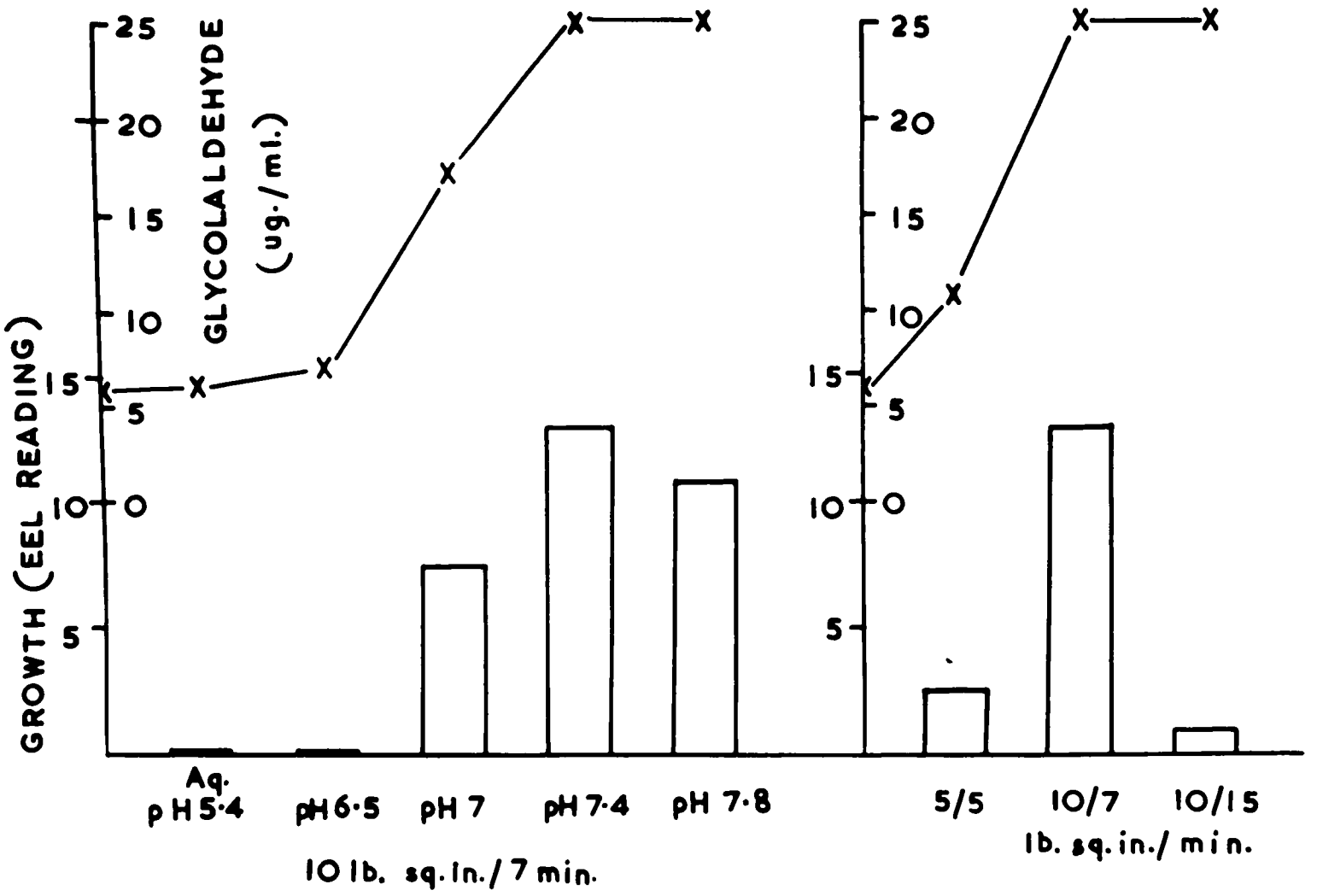
Other amino acids or substances metabolically related to serine and glycine and found to be inactive (over a range of concs. from 10^{-6} to 5×10^{-3} M)) were:- formiminoglycine, glycollic, glyoxylic, pyruvic, β-hydroxypyruvic and δ-aminolaevulic acids, ethanolamine,

DL-alanine, L-arginine, DL-asparagine, DL-aspartic acid, DL-glutamic acid, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, DL-tryptophan, DL-tyrosine and DL-valine.

Conclusions

1. Serine-glycine seems to have two apparently distinct effects on the growth of *E. coli* B166, viz.:-
   (a) supporting growth in the absence of pyridoxine - but only on glucose-containing medium;
   (b) on both glucose and lactate media and with pyridoxine present they reduce the delay in the onset of growth by 20 to 25 hr. and slightly increase the final cell density.
2. Although L-serine and glycine were individually active, they were better used in admixture.
3. Other amino acids and a number of chemically-related compounds including glyoxylic and β-hydroxypyruvic acids were unable to replace glycine and serine.

Comparison of 'activity' (block diagram) with content of substance assaying as glycolaldehyde



Activated glucose samples prepared by autoclaving glucose solutions (10 mg./ml.) in 0.04 M phosphate buffers.

Glycolaldehyde estimated by method of Dische and Borenfreund (1947). 'Activity' measured as extent of growth of E. coli B166 in Medium G, supplemented with sample plus serine-glycine (10^{-3} M).

FIG. 13

Chapter C3

An effect of Autoclaved Glucose

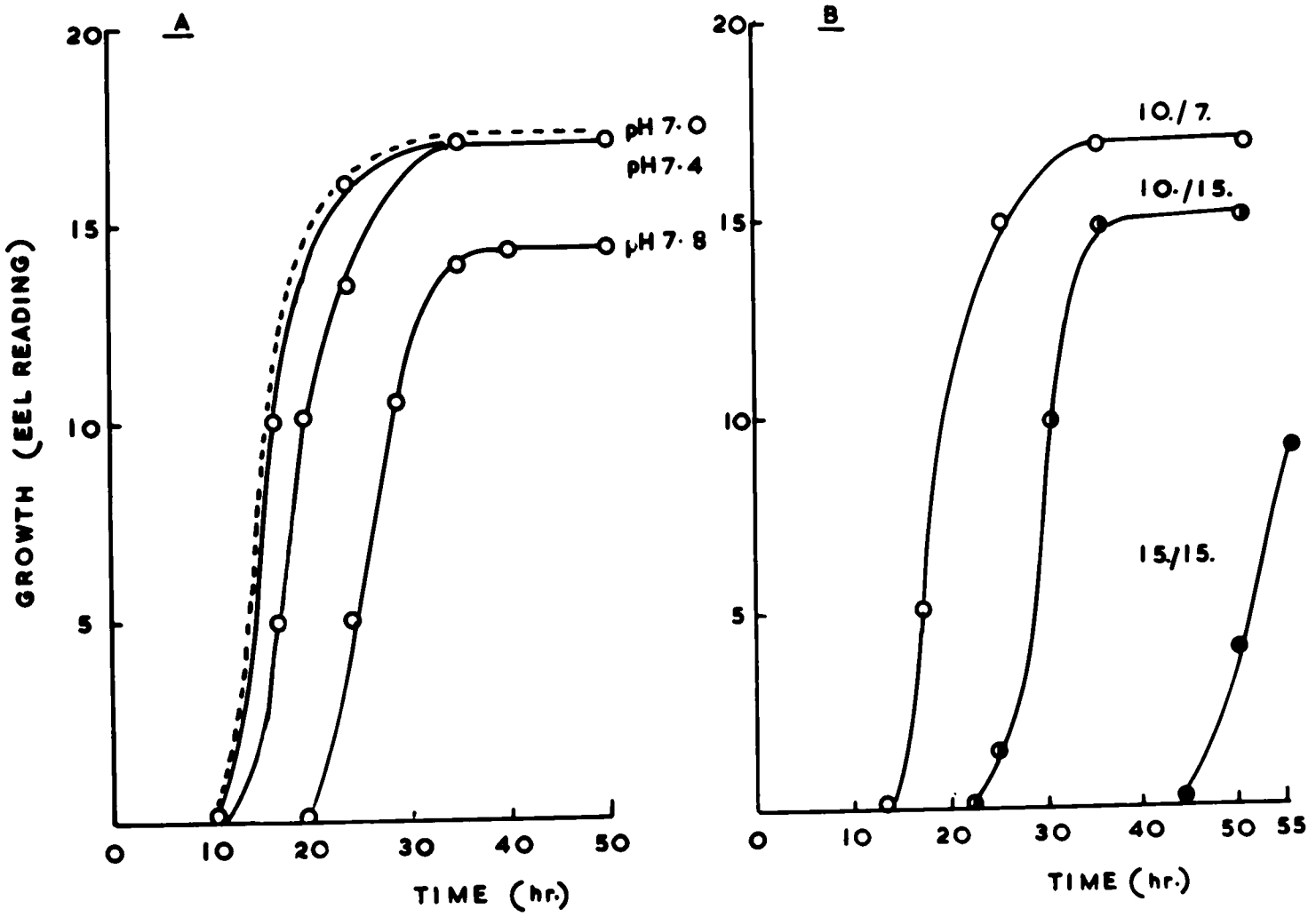
The fact that serine-glycine supported growth of Escherichia coli B166 in the absence of pyridoxine, but only on the glucose-containing medium, suggested that the activity of the amino acids was dependent on the concurrent presence of some substance formed as a result either of glucose metabolism by the organism or of the heat-sterilization of the medium.

In the above experiments, the glucose was added to the other constituents of the medium before autoclaving. When the glucose was separately sterilised by Seitz-filtration, and then aseptically added to the remainder of the medium (which had been autoclaved as usual), serine-glycine no longer supported growth. This was also the case when the glucose was separately sterilised by autoclaving in aqueous solution (in glass distilled water when pH was 5.4) (Fig. 16). Autoclaving in 0.04 M phosphate buffers of various pH values showed that only at about pH 7 and above was a solution of glucose obtained which permitted growth on serine-glycine (Fig. 16).

For present purposes, the term 'activated' glucose will be used in reference to glucose solutions which permit growth of the organism on serine-glycine

FIG. 17

Inhibition of growth of E. coli B166 by glucose solutions subjected to varying degrees of autoclaving



Key: --- = inactive glucose.

A - increasing pH of autoclaving at 10 lb.sq.in./7 min.

B - increasing severity of autoclaving at pH 7.4, e.g., 10/15 = 10 lb.sq.in./15 min.

The glucose solutions (10 mg./ml.) were autoclaved as shown in 0.04 M phosphate buffers.
5 ml. volumes of Medium G_1 supplemented with pyridoxine (10^{-6} M) serine-glycine (10^{-3} M) and test sample (to glucose conc. 1 mg./ml.), were inoculated with E. coli B166 and incubated in sloped tubes at 37°.

without added pyridoxine. 'Inactive' glucose will be used in the opposite sense. Untreated glucose was inactive, and remained so after sterilisation by Seitz-filtration or by autoclaving at pH 6.5 and below.

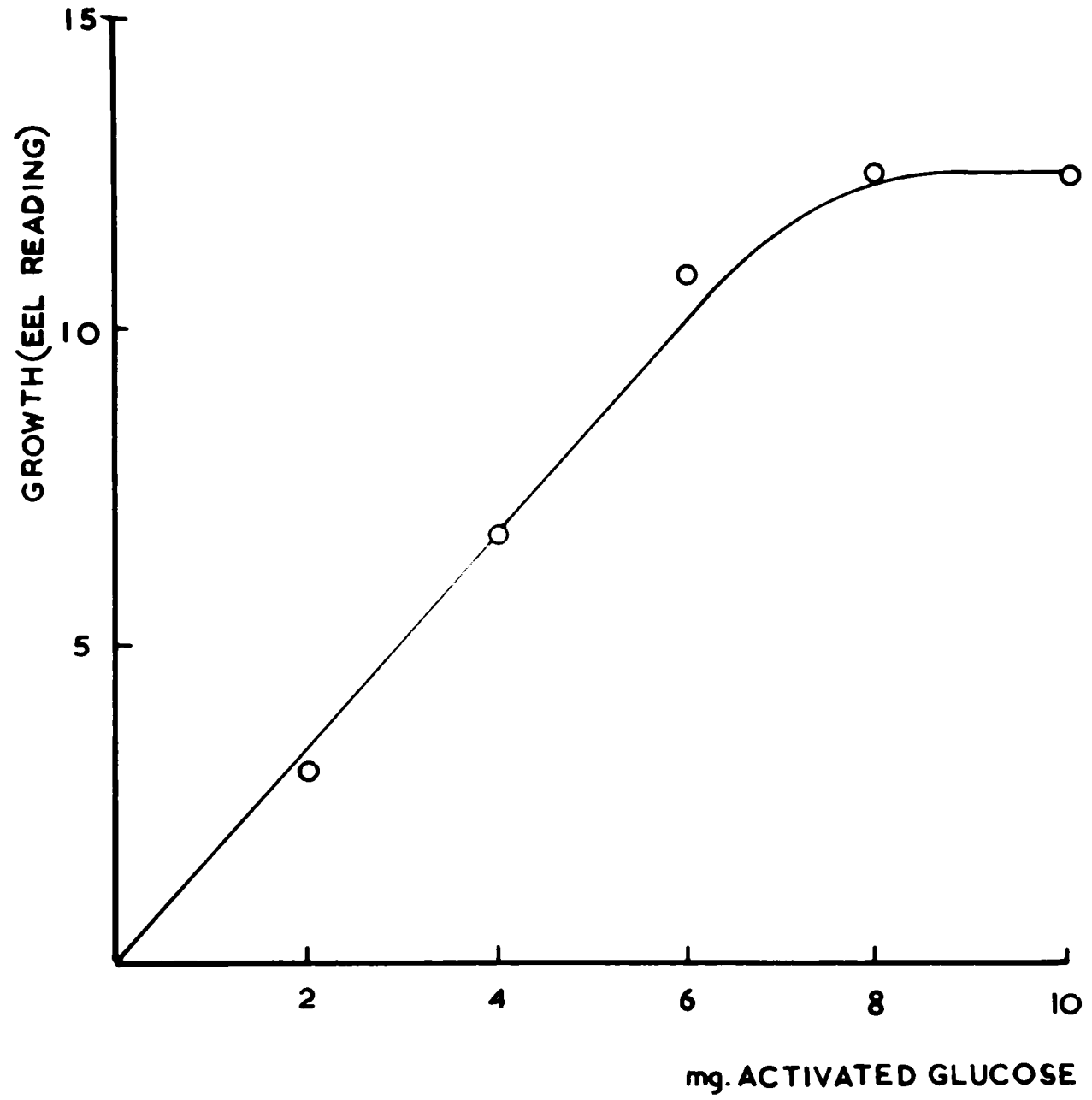
Conditions for the formation of activated glucose

Glucose (10 mg./ml.) was autoclaved at various combinations of pressure and time, at pH values ranging from 6 to 8. After neutralising, the solution was added to previously sterilised basal medium supplemented only with serine-glycine. A selection of the results are shown in Fig. 16.

Best activation occurred when autoclaving was carried out at 10 lb.sq.in./7 min. at pH 7.4. At higher pH or with more severe autoclaving, there was obvious caramelisation and a decrease in activity - probably due rather to the concurrent production of inhibitory substances than to decreased formation of the active factor. Onset of growth in the presence of serine-glycine plus pyridoxine was delayed 10, 10 and 30 hr. respectively if the glucose was treated at pH 7.8 (10 lb.sq.in for 7 min.) or at pH 7.4 for 15 min. at 10 and 15 lb.sq.in.(see also Fig. 17).

FIG. 18

'Titration' of a sample of activated glucose



The sample of activated glucose was prepared by autoclaving a glucose solution (20 mg./ml.) in 0.04 M phosphate buffer pH 7.4 at 10 lb.sq.in./7 min.

5 ml. volumes of Medium G_1 (containing inactive glucose in sufficient amount to bring the total glucose conc. in each tube to 2 mg./ml.), were supplemented with serine-glycine (10^{-3} M) and inoculated with E. coli B166. Incubation was in sloped tubes for 85 hr. at 37°.

Other effects of activated glucose

Both serine-glycine and activated glucose were required for growth in the absence of pyridoxine. Experiments with mixtures of inactive and activated glucose showed that the maximum effect was obtained if at least 80% of the glucose in the medium was provided in the activated form (Fig. 18).

When pyridoxine was present (either with or without serine-glycine), activated glucose was not required, nor did it increase the rate or final extent of growth (Fig. 19). The only effect was a slight delay in the onset of growth, possibly again due to the presence of inhibitory substances. Activated glucose was therefore not required for the demonstration of the second action of serine-glycine (i.e., decreasing the lag when pyridoxine is present) but only for the total replacement of pyridoxine.

Reducing agents

The activation of glucose might simply have been due to the development of non-specific reducing conditions in the autoclaved mixtures.

One powerful reducing agent known to be formed when alkaline solutions of glucose are heated, is glucose reductone (enoltartronaldehyde) (Pigmann and Goepp, 1948). This compound (10^{-10} to 10^{-9} M) did not replace activated

TABLE C3-1

Reducing agents and the growth of *E. coli* B166 on serine-glycine

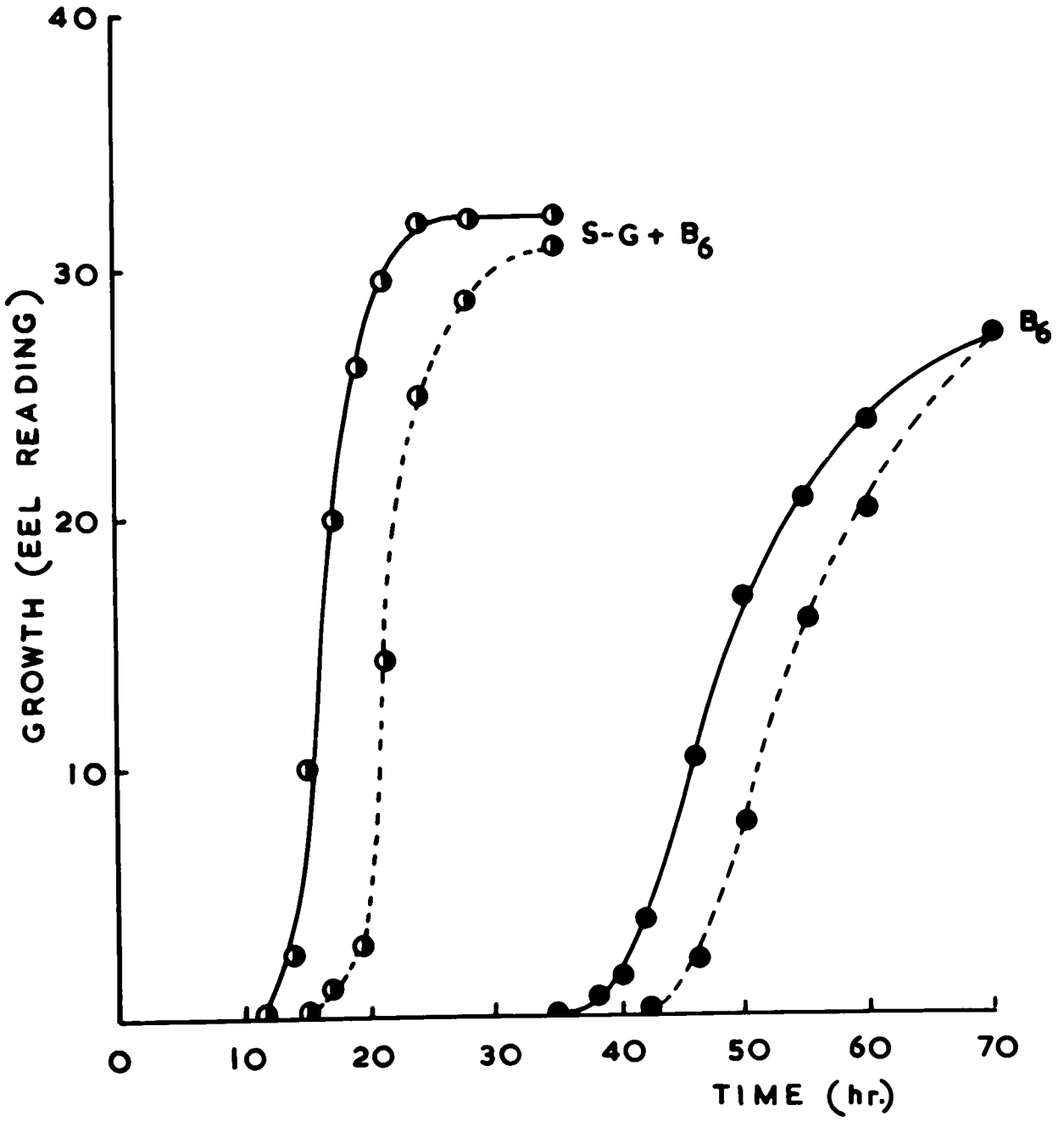
Supplement	Conc. (M)	Growth (EEL reading)	
		Serine-glycine	Serine-glycine plus vitamin B6
Nil	--	0	15
Ascorbic acid	2×10^{-3}	0	0
	4×10^{-4}	0	(15)
	8×10^{-5}	0	15
Glucose reductone	10^{-5}	0	(15)
Activated glucose*	5×10^{-4}	10	15

( ) signifies growth delayed by 10 hr. compared with control.
*Control in which glucose was added to basal medium before autoclaving.
The reducing agents were sterilised by Seitz-filtration and added aseptically post autoclaving. When present, serine-glycine (10^{-3} M), and vitamin B_6 (pyridoxine 10^{-6} M).

Five ml. volumes of Medium G_1 supplemented as shown, were inoculated with *E. coli* B166 and incubated in sloped tubes for 64 hr. at 37°.

FIG. 19

Comparison of effects of (a) inactive, and (b) activated glucose on growth of E. coli B166 on Medium G1 supplemented with pyridoxine



Key: —— = inactive glucose
--- = activated glucose
S-G = serine-glycine (10^{-3} M)
B_6 = pyridoxine (10^{-6} M)

5 ml. volumes rocked in 1-tubes at 37°.

glucose for growth of B166 on Medium G_1 supplemented with serine-glycine; at concentrations above 10^{-5} M it partly inhibited growth in the controls (Table C3-1). It may be partially responsible for the slight delay in growth obtained when activated glucose was present in the medium (see also Mondolfo and Monnie, 1948).

Ascorbic acid was chosen as a reducing agent of carbohydrate-like nature though one which is not formed from glucose on autoclaving. It too, failed to replace activated glucose at the concentrations tested (10^{-8} to 5×10^{-3} M) and was inhibitory at the higher levels (Table C3-1).

Activated glucose was also necessary for growth on serine-glycine under anaerobic conditions (Table C7-1).

The weight of evidence is therefore that the activation of glucose is due to the formation of some specific compound which together with serine-glycine permits growth of E. coli B166 in the absence of added vitamin B_6.

Conclusions

1. Glucose-containing media would only support growth of E. coli B166 on serine-glycine if the glucose had been previously 'activated'.
2. The growth-promoting action of serine-glycine in a

vitamin B_6-containing medium was not dependent upon the concurrent presence of activated glucose.

3. Autoclaving at pH 7.4 and at 15 lb.sq.in./7 min. resulted in maximal activation of a 10 mg./ml. glucose solution. More strenuous autoclaving produced growth-inhibitory substances.

4. Activated glucose samples were so distinguished not by virtue of their possession of a lowered redox potential, but by their content of some specific chemical compound formed during the activation procedure.

Chapter C4

Identification of the Active Factor in Alkaline Autoclaved Glucose Solutions

Although moderately stable at an acid pH, glucose is profoundly affected by the action of alkalies under even very mild conditions (Evans, 1942; Pigmann and Goepp, 1948). The possible reactions and consequent products are numerous. Two types of reaction are concerned:-

(a) isomerisation, chiefly taking place at C-1 and C-2;

(b) fragmentation, giving rise to products each containing less than six carbon atoms.

The simplest isomerisation reactions are of the Lobry de Bruyn-van Eckenstein type (Pigmann and Goepp, 1948). Amongst other products, mannose and fructose might so be formed when glucose is treated with dilute alkalies at room temperature. The reaction probably proceeds via the formation of an intermediate ene-diol; there is good evidence for the existence of such compounds in alkaline solutions of aldoses. More drastic rearrangements result in the generation of acidic materials including branched chain saccharinic acids (Scheibler, 1880; Kiliani, 1882).

Many more compounds are produced by the fragmentation of the glucose molecule. Cleavage of the carbon skeleton may give rise to formaldehyde and aldopentoses,

glycolaldehyde and aldotetroses, or dihydroxyacetone and glyceraldehyde. In turn each of these products may isomerise through the ene-diols to the corresponding ketoses and thence to the various saccharinic acids (Pigmann and Goepp, 1948).

One of the principal products of molecular cleavage seems to be DL-lactic acid. Some other identified products are dihydroxybutyric acid, methylglyoxal, acetol, diacetyl, glucose reductone, formic and acetic acids (Pigmann and Goepp, 1948), reductol and dihydroxyacrylic acid (Urnka, 1951).

