A STUDY ON PYRIMIDINE BIOSYNTHESIS

By George David Birnie, B.Sc.

The pathways of biosynthesis of the pyrimidine nucleotides uridine-5'-phosphate and cytidine-5'-phosphate have been elucidated in recent years. The object of the present study was the development of a cell-free system in which the synthesis of thymine, thymidine or thymidylic acid could be investigated, with particular reference to the mechanism whereby the pyrimidine ring undergoes methylation. The organisms used were Escherischia coli PA/15, Esch. coli 113/3, Esch. coli 15T- and Bacillus subtilis N.C.I.B. 8059.

The ability of Esch. coli PA/15 to catalyse the synthesis of methionine from homocysteine and serine, a reaction formally analagous to the methylation of uracil or deoxyuridine, was confirmed. Attempts were made to synthesise thymine or thymidine from uracil or deoxyuridine using Esch. coli PA/15 and B. subtilis 8059 under the same conditions as those involved in methionine synthesis. No evidence was obtained for the synthesis of thymine or thymidine in this system using (i) a microbiological assay system employing Esch. coli 15T- as assay organism or (ii) extensive paper chromatographic analysis, with the use of $3-^{14}C$-serine as the one-carbon unit precursor. The use of the cofactors folic acid, $N^{10}-$

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formylfolic acid and N^5-formyltetrahydrofolic acid did not stimulate any detectable synthesis of thymine or thymidine.

A detailed investigation of the enzyme activities present in Esch. coli PA/15 was undertaken. The ability of Esch. coli to catalyse the production of one-carbon units from serine and from formate was confirmed and evidence in corroberation of the involvement of N-hydroxymethyltetrahydrofolic acid as the immediate one-carbon unit precursor was obtained. Conditions were established under which the hydrolysis of nucleosides by nucleoside phosphorylase and inorganic phosphate (produced from ATP by adenosine-5'-triphosphatase) was held to a minimum. . The operation of regenerative TPNH and DPNH systems in Esch. coli under suitable conditions was also confirmed. On the basis of information derived from this investigation, a system was developed in which thymidylic acid was synthesised by cell-free extracts of Esch. coli PA/15 and Esch. coli 113/3 from (i) serine, formaldehyde or formate and (ii) deoxyuridine, uridine or deoxyuridylic acid, together with the cofactors ATP, Mg^{2+}, TPNH (or DPNH), tetrahydrofolic acid and pyridoxal phosphate. The evidence that thymidylic acid was synthesised in these systems was obtained by an extensive programme of paper chromatography and electrophoresis.

Extensive examination of these systems for inter-
mediates on the biosynthetic pathway to thymidylic acid
was carried out, but no intermediates of the 5-hydroxy-
methyl-pyrimidine type were identified.   Experiments
using extracts of Esch. coli 15T-, a thymine- or
thymidine-less mutant, as the enzyme source were carried
out but again no thymidylic acid precursors of the
5-hydroxymethyl-pyrimidine type were identified.
Successful chemical syntheses of 5-hydroxymethyluracil
and 5-hydroxymethyldeoxyuridine were achieved and the
structure of the latter was confirmed by degradation
studies.   Attempts to synthesise 5-hydroxymethyldeoxy-
uridylic acid were unsuccessful.   Evidence was obtained
that thymidine-5'-triphosphate was formed in systems in
which thymidylic acid was synthesised, but no in vitro
synthesis of DNA-thymine was detected in the same
systems.

A comparison of serine, formaldehyde and formate as
one-carbon unit precursors revealed that serine was a
much more prolific source of one-carbon units than either
formaldehyde or formate.   A similar comparison of
deoxyuridine, uridine and deoxyuridylic acid as one-
carbon unit acceptors showed that uridine was a less
efficient acceptor than either deoxyuridine or deoxy-
uridylic acid but no significant difference between

deoxyuridine and deoxyuridylic acid was detected. The effect of vitamin B_{12} on thymidylic acid biosynthesis was also investigated using Esch. coli 113/3, a methionine- or vitamin B_{12}-less mutant, but no unequivocal evidence for a vitamin B_{12} effect was obtained with extracts of Esch. coli 113/3 cells which had been depleted of vitamin B_{12} by serial sub-culturing in a methionine medium.

Further extensive investigation of the ^{14}C-thymidylic acid synthesised in these systems revealed that a large proportion of the ^{14}C-thymidylic acid isolated was not , in fact, authentic thymidylic acid. Attempts to elucidate the structure of the contaminant have been carried out. Degradative and autoradiographic studies have led to a tentative identification of β-amino-iso-butyric acid and β-ureido-iso-butyric acid as hydrolytic products of the thymidylic acid contaminant. On this evidence, the contaminant appears to be the 4:5-dihydro derivative of thymidylic acid. Parallel investigation of the degradation of thymine and thymidylic acid by extracts of Esch. coli PA/15 has provided spectrophoto-metric evidence that the organism catalyses the reduction of the pyrimidine ring across the 4:5-double bond. Thymidine does not appear to be a substrate for this reduction system.

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by

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I am indebted to Dr. G.W. Crosbie for Fig. IV, 12. The experimental results used in constructing Fig. II, 8 were obtained by a group of Honours students of this department (Miss J. Prosser, Miss B. Binnie, Miss M. Kellock and Mr. I Boyle) and I am indebted to them for permission to use their results.

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1. General Introduction

With one exception, the pathways by which the
nucleotides of deoxyribonucleic acid (DNA) are
synthesised have been elucidated in recent years and
have been reviewed by a number of authors (Reichard, 1955a;
Schlenk, 1955; Carter, 1956; Davidson, 1957a). The one
exception is the pathway by which thymidine-5'-phosphate
(thymidylic acid; TMP) is synthesised with particular
reference to the mechanism by which the methylation of
the pyrimidine ring is achieved. A study of this
biosynthetic pathway in vitro was the purpose of the work
described in this thesis.

A study of the biosynthesis of TMP involves
consideration of a number of different points, namely
(a) the biosynthesis of the pyrimidine ring; (b) the
biosynthesis of the deoxyribose component; and (c) the
methylation of the pyrimidine ring. This last point
(c) alone involves a number of interesting problems
including (i) the source of the one-carbon unit which
is transformed into the TMP-methyl group; (ii) the
structure of the one-carbon unit acceptor compound;
(iii) the mode of transfer of the one- carbon unit and
(iv) the mechanism by which the TMP-methyl group is

formed from this one-carbon unit.

The biosynthetic pathways to the pyrimidine nucleotides have been established on the basis of information obtained (i) from whole cell systems by isotope incorporation studies (using labelled small-molecule precursors) and by growth requirement studies (using microbial mutants) and (ii) from cell-free systems by studies of the relevant enzyme systems.

It was initially intended that this introductory section (Section I) should incorporate a review of the problems of TMP synthesis which had been elucidated at the time when the present study was commenced, followed by a brief synopsis of the results published during the time this work was proceeding. Unfortunately, this approach has been found to be exceedingly difficult if not, indeed, impossible since the field has broadened rapidly in the last few years. Results which have been published since October, 1956 (when this study was begun) have had an important bearing on the interpretation of previous observations, with the result that a review of the field up to the end of 1956 would be misleading in a number of aspects and would necessitate considerable amendment. This would lead inevitably to a very clumsy discussion of the published work and, accordingly, the introductory section incorporates results published by early 1959.

In section II, the section devoted to a description of the observations made during the study, it has been found necessary to include a short discussion of the results of each experiment in order that the logical sequence of the experiments may be clearly understood. A short general discussion of the overall significance of the experimental observations has been included also (Section III). The experimental techniques used are described in full in Section IV.

2. Biosynthesis of the pyrimidine ring.

(1) Studies in whole-cell systems.

The first attacks on the problem of pyrimidine nucleotide biosynthesis were made by studies on the incorporation of carbon and nitrogen isotopes, in the form of simple precursors, into the nucleic acid pyrimidines of whole animals, mammalian and avian tissue slices and homogenates and micro-organisms and by studies of the growth factors of various microbial mutants. The initial step was taken by Barnes and Schoenheimer (1943) when these authors found that ^{15}N-ammonium citrate was incorporated into the nucleic acids of mammalian and avian tissues thus showing that the nucleic acids could be derived from small molecule precursors. Incorporation of other labelled precursors into the nucleic acids of similar tissues, followed by isolation and

degradation of the pyrimidines, has shown that N_1
of the pyrimidine ring is derived from ammonia
(Lagerkvist, 1953), C_2 from CO_2 (Heinrich and Wilson,
1950) and N_3, C_4, C_5 and C_6 from aspartic acid
(Lagerkvist et al, 1952; Reichard and Lagerkvist,
1953) (See Fig. I, 1 for the definition of ring
numbering in the pyrimidines).

Both carbamylaspartic acid (ureidosuccinic
acid) and orotic acid (uracil-4-carboxylic acid) two
growth factors of Lactobacillus bulgaricus 09, were
shown to be implicated in the biosynthesis of the
pyrimidine ring when it was found that both compounds
were incorporated into the nucleic acids of that
organism (Wright et al, 1951). The rôle of orotic acid
as a precursor of the nucleic acid pyrimidines of a
large number of tissues has been confirmed both in vivo
and in vitro. Orotic acid has been shown to be
incorporated (a) into the nucleic acids of Escherichia
coli B (Weed and Cohen, 1951), yeast (Edmonds et al, 1952),
the rat (Hurlbert and Potter, 1952; Harrington and Lavik,
1955), the mouse (Lagerkvist and Reichard, 1954),
mammalian liver slices (Weed and Wilson, 1951; Reichard
and Borgström, 1951), cat spleen slices (Weed and Wilson,
1953), tumours (Weed, 1951) and Ehrlich ascites tumour
cells (Lagerkvist and Reichard, 1954) and (b) into the

uridine-5'-phosphate (uridylic acid; UMP) of the acid-soluble fraction of rat liver (Hurlbert and Potter, 1952; Hurlbert, 1952; Hurlbert, 1953).

The biosynthesis of orotic acid, so clearly implicated in pyrimidine biosynthesis by the evidence quoted above, was investigated very extensively by Reichard and Lagerkvist (1953). Those authors studied the incorporation of ^{15}N-ammonium chloride, ^{13}C-bicarbonate, ^{15}N-L-aspartic acid, 1:4-^{13}C-L-aspartic acid, 2:3-^{14}C-L-aspartic acid and ^{15}N-L-carbamylaspartic acid into an added pool of orotic acid and obtained results which both confirmed and amplified those obtained in previous investigations. The results, in conjunction with the other evidence obtained from isotope incorporation and microbiological experiments, showed clearly that aspartic acid, carbamylaspartic acid and orotic acid (or close derivatives of these compounds) were all involved as precursors in the biosynthesis of UMP.

One other compound, dihydro-orotic acid, was implicated in UMP biosynthesis by experiments of the same type. It was found that dihydro-orotic acid would support the growth of L. bulgaricus 09 in place of carbamylaspartic acid or orotic acid (Miller et al, 1953) and further, that rat liver homogenates would catalyse

the dehydrogenation of dihydro-orotic acid to yield orotic acid.

Experiments using whole cell systems have shown (a) that the pyrimidine ring can be derived from small molecule precursors and (b) that four compounds (aspartic acid, carbamylaspartic acid, dihydro-orotic acid and orotic acid) are intimately connected with the biosynthetic pathway to the pyrimidine nucleotides. The further details of this pathway were worked out by studying the enzyme systems involved in cell-free extracts and, in some cases, with purified enzymes.

(ii) Studies in cell-free systems

The synthesis of citrulline from ornithine,

$$NH_2.(CH_2)_3. CH(NH_2).COOH \longrightarrow NH_2.CONH.(CH_2)_3.CH(NH_2)COOH$$

ornithine   citrulline
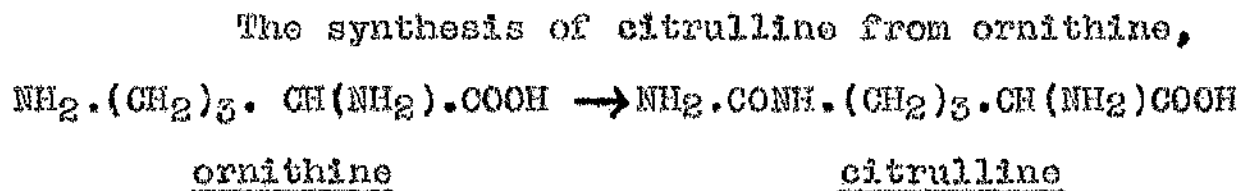
a reaction which is formally analogous to the formation of carbamylaspartic acid from aspartic acid, has been studied by Grisolia and Cohen (1952) and by Jones et al. (1955). Grisolia and Cohen showed that the formation of the "active" derivative of ammonia and CO_2 (Compound X) involved in the synthesis of citrulline is dependent on the presence of adenosine-5'-triphosphate (ATP), Mg^{2+} and acetylglutamate. Jones et al. found (i) that cell-free extracts of Streptococcus faecalis

will catalyse the formation of carbamyl phosphate
(compound X) from CO_2 and ammonia in the presence
of ATP :-

$$CO_2 + NH_3 + ATP \longrightarrow NH_2 - CO - OPO_3H_2 + ADP$$

and (ii) that the carbamyl phosphate so formed is a
substrate of the ornithine carbamyl transferase
activity which catalyses the synthesis of citrulline
from ornithine. The analogous condensation between
aspartic acid and carbamyl phosphate, catalysed by
the enzyme aspartate carbamyl transferase, has been
demonstrated by Reichard and Hanshoff (1956) using
(i) an extract of rat liver and (ii) a purified
Esch. coli enzyme. In addition, Reichard and
Hanshoff established that the ornithine- and
aspartate carbamyl transferase were not identical.
The aspartate carbamyl transferase activity isolated
from Esch. coli has been purified to a considerable
extent and, using the purified enzyme, good
stiochiometry for the reaction

carbamyl phosphate + aspartate $\longrightarrow$ carbamylaspartate
+ inorganic phosphate
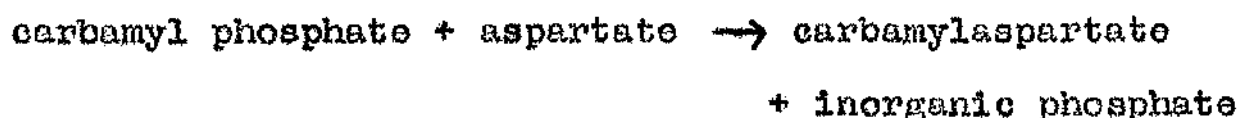
has been obtained. No evidence that this reaction is
reversible was obtained by the use of $^{32}PO_4^{3-}$. The
failure to demonstrate exchange between added

[14]C-aspartic acid and carbamylaspartic acid in this reaction indicates the non-involvement of a carbamyl-enzyme complex in the mechanism. The mechanism probably involves a nucleophilic attack on the carbonyl group of carbamyl phosphate by the N-atom of aspartic acid subsequent to substrate-binding by the enzyme.

The hydrolysis of carbamylaspartic acid to aspartic acid, ammonia and CO_2 has been described by Lieberman and Kornberg (1955). This degradation is effected by a ureidosuccinase (carbamylaspartase) enzyme which is obtained from extracts of Zymobacterium oroticum grown anaerobically on orotic acid as the sole carbon source. The hydrolysis is dependent on the present of Mn^{2+} and a sulphhydryl compound, but not adenosine-5'-diphosphate (ADP), whereas the corresponding hydrolysis of citrulline yields ATP. The reaction is both highly exergonic and virtually irreversible and these facts led to the suggestion that the mechanism of carbamylaspartic acid synthesis from aspartic acid involved arginosuccinic acid. It had been observed that citrulline-ureido-^{14}C was utilized (a) in the synthesis of nucleic acid pyrimidines in Neurospora (Heinrich et al, 1954) and in pigeon liver homogenates (Schulman and Badger, 1954) and (b) for orotic acid synthesis in rat

liver slices (Smith and Stettin, 1954). These
observations had been interpreted as indicating that
arginosuccinic acid was implicated in the biosynthesis
of the pyrimidine ring. However, the observed
formation of carbamylphosphate from citrulline by a
rat liver mitochondrial extract in the presence of
ATP, Mg^{2+} and acetylglutamate (Smith and Reichard,
1956) has enabled these observations on citrulline
utilization to be integrated with the pathway of
carbamylaspartic acid synthesis from carbamyl phosphate
and aspartic acid without the participation of
arginosuccinic acid.

Lieberman and Kornberg (1953) have shown
that cell-free extracts of Z. oroticum will catalyse
the interconversion of carbamylaspartic acid and orotic
acid. The extracts of this micro-organism contain
(a) dihydro-orotase which catalyses the formation of
dihydro-orotic acid from carbamylaspartic acid and
(b) dihydro-orotic acid dehydrogenase, a
diphosphopyridine nucleotide-(DPN-) linked enzyme which
catalyses the dehydrogenation of dihydro-orotic acid to
yield orotic acid. The possibility that these enzymes
may not be involved in normal pyrimidine biosynthetic
pathways must be considered since it has been demonstrated

that both are adaptive enzymes (Yates and Pardee, 1956a).
However, the corresponding enzymes have been shown to
be present in extracts of Esch. coli B which has been
grown on minimal medium (Yates and Pardee, 1956) and,
moreover, the activity of the isolated enzymes is
sufficient to account for their involvement in the
synthesis of the nucleic acid pyrimidines of the parent
cells.

The recent work of Lieberman et al. (1955) has
enabled the final stages in the synthesis of UMP:

orotic acid ⟶ orotidine-5'-phosphate ⟶ UMP

to be defined clearly. From yeast extracts, these
authors have isolated and purified an enzyme
(orotidine-5'-phosphate pyrophosphorylase) which
catalyses the formation of orotidine-5'-phosphate from
orotic acid and 5-phosphoribosylpyrophosphate in the
presence of Mg^{2+}. These yeast extracts also contain
orotidine-5'-phosphate decarboxylase which converts
orotidine-5-phosphate irreversibly to UMP. The presence
of these enzymes has been reported in mammalian liver
also (Hurlbert and Reichard, 1955), an observation which
explains the observed incorporation of ^{14}C-orotic acid
into the UMP of the acid-soluble fraction of rat liver
(Hurlbert and Potter, 1952; Hurlbert, 1952; Hurlbert, 1953;

Hurlbert and Reichard, 1955). The enzyme
orotidine-5'-phosphate pyrophosphorylase, isolated
from yeast, appears to be specific to orotic acid.
No corresponding reaction with DL-carbamylaspartic
acid, L-dihydro-orotic acid, uracil or cytosine has
been reported. Similarly, no evidence for the
involvement of an open-chain glycoside in reactions
leading to the formation of orotidine-5'-phosphate
has been obtained.

Fox et al. (1957) have recently substantiated
the structure of orotidine as 3-D-ribofuranosyluracil-
4-carboxylic acid by a spectrophotometric study of
N-alkyl derivatives of orotic acid. Lieberman et al.
(1955) have demonstrated that the orotidine-5'-phosphate
which they obtained was apparently identical with the
product of an enzymically-catalysed phosphate transfer
to orotidine (Michelson, 1951), thus indicating the
compound to be the 5'-phosphate ester of 3-D-ribofuranosyl-
uracil-4-carboxylic acid.

The biosynthetic pathway to the pyrimidines,
shown in Fig. I, 1, has been established on the basis of
this evidence. The formation of the free base from UMP
is catalysed by the action of uridylic acid
phosphomonoesterase on UMP to yield uridine, followed by
hydrolysis of the nucleoside to uracil by nucleoside
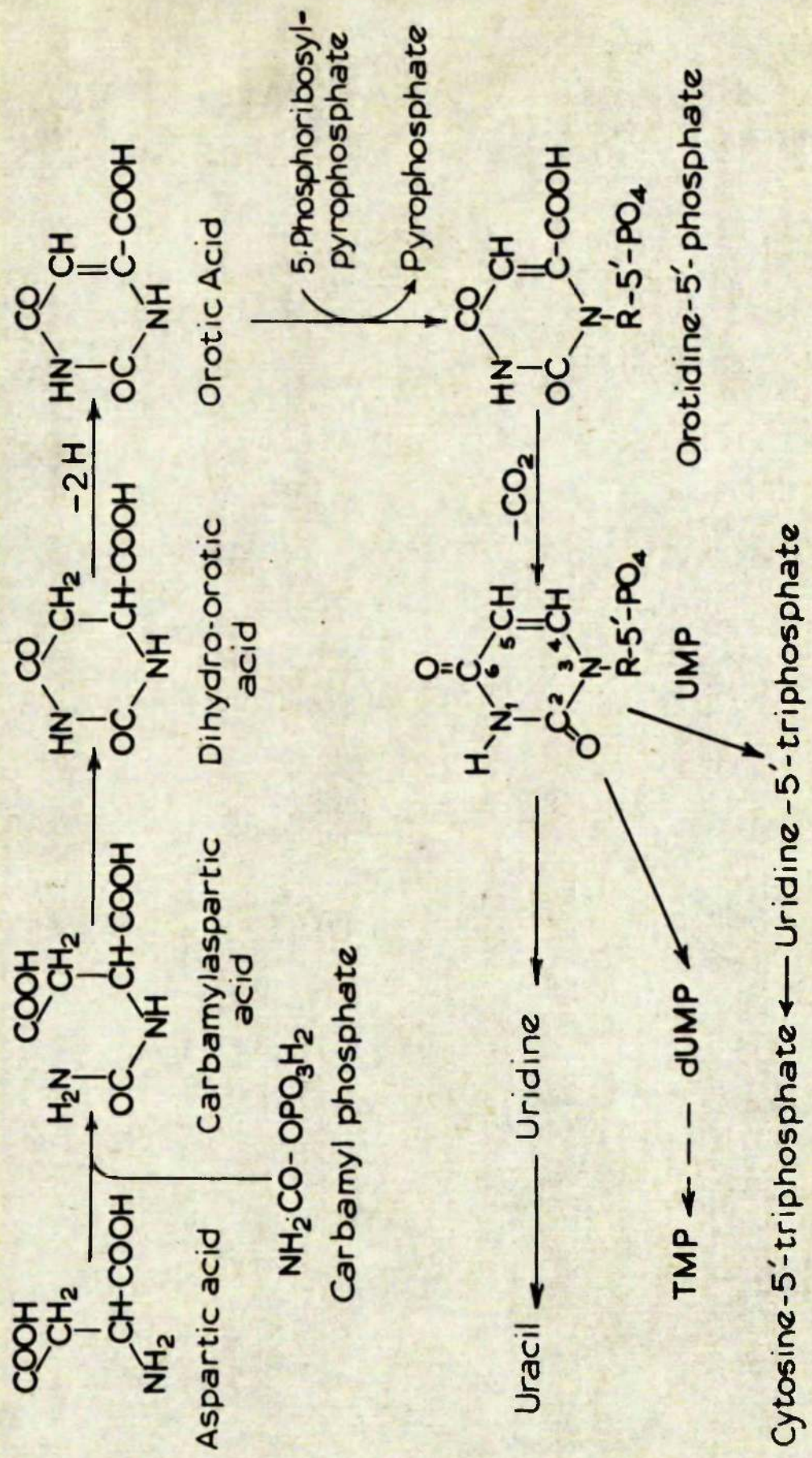
Figure I. 1

phosphorylase. The conversion of uracil to cytosine has been shown to take place at the nucleotide triphosphate level (Lieberman, 1955; Lieberman, 1956). The synthesis of thymine is more complex and will be discussed later at some length.

It is worthy of note that a control of pyrimidine nucleotide biosynthesis in Esch. coli by a feed-back inhibition mechanism has been demonstrated by Yates and Pardee (1956b). These authors have shown that cytidine-5'-phosphate is a competitive inhibitor in the formation of carbamylaspartic acid from carbamyl phosphate and aspartic acid. Cytidine will also inhibit this reaction though to a lesser extent whereas the presence of uracil, uridine, UMP, or cytosine does not cause any appreciable inhibition.

(iii) Alternative pathways of pyrimidine ring biosynthesis

Although the pathway outlined in Fig. I, 1, has been established very clearly, some evidence of the operation of alternative pathways of pyrimidine biosynthesis has been reported in recent years. A reversal of the reaction sequence by which uracil is degraded to β-alanine (Fig.I,2)(Grisolia and Wallach, 1955; Canellakis, 1956; Fink et al., 1956b; Fritzson, 1957; Fritzson and Pihl, 1957) has been considered as a possible pathway to the pyrimidines.

Boyd and Fairley (1958), working with the pyrimidine-less mutant Neurospora crassa 1298 which is capable of growth on propionic acid or α-aminobutyric acid, have shown that this mutant will incorporate propionic acid and α-aminobutyric acid into the pyrimidines of the nucleic acids. They have proposed that this incorporation involves a derivative of β-alanine. However, Cohen et al. (1957) have shown that dihydrouracil and dihydrocytosine (and the corresponding ribosides and deoxyribosides) do not support the growth of a variety of pyrimidine-less mutants of Esch. coli. Certain mutants of Esch. coli are capable of growth on dihydrouracil, but it has been shown recently that these organisms are pantothenate-less mutants (Slotnick and Weinfeld, 1957).

Fritzson (1957) has found that the only step in the reaction sequence in Fig. I, 2 which is effectively reversible in rat liver slices is the interconversion of dihydrouracil and β-ureidopropionic acid. However, as the utilization of uracil by rat liver for poly-nucleotide synthesis is negligible (Plentl and Schoenheimer, 1944; Rutman et al., 1954), a study of the anabolic potentialities of the degradative pathway in a tissue which is capable of utilizing uracil to a greater extent, such as rat intestinal mucosa or the

Flexner-Jobling carcinoma (Heidelberger et al., 1957),
would be of interest.   Lagerkvist et al. (1955) have
shown that ^{15}N-labelled dihydrouracil, β-ureidopropionic
acid and β-alanine are not incorporated into the
nucleic acids of Ehrlich ascites tumour cells to a
significant extent whereas uracil is utilized for
polynucleotide pyrimidine biosynthesis in the same
system.   On the other hand, 2-^{14}C-dihydrouracil has been
shown to be incorporated into the acid-soluble pyrimidine
nucleotides of a high-speed supernatant fraction of rat
liver or Flexner-Jobling carcinoma homogenates to a slightly
greater extent than is uracil (Heidelberger et al., 1957).
The significance of these observations is not clear, but
it must be borne in mind that the observed incorporation
of 2-^{14}C-dihydrouracil may have been due to the hydrolysis
of the dihydrouracil to carbamyl-β-alanine followed by
transfer of the carbamyl to aspartic acid and from thence
by the scheme outlined in Fig. I, 1.

The recent work of Mokrasch and Grisolia (1958)
has given the most significant indication of the rôle of
dihydro-pyrimidines in the synthesis of the nucleic acids.
These authors have obtained, from mammalian and avian
liver, a soluble enzyme system which catalyses the
incorporation of carbamyl-β- alanine, carbamyl-β- alanine
riboside, carbamyl-β-alanine riboside-5'-phosphate,

dihydrouridine and dihydrouridine-5'-phosphate into the ribonucleic acid (RNA) of the system. They have shown that the pathway of incorporation does not involve orotic acid and that the ribotides are utilized more extensively than the other derivatives. The latter observation strongly suggests that the reaction involves the ribotide derivatives, but further investigation of this system will be necessary in order that an evaluation of the observations may be obtained. On the other hand, it must be noted that Visser et al. (1957) have been able to detect only negligible incorporation of $^{14}C_9$-dihydrouridine-3'(2'-)-phosphate into the nucleic acids of rat intestinal mucosa and regenerating liver.

If the reverse of the reaction sequence from uracil to β-alanine (Fig. I, 2) can be demonstrated, the final steps of the biosynthesis from β-alanine to UMP can be completed by the formation of uridine from uracil and ribose-1-phosphate under the influence of nucleoside phosphorylase followed by the phosphorylation of the nucleoside by the action of uridine-5'-phosphokinase in the presence of ATP.

3. Biosynthesis of the pentose sugars.

(1) D-ribose biosynthesis.