Several approaches were available in attempting identification of the biologically active compound:

(a) Direct, i. concentration and isolation;

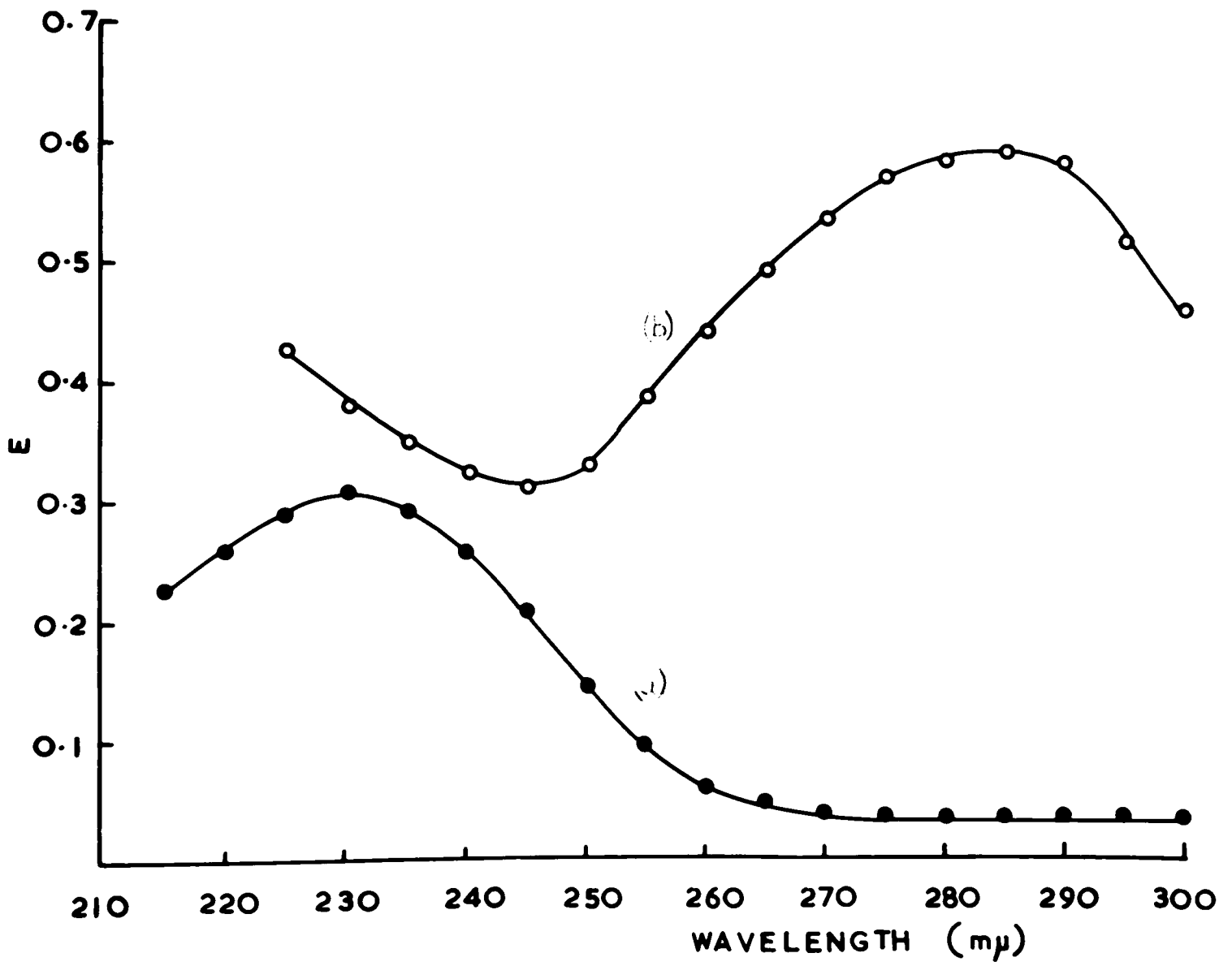
ii. testing of known products of glucose alkaline degradation.

(b) Indirect; examination of a number of sugars and their derivatives to see which could be activated in a manner similar to glucose by autoclaving at a slightly alkaline pH. A structural common denominator for potential activation could emerge from this survey. Stable substituted glucose derivatives such as the O-methylglucoses might be employed.

All three approaches were followed simultaneously. The criterion of activity used throughout was the ability of Seitz-filtered samples to replace activated glucose in

FIG. 20

Ultra-violet absorption spectra of mixtures formed by autoclaving glucose solutions (20 mg./ml.) in,
(a) glass-distilled water, pH 5.5,
(b) phosphate buffer (0.02 M), pH 7.4



Autoclaving was at 10 lb.sq.in. for 7 min.

supporting growth of E. coli B166 on Medium G_2 supplemented with serine-glycine; inactive glucose was routinely present as the main carbon and energy source. To prevent enhancement of growth due merely to non-specific increase in the concentration of carbon source, the amount of inactive glucose added was increased from the 2 mg./ml. previously used (Medium G_1) to the optimal 10 mg./ml. present in Medium G_2.

Enhanced activity when assayed microbiologically may not be a measure of increased concentration of active factor but may result from the removal of growth inhibitory compounds (as are known to be present in activated glucose). This was checked where necessary by investigating the effect on growth in the presence of serine-glycine and pyridoxine.

(1) Attempted concentration and purification

a. Ultra-violet absorption characteristics

The mixture obtained by autoclaving a glucose solution (20 mg./ml.) in 0.02 M phosphate buffer pH 7.4 at 10 lb.sq.in./7 min., was faint yellow in colour and possessed a distinctive absorption band in the ultra-violet (max. about 285 mμ) (Fig. 20). A product showing no absorption peak at 285 mμ but with a small peak at 230 mμ was produced when an aqueous solution of glucose

(30 mg./ml., pH 5.4) was similarly autoclaved. Evstigneev and Nikiforova (1950) studied the u.v. absorption spectra of glucose solutions boiled for long periods at various initial pH's and commented on the appearance of absorption maxima in the region of 230 and 280 mμ. They discriminated between two absorption maxima in the latter region, (a) at 275 to 280 representing glucose with a free carbonyl group, and (b) at 282.5 due to 5-hydroxymethylfurfural (this was not formed at pH's higher than 4.5 to 5.0).

The absorption at 285 mμ in activated glucose solutions might be due to glucose reductone which shows a very strong absorption at 287 mμ in alkaline solution. But glucose reductone was completely inactive (Table C3-1).

b. Concentration

In the preparation of activated glucose, the phosphate buffer concentration could be reduced to 0.005 M without affecting the resultant biological activity. A more active preparation was obtained by autoclaving dilute (20 mg./ml.) solutions of glucose in 0.005 M phosphate buffer and then concentrating these 20-fold, than by autoclaving a 400 mg./ml. solution in 0.1 M buffer when more caramelisation, with its attendant growth inhibitory products, occurred.

Concentration was first carried out by

distillation at room temperature under a reduced pressure of nitrogen, but it was subsequently found that there was no appreciable loss in activity during vacuum distillation at <40°.

It soon became apparent that the active factor was stable at neutral pH at room temperature, and at all temperatures was somewhat more stable at acid than at alkaline pH values.

c. Attempted purification

Activity was unchanged by passage of concentrated activated glucose through a column of Dowex 50 (H^+ form), but disappeared when the effluent was then batch-treated with Dowex 1 (OH^- form). No active fraction could subsequently be eluted from the Dowex 1 by treatment with aqueous HCl of increasing strength (up to 0.5 N). The 285 mμ-absorbing material also passed unchanged through the Dowex 50 column but could not be detected after Dowex 1 treatment. Disappearance of activity on treatment with Dowex 1 could be the result of some chemical reaction on the surface of the resin other than straightforward ion exchange, e.g., alkaline degradation or polymerisation (cf. Phillips and Pollard, 1953).

Some obvious purification of the yellow concentrate was achieved by batch treatment with acid-washed

norite. Activity remained undiminished in the now colourless filtrate.

Attempted extraction with ether and ethyl acetate over periods of 6 to 12 hr. at pH 2 or 7.4, gave disappointing results. Very little activity passed into the organic solvent phase though even unchanged glucose was extracted. It was concluded that the active factor was highly hydrophilic.

Paper chromatography (descending butanol:water or butanol:acetic acid:water) proved valueless. Due to the preponderance of glucose and the very large number of reducing compounds present in all samples examined, slight resolution of any was possible. No distinctive acid compounds could be detected (using the method of Benson and Phares, 1952).

Chromatograms of activated glucose concentrates developed with a variety of solvent mixtures and dried, were cut into bands, and these eluted with distilled water. No eluate possessed appreciable activity. Bioautography of such chromatograms on plates of Medium G_2 with serine-glycine solidified with washed agar, incorporating tetrazolium tetrachloride to enhance sensitivity, and seeded with E. coli B166 was also unsuccessful - perhaps due to dilution during chromatography.

TABLE C4-1

Ability of variously treated sugars and their derivatives to replace activated glucose

Activation = + No activation = -

Compound	Seitz-filtered	Aqueous sol. (pH 5.4-5.6)	Medium (pH 7)	PO_4 buffer (pH 7.4)
Galactose	-	-	+	+
Mannose	-	-	+	+
Fructose*	-	-	+	+
Sorbose	-	-	+	+
Arabinose	-	-	+	+
Xylose	-	-	+	+
Ribose	-	-	+	+
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
Erythritol	-	-	-	-
3-O-methyl-glucose	-	-	-	-

*Activated fructose was somewhat inhibitory (delayed growth).

Criterion of activity was growth of E. coli B166 on Medium C_2 supplemented with serine-glycine plus the test compound. All compounds under test were used at a final conc. of 4 mg./ml. and autoclaved as stated at a conc. of 20 mg./ml. (i.e., at 10 lb.sq.in./7 min.).

(2) Effect of autoclaving on other sugars and derivatives

Besides glucose, pentoses and other hexoses were able to form active material when autoclaved at slightly alkaline pH.

Solutions (20 mg./ml.) were (a) Seitz-filtered, (b) autoclaved at the existing pH (5.4 to 5.7), (c) autoclaved in 0.04 M phosphate buffer pH 7.4 and (d) added to the supplemented basal medium (pH 7) before autoclaving. The treated solutions were tested at a final concentration of 4 mg./ml. The additional carbohydrate did not affect the rate or extent of growth in control media supplemented with pyridoxine (± serine-glycine).

None of the sugars permitted growth with serine-glycine unless they had first been autoclaved at pH 7 or 7.4 (Table C4-1). The three sugar alcohols and 3-O-methyl-glucose remained inactive even after such treatment. Fructose gave rise more easily than any to inhibitory substances, and the growth which did occur on serine-glycine with autoclaved preparations of fructose was delayed 24 to 48 hr. compared with the other sugars.

A number of the carbohydrates are fermented by E. coli and could replace glucose as the main carbon source for growth of the mutant in the presence of pyridoxine. In these cases, development of activity was also assayed

TABLE C4-2

Growth of E. coli B166 in the presence of serine-glycine plus variously treated sugars

Growth = + No growth = -

Sugar etc.	Autoclaved in: Seitz-filtered	Aqueous soln. (pH 5.4-5.6)	Medium (pH 7)	PO_4 buffer (pH 7.4)
Galactose	-	-	+	+
Mannose	-	-	+	+
Fructose*	-	-	+	+
Arabinose	-	-	+	+
Xylose	-	-	+	+
Mannitol	-	-	-	-

All the above substrates supported growth in the presence of pyridoxine.
*Activated fructose was somewhat inhibitory (delayed growth even in the presence of pyridoxine).

Basal medium was as for Medium G_1 but with compound specified replacing glucose. Serine-glycine also added at usual (10^{-3} M) concentration. All autoclaving was at 10 lb.sq.in./7 min. Five ml. volumes of the supplemented medium were inoculated with E. coli B166, then incubated at 37° in sloped tubes for 30 hr.

by adding the carbohydrate to the medium (i.e., G_2 minus glucose) as the sole carbon source, either before autoclaving or as a Seitz-filtered solution after autoclaving. The sugar alcohol mannitol was inactive in both cases, the sugars as before, were active only after autoclaving (Table C4-2).

The active substance was thus formed from both hexoses and pentoses. Even with the relatively small number of carbohydrates tested, it would appear that the substance was formed only if there were an actual or potential aldehyde group at C-1 and an unsubstituted hydroxyl group on C-3.

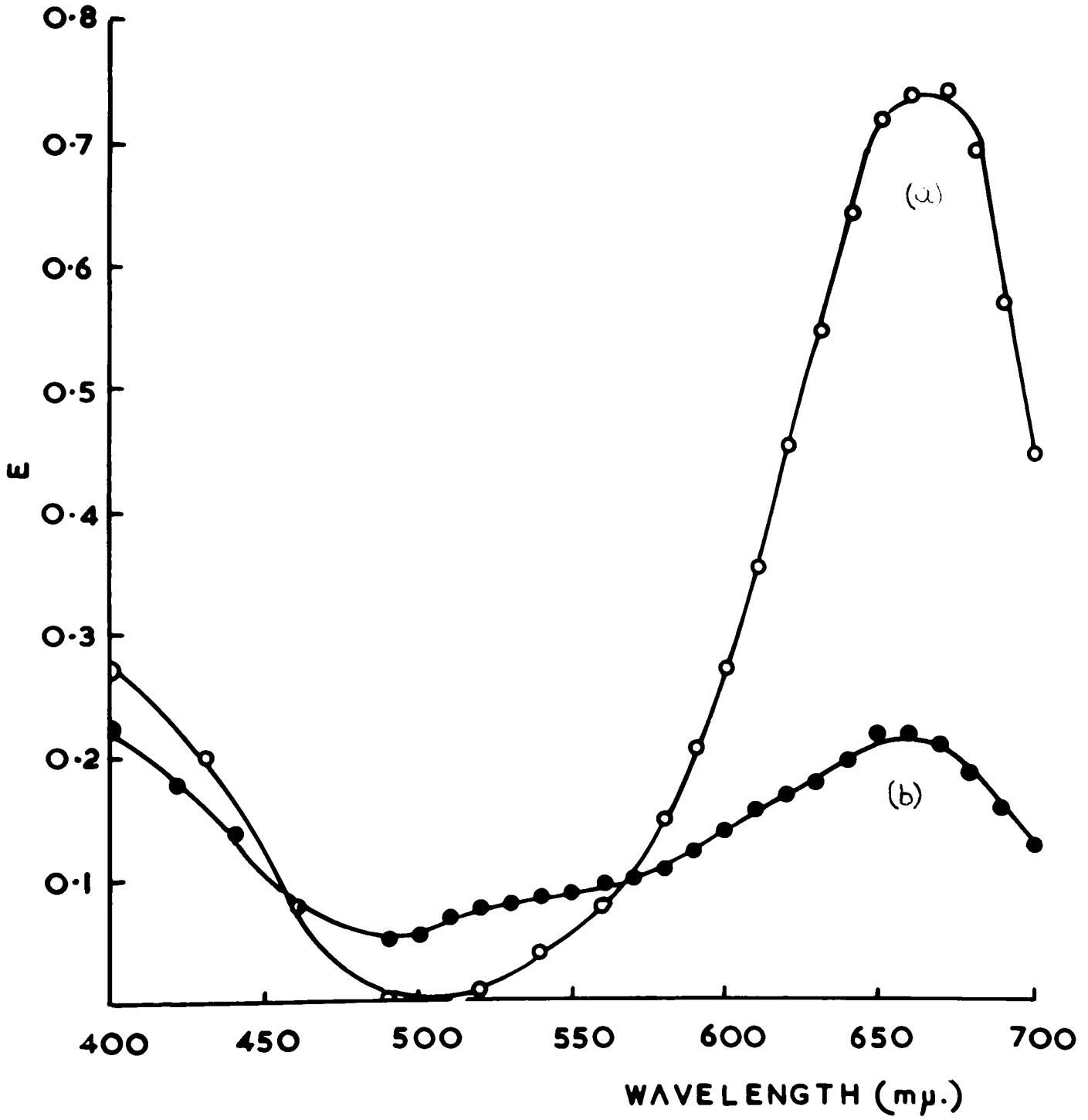
(3) Behaviour of known degradation products of glucose

Concurrently with the above experiments, a number of compounds reported to be amongst the products of alkaline degradation of glucose, were assayed for activity. These were added in Seitz-filtered solution over a range of concentrations (10^{-6} to 5 x 10^{-3} M), to Medium G_2 containing serine-glycine.

No growth was obtained with the following: lactic, pyruvic, acetic, formic and tartronic acids, methylglyoxal, dihydroxyacetone, DL-glyceraldehyde and formaldehyde. Also tested and found to be inactive was 5-hydroxymethylfurfural (which in any event is predominantly

FIG. 21a

Absorption spectra of colours given in Dische and Borenfreund assay by (a) glycolaldehyde
(b) activated glucose



Similarly treated inactive glucose sample as blank.

a product of acidic degradation of glucose).

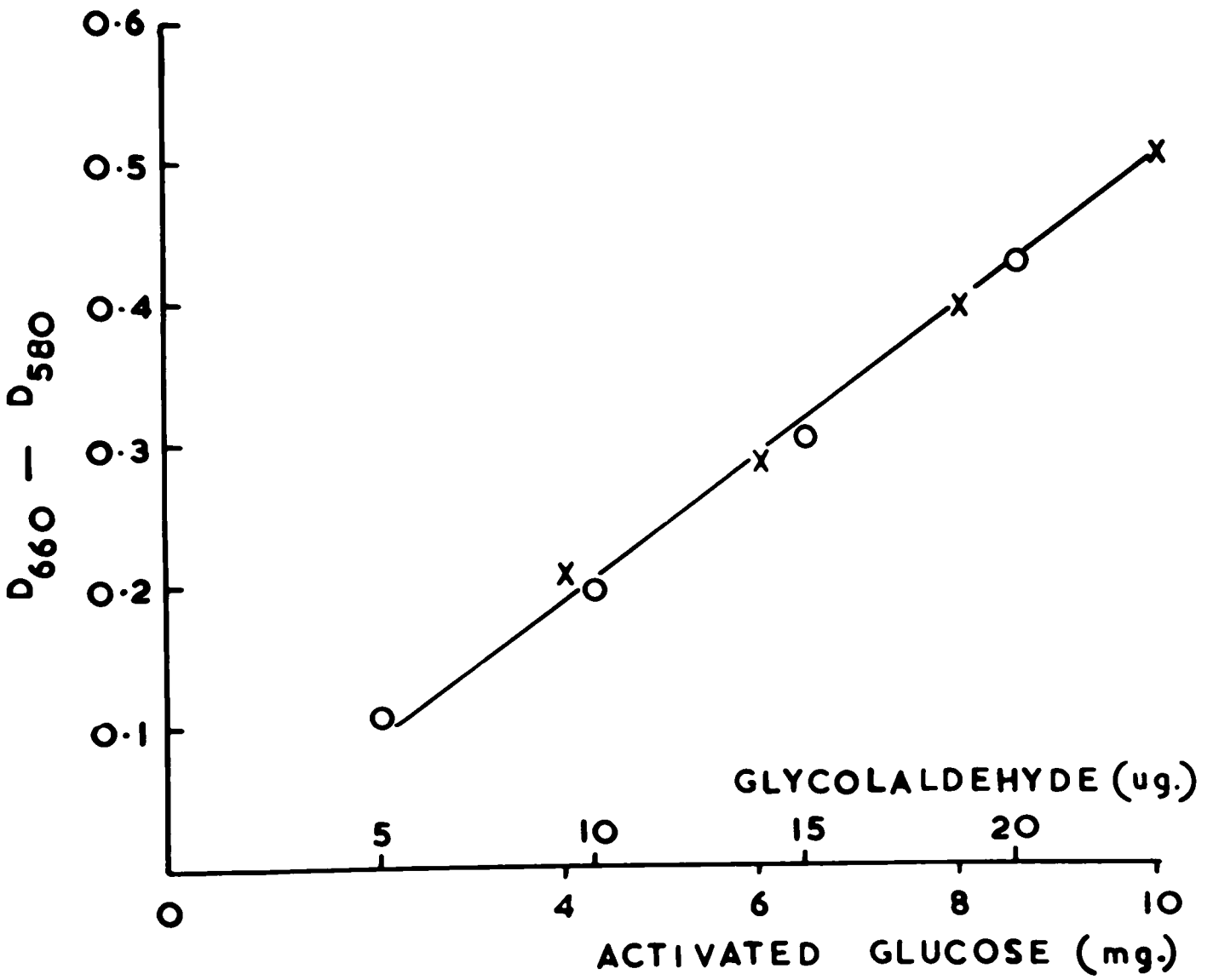
Glycolaldehyde (5×10^{-5} to 10^{-3} M) supported good growth of B166 in the presence of serine-glycine and absence of added pyridoxine. Both a recrystallised commercial specimen, and material prepared from dihydroxy-fumaric acid and recrystallised to M.P. 94.5 to 95° were quantitatively as active as the commercial product used in all subsequent experiments.

Apparent glycolaldehyde content of activated glucose

A glucose solution of strength and pH identical with that of Medium G_2A (10 mg./ml.; pH 7.0) was autoclaved under the standard conditions of 7 min./10 lb.sq.in. and the glycolaldehyde estimated colorimetrically by the method of Dische and Borenfreund (1949). About 2×10^{-4} M was found to be present (using an unautoclaved solution as blank: Fig. 21a). The dose-response curve with increasing concentrations of activated glucose could be superimposed upon the standard curve (Fig. 21b). Glycolaldehyde formation under other conditions of autoclaving was also determined (Fig. 16). Increasing ability to support growth on serine-glycine was seemingly matched by increasing glycolaldehyde content. Conditions which decreased biological activity (greater heat or alkalinity) still produced the maximum glycolaldehyde concentration; this may be a further indication that the diminished activity

FIG. 21b

Dische and Borenfreund assay - superimposition of assay curve obtained with activated glucose upon the glycolaldehyde standard curve



o—o = glycolaldehyde

x—x = activated glucose

was due to concurrent formation of inhibitory substances.

Conclusions

1. For maximal growth of E. coli B166, the concentration of inactive glucose in the basal medium should be increased to 10 mg./ml.
2. Activated glucose solutions showed a broad u.v. absorption peak at about 285 mμ.
3. The active factor was quite stable at neutral pH and room temperature, but became more labile as the pH was raised.
4. Activated glucose was best prepared by autoclaving (10 lb.sq.in. for 7 min.) a 20 mg./ml. solution in 0.005 M phosphate buffer (pH 7.4) and then concentrating by vacuum distillation at <40°.
5. Although activated glucose concentrates could be decolorised without loss of activity by treatment with norite at room temperature, further attempts at purification were unsuccessful.
6. From its behaviour with Dowex 50 and organic solvents, it was concluded that the active factor was non-basic and highly hydrophilic.
7. From the ability of a limited number of hexoses and pentoses and the inability of sugar alcohols and 3-O-methylglucose to be activated by autoclaving at

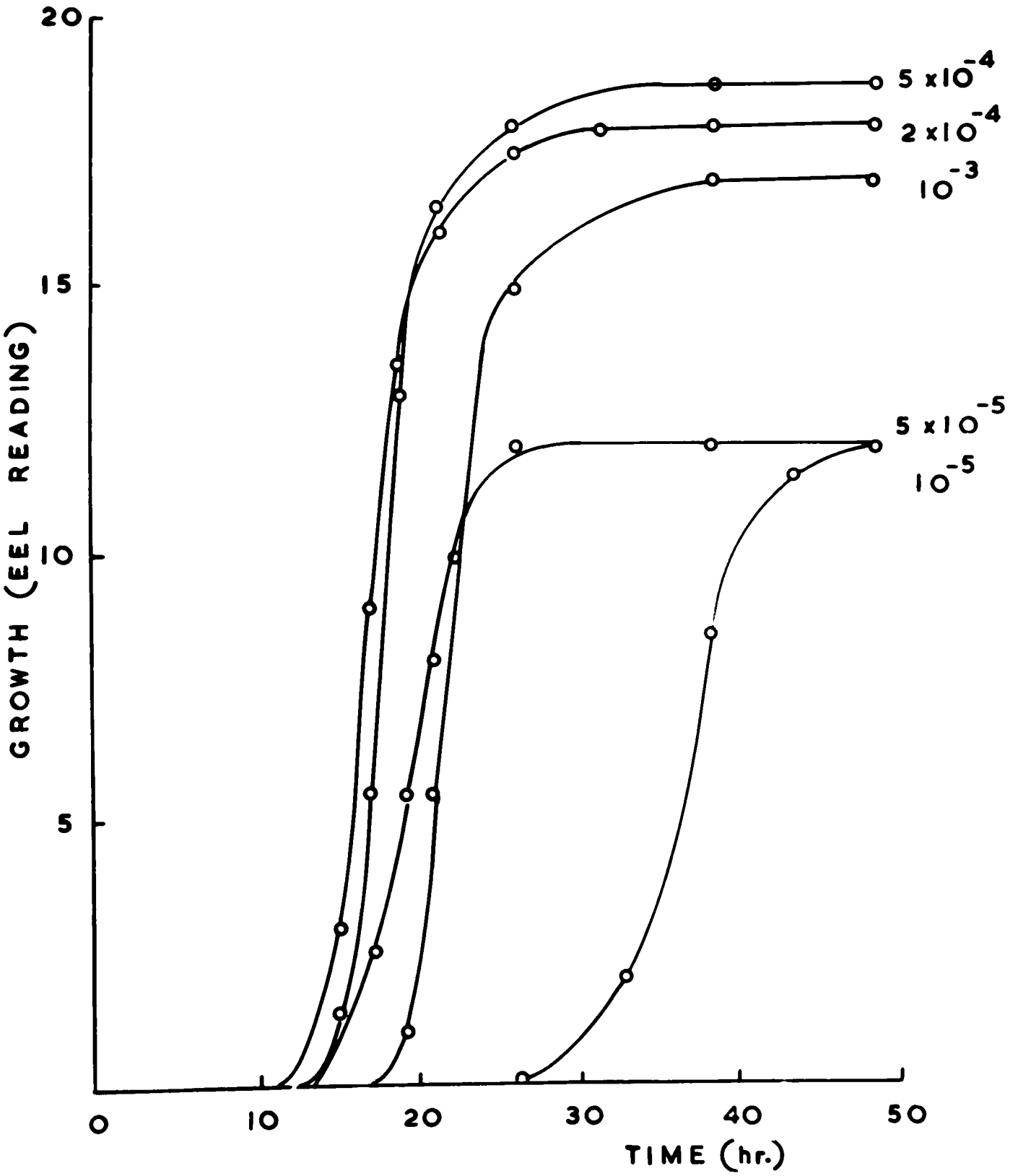
pH 7 to 7.4, it was deduced that a potential aldehyde group at C-1 and an unsubstituted hydroxyl group at C-3 were necessary attributes of any compound liable to activation.

8. Approaches (5) and (7) were discontinued when it was found that glycolaldehyde in reasonable concentrations (5×10^{-5} to 10^{-3} M) could substitute for activated glucose. It alone of many degradation products of glucose tested, showed such activity.

9. That the active factor present in activated glucose is in all probability glycolaldehyde is compatible with the conclusions reached in (7) above. Using a colorimetric assay, it was found that an activated glucose sample contained sufficient glycolaldehyde to account for its biological activity. No glycolaldehyde was formed when glucose solutions were autoclaved at pH 6.5 and formation of a compound assaying as glycolaldehyde paralleled the development of activity in glucose solutions.

FIG. 22

Growth of E. coli B166 on Medium G_2 supplemented with serine-glycine (10^{-3} M) and various concentrations of glycolaldehyde



Glycolaldehyde concs. as shown (M).
Incubation was in 1-tubes rocked at 37°.

Chapter C5

Glycolaldehyde and the Growth of E. coli B166

1. On glucose-containing media

The ability of glycolaldehyde to support growth of E. coli B166 on Medium G_2 supplemented with serine-glycine, was studied in aerobic, shaken culture (Fig. 22). Delayed and suboptimal growth was obtained with a glycolaldehyde concentration of 10^{-5} M; 2×10^{-4} M supported most rapid, and 5×10^{-4} M, greatest growth. Above these concentrations, progressive retardation and diminution of growth was noted, until at 5×10^{-3} M it was completely inhibited (even in the presence of pyridoxine). Glycolaldehyde (5×10^{-4} M) was more effective than activated glucose in supporting growth on serine-glycine (Fig. 22, compare Fig. 14), the delay in growth being greatly reduced, i.e., from 30 to 15 hr. The presence of glycolaldehyde, however, had no effect on growth of the organism in the presence of pyridoxine, altering neither the lag period nor the final yield of organisms (Fig. 23, compare Fig. 19).

Although final growth was better in shaken than in stationary culture under all conditions, the effect of aeration was relatively greater for growth on serine-glycine. Thus the final mass on serine-glycine and

TABLE C5-1

Growth of E. coli B166

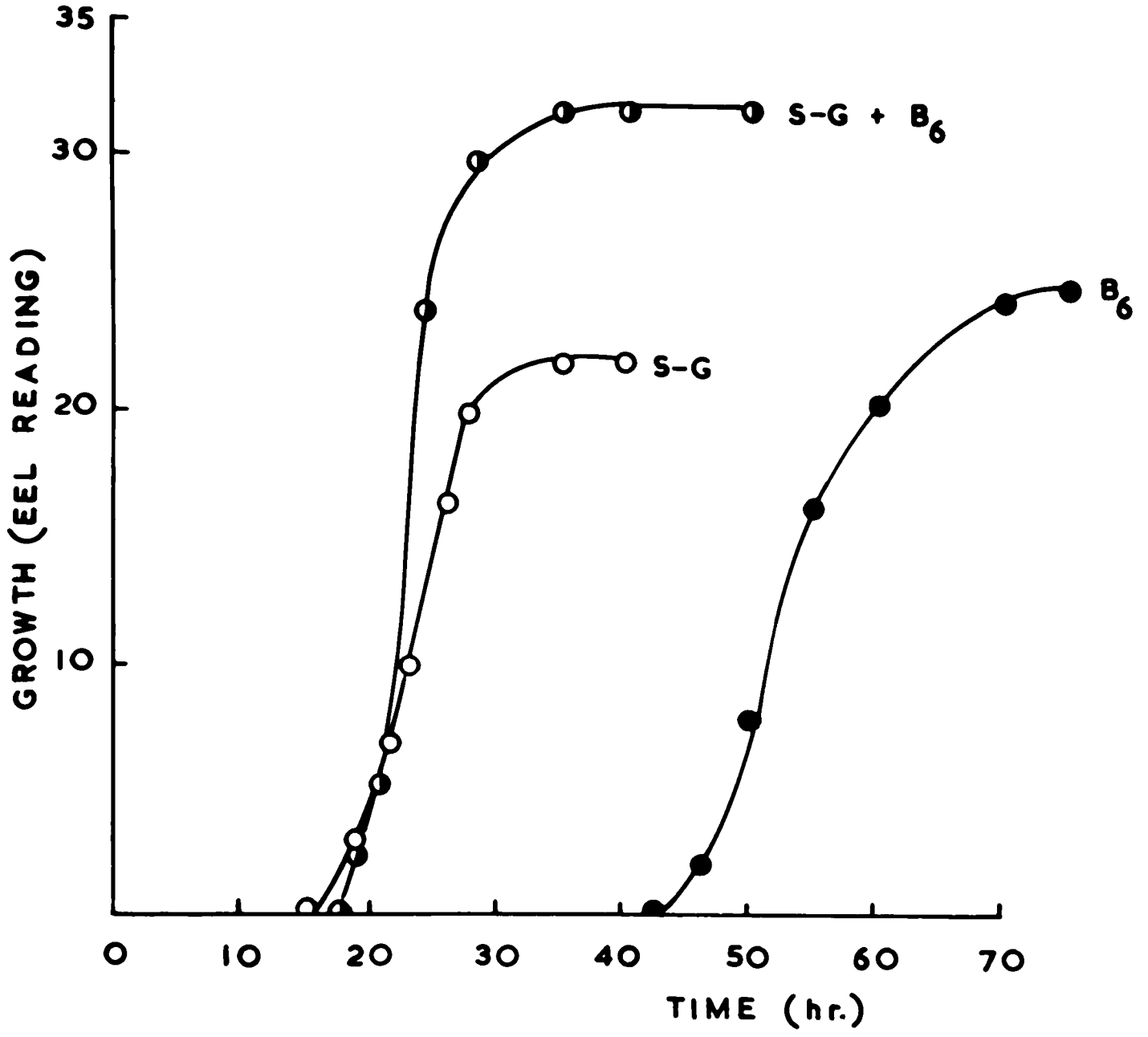
in the presence of amino acids

	Growth (KSL reading)
Glycolaldehyde	[illegible]
Plus serine-glycine	12
Plus AA-SG	7
Plus DL-threonine	8
Pyridoxine	21
Plus serine-glycine	24
Plus AA + PP + V	22

Supplements: glycolaldehyde 10^{-3} M; pyridoxine 10^{-6} M; serine-glycine 10^{-3} M; DL-threonine 10^{-3} M.
AA-SG amino acid mixture without serine-glycine used in 1:10 dilution.
AA + PP + V amino acids, plus purines, pyrimidines and vitamins used in 1:10 dilution.
Five ml. volumes of Medium G2, supplemented as shown, were inoculated with E. coli B166 and incubated in sloped tubes for 84 hr. at 37°.

FIG. 23

Effect of glycolaldehyde on growth of E. coli B166 on Medium G_2 in presence and absence of pyridoxine



S-G = serine-glycine (10^{-3} M).
B_6 = pyridoxine (10^{-6} M).
Glycolaldehyde (5×10^{-4} M) present throughout.
Incubated in rocked 1-tubes at 37°.

glycolaldehyde in shaken tubes was 65-70% of that with pyridoxine also present but only 50-55% in stationary tubes (from data of Fig. 23 and Table C5-1).

The optimal glycolaldehyde requirement for growth in stationary culture was somewhat higher (10^{-3} M) than in shaken culture.

2. On ammonium lactate medium

E. coli B166 did not respond to serine-glycine alone in Medium L (Fig. 15). Addition of activated glucose permitted limited growth; the concentration of activated glucose required being approximately the same as with the wholly glucose-containing basal medium. Glycolaldehyde (5×10^{-4} M) again replaced activated glucose, allowing suboptimal growth to occur with no greater delay than when pyridoxine was also present (Fig. 24). The final growth was not improved by increasing the concentration of glycolaldehyde; indeed, this was somewhat toxic at a concentration (10^{-3} M) which gave good growth on Medium G_2.

The response in lactate medium to pyridoxine alone, or together with serine-glycine, was not affected by glycolaldehyde (Fig. 24, compare Fig. 15).

Whilst 5×10^{-4} M glycolaldehyde bettered the action of activated glucose in Medium G_2, it seemed less effective in Medium L (Fig. 24). However, when inactive

TABLE C5-2

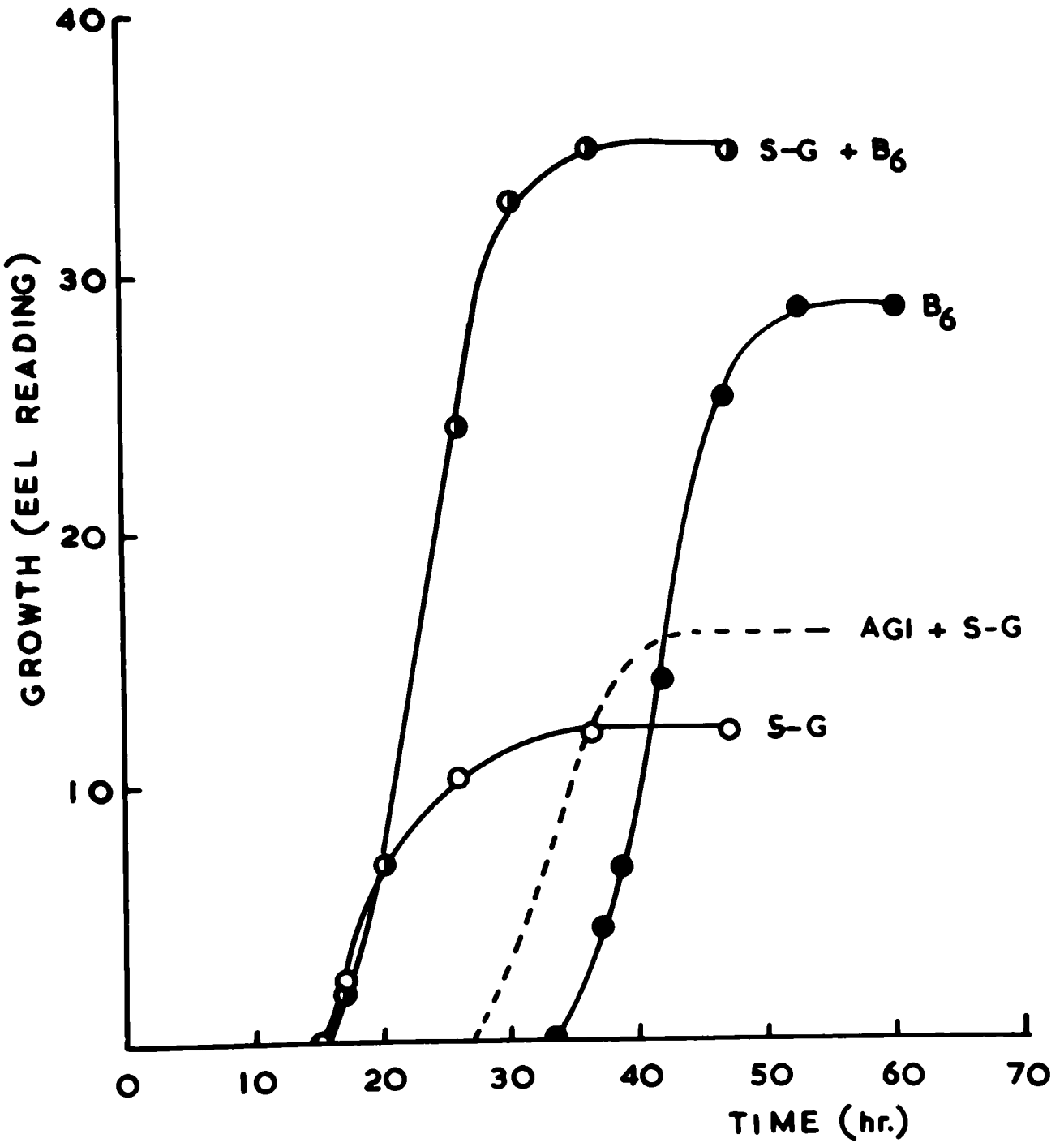
Pyridoxine requirement of E. coli B166 in the presence of activated glucose or glycolaldehyde

Pyridoxine conc. (M)	Growth (EEL reading) with Nil addition	Activated glucose	Glycolaldehyde (10^{-3} M)
10^{-6}	18	16	17.5
3×10^{-7}	15.5	14	12
10^{-7}	5	7	7.5
3×10^{-8}	0	(2)	(5)
10^{-8}	0	(2)	(2)
Nil	0	0	0

( ) signifies delay in growth.
Activated glucose was used in 10 mg./ml. conc.
Five ml. volumes of Medium G2, supplemented as shown, were inoculated with E. coli B166 and incubated in sloped tubes at 37° for 90 hr.

FIG. 24

Effect of glycolaldehyde (5×10^{-4} M) on the growth of *E. coli* B166 in ammonium lactate basal medium



Supplements: AGl = activated glucose.
S-G = serine-glycine (10^{-3} M).
B_6 = pyridoxine (10^{-6} M).

5 ml. volumes of Medium L supplemented as shown and inoculated with *E. coli* B166 rocked in 1-tubes at 37°.

glucose (10 mg./ml.) was added with the glycolaldehyde, whereas there was no effect on the ultimate mass achieved in the presence of pyridoxine, the suboptimal growth obtained in the absence of added vitamin was doubled (Fig. 25, compare Fig. 24). Galactose, gluconate and fructose substituted for glucose, but other possible carbon sources, e.g., arabinose, ribose, pyruvate, glyceraldehyde and acetate, were inactive or much less active.

3. Other effects

Addition of glycolaldehyde alone to Media G_2 or L did not allow of growth of E. coli B166 or reduce its pyridoxine requirement (Table C5-2).

From the growth response to a range of glycolaldehyde concentrations in the presence of serine-glycine, activated glucose (10 mg./ml.) would seem to contain about 1 to 4 x 10^{-4} M glycolaldehyde (when due allowance was made for concurrent inhibitory effects of the mixture in Medium G_2) (Fig. 14, compare Fig. 22). This value is in good agreement with the results obtained by colorimetric estimation.

Re-examination of the serine-glycine requirement

Using glycolaldehyde, it was possible to investigate the quantitative requirements of the mutant for serine-glycine in a chemically defined medium. These were

TABLE C5-3

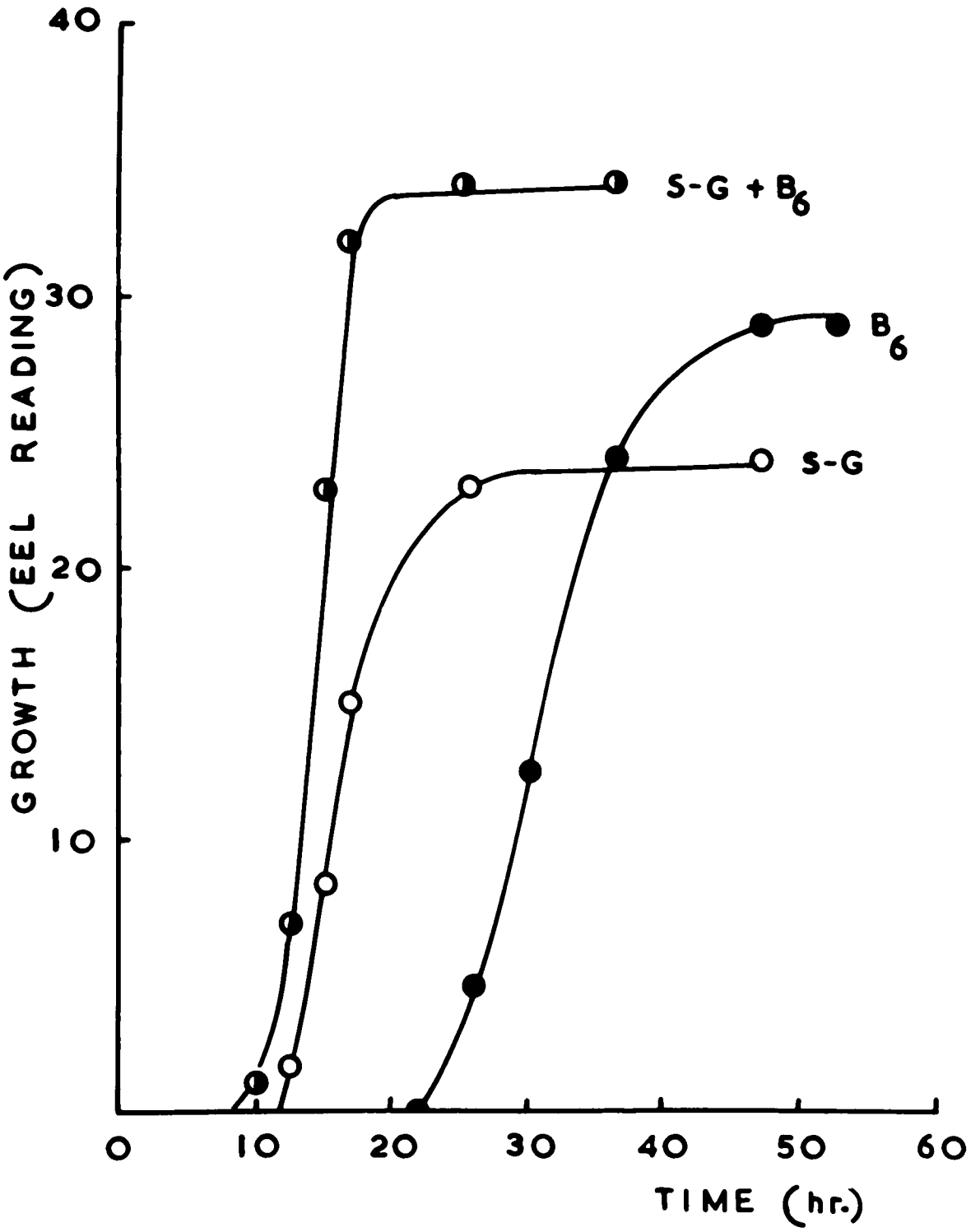
Titration of L-serine and/or glycine requirements of E. coli B166 in the presence of 5×10^{-4} M glycolaldehyde

Amino acid	Conc. (M)	Growth (EEL reading) in duplicates, after 40 hr.		64 hr.	
L-serine	10^{-3}	14.5	14.5	16	17
	2.5×10^{-4}	4.5	3.5	(28)	(29)
	5×10^{-5}	4	4	10	(42)
	10^{-5}	Tr.	Tr.	2	(26)
Glycine	10^{-3}	22	21	25	23
	2.5×10^{-4}	3	3	3	(30)
	5×10^{-5}	1	0.5	3	(30)•
	10^{-5}	Tr.-	Tr.-	Tr.	(9)
L-serine plus glycine	10^{-3}	22	22	22	22
	2.5×10^{-4}	4	3.5	5	(41)
	5×10^{-5}	1	1	3	3
	10^{-5}	Tr.	Tr.	3	3

( ) signifies reversion.
Five ml. volumes of Medium G2 with 5×10^{-4} M glycolaldehyde and other supplements shown, were inoculated with E. coli B166 and incubated in L-tubes rocked at 37°.

FIG. 25

Growth of E. coli B166 on Medium L supplemented with glycolaldehyde (5×10^{-4} M) and inactive glucose (10 mg./ml.)



Details as in Fig. 24.

unchanged when 10^{-3} M glycolaldehyde was used in place of activated glucose in Medium G_2 with or without added pyridoxine (Table 05-3).

In mammalian tissues, glycolaldehyde may undergo oxidation giving first glycollic acid and then glyoxylic acid from which glycine may be formed (Nelsonach and Sprinson, 1953, Friedmann, Levin and Kelmhouse, 1956). However, glycolaldehyde (1^{-3} M) did not reduce the glycine requirement of the serine or glycine mutant E. coli PA15.

The specificity of the serine-glycine requirement of E. coli 3156 was unchanged when glycolaldehyde was used in place of activated glucose. Of all the compounds again tested, DL-threonine alone showed any activity, supporting suboptimal growth after a delay of some 60 hr. (Table 05-1).

In the presence of an optimal concentration of glycolaldehyde and suboptimal concentrations of DL-serine or glycine, growth became very erratic after 40 hr. incubation. The use of the amino acids in admixture always decreased the frequency of these abnormalities but did not altogether eradicate them (Table 05-3).

Strains obtained from a culture supporting abnormally heavy growth on a suboptimal concentration of glycine, and isolated by successive plating on nutrient agar with the isolation of single colonies, all grew on unsupplemented Medium G_2 (Table 05-4). Growth was, however,

TABLE C5-4

Growth of a 'partially reverted' substrain of E. coli B166

Key:- +, ±, +±, etc., index of increasing growth.

Supplement	Growth (EEL reading) at 15 hr.	24 hr.	36 hr.	48 hr.
Nil	0	0	±	22
Serine-glycine	+++	+++	+++	29
Glycolaldehyde	+	+±+	+±+	22
Serine-glycine plus glycolaldehyde	+++	+++	+++	29
Pyridoxine	±+	+++	+++	26
Serine-glycine plus pyridoxine	+++	+++	+++	29

Concs. of supplements: DL-serine-glycine 10^{-3} M; pyridoxine 10^{-6} M; glycolaldehyde 10^{-3} M.

The substrain used was isolated from the culture marked * in Table C5-3.

Five ml. volumes of Medium G2, supplemented as shown, were inoculated with the isolated substrain of E. coli B166 and incubated in sloped tubes at 37°.

more rapid in the presence of pyridoxine or glycolaldehyde, and most rapid with serine-glycine. Only 'partial' reversion had occurred. The properties of these sub-strains were not further examined.

Other amino acids

The response of E. coli B166 to amino acid mixtures was investigated at various temperatures since E. coli DW was known to grow in the absence of added vitamin B_6 if provided with a comprehensive mixture of amino acids at 25° (Wijesundera, 1954). When incubated at both 25 and 37°, growth of B166 took place on Medium G_2 supplemented with casein hydrolysate only when glycolaldehyde (10^{-3} M) was also added. With a mixture of amino acids not including serine and glycine (AA-SG), growth in the presence of glycolaldehyde was less than when serine-glycine was present (Table C5-1). The growth obtained in the absence of serine-glycine was due entirely to the DL-threonine content of the amino acid mixture.

The specificity of the response to serine-glycine was further emphasised by the finding that in the presence of added pyridoxine, growth in a complex mixture of amino acids, purines, pyrimidines and other vitamins, was less good than on serine-glycine (Table C5-1). The concurrent provision of the amino acid mixture without serine-glycine (AA-SG) reduced the pyridoxine requirement of this mutant

only very slightly.

Conclusions

1. Glycolaldehyde (5×10^{-4} M) supported suboptimal growth of E. coli B166 on Medium G_2 with serine-glycine, in rocked aerobic culture. Concentrations above 10^{-3} M were increasingly growth inhibitory.
2. On Medium L with serine-glycine, 5×10^{-4} M glycolaldehyde appeared to be less active than pH 7.4 autoclaved glucose. However, concurrent addition of inactive glucose doubled the growth response.
3. Glycolaldehyde did not appreciably reduce the vitamin B_6 or serine-glycine requirements of E. coli B166. Nor did it affect the serine-glycine requirements of E. coli PA15.
4. The requirement for serine-glycine remained specific in the presence of glycolaldehyde, but there was a marked tendency towards reversion at suboptimal concentrations of these amino acids.
5. At no tested temperature was E. coli B166 able to grow on a comprehensive amino acid mixture in the absence of added vitamin B_6 or glycolaldehyde - in contradistinction to E. coli DW.

Chapter C6

The Specificity of the Requirement for Free Glycolaldehyde shown by E. coli B166 in the Absence of Added Vitamin B6

Some early study was made of glycolaldehyde as a possible metabolite (Mayer, 1903; Fincke, 1914; Lob, 1914) following the observation that it could be chemically transformed into a hexose (Fenton, 1897). Interest in the biochemical potentialities of the compound has recently been revived by the discovery of transketolase. This enzyme, concerned in the 'pentose oxidative cycle' catalyses the transference of a C_2-fragment (currently regarded as 'active glycolaldehyde' or glycolaldehyde-thiamine pyrophosphate), from one of a series of possible 'active glycolaldehyde donors' to one of a number of possible 'acceptors' (see Gunsalus, Horecker and Wood, 1955); the actual structure of the C_2 compound has not been determined.

A number of substances either related structurally to glycolaldehyde, or known to function under other circumstances as donors or acceptors of 'active glycolaldehyde' (Horecker and Mehler, 1955) did not replace glycolaldehyde in promoting growth of E. coli B166 on serine-glycine. The compounds so tested over a range of concentrations (10^{-6} to 2×10^{-3} M) in Seitz-filtered

TABLE C6-1

Glycolaldehyde, dihydroxyfumaric acid and β-hydroxypyruvic acid-supported growth of E. coli B166 on Medium G2 with serine-glycine

Final conc. (M)	Growth (EEL reading) with Glycolaldehyde	Dihydroxy-fumaric acid	β-Hydroxy-pyruvic acid
4×10^{-3}	0	—	7
10^{-3}	14	12	10
5×10^{-4}	13.5	10	8.5
2×10^{-4}	11	(5)	(5)
5×10^{-5}	9	0	0
10^{-5}	(7)	0	0
10^{-5}	0	0	0

( ) signifies delayed growth.
All compounds were added aseptically in Seitz-filtered solution.
Five ml. volumes of Medium G_2 plus serine-glycine (10^{-3} M) and supplemented as above, were inoculated with E. coli B166 and incubated in sloped tubes for 80 hr. at 37°.

solution, included: ethanolamine, ethylene glycol, glycollic, glyoxylic and oxalic acids, glycolaldehyde-2-phosphate, D-erythrose-4-phosphate, L-xylulose-5-phosphate, sedoheptulose-7-phosphate and L-erythrulose. D-Ribulose-1:5-diphosphate was also tested and found to be without activity. It is possible that the phosphate esters do not permeate the organism.

Dihydroxyfumaric acid and β-hydroxypyruvic acid were active (Table C6-1); since the required concentration was about 10 times that of free glycolaldehyde, this activity might have been due to spontaneous or metabolic conversion to glycolaldehyde.

A solution of dihydroxyfumaric acid kept at 60° for 30 min. before use, assayed with E. coli B166 as if it had been converted to its molar equivalent of free glycolaldehyde. Some difficulty was encountered with the colorimetric assay for glycolaldehyde when solutions of β-hydroxypyruvic acid were tested, but maximum and minimum values for free glycolaldehyde in a 5×10^{-3} M solution of this acid were 5×10^{-4} and 3×10^{-4} M respectively (see also Milhaud, Benson and Calvin, 1956).

Conclusion

The ability to promote growth of *E. coli* B166 in the absence of added vitamin B_6 and presence of serine-glycine appears to be a specific attribute of free glycolaldehyde.

TABLE C7-1

Effect of changes in cultural conditions on growth of E. coli B166 in the presence and absence of pyridoxine

	Growth (EEL reading)			
	$N_2 + CO_2$	Air		
	37°	37°	25°	37°
Supplement	pH 7	pH 7	pH 7	pH 6.5
Nil	0	0	0	0
Pyridoxine	8	13	0*	8.5
Serine-glycine plus:				
Activated glucose	2.5	10.5		
Glycolaldehyde	4.5	11	9	10.5
Pyridoxine	11	20	15	15

*Some growth on prolonged incubation.