The pathway of ribose biosynthesis from glucose

UMP

Uridylic acid phosphomonoesterase

Uridine

nucleoside phosphorylase

Dihydrouracil

Uracil

Carbamyl-β-alanine
(β-ureidopropionic acid)

β-alanine

[R = ribose]
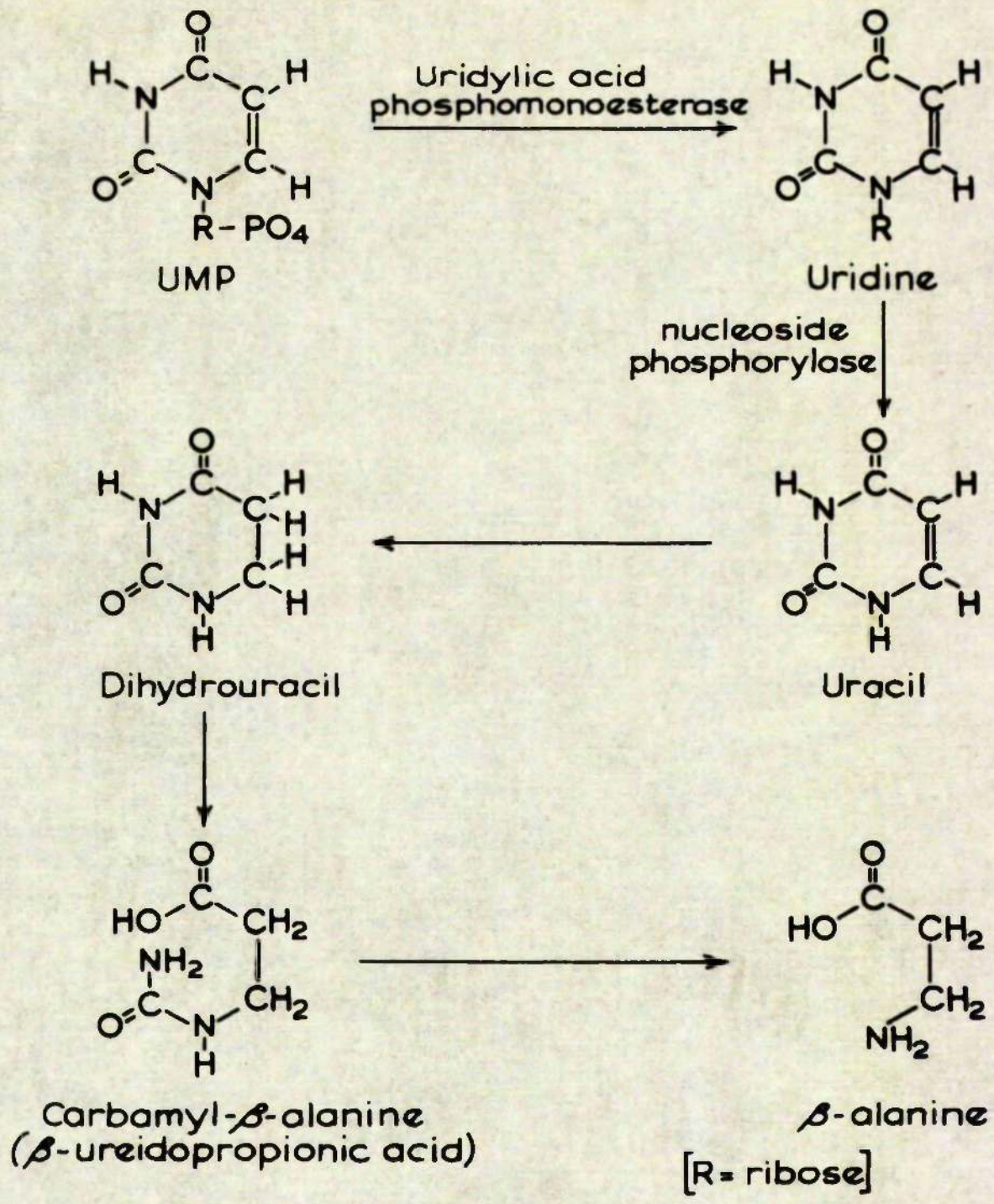
Figure I, 2

(Fig. I, 3) has been elucidated by the work of McNair-Scott and Cohen (1951), Horecker (1951) and Dickens (1953). The hydroxyl group on C_6 of the glucose molecule is phosphorylated by the action of hexokinase in the presence of ATP. The glucose-6-phosphate is oxidised by hexosemonophosphate dehydrogenase action in the presence of triphosphopyridine nucleotide (TPN) to yield the straight-chain compound 6-phosphogluconic acid which is decarboxylated at C_1 and oxidised at C_3 to yield ribulose-5-phosphate (D-2-oxoribose-5-phosphate). The action of phosphoribose isomerase converts ribulose-5-phosphate to the isomeric aldehyde with asymmetric reduction of C_2 keto-group and ring closure takes place to form D-ribose-5-phosphate (D-ribofuranose-5-phosphate).

An alternative pathway for the synthesis of ribose-5-phosphate has been found in certain bacteria. These bacteria are able to couple glycolaldehyde and glyceraldehyde-3-phosphate, both of which can be derived from glucose, to form ribose-5-phosphate.

There are three pathways known by which the ribose-5-phosphate formed by either of the above routes may be incorporated into the pyrimidine nucleotides. The mechanisms of these pathways involve (a) direct condensation of the pentose derivative with the pyrimidine

glucose → **glucose-6-phosphate**

ATP → ADP

TPN → TPNH

ribulose-5-phosphate ← **6-phosphogluconic acid**

glyceraldehyde-3-PO

glycolaldehyde

D-ribose-5-phosphate
(D-ribofuranose-5-phosphate)

---?---→ 2-deoxy-D ribose

ATP → AMP

5-phosphoribosylpyrophosphate   **D-ribose-1-phosphate**
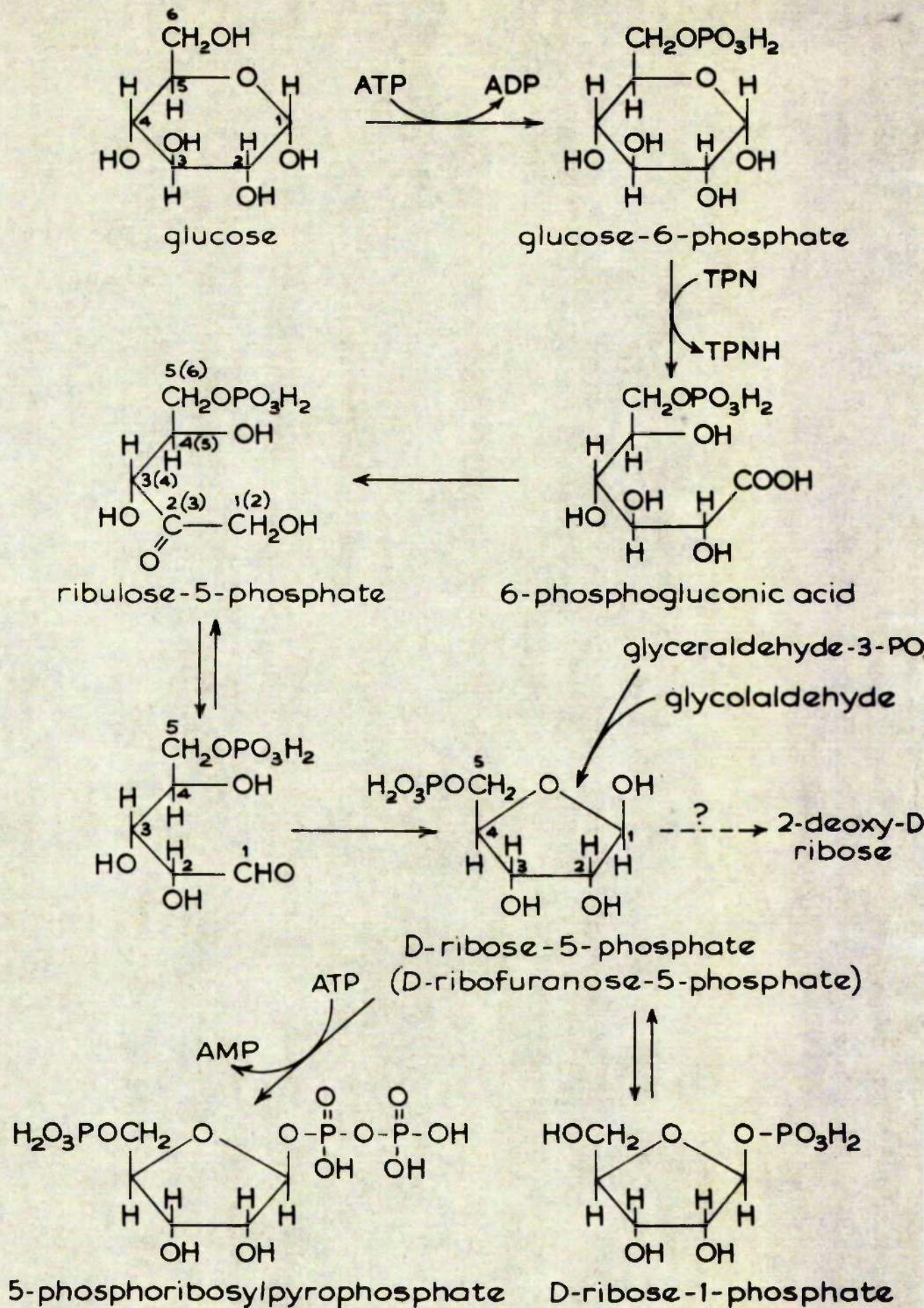
Figure I, 3

bases to form the pyrimidine nucleotides (Davidson, 1957); (b) isomerisation to ribose-1-phosphate by the action of phosphoribomutase followed by condensation with the pyrimidine base under the influence of nucleoside phosphorylase to form the pyrimidine nucleoside; and (c) reaction with ATP to form an "active" derivative which will condense with orotic acid. Kornberg et al.(1954 and 1955) and Remy et al.(1955) have identified this "active" derivative as 5-phosphoribosylpyrophosphate. As has been described previously, Lieberman et al.(1955) have reported the isolation from yeast of orotidine-5'-phosphate pyrophosphorylase under the influence of which orotic acid and 5-phosphoribosylpyrophosphate couple to form orotidine-5'-phosphate and pyrophosphate (Fig. I, 1).

The evidence obtained from the systems studied so far indicate that mechanism (c) is the predominating pathway by which the ribose moiety of the molecule is introduced into pyrimidine (and purine) nucleotides synthesised de novo. The other mechanisms appear to operate only in the cases where preformed pyrimidines are supplied to a system synthesising polynucleotides.

(ii) D-2-deoxyribose biosynthesis.

Racker (1952) has described a synthesis of deoxyribose-5-phosphate, which is analagous to the formation of ribose-5-phosphate from glyceraldehyde-3-phosphate and glycolaldehyde, under the influence of an enzyme found in extracts of Esch. coli. This enzyme, described as an aldolase, catalyses the condensation of glyceraldehyde-3-phosphate and acetaldehyde to form D-2-deoxyribose-5-phosphate. However, the exact rôle of the Racker aldolase in de novo deoxyribose synthesis is not clear. Lanning and Cohen (1954), using 1-^{14}C-glucose in the presence of Esch.coli, have shown that the pathway of deoxyribose formation is predominantly by way of 6-phosphogluconate and ribose (Fig. I, 3) whereas, in the presence of bacteriophage-infected Esch. coli, the distribution of the labelling in the deoxyribose formed is altered to a pattern which appears to indicate the operation of a pathway involving the Racker aldolase rather than the normal pathway (Lanning and Cohen, 1955). In these organisms there is the possibility that the pyrimidine deoxyribotides may be synthesised by the same type of pathway as is UMP, that is by interaction of 5-phosphodeoxyribosylpyrophosphate and orotic acid to form deoxyorotidine-5'-phosphate but as yet there have been no reports of these intermediates or reactions having

been observed. An alternative pathway is the formation
of the nucleosides by the interaction of deoxyribose-
1-phosphate and a pyrimidine under the influence of
nucleoside phosphorylase, but again this depends on the
presence of preformed pyrimidines.

Information gleaned from studies of the
distribution of activity in the nucleic acid ribose
and deoxyribose chains of Esch. coli grown on a variety
of ^{14}C-labelled substrates including $1-^{14}C$-lactate and
$1-^{14}C$-acetate have shown that deoxyribose is derived
from ribose, or from a precursor of ribose, even more so
than the comparable studies using $1-^{14}C$-glucose (Lanning
and Cohen, 1954 and 1955). This was demonstrated very
recently by Bernstein and Sweet (1958) who grew Esch.
coli on $1-^{14}C$-lactate and found that the pattern of
activity in C_1 to C_5 of the polynucleotide deoxyribose
chains in the organism to be 21: 12: 62: 4: 3 compared
with 10: 15: 69: 1: 1 for the corresponding ribose
chains. Bagatell et al. (1958), using $1-^{14}C$-acetate as
a carbon source in a similar system, confirmed the
conclusion that deoxyribose is derived from ribose or a
ribose precursor.

The vast proportion of the evidence on
deoxyribose biosynthesis which has accumulated clearly
indicates that the conversion of ribose to deoxyribose

takes place at the nucleoside or nucleotide level without either fission of the N-glycoside linkage or rupture of the pentose chain. Rose and Schweigert (1953) have shown that $^{14}C_9$-cytidine is incorporated into the deoxycytidine residues of rat tissue DNA with no loss of specific activity. Very recently McNutt (1958) has confirmed this result using a cytidine-less mutant of Neurospora, and the intact incorporation of $^{14}C_9$-cytidine and $^{14}C_9$-uridine into the polynucleotide pyrimidine deoxynucleotide residues of mammalian and avian tissues also has been demonstrated (Roll et al., 1956; Reichard, 1957 and 1958). Whether the ribose-deoxyribose interconversion takes place at the nucleoside or the nucleotide level is not clear, but some evidence to indicate that the reaction takes place at the level of the nucleoside-5'-phosphate has been obtained recently by Reichard (1958) who has described briefly the formation of deoxyuridylic acid (deoxyuridine-5'-phosphate;dUMP) from UMP by a soluble enzyme from chick embryo homogenates. No evidence of the interconversion taking place at the nucleoside level has been obtained with the exception of a dithiol-activated soluble enzyme of Salmonella tymphimurium LT-2 which has recently been reported by Grossman (1958) without details and which is stated to effect a reduction of uridine to deoxyuridine.

The mechanism of the reduction at the 2'
position in ribonucleotides has not been elucidated
but recently Brown et al.(1958) have suggested
tentatively that a pyrimidine O^2:2'-cyclo-nucleoside
structure may be involved in the biosynthesis of
deoxyribose.   However, the stereochemistry of the
analagous purine derivatives is such that it would
appear impossible for a purine O^2:2'-cyclo-nucleoside
to participate in purine deoxynucleotide synthesis.

Although vitamin B_{12} appears to be intimately
concerned in the biosynthesis of the nucleic acids in
some way, the rôle played by this vitamin is still
obscure.   Vitamin B_{12} has been implicated (a) in the
synthesis of the thymine-methyl group (Elwyn and
Sprinson, 1950; Totter, 1954; Smith, 1956) and (b) in
the synthesis of the deoxyribose moiety of DNA(Downing
and Schweigert, 1956).   These aspects will be discussed
later at some length.

Another possible pathway of dUMP biosynthesis
warrants some consideration.   Lieberman (1955 and 1956)
has demonstrated that the amination of uracil, to yield
cytosine, takes place at the nucleotide triphosphate level
but there is no evidence to suggest that deoxycytidine-5'-
triphosphate is formed by the analagous amination of
deoxyuridine-5'-triphosphate.   Reichard (1955) has shown

that ^{14}C-deoxyuridine is not incorporated into the
cytosine of DNA of regenerating liver or intestinal
mucosa and Friedkin and Roberts (1956) have confirmed
this observation with minced chick embryo and
suspensions of rabbit and chicken bone marrow cells.
Moreover, Friedkin and Kornberg (1957) were unable to
demonstrate 5'-deoxynucleotide kinase activity towards
dUMP in extracts of Esch. coli.   Thus the de novo
synthesis of deoxycytidine nucleotides probably
involves a pathway of the type: UMP $\rightarrow$ uridine -5'-
diphosphate $\rightarrow$ uridine-5'-triphosphate $\rightarrow$ cytosine-
5'-triphosphate $\rightarrow$? $\rightarrow$ deoxycytidine-5'-phosphate.
The deoxycytidine-5'-phosphate formed by these reactions
may be deaminated to dUMP by the action of deoxycytidine-
5'-phosphate deaminase, a new deaminase found recently
in homogenates and acetone powder extracts of
Paracentrotus lividus (Scarano, 1958).   The dUMP formed
in this way is a possible source of pyrimidine
deoxynucleotides for DNA biosynthesis. However, it is
possible that the sugar interconversion takes place at
the triphosphate level.   In this case, the deamination
of deoxycytidine-5'-phosphate may be a mechanism for
salvaging a pyrimidine ring by synthesising dUMP from a
compound which is not on the direct DNA synthetic pathway

rather than a mechanism whereby pyrimidine deoxy-
nucleotides are synthesised de novo.

4. Methylation of the pyrimidine ring.

(1) Sources of the methyl group.

The origin of the thymine-methyl group in DNA
has been investigated by a number of workers and has
been shown to be in a one-carbon unit which can be
derived from a number of simple precursors including
formate, formaldehyde, glycine, serine and methionine.

The in vivo incorporation of ^{14}C-formate into
the thymine-methyl group of DNA of the internal organs
(liver, intestinal mucosa, spleen, etc.) of the rat and
the chick has been demonstrated by Totter et al.(1951),
Elwyn and Sprinson(1954), Harrington and Lavik (1955),
and Herrmann et al. (1955). Mannell and Rossiter(1955)
have shown that ^{14}C-formate labels the methyl group of
DNA-thymine in tissue slices of rat liver and spleen.
The in vitro incorporation of ^{14}C-formate into the thymine-
methyl group has been demonstrated also by Totter(1954)
and Totter and Best (1955), using suspensions of rabbit
bone marrow cells and Prusof and Lajtha (1956) and
Prusof et al. (1956) have shown that ^{14}C-formate is
incorporated into DNA-thymine by suspensions of Ehrlich
ascites tumour cells. Kit(1957) has confirmed these

observations with Ehrlich ascites tumour cells and
Kit et al.(1958) have also shown that ^{14}C-formate is
incorporated into the thymine compounds present in
the acid-soluble fraction of both normal and malignant
lymphatic tissues.   However, it must be noted that it
has been shown that ^{14}C-formate is not incorporated
into the thymine-methyl group of the DNA of a growing
culture of Esch.coli although, in the same system, the
[14]C-formate will label positions 2 and 8 of the purine
rings (Crosbie, 1958).

Lowy et al. (1956) have demonstrated the
incorporation of ^{14}C-formaldehyde into the thymine-
methyl group of mammalian DNA, a result which has been
confirmed by the observations of Hamill et al. (1956).
Recently Kit et al.(1958) have shown clearly that normal
or neoplastic lymphatic cell suspensions will convert
[14]C-formaldehyde to the thymine-methyl group of acid-
soluble thymine compounds and of DNA-thymine.   [14]C-
formaldehyde has also been shown to be converted to the
methyl group of thymine nucleotides by cell-free
extracts of Esch. coli (Friedkin and Kornberg, 1957;
Flaks and Cohen, 1957) and by cell-free extracts of rat
thymus tissue (Phear and Greenberg, 1957).

Totter et al. (1951) have shown that 2-[14]C-
glycine gives rise to a labelled one-carbon unit which

is incorporated _in vivo_ into the thymine-methyl group
of mammalian DNA. This observation has been confirmed
by Elwyn and Sprinson (1954) who isolated and degraded
the DNA of the internal organs (liver, spleen, kidneys,
heart, intestine, pancreas, gonads and lungs) of rats
which had been fed $2-^{14}C$-glycine or $3-^{14}C$-serine. They
found, in both cases, that the thymine-methyl group was
labelled. Their results indicated that the hydroxy-
methyl group of serine is a major source of the methyl
group of DNA-thymine and that the α-carbon of $2-^{14}C$-
glycine, which is converted to the thymine-methyl
group also, is a much less effective precursor. Degrad-
ation of the isolated pyrimidine showed that more than
90% of the ^{14}C present in the thymine was located in the
5-methyl group, both when $2-^{14}C$-glycine and when $3-^{14}C$-
serine was the one-carbon unit source. An _in vitro_
system in which deoxyuridine is "methylated" by $3-^{14}C$-
serine under the influence of a soluble enzyme extract
of rabbit thymus tissue has been described by Blakley
(1957).

The conversion of the methyl group of methionine
to mammalian DNA-thymine has been demonstrated by Herrmann
et al. (1955) and by Kit et al. (1958). Kit et al.(1958)
noted that the utilization of methionine-methyl-^{14}C was
reduced markedly by the addition of formaldehyde but that

the incorporation of ^{14}C-formaldehyde was unaffected by unlabelled methionine. Green and Cohen (1957) and Crosbie (1958) have shown that methionine is not a methyl donor for DNA biosynthesis in methionine-less mutants of Esch. coli. Green and Cohen (1957) confirmed this with the observation that methionine-methyl-^{14}C is not utilized for DNA-thymine synthesis in a uracil-less mutant of Esch. coli. On the other hand, Dinning et al. (1958) have described the incorporation of methionine-methyl-^{14}C into the DNA-thymine of Lactobacillus leichmannii and they have reported that the pathway of incorporation is not influenced by vitamin B_{12}. Kit et al. (1958) have suggested that the pathway of methionine-methyl utilization in this reaction involves prior oxidation of the methyl group to form an active donor which is probably a folic acid derivative, thus agreeing with the conclusions drawn by Herrmann et al. (1955).

(ii) The one-carbon unit acceptor.

Although the precursors of the one-carbon unit (the one-carbon unit donors) have been elucidated the nature of the compounds which act as acceptors of the one-carbon unit has remained obscure until recently. At different times orotic acid, uracil, cytosine, the ribo- and deoxyribonucleosides and nucleotides of these pyrimidines

and the 4:5-dihydroderivatives of these compounds have
been suggested as possible one-carbon unit acceptors.

It has been shown that free orotic acid is
utilized for the _in vivo_ synthesis of DNA-thymine in
the rat (Hurlbert and Potter, 1952; Harrington and
Lavik, 1955) and in the mouse (Lagerkvist and Reichard,
1954). The _in vitro_ utilization of orotic acid for
DNA-thymine synthesis has been demonstrated by Weed and
Wilson(1953) who showed that $2-^{14}C$-orotic acid was
incorporated into DNA-thymine in tissue slices of rat
liver and cat spleen. Lagerkvist and Reichard (1954)
have observed the incorporation of orotic acid into the
DNA-thymine of Ehrlich ascites tumour cells. Other
free pyrimidines have been shown to be DNA-thymine
precursors in some cases. Free thymine, uracil and
cytosine are incorporated into the DNA-thymine of the
normal rat to a very small extent (Holmes et al., 1954;
Reichard, 1955) and Rutman et al.(1954) have shown that
$2-^{14}C$-uracil is a precursor of DNA-thymine in rat
hepatoma tissue. The mouse has been shown to be rather
an exceptional animal in that it will utilize uracil for
DNA-thymine biosynthesis to a much larger extent
(Lagerkvist and Reichard, 1954). Uracil is also
utilized for DNA-thymine biosynthesis by some uracil-
less mutants of Esch. coli. (Moore and Boylen, 1955;

Green and Cohen, 1957) and Rege and Sreenivasan(1954)
have reported that uracil is converted to thymine in
the presence of a washed whole-cell suspension of
Bacillus subtilis.

Studies on the utilization of nucleosides
for DNA-thymine biosynthesis have proved more rewarding.
Both ribonucleosides and deoxyribonucleosides have been
observed many times to be incorporated as intact units
into the thymine of mammalian and bacterial DNA. Rose
and Schweigert (1953) have shown that $^{14}C_9$-cytidine is
incorporated into the DNA-thymine of rat tissues and both
$^{14}C_9$-cytidine and $^{14}C_9$-uridine have been shown to be
utilized for the synthesis of the DNA-thymine of
mammalian and avian tissues (Roll et al., 1956; Reichard,
1957 and 1958). McNutt (1958) has confirmed this with
Neurospora mutants. Grossman and Visser (1954) have
demonstrated the incorporation of $4-^{14}C$-cytidine into
DNA-thymine in in vitro experiments with rat liver
slices. The in vitro utilization of $2-^{14}C$-deoxyuridine
for the synthesis of acid-soluble thymine compounds and
of DNA-thymine has been observed by Friedkin and Roberts
(1955 and 1956) using suspensions of chick embryo or
rabbit bone marrow cells. These authors noted that this
reaction was inhibited by aminopterin and that the $2-^{14}C$-

deoxyuridine was not incorporated into the DNA-cytosine
or into the RNA-pyrimidines. Moreover, the incorpor-
ation of the labelled nucleosides into the DNA-thymine
was reduced significantly by the addition of unlabelled
thymidine. Similar results were obtained by Prussof
and Lajtha (1956), Prussof et al. (1956) and Prussof
(1958) during experiments on the incorporation of ^{14}C-
formate into the DNA-thymine of rabbit bone-marrow and
Ehrlich ascites tumour cells. These workers found
that the specific activity of the DNA-thymine was
increased by the addition of deoxyuridine, deoxycytidine
and the corresponding ribosides and was decreased
markedly by the presence of thymidine, aminopterin or
azathymidine, but not by azathymine. They also found
that the cytosine nucleosides were considerably more
efficient in boosting the synthesis of DNA-thymine than
were the corresponding uracil nucleosides. In similar
experiments, Kit et al. (1958) found that the addition
of 4:5-dihydrouracil deoxyriboside or 5-methylcytosine
deoxyriboside did not reduce the extent to which ^{14}C-
formaldehyde was converted to acid-soluble thymine
compounds.

A comparison of the incorporation patterns of
a number of labelled nucleosides into DNA-thymine has
shed some light upon the problem of the nature of the

one-carbon unit acceptor. Reichard (1955) has studied the _in vivo_ incorporation of $2-^{14}C-$ deoxyuridine and $2-^{14}C-5-$methyluridine into the DNA-thymine of regenerating rat liver and intestinal mucosa. Friedkin _et al._ (1956), using embryonic tissues and Friedkin and Wood (1956), using bone marrow cells and isolated thymus nuclei, have demonstrated that $2-^{14}C-$thymidine is incorporated into DNA-thymine but not into any other nucleic acid pyrimidine, observations which have been confirmed with bacteria by Downing and Schweigort (1956). A comparison of the pattern of $2-^{14}C-5-$methyluridine, $2-^{14}C-$thymine, $2-^{14}C-$deoxyuridine, $2-^{14}C-$thymidine and $2-^{14}C-$uridine, as determined by these authors, has confirmed and co-ordinated results obtained by a large number of workers. The utilization of deoxyuridine for DNA-thymine biosynthesis takes place rapidly under conditions where uracil is not utilized and the pattern of $2-^{14}C-$deoxyuridine incorporation resembles that of $2-^{14}C-$thymidine rather than $2-^{14}C-$uridine. In no case was $2-^{14}C-$deoxyuridine observed to be utilized for DNA-cytosine synthesis. The pattern of the small but significant incorporation of $2-^{14}C-5-$methyluridine into the DNA-thymine of regenerating rat liver when administered at high dose

levels resembles that of $2-^{14}C$-thymine, suggesting that 5-methyluridine is utilized for DNA-thymine synthesis only after conversion to free thymine.

The evidence obtained from these results indicates that deoxyuridine (or a nucleotide derivative thereof) is the primary one-carbon unit acceptor in the biosynthesis of DNA-thymine.   In confirmation of this, Friedkin and Kornberg (1957) have described a system in which a cell-free extract of Esch. coli effects the synthesis of thymidine-5'-triphosphate from dUMP.   Friedkin(1957) has also described an Esch. coli system which synthesises TMP from dUMP and formaldehyde or serine.   Flaks and Cohen (1957) have used a similar system to synthesise TMP from dUMP and formaldehyde in the presence of extracts from normal or bacteriophage-infected Esch. coli cells.   A comparable mammalian system has been described by Phear and Greenberg (1957) who observed the coupling of deoxyuridine and ^{14}C-formaldehyde to yield ^{14}C-TMP in the presence of ATP and a cell-free extract of rat thymus tissue.   In contrast to this, Blakley (1957) has observed the synthesis of ^{14}C-thymidine from deoxyuridine and $3-^{14}C$-serine in the presence of the soluble enzymes from a rabbit thymus homogenate.   Blakley has stated that dUMP is not

utilized as effectively as deoxyuridine in this system,
an observation which is difficult to reconcilewith the
results obtained by the other groups in the same field.
Blakley (1957) has also found that uridine, 4:5-
dihydrouridine and 4:5-dihydrodeoxyuridine are not
involved in the methylation reaction.

However, there is some evidence to suggest
that deoxycytidine can act as a one-carbon unit
acceptor in DNA-thymine synthesis. Elwyn and Sprinson
(1954) interpret the results obtained in a study of
$2-^{14}C$-glycine and $3-^{14}C$-serine incorporation into DNA-
thymine in the rat to suggest that thymine arises by
methylation of a cytosine derivative. The results
obtained by the Prusof school (1956 and 1958), showing
that cytosine nucleosides are more effective than
uracil nucleosides in increasing the labelling of DNA-
thymine by ^{14}C-formate, together with the observation
that Esch. coli extracts contain 5-methyl- deoxycytidine
deaminase activity (Cohen and Barner, 1957), have been
interpreted as indicating that deoxycytidine, rather than
deoxyuridine, is the one-carbon unit acceptor. However,
since the interpretation of evidence obtained by isotope
incorporation studies in whole-cell systems involves
difficulties raised by the operation of a number of
factors (permeability, pool size and kinetic) of unknown

character and magnitude, the isotopic evidence upon
which this conclusion is based cannot be considered
unequivocal.   When attempting to assess the significance
of these results it is advisable to bear in mind the
observations made by Kit et al. (1958) who found that
acid-soluble 5-methyldeoxycytidine and the corresponding
5'-deoxynucleotide are not labelled during the
incorporation of ^{14}C-formaldehyde into the DNA-thymine
of lymphatic cell suspensions in the presence of an
added pool of 5-methyldeoxycytidine.

It must be noted, however, that deoxycytidine-
5'-phosphate does function as a one-carbon unit acceptor
molecule in the system described by Flaks and Cohen
(1957) who showed that 5-hydroxymethyldeoxycytidylic
acid is synthesised from formaldehyde and deoxycytidine-
5'-phosphate under the influence of a cell-free extract
of T6r+ bacteriophage-infected Esch. coli cells.   The
problem raised by these observations remains to be
resolved.

Other compounds cited as possible one-carbon
unit acceptors are the 4:5-dihydro derivatives of the
pyrimidines and their nucleosides and nucleotides.
However, no evidence to indicate that the 4:5-dihydro-
pyrimidine derivatives function as one-carbon unit
acceptors in the synthesis of thymine nucleotides or

DNA-thymine has been obtained. Indeed, a considerable amount of evidence to suggest that they do not function in this way has been amassed. Cohen et al. (1956) found that the 4:5-dihydro derivatives of uracil, thymine, uridine, deoxyuridine, thymidine, cytosine and deoxycytidine do not support the growth of the thymine-less mutant Esch. coli 15 T-. Later, Cohen et al. (1957) reported that 4:5-dihydrodeoxyuridine and 4:5-dihydro-deoxycytidine are not utilized for DNA-thymine synthesis in Esch. coli B or Esch. coli 15 T- or for viral DNA-thymine and 5-hydroxymethylcytosine in T6r bacteriophage grown in Esch. coli B or Esch. coli BU- (a uracil-less mutant). Blakley (1957) similarly has reported that 4:5-dihydrodeoxyuridine and 4:5-dihydrodeoxycytidine are not involved in thymidine synthesis in his cell-free thymus system. Kit et al. (1958) have shown that 4:5 dihydrodeoxyuridine is not involved in DNA-thymine synthesis in lymphatic tissues and tumours. It has been shown that $2-^{14}C-4:5$-dihydrodeoxyuridine will penetrate the Esch. coli cell by Cohen et al. (1957) who isolated a phosphorylated derivative of the nucleoside from the acid-soluble fraction of Esch. coli 15 T-, so that the non-involvement of the dihydro derivative in this system is not merely a reflection of a permeability effect. However, the evidence is not conclusive since

it is probable that the "methylation" reaction is taking place at a nucleotide level in a system which may not possess any kinase activity towards the 4:5-dihydrodeoxynucleoside.