Conc. of supplements: DL-serine-glycine 10^{-3} M; activated glucose 10 mg./ml.; glycolaldehyde 10^{-3} M; and pyridoxine 10^{-6} M.
Five ml. volumes of Medium G_2, supplemented as shown, were inoculated with 0.1 ml. of a 1:100 EEL 10 suspension of E. coli B166 and incubated for 80 hr. at the temps. and in the atmospheres shown;
in air - sloped tubes,
in $N_2 + CO_2$ - standing tubes in modified Kilner jar containing N_2/CO_2 mixture 95/5% (v/v).
When pH 6.5 basal medium was used, this was Medium G_2 brought to pH 6.5 with N HCl.

Chapter C7

Growth of E. coli B166 under Changed Cultural Conditions

Under the usual conditions of aerobic culture at 37° and pH 7, there is an evident difference in the response of the organism to vitamin B_6 and to glycolaldehyde plus serine-glycine (Fig. 23). It was of interest to see whether common changes in cultural conditions could accentuate or eradicate this difference.

(a) Anaerobic incubation

Although anaerobic growth was overall less good than that obtained aerobically, the response of E. coli B166 to the compounds of interest, remained relatively unchanged. The superiority of 10^{-3} M glycolaldehyde to activated glucose in Medium G_2 showed up a little better under these conditions (Table C7-1).

(b) Incubation at 25°

Growth in the presence of added pyridoxine alone was extremely delayed (110 hr.), but the decrease in incubation temperature had only a slight effect upon growth in the absence of added vitamin B_6 (Table C7-1).

TABLE C7-2

Inhibition of growth of E. coli B166

by 4-deoxypyridoxine

4-Deoxy-pyridoxine conc.(M)	Growth (KEL reading)		
	Serine-glycine plus glycolaldehyde	Serine-glycine plus pyridoxine	Pyridoxine
10^{-2}	4	0	0
10^{-3}	8.5	0	0
10^{-4}	7.5	7	0
10^{-5}	7.5	12.5	(7)
10^{-6}	8.5	16	10
Nil	11	16	12.5

( ) signifies delayed growth.

The 4-deoxypyridoxine was added aseptically in Seitz-filtered solution, other supplements were serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M; and pyridoxine 10^{-6} M. Five ml. volumes of Medium G_1 supplemented as above, were inoculated with E. coli B166 and incubated in sloped tubes for 80 hr. at 37°.

TABLE C7-3

Inhibition of growth of E. coli B166 by isoniazid

Isoniazid conc. (M)	Growth (EEL reading) with Serine-glycine plus glycolaldehyde	Serine-glycine plus pyridoxine	Pyridoxine
10^{-2}	0	(11)	0
6×10^{-3}	0	(13.5)	0
4×10^{-3}	(6)	14	0
2×10^{-3}	9	20	0
10^{-3}	13	22	(14)
3×10^{-4}	13	24	20
10^{-4}	15	24	21
Nil	16	24	21

( ) signifies delayed growth.
The isoniazid was added aseptically in Seitz-filtered solution. Other supplements were, serine-glycine 10^{-3} M; pyridoxine 10^{-6} M; and glycolaldehyde 10^{-3} M (aseptically). Five ml. volumes of Medium G_2 supplemented as shown, were inoculated with E. coli B166, and incubated in sloped tubes for 84 hr. at 37°.

(c) pH of medium lowered to 6.5

Again, growth in the presence of pyridoxine was more adversely affected than that obtained on serine-glycine plus glycolaldehyde (Table C7-1).

(d) 4-Deoxypyridoxine

E. coli B166 was most sensitive to inhibition by 4-deoxypyridoxine when growing in the presence of pyridoxine. Addition of serine-glycine decreased the sensitivity, while growth in the absence of vitamin B_6 was least affected (Table C7-2).

(e) Isoniazid

Again, the organism was most sensitive to inhibition when growing on vitamin B_6 (Table C7-3).

The addition of serine-glycine to the pyridoxine-containing medium decreased sensitivity to the farthest extent. Growth on serine-glycine and glycolaldehyde showed an intermediate degree of resistance to inhibition.

Conclusion

The growth response of B166 to (a) vitamin B_6 and (b) serine-glycine and glycolaldehyde, was affected differently by various changes in other cultural conditions.

TABLE D1-1

Growth of *E. coli* B167 on Medium G_2

with various supplements

Supplement	Growth 16 hr.	40 hr.	48 hr.	64 hr.	EEL reading 88 hr.
Nil	0	0	0	0	0
Activated glucose	0	0	0	0	<1
Serine-glycine	0	0	0	0	0
Serine-glycine + activated glucose	0	±	±	±	10
Serine-glycine + glycolaldehyde	Tr.	±	±	±	11.5
Glycolaldehyde	0	0	0	0	0
Pyridoxine	0	Tr.	±	±	12
Serine-glycine + pyridoxine	++	+±+	+±+	+±+	20

Key: +, ±, ±, ++, etc., signify increasing growth.

Conc. of supplements: Serine-glycine 10^{-3} M; activated glucose 10 mg./ml.; glycolaldehyde 10^{-3} M; pyridoxine 10^{-6} M.

Medium G_2 supplemented as shown, was inoculated with *E. coli* B167 and incubated in sloped tubes at 37°.

SECTION D

The Growth Characteristics of Other Vitamin B_6 Auxotrophs of Escherichia coli

1. Mutants showing a serine-glycine effect

(a) E. coli B167

This mutant closely resembled B166 under all conditions tested, i.e., in its ability to grow in the absence of added vitamin B_6 only if serine-glycine and glycolaldehyde were provided, and in the enhancement and stimulation of its growth on pyridoxine by serine-glycine. There was no growth on Media G_2 or L with serine-glycine or glycolaldehyde as sole supplements (Table D1-1).

(b) E. coli 22/99

This differed from the other two members of this group in being able to grow on serine-glycine alone, and eventually, though only slightly, when glycolaldehyde was the sole supplement to Medium G_2. Growth on pyridoxine was again found to be belated and suboptimal, best growth being observed with a mixture of serine-glycine plus either pyridoxine or glycolaldehyde (Fig. 26).

TABLE D1-2

Growth of E. coli DW at 25° on supplemented Medium G_2

Supplement	Growth (EEL reading)
Nil	0
Pyridoxine	17.5
Casein hydrolysate	16
Amino acid mixture (AA)	17
Amino acid mixture (AA - SG)	20
DL-Alanine plus DL-methionine	(15)
DL-Alanine	(<1)
DL-Methionine	0

( ) signifies somewhat delayed growth.

Concentration of supplements: pyridoxine 10^{-6} M; amino acid mixtures used in 1:10 dilution to give final amino acid concs. of 5×10^{-4} M (each); DL-alanine 10^{-3} M; DL-methionine 5×10^{-4} M.

Five ml. volumes of Medium G_2 supplemented as shown, were inoculated with E. coli DW and incubated in sloped tubes for 86 hr. at 25°.

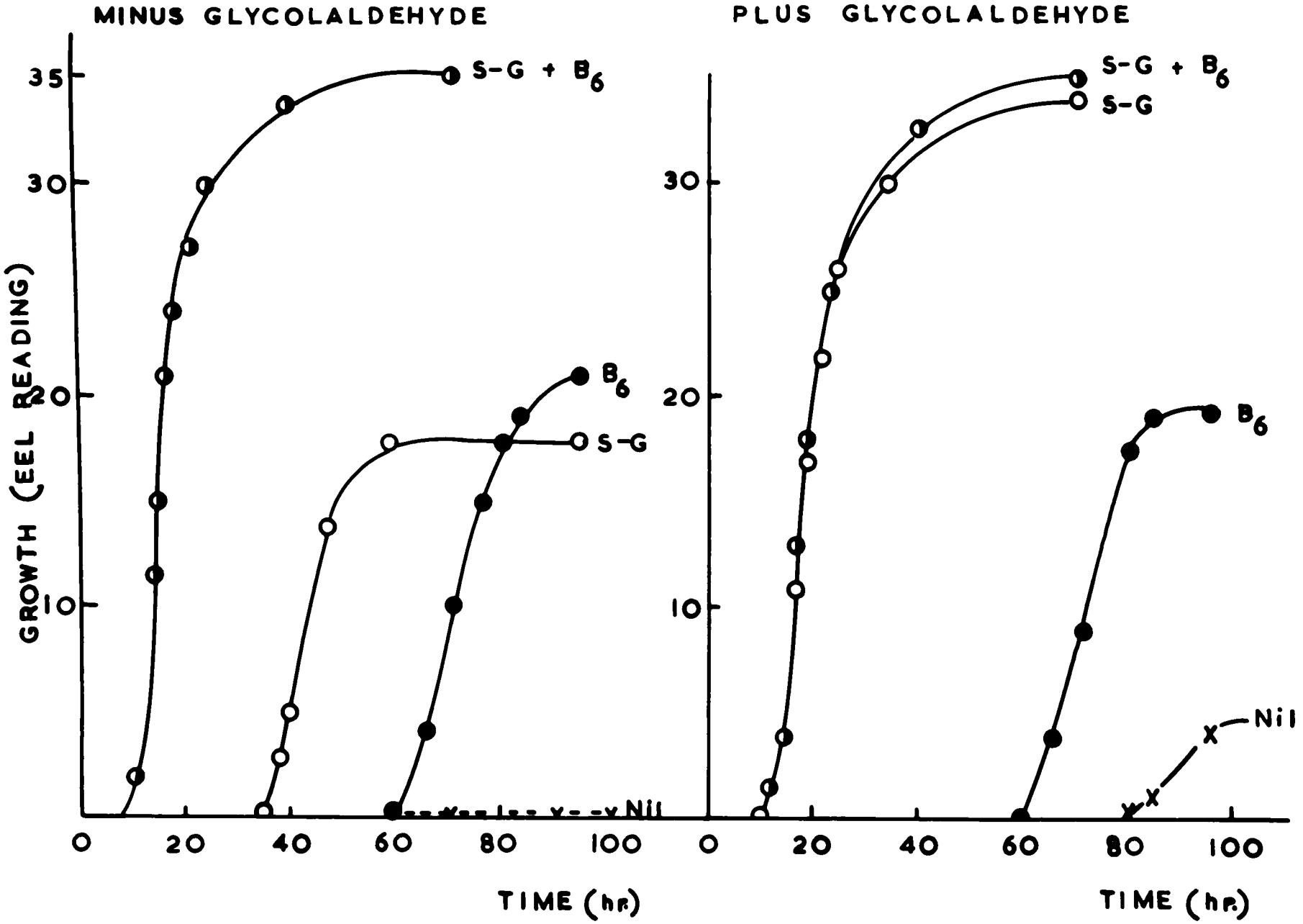


FIG. 26

Growth of E. coli 22/99 on Medium G_2 ± glycolaldehyde (10^{-3} M).

Nil = no supplement. Other details as in Fig. 24.

2. Mutants showing no serine-glycine effect

(a) E. coli DW

Vitamin B_6 was required by this organism for growth at 37°. At this temperature, it grew well with only short delay on Medium G_2 supplemented with 10^{-6} M pyridoxine. The final extent of growth was increased by the concurrent addition of a comprehensive amino-acid mixture (Mixture AA) but in this serine and glycine played no special role. Glycolaldehyde (10^{-3} M) alone, or in admixture with serine-glycine (10^{-3} M) was completely without effect.

At 25° growth was possible in the absence of added vitamin B_6 if an acid-hydrolysate of casein, or a comprehensive amino acid mixture (Mixture AA) was supplied (Wijesundera, 1954). Serine and glycine seemed to have no part in this replacement action; the one amino acid whose presence was found to be essential for this effect was in fact D-alanine. Fairly good growth of E. coli DW was obtained by incubating at 25° in Medium G_2 supplemented with DL-alanine plus DL-methionine (5×10^{-4} M) (Table D1-2).

Glycolaldehyde (at concentrations below 10^{-3} M) had no observable effect on the growth of this mutant at 25° in the presence of absence of added vitamin B_6.

TABLE D1-3

The pyridoxine requirement of E. coli M_2 in the presence of serine-glycine and glycolaldehyde

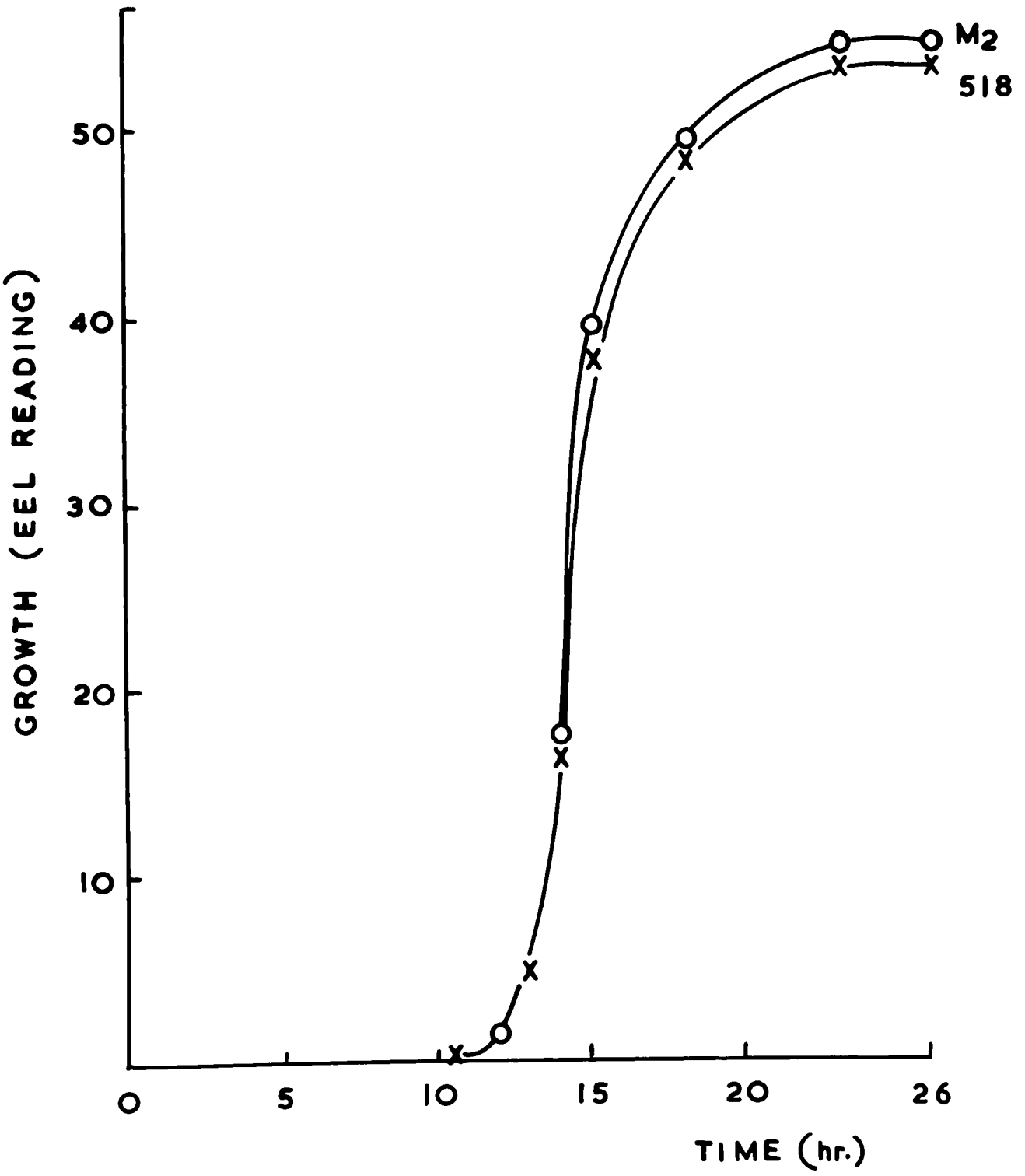
Pyridoxine conc. (M)	Growth (EEL reading) with Nil addition	Serine-glycine	Glycol-aldehyde	Serine-glycine + glycolaldehyde
10^{-6}	27	27	27	27
10^{-7}	27	27	27	27
5×10^{-8}	(22)	(22)	(8)	(<1)
2×10^{-8}	0	0	0	0

( ) signifies delayed growth.

Supplements: serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M.
Five ml. volumes of Medium G_2, supplemented as shown, were inoculated with E. coli M_2 and incubated in sloped tubes for 42 hr. at 37°.

FIG. 27

To compare growth of E. coli M_2 and prototroph E. coli 518



Basal medium was Medium G_2, supplemented with 10^{-7} M pyridoxine for Strain M_2.
Incubated in rocked 1-tubes at 37°.

(b) E. coli M2

On Media G_2 and L, this mutant showed an absolute requirement for vitamin B_6 over a temperature range of 25 to 37°. Excellent and rapid growth was obtained with 10^{-7} M pyridoxine (Fig. 27). Rarely, heavy growth appeared in one of several tubes containing a casein hydrolysate supplement, but whenever this took place, examination proved that reversion had occurred. In the usual media the organism was completely stable even at very suboptimal concentrations of pyridoxine. Serine-glycine (10^{-3} M) had no observable effect either alone, or in admixture with 10^{-3} M glycolaldehyde (Table VI-3).

When growth was limited by the use of sub-optimal concentrations of pyridoxine, the organisms clumped together producing an abnormal appearance in shaken culture. Poor growth (EEL 4) was obtained by incubating in Medium G_2 supplemented with 4×10^{-8} M pyridoxine in shaken culture at 37° for 16 hr. The culture medium was found to contain abnormally high concentrations of keto acids (detected as their dinitrophenyl hydrazones).

Conclusions

The findings are summarised in Table VI-4. It is interesting that an observable effect of glycolaldehyde is confined to those mutants showing an alternative serine-glycine requirement.

TABLE D1-4

Summarised characteristics

of vitamin B_6 mutants of E. coli

Mutant	Nil addition	Pyridoxine	Serine-glycine & pyridoxine	Serine-glycine & glycolaldehyde	Serine-glycine	Glycolaldehyde
B166	0	(++)	+++	++	0	0
B167	0	(++)	+++	++	0	0
22/99	0	(++)	+++	+++	(++)	(±)
DW	0	++	++	0	0	0
M_2	0	+++	+++	0	0	0

Key: growth response indicated by +++, ++, ±, etc.

( ) denotes appreciable delay before commencement of growth.
Supplements: serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M; pyridoxine 10^{-6} M.

SECTION E

Chapter 1

Concerning the Mode of Action of Serine-Glycine and Glycolaldehyde

Effect on other B_6-requiring microorganisms

The vitamin B_6 requirements of Saccharomyces carlsbergensis 4228c were unchanged in the presence of serine-glycine and glycolaldehyde (10^{-3} M each, in Medium A). Nor would these compounds substitute for vitamin B_6 in supporting the growth of vitamin B_6 auxotrophs of Aspergillus nidulans.

Attempted production of glycolaldehyde mutants of E. coli

Several attempts were made to produce glycolaldehyde or serine-glycine plus glycolaldehyde auxotrophs of Escherichia coli. E. coli 518 was subjected to u.v. irradiation followed by selection by the penicillin plate technique using elective layers containing glycolaldehyde (5×10^{-4} M final) alone, or together with serine-glycine (10^{-3} M). No glycolaldehyde auxotroph was obtained.

Ninhydrin-positive compounds excreted by E. coli B166 and M2

While in ignorance of the possible structure of any vitamin B_6 precursor, it was thought that supernatants

obtained by incubation of washed suspensions of E. coli B166 and E. coli M_2 with glucose, glycine and glycolaldehyde might be examined for their content of ninhydrin-positive compounds. These could be of potential importance as it is possible that the pyridine ring could arise by the ring closure of an aliphatic compound carrying a free amino group ('Introduction', Chapter 2). At the same time, it was of general interest to see whether the pattern of amino acid excretion was at all different in the vitamin B_6 auxotrophs as compared with the parent prototrophs. A changed pattern could reflect a reorientated metabolism of the mutant organism.

Accordingly, organisms harvested after optimal aerobic growth on Medium G_2 (supplemented when necessary with 10^{-6} M pyridoxine) were well washed, and suspensions (10 mg./ml. dry wt.) made in 0.04 M phosphate buffer pH 7 containing glucose NH_4^+ and Mg^{++}. These were incubated aerobically with shaking for 3 hr. at 37°, the organisms removed by centrifugation, and samples of the supernatants chromatographed on Whatman No. 3 paper with a descending solvent mixture of phenol/NH_3/water. After drying, the paper was treated with ninhydrin in the usual manner.

Three main ninhydrin-positive components were detected in all supernatants, identified by subsequent two-dimensional paper chromatography in the presence of

TABLE E1-1

Ninhydrin-reactive substances formed by washed suspensions of vitamin B_6 auxotrophs of E. coli

Washed suspensions of organisms (10 mg./ml. dry wt.) were incubated for 3 hr. at 37° in 0.04 M phosphate buffer pH 7 supplemented with 0.01% $MgSO_4.7H_2O$, 1% glucose and where shown 0.1% $(NH_4)_2SO_4$, 10^{-2} M glycolaldehyde and 10^{-2} M glycine. 0.15 ml. samples of the resulting supernates were chromatographed (descending development with phenol/NH_3/water) and sprayed, after drying, with ninhydrin.

(a) Incubated with glucose and NH_4^+.

Organism	R_f values of ninhydrin +ve spots
E. coli B	0.24 ; 0.62 ; 0.77
E. coli B166	0.24 ; 0.62 ; 0.77
E. coli 518	0.24 ; 0.62 ; 0.77
E. coli M2	0.24 ; 0.62 ; 0.77

(b) Incubated with glucose, glycine and glycolaldehyde

Exactly as for (a), except for the additional appearance of residual glycine (Rf. 0.43).

Identification of the excreted amino acids

Two-dimensional chromatography of 0.15 ml. samples on Whatman No. 1 paper - with markers.

Component (R_f)	Marker	Nil addition		Plus marker	
		Phen./NH_3	But./Ac.	Phen./NH_3	But./Ac.
0.24	Glutamic	0.23	0.12	0.24	0.14
0.62	Alanine	0.58	0.14	0.57	0.16
0.77	Valine	0.76	0.26	0.75	0.28
?		0.80	0.40		

The fourth spot was rather weak - probably leucine or isoleucine.

'markers' as glutamic acid, alanine and valine. A fourth and weaker component became evident only in two-dimensional chromatography of large samples - this was tentatively identified as leucine or isoleucine.

E. coli B166, but not M_2, excreted an unusually high concentration of glutamic acid under these conditions. Apart from the presence of residual glycine, exactly the same ninhydrin-reactive substances were identified when the suspensions were incubated with glycolaldehyde (10^{-2} M) and glycine (10^{-2} M) also present (Table B1-1).

Inability of glycolaldehyde to substitute directly for pyridoxal

Glyoxylic acid has been shown to substitute directly for vitamin B_6 in certain in vitro pyridoxal-catalysed reactions (Metzler, Olivard and Snell, 1954). It was of interest to see whether glycolaldehyde or its phosphate could reactivate a pyridoxal phosphate-dependent apoenzyme. It was found that glycolaldehyde and glycolaldehyde-2-phosphate were unable to replace pyridoxal and pyridoxal-5-phosphate in reactivating the tyrosine decarboxylase of washed organisms or an acetone-dried preparation respectively of Streptococcus faecalis R which had been grown on a medium devoid of vitamin B_6. These compounds at the concentrations tested did not affect

TABLE E1-2

Inhibition of growth of E. coli PA15 by glycolaldehyde

Glycolaldehyde conc. (M)	Growth (EEL reading)
10^{-2}	0
5×10^{-3}	0
2×10^{-3}	(22)
10^{-3}	24
Nil	24

( ) signifies delayed growth.

Glycolaldehyde was (as usual) added aseptically in Seitz-filtered solution.
Five ml. volumes of Medium Gg supplemented as shown and with 10^{-2} M DL-serine, were inoculated with E. coli PA15 and incubated in sloped tubes for 40 hr. at 37°.

TABLE El-3

Inability to overcome glycolaldehyde inhibition of growth of E. coli 518 by high concentrations of serine-glycine

Glycolaldehyde conc. (M)	Growth (EEL reading) with	
	Nil addition	Serine-glycine
5×10^{-3}	0	0
4×10^{-3}	0	0
3×10^{-3}	(28)	(28)
2×10^{-3}	29	29
Nil	29	30

( ) signifies delayed growth.
Five ml. volumes of Medium G_2 supplemented as shown were inoculated with E. coli 518 and incubated in sloped tubes for 64 hr. at 37°.

the reactivation of the apoenzyme by the vitamin B_6 derivatives.

Possible antimetabolite action of glycolaldehyde

In concentrations exceeding 10^{-3} M, glycolaldehyde was inhibitory to the growth of both E. coli 518 and E. coli B166. If free glycolaldehyde could act as a specific inhibitor of serine-glycine biosynthesis (which might explain some of the observations - see 'Discussion'), its inhibitory action might be overcome by high concentrations of serine-glycine. However, glycolaldehyde proved inhibitory in the presence of optimal concentrations of serine-glycine to the growth of E. coli PA15 which is not able to synthesise these amino acids (Table III-2). Thus, glycolaldehyde does not exercise its inhibitory effects solely, if at all, via the inhibition of serine-glycine synthesis. This conclusion is confirmed by the observation that relatively high concentrations of serine-glycine had no effect on the inhibition of growth of E. coli 518 by glycolaldehyde (Table III-3).

Conclusions

1. Serine-glycine and glycolaldehyde had no effect upon the vitamin B_6 requirements of Saccharomyces carlsbergensis 423°c, and a number of B_6 auxotrophs of

Aspergillus nidulans.

2. No glycolaldehyde or serine-glycine plus glycolaldehyde auxotroph could be isolated after u.v. irradiation of E. coli 518.

3. No novel ninhydrin-positive compound was accumulated by washed suspensions of E. coli B166 and E. coli M_2 incubated with glucose, glycolaldehyde and glycine. E. coli B166 excreted significantly greater amounts of glutamic acid than did E. coli B (its parent) when both were incubated in suspension with glucose and NH_4^+.

4. Glycolaldehyde did not substitute for pyridoxal in reactivating the tyrosine apodecarboxylase of vitamin B_6-deficient Strep. faecalis R, nor did glycolaldehyde-2-phosphate compete with pyridoxal-5-phosphate for the tyrosine apodecarboxylase of acetone-dried preparations of the same organism.

5. The growth inhibition brought about by high concentrations of glycolaldehyde is not due to interference with serine-glycine biosynthesis.

TABLE E2-1

Serine-glycine and glycolaldehyde and the synthesis of vitamin B_6 by competent organisms

Organism	Medium	Vitamin B_6 synthesis (mμmoles pyridoxine /g. dry wt.)
E. coli 518	G_2	454
	G_2 plus serine-glycine	495
	G_2 plus serine-glycine plus glycolaldehyde	570
E. coli B	L	450
	L plus glucose	449
	L plus serine-glycine & glycolaldehyde	452
	L plus glucose & serine-glycine & glycolaldehyde	600
Proteus morganii 231	E	1840
	E plus serine-glycine and glycolaldehyde	1930

Supplements: serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M; glucose 1%.
Fifty ml. volumes of supplemented media were inoculated with 0.1 ml. of 1:10 EEL 10 suspensions of the organisms taken from 24 hr. 37° slopes on Medium S1, and incubated with rocking in large L-tubes at 37° until full growth was achieved.

Chapter E2

Serine-Glycine and Glycolaldehyde in relation to Vitamin B_6 Biosynthesis

A possible explanation for the growth of E. coli B166 on serine-glycine and glycolaldehyde, would be that these compounds are, in this organism, direct or indirect precursors of vitamin B_6. The manner in which provision of serine-glycine and glycolaldehyde affects vitamin B_6 biosynthesis was examined both during growth, and with washed suspensions, of several organisms.

1. With competent organisms

(a) During growth

Glycolaldehyde (10^{-3} M) and serine-glycine (10^{-3} M) either alone or in admixture, had little effect on the amount of vitamin B_6 synthesised during growth of E. coli 518 or E. coli E on Medium G_2, or of Proteus morganii 231 on Medium E (Table E2-1).

When the serine-glycine mutant E. coli PA15 was grown on growth-limiting concentrations of glycine, the amount of vitamin B_6 synthesised (expressed in terms of amount/g. dry wt. organism) was greater than when optimal growth was achieved in the presence of an excess of the amino acid (Table E2-2).

TABLE E2-2

Synthesis of vitamin B_6 by E. coli PA15

(a) During growth

Glycine conc. (M)	Growth (EEL reading)	Vitamin B_6 synthesis conc. (M)	Vitamin B_6 synthesis mμmoles/g. dry wt.
5×10^{-2}	66	10^{-6}	500
10^{-2}	60	1.1×10^{-6}	565
5×10^{-3}	35	1.1×10^{-6}	1100
2×10^{-3}	15.5	5.5×10^{-7}	1550
10^{-3}	6	2.8×10^{-7}	1870
5×10^{-4}	3	1.4×10^{-7}	1870

(b) Using 'pre-depleted' suspensions

Supplement	Vitamin B_6 synthesis (mμmoles/g. dry wt.)
Nil	0
Glucose plus NH_4^+	800
Glucose plus NH_4 and glycine	720
Glucose plus NH_4 & glycolaldehyde	800
Glucose plus NH_4 & glycine and glycolaldehyde	960

Growth: 5 ml. volumes of Medium G_2 supplemented with glycine as shown, were inoculated with E. coli PA15 and incubated with rocking in 1-tubes at 37° until maximum growth was attained.

'Pre-depleted' suspensions: Organisms were harvested after growth on Medium G_2 plus 10^{-3} M glycine (a growth-limiting concentration), washed in 0.1 M phosphate buffer pH 7 and resuspended in the same strength buffer supplemented with 0.01% $MgSO_4.7H_2O$ and other substrates shown. Incubation (aerobic in rocked 1-tubes) was for 6 hr. at 37°.

TABLE E2-3

Serine-glycine and glycolaldehyde and vitamin B_6 synthesis by E. coli EW and E. coli 22/99 during growth in the absence of the vitamin

Supplements	Growth (EEL reading)	Total vit.B_6 synthesis (mμmoles pyridoxine /g. dry wt.)
(a) E. coli EW (25°)		
DL-alanine and DL-methionine	26	33
Amino acid mixture (AA-SG)	22	28
Amino acid mixture (AA-SG) & serine-glycine & glycolaldehyde	23	73
(b) E. coli 22/99		
Serine-glycine	21	180
Serine-glycine and glycolaldehyde	28.5	424

Supplement conc: AA-SG was used at 1:10 dilution giving a final conc. per amino acid of 5×10^{-4} M; serine-glycine 10^{-3} M; and glycolaldehyde 10^{-3} M.

Fifty ml. volumes of Medium G_2 supplemented as shown, were inoculated with 0.1 ml. of a 1:10 suspension of organism taken from a 24 hr. 37° slope on Medium S_1 and incubated in large 1-tubes with rocking at the temperature indicated until maximum growth was achieved.

(b) In suspension

Serine-glycine and glycolaldehyde (10^{-3} M of each), did not enhance the 'basal' synthesis of vitamin B_6 obtained when suspensions of E. coli B or Proteus morganii 231 were incubated with glucose and NH_4^+. Preliminary 'adaptation' to the use of serine-glycine and glycolaldehyde by growth in the presence of these compounds, had no observable effect on subsequent vitamin B_6 biosynthesis. Vitamin B_6 synthesis from glucose and NH_4^+ by washed suspensions of glycine-depleted E. coli PA15 was not increased by further addition of glycine (Table 32-2).

2. Vitamin B6 synthesis by a 'leaky' pyridoxine auxotroph which demonstrates no serine-glycine effect

E. coli 3W was grown aerobically at 25° in Medium G_2 supplemented with (a) an amino acid mixture not including serine and glycine (AA-SG), and (b) the same amino acid mixture but with 10^{-3} M each of serine-glycine and glycolaldehyde. More vitamin B_6 was formed during growth in the presence of serine-glycine and glycolaldehyde (Table 32-3).

TABLE E2-4

Biosynthesis of vitamin B_6 during growth of prototrophic E. coli B and auxotrophic E. coli B166

Supplements	Vitamin B_6 synthesis (mμmoles pyridoxine/g. dry wt.)	
	Prototroph (B)	Auxotroph (B166)
Nil	465	No growth
Serine-glycine and activated glucose	400	255
Serine-glycine and glycolaldehyde	470	420

Note: Amount of vitamin B_6 within the organisms was about 2% of the whole, but varied with the age of the culture.

Supplements: serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M; activated glucose 10 mg. glucose/ml.
500 ml. volumes of Medium G2 supplemented as shown were inoculated with 0.1 ml. of an EEL 10 suspension of organism taken from a 24 hr. 37° slope on Medium S_1, and incubated with constant rotary shaking at 37° for 40 hr.

3. Vitamin B_6 biosynthesis by those pyridoxine auxotrophs of E. coli which respond alternatively to serine-glycine plus glycolaldehyde

E. coli B166 -

(a) In growth

The ability of mutant B166 to synthesise vitamin B_6 when growing on serine-glycine and glycolaldehyde (or activated glucose) was tested, and compared with that of the prototroph E. coli B (Table F2-4). The results given, are in terms of the total vitamin B_6 content of the culture (organisms and medium) at full growth. Synthesis of vitamin B_6 by the mutant under these conditions was of the same order of magnitude as that of the parent strain on unsupplemented basal medium.

When E. coli B166 was grown on Medium G_2 containing serine-glycine and glycolaldehyde, the distribution of vitamin B_6 between organisms and culture fluid varied with the age of the culture in a similar fashion to the behaviour already noted with E. coli B18.

There was no change in the amount of vitamin B_6 synthesised when Medium G_2 plus serine-glycine and glycolaldehyde was further supplemented with casein hydrolysate, a mixture of purines and pyrimidines and vitamins other than vitamin B_6. In particular, the synthesis was unaffected

by addition to the medium of thiamine (10^{-5} M) or adenine plus nicotinic acid (10^{-4} M).

(b) In suspension

Although after growth of E. coli B166 in the presence of 10^{-6} M pyridoxine, about 50% only was recovered as assayable vitamin B_6, there was 100% recovery when a washed suspension of B166 was incubated aerobically with 10^{-6} M pyridoxine for 8 hr. at 37°. It seemed, therefore, that possible degradation of synthesised vitamin B_6 would not be sufficiently great to interfere with the study of its biosynthesis by washed suspensions.

Well-washed suspensions of E. coli B166 harvested from pyridoxine-containing media, synthesised little vitamin B_6 when subsequently incubated in phosphate buffer containing Mg^{++}, NH_4^+, glucose, serine-glycine and glycolaldehyde. Organisms grown in the absence of added vitamin B_6 were able to synthesise the vitamin in much better yield. The actual magnitude of the synthesis varied from experiment to experiment even though great care was taken to grow, harvest and treat each batch of organisms identically. With such washed suspensions, some degree of synthesis of vitamin B_6 was always obtainable with glucose and NH_4^+, but this was invariably enhanced by the addition of glycolaldehyde. With serine-glycine and glycolaldehyde

TABLE E2-5

Synthesis of vitamin B_6 by washed suspensions of E. coli B166 grown on Medium G_2 with serine-glycine and glycolaldehyde

(a) Used directly after harvesting

Supplement	Vitamin B_6 biosynthesis (100 x μmoles pyridoxine/g. dry wt.)			
Expt.	(a)	(b)	(c)	(d)
Nil	0	0	0	
Serine-glycine plus glycolaldehyde	21	9	5	
Glucose plus NH_4^+ plus:-				
Nil addition	33	2	6	
Serine-glycine	-	-	6	
Glycolaldehyde	-	-	17	
Serine-glycine and glycolaldehyde	51	31	18	

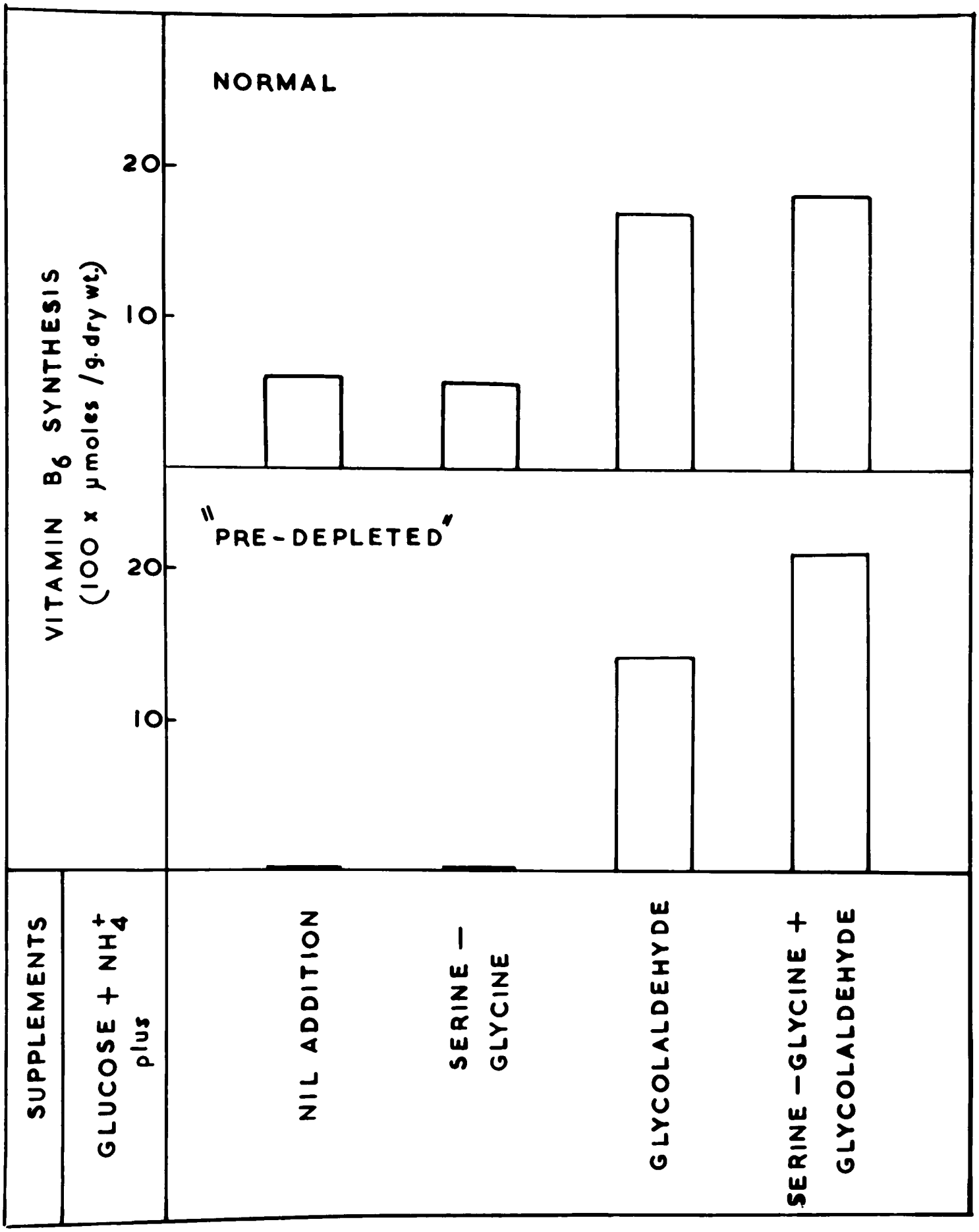
(b) Using 'pre-depleted' organisms

Nil addition	0	0	0	0
Glucose + NH_4^+	1	0	0	0
Glucose + glycolaldehyde	2	0	0	0
Glucose + serine-glycine	2	0	0	0
Glucose + NH_4^+ & glycolaldehyde	-	10	18	14
Glucose + serine-glycine and glycolaldehyde	32	14	22	12
Glucose + NH_4^+ and serine-glycine and glycolaldehyde	-	-	37	21
Serine-glycine + glycolaldehyde	-	-	-	4

Supplement conc.: glucose 1%; $(NH_4)_2SO_4$ 5×10^{-2} M; serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M.

Organisms were grown on Medium G_2 with serine-glycine and glycolaldehyde, and washed in 0.1 M phosphate buffer pH 7. 'Pre-depletion' was by further incubation in this buffer for 3 hr.

FIG. 18



Synthesis of vitamin B_6 by washed suspensions of E.coli B166

For further details see Table E2-5.

as sole substrates, some vitamin B_6 was formed, though not as much as when glucose was concurrently present (Fig. 28 and Table B2-5).

It was thought that the 'basal' synthesis obtained on glucose and NH_4^+ might be due to the carry-over of some precursor(s) formed by the organisms during their growth on serine-glycine and glycolaldehyde - the substrates whose effect on vitamin B_6 biosynthesis it was desired to test. An attempt was made to remove the 'precursors' and so decrease the blank synthesis, by starvation of the organisms prior to use. After harvesting from the serine-glycine plus glycolaldehyde medium, the cells were washed twice with 0.1 M phosphate buffer pH 7. Suspensions (5 mg./ml.) in phosphate buffer of the same strength and pH, containing 0.2% $MgSO_4.7H_2O$ were rocked aerobically at 37° for 2 to 3 hr. Organisms so treated were termed 'depleted' organisms. They were wholly unable to synthesise vitamin B_6 from glucose and NH_4^+ but were able to do so when glycolaldehyde (10^{-3} M) was further added. Serine-glycine enhanced the synthesis (Fig. 28 and Table B2-5).

The actual yield of vitamin B_6 again varied from batch to batch of depleted cells used. All showed an absolute requirement for glycolaldehyde for any synthesis of the vitamin. The synthesis in the presence of glycol-aldehyde and/or serine-glycine, was much increased by the

TABLE E2-6

Synthesis of vitamin B_6 by pre-depleted, washed suspensions of E. coli 22/99

Supplements	Vitamin B_6 synthesis (100 x µmoles pyridoxine /g. dry wt.)	
	A	B
Nil	0	0
Glucose and NH_4^+	7.5	0
NH_4^+ + glycolaldehyde	0	0
Glucose + NH_4^+ & glycolaldehyde	13.5	0
Glucose & serine-glycine	6	0
Serine-glycine + glycolaldehyde	0	7
Glucose + serine-glycine & glycolaldehyde	18	18

Key: organisms A - grown on Medium G_2 with serine-glycine.
B - grown on Medium G_2 with serine-glycine and glycolaldehyde.

Supplement conc.: Glucose 1%; serine-glycine 10^{-3} M; glycolaldehyde 10^{-3} M; NH_4^+ - 5×10^{-2} M $(NH_4)_2SO_4$.

The organism was grown on the media indicated, in 500 ml. volumes shaken at 37° until maximum growth was attained, harvested, washed and pre-depleted by aerobic incubation in 0.1 M phosphate buffer pH 7 for 3 hr. at 37°. The reharvested organisms were suspended in the same conc. of phosphate buffer containing 0.01% $MgSO_4.7H_2O$ plus the supplements shown, and incubated aerobically with rocking at 37° for 6 hr.
Samples were acid-extracted and assayed for vitamin B_6 at time 0 and after incubation.

addition of glucose. This might serve both as an energy supply and as the source of another carbon fragment which would otherwise have to be provided by endogenous metabolism.

E. coli 22/99 - This 'vitamin B_6 auxotroph' grows optimally on serine-glycine and glycolaldehyde and less well on serine-glycine (Fig. 26). More vitamin B_6 was synthesised when it was grown on glycolaldehyde plus serine-glycine than when it was grown on serine-glycine only (Table 32-3).

Since good growth was obtainable on Medium G_2 plus serine-glycine, it was thought that suspensions of organisms so grown might not show an obligatory requirement for glycolaldehyde for vitamin B_6 synthesis. This proved to be correct, for suspensions of E. coli 22/99 which had been grown on serine-glycine and depleted by aerobic incubation in phosphate buffer, were capable of synthesising vitamin B_6 to some extent from glucose and NH_4^+. Synthesis was still enhanced by the further provision of glycol-aldehyde.

Conversely, suspensions obtained by 'depleting' organisms grown on serine-glycine plus glycolaldehyde, would synthesise vitamin B_6 only when provided with both glycolaldehyde and serine-glycine (Table 32-6).

Conclusions

1. Vitamin B_6 synthesis by the competent organisms tested, both during growth and in suspension, was not enhanced by the addition of serine-glycine and glycolaldehyde.
2. Vitamin B_6 synthesis by E. coli DW growing on an amino acid mixture at 25° was increased by the further addition of serine-glycine and glycolaldehyde.
3. There was no marked difference between the amounts of vitamin B_6 synthesised by wild-type E. coli B and the mutant strain B166 when growing on Medium G_2 supplemented with serine-glycine and glycolaldehyde.
4. Suspensions of E. coli B166 harvested and washed after growth on Medium G_2 supplemented with serine-glycine plus glycolaldehyde, would synthesise vitamin B_6 from glucose and NH_4^+. The synthesis was much improved by the provision of glycolaldehyde.
5. If the organisms used were first depleted by incubating 2 to 3 hr. in aerobic suspension at 37° in phosphate buffer plus Mg^{++}, then no vitamin B_6 synthesis was obtained when the cells were subsequently incubated with glucose and NH_4^+. Synthesis of the vitamin was then wholly dependent upon glycolaldehyde. Further addition of serine-glycine somewhat improved the synthesis.

6. Suspensions of *E. coli* 22/99 harvested after growth on serine-glycine, and 'depleted' prior to use, were still able to synthesise vitamin B_6 from glucose and NH_4^+. The synthesis was again enhanced by serine-glycine and glycolaldehyde.

7. Suspensions of *E. coli* 22/99 grown on serine-glycine plus glycolaldehyde and 'depleted' before use, were unable to synthesise vitamin B_6 unless both serine-glycine and glycolaldehyde were supplied.

DISCUSSION

During the course of this study, all but one of the possible approaches discussed above (pp. 31 to 40), were followed. The exception was that employing isotopes. Application of the isotope-dilution technique to the identification of possible intermediates, and incorporation studies with labelled precursors, were held up by the non-availability of an organism producing sufficiently large quantities of vitamin B_6. Methods would first have to be developed to obtain optimal extraction of the comparatively small amounts of the vitamin synthesised by the available organisms. Instead, attention was directed towards the vitamin B_6 auxotrophic organisms. The growth of E. coli B166 in the absence of vitamin B_6 in a glucose-containing medium supplemented with serine-glycine, but not in a lactate medium containing the same amino acids, was particularly intriguing, and was studied in detail. The resulting discovery of the essential role of glycolaldehyde was encouraging, particularly in view of the current interest in 'active glycolaldehyde' metabolism. When it was further found that the amount of vitamin B_6 synthesised by E. coli B166 grown on serine-glycine and glycolaldehyde was not significantly less than that amount normally synthesised by the prototroph E. coli B, it was thought

possible that a study of the glycolaldehyde requirement of this organism might lead to the identification of a more closely related vitamin B_6 precursor. This explains why so much attention is here devoted to an appraisal of the growth characteristics of E. coli B166.

RESULTS WITH COMPETENT ORGANISMS

Work with these organisms was handicapped by their limited synthesis of vitamin B_6. Thus, no conclusion may be drawn from the observation that production of the vitamin by Escherichia coli was not enhanced by addition to the minimal media of any plausible supplement, for it is possible that the adequate de novo synthesis from glucose plus NH_4^+ could not be surpassed. Even the vitamin B_6 synthesis of the most productive organism used, i.e., Proteus morganii 235 at 4 μmole/g. dry wt., is much too small to represent anything but a minor commitment of the main carbon source. More immediate precursors might be diverted under such circumstances along alternative metabolic routes.

The inability of casein hydrolysate, purines, pyrimidines and other supplements such as shikimic and 3-hydroxyanthranilic acids, to enhance the 'basal' synthesis of vitamin B_6 from glucose plus NH_4^+ by wild-type E. coli and Proteus morganii, might indicate a precursor role for

direct products of carbohydrate metabolism, as in shikimic acid biosynthesis. Were formation of such a carbohydrate-derived precursor, the rate-limiting step in the biosynthesis, provision of a second fragment, e.g., in the form of an amino acid, need not result in increased vitamin B_6 formation.

Aerobic incubation favoured vitamin B_6 synthesis from glucose plus NH_4^+. That nicotinic acid might be required, was to be expected; the need for thiamine might be more significant - this vitamin plays an important role in the transketolase reactions of glucose oxidative catabolism.

The inability to obtain vitamin B_6 synthesis from simple precursors in more than slight yield with cell-free extract preparations, and to enhance even that little which was obtained by addition of a number of plausible substrates and cofactors, was again to be expected, since the proper functioning of a multitude of sequential reactions was demanded. Cell-free extracts are of greatest value when one or two-step transformations only are studied.

RESULTS OBTAINED WITH VITAMIN B_6 AUXOTROPHS

It appears that vitamin B_6 mutants of *E. coli* are difficult to produce, and consequently rather rare. Perhaps the difficulty is restricted to this organism for

pyridoxine auxotrophs have appeared frequently amongst the products of irradiation of Aspergillus nidulans and Neurospora (Pontecorvo, 1953). Vitamin B_6 mutants of E. coli might die off very rapidly in media unsupplemented with the vitamin (cf. Pontecorvo, 1953; Bauman and Davis, 1957).

No evidence for a common route of biosynthesis of nicotinic acid and vitamin B6

No multiple mutants of E. coli requiring both nicotinic acid and pyridoxine were obtained, and no sparing effect was demonstrable of one vitamin on the requirement shown for the other by any auxotroph.

It might yet be found that the apparently novel route of nicotinic acid biosynthesis in E. coli, Bacillus subtilis (Yanofsky, 1954), and Aspergillus nidulans (Pontecorvo, 1953) is due to no more than the existence in these organisms of a mechanism for the direct hydroxylation of anthranilic acid (McCullough, 1957).

3-Hydroxyanthranilic acid, anthranilic acid and shikimic acid did not increase vitamin B_6 production by E. coli, nor, on the other hand, did serine-glycine and glycolaldehyde have any apparent effect upon the quantitative requirements of a variety of indole, tryptophan and nicotinic acid mutants of E. coli.

PROPERTIES OF THE 'ALTERNATIVE' SERINE-GLYCINE OR VITAMIN B_6 MUTANTS

1. Glycolaldehyde as the active factor in activated glucose

Glycolaldehyde was able to reproduce in every particular the biological activity of activated glucose with these mutants; an optimal concentration consistently improved upon these actions. Even the observation that glycolaldehyde could at no concentration satisfactorily substitute for activated glucose in Medium L, was fully explained by the concurrent action of unchanged glucose. Although absolute reliance may not be placed upon a colorimetric reaction as unspecific as the diphenylamine reaction used in the Dische-Borenfreund method, the assay of the glycolaldehyde content of activated glucose samples by this means is sufficiently accurate for it to be concluded that,

(a) sufficient glycolaldehyde is probably present to account for the biological activity of the samples;

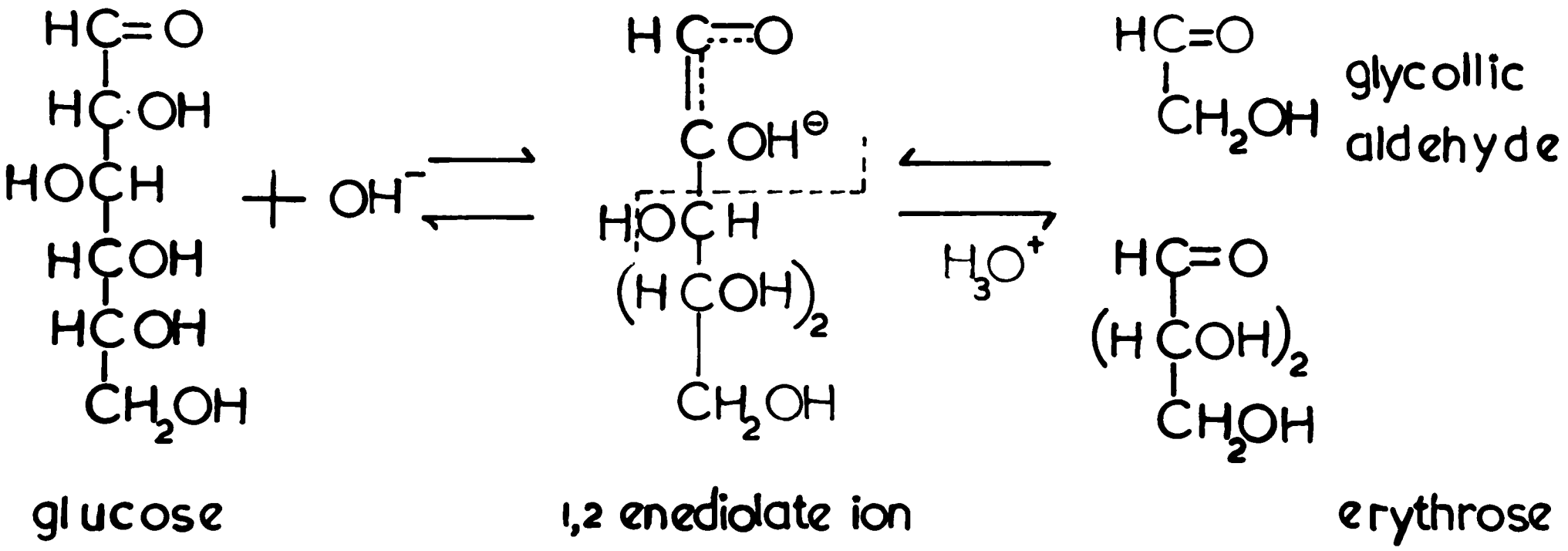
(b) the glycolaldehyde is formed only during the specific autoclaving procedure necessary for the production of activated glucose.