(iii)   Mechanisms of production and transfer of the one-carbon unit.

(a)   The structure of the one-carbon unit pool complex

As previously discussed, the precursors of the methyl group of DNA-thymine and acid-soluble thymine compounds have been elucidated in a large number of mammalian, avian and bacterial systems, both in vivo and in vitro.   However, these precursors are so diverse in their nature that it was very soon obvious that they must possess a common denominator, which became known as the "one-carbon unit pool".   It became obvious that each one-carbon unit precursor donated one-carbon units to the common pool which then acted as the immediate one-carbon unit donor.   The nature of the material forming the one-carbon pool and the mode of its formation and utilization has been a point of interest for some years.   It is only recently that the structure of the compound which takes up the one-carbon unit from the precursors and acts as the common one-carbon unit donor has been elucidated. Indeed, there is still some discussion as to the precise structure of the intermediate one-carbon unit carrying

complex.

A number of workers have found that
biosynthetic pathways involving one-carbon units
are dependent on the presence of folic acid (or its
derivatives).   Using bacterial mutants which
required folic acid for growth, Stokes (1944) and
Rogers and Shive (1948) obtained results from which
it was inferred that folic acid is involved in the
biosynthesis of nucleic acid purines and DNA-thymine.
Prussof et al. (1948) have shown more directly that
folic acid is intimately concerned in the biosynthesis
of Lactobacillus casei nucleic acids, an observation
which was confirmed later by the work of Rege and
Sreenivasan (1950). Skipper et al. (1950) have shown
that the incorporation of ^{14}C-formate into the nucleic
acid purines is influenced by dietary folic acid in
the mouse.   They found that the folic acid antagonists
aminopterin and amethopterin inhibited the incorporation
of ^{14}C-formate into mouse viscera nucleic acids.   Later,
Skipper et al. (1952) extended this work to show that
the incorporation of ^{14}C-formate into the DNA and RNA
purines and the methyl-group of DNA-thymine of mouse
viscera and leukaemic cells was inhibited by amethopterin.
Goldthwait and Bendich (1952) also observed this
inhibition of ^{14}C-formate incorporation into rat intestinal

nucleic acids by aminopterin. By comparing the incorporation of ^{14}C-adenine and ^{14}C-formate in the presence of aminopterin these authors also demonstrated that the folic acid antagonist acts at some point on the purine ring synthetic pathway and not after completion of the purine ring had been effected. This was confirmed by Buchanan and Schulman (1953) who showed that the incorporation of ^{14}C-formate into position 2 of the purine ring of inosinic acid was dependent on the presence of a folic acid derivative, leucovorin (N^5-formyltetrahydrofolic acid). It must be noted, however, that Totter and Best (1955) have described an in vitro rabbit bone marrow system in which aminopterin exerts no great effect on the incorporation of ^{14}C-formate into RNA and DNA purines although it does inhibit the incorporation of the ^{14}C into the methyl group of DNA-thymine.

Other systems which involve the transfer of a one-carbon unit have been shown also to be dependent on folic acid or on a derivative of folic acid. Blakley (1954) has demonstrated the synthesis of 2-^{14}C-serine from 2-^{14}C-glycine and formaldehyde under the influence of a pigeon liver extract. He found that the presence of folic acid was essential for this reaction to take place and that 5:6:7:8-tetrahydrofolic acid was a more

efficient cofactor than folic acid itself, suggesting a prior reduction of folic acid to tetrahydrofolic acid which is the active cofactor. Kisluik and Sakami (1955) also demonstrated a requirement for tetrahydrofolic acid in the transfer of a ^{14}C-label from glycine to serine. Also, folic acid has been implicated in the synthesis of the methyl groups of choline and methionine (Bennett, 1950; Jukes et al., 1950). Woods (1958) has recently demonstrated a requirement for tetrahydrofolic acid in his cell-free Esch. coli system which effects the synthesis of methionine from homocysteine.

Results obtained from experiments designed to elucidate the nature of the folic acid derivative which acted as the immediate one-carbon unit donor quickly made it clear that the folic acid derivative concerned with purine ring synthesis differs from that involved in thymine-methyl synthesis. Greenberg (1954a and 1954b), using pigeon liver extracts and Jaenicke (1954), using pig liver extracts, demonstrated an ATP-dependent reaction between tetrahydrofolic acid and ^{14}C-formate to yield a derivative which transformylates directly to 5-amino-4-imidazolecarboxamide-5'-phosphoriboside in the absence of ATP to yield 2-^{14}C-inosine-5'-phosphate (inosinic acid). Greenberg (1954a

and 1954b) also noted that the formylation of 5-amino-
4- imidazolecarboxamide-5'-phosphoribotide by N^5-
formyltetrahydrofolic acid was dependent on the presence
of ATP. The activation of N^5-formyltetrahydrofolic
acid was shown to be due to its conversion to N^{10}-
formyltetrahydrofolic acid or N^5, N^{10}-methenyl-
tetrahydrofolic acid (Greenberg, 1954b; Greenberg et al.,
1955; Goldthwait et al., 1955). Jaenicke (1955) has
isolated and identified N^{10}-^{14}C-formyltetrahydrofolic
acid as a product of (i) the interaction of ^{14}C-formate,
ATP and tetrahydrofolic acid and (ii) the conversion of
3-^{14}C-serine to glycine under the influence of the
tetrahydrofolate formylase activity present in pig liver
extracts.

The difference between the folic acid
derivatives concerned in purine ring synthesis and
thymine-methyl synthesis has been demonstrated very
neatly by Elwyn and Sprinson (1954). These authors
have shown that, in the synthesis of the methyl group
of DNA-thymine of rat tissues from ingested L-(3-^{14}C:
2, 3-$^2H_2$: ^{15}N)-serine or ^{14}C:^2H-sodium formate, 1.5 and
0.9 atoms of deuterium, respectively, accompanied the
labelled carbon atom. In contrast to this, during the
conversion of the β-carbon of L-(3-^{14}C:2,3-$^2H_2$:^{15}N)-
serine or ^{14}C:^2H-sodium formate to carbon atoms 2 and 8

in the purine ring, extensive labilisation of the
deuterium took place. It was inferred from these
results that the pathway of incorporation of the
β-carbon of serine into the thymine-methyl group
does not involve a derivative at the oxidation level
of N^{10}-formyltetrahydrofolic acid. On the other
hand, Lowy et al. (1956) have shown that ^{14}C, D-labelled
formaldehyde (a mixture of H.^{14}CHO, D.CHO, H.CDO and
DCDO) is incorporated into the methyl group of rat
DNA-thymine with considerable loss of D relative to
^{14}C. However, as the authors themselves have pointed
out, the significance of the results may be vitiated by
isotope selection effects of the type which had been
noted already by Rachele et al.(1956) and by Abeles
(1955). Crosbie (1958) has shown that ^{14}C-formate is
incorporated into positions 2 and 8 of DNA-purine rings
in a growing culture of Esch. coli but that the isotope
is not utilized in the synthesis of the methyl group of
DNA-thymine in the same system. A number of investigators
studying the in vitro synthesis of thymidine or of thymine
nucleotides, have observed that the "methylation" of
deoxyuridine or dUMP by ^{14}C-formaldehyde or 3-^{14}C-serine
in the presence of cell-free extracts of Esch.coli or
rabbit or rat thymus tissue is dependent on the presence
of tetrahydrofolic acid (Friedkin, 1957; Friedkin and

Kornberg, 1957; Blakley, 1957; Phear and Greenberg, 1957; Flaks and Cohen, 1957).

The evidence of these experiments clearly indicates that N-hydroxymethyltetrahydrofolic acid (or, by analogy with the corresponding formyl derivative in purine synthesis, N^5, N^{10}-methylene-tetrahydrofolic acid) is the folic acid derivative involved in the synthesis of the thymine-methyl group. Evidence in confirmation of this has been presented by Friedkin (1957) and Friedkin and Kornberg (1957) who observed that N-hydroxymethyltetrahydrofolic acid could replace serine and tetrahydrofolic acid in the synthesis of thymine nucleotides from deoxyuridylic acid. A number of authors have reported that N-hydroxymethyltetrahydrofolic acid is a product of (i) the action of serine hydroxymethylase on serine in the presence of tetrahydrofolic acid (Jaenicke, 1956; Huennekens et al., 1957; Blakley, 1958); (ii) the non-enzymic interaction of formaldehyde and tetrahydrofolic acid (Jaenicke, 1956; Kisluik, 1957; Blakley, 1958); and (iii) the action of N-hydroxymethyltetrahydrofolic acid dehydrogenase on N^{10}-formyltetrahydrofolic acid (or N^5, N^{10}-methenyl-tetrahydrofolic acid) (Jaenicke, 1955 and 1956; Hatefi et al., 1957).

The precise structure of the immediate one-carbon unit donor (written here as N-hydroxymethyltetrahydrofolic acid) is still under discussion. Blakley (1954) first postulated that the N^5, N^{10}-methylene-bridge structure (Fig. I, 4 (c)) was the form taken by the derivative although Kisluik and Sakami (1954) have suggested that N^5-hydroxymethyltetrahydrofolic acid was a possible structure (Fig. I, 4 (b)). Kisluik (1957) subsequently demonstrated that the presence of unsubstituted N^5- and N^{10} positions in the molecule was necessary for formaldehyde binding by tetrahydrofolic acid (Fig. I, 4(a)). Blakley (1958) has recently re-investigated this question and has obtained evidence which indicates that the primary site of interaction of formaldehyde with tetrahydrofolic acid is at the N^5-position and that the adduct contains an N^5, N^{10}-methylene bridge. Moreover, he has shown that the only difference between synthetic N^5, N^{10}-methylene - tetrahydrofolic acid and the product of serine hydroxymethylase action on L-serine in the presence of tetrahydrofolic acid is in a stereoisomeric aspect - the synthetic product is a racemate while the enzymic product is a single stereoisomer. The results obtained, however, do not rule out the possibility that the N^5, N^{10}-methylene-bridge derivative is in equilibrium with an N-hydroxymethyl-
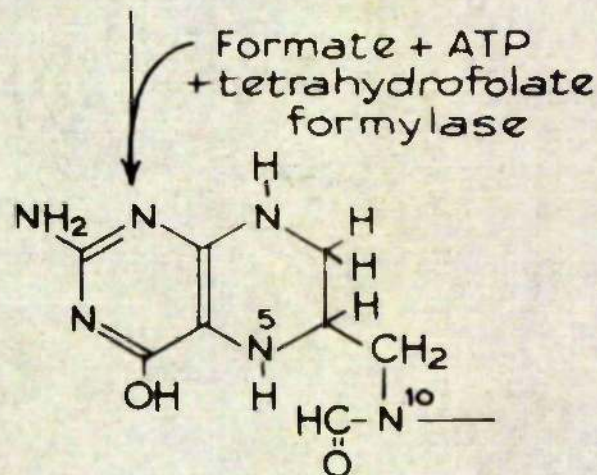
Figure I, 4
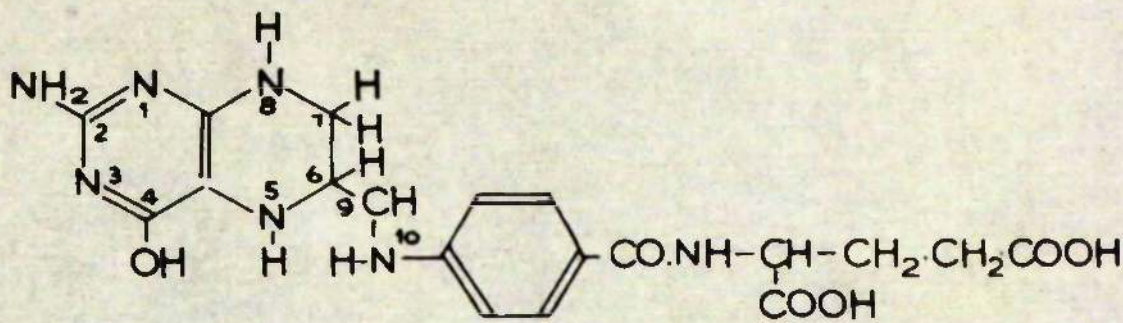
(a) 5:6:7:8 – tetrahydrofolic acid
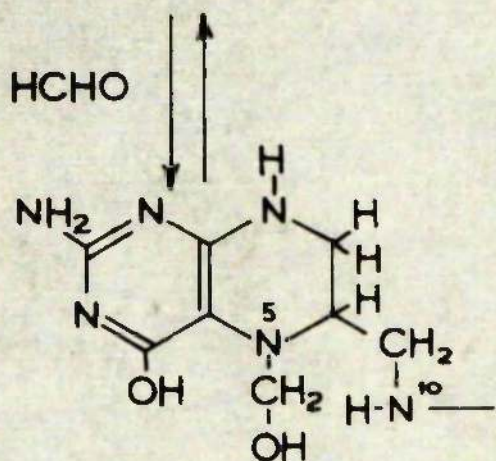
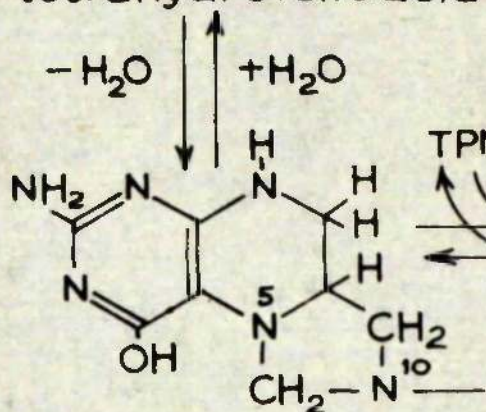(b) N^5-hydroxymethyl-tetrahydrofolic acid

(c) N^5, N^{10}-methylene-tetrahydrofolic acid

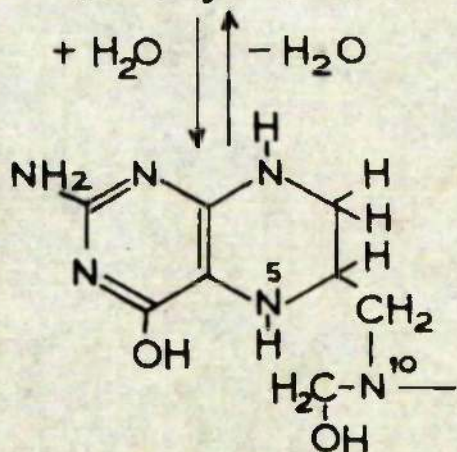(d) N^{10}-hydroxymethyl-tetrahydrofolic acid

(e) N^{10}-formyl-tetrahydro-folic acid
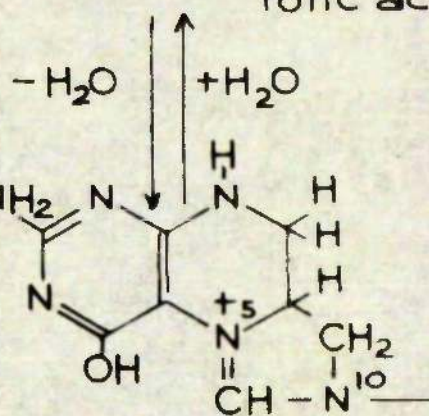
(f) N^5, N^{10}-methenyl-tetrahydro-folic acid (anhydroleucovorin)
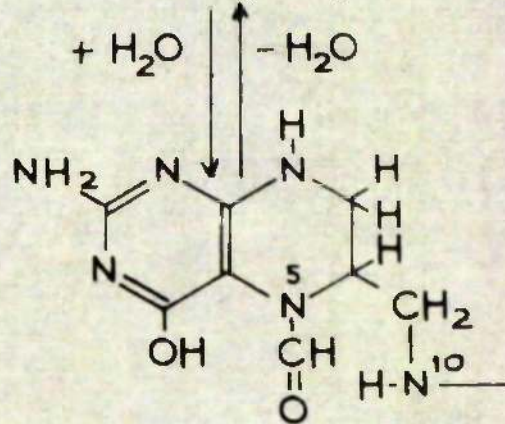
(g) N^5-formyl-tetrahydrofolic acid

tetrahydrofolic acid which is the form of the
derivative actually involved in the transfer of a
one-carbon unit to the acceptor molecule.   Indeed,
there is some evidence to indicate that N^5, N^{10}-
methylenetetrahydrofolic acid dissociates readily
to form N-hydroxymethyltetrahydrofolic acid.   On
the other hand, as Blakley (1959) points out, the
enzymic interconversion of N^{10}-formyl-and N-hydroxy-
methyltetrahydrofolic acid (Fig. I, 4) is more easily
conceived as involving the methenyl-bridge and
methylene-bridge compounds as substrates (Jaenicke,
1955; Hatefi et al., 1957). However, as the scheme in
Fig. I, 4 shows, the concept of a ready interconversion
of the three forms of N-hydroxymethyltetrahydrofolic
acid is quite feasible. For comparison, Fig. I, 4 also
includes the scheme of the analagous N-formyltetra-
hydrofolic acid interconversions.

(b) Pathways of one-carbon unit pool formation from
different one-carbon unit precursors.

As has been discussed already, the sources of
one-carbon units utilized for the synthesis of thymino-
methyl groups have a common denominator in that they all
contribute a one-carbon unit to a common one-carbon pool
in the form of N-hydroxymethyltetrahydrofolic acid, which
then acts as the immediate one-carbon unit donor.

However, the pathways by which the precursors donate
their one-carbon units to the tetrahydrofolic acid
derivative differ quite widely from each other.

The most simple pathway is that of formaldehyde
utilization. Formaldehyde has been shown by a number
of workers to condense non-enzymically with tetrahydro-
folic acid to form the N-hydroxymethyl derivative
(Jaenicke, 1956; Kisluik, 1957, Blakley, 1958). The
derivative formed in this way has been shown to differ
from the enzymically synthesised derivative in
stereoisomeric respects only.

The case of formate incorporation into thymine-
methyl groups is not quite so simple. However, it
appears likely that the first step is the formation of
N^{10}-formyltetrahydrofolic acid (Jaenicke, 1955;
Greenberg et al., 1955) which is converted to the N^5,
N^{10}-methenyl derivative by the loss of one molecule of
water. Jaenicke (1956) and Hatefi et al. (1957) have
demonstrated the hydrogenation of this derivative to
form N-hydroxymethyltetrahydrofolic acid, presumably by
way of the N^5, N^{10}-methylene-bridge derivative (Fig. I,
4.) Crosbie (1958) has shown that ^{14}C-formate is
utilized for the synthesis of C_2 and C_8 of the purine
ring but not for the thymine-methyl group in the DNA of
a growing culture of Esch. coli. This observation has
been attributed to (1) the rapid oxidation of formate

to CO_2 and (ii) the very rapid utilization of the N^{10}-formyltetrahydrofolic acid for purine synthesis in the growing culture with the result that the interconversion of the formyl- and hydroxymethyl-derivatives (by way of the N^5, N^{10}-bridge compounds) does not take place. It must be noted that Dinning et al. (1958) have interpreted results obtained during a study of the effect of vitamin B_{12} on the incorporation of ^{14}C-formate into the DNA-thymine methyl groups of Lactobacillus leichmannii to indicate that formate is converted to thymine-methyl by a pathway which does not involve a hydroxymethyl group in equilibrium with carbon-3 of serine. However, the validity of the authors' interpretation of their results is questionable.

Serine has been shown to give rise to N-hydroxymethyltetrahydrofolic acid by the action of serine hydroxymethylase in the presence of tetrahydrofolic acid (Jaenicke, 1956, Huennekens et al.; 1957; Blakley, 1958). A kinetic study of the incorporation of $2-^{14}C$-glycine and $3-^{14}C$-serine into the thymine-methyl group of the DNA of exponentially growing cells of Esch. coli has revealed that glycine and serine do not lie on the pathway of incorporation of $3-^{14}C$-serine and $2-^{14}C$-glycine, respectively (Crosbie, 1958). The

pathway of serine utilization has been shown to involve pyridoxal phosphate (Vitamin B_6). Blakley (1955) has shown that the interconversion of serine and glycine - a reaction which requires the transfer of a one-carbon unit from serine - is dependent on pyridoxal phosphate. Snell and Metzler (1954) have discussed the mechanism of pyridoxal phosphate-catalysed reactions of this type and they and Blakley (1955) have concluded that amino acids undergo reaction while bound to pyridoxal phosphate through the amino group of the amino acid and the aldehyde group of pyridoxal phosphate, in effect in the form of Schiff bases.

One other point in the interaction of serine and tetrahydrofolic acid is of sufficient interest to mention. Jaenicke (1956) has observed the formation of a serine-tetrahydrofolic acid complex during the interaction of serine and tetrahydrofolic acid. The significance of this observation is not clear, although it is possible that the formation of this complex is the initial step in the synthesis of N-hydroxymethyltetra-hydrofolic acid from serine.

The route by which glycine gives rise to a one-carbon unit has not been completely clarified as yet. The previously discussed observations noted by Crosbie (1958) have shown that serine does not lie on the

pathway of $2\text{-}^{14}C$-glycine incorporation into the thymine-methyl group of <u>Esch. coli</u> DNA. The results obtained by Nakada and Weinhouse (1953) can be interpreted to indicate that the pathway by which the α-carbon of $2\text{-}^{14}C$-glycine is incorporated into the thymine-methyl group involves an initial transamination to yield $2\text{-}^{14}C$-glyoxylate followed by oxidation to ^{14}C-formate and then by way of the steps outlined previously via $N^{10}\text{-}^{14}C$-formyltetrahydrofolic acid. However, the observation that ^{14}C-formate is incorporated into positions 2 and 8 of the purine ring but not into DNA-thymine in an organism <u>Esch. coli</u>, in which $2\text{-}^{14}C$-glycine is utilized for all three "one-carbon" positions under the same conditions (Crosbie, 1958) strongly indicates that free formate (and probably N^{10}-formyltetrahydrofolic acid) is not involved in the pathway of $2\text{-}^{14}C$-glycine incorporation. Moreover the evidence obtained by Nakada and Weinhouse (1953), indicating that glyoxylate is involved in formate production from glycine, is based solely on experiments employing the trapping technique and, accordingly, this evidence has been rendered suspect by the recent observation of the randomisation of activity between $2\text{-}^{14}C$-glycine and glyoxylate due to facile non-enzymic transamination (Crosbie, 1959). Indeed, this recent

evidence obtained by Crosbie (1958 and 1959) has cast doubt upon the details of this proposed incorporation pathway.

An alternative pathway of one-carbon unit formation from glycine has been proposed. In this pathway it is postulated that the α-carbon of glycine condenses with a succinyl-coenzyme A complex to form α-amino-β-oxoadipic acid which is decarboxylated to yield δ-aminolevulinic acid. The δ-aminolevulinic acid can be utilized for pophyrin synthesis or the δ-carbon (originally the α-carbon of glycine) can give rise to formaldehyde which acts as the one-carbon unit donor (Shemin, 1955).

It seems likely therefore, from the evidence published that an N-hydroxymethyl derivative of tetrahydrofolic acid (the precursor of the DNA-thymine methyl group) is the primary one-carbon unit complex formed since this derivative may be dehydrogenated readily to form N^{10}-formyltetrahydrofolic acid (the precursor of C_2 and C_8 in nucleic acid purines) whereas the reverse reaction does not take place in a growing culture of Esch. coli in which $2-^{14}C$-glycine readily labels all three "one-carbon" positions. This scheme can be inferred, from the work of Elwyn and Sprinson (1954), to take place in the case of ^{14}C-labelling of

C_2 and C_8 of DNA-purines and the methyl group of DNA-thymine by $3-^{14}C$-serine.

The pathway of one-carbon unit formation from methionine is also somewhat obscure as yet. Methionine does not donate a one-carbon unit for the synthesis of DNA-thymine in many systems. For example, methionine is not a thymine-methyl group donor in methionine-less mutants of Esch. coli (Green and Cohen, 1957; Crosbie, 1958), an observation which has been confirmed for a uracil-less mutant of Esch. coli (Green and Cohen, 1957). Kit et al. (1958) have pointed out that the transfer of the methyl group of methionine to thymine may be by way of a transmethylation reaction or oxidation of the methionine-methyl group to a one-carbon unit which forms the usual complex with tetrahydrofolic acid. In support of the first possibility, the observed transfer of methionine-methyl by transmethylation to form a C-C linkage in sterol synthesis (Alexander et al., 1957; Alexander and Schwenk, 1957) has been cited. However, Kit et al. (1958) observed that the utilization of methionine-^{14}C-methyl for DNA-thymine methyl group synthesis was greatly decreased by incubation under anaerobic conditions, suggesting that the pathway involves oxidation of the methionine-methyl group in some way. These observations confirmed conclusions previously

reached by Herrmann et al. (1955). The stage at which this oxidation takes place is not clear since Green and Cohen (1957) have shown that S -hydroxymethylhomocysteine (a possible product of methionine-methyl oxidation) is not a precursor of the methyl group of thymine or of the 5- hydroxymethyl group of 5-hydroxymethylcytosine in viral DNA. It would appear, however, that the pathway of methionine-methyl utilization involves a folic acid derivative since methionine-methyl-^{14}C is incorporated into the adenine, guanine and thymine of DNA in lymphatic tissues and tumours and, moreover, this incorporation is depressed to some extent by the presence of aminopterin (Kit et al., 1958). These authors also found that formate incorporation was inhibited to a greater extent than was methionine-methyl incorporation by the addition of aminopterin, suggesting that formate does not lie on the pathway of methionine-methyl utilization. Conversely, Dinning et al. (1958) have obtained evidence which shows that methionine is not an intermediate in the formate utilization pathway. However, it must be noted that Dinning et al.(1958) found that methionine-methyl is a major precursor of the DNA-thymine methyl group in L. leichmannii whereas Kit et al. (1958) found that the methyl group of methionine serves only to a minor extent as a precursor of the one-carbon

unit pool in lymphatic tissues and tumours. The
integration of these observations and the pathway of
methionine-methyl utilization in one-carbon unit
reactions awaits illumination. It appears likely,
however, that the pathway of methionine-methyl
utilization is of the type:

$$\text{-S-Me} \rightleftharpoons \text{N-hydroxymethyltetrahydrofolic acid} \longrightarrow \text{thymine-methyl}$$

$$\text{N-formyltetrahydrofolic acid} \longrightarrow \text{purines}$$
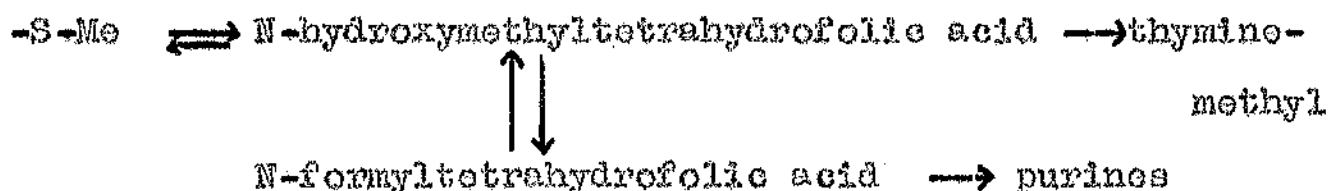
5. The mechanism of thymine-methyl group synthesis.

The observations which have been discussed
already clearly implicate deoxyuridine (or deoxyuridylic
acid) as the one-carbon unit acceptor and N-hydroxymethyl-
tetrahydrofolic acid (or the N^5, N^{10} -methylene-bridge
derivative) as the immediate one-carbon unit donor and
that it is these two compounds which interact to form
TMP. Integration of the pathway of de novo UMP synthesis
with the synthesis of TMP has been achieved by the recent
results obtained by Reichard (1958) who has described the
formation of dUMP from UMP under the influence of an
enzyme present in chick embryo homogenates, thus defining
a pathway of thymine nucleotide synthesis from aspartic
acid, carbamyl phosphate, 5-phosphoribosyl pyrophosphate
and a one-carbon unit donor.