Glycolaldehyde, too, would possess those chemical properties attributed to the active factor as a consequence of its behaviour during attempted extraction, e.g., a highly

FIG. 29

Theoretical Scheme for the Scission of Glucose giving rise to Glycolaldehyde plus a Tetrose.

At an Alkaline pH :



hydrophilic character with a tendency towards instability in alkaline solution.

Any formation of glycolaldehyde from glucose during alkaline autoclaving would probably proceed by way of scission of the 1:2-enediol intermediate into C_2 and C_4 fragments (Fig. 29, after Wolfram and Schumacher, 1955). This accords with the finding that the sugar alcohols could not be activated by autoclaving, for these compounds would be unable to form the enediolate ion. Then again, scission at this position apparently demands the migration of the hydrogen atom from the C-3 hydroxyl group. This could not occur in 3-O-methylglucose, which was significantly unable to form 'active factor'.

All the findings therefore support the hypothesis that the activation of glucose solutions by autoclaving at a slightly alkaline pH, is a function of the concurrent formation of glycolaldehyde.

Several cases are known of the development of biological activity during heat-sterilisation of glucose-containing media (Orla-Jensen, 1933; Dyson and Peterson, 1949; Cheldelin, 1954; Hachisuka, Kato, Asano and Kuno, 1955; Ramsey and Lankford, 1956; Field and Lichstein, 1957; Sergeant, Lankford and Traxler, 1957). When the medium contains amino acids, Maillard-type, or other, interactions between these and the hexose have been

described. For example, N-D-glucosylglycine has been found to possess the same growth stimulating activity for Lactobacillus gavonii as medium-autoclaved glucose (Rogers, King and Cheldelin, 1953). Scission products have occasionally been held responsible for the growth stimulatory or inhibitory effects of autoclaved glucose; pyruvate replaced autoclaved glucose for Streptococcus salivarius and Lactobacillus bulgaricus (Smiley, Niven and Sherman, 1943; Snell, Kitay and Hoff-Jorgensen, 1948), while reductone was a bactericidal component of glucose caramels (see also Mondolfo and Honnie, 1948). The present study appears to be the first to implicate free glycolaldehyde as a component of alkaline autoclaved glucose of possible biological significance.

Escherichia coli B166

This is not a true vitamin B_6 auxotroph, for no concentration of any known form of vitamin B_6 could reproduce wild-type growth characteristics; instead, only suboptimal growth occurred after long delay. It is obvious that the lesion in the organism is not confined to interference with vitamin B_6 biosynthesis; some other biosynthetic pathway is manifestly affected. It seems that this might be the biosynthesis of serine-glycine, for when these amino

acids were present with pyridoxine, then rapid and good growth resulted. The organism is therefore multiply blocked - being at least, a serine-glycine plus vitamin B_6 auxotroph. Even so, the growth attained on supplemented simple media never equalled that achieved by the wild-type parent organism on the unsupplemented media. Another peculiarity is that the vitamin B_6 requirement of this strain was some ten times that of E. coli M_2 which showed an absolute requirement for the vitamin.

Acceleration and enhancement of growth by serine-glycine in the presence of pyridoxine, could conceivably be a result of conversion of the vitamin into an even more active form, or of increased permeability of the organism to the vitamin. This is hardly likely though, since even very high concentrations of all known forms of the vitamin could not improve growth of the organism in the absence of the amino acids. Alternatively, there might exist a growth-limiting state of serine-glycine, or other, deficiency during growth on the vitamin.

Serine-glycine must serve a double function, for, besides the growth-enhancement effect, together with glycolaldehyde these amino acids effect a vitamin B_6 replacement. The amino acids must be concerned with glycolaldehyde in this second function, else glycolaldehyde alone would be expected to evoke at least the suboptimal growth achieved on vitamin B_6.

In contrast to serine-glycine, glycolaldehyde seems to have no specific action on the organism other than in its role in vitamin B_6 replacement: under no circumstance did glycolaldehyde exercise any effect in the presence of pyridoxine. If this is the case, then, as free glycolaldehyde was required at a concentration some 500 times that optimal for vitamin B_6, either it must be very rapidly metabolised and destroyed by the organism, or it acts only indirectly.

As growth of the organism was possible on certain supplements in the absence of added vitamin B_6, the first question to be answered was whether the supplements reduce the vitamin B_6 requirement of the organism, or partake in its synthesis.

Concerning the vitamin B_6-sparing abilities of serine-glycine and glycolaldehyde

Serine-glycine and glycolaldehyde could replace vitamin B_6 for this organism if they were the sole products of function of the vitamin when growth was taking place on an otherwise minimal medium. What is known of the mode of action of the vitamin (see 'Introduction', Chapter 1), is evidence enough that this is impossible.

The alternative remains that the mutant is partially able to synthesise the vitamin, i.e., it is a

'leaky' vitamin B_6 mutant and the amount formed is sufficient to satisfy all its functions except those of synthesising the essential metabolites serine-glycine and glycolaldehyde (Fig. 30). Provision of these would allow of growth of bacteria with a suboptimal content of vitamin.

There are some indications that serine-glycine might in fact have some sparing effect on the vitamin B_6 requirements of this organism, e.g., the reduced pyridoxine requirement for growth, and the increased resistance to inhibition by 4-deoxypyridoxine and isoniazid in the presence of serine-glycine (Tables C2-1, C7-2 and C7-3). This agrees with the known function of vitamin B_6 in the synthesis of these, as of all other amino acids.

The activity of serine-glycine was not solely, if at all, a function of its vitamin B_6-sparing ability, for it would be impossible so to explain its marked growth promoting effect in the presence of excess pyridoxine. Besides, even if only the vitamin B_6 replacement action is considered, the possibility that it was due to a reduced requirement for the vitamin must be rejected for:-

(a) growth in the presence of serine-glycine and glycolaldehyde occurred with much less delay than with an excess of any form of vitamin B_6;

(b) glycolaldehyde alone had no pyridoxine-sparing effect;

(c) serine-glycine and/or glycolaldehyde, had no marked

pyridoxine-sparing action with E. coli M_2 or E. coli DW at 37°;

(d) serine-glycine and/or glycolaldehyde, had no beneficial effect on the alanine plus methionine supported growth of E. coli DW at 25°;

(4) when the mutant was grown on serine-glycine and glycolaldehyde the amount of vitamin B_6 synthesised was practically identical with that produced by the parent wild-type organism growing on unsupplemented medium.

This last observation alone would seem to exclude the hypothesis of sparing action, for, in cases of genuine vitamin-sparing action, synthesis of the vitamin is generally obviously subnormal. Numerous examples might be given, but those most relevant to the present study are the behaviour of Streptococcus faecalis R and E. coli DW. When Strep. faecalis R was grown on a full replacement medium containing D-alanine, the synthesis of vitamin B_6 was very small and the organisms consequently strikingly deficient in pyridoxal phosphate (Holden and Snell, 1949). E. coli DW grown on a mixture of amino acids at 25° showed diminished vitamin B_6-enzyme activity, restored by supplementation with a suitable form of the vitamin (Wijesundera, 1954; Denman, Hoare and Work, 1955). Even

when growing at 25° on DL-alanine plus DL-methionine, synthesis of vitamin B_6 by the organism was markedly suboptimal.

It is assumed of course that the substance which was assayed by Saccharomyces carlsbergensis 4228c, in extracts of B166, was actually vitamin B_6 and not a precursor as effective as the vitamin in supporting growth of the yeast. Confidence in the assay was partially justified by the finding that assays of B166 extracts gave approximately the same results when performed (rather less accurately) with E. coli M_2.

Possible functioning of serine-glycine and glycolaldehyde as precursors in vitamin B_6 biosynthesis by E. coli B166

The evidence by direct assay of normal vitamin B_6 synthesis during growth of E. coli B166 on serine-glycine and glycolaldehyde, whilst being the most powerful argument against the functioning of these substrates by vitamin B_6-sparing ability, is the most suggestive of a precursor role for these compounds in vitamin B_6 biosynthesis. The great increase in resistance of the organism to 4-deoxypyridoxine, when growing on serine-glycine and glycolaldehyde, if interpreted along the lines suggested by the observations of Rabinowitz and Snell (1953), would support this view. However, it seems that this is not conclusive evidence,

for a vitamin-sparing action would also be expected to decrease sensitivity to the antimetabolite. To assess the contribution made by serine-glycine and glycolaldehyde to any synthesis of vitamin B_6, they are best considered individually.

(a) Serine-glycine

The position with regard to these amino acids, is complicated by their playing more than one metabolic role. They are essential metabolites, and besides any proposed function in vitamin B_6 synthesis, might to some extent be able to reduce the requirement for this vitamin.

From close examination of the growth properties of *E. coli* B166, the conclusion seems inescapable that serine-glycine provision is essential for the synthesis of vitamin B_6 by this organism. This need not necessarily mean the direct incorporation of either of these amino acids into the vitamin B_6 molecule; the mechanism of action could be indirect and peculiar to these auxotrophs (*vide infra*).

The inability of serine-glycine to influence vitamin B_6 synthesis by those competent organisms tested, might, as suggested earlier, be only a consequence of the small and apparently restricted synthesis obtainable and cannot be regarded as excluding their functioning as

precursors of the vitamin. Nor in view of their relative quantitative requirements, can any certain conclusion be drawn from the finding that vitamin B_6 synthesis by E. coli PA15 in growth and suspension was unaffected by (assumed) serine-glycine deficiency.

With washed suspensions of E. coli B166 (even those depleted of precursors by preincubation), NH_4^+ could adequately substitute for serine-glycine in vitamin B_6 biosynthesis, though for maximal synthesis, both serine-glycine and NH_4^+ were required. Perhaps with the major quantitative requirement for the amino acids already satisfied, the organism is capable of sufficient de novo serine-glycine synthesis to furnish the requirement for vitamin B_6 formation. More probable is the existence of an endogenous pool of serine-glycine developed during growth on the amino acids and more than adequate to meet the demands of a very small vitamin B_6 synthesis. It is therefore somewhat surprising, if gratifying, to have been able to demonstrate an absolute requirement for serine-glycine in the synthesis of vitamin B_6 by pre-depleted suspensions of E. coli 22/99 grown on the amino acids plus glycolaldehyde.

(b) Glycolaldehyde

All the evidence suggests a function for

glycolaldehyde in the biosynthesis of vitamin B_6 by E. coli B166.

It was unfortunately impossible to produce suspensions of the organism harvested after growth in the absence of glycolaldehyde which were able to synthesise a significant amount of vitamin B_6. It is therefore probable that the more limited vitamin B_6 synthesis from glucose and NH_4^+ was made possible by the participation of endogenous intermediates formed from glycolaldehyde during growth. Even so, addition of free glycolaldehyde invariably increased the yield of vitamin. By 'starvation' after harvesting, suspensions of organisms were obtained which were incapable of synthesising vitamin B_6 from glucose and NH_4^+; glycolaldehyde had become an obligatory requirement. Although use of pre-depleted organisms could be deplored on the grounds that an unknown amount of irreparable harm might concurrently have been done them, normal synthesis of the vitamin was obtainable on provision of glycolaldehyde, and the most acceptable explanation is that during the pre-incubation in phosphate buffer, utilisation with consequent depletion of endogenous supplies of glycolaldehyde or/and its metabolic products had been achieved.

Results with washed suspensions of E. coli 22/99 support these findings. Here, glycolaldehyde was even required for optimal vitamin B_6 synthesis by suspensions

of organisms which had been grown with serine-glycine, and which, it must be assumed, were capable of limited synthesis of glycolaldehyde products. Synthesis of vitamin B_6 during the growth of this mutant was greater with serine-glycine plus glycolaldehyde than with serine-glycine alone, (under which conditions it must be regarded as 'glycolaldehyde-deficient').

As with serine-glycine, the inability of glycolaldehyde to increase the amount of vitamin B_6 formed by competent microorganisms does not exclude the possibility that it may participate in its synthesis.

It was interesting to find that supplementation of the medium with serine-glycine and glycolaldehyde enhanced the suboptimal synthesis of vitamin B_6 by E. coli DW at 25° but had no apparent effect on the growth of the organism at 37°, at which temperature the block in vitamin B_6 formation seems to be complete. Again, there was no detectable action of these compounds on E. coli M_2, Saccharomyces carlsbergensis 4228c and Aspergillus nidulans bi.pyro$_1$. It is reasonable to assume that the lesion in vitamin B_6 synthesis is in these organisms subsequent to the point of action of serine-glycine and glycolaldehyde.

In summary, the evidence for the participation of glycolaldehyde in vitamin B_6 biosynthesis is somewhat stronger than that supporting a similar role for serine-glycine. However, it appears that these amino acids must

be concerned in the synthesis, if only indirectly.

Possible importance of a third factor

The magnitude of the growth response of E. coli B166 to serine-glycine and glycolaldehyde was dependent upon the basal medium used. It seems that some further substance is concerned with these compounds in the nutrition of the organism, and in particular, in the vitamin B_6 replacement action. This substance is more readily available when glucose rather than lactate is the main carbon source. Perhaps, could the factor be provided preformed, growth on serine-glycine plus glycolaldehyde would at least attain the level found with serine-glycine plus pyridoxine, whilst growth under both conditions might achieve that maximum reached by the prototroph (i.e., assuming that the factor possessed important functions besides its role in vitamin B_6 biosynthesis).

Alternatively, the nature of the carbon source might otherwise affect the properties of the organism.

NATURE OF THE METABOLIC LESION IN E. Coli B166

Assuming that, as the product of a single irradiation, E. coli B166 suffers from a single mutational change, the lesion must induce a number of metabolic defects, for the organism possesses, -

(a) a block in vitamin B_6 biosynthesis which may be overcome by supplying the organism with serine-glycine and glycolaldehyde (and another factor?);

(b) a partial block in serine-glycine synthesis. Partial, because the organism can grow in the absence of added serine-glycine; to consider pyridoxine (10^{-6} M) as a precursor of the required concentrations of serine-glycine (10^{-3} M), or as the sole product of utilisation of the amino acids, is absurd. Indirect evidence suggesting the existence of a partial blockage only in the synthesis of these amino acids, comes from the finding that the serine-glycine requirements of E. coli B166 are less than one-tenth those of E. coli PA15;

(c) a defect in the synthesis of glycolaldehyde, and of the hypothetical third factor, if these are normal metabolites. If the utilisation of these substrates is an abnormal (artificial) process, then it is the inferred natural metabolite, i.e., the product of their assimulation, whose biosynthesis is obstructed. This metabolite need not function solely in vitamin B_6 biosynthesis.

Now, there are several possible methods whereby a single metabolic lesion might injuriously affect more than one route of biosynthesis -

(1) by cross-inhibition whereby an accumulated precursor

could inhibit the utilisation of other metabolites;
(2) by the functioning in each biosynthetic route of a reaction-specific catalyst (enzyme or coenzyme) whose provision or activity is curtailed;
(3) as a consequence of the existence of a common precursor.

1. Cross inhibition

The possible mechanisms of cross inhibition in this mutant may be divided into two main types: (a) where a precursor in vitamin B_6 synthesis interferes with serine-glycine biosynthesis; and (b), where an accumulated serine-glycine precursor completely prevents vitamin B_6 biosynthesis (Fig. 31). Glycolaldehyde might under these circumstances be considered as acting by circumventing or overcoming the inhibition. It obviously does not function by circumvention, i.e., by feeding into the stream of reactions after the blocked step, for this would mean that in case (a) vitamin B_6 would be an obligatory requirement, and in case (b) that growth should occur equivalent to that obtained with pyridoxine alone when glycolaldehyde is the sole supplement.

Alternatively, glycolaldehyde might act as an antimetabolite (perhaps removing metabolic restriction by combining with the inhibitory precursor I, and so rendering it ineffectual). The marked growth-inhibitory properties

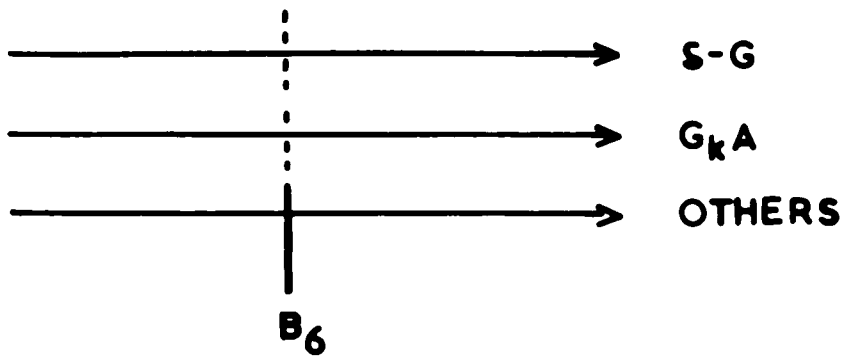


FIG. 30

(a) (b)

FIG. 31

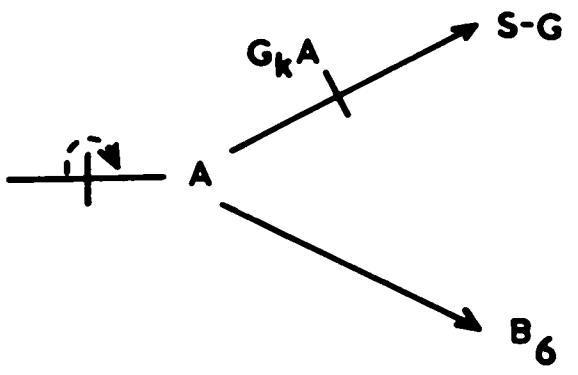
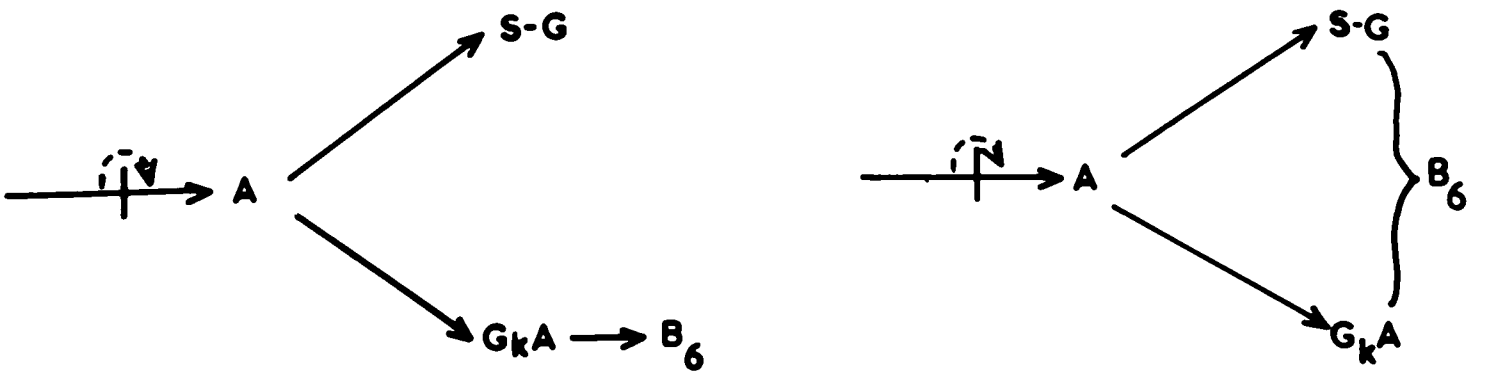


FIG. 32



(a) (b)

FIG. 33

of concentrations of glycolaldehyde little greater than those optimal for the observed biological activity, and an inhibitory action of glycolaldehyde-2-phosphate on the growth of Neisseria gonorrhoeae and B. proteus (Racker, 1954), might seem to support such a hypothesis. That glycolaldehyde is acting only by this mechanism is impossible, for in case (a) no growth could occur in the absence of vitamin B_6. In case (b), removal by this mechanism of the inhibition to vitamin B_6 biosynthesis would certainly be at the expense of introducing an absolute serine-glycine requirement, but no growth should be possible on pyridoxine plus glycolaldehyde.

It is not obvious, therefore, how the behaviour of B166 could be explained on the basis of any cross inhibition mechanism and only one possibility remains (if an antimetabolite effect of glycolaldehyde is postulated) which will take account of the concurrent requirement for serine-glycine; this is to suppose a common precursor for vitamin B_6 and serine-glycine and a specific antimetabolite action of glycolaldehyde in inhibiting the biosynthesis of the amino acids (Fig. 32). A partial blockage of the synthesis of common precursor A should have to be assumed, together with preferential synthesis of serine-glycine from the small amount of A formed thereby. Glycolaldehyde, by preventing the synthesis of serine-glycine, would effectively

direct the available supplies of A to vitamin B_6 formation, but only at the expense of the imposition of an absolute serine-glycine requirement. This scheme can be discounted on the basis of:

(a) the great disparity in the quantitative requirements for vitamin and amino acids respectively;

(b) the fact that the serine-glycine requirement is not increased (e.g., to the level optimal for E. coli PA15) by the addition of glycolaldehyde;

(c) glycolaldehyde-induced inhibition of growth of E. coli 518 is not overcome by high concentrations of serine-glycine;

(d) glycolaldehyde is equally inhibitory to the growth of E. coli 518 (on Medium G_2) and E. coli PA15 (on Medium G_2 with 10^{-2} M serine-glycine).

2. Dependence upon a common catalyst

This explanation proposes the existence of a catalyst of reaction-specificity only and which is common to all the otherwise independent routes of biosynthesis, e.g., as in the 'transaminase mutants'. If the formation of the catalyst by the organism is totally prevented, then the affected reactions represent its sole functions under the conditions of growth used. If the mutant is 'leaky' and only partially deficient in the catalyst, then the required supplements are products of reactions which are

preferentially afflicted by the deficiency; sparing effects by other products of function of the catalyst might be demonstrable.

There is no evidence in the behaviour of E. coli B166 to suggest the existence of this form of interrelation.

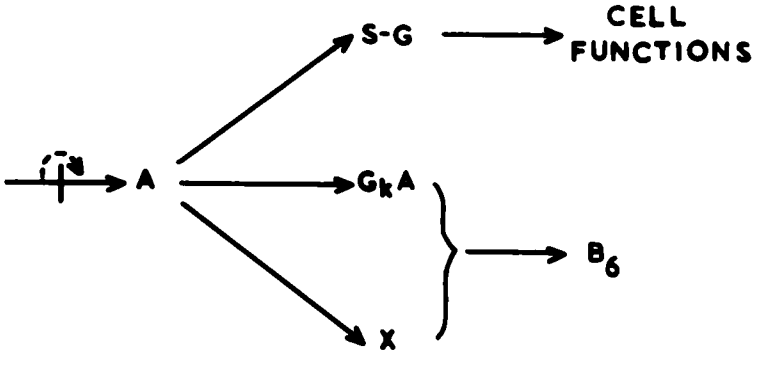
An extreme example is the case where the common catalyst possesses strict substrate specificity, i.e., all affected products arise from the same precursor.

3. Origin from a common precursor

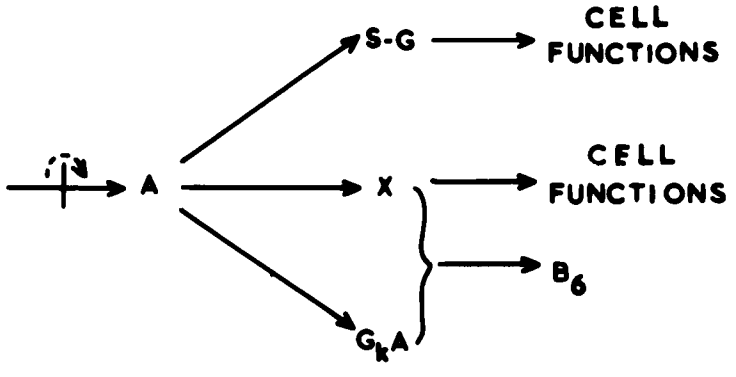
This remains as the most probable explanation of the properties of E. coli B166. Even so there appear to be insuperable objections to any explanation based on the assumption of a precursor common to serine-glycine and glycolaldehyde only, with vitamin B_6 arising from the utilisation of one or both of these products, e.g., Fig. 33a & b. To account for growth being obtainable with vitamin B_6 alone, it is necessary to postulate an ability to synthesise some serine-glycine, and it is then not obvious why the organism should be incapable of growth on glycolaldehyde alone, or alternatively on serine-glycine alone. Perhaps viable counts would show that some reproduction does occur under these conditions though it is insufficient to produce visible growth.

FIG. 34

Key: A = common precursor
S-G = serine-glycine
G_kA = glycolaldehyde
X = unknown factor
B_6 = vitamin B_6



(a)



(b)

Case (a), when insufficient A is formed to satisfy the S-G requirements of the organism

Then with,

1. S-G only, no growth for A → G_kA and no X available.
2. S-G & G_kA, growth, for A → X. Should equal growth found on S-G plus B_6.
3. B_6, suboptimal growth for S-G suboptimal.
4. S-G plus B_6, optimal growth.
5. G_kA, no growth, for no X available.

Case (b), when sufficient A is formed to fully satisfy the S-G requirements of the organism

Now with,

1. S-G only, no growth, for A → X but no G_kA available.
2. S-G plus G_kA, growth, for A → X; not as good as on S-G plus B_6 for B_6 spares X.
3. B_6, suboptimal growth due to X-deficiency.
4. S-G plus B_6, optimal growth.
5. G_kA, no growth, for no X available.

To account for all the vagaries, it seems that the scheme must be made even more complex by the supposed participation of another product (X) arising from the common precursor. A situation similar to those represented in Fig. 34a & b would result.