The details of the "methylation" reaction mechanism are not yet clear, but there are several possible pathways which have been considered. Three of these mechanisms postulate an initial condensation of N-hydroxymethyltetrahydrofolic acid with the slightly-activated C_5 of dUMP to form a derivative of dUMP and tetrahydrofolic acid linked through a methylene bridge (I, Fig. I, 5). The first of these three mechanisms, outlined in Scheme A (Fig. I, 5), was postulated by Cohen et al.(1956) and Hamill et al. (1956) and involves hydrolysis of the intermediate to form 5-hydroxymethyldeoxyuridylic acid (II) and regenerated tetrahydrofolic acid. The 5-hydroxymethyl derivative undergoes hydrogenation of the 4:5 double-bond of the pyrimidine ring to yield the 4:5-dihydro derivative (III) followed by dehydration to form the 5-methene derivative (IV) which rearranges spontaneously to form the 5-methyl derivative TMP (V). Cohen et al. (1956) obtained some evidence in support of this mechanism when they isolated thymine and thymidine from the products of catalytic hydrogenation of 5-hydroxy-methyluracil and the corresponding deoxynucleoside, respectively. The catalytic hydrogenation also yielded the 4:5-dihydro derivatives in addition to thymine and thymidine. However, no evidence to indicate that this

Figure I, 5

FAH₄ = tetrahydrofolic acid
N-CH₂OH-FAH₄ = N-hydroxymethyl-tetrahydrofolic acid
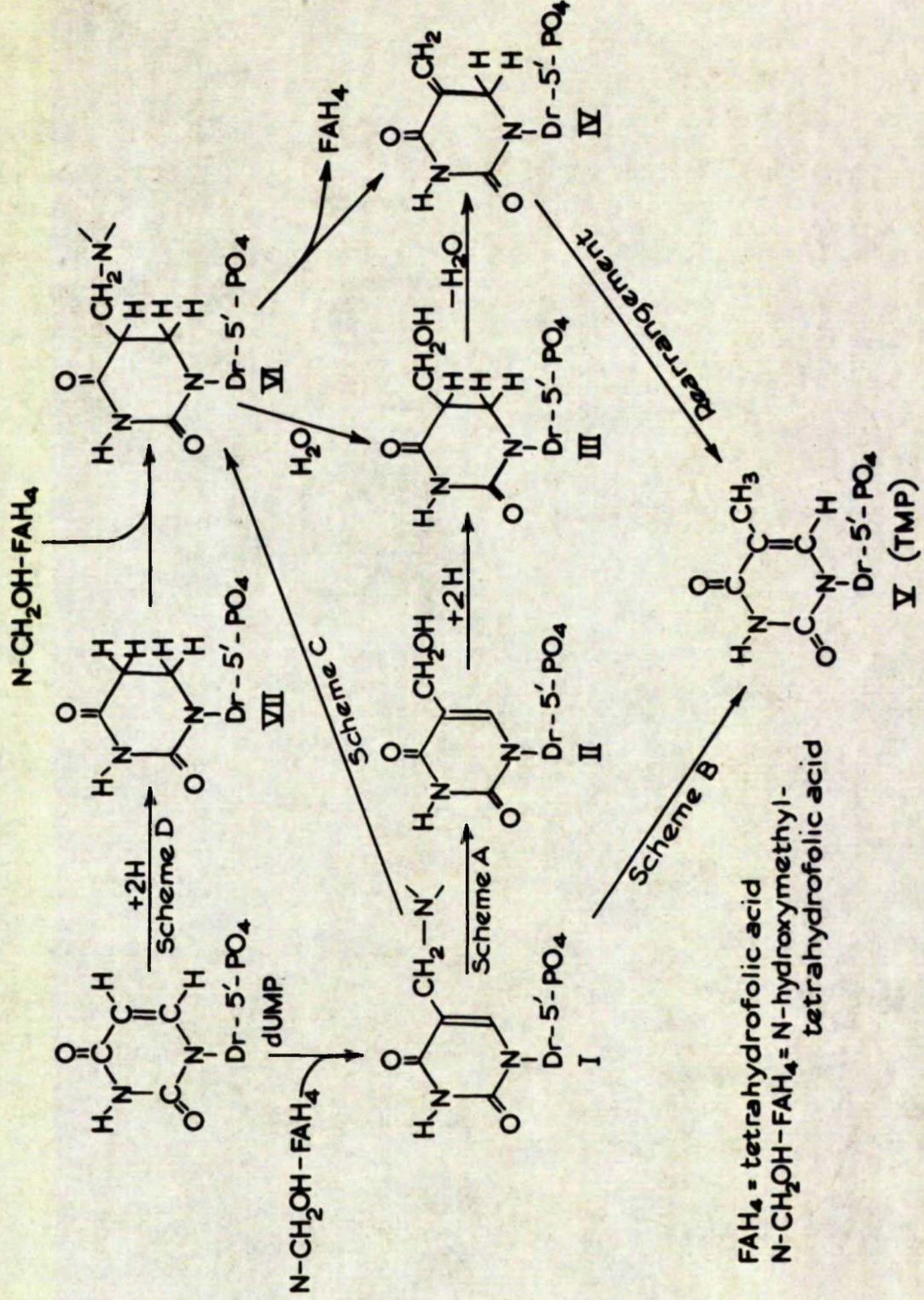
mechanism operates in biological systems has been obtained. Cohen et al. (1957), investigating the rôle of 5-hydroxymethyluracil, 5-hydroxymethyl-cytosine and the corresponding deoxynucleosides in bacterial and bacteriophage DNA-pyrimidine biosynthesis by the isotope competition technique, failed to obtain any evidence for the utilization of 5-hydroxymethyl-pyrimidine derivatives in DNA-thymine biosynthesis. Furthermore, Green et al. (1957) have shown that the 4:5-dihydro derivatives of 5-hydroxymethyluracil, 5-hydroxymethylcytosine and the corresponding deoxy-nucleosides do not support the growth of Esch. coli 15T- (a thymine-less mutant). This evidence indicates that any involvement of 5-hydroxymethyl- or 4:5-dihydro-5-hydroxymethylpyrimidine derivatives in thymine biosynthesis must occur at a nucleotide level. However, the mechanism outlined in Scheme A appears to be the most likely pathway of 5-hydroxymethyldeoxycytidylic acid biosynthesis (Flaks and Cohen, 1957). This reaction is not inhibited by 5-fluorouracil deoxyribose-5'-phosphate whereas the Esch. coli thymidylate synthetase reaction is inhibited powerfully and irreversibly by this antimetabolite (Cohen et al., 1958). This observation would appear to argue against the mechanism of Scheme A being involved in TMP biosynthesis.

The second of these three mechanisms, Scheme B (Fig. I, 5) suggested by Friedkin and Kornberg (1957), involves either (i) a reductive fission of the CH_2-N linkage of the intermediate (I) to yield TMP (V) and regenerated tetrahydrofolic acid or (ii) an elimination reaction within the intermediate (I) to yield TMP and 7:8-dihydrofolic acid. In case (ii) the point of action of the reduced phosphopyridine nucleotide (an essential cofactor for TMP synthesis - Blakley, 1957; Phear and Greenberg, 1957) will be in the regeneration of tetrahydrofolic acid from dihydrofolic acid. Greenberg and Humphreys (1958) provided some evidence in support of the latter possibility when they observed a stiochiometric relationship between thymine-methyl group synthesis and tetrahydrofolic acid concentration in the system described by Phear and Greenberg (1957). They also found that reduced diphosphopyridine nucleotide will stimulate thymine-methyl group synthesis in the presence of sub-optimal concentrations of tetrahydrofolic acid.

The third mechanism, Scheme C (Fig. I, 5) envisages the reduction of the 4:5-double bond of the intermediate (I) followed (i) by hydrolysis to yield the 4:5-dihydro-5-hydroxymethyl derivative (III) which forms TMP by the steps outlined in Scheme A or (ii) by

an elimination reaction to yield the 4:5-dihydro-
5-methene derivative (IV) which forms TMP by the
steps outlined in Scheme A.   A possible variation
of this is shown in Scheme D where the hydrogenation
of the pyrimidine 4:5-double bond takes place to
yield 4:5-dihydrodeoxyuridylic acid (VII) which
condenses with the N-hydroxymethyltetrahydrofolic
acid to form an intermediate (VI).   However, as
discussed previously, there is a considerable body of
evidence which indicates that 4:5-dihydropyrimidines
are not involved in the synthesis of TMP, at least at
the level of the free base or the nucleoside (Cohen
et al., 1957; Blakley, 1957).

Each of the pathways outlined in Fig. I, 5
form quite feasible mechanisms for the "methylation"
reaction although no strong evidence to indicate the
operation of any one of them has been obtained.   At
best, only negative evidence or evidence best described
as circumstantial evidence concerning each mechanism
has been published.   However, a consideration of the
problem on this basis indicates that the mechanism
described in Scheme B appears to be the most likely
of the mechanisms postulated.   It must be pointed out,
however, that this is no more than an interim
conclusion and that the problem has still to be resolved.

6. The rôle of vitamin B_{12} in nucleic acid biosynthesis.

The role of vitamin B_{12} in nucleic acid biosynthesis is still obscure and little evidence with regard to the effect of this vitamin has been reported. Vitamin B_{12} has been implicated, under some conditions, in (i) the biosynthesis of the deoxyribose moiety (Downing and Schweigert, 1956) and (ii) the production of the one-carbon unit utilized for DNA-thymine methyl group synthesis (Elwyn and Sprinson, 1950; Totter, 1954; Smith, 1956, Dinning et al., 1958).

Lactobacillus leichmannii will grow on a medium supplemented with a very small amount of vitamin B_{12}. This growth requirement can be replaced by deoxynucleosides in much larger amounts - the ratio of vitamin B_{12}:deoxynucleosides required is 1:10,000. Downing and Schweigert(1956) have studied the incorporation of $^{14}C_9$-thymidine into the DNA of this micro-organism and have shown that the deoxyribose of the $^{14}C_9$-thymidine is utilized for DNA-deoxyribose synthesis without dilution when vitamin B_{12} is absent. When vitamin B_{12} is present, however, the $^{14}C_5$-deoxyribose from $^{14}C_9$-thymidine is incorporated into the DNA with considerable dilution. These results strongly suggest that vitamin B_{12} is involved in deoxynucleoside synthesis although participation in the Racker aldolase reaction

(Racker, 1952) or in the synthesis of the N-glycoside linkage does not appear to occur. The point at which vitamin B_{12} acts is not known.

The rôle of vitamin B_{12} in thymine-methyl synthesis is similarly obscure. Dinning et al. (1958) have shown that vitamin B_{12} has no effect on the utilization of $1-^{14}C$-glycine, $2-^{14}C$-glycine, $3-^{14}C$-serine or methionine-methyl-^{14}C for RNA-purines or DNA-thymine synthesis in L. leichmannii but that the vitamin does have a profound effect on the incorporation of ^{14}C-formate into DNA-thymine in the same organism. The authors suggest that vitamin B_{12} is involved in the reduction of formate during the methyl group synthesis. However, no unambiguous evidence of the extent of ^{14}C-formate incorporation into the thymine-methyl group as compared with the ring positions was presented, making an assessment of the significance of the results difficult.

However, vitamin B_{12} does not appear to have an effect on RNA or DNA synthesis in all systems. In complete contrast to the above results, Wagle et al. (1958), using vitamin B_{12}-deficient piglets and chicks, have found that vitamin B_{12} has no effect whatsoever on (i) the incorporation of ^{14}C formate, ^{14}C-formaldehyde, $2-^{14}C$-glycine, $3-^{14}C$-serine or methionine-methyl-^{14}C

into the polynucleotide bases or (ii) the utilization
of ^{14}C-glucose for the synthesis of the polynucleotide
pentoses.

The problem raised by these contrasting
results and the elucidation of the actual rôle of
vitamin B_{12} in the synthesis of the nucleic acids are
questions which remain to be solved.

Section II : Results.

Contents.

16. Acid hydrolysis of thymidylic acid.

17. Attempted syntheses of thymine and thymidine by Bacillus subtilis 8059 and Esch. coli PA/15.

18. Synthesis of thymidine by extracts of rabbit thymus glands.

19. Synthesis of thymidylic acid by cell-free extracts of Esch. coli PA/15 and Esch. coli 113/3.

20. Investigation of the thymidylic acid synthetic system for the presence of intermediates.

21. Comparison of deoxyuridine, uridine and deoxyuridylic acid as one-carbon unit acceptors in the synthesis of ^{14}C-TMP.

22. Comparison of 3-^{14}C-serine, ^{14}C-formaldehyde and ^{14}C-formate as one-carbon unit donors in the synthesis of ^{14}C-TMP.

23. Synthesis of ^{14}C-TMP by cell-free extracts of Esch. coli 15T-.

24. Synthesis of ^{14}C-thymidine-5'-triphosphate and DNA-thymine by cell-free extracts of Esch. coli PA/15.

25. The catabolism of thymine, thymidine and thymidylic acid.

26. ^{14}C-labelled materials produced by the catabolism of ^{14}C-TMP synthesised in vitro by cell-free extracts of Esch. coli PA/15.

1. Introduction.

The aim of this work was to find a cell-free bacterial system in which thymine, thymidine or thymidylic acid (TMP) was synthesised, to study the effects of various factors on the system and to elucidate the mechanisms by which one-carbon units were transferred and reduced to thymine-methyl groups. A number of compounds were used as one-carbon acceptors, namely uracil, uridine (UR), deoxyuridine (UDr) and deoxyuridylic acid (dUMP). The one-carbon donor systems studied were (a) ^{14}C-formaldehyde in the presence of 5: 6: 7: 8-tetrahydrofolic acid (FAH_4); (b) ^{14}C-formate and ATP in the presence of FAH_4; and (c) $3-^{14}C$-serine in the presence of FAH_4 and serine hydroxymethylase. The enzyme sources were (i) Escherischia coli PA/15, a serine- or glycine-less mutant; (ii) Esch. coli 15T-, a thymine-or thymidine-less mutant; (iii) Esch. coli 113/3, a methionine- or vitamin B_{12}-less mutant; and (iv) Bacillus subtilis N.C.I.B. 8059.

Initial work was carried out with washed whole-cell suspensions and later work with cell-free extracts. In the case of cell-free extracts the enzyme system was a crude cell extract possessing a large variety of enzyme

activities, some of which are involved in pathways
resulting in degradations or side-reactions of the
substrates and others of which are concerned in the
synthetic pathways being studied.   The bacterial
extracts were examined for the presence of some of
these enzymes, in particular for deoxycytidine deaminase,
nucleoside phosphorylase, adenosine-5'-triphosphatase
deoxyuridylic acid and thymidylic acid phosphomonoesterases
serine deaminase and pyruvic transaminase activities on
the substrates degradative pathways and for glucose-6-
phosphate dehydrogenase, pyridine nucleotide transhydro-
genase, deoxyuridine kinase, serine hydroxymethylase
and methionine synthetase activities on the synthetic
pathways.

Each of these enzymes or enzyme systems was
shown to be present in the crude cell-free extracts.
The results obtained from these studies are discussed
more fully in Sections II, 2 to II, 12 (inclusive). From
information derived from these results the cell-free
systems in which TMP synthesis was investigated (Sections
II, 17 to II, 23, inclusive) were constructed.

2.   Deamination of deoxycytidine by Esch. coli PA/15

Cohen et al. (1956) found that Esch. coli 15T-,
a mutant requiring thymine or thymidine for growth, was
capable of growing when 5-methyldeoxycytidine was

substituted for thymidine and they succeeded in
isolating from Esch. coli an enzyme which catalyses
the deamination of 5-methyldeoxycytidine to thymidine.
The presence of this enzyme in Esch. coli 15T- explains
the organism's ability to utilize 5-methyldeoxycytidine
for growth.   Earlier, Wang et al. (1950) isolated a
deoxycytidine deaminase from Esch. coli and from yeast.
Under the influence of this enzyme, deoxycytidine is
deaminated to UDr and, accordingly, a cell-free extract
of Esch. coli PA/15 was examined for the presence of
this enzyme activity.

The deamination of deoxycytidine was followed.
spectrophotometrically by making use of the relatively
high extinction of deoxycytidine at 280 mμ as compared
with that of UDr and the enzyme solution at the same
wavelength.   Fig. II, 1 shows clearly that deoxycytidine
is deaminated rapidly even at 25° C. and that the
deoxycytidine deaminase activity of cell-free extracts
of Esch. coli PA/15 is high.   Thus deoxycytidine is a
possible substitute for UDr in the study of the transfer
of one-carbon units to the pyrimidine ring.   In this
case the reaction will proceed by the initial deamination
of deoxycytidine to UDr.

Figure II, 1

The deamination of deoxycytidine at 25° C. by a cell-free extract of Esch. coli PA/15.

Test - 0.25 μmoles deoxycytidine in 3 ml. phosphate
buffer (0.1M, pH 7.2) + 0.2 ml. cell-free
extract of Esch. coli PA/15 (0.1 mg. protein);

Control - 3 ml. phosphate buffer (0.1M, pH 7.2) +
0.2 ml. cell-free extract of Esch. coli PA/15
(0.1 mg. protein).

The extinction of both test and control at
280 mμ was determined in a u.v. spectrophotometer at
fixed time intervals over a period of 120 minutes and
the difference in extinction between test and control
($\triangle E_{280}$) plotted against time.   The rate of decrease
in $\triangle E_{280}$ with time is a measure of the
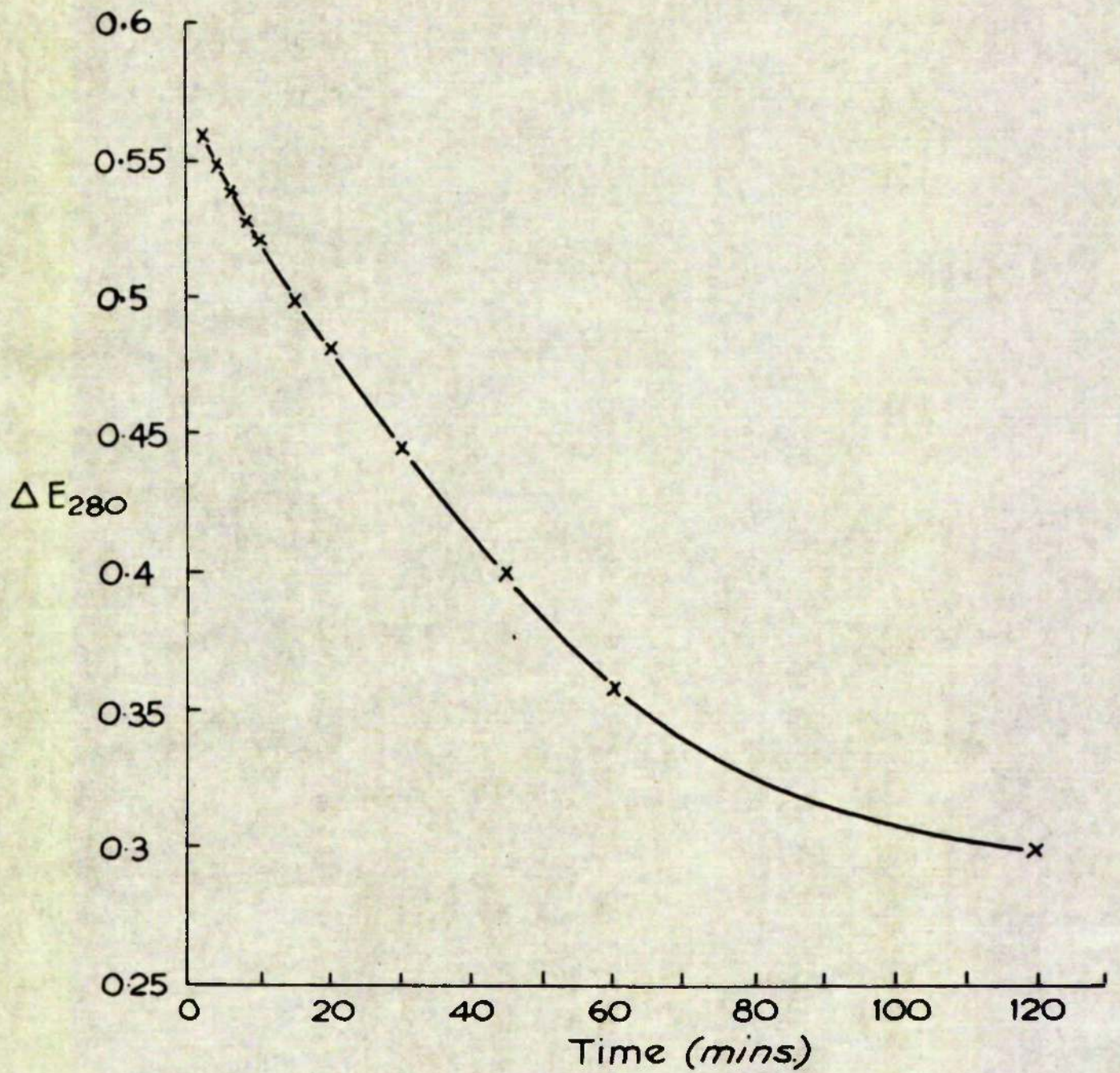deoxycytidine deaminase activity of the cell-free
extract.

Figure II, 1

3. Nucleoside phosphorylase activity in Esch. coli PA/15

Although it was known that Esch. coli contained
a nucleoside phosphorylase, the great activity of this
enzyme in Esch. coli PA/15 was realised first when it was
noticed that, after UDr or UR had been incubated with
whole-cell suspensions or cell-free extracts of Esch. coli
PA/15 in phosphate buffer, very little nucleoside could
be isolated from the reaction mixture but that uracil was
isolated in a very large yield. Nucleoside phosphorylase
activity is dependent on the presence of inorganic phosphate
and an investigation was carried out to determine whether
there was sufficient inorganic phosphate in the cells to
promote the activity or whether an external supply of
phosphate was necessary.

This investigation was carried out by comparing
the rate at which thymidine was degraded by nucleoside
phosphorylase in phosphate buffer with that in phosphate-free
buffer, using both washed whole-cell suspensions and cell-
free extracts of Esch. coli PA/15 as sources of nucleoside
phosphorylase. Tris-(hydroxymethylamino-) methane (Tris)
buffer was chosen as the phosphate-free buffer. The
reaction was followed spectrophotometrically, making use
of the relatively high extinction of thymine at 290 mμ
in alkaline solution as compared with that of thymidine
and the enzyme solution at the same wavelength and pH.

Although there is considerable scatter of the points on the graphs, Figs. II, 2(a) and II, 2 (b) show clearly that an external source of inorganic phosphate is essential to the nucleoside phosphorylase activity present in Esch. coli PA/15. This difference is especially evident in the case of cell-free extracts (Fig. II, 2 (b)) in which case the degradation of thymidine was reduced to a vanishingly small degree in Tris buffer. Subsequent to this observation Tris buffer was used in all systems involving nucleosides to obviate the possibility of substrate degradation by nucleoside phosphorylase action.

4. Degradation of adenine-5'-triphosphate by Esch. coli PA/15 and Esch. coli 15T-.

In simple reaction mixtures, degradation of nucleosides by the nucleoside phosphorylase activity present in Esch. coli is avoided by carrying out the incubations in phosphate-free buffer. However, in more complex reaction mixtures which contain adenosine-5'-triphosphate (ATP) the possible presence of adenosine-5'-triphosphatase in the cell-free extract could provide a source of inorganic phosphate. Accordingly, cell-free extracts of Esch. coli PA/15 and Esch. coli 15T- were tested for adenosine-5'-triphosphatase activity under conditions parallel to those under which TMP synthesis

Degradation of thymidine by <u>nucleoside phosphorylase</u>

(a) <u>in washed whole-cell suspensions of Esch. coli PA/15.</u>

Tests - 5 μmoles TDr + 0.2ml. washed whole-cell

    suspension of Esch. coli PA/15 (10 mg.

    dry wt.) in 5 ml. of (i) phosphate buffer

    (0.1M, pH 7.2) or (ii) Tris buffer

    (0.1M, pH 7.2);

Controls - 0.2 ml. washed whole-cell suspension of

    Esch. coli PA/15 (10 mg. dry wt.) in 5 ml.

    of (i) phosphate buffer (0.1M, pH 7.2) or

    (ii) Tris buffer (0.1M, pH 7.2).

(b) <u>in cell-free extracts of Esch. coli PA/15.</u>

Tests - 5 μmoles TDr + 0.2 ml. cell-free extract of

    Esch. coli PA/15 (1 mg. protein) in 5 ml.

    of (i) phosphate buffer (0.1M, pH 7.2) or

    (ii) Tris buffer (0.1M, pH 7.2).

Controls - 0.2 ml. cell-free extract of Esch. coli

    PA/15 (1 mg. protein) in 5 ml. of

    (i) phosphate buffer (0.1 M, pH 7.2) or

    (ii) Tris buffer (0.1M, pH 7.2)

Incubation was at 37° C. under aerobic conditions.

Each test and control was sampled at fixed time

intervals and each sample (0.5 ml.) was mixed with

2N-NaOH (2 ml.) immediately on withdrawal. These mixtures were diluted to 5 ml. with water and the extinction of each at 290 mμ was determined. The difference in extinction between test and control (ΔE_{290}) was plotted against time. The rate of increase in ΔE_{290} is a measure of the rate of thymine production and hence of the nucleoside phosphorylase activity of (a) the washed whole-cell suspension and (b) the cell-free extract of Esch. coli PA/15.
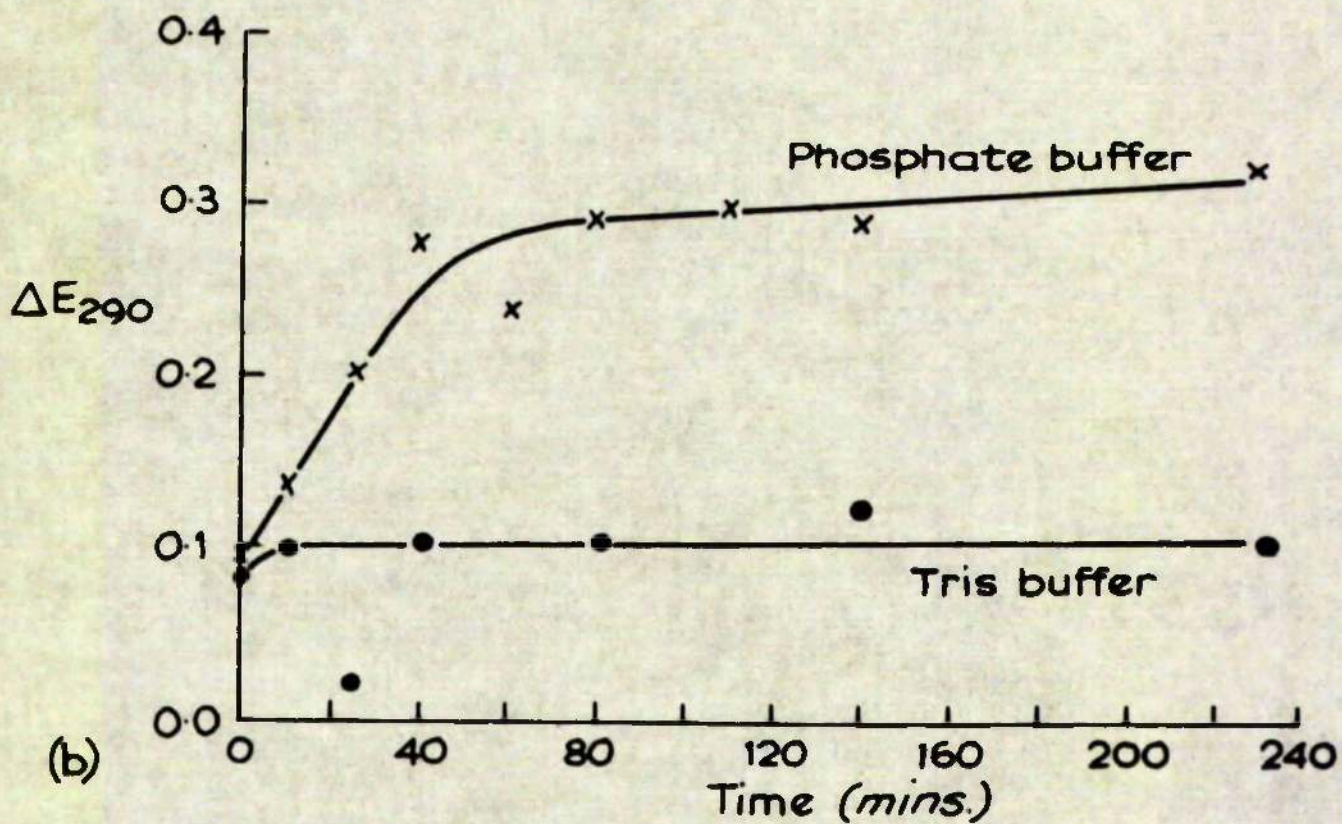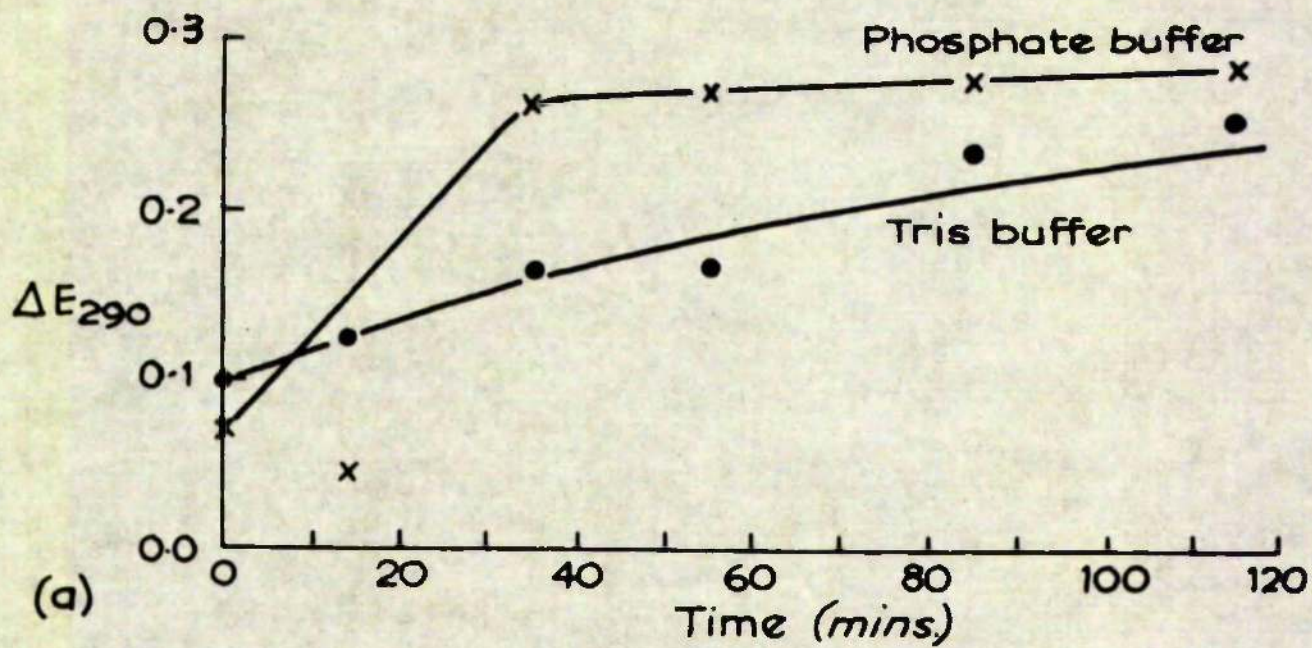
Figure II, 2

was investigated.

The degradation of ATP by adenosine-5'-triphosphatase activity present in cell-free extracts of Esch. coli PA/15 and Esch. coli 15T- was followed by incubating ATP with cell-free extracts of these organisms in Tris buffer and determining the concentration of inorganic phosphate present at various time intervals over a period of 6 hours. The presence of adenosine-5'-triphosphatase in cell-free extracts of Esch. coli PA/15 and Esch. coli 15T- is shown clearly by Fig. II, 3. The graph also shows that the degradation of ATP proceeds slowly under these conditions, incubation for 6 hours resulting in less than 30% degradation of ATP by Esch. coli PA/15 and just over 50% by Esch. coli 15T-.

Further investigation of the nucleoside phosphorylase activity in Esch. coli under conditions where the sole source of inorganic phosphate was from ATP, degraded by the adenosine-5'-triphosphatase present in the organism, showed that 20% or less of the added nucleoside was hydrolysed to the free base in 5 hours, even when the ATP was present in concentrations as high as 30 μmoles/ml. Thus the adenosine-5'-triphosphatase activity present in Esch. coli does not cause serious degradation of nucleoside substrates in incubation mixtures containing ATP.

Figure II, 3

Degradation of adenosine-5'-triphosphate by adenosine-5'-triphosphatase in cell-free extracts of Esch. coli PA/15 and Esch. coli 15T-.

Tests - 10 μmoles ATP + 100 μmoles MgSO$_4$ in Tris buffer
(0.1M, pH 7.2) with (a) a cell-free extract
of Esch. coli PA/15 (0.7 mg. protein); and
(b) a cell-free extract of Esch. coli 15T-
3.3 mg. protein).

Controls - 100 μmoles MgSO$_4$ in Tris buffer (0.1M,
pH 7.2) with (i) a cell-free extract of
Esch. coli PA/15 (0.7mg.
protein);
(ii) a cell-free extract of
Esch. coli 15T- (3.3mg.
protein); and
(iii) 10 μmoles ATP.

Total volume of the incubation mixtures was 5 ml. Incubation was carried out under aerobic conditions at 37° C. Each test and control was sampled at fixed time intervals over a period of six hours and each sample (0.5 ml.) treated with 2 ml. 30% trichloroacetic acid. The precipitated protein was centrifuged, washed with 5 ml. 5% trichloroacetic acid and recentrifuged. The combined supernatants were diluted

to 10 ml. with water and 8 ml. of each was assayed for inorganic phosphate by the method of Borenblum and Chain (1938).

The concentration of inorganic phosphate was plotted against time. The rate of rise of this concentration is proportional to the rate of degradation of ATP and hence to the adenosine-5'-triphosphatase activity of the cell-free extracts.
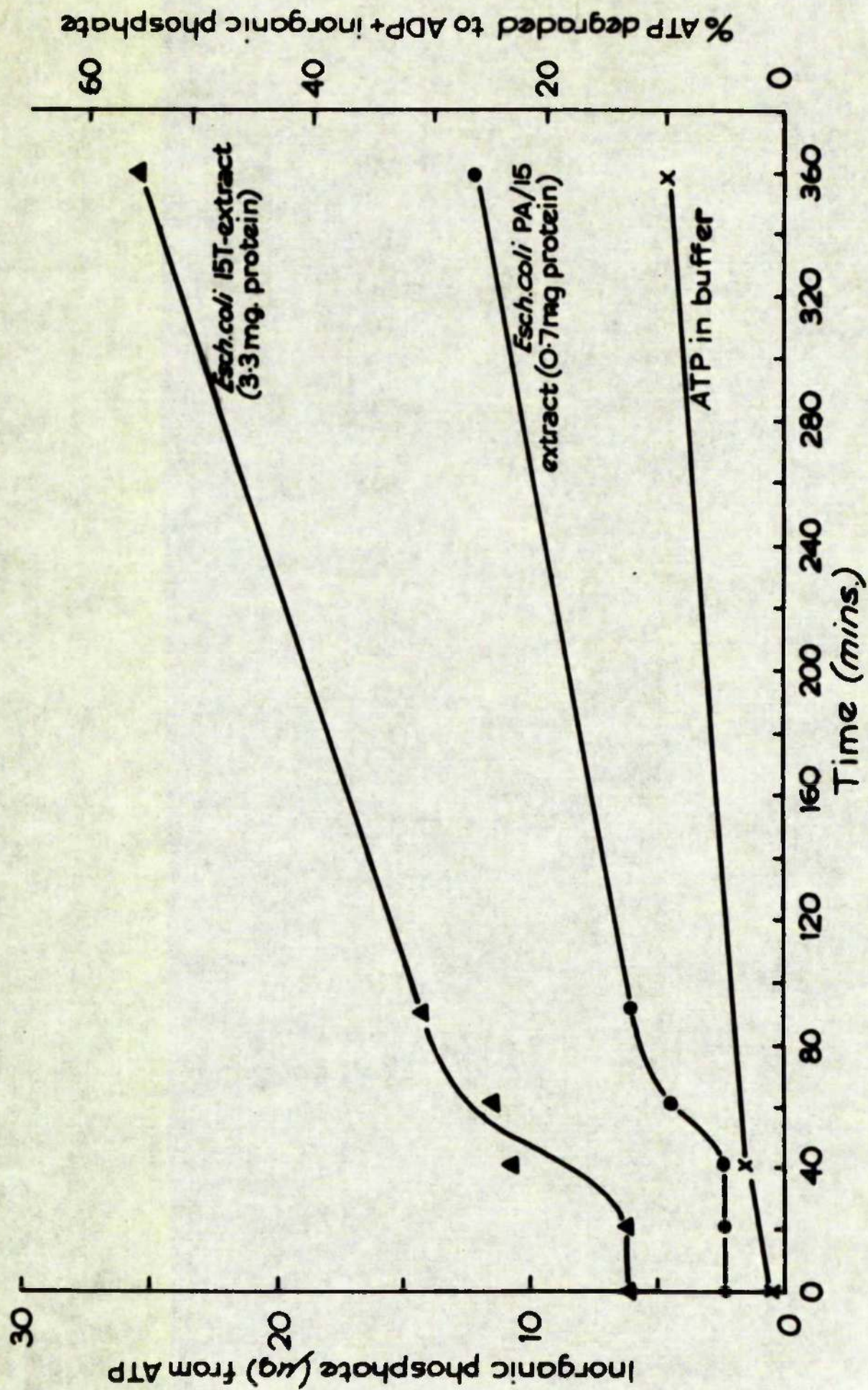
Figure II, 3

5. Deoxyuridylic acid and thymidylic acid phosphomonoesterase activity in Esch. coli PA/15.

The presence of deoxyuridylic acid phosphomonoesterase activity in Esch. coli PA/15 was confirmed by incubating dUMP (30 μmoles), MgSO$_4$ (50 μmoles) and a cell-free extract of Esch. coli PA/15 in Tris buffer (0.1M, pH 7.2), total volume 3 ml. After incubation at 37°C. for 2$\frac{1}{2}$ hours under aerobic conditions the reaction was stopped by addition of 0.3 ml. 10N-HCl and heating at 100° C. for 10 minutes. The reaction supernatant was separated by chromatography on paper in n-butanol-water and the area carrying adenine, uracil and UDr was eluted with water. The material so obtained was rechromatographed in two dimensions (n-butanol-water and iso-propanol-water-HCl). A small amount of material was found in the uracil position but none in the UDr position. The ultra-violet absorption spectra of the material in acid and alkali confirmed that it was uracil and that it was uncontaminated with UDr.

The presence of thymidylic acid phosphomonoesterase activity in Esch. coli PA/15 was confirmed by incubating TMP (6 μmoles) MgSO4 (50 μmoles) and a cell-free extract of Esch. coli PA/15 (6.3 mg. protein) in Tris buffer (0.1 M, pH 7.2), total volume 3 ml. After incubation at 37° C. for 3 hours under aerobic conditions

the reaction was stopped by the addition of 0.3 ml.
10N-HCl and heating at 100° C. for 10 minutes.   The
reaction supernatant was separated by chromatography
on paper in n-butanol-water and the area carrying
thymine and thymidine was eluted with water.   The
material so obtained was rechromatographed in the same
solvent system.   The ratio of thymine and thymidine
to TMP was determined by extinction measurements in
acid at 260 mμ, and it was found that 15% of the TMP
had been degraded to thymine or thymidine.   The ratio
of thymine to thymidine was determined also by
differential spectrophotometry and it was found that the
mixture consisted of 85% thymine.   It was found that
treatment of TMP with N-HCl at 100° C. for 10 minutes
effects hydrolysis of the TMP to the extent of 7% and
that the hydrolysis product consisted of 50% thymine
(Fig. II, 20).

In the first experiment uracil had been
produced by the action of the phosphomonoesterase on
dUMP followed by the degradation of the UDr produced
by nucleoside phosphorylase.   In the second experiment
an analogous pathway has been followed, as is shown by
the increased proportion of thymine in the product as
compared with that in the product of N-HCl hydrolysis of
TMP.

Table II, 1(b)

Omissions	Incubation atmosphere	μmoles/ml. keto-acids [1]	Percentage yield of keto-acids from ser
None	air	0.82	)
None	air	0.76	) 0.13
None	nitrogen	0.47	)
None	nitrogen	0.54	) 0.05
Serine	air	0.01	)
Serine	air	0.03	) —
Serine	nitrogen	0.20	)
Serine	nitrogen	0.18	) —

1. Calculated on assumption that yield is 100% pyruvic acid.

2. Corrected for keto-acids produced by cells alone.

Table II, 1(b)

Keto-acids liberated by washed whole-cell suspensions of Esch. coli PA/15 in the presence and absence of serine.

Tubes were filled and the reaction mixtures treated as in Table II, 1(a). The reaction supernatants were assayed for keto-acids by the method of Friedemann and Hangen (1943).

Table II, 1 (a)

Omissions	Incubation atmosphere	μmoles/ml. NH_4^+	Percentage yield NH_4^+ from serine[1]
None	air	123.8	
None	air	120.0	20. 1
None	nitrogen	100.6	
None	nitrogen	100.6	16.4
Serine	air	1.06	
Serine	air	1.02	—
Serine	nitrogen	2.12	
Serine	nitrogen	2.12	—

1 Corrected for NH_4^+ produced by cells alone.

Table II, 1(a)

Ammonia liberated by washed whole-cell suspensions of Esch. coli PA/15 in the presence and absence of serine.

       Tubes contained washed whole-cell suspensions of Esch. coli PA/15 (6 mg. dry wt.) and serine (600 μmoles) in phosphate buffer (0.1M, pH 7.2) total volume 6 ml. Incubation was carried out under aerobic and anaerobic conditions at 37^{0} C. for $2\frac{1}{2}$ hours. At the end of the incubation period reaction mixtures were centrifuged (20,000 g) and the supernatants assayed for NH_4^+ by the method of Johnson (1941).

6.   Deamination of serine by Esch. coli PA/15

The presence of serine deaminase activity in
Esch. coli PA/15 was confirmed by incubating washed
whole-cell suspensions of Esch. coli PA/15 with serine
in phosphate buffer.   The deamination was followed by
assaying the reaction mixtures for NH_4^+ and for keto-
acids.

Table II,1(a) shows that the liberation of
NH_4^+ was detected in (i) the presence and (ii) the
absence of serine when incubation was carried out under
aerobic or anaerobic conditions.   In the absence of
serine, the cell suspensions released twice as much
NH_4^+ under anaerobic conditions as they did under
aerobic conditions whereas, when serine was present, the
amount of NH_4^+ released under anaerobic conditions was
only 87% of that released under aerobic conditions.   The
addition of serine caused a 100-fold increase in the
amount of NH_4^+ liberated by the cells so that the origin
of the NH_4^+ when serine was included in the incubation
mixture is beyond doubt by the action of serine deaminase
on the amino acid.   A smaller proportion (16%) of added
serine was deaminated under anaerobic conditions than under
aerobic conditions (20%).

Table II, 1(b) shows that the production of
keto-acids was detected in (i) the presence and (ii) the

absence of serine when incubation was carried out under aerobic or anaerobic conditions. In the absence of serine, there was a ten-fold increased in keto-acid production under anaerobic conditions as compared with aerobic conditions whereas, when serine was present, the amount of keto-acids produced under anaerobic conditions was only 40% of that produced under aerobic conditions. The addition of serine caused a 40-fold increase in the yield of keto-acids so that the origin of the keto-acids when serine was included in the incubation mixture is beyond doubt by the action of serine deaminase on the amino acid. A smaller proportion (0.05%) of added serine was deaminated under anaerobic conditions than under aerobic conditions.

Comparisons of corresponding assays for NH_4^+ and keto-acids in Tables II, 1(a) and II, 1 (b) show immediately vast differences between the concentrations of NH_4^+ and keto-acids detected in the same system, aerobic deamination of serine giving rise to a 20% yield of NH_4^+ but only 0.1% keto-acid and anaerobic deamination giving rise to a 16% yield of NH_4^+ but only 0.05% keto-acid.

The nature of the keto-acids synthesised in this system was elucidated by incubating washed whole-cell suspensions of Esch. coli PA/15 with $3-^{14}C$-serine in

phosphate buffer. The 2:4-dinitrophenylhydrazones of
the keto-acids were prepared and separated by paper
chromatography. Figs. II, 4 (a) and II, 4 (b) show
radioactivity coinciding in position with the two
isomers of pyruvic 2:4-dinitrophenylhydrazone (R_F values
0.52 and 0.56 respectively). The natures of the other
radioactive materials (R_F values 0.16 and 0.05 respectively)
were not determined. Since neither is coincident with
any ultra-violet absorbing or ninhydrin-positive material
it is unlikely that they are keto-acid 2:4-dinitrophenyl-
hydrazones or amino acids.

7. Synthesis of alanine from serine by Esch. coli PA/15.

The presence of pyruvic transaminase activity
in Esch. coli PA/15 was confirmed by incubating serine
with washed whole-cell suspensions of Esch. coli PA/15
under both aerobic and anaerobic conditions. Fig. II,
5 shows that alanine was synthesised by Esch. coli PA/15
under these conditions. When 3-^{14}C-serine was incubated
with washed whole-cell suspensions of Esch. coli PA/15
under the same conditions, chromatography of the reaction
mixtures followed by autoradiography showed that alanine
and glutamic acid were both highly labelled (Figs. II,
6 (a) and II, 6 (b)).

Thus the origin of the alanine synthesised in
this system is beyond doubt by the action of serine

Figure II, 4

Deamination of serine by serine deaminase of Esch. coli PA/15.

(a) Ultra-violet photograph and (b) autoradiograph of paper chromatographic separation of 2:4-dinitrophenyl-hydrazones prepared from reaction mixtures containing 3-^{14}C-serine and a washed whole-cell suspension of Each. coli PA/15 incubated under anaerobic (2) and aerobic (3) conditions, together with markers of 2:4-dinitrophenylhydrazine (1), pyruvic 2:4- dinitro-phenylhydrazone (4) and glyoxylic 2:4-dinitrophenyl-hydrazone (5).

Note "A" is an impurity present in 2:4-dinitrophenyl-
             hydrazine.

Complete incubation mixture - 3-^{14}C-serine (200 µmoles, 10µc) and a washed whole-cell suspension of Esch. coli PA/15 (2 mg. dry wt.) in phosphate buffer (0.1M, pH 7.2). Total volume of the incubation mixture was 2 ml. Incubation was carried out under (i) aerobic and (ii) anaerobic conditions at 37°C. for 2½ hours.

Figure II.4(a)

Figure II.4(b)
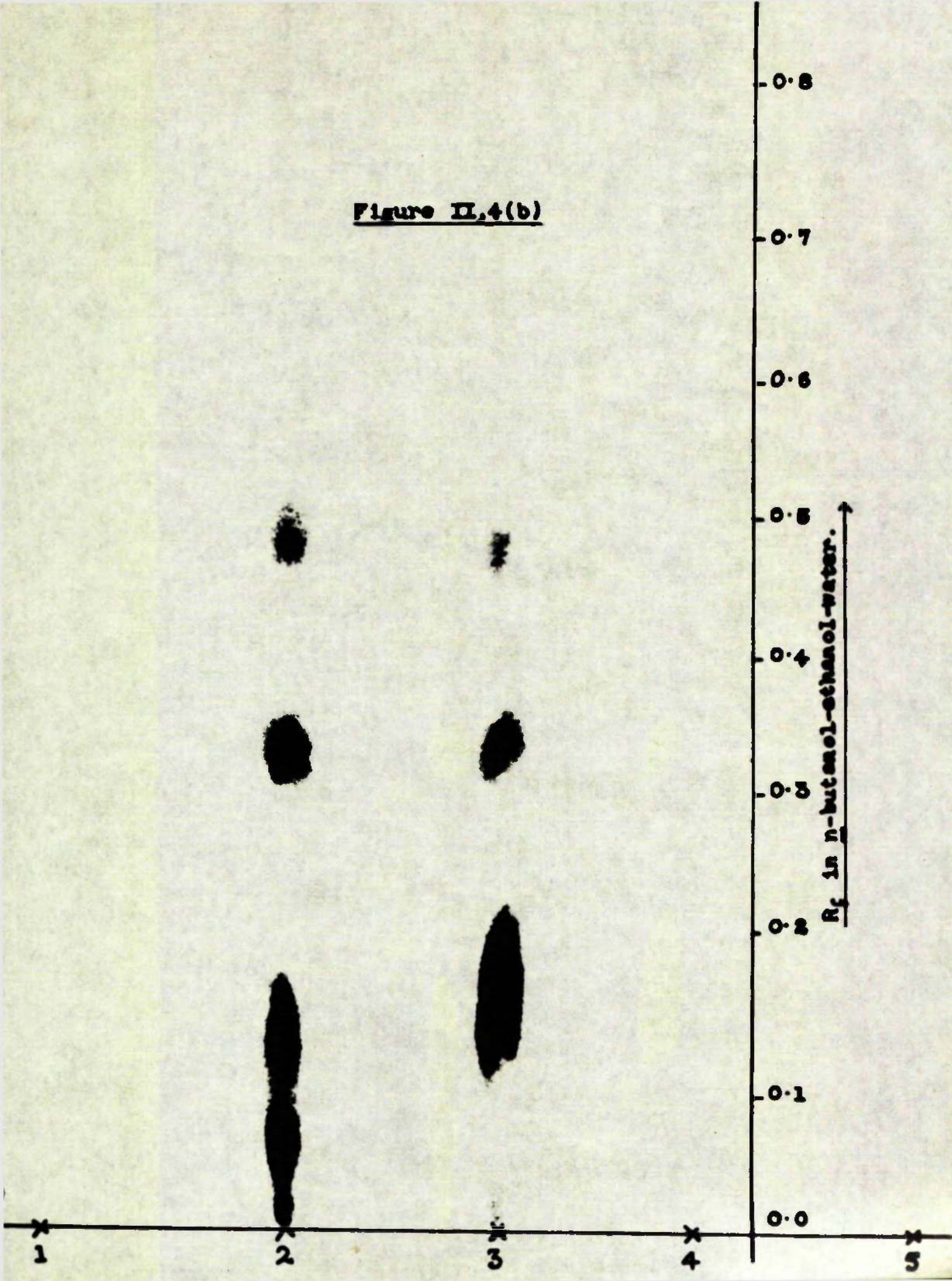
Figure II, 5.

Synthesis of alanine from serine by Esch. coli PA/15

Photograph of paper chromatographic separation of reaction mixtures containing washed whole-cell suspensions of Esch. coli PA/15 and serine in phosphate buffer incubated under anaerobic conditions (1) or aerobic Aminoacids (2) with markers of serine and alanine (3). Aminoacids spots were developed by spraying with ninhydrin.

Complete reaction mixture - Serine (600 μmoles) and a washed whole-cell suspension of Esch. coli PA/15 (6mg. dry wt.) in phosphate buffer (0.1M, pH 7.2). Total volume of the incubation mixture was 6 ml. Incubation was carried out under aerobic and anaerobic conditions at 37° C. for $2\frac{1}{2}$ hours. After incubation the cell suspension was centrifuged (20,000 g.).

Figure IX.5.

Figure II, 6

Synthesis of alanine from serine by Esch. coli PA/15

(a) Photograph and (b) autoradiograph of paper chromatographic separation of reaction mixtures containing washed whole-cell suspensions of Esch. coli PA/15 and 3-^{14}C-serine in phosphate buffer incubated under aerobic conditions (2) or anaerobic conditions (3) with markers of glutamic acid, serine and alanine(1). Amino acid spots were developed by spraying with ninhydrin.

Complete reaction mixture - 3-^{14}C-serine (200 μmoles, 10 μc) and a washed whole-cell suspension of Esch. coli PA/15 (2 mg. dry wt.) in phosphate buffer (0.1M, pH 7.2). Total volume of the incubation mixture was 2 ml. Incubation was carried out under aerobic and anaerobic conditions at 37° C. for 3 hours. After incubation the cell suspension was centrifuged (20,000 g.).
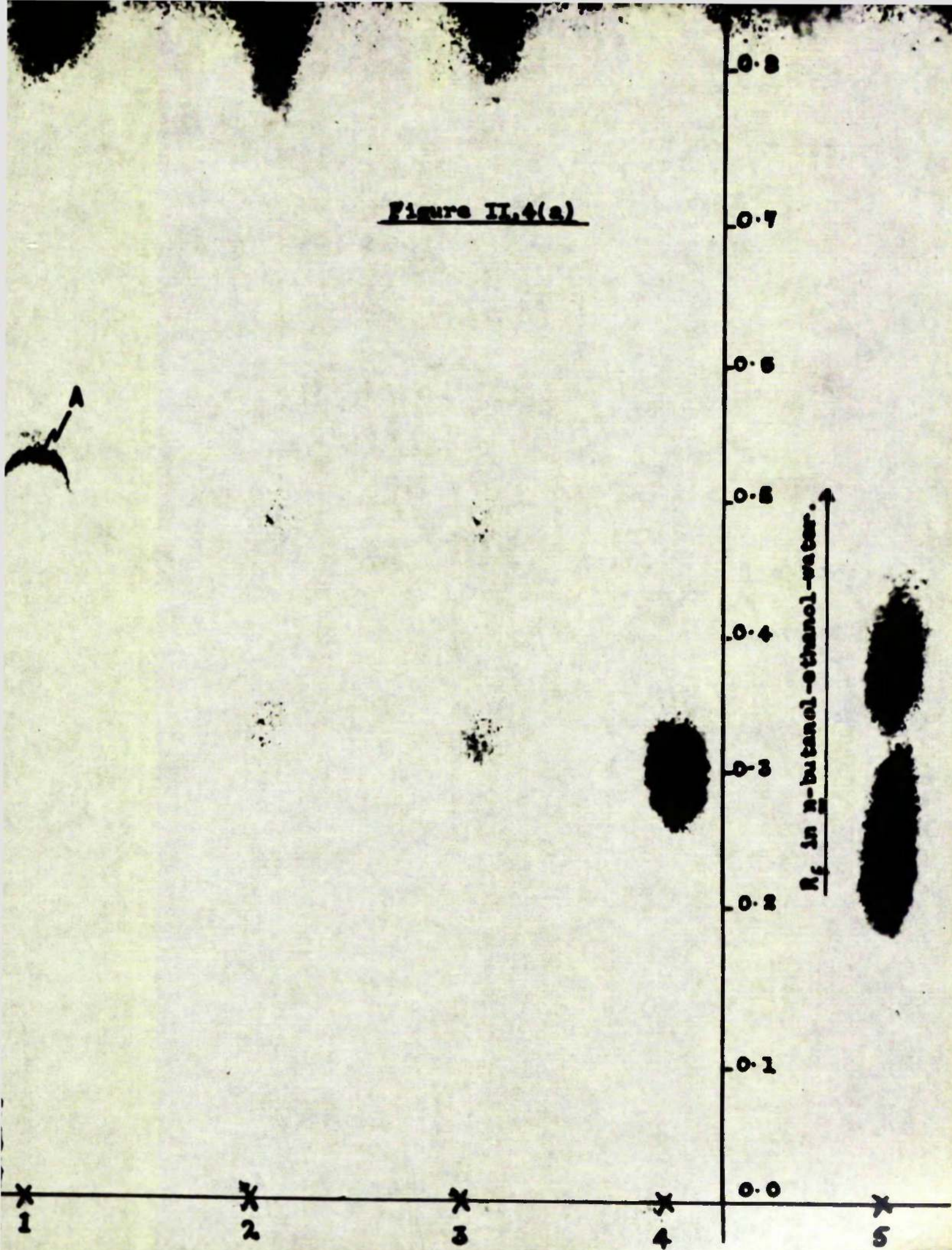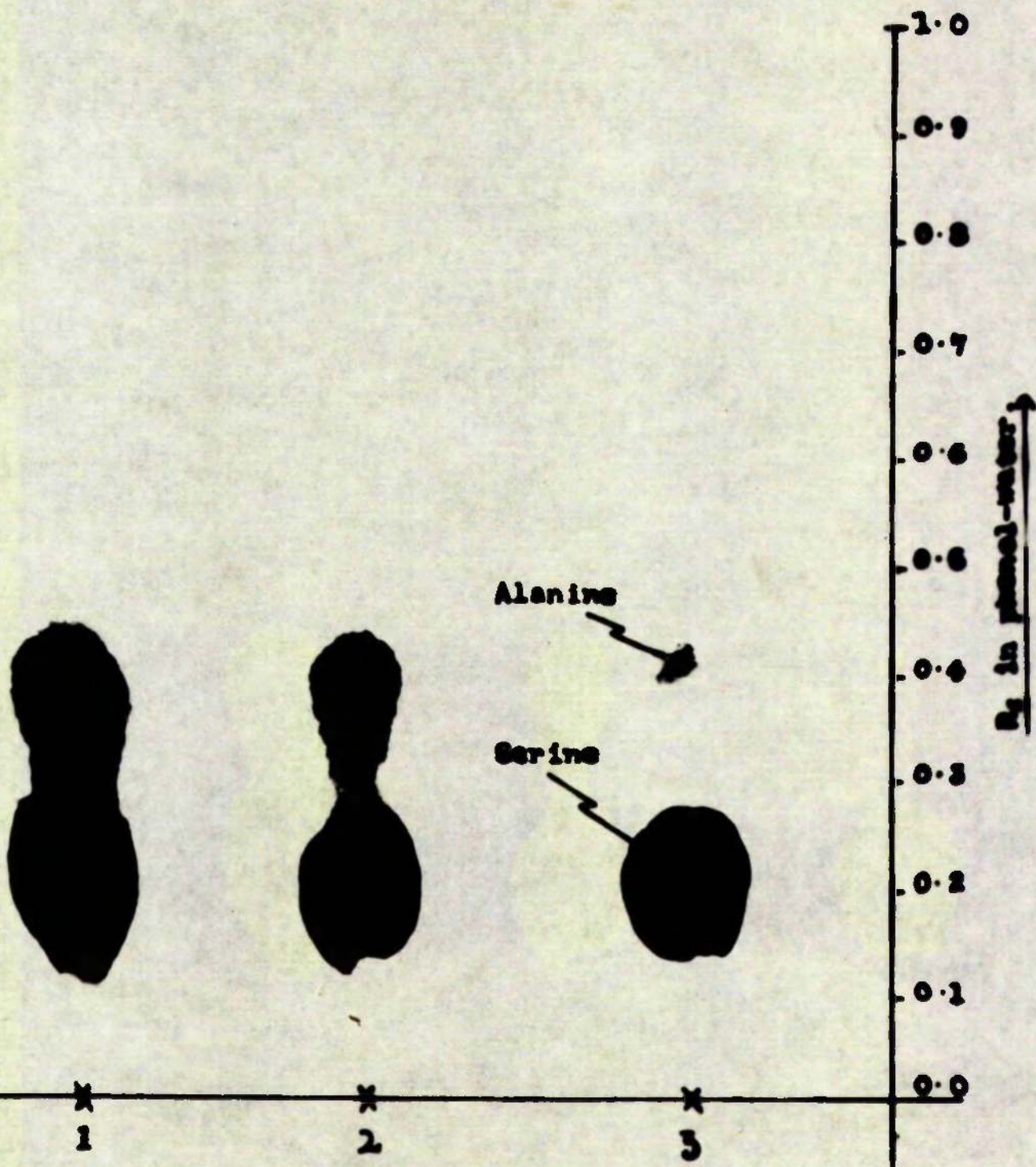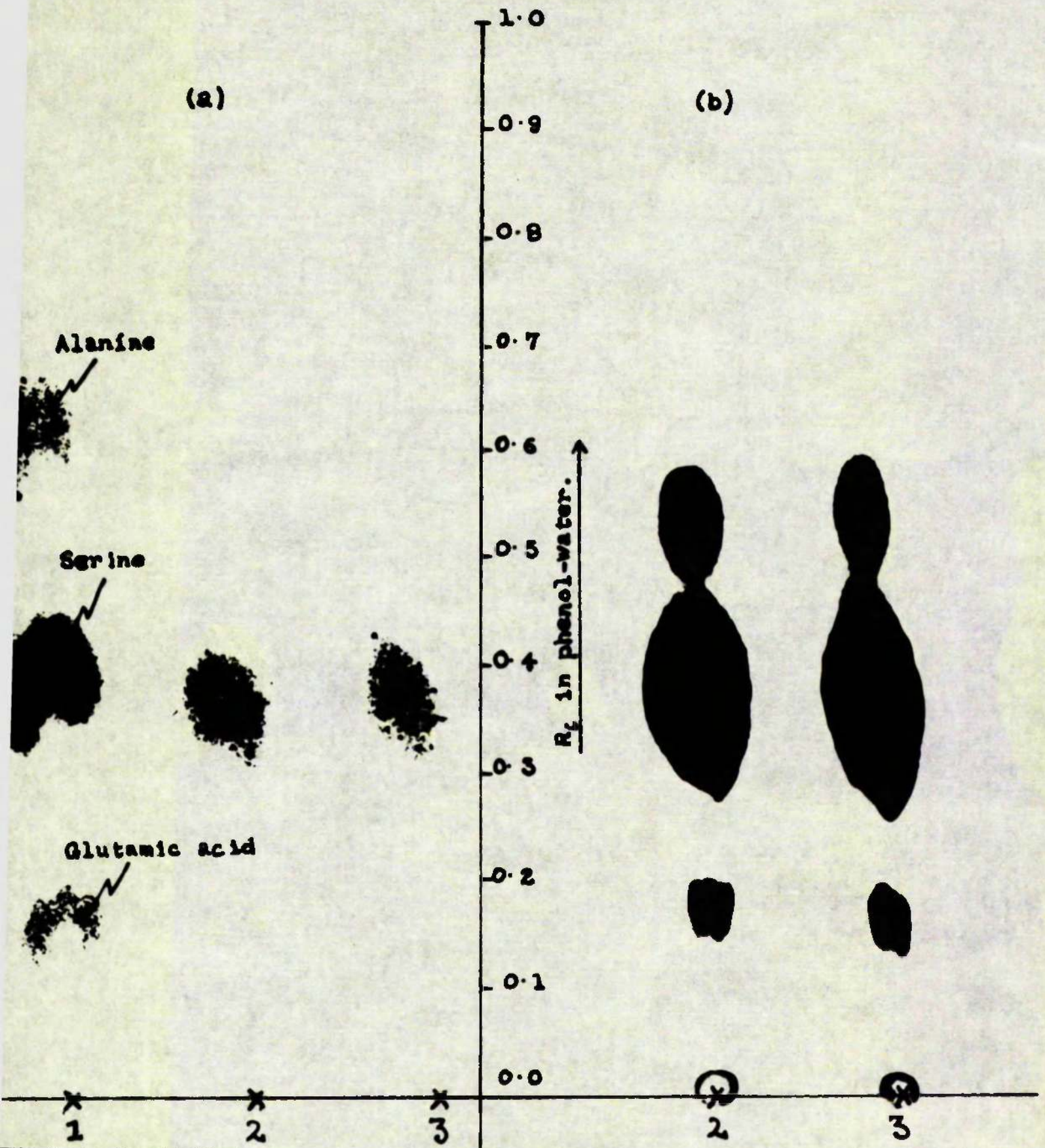
Figure II, 6.