Serine-glycine could be directly concerned in the synthesis of the vitamin. On the other hand, presence of the amino acids might merely allow diversion of what A is synthesised to the formation of X, and thence in the presence of glycolaldehyde to vitamin B_6. If organisms grown on pyridoxine alone were serine-glycine deficient, then the likelihood is that the only function of X is in vitamin B_6 synthesis; on the other hand, if sufficient A was being formed to satisfy the serine-glycine requirements of the organism, then the suboptimal growth on vitamin B_6 alone, must be the result of X-deficiency. The former situation would be represented by Fig. 34a, and the latter by Fig. 34b.

Assuming that vitamin B_6 synthesis is optimal when the organism is growing on serine-glycine and glycolaldehyde, then it is difficult to explain the less good growth of B166 under these conditions as compared with that obtained on serine-glycine and pyridoxine, by any other means than by assuming a deficiency of another factor, X. Pyridoxine might reduce the requirement for this factor.

With *E. coli* 22/99 the same difficulty does not arise, for growth on serine-glycine and glycolaldehyde is quite as good as on serine-glycine and pyridoxine. It appears that the lesion in serine-glycine synthesis is quite as severe in this mutant as in B166, for growth on pyridoxine is suboptimal; 22/99 is seemingly better able, however, to synthesise X and glycolaldehyde, so that growth is now possible (though slow) on serine-glycine alone. Suboptimal and long delayed growth on glycolaldehyde alone is possibly due to the ability of this strain to synthesise X rather than serine-glycine.

In summary, it must be stated that although the most likely hypothesis is one which proposes the origin of the critical compounds from a common precursor, the interrelationship is at present too confused for any metabolic scheme to be assigned it with certainty. Especially is this the case when there is evidence to suggest the participation of another as yet unidentified compound.

POSSIBLE MODE OF ACTION

(a) Serine-glycine

It has been suggested that, while in *E. coli* B serine can be converted to glycine, the reverse reaction does not normally occur (Roberts et al., 1955), though the

existence of auxotrophic strains of E. coli responsive to serine or glycine is proof that serine may at least be formed from high concentrations of glycine (Meinhart and Simmonds, 1955). The existence of such mutants might suppose the functioning of only one route of glycine biosynthesis, yet in some strains at least one-third of the glycine arising from glucose was not formed via L-serine (Meinhart and Simmonds, 1955). Serine can arise directly from products of glucose metabolism, probably at the C_3 level (Arnstein and Keglević, 1956). The suggestion has recently been made that in animal tissues triose phosphate may be convertible to β-hydroxypyruvate phosphate and thence to serine phosphate (Ichihara and Greenberg, 1955; 1957). In E. coli grown under certain conditions, glyoxylic acid may be formed by an active isocitritase (Smith and Gunsalus, 1954) and be convertible to hydroxypyruvate (Krakow and Barkulis, 1956).

Boll (1954a & b) has described a synergistic action of glycine and pyridoxine upon the growth of excised tomato roots. It appeared that this was the result of vitamin-sparing action, as many other unrelated amino acids possessed similar activity to glycine; ethanolamine and choline were even more active.

In animal tissues, a direct relation may exist between serine, glycolaldehyde and glycine, i.e.,

serine → ethanolamine → glycolaldehyde → glycollic acid → glyoxylic acid → glycine (Weissbach and Sprinson, 1953). There has been no report of the operation of this reaction sequence in bacteria; apart from Nord (1919), no-one has reported bacterial ethanolamine formation by serine decarboxylation. There is certainly no suggestion that such reactions are operative in E. coli B166; thus ethanolamine did not substitute for glycolaldehyde, nor glyoxylic acid for glycine. If a common precursor for serine-glycine and glycolaldehyde need be postulated, it might be a C_3 compound closely related to triose and perhaps to hydroxypyruvate. Whilst possessing some glycolaldehyde-like activity, hydroxypyruvate was less active than free glycolaldehyde, and would not substitute for serine-glycine. There is no strong reason, however, to believe that free hydroxypyruvate is in equilibrium with the carbohydrate-derived precursors of serine-glycine and glycolaldehyde.

From the mere fact of their being amino acids, any participation of serine-glycine in the biosynthesis of vitamin B_6 carries with it the suggestion that one of these is the vehicle for the introduction of the pyridine nitrogen atom, but no evidence of this has been obtained. If ^{14}C-labelled amino acid is found to be incorporated into the vitamin B_6 molecule, then ^{15}N-labelling must be

used to confirm the simultaneous origin of the nitrogen atom.

It has been pointed out above that the serine-glycine requirements of E. coli B166 are rather less than one-tenth those of E. coli PA15. Rapid degradation has been held to account for the abnormally high serine-glycine requirements of such mutants as the latter, together with the quantitative importance of these amino acids in C_1 metabolism and purine and porphyrin biosynthesis (Meinhart and Simmonds, 1955). Perhaps the rate of this destruction is for some reason reduced in B166, or more probably, this strain is capable of synthesising part of its own requirements.

With indirect evidence for a function of p-aminobensoic acid in vitamin B_6 biosynthesis by yeast (Moulder and Woods - unpublished), the possible hydroxymethylation or methylation of either an aliphatic precursor or, less likely, the pyridine ring, must be considered. Serine could act as the C_1 unit donor in such a reaction, but this would not explain why vitamin B_6 synthesis should be preferentially affected by its deficiency; indeed, good synthesis of vitamin B_6 could be carried out by serine-glycine depleted E. coli PA15.

No explanation is offered for the enhanced rate of reversion of B166 when growing on suboptimal serine or glycine concentrations - nor consequently, for the

somewhat lower rate when the amino acids were used in admixture. This behaviour is not unique to B166, other examples having been reported of increased reversion rates on suboptimal concentrations of required metabolite or, alternatively, decreased reversion rate in the presence of optimal concentrations of the nutrilite (Ryan and Schneider, 1948).

(b) Glycolaldehyde

There is nothing known of glycolaldehyde to suggest its functioning as a precursor of vitamin B_6. As described earlier (Chapter C6) the activated C_2 fragment transferred in transketolase-catalysed reactions, has tentatively been identified with 'active glycolaldehyde' or glycolaldehyde-thiamine pyrophosphate, though the actual evidence for this is slight (Horecker, Smyrniotis and Klenow, 1953).

Glycogenic activity of free glycolaldehyde in animals (Mayer, 1903) and the appearance of free glycolaldehyde in the products of yeast fermentations (Neuberg and Rosenthal, 1914) were early reported. A species of Fusarium produced glycolaldehyde when metabolising pentoses (Wirth and Nord, 1942), as did also Acetobacter acetigenum when grown on ethylene glycol, D-xylose or L-arabinose (Kaushal, Jowett and Walker, 1951); similarly, glycolaldehyde

phosphate was a product of pentose phosphate metabolism by haemolysed human erythrocytes (Dische, 1938). The reverse reaction, i.e., the utilisation of free glycolaldehyde or its phosphate for the biosynthesis of pentose phosphate has been reported on many occasions (Forrest, Hough and Jones, 1951; Marmur and Schlenk, 1951; McGeown and Malpress, 1954).

Sable (1952) was unable to show glycolaldehyde production during the action of several cell-free extracts (e.g., of yeast) upon ribose-5-phosphate. He showed, however, an oxidation of reduced DPN or TPN in the presence of added glycolaldehyde, which he attributed to the occurrence of a dismutation reaction catalysed by alcohol and aldehyde dehydrogenases.

The formation of glycolaldehyde has been suggested as the first step in ethanolamine metabolism in animal tissues - to give eventually glycine (Weissbach and Sprinson, 1953), or acetate (Sprinson and Weliky, 1954). Using 2-^{14}C-labelled glycolaldehyde, it was shown that free glycolaldehyde injected intraperitoneally into rats was very rapidly metabolised to glycine, with minor conversions to formate and carbon dioxide; some 13% was excreted in the urine as an unidentified derivative (Friedmann, Levin and Weinhouse, 1956). Kun, Dechary and Pitot (1954) demonstrated the oxidation of glycolaldehyde to glycollic and glyoxylic acids in rat liver, while Buyze (1955)

obtained indirect evidence of its reduction to ethylene glycol by certain Lactobacilli. Free glycolaldehyde has been reported to participate in two further metabolic reactions, (a) as substrate with triose phosphate of aldolase, giving a ketopentose phosphate (Glock, 1952), identified as D-xylulose-1-phosphate (Byrne and Lardy, 1954), and (b), as an 'active glycolaldehyde' acceptor giving rise presumably to erythrulose (Horecker and Mehler, 1955). Whether free glycolaldehyde can enter into equilibrium with the 'active glycolaldehyde' fragment is not known. The isotopic data of Friedmann et al. (1956) suggest that in animal tissues it cannot.

In the light of these metabolic transformations, it is significant that ethanolamine, ethylene glycol, glycollic and glyoxylic acids had no glycolaldehyde-like activity for E. coli B166. Nor was any of the 'active glycolaldehyde donors' tested capable of substituting for free glycolaldehyde. Most of these were phosphate esters which it is conceivable were unavailable to intact organisms (though see Racker, 1954). Dihydroxyfumaric and β-hydroxypyruvic acids probably possessed activity as a result of spontaneous or metabolic conversion to glycolaldehyde (Neuberg and Schwenk, 1915; Powers, Tabakoglu and Sable, 1956; Uehara, Muramatsu and Mori, 1953; Milhaud, Benson and Calvin, 1956). Perhaps the most

significant result of all was the inactivity of free L-erythrulose.

It has been concluded from the high optimal concentration of glycolaldehyde (5×10^{-4} to 10^{-3} M) that if its sole action is in vitamin B_6 biosynthesis, it acts indirectly (even by a mass action effect?), or else that it is very rapidly destroyed by the organism. Free glycolaldehyde in the presence of glucose is metabolised extensively and rapidly in growth and in suspension by E. coli B166 (Mulder - unpublished).

There is no evidence that glycolaldehyde itself is a natural metabolite in Escherichia coli, or, even if it were, that it shares a common precursor with serine-glycine. The finding that the rate of reversion of E. coli PA15 in suboptimal concentrations of serine was particularly high when glycolaldehyde was also present might serve as evidence for some relationship between these compounds. No glycolaldehyde mutant of E. coli 518 was found, but this is of course no evidence that they cannot exist.

It was to be expected that glycolaldehyde would not directly substitute for pyridoxal, or its 2-phosphate for pyridoxal-5-phosphate, in reactivating tyrosine apodecarboxylase. On purely chemical considerations, even though glyoxylic acid can emulate pyridoxal in certain of its 'model' reactions, glycolaldehyde should be unable to

substitute for either. Although it possesses the necessary free aldehyde group, it does not carry an opposed electrophilic grouping - supplied in glyoxylic acid by the carbonyl group.

Theoretical chemistry

Although there is no evidence for the participation of alanine in vitamin B_6 biosynthesis, the scheme of three-fragment synthesis envisaged originally by Snell and Guirard (1943), and used chemically with great success by Cohen, Haworth and Hughes (1952), remains an attractive hypothesis if only because the postulated C_4 fragment (giving rise to C-4 and C-5 of the pyridine ring with their hydroxymethyl substituents) could resemble a possible direct product of carbohydrate and more particularly glycolaldehyde metabolism. A formyl substituent on the predestined C-5 would be strategically placed for spontaneous ring closure with a free amino group which might be supplied by glycine. All that would now be required would be methylation at the C-2 position and dehydrogenation (compare Fig. 2b).

In the present state of knowledge, speculation of this nature, though fascinating, is of no value except as a guide to future experimentation. At present it must not even be assumed that pyridoxine is the first product of synthesis from which all other forms of the vitamin

arise. The 5-hydroxymethyl group might be phosphorylated prior to ring closure even though a potent pyridoxalkinase is known to exist (Hurwitz, 1955). The 4'-substituent might be a hydroxymethyl, aldehyde or aminomethyl group. Although there is no evidence of ribotidation of the vitamin, the precursor might be synthesised as a N'ribotide with the amino group arising not after all from an amino acid, but from phosphoribosylamine.

SUMMARY AND CONCLUSIONS

We are little nearer to the solution of the problem of vitamin B_6 biosynthesis. Yet it seems that the foundations may now have been laid for a more rapid and successful assault on the problem, i.e., with the discovery of a possible precursor role for a glycolaldehyde metabolite, at least in select mutants of Escherichia coli, together with some implication of serine and/or glycine. It is possible that the production of vitamin B_6 from glycolaldehyde, serine-glycine and glucose, is an alternative to the normal route of biosynthesis which is available to these mutants, but by use of these compounds suitably ^{14}C-labelled, some intermediates along the biosynthetic route, might yet be identified even in the continued absence of an organism which synthesises the vitamin in large amount.

APPENDIX 3

APPENDIX 1

CHEMICALS

PREPARATIONS

Dihydroxyfumaric acid - prepared from tartaric acid by the method of Powers, Tabakoglu and Sable (1955) and the hydrate recrystallised from aqueous methanol.

Formamidine - prepared from thiourea by the method of Brown (1952). Hydrochloride M.P. (uncorr.) 78°.

Formiminoglycine - from formamidine and glycine by the method of von Fritz, Micheel and Flitsch (1952). Recrystallised from alcohol-ether-water mixture. M.p. (uncorr.) 217°.

Glucose reductone (enoltartronaldehyde:hydroxymalonic aldehyde) - isolated from products of action of alkali on glucose (von Euler and Martius, 1933). Recrystallised from dry ethyl acetate and then three times from boiling water. Cream-coloured needles obtained; solution showed pronounced u.v. absorption maxima at 267 mμ (pH 2) or 287 mμ (pH 10).

Glycolaldehyde - based on the method of Powers, Tabakoglu and Sable (1955). Fifty g. of recrystallised dihydroxyfumaric acid were added to 100 ml. of pyridine and heated in a water bath at 50-55° with stirring. Evolution

of gas occurred and stirring and warming were continued until a clear solution was obtained. The solution was transferred to a 200 ml. flask, and the pyridine removed under reduced pressure at 35°. The distillation was continued until the temperature of the distillate rose to 25-27°, indicating that most of the pyridine had been removed. An oil bath was substituted for the water bath and the distillation continued with the receiver packed in ice and the bath temperature raised to 160°. The distillate obtained at a temperature of about 90° was glycolaldehyde. The syrupy distillate was placed in a vacuum desiccator over conc. H_2SO_4, and NaOH pellets. Crystallisation started spontaneously. When complete, the product was dispersed in a small volume of acetone, filtered and air dried. The product, (m.p. 88°) was replaced in the desiccator.

To recrystallise, 500 mg. of the crude product was heated under reflux with 30 ml. acetone until solution was complete. It was filtered and the filtrate allowed to stand over silion gel in a desiccator at 2°. After a month, crystals of glycolaldehyde (m.p. 94.5-95°) were deposited.

5-hydroxymethylfurfuraldehyde - from sucrose, by the method of Haworth and Jones (1944). In the final distillation at 110-115° (bath temp.), and 0.5-0.6 mm. Hg pressure,

2-(hydroxyacetyl)furan sublimed over (Miller and Cantor, 1952). At 145°, a yellowish, mobile oil was obtained. When kept overnight at 2°, this solidified. Unsuccessful attempts were made to recrystallise this manifestly impure product from an ether/petroleum ether mixture. Aqueous solutions showed a u.v. absorption peak at 225 mμ, and another much stronger peak at 280 mμ. Molarity of the stock solutions was determined assuming a molar extinction coefficient of 16,200.

β-hydroxypyruvic acid - by controlled alkaline hydrolysis of β-bromopyruvic acid (Sprinson and Chargaff, 1946). β-Bromopyruvic acid was recrystallised twice from hot chloroform, when a white, crystalline solid of m.p. 59° was obtained. 835 mg. of β-bromopyruvic acid was weighed into a 250 ml. beaker and dissolved in 3 ml. of water. The resulting solution was now titrated against 0.107 N-NaOH. For complete neutralisation, and hydrolysis 93.5 ml. would be required. The pH was followed throughout with a glass electrode. The first portion of 60 ml. was added quite quickly, the pH remaining below 7 throughout. The remnant had to be added very slowly (1 ml. every 15 min.), so that the pH at no time exceeded 8.5. The final molarity of the solution was calculated from the amount of standard alkali consumed. The β-hydroxypyruvic acid was used as produced, i.e., in solution containing an equimolar

concentration of NaBr.

Methylglyoxal - this was produced in solution from dihydroxyacetone (Umbreit, Burris and Stauffer, 1945) and assayed by the method of Friedmann (1927).

Ribose-5-phosphate - prepared in solution with adenine and phosphate, by the controlled acid hydrolysis of ATP. A 0.05 M solution of ATP was made 0.01 N with respect to HCl, and kept in a boiling water bath for 3 hr. After cooling, the solution was neutralised with NaOH and diluted.

SOURCES OF OTHER SPECIAL CHEMICALS

δ-aminolaevulic acid - gift from Prof. A. Neuberger.

ATP - as the disodium, trihydrate salt crystalline, from muscle. Sigma Chemical Corp. Kept at -15° over silica gel.

DPN - cozymase '75' of the Sigma Chemical Corp. Neutralised solution used. Stored at -15°.

4-deoxypyridoxine - (Nutritional Biochemicals Corp.). Seitz-filtered stock solution (10^{-1} M) at 2°.

Dihydroxyacetone - prepared by Dr. R.G. Tucker by the partial oxidation of glycerol with Acetobacter suboxydans 621.

D-erythrose-4-phosphate - a specimen of the cyclohexylamine salt of the acetal, was donated by Dr. Clinton E. Ballou.

A solution of free D-erythrose-4-phosphate was obtained from it by the method of Ballou, Fischer and MacDonald (1955), i.e., a solution of 10 mg. of the salt in 5 ml. of water was swirled for a minute with 2 ml. of Dowex 50 (H^+ form; 0.2 m.eq./ml.). The resin was centrifuged off and the supernate kept in a tightly stoppered container at 40° for 18 hr. The 0.1% solution of the free ester was neutralised before use.

L-erythrulose - a sample in the form of its o-nitrophenylhydrazine was given by Dr. B.L. Horecker. A solution of free L-erythrulose was prepared according to the method of Müller, Montigel and Reichstein (1937), modified for use on a micro scale.

Glucose-6-phosphate - a sample of the barium salt was given by Mr. R.W. Wakelin. The free phosphate ester was liberated by quantitative precipitation of the barium with H_2SO_4. The stock solution was neutralised and Seitz-filtered.

Glycolaldehyde - (Light and Co., Ltd.; and Fluka, A.G.).

Glycolaldehyde-2-phosphate - a specimen of the barium salt was given by Dr. E. Racker. The soluble sodium salt was prepared by batch treatment with Amberlite IR 50 in the Na^+ form. The molarity of the solutions used was checked by colorimetric estimation of phosphate liberated by perchloric acid digestion, by the method

of Fiske and Subbarow (1925).

Isoniazid - (Roche Products, Ltd.). Stock (10^{-1} M) solution was Seitz-filtered and stored at 2°.

Penicillin - crystalline penicillin G (Glaxo Labs.). 1650 units/mg.; weighed out into sterile water, and used immediately.

Penicillinase - standard preparation supplied by Distillers Co. as either (a) sterile solution, kept at 2°, or (b) freeze-dried material kept at -15° and made up in sterile water just before use.

3-Phosphoglyceric acid - a 10^{-1} M solution of the sodium salt given by Dr. K.M. Jones.

Ribulose-1:5-diphosphate and sedoheptulose-7-phosphate - samples of the barium salts were given by Dr. B.L. Horecker. The sodium salts were prepared in solution by batch treatment with Amberlite IR 50 (in the Na^+ form).

Shikimic acid - (Merck and Co., Inc.).

Xylulose-5-phosphate - a specimen of the barium salt was donated by Dr. B.L. Horecker. The sodium salt was prepared in solution by batch treatment with Amberlite IR 50.

VITAMINS

B12 - dilutions were made from a standard 2 mg./ml. solution

in physiological saline, stored at 2° ('Anacobin', B.D.H., Ltd.).

PtG - pteroylglutamic acid (Lederle Laboratories Division of the American Cyanamide Co.). Dilutions made in sterile water from a stock (10^{-3} M) sterile solution kept at -15°.

Pyridoxal-HCl, pyridoxamine-HCl and calcium salts of pyridoxal-5-phosphate and pyridoxamine-5-phosphate - (Merck and Co., Inc.).

Pyridoxal-5-phosphate and pyridoxine-HCl - (Roche Products, Ltd.).

APPENDIX 2

MEDIA

Nutrient broths

TMB - tryptic meat broth.

Bullocks' hearts were defatted, minced and weighed, then 1.1 times their weight of hot tap water added. The mixture was brought to 80° and maintained at this temperature and at pH 8 for 30 min. After cooling to 45°, pancreatic extract was added (0.1 volume of water used). After 4 hr. constant stirring at pH 8 and 45°, the pH was brought to 6 and the temperature raised to 95°. Following 1 hr. steaming in an autoclave, the mixture was filtered through muslin and Green's FF904$^{1/2}$ while still hot, then cooled and its pH adjusted to 7.6. Storage was at room temp. in the dark over $CHCl_3$.

TGB - tryptose glucose broth

Bacto-tryptose (Difco) ..	10 g.
Glucose	10 g.
K_2HPO_4	5 g.
Yeast extract (Difco)	2 g.
Salts solution (mixture B)..	5 ml.
16% liver extract (Oxoid) ..	10 ml.
Final volume to	1000 ml., pH 6.8

Procedure: warmed to dissolve, made up to volume with water, boiled and filtered through Green's FP904 1/2. pH adjusted to 6.8.

Solid media

Agar - several grades, viz.,
BDH - used for solidifying non-defined media.
Difco - used when fairly high degree of purity required.
Washed agar - used when growth-factor-free agar required.

To prepare - Difco agar (100 g.) was placed in a 5 litre glass jar and washed thoroughly with 4 litre glass distilled water. The agar was allowed to settle and the supernatant carefully decanted. This procedure was repeated 10 times. The wet agar was sucked dry on a Buchner funnel and further washed with two 4 litre volumes of redistilled azeo-alcohol, again filtered on a Buchner funnel (4 hr.) and then placed in a vacuum oven at 38° over anhydrous $CaCl_2$ for two days.

Nutrient agar

(a) Oxoid nutrient agar - 28 g. of granules in 1 litre water, pH 7.2. Sterilised by autoclaving 15 lb.sq.in./20 min.

(b) Casein-marmite agar -

Tryptic digest of casein ..	66 ml.
10% Marmite solution	2 ml.
BDH agar	5 g.
Water	200 ml.

The marmite and casein hydrolysate were dissolved in 100 ml. water, the agar melted by steaming in the other 100 ml. and both mixed when hot. Auto. 10 lb.sq.in for 20 min.

Medium S_1 - tryptic meat agar.

Tryptic meat broth	500 ml.
BDH agar	20 g.
Water	500 ml.

Procedure: agar melted by steaming with the water, the TMB also heated and thoroughly mixed with the agar. Distributed, autoclaved 15 lb.sq.in./10 min. and sloped.

Medium S_2 - tryptose glucose agar.

Bacto tryptose (Difco) ..	10 g.
Glucose	10 g.
K_2HPO_4	2 g.
$CaCO_3$	3 g.
Salts soln. (mixture 3) ..	5 ml.
16% liver extract (Oxoid)..	10 ml.
Agar (BDH)	15 g.
Water to	1000 ml.

Procedure: dissolved by steaming, mixed and distributed. Autoclaved 15 lb.sq.in./10 min. Solidified. Stabs.

Medium S_3 - tryptic casein agar.

Tryptic casein digest ..	330 ml.
Agar (BDH)	20 g.
Water	670 ml.

Procedure: agar melted in water by steaming, then casein digest added hot. Mixed, distributed, autoclaved 15 lb.sq.in./10 min. Sloped.

Medium S_4 - glycerol yeast agar.

Glycerol	40 ml.
Yeast extract (Difco)	5 g.
Agar (BDH)	14 g.
Water to	1000 ml., pH 6.0

Procedure: agar melted in 500 ml. water by steaming, the remaining water (460 ml.) added to the yeast extract and glycerol and heated. The pH was adjusted to 6.0.

Distributed, autoclaved 15.lb.sq.in./15 min. Sloped.

Medium S_5 - mould medium agar.

Malt extract ..	22.5 g.	KH_2PO_4	1.0 g.
Ammonium tartrate	5.0 g.	$MgSO_4.7H_2O$ ..	0.5 g.
Yeast extract (Difco)	5.0 g.	NaCl	0.1 g.
Sucrose	5.0 g.	$CaCl_2$ (anhyd.)	0.05 g.
Fe^{++} as iron citrate ..	2×10^{-5} M	NH_4NO_3	1.0 g.

Agar 20 g. and water to 1000 ml. pH 5.5.

Autoclaved 10 lb.sq.in./10 min. Sloped.

Chemically defined and semi-defined media

Medium A - for the assay of vitamin B_6 with Saccharomyces carlsbergensis 4228c. 500 ml. of double strength medium contains,

Glucose	50.0 g.
KH_2PO_4	550 mg.
$CaCl_2$ (anhyd.)	100 mg.
KCl	425 mg.

$MgSO_4.7H_2O$	125 mg.
$MnSO_4.4H_2O$	2.5 mg.
Citric acid	1.0 g.
Sodium citrate.$3H_2O$ ..	5.0 g.
Acid-hydrolysed casein (10%) ..	20.0 ml.
$FeCl_3$	2.5 mg.

Vitamins:

Biotin	8.0 µg.
Inositol	25.0 mg.
Calcium pantothenate	2.5 mg.
Thiamine	0.25 mg.
Nicotinic acid	2.5 mg.

Procedure: all except vitamins added to about 400 ml. water, brought to boil, simmered 3 min., cooled and filtered if necessary. Vitamins added to filtrate and sufficient N-HCl (about 1 ml.) to bring pH to 5-5.2. Volume made up to 500 ml.

Medium B - for growth of vitamin B_6-deficient Strep. faecalis R. 1,000 ml. final volume contains,

Glucose	10.0 g.
KH_2PO_4	8.0 g.
DL-alanine	200 mg.
L-cysteine.HCl	130 mg.
DL-tryptophan	100 mg.
Acetic acid (glacial) ..	0.85 ml.
Thioglycollic acid (redist.)	0.08 ml.
Acid-hydrolysed casein (10%)	50.0 ml.
Salts solution (mixture B)	5.0 ml.
Adenine & guanine (1 mg./ml.)	1.0 ml.
Uracil (10 mg./ml.)	0.5 ml.