deaminase on the serine followed by transamination of
the pyruvic acid so produced.   The scheme of the
reactions involved may be drawn out as follows:-

Serine

serine

deaminase

NH_3 + pyruvic acid ————→

pyruvic   glutamic acid   tricarboxylic acid

transaminase   cycle

                    α-oxo-glutaric acid ←——

alanine
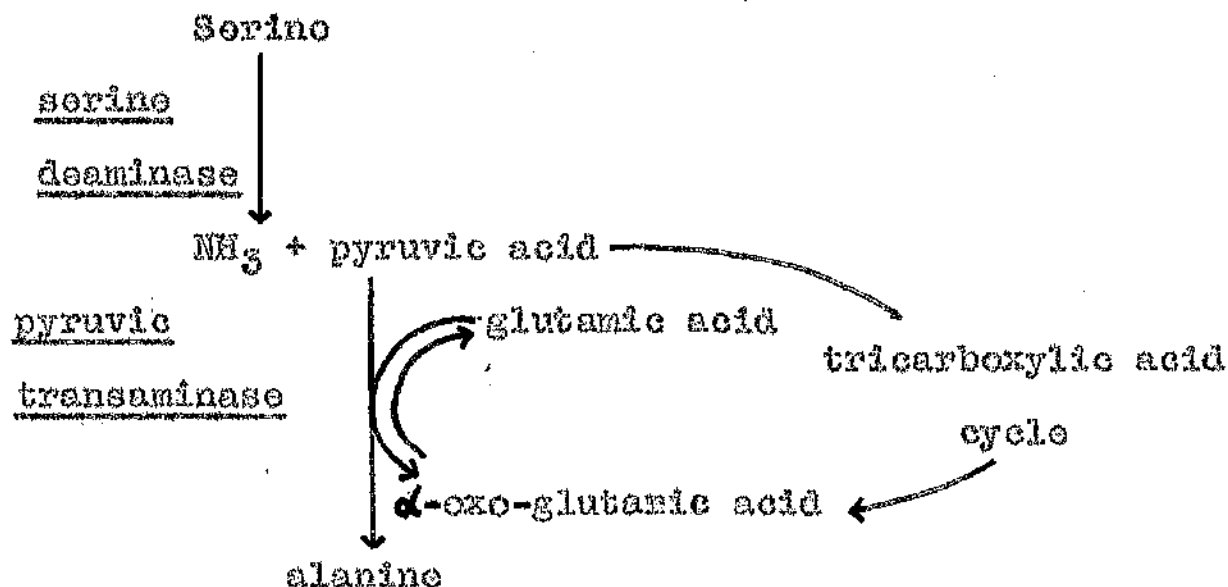
8.   Synthesis of reduced triphosphopyridine nucleotide and transhydrogenation of diphosphopyridine nucleotide by Esch. coli.

The dehydrogenation of glucose-6-phosphate is
a triphosphopyridine nucleotide (TPN)-linked reaction.
Incubation of glucose-6-phosphate and TPN with glucose-
6-phosphate-dehydrogenase is therefore a convenient method
of preparing reduced triphosphopyridine nucleotide (TPNH)
in situ. The presence of glucose-6-phosphate dehydrogenase
activity in Esch. coli was confirmed by incubating glucose-
6-phosphate, TPN and a cell-free extract of Esch. coli PA/15.
The synthesis of TPNH was followed spectrophotometrically

by making use of the relatively high extinction of
TPNH at 340 mµ compared with that of TPN.   Fig. II, 7
shows clearly that TPNH is synthesised rapidly in this
system and hence that cell-free extracts of Esch. coli
PA/15 contain very high glucose-6-phosphate dehydrogenase
activity.

The nature of the buffer used does not appear
to affect this reaction since phosphate buffer (0.1 M,
pH 7.2), Tris buffer (0.1 M, pH 7.2) and glycylglycine
buffer (0.027 M, pH 7.2) can all replace the NaHCO₃
buffer used in this case without causing any noticeable
change in the rate of the reaction.   Using the same
technique, cell-free extracts of Esch. coli 15T- and
Esch. coli 113/3 have been shown to possess the same
activity.

The presence of pyridine nucleotide transhydro-
genase activity in Esch. coli was confirmed by adding
diphosphopyridine nucleotide (DPN) to a reaction mixture
containing TPNH which had been synthesised in situ from
TPN and a limiting amount of glucose-6-phosphate by the
action of glucose-6-phosphate dehydrogenase. Conditions
chosen were those under which a limited amount of TPNH
had been synthesised by including less than a molar
proportion of glucose-6-phosphate in the incubation
mixture.   The reaction was followed spectrophotometrically

Figure II, 7

The reduction of triphosphopyridine nucleotide at
25° C. by a cell-free extract of Esch. coli PA/15.

Test- 0.5 μmoles TPN in 3 ml. NaHCO$_3$ solution (0.2%)
+ 0.4 ml. Ba^{2+}-free glucose-6-phosphate
(18 μmoles) + 0.4 ml. cell-free extract of
Esch. coli PA/15 (0.2 mg. protein);

Control- 0.5 μmoles TPN in 3 ml. NaHCO$_3$ solution
(0.2%) + 0.4 ml. glass-distilled water +
0.4 ml. cell-free extract of Esch. coli
PA/15 (0.2 mg. protein).

The extinction of both test and control at
340 mμ was determined in a u.v. spectrophotometer
at intervals of one minute over a period of 22
minutes and the difference in extinction between
test and control (ΔE_{340}) plotted against time.
The rate of increase in ΔE_{340} is directly proportional
to the glucose-6-phosphate dehydrogenase activity of
the cell-free extract, and to the rate of TPNH synthesis.

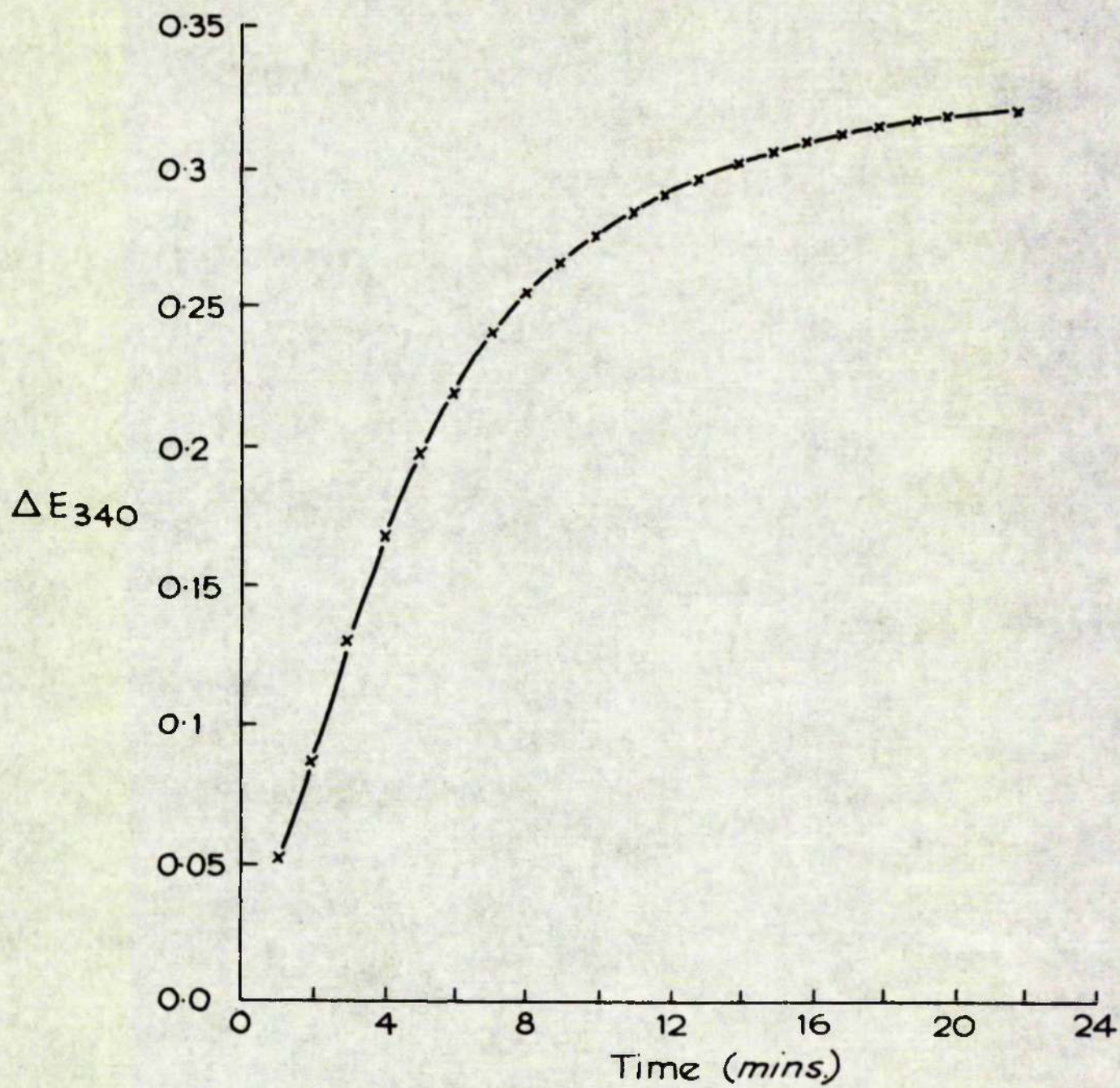Figure II, 7

by making use of the relatively high extinction of
TPNH at 340 mμ compared with that of a mixture of TPN,
DPN, reduced diphosphopyridine nucleotide (DPNH) and
the enzyme solution at the same wavelength.   Fig. II,
8 shows clearly that a cell-free extract of Esch. coli
PA/15 readily catalyses the hydrogenation of DPN by
TPNH and hence that cell-free extracts of Esch. coli
PA/15 contain pyridine nucleotide transhydrogenase
activity.

As cell-free extracts of Esch. coli possess
glucose-6-phosphate dehydrogenase and pyridine nucleotide
transhydrogenase activities, the addition of TPN, DPN
and excess (5 to 10 molar equivalents) glucose-6-phosphate
to incubation mixtures containing cell-free extracts of
Esch. coli will set up a regenerative system in which
TPNH and DPNH are synthesised in situ, thus ensuring that
a large supply of DPNH and TPNH is available in those
incubation mixtures.

9.   Deoxyuridine kinase activity of Esch coli.

The phosphorylation of UDr by ATP in the presence
of cell-free extracts of Esch. coli PA/15 or Esch. coli
15T- was studied in incubation mixtures used to synthesise
TMP.   After 2$\frac{1}{2}$ hours incubation at 37° C. under anaerobic
conditions the nucleotide material was isolated from the
deproteinised reaction mixtures by paper chromatography.

Figure II, 8

Pyridine nucleotide transhydrogenation by Esch. coli PA/15.

A solution of TPNH was prepared by mixing 80 μmoles of $MgCl_2$, 0.18 μmoles glucose-6-phosphate, 0.4 μmoles TPN and a cell-free extract of Esch. coli PA/15 in 6 ml. glycylglycine buffer (0.027M, pH 7.2).

Test - 2.9 ml. "TPNH solution" + 0.1 ml. DPN solution (2 μmoles).

Control - 2.9 ml. "TPNH solution" + 0.1 ml. glass-distilled water.

The extinction of the test and control was determined at 340 mμ at fixed time intervals over a period of $2\frac{1}{3}$ hours and plotted against time.

x — x — x   control

. — . — .   test

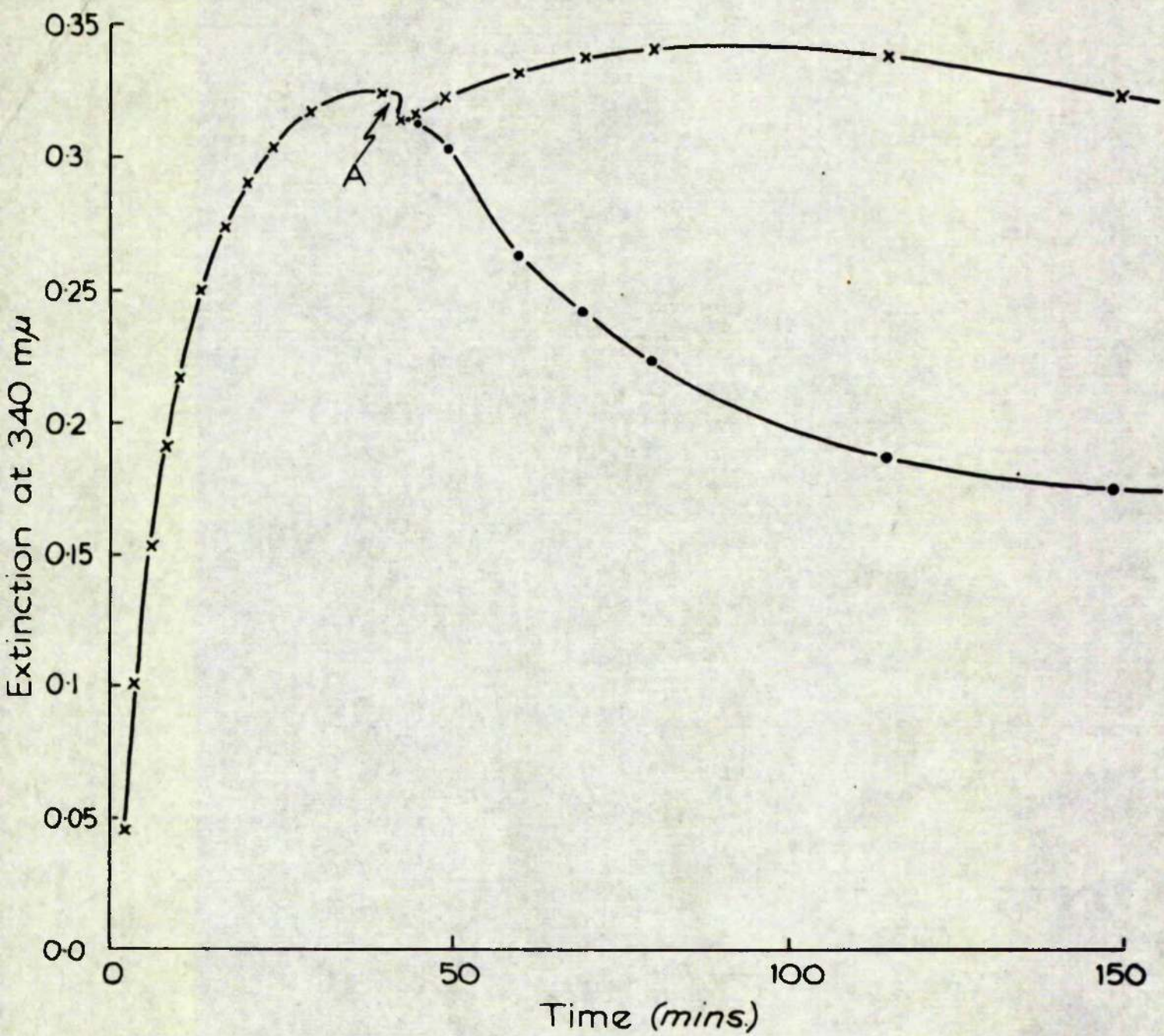At point A, glass-distilled water was added to the control and DPN solution to the test.

Figure II, 8

This nucleotide material was treated with 2N- HCl at 100° C. for 1 hour and the hydrolysis products were separated by paper chromatography.

Fig. II, 9 clearly shows material in the uracil position from the reaction mixture which contained UDr, ATP and a cell-free extract of Esch. coli 15T- (2). A corresponding ultra-violet absorbing spot was detected from the reaction mixture which contained UDr, ATP and a cell-free extract of Esch. coli PA/15 (4), but it was very faint and was not reproduced on this photograph. For comparison the corresponding hydrolysate of material isolated from a reaction mixture which contained dUMP, ATP and a cell-free extract of Esch. coli PA/15 was run in parallel and it also shows the ultra-violet absorbing material in the uracil position (3).

The ultra-violet absorption spectra of the material in the uracil position corresponded exactly with that of an authentic sample of uracil at all pH values investigated. Moreover, no material corresponding to this uracil was obtained from a similar incubation mixture from which pyrimidine derivatives had been excluded.

Uracil and UDr are well separated from the nucleotide material by the chromatographic analysis carried out on the deproteinised reaction mixtures. Thus the uracil appearing after hydrolysis of the nucleotide fraction of the reaction mixtures must have its origin in

Figure II, 9.

The phosphorylation of deoxyuridine by Esch. coli PA/15
and Esch. coli 15T-

Ultraviolet photograph of paper chromatographic
separation of hydrolysis products of the base bands
(R_F 0.0 to 0.05) of n-butanol-water separations of
deproteinised reaction mixtures containing UDr and a
cell-free extract of Esch. coli 15T- (2); dUMP and
a cell-free extract of Esch. coli PA/15 (3); and UDr
and a cell-free extract of Esch. coli PA/15 (4),
together with marker adenine and uracil (1).
Complete phosphorylation system - The phosphorylation
of UDr was studied in systems used to synthesise TMP, that is
ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles),
pyridoxal phosphate (0.6 μmoles), DPNH (2 μmoles),
TPN (2 μmoles), glucose-6-phosphate (20 μmoles),
3-^{14}C-serine (30 μmoles, 15 μc) in Tris buffer
(0.1M, pH 7.2) with (2) UDr (30 μmoles) + a cell-free
extract of Esch. coli 15T- (5 mg. protein); (3) dUMP
(30 μmoles) + a cell-free extract of Esch. coli PA/15
(3.6 mg. protein); and (4) UDr (30 μmoles) + a cell-
free extract of Esch. coli PA/15 (3.6 mg. protein).
Incubation was anaerobic, at 37° C. for 2⅓ hours.

The hydrolysis of the base-bands was carried
out by heating with 2N-HCl at 100° C. for one hour.
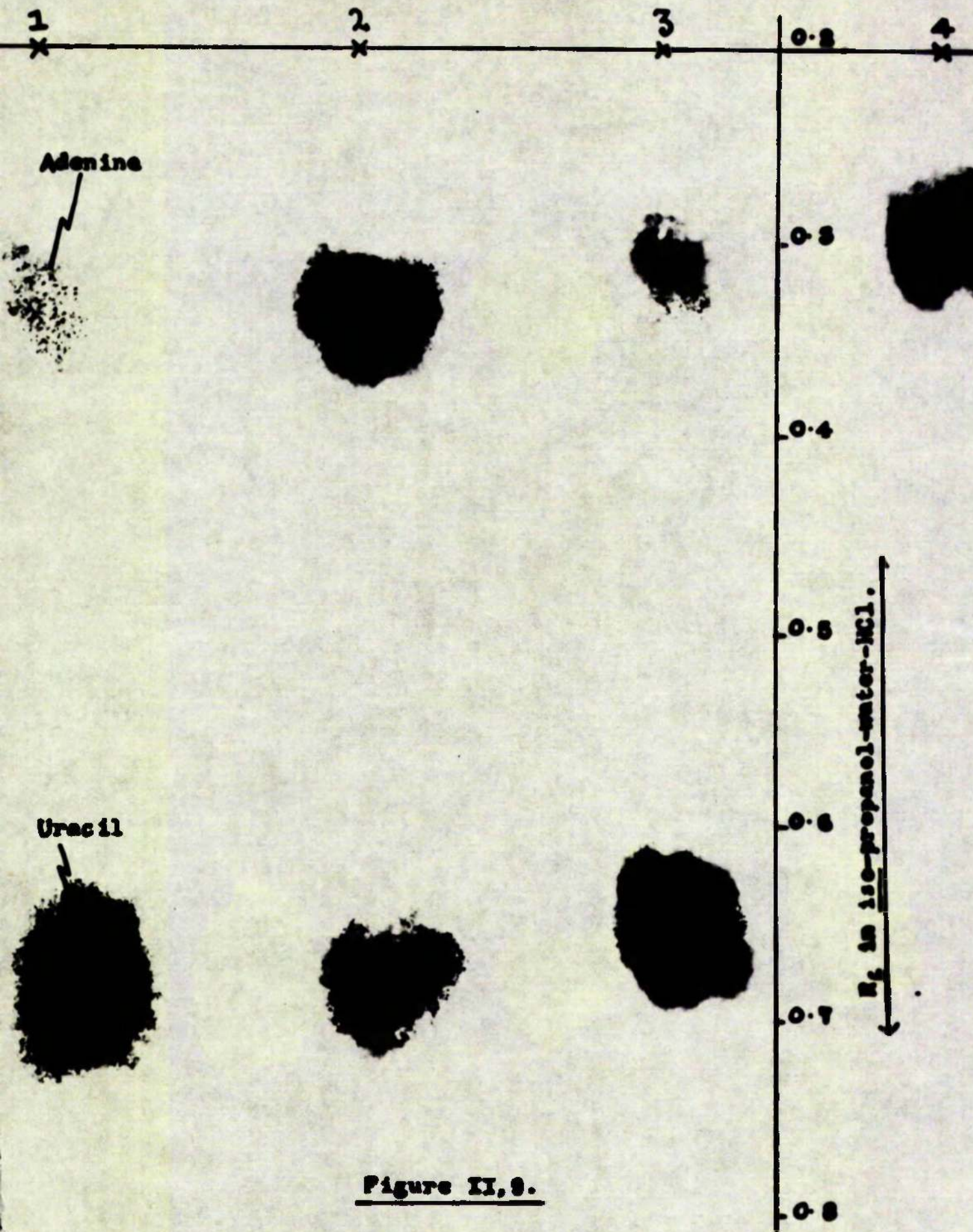
Figure II.9.

a uracil nucleotide, most probably dUMP. This shows
that cell-free extracts of Esch. coli PA/15 and Esch.
coli 15T- are capable of effecting the phosphorylation
of UDr by ATP.

Extinction measurements at 260 mμ indicated
that 18% of added UDr was degraded to uracil by
nucleoside phosphorylase and 14% phosphorylated to dUMP
in the case of cell-free extracts of Esch. coli 15T-.
Insufficient phosphorylation of UDr took place with Esch.
coli PA/15 for measurements to be made but it was found
that 20% of added UDr was degraded to uracil by
nucleoside phosphorylase.

10. Interconversion of serine and glycine.

Blakley (1954 and 1955) has confirmed the
presence of serine hydroxymethylase in extracts of
pigeon liver by demonstrating the conversion of 2-^{14}C-
glycine to 2-^{14}C-serine and the synthesis of 2-^{14}C-
serine from 2-^{14}C-glycine and formaldehyde. He found
that these reactions were dependent on FAH$_4$ and pyridoxal
phosphate. These observations have been confirmed by
Kisluik and Sakami (1955).

As a first step to confirming the presence of
serine hydroxymethylase in Esch. coli PA/15 the inter-
conversion of serine and glycine was studied. Cell-free
extracts of Esch. coli PA/15 (0.5 mg. protein) were

incubated in phosphate buffer (0.1M, pH 7.2) at
37° C. for 5 hours with 2-^{14}C-glycine (100 μmoles, 2 μc)
and (i) serine (100 μmoles); (ii) FAH$_4$ (0.5 μmoles);
(iii) serine (100 μmoles) and FAH$_4$ (0.5 μmoles); (iv)
serine (100 μmoles), FAH$_4$ (0.5 μmoles) and pyridoxal
phosphate (0.1 μmole); (v) formaldehyde (0.5 μmoles)
and FAH$_4$ (0.5 μmoles); (vi) formaldehyde (0.5 μmoles),
serine (100 μmoles) and FAH$_4$ (0.5 μmoles).   The total
volume of each incubation mixture was 5 ml. and
incubation was carried out under anaerobic conditions.
The deproteinised reaction mixtures were treated with
1-fluoro-2:4-dinitrobenzene and the 2:4 dinitrophenyl-
(DNP-) derivatives of serine and glycine isolated and
separated by chromatography on celite columns (section
(IV, 21).   In no case was the DNP-serine found to be
radioactive.

Cell-free extracts of Esch. coli PA/15 (0.5
mg protein) were incubated under exactly the same
conditions with 3-^{14}C-serine (100 μmoles, 2 μc.) and
(i) FAH$_4$ (0.5 μmoles); (ii) folic acid (0.5 μmoles);
(iii) N^5-formyl-FAH$_4$ (0.5 μmoles); (iv) glycine (100
μmoles); (v) glycine (100 μmoles) and FAH$_4$ (0.5 μmoles).
The deproteinised reaction mixtures were treated with
1-fluoro-2:4-dinitrobenzene and the DNP-derivatives of
serine and glycine isolated and separated by
chromatography on celite columns.   In no case was the

DNP-glycine found to be radioactive.

The presence of serine hydroxymethylase in
Esch. coli PA/15 was demonstrated, however, when cell-
free extracts of that organism were incubated under
anaerobic conditions in Tris buffer with $^{14}C_3$-serine,
DPNH and (i) glycine; (ii) glycine and FAH_4; (iii)
FAH_4. Table II, 2 shows that, in each case, the DNP-
derivative of glycine was radioactive. The synthesis
of $^{14}C_2$-glycine from $^{14}C_3$-serine in these systems was
confirmed by paper chromatographic separation of
(i) the free amino acids of the deproteinised reaction
mixtures and (ii) the DNP-amino acids prepared from the
deproteinised reaction mixtures by treatment with
1-fluoro-2:4-dinitrobenzene. Autoradiography of these
chromatograms showed radioactive spots coincident with
(i) carrier glycine and carrier serine (Figs. II, 10 (a)
and II, 10 (b)) and (ii) carrier DNP-glycine and DNP-
serine (Figs. II, 11(a) and II, 11(b)), respectively .

The presence of serine hydroxymethylase in
Esch. coli PA/15 was also confirmed when cell-free
extracts of this organism were incubated with ^{14}C-
formaldehyde and glycine in the presence of DPNH and
FAH_4. DNP-serine isolated from this incubation was
radioactive (Table II, 2). The failure to demonstrate
the presence of this enzyme in Esch. coli PA/15 in
earlier experiments using 2-^{14}C-glycine and formaldehyde

Table II, 2.

Specific activities (counts/min./μmole) of
DNP-glycine and DNP-serine isolated from
reaction mixtures containing $^{14}C_3$-serine and
a cell-free extract of Esch. coli PA/15.

Tubes contained cell-free extracts of
Esch. coli PA/15 (4 mg. protein) and DPNH
(0.4 μmoles) in Tris buffer (0.1M, pH 7.2) with
(i) $^{14}C_3$-serine (40 μmoles, 4 μc) and glycine
(40 μmoles); (ii) $^{14}C_3$-serine (40 μmoles, 4 μc),
glycine (40 μmoles) and FAH$_4$ (2.5 μmoles);
(iii) $^{14}C_3$-serine (40 μmoles, 4 μc) and FAH$_4$
2.5 μmoles); (iv) ^{14}C-formaldehyde (3 μmoles,
4 μc), glycine (40 μmoles) and FAH$_4$ (2.5 μmoles).
Total volume of the incubation mixtures was 5 ml.
Incubation was carried out under anaerobic
conditions at 37° C. for 2 hours. The deprotein-
ised reaction mixtures were treated with 1-fluoro-
2:4-dinitrobenzene and the DNP-derivatives of
serine and glycine isolated and separated by
chromatography on celite columns.

Table II, 2.

Additions to cell-free extract of Esch. coli PA/15 in Tris buffer	Specific activity of DNA derivatives isolated	
	DNP-serine	DNP-glycine
$^{14}C_3$-serine + glycine + DPNH	28,420	2,640
$^{14}C_3$-serine + glycine + DPNH +FAH$_4$	21,420	2,560
$^{14}C_3$-serine + DPNH + FAH$_4$ [1]	15,490	1,000
H^{14}CHO + glycine + DPNH + FAH$_4$ [2]	500	50

1. Carrier DNP-glycine added before isolation of the DNP-derivatives.

2. Carrier DNP-serine added before isolation of the DNP-derivatives.

Figure II, 10

Interconversion of serine and glycine.

(a) Photograph and (b) autoradiograph of paper chromatographic separation of deproteinised reaction mixtures containing $^{14}C_3$-serine, DPNH, a cell-free extract of Esch. coli PA/15 and (i) glycine (2), (ii) glycine + FAH$_4$ (3) and (iii) FAH$_4$ (4) together with a marker of glycine (1).

Complete reaction mixture - $^{14}C_3$-serine (40 μmoles 4 μc); glycine (40 μmoles), DPNH (0.4 μmoles), FAH$_4$ (5 μmoles) and a cell-free extract of Esch. coli PA/15 (4mg. protein) in Tris buffer (0.1M, pH 7.2). Total volume of the incubation mixture was 5 ml. Incubation was carried out under anaerobic conditions at 37° C. for two hours.

Figure II, 10 (a).

Figure II. 10 (b).

Figure II, 11

Interconversion of serine and glycine

(a) Ultraviolet photograph and (b) autoradiograph of paper chromatographic separation of the DNP-derivatives of serine and glycine prepared from deproteinised reaction mixtures containing $^{14}C_3$-serine, DPNH, a cell-free extract of Esch. coli PA/15 and (i) glycine (3), (ii) glycine + FAH$_4$ (2) and (iii) FAH$_4$ (1) together with marker DNP-glycine (4) and DNP-serine (5).

Complete incubation mixture - $^{14}C_3$-serine (40 μmoles, 4 μc), glycine (40 μmoles), DPNH (0.4 μmoles), FAH$_4$ (5 μmoles) and a cell-free extract of Esch. coli PA/15 (4 mg. protein) in Tris buffer (0.1M, pH 7.2). Total volume of the incubation mixture was 5 ml. Incubation was carried out under anaerobic conditions at 37° C. for two hours.
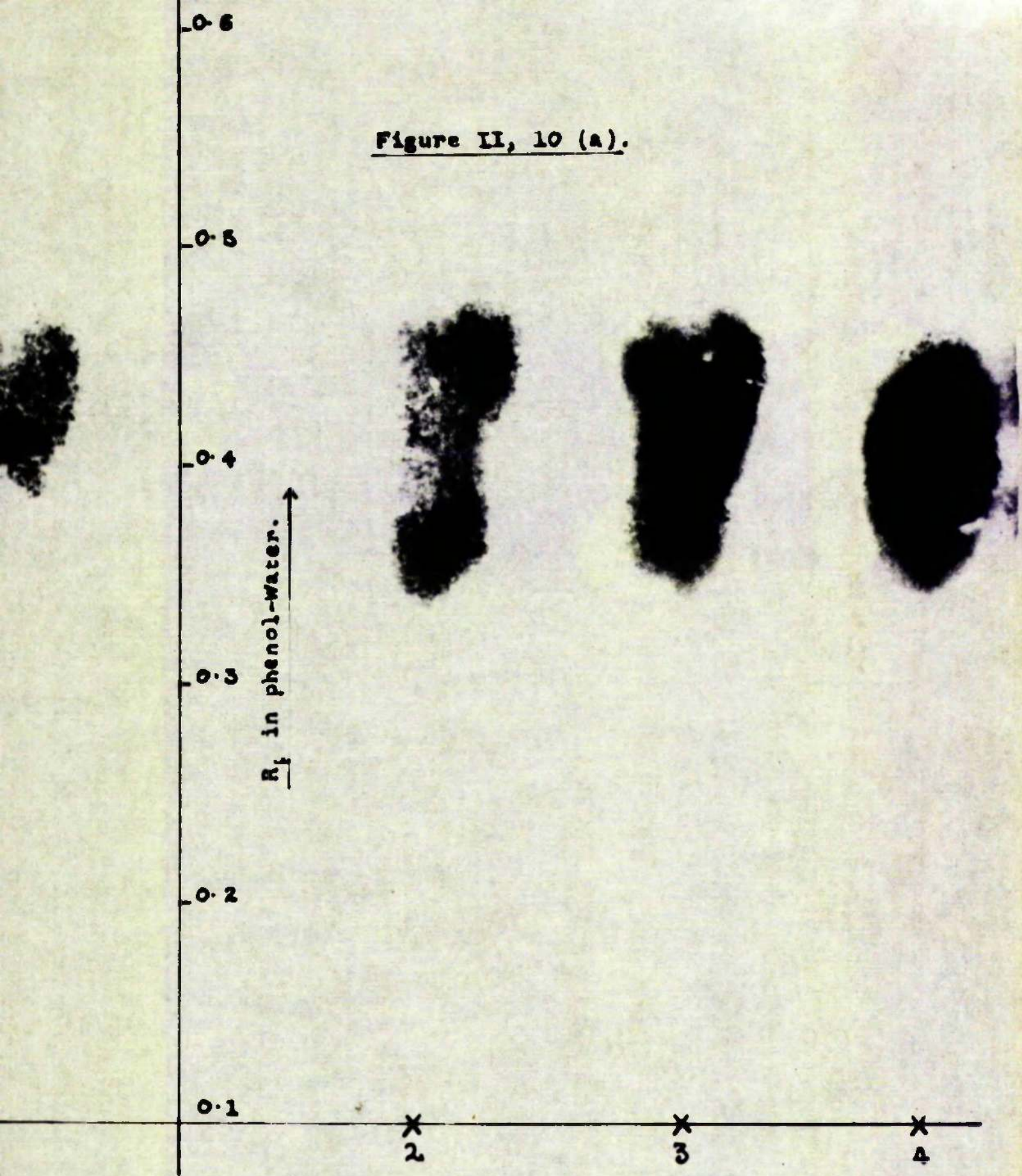
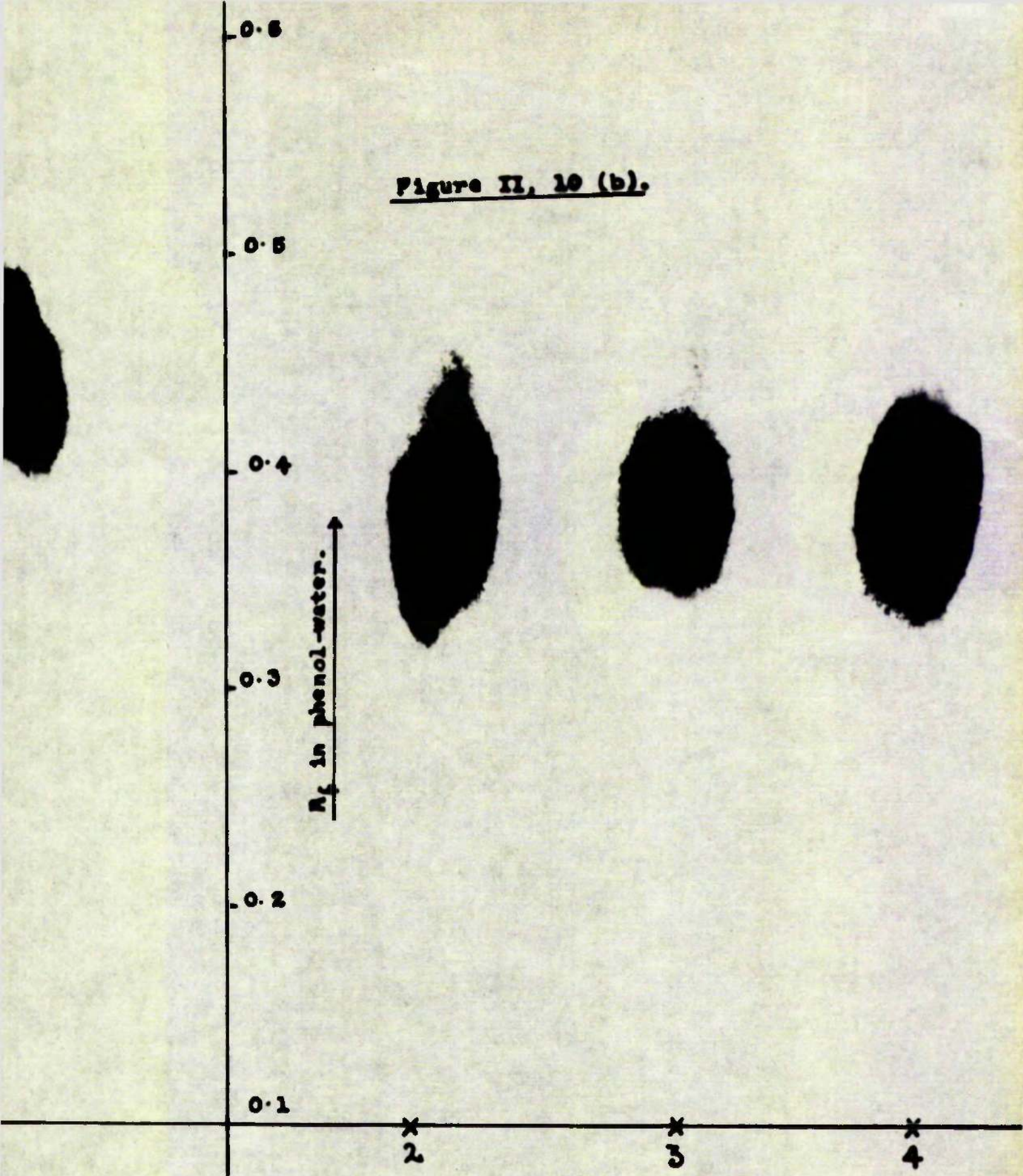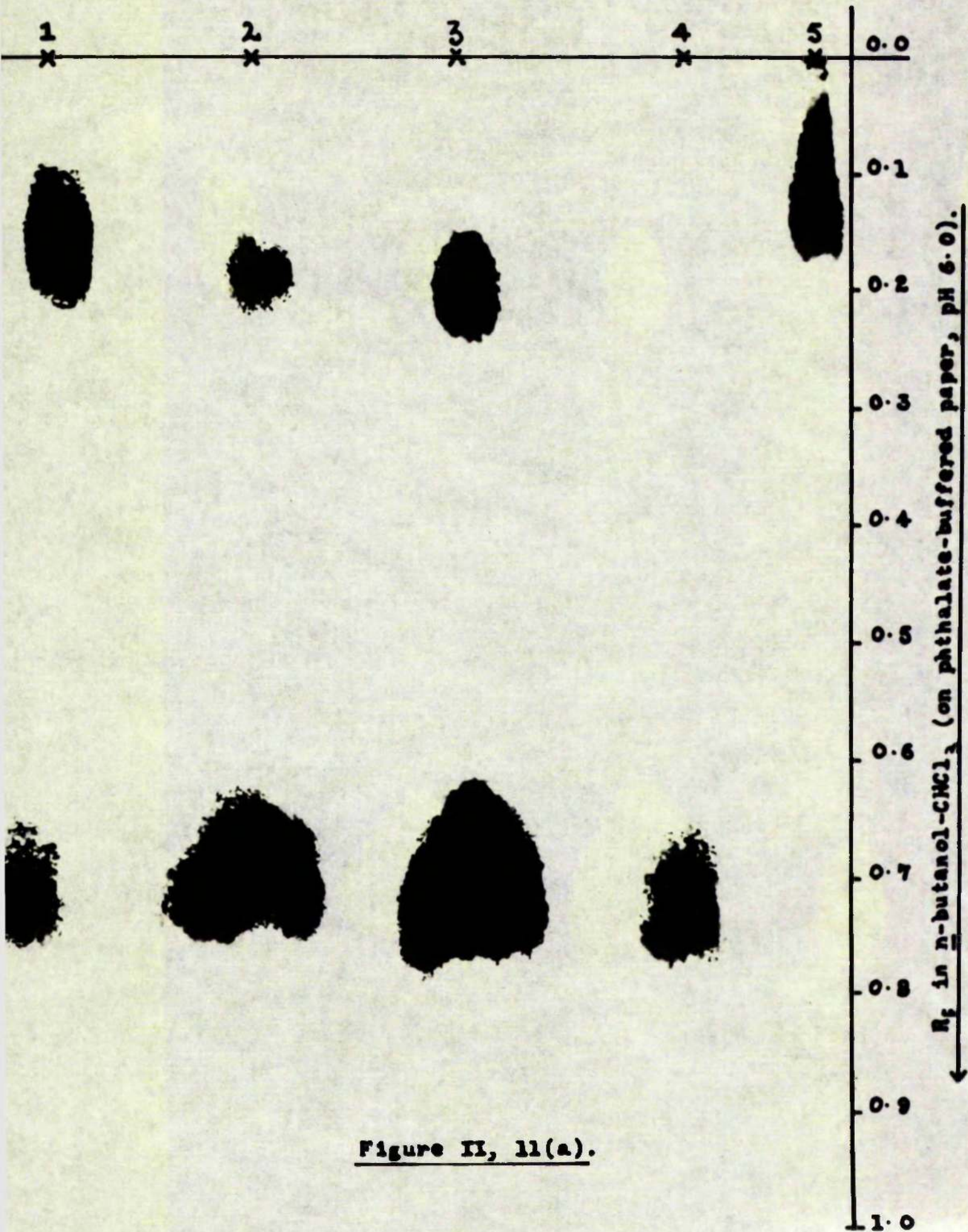Figure II, 11(a).
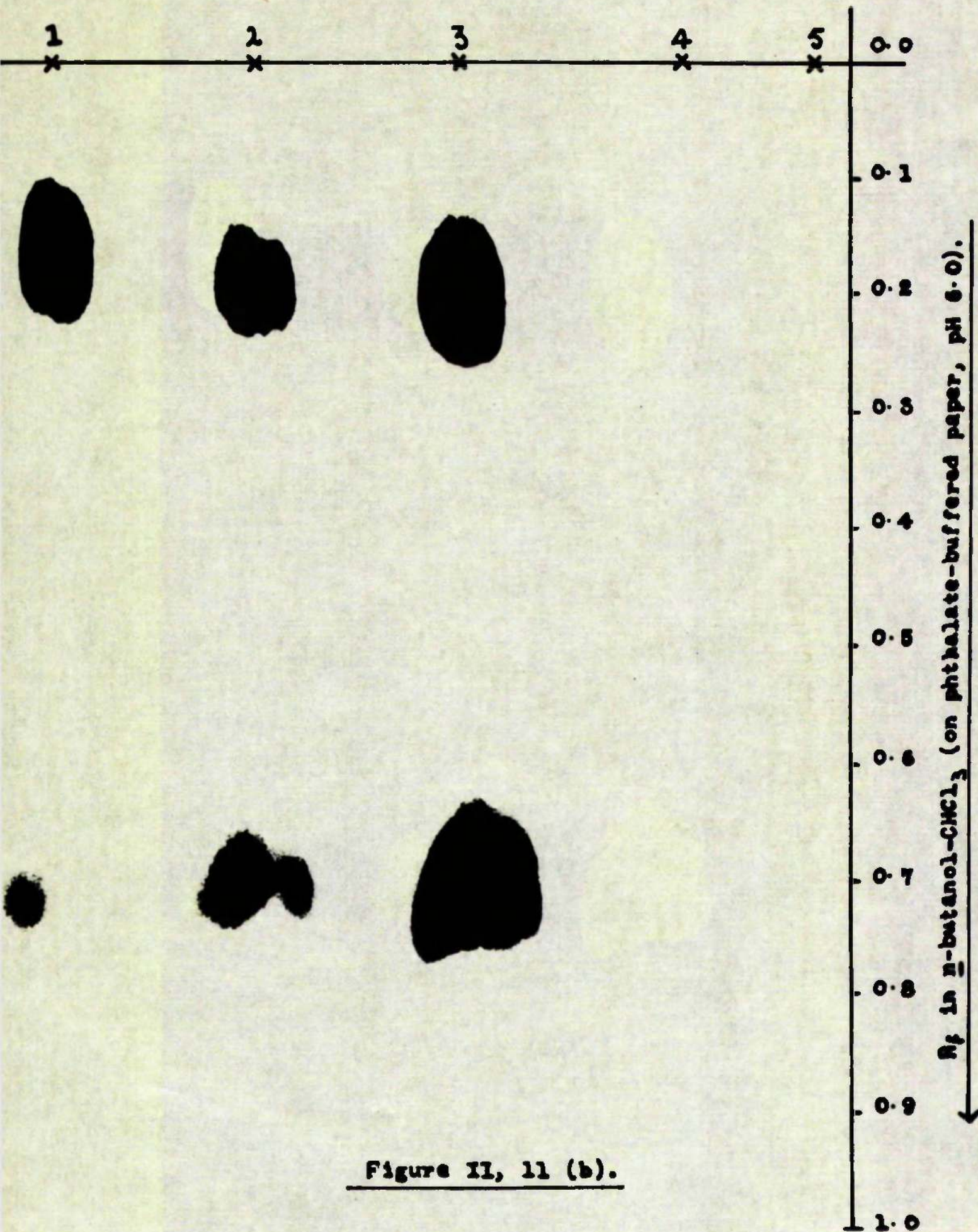
Figure II, 11 (b).

is due entirely to the very low specific activity of
the $2-^{14}C$-glycine used compared to that of the ^{14}C-
formaldehyde used in this latter experiment.

11. The synthesis of N-hydroxymethyltetrahydrofolic
acid by Esch. coli.

FAH_4 has been shown to be an essential co-
factor in a number of reactions involving the transfer
of one-carbon units.   Blakley (1954) has found that
the synthesis of $2-^{14}C$-serine from $2-^{14}C$-glycine and
formaldehyde, using pigeon liver extracts, was dependent
on added FAH_4.   Kisluik and Sakami (1955) have
confirmed this observation.   Woods (1958) also has
reported that FAH_4 is a co-factor required for one-
carbon unit transfer in the synthesis of methionine
by cell-free extracts of Esch. coli PA/15.   In the
study of serine-glycine interconversion (section II,
10) it has been shown that the addition of FAH_4
increases the rate at which $^{14}C_3$-serine disappears from
from incubation mixtures containing cell-free extracts
of Esch. coli PA/15.

The derivative of FAH_4 involved in the transfer
of one-carbon units has been the subject of much work
and discussion.   Kisluik (1957) and Blakley (1958) have
shown that the binding of formaldehyde by FAH_4 is

dependent on the presence of unsubstituted N^5-and N^{10}-positions in FAH_4. These authors have shown that formaldehyde and FAH_4 condense non-enzymically to form N-hydroxymethyltetrahydrofolic acid (N-CH_2OH-FAH_4). Kisluik and Sakami (1954) have postulated that the structure of the formaldehyde-FAH_4 complex is N^5-CH_2OH-FAH_4 and that it is this compound which acts as the intermediate one-carbon unit donor. Blakley (1958), on the other hand, has indicated that the N^5-CH_2OH-FAH_4 formed initially is transformed to a compound containing an N^5, N^{10}-methylene bridge. However, the evidence presented does not rule out the possibility that the derivative involved is an N-CH_2OH-FAH_4 (either N^5 or N^{10}-CH_2OH) which is in equilibrium with N^5, N^{10}-methylene-FAH_4. For the purpose of the present work it can be assumed that N^5-CH_2OH-, N^{10}-CH_2OH and N^5, N^{10}-methylene-FAH_4 are freely interchangeable and the structure of the derivative involved will be written as N-CH_2OH-FAH_4.

Friedkin (1957) has shown that a mixture of formaldehyde and FAH_4 (which are known to condense non-enzymically to form N-CH_2OH-FAH_4) will replace serine and FAH_4 in the synthesis of TMP from dUMP by cell-free extracts of Esch. coli and a number of authors (Jaenicke, 1955 and 1956; Huennekens et al., 1957; Blakley, 1958)

have reported that $N-CH_2OH-FAH_4$ is a product of the action of serine hydroxymethylase on serine. The synthesis of $N-CH_2OH-FAH_4$ from serine and FAH_4 by the serine hydroxymethylase activity present in cell-free extracts of (i) rabbit thymus glands, (ii) Esch. coli PA/15, (iii) Esch. coli 15T- and (iv) Esch. coli 113/3 was investigated by the steam distillation technique described in Section IV, 19 and by paper chromatography.

Table II, 3 (a) shows that incubation of $3-^{14}C$-serine in the presence of a rabbit thymus extract results in the production of volatile ^{14}C-material. Incubation under anaerobic conditions increases the yield of this volatile ^{14}C-material whereas the omission of ATP, DPNH, FAH_4 and $MgSO_4$ from the incubation mixture causes a large decrease in volatile ^{14}C-material. Table II, 3(b) shows that FAH_4 is an essential co-factor for the production of one-carbon units (assayed as volatile ^{14}C-material) from $3-^{14}C$ serine in the presence of a rabbit thymus extract which had been treated with Dowex-1-(chloride) before incubation.   In the same system the presence of Mg^{2+} ions has been shown to increase the yield of one-carbon units.

One-carbon unit production has been shown also to be affected by substituting TPNH (TPN + glucose-6-phosphate in the presence of glucose-6-phosphate dehydro-

Table II, 3 (a)

Steam-volatile ^{14}C-material isolated from incubation mixtures containing 3-^{14}C-serine and rabbit thymus extracts (untreated).

The synthesis of N-CH_2OH-FAH$_4$ from FAH$_4$ and one-carbon units produced by the action of serine hydroxymethylase on 3-^{14}C-serine was studied in systems used to synthesise thymidine. Tubes contained ATP (4 umoles), DPNH (0.3 μmoles), UDR (40 μmoles), 3-^{14}C-serine (8 μmoles, 1μc), FAH$_4$ (5 μmoles), MgSO$_4$ (40 μmoles) and an extract of rabbit thymus gland in Tris buffer (0.1M, pH 7.2), total volume 4 ml. Incubation was carried out under (i) aerobic and (ii) anaerobic conditions at 37^0 C. for 4 hours. 1.0 ml. of the reaction mixture was treated with 10 ml. 5N-H_2SO_4 and steam-distilled. Volatile ^{14}C-material was trapped in 2:4-dinitrophenylhydrazine solution. The solution was extracted with CCl_4 and this extract plated and counted.

Table II, 3 (a)

Omissions	Incubation atmosphere	counts/min./ml. incubation mixture
None	air	40
None	nitrogen	150
None [1]	nitrogen	60
ATP, DPNH, FAH$_4$ and MgSO$_4$	air	20
ATP, DPNH, FAH$_4$ and MgSO$_4$	nitrogen	20

1. The extract used in this case was treated with Dowex-1-Cl" before incubation.

Table II, 3(b)

Steam volatile ^{14}C-material isolated from incubation mixtures containing 3-^{14}C-serine and rabbit thymus extracts (treated).

The synthesis of N-CH$_2$OH-FAH$_4$ from FAH$_4$ and one-carbon units produced by the action of _serine hydroxymethylase_ on 3-^{14}C-serine was studied in systems used to synthesise thymidine. Tubes contained (ATP (4 μmoles), DPNH (0.3 μmoles), UDr (40 μmoles), 3-^{14}C-serine (40 μmoles, 4 μc), FAH$_4$ (5 μmoles), MgSO$_4$ (40 μmoles) and an extract of vex-1-Cl$^-$ prior to incubation, in Tris buffer (0.1M, pH 7.2), total volume 2 ml. Incubation was carried out under anaerobic conditions at 37^0 C. for 4 hours. Steam-volatile ^{14}C-material was isolated from 1.0 ml. incubation mixture as in Table II, 3(a).

Table II, 3(b)

Omissions	Incubation atmosphere	counts/min./ml. incubation mixture.
None	nitrogen	180
$MgSO_4$	nitrogen	110
$MgSO_4$ and FAH_4	nitrogen	10

genase) for DPNH. Table II, 4(a) shows that there was a five-fold increase in the amount of volatile ^{14}C-material produced by the serine hydroxymethylase of Esch. coli PA/15 when TPNH replaced DPNH in the incubation mixture. There was a similar increase (eight-fold) in the amount of volatile ^{14}C-material produced by the serine hydroxymethylase of Esch. coli 113/3 when TPNH replaced DPNH in the incubation mixture (Table II, 4(b)).

Table II, 5 shows that cell-free extracts of Esch. coli 15T- will catalyse the production of one-carbon units from $3-^{14}C$-serine and so confirms the presence of serine hydroxymethylase activity in this mutant. Table II, 5 also shows that the inclusion of dUMP in the incubation mixture made no significant difference to the specific activity (counts/min./μmole) of ^{14}C-formaldehyde 2:4-dinitrophenylhydrazone isolated whereas the inclusion of UDr caused a five-fold increase. It would appear that a labile derivative of UDr, which yields ^{14}C-formaldehyde on hydrolysis, is involved but no such derivative was isolated following extensive paper chromatography.

The production of one-carbon units from ^{14}C-formate in the presence of cell-free extracts of Esch. coli PA/15 was also observed. The specific activity

Steam-volatile ^{14}C-material isolated from
incubation mixtures containing 3-^{14}C-serine and
a cell-free extract of Esch. coli PA/15.

The synthesis of N-CH$_2$OH-FAH$_4$ from FAH$_4$
and one-carbon units produced by the action of
serine hydroxymethylase on 3-^{14}C-serine in the
presence of (i) DPNH and (ii) TPNH was studied
in systems used to synthesise TMP. Tubes
contained ATP (4 μmoles), UDR (40 μmoles,
3-^{14}C-serine (8 μmoles, 4 μc), FAH$_4$ (5 μmoles),
MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.1
μmoles), a cell-free extract of Esch. coli PA/15
and (i) DPNH (0.3 μmoles) or (ii) TPN (0.3 μmoles)
+ glucose-6-phosphate (3 μmoles) in Tris buffer
(0.1M, pH 7.2), total volume 4 ml. Incubation was
carried out under anaerobic conditions at 37° C. for
4 hours. Steam-volatile ^{14}C-material was isolated
from 1.0 ml. incubation mixtures in Table II, 3(a).

Table II, 4(a)

System containing	counts/min/ml. incubation mixture
(i) DPNH	160
(ii) TPN + glucose-6-phosphate	850

Steam-volatile ^{14}C-material isolated from incubation
mixtures containing 3-^{14}C-serine and a cell-free
extract of Esch. coli 113/3.

The synthesis of N-CH$_2$OH-FAH$_4$ from FAH$_4$
and one-carbon units produced by the action of
serine hydroxymethylase on 3-^{14}C-serine in the
presence of (i) DPNH and (ii) TPNH was studied in
systems used to synthesise TMP.   Tubes contained
ATP (60 μmoles), UR (20 μmoles), 3-^{14}C-serine
(20 μmoles, 10 μc), FAH$_4$ (5 μmoles), MgSO$_4$
(40 μmoles), pyridoxal phosphate (0.1 μmoles),
vitamin B$_{12}$ (2 μg.), a cell-free extract of Esch.
coli 113/3 (12 mg. protein) and (i) DPNH (2 μmoles)
or (ii) TPN (2 μmoles) + glucose-6-phosphate
(20 μmoles) in Tris buffer (0.1M, pH 7.2), total
volume 2 ml.   Incubation was carried out under
anaerobic conditions at 37^{0} C. for 4 hours.   Steam-
volatile ^{14}C-material was isolated from 1.0 ml.
incubation mixtures in Table II, 3(a).

Table II, 4(b)

System containing	counts/min./ml. incubation mixture
(i) DPNH	250
(ii) TPN + glucose-6-phosphate	2,000

Steam-volatile ^{14}C-material isolated from incubation mixtures containing (i) 3-^{14}C-serine and a cell-free extract of (a) Esch. coli PA/15 and (b) Esch. coli 15T- and (ii) ^{14}C-formate and a cell-free extract of Esch. coli PA/15.

The synthesis of N-CH_2OH-FAH$_4$ from FAH$_4$ and one-carbon units produced from (i) 3-^{14}C-serine by serine hydroxymethylase activity in cell-free extracts of (a) Esch. coli PA/15 and (b) Esch. coli 15T- and (ii) ^{14}C-formate by cell-free extracts of Esch. coli PA/15 was studied in systems used to synthesis TMP. Tubes contained ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), DPNH (2 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles) and a cell-free extract of (a) Esch. coli PA/15 with (1) 3-^{14}C-serine (30 μmoles, 15 μc); (2) 3-^{14}C-serine (30 μmoles, 15 μc) and UDr (30 μmoles); (3) 3-^{14}C-serine (30 μmoles, 15 μc) and dUMP (30 μmoles); (4) ^{14}C-formate (30 μmoles, 15 μc) and UDr (30 μmoles); or (b) Esch. coli 15T- with 3-^{14}C serine (30 μmoles, 15 μc) and UDr (30 μmoles) in Tris buffer, (0.1M, pH 7.2) total volume 5 ml. Incubation was carried out under anaerobic conditions at 37⁰ C. for 2½ hours or 5 hours. The specific activities (counts/min./μmoles) of the ^{14}C-formaldehyde 2:4-dinitrophenylhydrazone isolated by

steam distillation after the addition of
formaldehyde (3.3 μmoles) as carrier to
1.0 ml. of each incubation mixture were
determined.