Vitamins:

Nicotinic acid	11.0 mg.
Riboflavine	1.0 mg.
Calcium pantothenate	1.0 mg.
Biotin	1.0 µg.

Procedure: solids (except vitamins) dissolved in 350 ml. water. All solutions now added and pH adjusted to 7.2 with 40% NaOH (w/v). Boiled, simmered 10 min., cooled, filtered if necessary, vitamins added and volume made up to 800 ml.

Medium C - for growth of *Azotobacter*. 500 ml. double strength.

Mannitol	15.0 g.
K_2HPO_4	0.2 g.
$MgSO_4.7H_2O$	0.2 g.
$CaCl_2$ (anhyd.)	0.02 g.
$FeCl_3$.aq. (10%)	0.05 ml.
Molybdate (Na^+ or K^+)	Trace

Procedure: dissolved in 500 ml. water and pH checked (7.2).

Medium SC - Medium C solidified with 15 g./litre (final) agar.

Medium C_1 - as for Medium C but with 0.02% NH_4NO_3.

Medium D - for *Pseudomonas fluorescens*. 500 ml. double strength, pH 7.0, contains -

Glucose	2.5 g.
KH_2PO_4	1.0 g.
NH_4NO_3	1.0 g.
$MgSO_4.7H_2O$	0.5 g.

Procedure: dissolved in 500 ml. water, pH brought to 7.0, boiled, simmered 3 min., and filtered hot. pH checked on cooling.

Medium E - for Proteus morganii. 500 ml. double strength (pH 7.4) contains,

KH_2PO_4	4.5 g.
NaOH (40% w/v)	2.4 ml.
Acid-hydrolysed casein (10%)	50 ml.
L-cysteine.HCl	20 mg.
Glucose	10 g.

Vitamins:

Calcium pantothenate	to 10^{-6} M
Nicotinic acid	to 10^{-4} M

Procedure: salts and casein hydrolysate dissolved in 400 ml. water by boiling; cooled and vitamins added, then volume made up to 500 ml. and pH checked. In the present work, the glucose and L-cysteine.HCl were added in sterile solution after autoclaving.

Medium F - for Bacillus spp. 500 ml. double strength (pH 7.0) contains,

Glucose	30.0 g.
KH_2PO_4	1.5 g.
$(NH_4)_2HPO_4$	7.0 g.
$MgSO_4.7H_2O$	0.5 g.
$CaCl_2.2H_2O$	0.3 g.
$MnSO_4.4H_2O$	40.0 mg.
$FeSO_4.7H_2O$	2.5 mg.
Ammonium molybdate	2.0 mg.

Procedure: salts dissolved in 500 ml. water, boiled, simmered for 3 min. and filtered hot (Whatman No. 50). In the present work, the glucose was added in sterile solution after autoclaving.

Media G - glucose-containing minimal media for E. coli.

Medium G1 - 500 ml. double strength (pH 7.0) contains,

K_2HPO_4	7.0 g.
KH_2PO_4	3.0 g.
$(NH_4)_2SO_4$	1.0 g.
Sodium citrate.$3H_2O$	0.5 g.
$MgSO_4.7H_2O$	0.1 g.
Glucose	2.0 g.

Procedure: salts dissolved in 500 ml. water and pH checked. The glucose is added in sterile solution (Seitz-filtered, or autoclaved in solution in glass-distilled water, pH 5.4), after remainder of the medium has been autoclaved.

Medium G1A - as for Medium G_1, but glucose added before autoclaving.

Medium G_2 - as for Medium G_1, but with glucose conc. increased from 2 to 10 g./litre final volume.

Medium G_2A - as for Medium G_2, but glucose added before autoclaving.

Medium H - for Chlorella pyrenoidosa. 500 ml. double strength medium (pH 6.5) contains,

Glucose	96.0 g.
Urea	6.8 g.
$MgSO_4.7H_2O$	1.28 g.
KH_2PO_4	1.8 g.
Ethylenediamine-tetraacetic acid	1.0 g.
$CaCl_2$ (anhyd.)	50 mg.
$FeSO_4.7H_2O$	110 mg.
$ZnSO_4.7H_2O$	226 mg.
$CuSO_4.5H_2O$	11 mg.
$Na_2MoO_4.2H_2O$	2 mg.

Procedure: constituents dissolved in 500 ml. water, boiled, filtered, cooled and pH checked.

Medium I - for Sarcina lutea. As described by Brown and Binkley (1954).

Medium J - for Corynebacterium erythrogenes A1062. For 500 ml. double strength medium,

Acid-hydrolysed casein (10%) ..	200 ml.
$MgSO_4.7H_2O$	1.0 g.
Phosphate buffer (pH 7)	300 ml. of 0.165 M
Glucose	2 g.

Procedure: the glucose was added after autoclaving.

Medium K - for Rhodopseudomonas capsulatus (Oxford). 500 ml. of double strength medium contains,

Sodium malate	3.25 g.
Sodium glutamate.H_2O	3.50 g.
$(NH_4)_2HPO_4$	730 mg.
$MgSO_4.7H_2O$	500 mg.
K_2HPO_4	450 mg.
KH_2PO_4	450 mg.
$CaCl_2$ (anhyd.)	36 mg.

Thiamine	1 mg.
Nicotinic acid	1 mg.
Biotin	10 µg.

The organism requires vitamin B_{12}, but commercial sodium glutamate was sufficiently contaminated with B_{12} to render its separate addition unnecessary.

Medium K and casein - contains 40 ml./litre acid-hydrolysed casein (10% w/v).

Medium L - ammonium lactate minimal medium for E. coli.

500 ml. double strength medium (pH 7.4) contains,

KH_2PO_4	2.0 g.
$Na_2HPO_4.12H_2O$	8.0 g.
$(NH_4)_2SO_4$	2.5 g.
NH_4Cl	2.5 g.
$MgSO_4.7H_2O$	50.0 mg.
Iron citrate (0.004 M) ..	1.0 ml.
Lactic acid	8.0 ml.
NaOH	4.0 g.

Procedure: after dissolving salts and lactic acid in about 450 ml. of water, NaOH added and the pH adjusted to 7.4. Water was then added to bring volume to 500 ml.

Medium L plus nicotinic acid - for Proteus vulgaris. Contains 10^{-6} M final conc. of nicotinic acid.

Medium L plus asparagine - contains 0.5% w/v asparagine.

Medium M - for Aspergillus nidulans. 500 ml. double strength medium (pH 6.5) contains,

$NaNO_3$	5.0 g.
KCl	0.52 g.
$MgSO_4.7H_2O$	0.52 g.
KH_2PO_4	1.52 g.
Fe^{++} and Zn^{++}	Traces
Glucose	10 g.

Procedure: salts dissolved in 500 ml. water and pH brought to 6.5 with N-NaOH. The glucose was added after autoclaving.

Medium M1 - for biotin auxotrophs of Aspergillus nidulans. Contains 10 mμg./ml. final biotin in Medium M.

Medium SM - Medium M supplemented with, per litre final volume,

Biotin	10 μg.
Pyridoxine.HCl	100 μg.
Agar (Difco)	25 g.

Procedure: steamed to dissolve agar, then vitamins added.

Solutions

Acid-hydrolysed casein. Vitamin-free, prepared according to the method of Snell and Rannefeld (1945).

Amino acid mixture (AA). Contained the following amino acids, all at 5×10^{-3} M except serine and glycine at 5×10^{-2} M:-

DL-alanine, L-arginine, DL-aspartic acid, L-cysteine.HCl, DL-glutamic acid, glycine, DL-histidine, L-hydroxypyroline, DL-isoleucine, DL-leucine, DL-lysine.diHCl, DL-methionine,

DL-phenylalanine, DL-proline, DL-serine, DL-threonine, DL-tryptophan, DL-tyrosine, DL-valine.

When required, p-aminobenzoic and p-hydroxybenaoic acids were added to give 10^{-5} M each.

Amino acid mixture minus serine-glycine (AA-SG). As for AA but without the serine and glycine.

Purines and pyrimidines mixture (PP). Contained 2×10^{-4} M adenine, and 10^{-4} M each of guanine, uracil, thymine and hypoxanthine. Addition of a little N-HCl facilitates solution (later neutralised).

Vitamin mixture (V)

Riboflavin (0.5 mg./ml.)	5 ml.
Thiamine (1.0 mg./ml.)	3 ml.
Calcium pantothenate (1 mg./ml.) ..	3 ml.
Nicotinic acid (5 mg./ml.)	1 ml.
Biotin (1 µg./ml.)	5 ml.
p-Aminobenzoic acid (10^{-3} M)	0.5 ml.
Vitamin B_{12} (1 µg./ml.)	5.0 ml.
Water to	50.0 ml.

When vitamin B_6 required, then 5 mg. pyridoxine.HCl added.

Salts mixture B

$MgSO_4.7H_2O$	20 g.
$MnSO_4.4H_2O$	1 g.
NaCl	1 g.
$FeSO_4.7H_2O$	1 g.

Dissolved in 200 ml. water containing 0.5 ml. conc. HCl aq. Volume made up to 500 ml.

Iron citrate (0.004 M).

$(NH_4)_2Fe(SO_4)_2.6H_2O$	504 mg.
Sodium citrate.$3H_2O$	708 mg.
Water	500 ml.

APPENDIX 3

Organisms

Acetobacter suboxydans 621. A.T.C.C. 621. Maintained on slopes of Medium S_4. Grown at 30°.

Aerobacter aerogenes 64 - Strain C.74. N.C.I.B. 8264: N.C.T.C. 8173. 71 - Strain C.75. N.C.I.B. 8271. N.C.T.C. 8197. Maintained on slopes of Medium S_1. Grown at 30°.

Agrobacterium radiobacter. Strain 36. N.C.I.B. 8149. A.T.C.C. 6466. Maintained on slopes of Medium S_1. Grown at 25°.

Agrobacterium tumefaciens. Strain A_1. N.C.I.B. 8150. A.T.C.C. 4720. Maintained on slopes of Medium S_1. Grown at 25°.

Alcaligenes faecalis. Strain 16. N.C.I.B. 8156. A.T.C.C. 8750. Maintained on slopes of Medium S_1. Grown at 37°.

Azotobacter. nT - isolated from soil by Dr. R.G. Tucker. Vinelandii - N.C.I.B. 8004: A.T.C.C. 9104. Maintained on slopes of Medium SC. Grown at 30°.

Aspergillus nidulans - pyridoxine auxotrophs, obtained from Prof. Pontecorvo. Bi $pyro_{1-3}$ - isolated after X-irradiation.
Bi $pyro_{4-8}$ - isolated after u.v. irradiation.
wn ACR bi $pyro_{9-10}$ - isolated after u.v. irradiation.

i.e., $pyro_{1-8}$ represent additional mutation in an already mutant strain bi (an X-ray mutant requiring biotin).

wn ACR bi $pyro_{9 \& 10}$ represent additional mutation in a complex strain differing from wild-type in colour of conidia (wn - white instead of green), biotin requirement, and acriflavine resistance (ACR).

$pyro_2$, $pyro_4$ and $pyro_5$ are allelic, hence presumably representing one block. The others had not been tested.

Maintained on slopes of Medium SM. Grown at 37°. Sub-cultured six-monthly.

Bacillus megatherium - N.C.I.B. 8508: N.R.R.L. B-938.

This strain is known to produce vitamin B_{12} and riboflavin in quite high yield. Maintained on slopes of Medium S_1. Grown at 37°.

Bacillus subtilis RT - isolated from soil by Dr. R.G. Tucker.

Porton
Pope } - from Ministry of Supply. Porton.

6346 - Strain S.243. N.C.I.B. 6346: N.C.T.C. 6346.

Maintained on slopes of Medium S_1. Grown at 37°.

Chlorella pyrenoidosa - obtained from Dr. L.A. Griffiths.

Maintained on slopes of Medium SH. Grown on the light at 30°.

Corynebacterium erythrogenes - A.1062 - laboratory stock culture. Maintained on slopes of Medium S_1. Grown at 25°.

Erwinia carotovora - N.C.I.B. 8057: A.T.C.C. 495.

Maintained on slopes of Medium S_1. Grown at 25°.

Mycobacterium phlei - Strain Crottin. N.C.I.B. 8573. Maintained on slopes of Medium S_1. Grown at 37°.

Proteus mirabilis - N.C.I.B. 5887: N.C.T.C. 5887. Maintained on slopes of Medium S_1. Grown at 37°.

Proteus morganii 231 - laboratory stock culture.

232 - Strain Spencer. N.C.I.B. 232: N.C.T.C. 232.

235 - Strain 33M. N.C.I.B. 235 N.C.T.C. 235.

8168 - Strain 21. N.C.I.B. 8168: A.T.C.C. 8019. Maintained on slopes of Medium S_1. Grown at 37°.

Proteus vulgaris X19 - Strain X19 (positive Weil-Felix). N.C.T.C. 67. N.C.I.B. 67.

R - laboratory stock culture. Maintained on slopes of Medium S_1. Grown at 37°.

Pseudomonas Sc/30 - isolated by Dr. R.B. Nimmo-Smith.

fluorescens 950 - Strain 0.45. N.C.I.B. 950: N.C.T.C. 950.

6751 - Strain Radlett. N.C.I.B. 6751: N.C.T.C. 6751. Maintained on slopes of Medium S_3. Grown at 25-30°.

Rhodopseudomonas capsulatus. Strain Oxford. Isolated by Dr. R. Cooper. Maintained on stab culture in Medium S. Grown at 30°.

Saccharomyces carlsbergensis. Strain 4228, sub-strain c. N.C.Y.C. 355. Maintained on slopes of Medium S_5. Grown at 30-37°.

Sarcina lutea. Strain PCl,1001. N.C.I.B. 8553: A.T.C.C. 9341. Maintained on slopes of Medium S_1. Grown at 25-30°.

Streptococcus faecalis R - Strain Rogers, sub-strain SLR(C). Obtained from Dr. I.C. Gunsalus. Maintained in stab culture in Medium S_2. Grown at 30°.

Vibrio. O_1 - N.C.I.B. 8250. Obtained from Prof. F.C. Happold. Maintained on slopes of Medium S_1. Grown at 30°.

Strains of Escherichia coli

Parent wild-type strains

B - from Dr. M.G. Tucker.

518 - Strain 15 N.C.I.B. 8114: A.T.C.C. 9723. From Dr. Roblin.

Auxotrophs

PA.15 - requires L-serine or glycine. From Dr. B. Wright.

2/57 - requires thiamine. Produced in this laboratory by u.v.-irradiation of Strain 15.

9/8 - requires nicotinic acid. Produced in this laboratory by u.v. irradiation of Strain 15.

Vitamin B_6 auxotrophs

B166 - from Dr. Joseph Gots.

B167 - from Dr. Joseph Gots.

22/99 - from Prof. B.D. Davis.

DW - a substrain of mutant M.154-59L of B.D. Davis provided by Dr. Elizabeth Work (Denman, Hoare and Work, 1953).

M_2 - produced by u.v.-irradiation of Strain 15.

All were maintained on slopes of Medium S_1 and grown at 37°.

Culture Collections

N.C.I.B. - National Collection of Industrial Bacteria. Chemical Research Laboratory, Teddington, Middlesex.

N.C.T.C. - National Collection of Type Cultures. Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

N.C.Y.C. - National Collection of Yeast Cultures. Brewing Industry Research Foundation, Nutfield, Surrey.

A.T.C.C. - American Type Culture Collection. Washington, D.C.

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ABSTRACT

INTRODUCTION

Although much attention has been paid to the group of closely related compounds making up 'vitamin B_6' as a consequence of the great importance of this vitamin in amino acid metabolism, nothing is known of its biogenesis by competent organisms. That D-alanine might be incorporated into the pyridine ring of pyridoxine in Streptococcus faecalis, was disproved by the discovery that in fact D-alanine merely showed a vitamin B_6-sparing action in an otherwise complete replacement medium. Although the suggestion has been made that in a certain strain of yeast, thiamine and pyridoxine were interconvertible via a common pyrimidine precursor, the supporting evidence was not convincing.

A survey of the known routes of synthesis of N-heterocyclic compounds suggests that incorporation of the N atom is achieved by spontaneous condensation of ketone with amine groups so that the immediate aliphatic precursor of vitamin B_6 might be expected to carry these two reactive groupings. At present, the only known route of pyridine ring biogenesis has as starting material the already aromatic benzene ring, i.e., origin of nicotinic,

quinolinic and picolinic acids from 3-hydroxyanthranilic acid. There is no evidence, however, to suggest a common origin of nicotinic acid and vitamin B_6, though it is true that in some microorganisms the pathway of nicotinic acid formation is still unknown. Appraisal of the possible methods available for the use of microorganisms in a study of growth factor biosynthesis, suggests that the first objective should be the isolation of an organism capable of forming large amounts of vitamin B_6. Failing this, attention should be directed to an examination of selected vitamin B_6 auxotrophs.

Experimental

A modification of the reported assay of vitamin B_6 using Saccharomyces carlsbergensis 4228 allowed smaller volumes to be used in static culture. As a direct consequence of its being a 'rate assay' (where rate of growth rather than its final possible extent is proportional to the concentration of growth factor), it was essential to maintain a standard time and steady temperature of incubation, and a constant degree of aeration and inoculum size, though it had the advantage that the sensitivity of the method could (within limits) be varied by adjusting the time of incubation. With the strain of S. carlsbergensis 4228 used, thiamine 'spared' vitamin B_6

at low concentrations of the latter, and was added in excess to the culture medium.

Using this method of assay, it was discovered that all of a number of various bacteria grown in chemically-defined media synthesised uniformly small amounts of vitamin B_6. The best synthesis obtained was with a strain of Proteus morganii whose production of 4μmole vitamin B_6/g. dry wt. of organism was only about ten times greater than average.

A cross-feeding, plate technique was devised, using S. carlsbergensis 4228e and the vitamin B_6 auxotroph E. coli M_2 as indicator organisms, the sensitivity of response being adjusted by incorporating 4-deoxypyridoxine into the vitamin B_6-free basal medium. Even when using this method of rapid screening, no organism was isolated from soil washings capable of appreciably better vitamin B_6 synthesis than E. coli 518. An isoniazid-resistant strain of E. coli 518 was isolated, but this only synthesised twice as much vitamin B_6 when grown in the presence of isoniazid (10^{-2} M) as in its absence. The amount of vitamin B_6 produced by wild-type E. coli 518 and B, could not be appreciably increased by addition to the growth medium of casein hydrolysate, and mixtures of purines, pyrimidines, growth factors or selected compounds such as 3-hydroxyanthranilic acid. Washed suspensions of E. coli

and Proteus morganii synthesised vitamin B_6 from glucose plus NH_4^+, the amount produced not being further increased by any supplement tested. Only very slight synthesis of vitamin B_6 was obtained with an ultra-sonically produced extract of Proteus morganii, and that only in the presence of a boiled extract of organisms.

Of four vitamin B_6 mutants of E. coli available, not one showed an absolute requirement for the vitamin under all conditions of growth. However, one such auxotroph, E. coli M_2, was produced by u.v.-irradiation of E. coli 518. Unsuccessful attempts were concurrently made to isolate 'multiple' mutants requiring both vitamin B_6 and nicotinic acid; no evidence of mutual sparing actions of these two vitamins could be obtained with the nicotinic acid-requiring Proteus vulgaris and E. coli 9/8, and the vitamin B_6 mutants E. coli B166 and M_2.

A detailed study was made of the properties of E. coli B166 which was reported to respond alternatively to vitamin B_6 or serine, or glycine. No other amino acid (apart from DL-threonine) or other compound of related structure including glyoxylic and β-hydroxypyruvic acids, could replace L-serine and glycine which were individually active but better used in admixture. Serine-glycine affected E. coli B166 in two distinct ways: (a) on both

glucose and lactate media with added pyridoxine, it reduced the delay in the onset of growth by 20-25 hr. and increased the final cell mass; (b) it supported suboptimal growth in the absence of pyridoxine - but only in a medium containing 'activated' glucose. Solutions of glucose of pH <6.5 (Seitz-filtered or autoclaved) were inactive. Autoclaving of a glucose solution (10 mg./ml.) at pH 7.4 and at 10 lb.sq.in./7 min. resulted in maximal activation, the product being so distinguished by its content of some specific chemical compound formed during the activation procedure. Attempts to effect purification and isolation of the 'active factor' were ended when, alone of a number of products of alkaline degradation of glucose tested, glycolaldehyde (5×10^{-5} to 10^{-3} M) was discovered to possess activity.

Glycolaldehyde (5×10^{-4} M) bettered the action of activated glucose in supporting the growth of E. coli B166 on an inactive glucose medium with serine-glycine, though concentrations above 10^{-3} M were growth inhibitory. On the lactate-containing medium, the same concentration of glycolaldehyde at first appeared to be less active than pH 7.4-autoclaved glucose, but addition of inactive glucose together with the glycolaldehyde doubled the response. In every other respect, glycolaldehyde behaved similarly to activated glucose, having no apparent effect

on growth in the presence of pyridoxine with or without serine-glycine. Of a number of chemically related compounds and 'active glycolaldehyde' donors and acceptors tested, only dihydroxyfumaric and β-hydroxypyruvic acids possessed glycolaldehyde-like activity, and this to a lesser degree suggestive of conversion to free glycolaldehyde. Colorimetric estimation showed the biological activity of samples of activated glucose to be paralleled by their content of glycolaldehyde.

The remaining vitamin B_6 mutants fell into two groups distinguished by their response to serine-glycine and glycolaldehyde. E. coli B167 and 22/99, like B166, grew in the absence of added vitamin B_6 if supplied with serine-glycine and glycolaldehyde. B167 behaved similarly in all respects to B166. Optimal growth of 22/99 was obtained on serine-glycine with either pyridoxine (10^{-6} M) or glycolaldehyde (5×10^{-4} M), suboptimal growth after delay was achieved with either serine-glycine or vitamin B_6, only very slight growth after prolonged delay being obtained on glycolaldehyde alone. Strains DW and M_2 did not respond to serine-glycine and glycolaldehyde. M_2 showed an absolute requirement for vitamin B_6 (to which it responded rapidly and maximally), at all temperatures and under all conditions tested. DW, on the other hand, grew in the absence of vitamin B_6 when provided with

DL-alanine plus DL-methionine at 25° (due probably to a vitamin B_6-sparing action of these amino acids), but showed an absolute requirement for the vitamin at 37°.

Although serine-glycine and glycolaldehyde did not markedly enhance vitamin B_6 synthesis in growth, or in washed suspensions (with glucose plus NH_4^+), of wild-type E. coli and Proteus morganii, the amount of vitamin B_6 formed at 25° by E. coli DW was somewhat increased in their presence. E. coli B166 harvested from growth on serine-glycine and glycolaldehyde, synthesised about as much vitamin B_6 as did its parent E. coli B, while E. coli 22/99 produced more vitamin B_6 when grown in the presence of glycolaldehyde than in its absence.

Unfortunately, significant vitamin B_6 synthesis by washed suspensions of E. coli B166 was only obtainable after growth of the organism in the absence of added vitamin. The formation of vitamin B_6 from glucose plus NH_4^+ by freshly harvested suspensions could therefore have been due to their possession of endogenous reserves of serine-glycine and glycolaldehyde (and/or their metabolic products). Even so, further addition of glycolaldehyde invariably enhanced the synthesis. When the organisms were 'depleted' by preliminary aerobic incubation in phosphate buffer, no vitamin B_6 synthesis was obtained in the absence of added glycolaldehyde; good synthesis being

achieved from glucose plus NH_4^+ plus glycolaldehyde. Synthesis became maximal only when serine-glycine was also present. With 'pre-depleted' suspensions of E. coli 22/99 grown on serine-glycine plus glycolaldehyde, these amino acids, as well as glycolaldehyde, were obligatory requirements for vitamin B_6 biosynthesis.

Discussion

No evidence has been obtained to suggest the common origin of the two pyridine B-vitamins, vitamin B_6 and nicotinic acid.

Most attention is here devoted to an analysis of the properties of several vitamin B_6 auxotrophs of E. coli, in particular mutant B166, this being considered as the most rewarding outcome of the study.

It is noted that although medium-autoclaved glucose has often been reported to possess growth-stimulatory properties for many microorganisms, never before has glycolaldehyde been implicated as a component of potential biological activity.

E. coli B166 appears to bear a partial blockage in serine-glycine biosynthesis as well as a lesion in vitamin B_6 formation. It is uncertain whether free glycolaldehyde is a normal metabolite in B166, but its metabolism must give rise to a compound whose synthesis is

limiting in this organism. There are several possible mechanisms whereby the demonstrated requirements could arise from a single mutation. The possibility that serine-glycine and glycolaldehyde act as products of the function of the vitamin, so reducing the organism's requirement for this to a level which may be produced by synthesis, is rejected in favour of the view that these compounds may be concerned (perhaps together with a third substance X) in vitamin B_6 biosynthesis. All these compounds are regarded as arising from a common precursor whose synthesis is suboptimal in this organism which must consequently be considered as being a 'leaky' mutant.

The behaviour of E. coli 22/99 may now be explained by supposing it to be better able than 3166 to synthesise the glycolaldehyde and Compound X components, whilst the metabolic lesion in E. coli DW and E. coli M_2 could be subsequent to the point of action of these compounds.

The possibility remains that the production of vitamin B_6 from glycolaldehyde, serine-glycine and glucose, is an alternative to the normal route of biosynthesis which is available to E. coli 3166.

A study of the route of incorporation of glycolaldehyde might lead to the identification of a precursor more nearly related to vitamin B_6.

TABLE C4-1

Ability of variously treated sugars and their derivatives to replace activated glucose

Activation = + No activation = -

Compound	Seits-filtered	Aqueous sol. (pH 5.4-5.6)	Medium (pH 7)	PO_4 buffer (pH 7.4)
Galactose	-	-	+	+
Mannose	-	-	+	+
Fructose*	-	-	+	+
Sorbose	-	-	+	+
Arabinose	-	-	+	+
Xylose	-	-	+	+
Ribose	-	-	+	+
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
Erythritol	-	-	-	-
3-O-methyl-glucose	-	-	-	-

*Activated fructose was somewhat inhibitory (delayed growth).

Criterion of activity was growth of *E. coli* B166 on Medium G_2 supplemented with serine-glycine plus the test compound. All compounds under test were used at a final conc. of 4 mg./ml. and autoclaved as stated at a conc. of 20 mg./ml. (i.e., at 10 lb.sq.in./7 min.).