Table II. 5

System containing	Incubation time (hrs.)	Esch. coli mutant	Protein mg./ml.	Specific activity of $H^{14}CHO-2:4-$ dinitrophenyl-hydrazones
3-^{14}C-serine	5	PA/15	1.38	9,360
3-^{14}C-serine + UDr	2⅓	PA/15	1.20	42,100
3-^{14}C-serine + dUMP	2⅓	PA/15	1.20	8,650
^{14}C-formate + UDr	5	PA/15	1.20	508
3-^{14}C-serine + UDr	5	15T-	1.65	11,080

of the ^{14}C-formaldehyde 2:4 dinitrophenylhydrazone isolated from this incubation mixture was very low, being about 5% of that from serine hydroxymethylase action on 3-^{14}C-serine.

The data presented show that rabbit thymus extracts and cell-free extracts of Esch. coli PA/15, Esch. coli 15T- and Esch. coli 113/3 all catalyse the production of one-carbon units from 3-^{14}C-serine and thus confirm the presence of serine hydroxymethylase activity in these extracts. The action of this enzyme has been shown to be dependent on FAH_4 and its activity to be increased by incubation under anaerobic conditions and by the addition of Mg^{2+} ions.

The rôle of FAH_4 in these systems was investigated by paper chromatographic separations of incubation mixtures containing serine hydroxymethylase, 3-^{14}C-serine and FAH_4. Paper chromatography of incubation mixtures in phosphate buffer (0.1M, pH 6.9) and in iso-amyl alcohol -5% KH_2PO_4 followed by auto-radiography merely indicated the presence of a labile ^{14}C-compound which was degraded during development of the chromatogram. Better results were obtained when the chromatograms were developed in the cold room (4° C.). Autoradiography revealed ^{14}C-material coincident with spots which fluoresced in ultra-violet light (Fig. II,12).

The synthesis of N-hydroxymethyl-5:6:7:8-tetrahydrofolic acid from 5:6:7:8-tetrahydrofolic acid and $3-^{14}C$-serine

Autoradiograph of paper chromatographic separation of reaction mixtures containing FAH_4, $3-^{14}C$-serine, pyridoxal phosphate, a cell-free extract of Esch. coli PA/15 and DPNH (1) or TPNH (2), together with markers consisting of mixtures of FAH_4 and $3-^{14}C$-serine (3) and FAH_4 and ^{14}C-formaldehyde (4). The dotted lines enclose areas corresponding to those which, on the original chromatogram, were fluorescent in ultra-violet light. The chromatography was carried out in the cold room ($4^{o}C$.).

Complete incubation mixture - The synthesis of $N-CH_2OH-FAH_4$ was studied in systems used to synthesis TMP, that is ATP (60 μmoles), FAH_4 (3 μmoles), $MgSO_4$ (40 μmoles), pyridoxal phosphate (0.2 μmoles), $3-^{14}C$-serine (40 μmoles, 20 μc), UDr (40 μmoles) and (i) DPNH (4 μmoles) or (ii) TPNH (4 μmoles) in Tris buffer (0.1M, pH 7.2) with a cell-free extract of Esch. coli PA/15 (9 mg. protein). The total volume of the incubation mixture was 4 ml. Incubation was carried out under anaerobic conditions at 37° C. for 4 hours.

Figure II, 12.
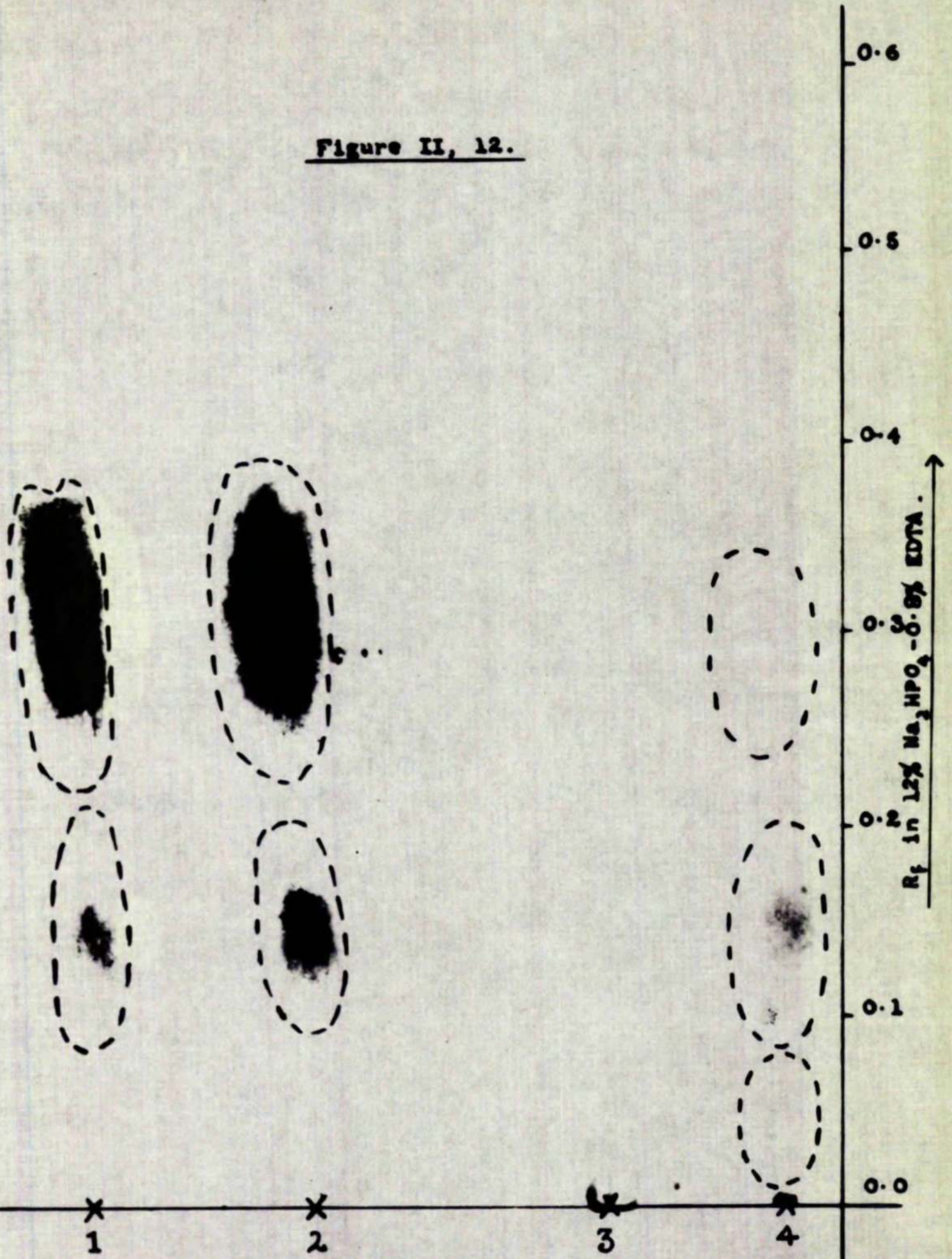
A mixture of FAH_4 and ^{14}C-formaldehyde, which condense non-enzymically to yield $N-CH_2OH-FAH_4$, run in parallel gives a radioactive fluorescent spot with the same R_F value (0.14) as the first of the two spots obtained from the incubation mixtures containing $3-^{14}C$-serine, FAH_4 and serine hydroxymethylase. The second of the two spots (R_F 0.30) shown in Fig. II, 12 did not give a positive reaction to ninhydrin and accordingly does not appear to be the FAH_4-serine complex reported by Jaenicke (1956) to be present in similar systems.

Miller and Waelsh (1957) have reported that N^5-formyl-FAH_4, on treatment with perchloric acid, will cyclise to form the N^5, N^{10}-imidazolium derivative (ACF) with a characteristic change in spectrum. Accordingly, a mixture of FAH_4 and $3-^{14}C$-serine incubated in Tris buffer with a cell-free extract of Esch. coli PA/15 was acidified with perchloric acid and examined spectrophotometrically. No significant peak developed at 350 mμ. Chromatography in 0.5 N-formic acid also failed to reveal the presence of ^{14}C-ACF. Thus no detectable amounts of N^5-formyl-FAH_4 was synthesised from FAH_4 and $3-^{14}C$-serine in the presence of serine hydroxymethylase.

These results indicate that the action of serine hydroxymethylase on $3-^{14}C$-serine in the presence

of FAH_4 is to synthesise $N-^{14}CH_2OH-FAH_4$ which acts as the intermediate one-carbon unit carrier in reactions involving the transfer of one-carbon units.   The synthesis of corresponding one-carbon units from formate by cell-free extracts of Esch. coli PA/15 proceeds, presumably, by the slow reduction of (i) formate to formaldehyde or (ii) N-formyl-FAH_4 to $N-CH_2OH-FAH_4$.   The effect of substituting TPNH for DPNH on the synthesis of $N-^{14}CH_2OH-FAH_4$ from $3-^{14}C$-serine is marked.   The synthesis of $N-CH_2OH-FAH_4$ from FAH_4 and serine is not TPN- or DPN-linked, but the oxidation of $N-CH_2OH-FAH_4$ to N^5-formyl-FAH_4 is TPN-dependent (Hatefi et al., 1957)

$$N-CH_2OH-FAH_4 \rightleftharpoons N^5-formyl-FAH_4 \rightleftharpoons formate \longrightarrow CO_2$$

TPN   TPNH
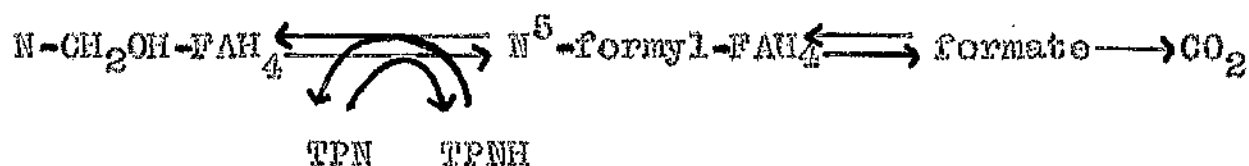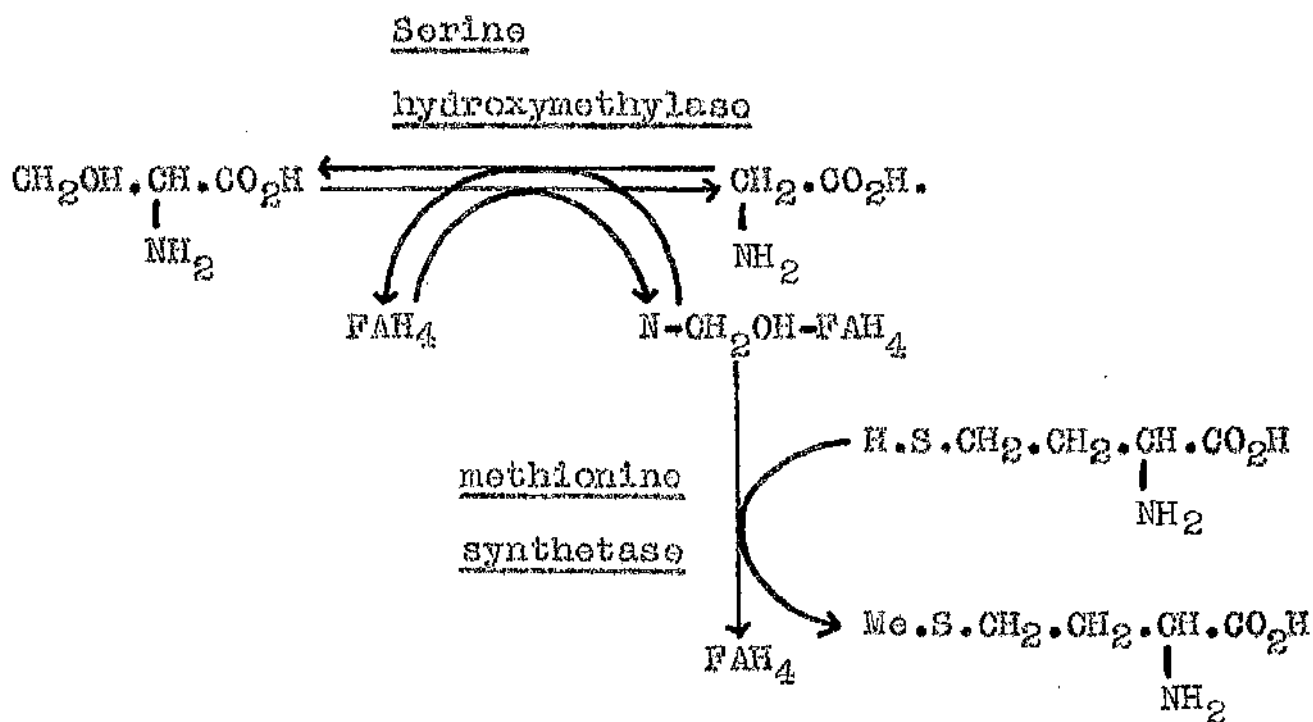
Thus, in the presence of TPN the equilibrium will lie more to the side of N^5-formyl-FAH_4 resulting in a reduction in the concentration of $N-CH_2OH-FAH_4$.   In the incubation mixtures under discussion there is a regenerative system for the synthesis of TPNH in operation so that the oxidation of $N-CH_2OH-FAH_4$ to N^5-formyl-FAH_4 and hence to CO_2 proceeds at a very slow rate.

12.   Synthesis of methionine by Esch. coli PA/15.

         Gibson and Woods (1952) have demonstrated the
synthesis of methionine from homocysteine by washed
whole-cell suspensions of various mutants of Esch. coli.
When a mutant, which required serine or glycine for
growth, was used in these studies, the authors showed
that either serine or glycine was essential for methionine
synthesis.   The addition of vitamin B_{12} was found to
stimulate synthesis.   Cross and Woods (1954) have also
demonstrated this synthesis with cell-free extracts of a
serine- or glycine-less mutant of Esch. coli and Guest
(1959) has shown a vitamin B_{12} requirement in this
system.

         Methionine synthesis from homocysteine and one-
carbon units, produced by serine hydroxymethylase activity
on serine, involves the transfer of a one-carbon unit
from serine to the sulphhydryl group of homocysteine
followed by the reduction of this one-carbon unit to form
the methyl group of methionine.   The mechanism of this
reaction may be represented by the following scheme:-

Serine

hydroxymethylase

$$CH_2OH.CH.CO_2H \rightleftharpoons CH_2.CO_2H.$$
$$| \qquad\qquad\qquad\qquad |$$
$$NH_2 \qquad\qquad\qquad\qquad NH_2$$

$$FAH_4 \qquad N\text{-}CH_2OH\text{-}FAH_4$$

$$H.S.CH_2.CH_2.CH.CO_2H$$
$$|$$
$$NH_2$$

methionine

synthetase

$$Me.S.CH_2.CH_2.CH.CO_2H$$
$$|$$
$$NH_2$$

$$FAH_4$$

This mechanism is analagous to that postulated for the "methylation" of UDr (or dUMP). Thus there is a possibility that replacement of homocysteine by UDr in systems containing methionine synthetase activity will lead to the synthesis of thymidine. Accordingly, washed whole-cell suspensions and cell-free extracts of Esch. coli PA/15 were investigated for methionine synthetase activity.

The presence of methionine synthetase activity in (a) washed whole-cell suspensions and (b) cell-free extracts of Esch. coli PA/15 was confirmed by incubating (i) washed whole-cell suspensions of Esch. coli PA/15 with glucose, vitamin B_{12}, serine and homocysteine in phosphate buffer and (ii) cell-free extracts of Esch. coli PA/15 with ATP, fructose-1:6-diphosphate, $MgSO_4$,

pyridoxal phosphate, serine and homocysteine in phosphate buffer. The synthesis of methionine was followed by microbiological assay of the reaction supernatants, after removal of cells or protein, using Esch. coli CW194, a methionine-less mutant, not capable of growth on homocysteine, as assay organism.

The results summarised in Tables II, 6(a) and II, 6(b) show respectively that washed whole-cell suspensions and cell-free extracts of Esch. coli PA/15 effect the synthesis of methionine under the described conditions. The presence of homocysteine was essential for methionine synthesis in both whole-cell and cell-free extract systems but in neither case was the addition of serine found to be necessary for synthesis to take place. However, the rôle of serine as a one-carbon unit donor is indicated by the decrease in methionine synthesised when serine was omitted from the incubation mixture. The addition of formaldehyde, on the other hand, partially inhibits the reaction in cell-free extracts in the presence of serine.

13. Syntheses of 5-hydroxymethyl derivatives of uracil, deoxyuridine, uridylic acid and deoxyuridylic acid.

Fink, Cline and Fink (1956) have described the synthesis of 5-hydroxymethyl derivatives of uracil, UDr and uridylic acid (UMP) by condensing formaldehyde

Table II, 6(a)

Synthesis of methionine by washed whole-cell suspension of Esch. coli PA/15.

The complete reaction mixture consisted of a washed whole-cell suspension of Esch. coli PA/15 (6 mg. dry wt.), glucose (100 μmoles), vitamin B_{12} (0.05 μg), serine (100 μmoles) and homocysteine (100 μmoles) in phosphate buffer (0.1M, pH 6.9), total volume 5ml. Incubation was carried out under aerobic conditions at 37° C. for 7 hours. At the end of the incubation period reaction mixtures were centrifuged (20,000 g) and the supernatants assayed for methionine using Esch. coli CW 194, a methionine-less mutant, as assay organism.

Table II 6(a)

Omissions	Methionine synthesised µmoles/hr./mg. cells (dry wt.)
None	0.26
Serine	0.14
Homocysteine	0.0
Serine and homocysteine	0.0

Table II. 6(b)

Synthesis of methionine by cell-free extracts of
Esch. coli PA/15.

The complete reaction mixture consisted
of a cell-free extract of Esch. coli PA/15(5 mg.
protein), ATP (120 μmoles), fructose-1:6-diphosphate
(180 μmoles), $MgSO_4$ (20 μmoles), pyridoxal phosphate
(0.4 μmoles), serine (100 μmoles), homocysteine
(100 μmoles) and formaldehyde (3.5 μmoles) in phosphate
buffer (0.1M, pH 6.9), total volume 5 ml. Incubation
was carried out under aerobic conditions at 37° C. for
7 hours. At the end of the incubation period
reaction mixtures were deproteinised by heating at
100° C. for 30 minutes and centrifuged. The reaction
supernatants were assayed for methionine using Esch.
coli CW194, a methionine-less mutant, as assay organism.

Table II, 6(b)

Omissions	Methionine synthesised μmoles/hr./mg. protein
None	0.56
HCHO	1.07
HCHO and serine	0.83
HCHO and homocysteine	0.0
HCHO, homocysteine and serine	0.0

and the pyrimidine at 100°C in the presence of 0.08N-
HCl.   As the 5-hydroxymethyl derivatives of UDr and
dUMP were of interest, because of their possible
involvement as intermediates on the thymidine/TMP
biosynthetic pathway, attempts were made to synthesise
5-hydroxymethyldeoxyuridine and 5-hydroxymethyl-
deoxyuridylic acid.   The conditions used for condensation
were those described by Fink, Cline and Fink (1956)
(Section IV, 13).   In addition, a variety of other
condensation conditions were investigated.

As a preliminary step the synthesis of
5-hydroxymethyluracil was investigated.   Uracil,
formaldehyde and HCl were heated in a sealed tube at
100° C. for 24 hours and the reaction products were
separated by paper chromatography.   Fig. II, 13 shows
that uracil and formaldehyde condense readily under
these conditions, yielding three products separable by
one-dimensional chromatography (solvent, n-butanol-
water).   One of these products migrated at the same
rate as did authentic 5-hydroxymethyluracil (R_F 0.28)
and, in addition, the ultra-violet absorption spectra
of this material at pH 1 and pH 13 corresponded exactly
with those of authentic 5-hydroxymethyluracil.   The
structures of the other materials shown in Fig. II, 13
were not elucidated.

Figure II, 13

Synthesis of 5-hydroxymethyluracil by the condensation of formaldehyde and uracil.

Ultraviolet photograph of paper chromatographic separation of the products of uracil and formaldehyde condensation (1) together with markers of uracil (2) and uracil and 5-hydroxymethyluracil (3).

Condensation system - 60 μmoles uracil + 1.3 mmoles formaldehyde in 0.1 ml. 0.08 N-HCl. The mixture was sealed in a capillary tube and heated at 100° C. for 24 hours.

Figure II, 13.
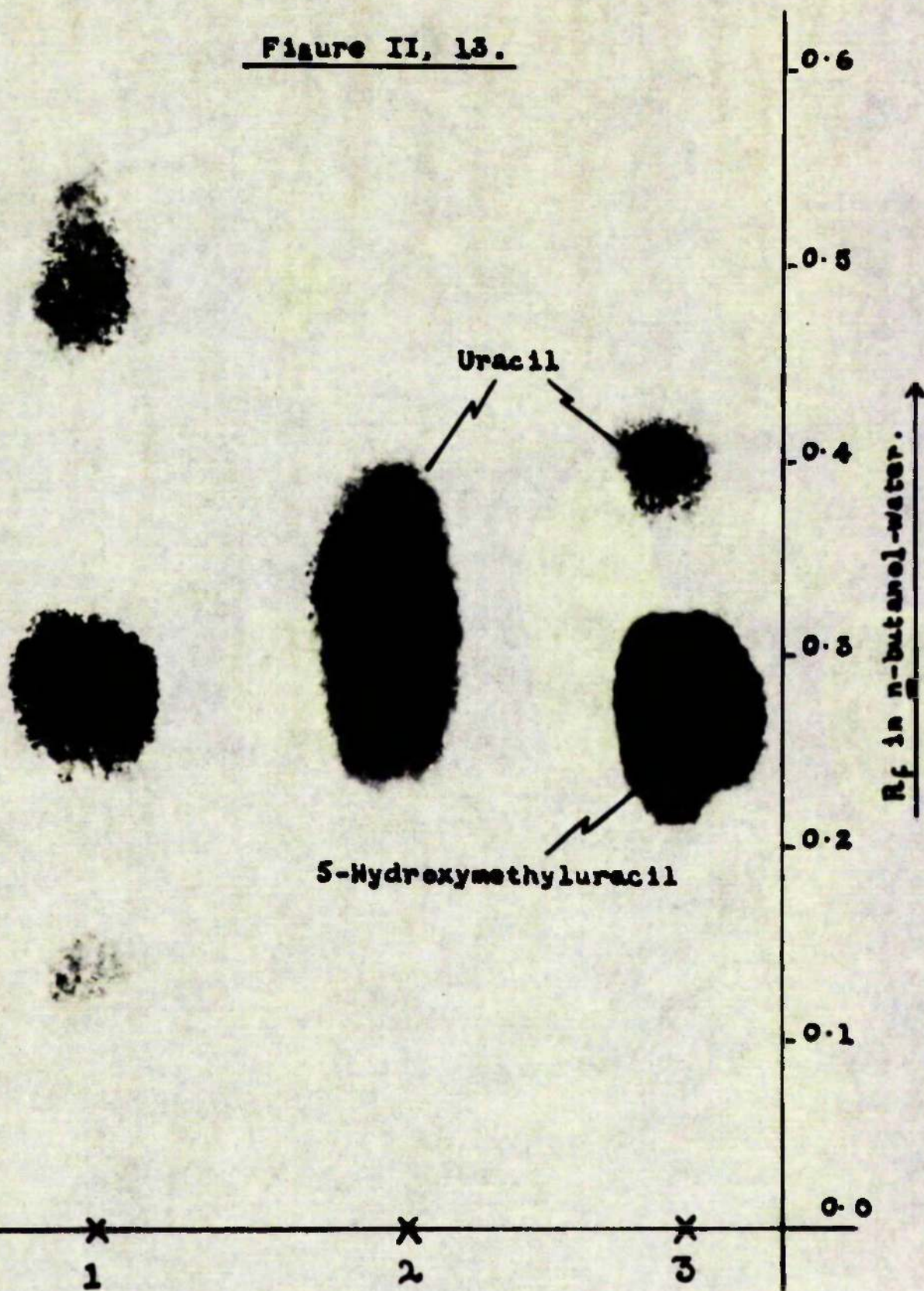
When UDr was substituted for uracil in this system the main condensation product was again 5-hydroxymethyluracil (Fig. II, 14). A trace of UDr (R_F 0.40), a fast-moving material (R_F 0.83) which is probably a polymer and a faint spot of material (R_F 0.12) are also shown by Fig. II, 14. Larger quantities of the material of R_F 0.12 were isolated by chromatography of the products of this condensation as bands in n-butanol-water. The band of R_F 0.05 to 0.15 was eluted with water and rechromatographed in two dimensions (n-butanol-water and acetone-water-formic acid). The material migrated as a single spot of R_F values 0.12 and 0.77, in n-butanol-water and acetone-water-formic acid, respectively. Authentic 5-hydroxy-methyldeoxyuridine, prepared by deamination of 5-hydroxy-methyldeoxycytidine from phage deoxyribonucleic acid (DNA), has an R_F value of 0.11 to 0.21 in n-butanol-water (Cohen, 1958).

The structure of the material of R_F 0.12 which was isolated from the products of condensation of UD_r and formaldehyde, and which was suspected to be 5-hydroxymethyldeoxyuridine, was investigated further by a study of the ultraviolet-absorption spectra at pH 1 and pH 13. Comparison of the ultraviolet-absorption spectra of uracil and 5-hydroxymethyluracil, at pH 1 and pH 13, shows that the effect of the hyperconjugation of a

Figure II, 14

Condensation of (i) deoxyuridine and formaldehyde at
100° C.; (ii) deoxyuridine and formaldehyde at room
temperature; (iii) uridylic acid and formaldehyde at
100° C.

Ultraviolet photograph of paper chromatographic
separation of the products of (i) UDr and formaldehyde
condensation at 100° C (1); (ii) UDr and formaldehyde
condensation at room temperature (3); and (iii) UMP
and formaldehyde condensation at 100° C (4) together
with a marker of UDr (2).

Condensation system - (a) 60 μmoles UDr or (b) 60 μmoles
UMP + 1.3 mmoles formaldehyde in 0.1 ml 0.08 N-HCl.
For condensations at 100° C. the mixtures were sealed in
capillary tubes and heated for 24 hours.   For
condensation at room temperature, the mixture was kept
in a sealed tube for 72 hours.

Figure II, 14.

R$_f$ in n-butanol-water.

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0.0

1   2   3   4
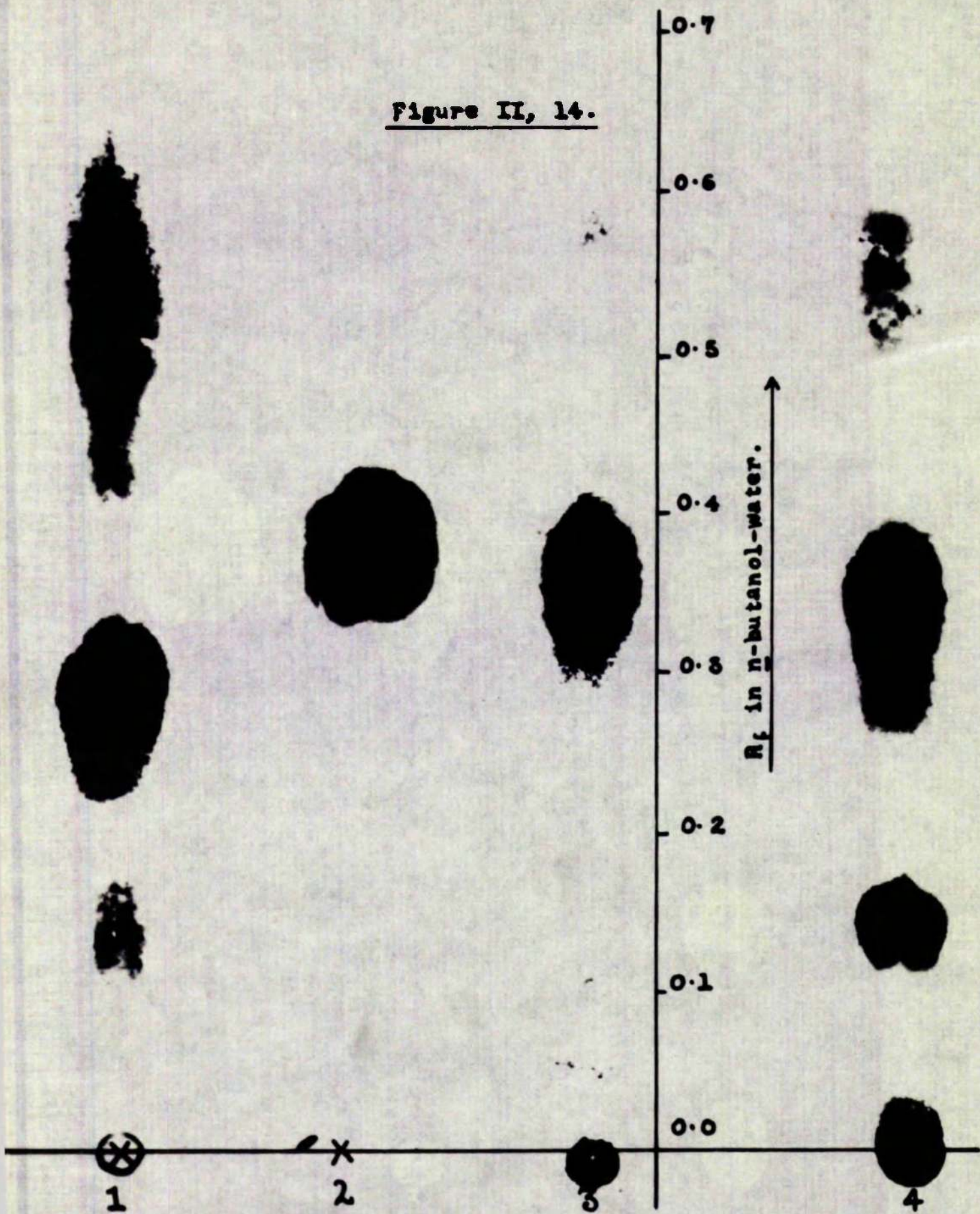
hydroxymethyl group on position 5 of the pyrimidine ring is to shift the wavelengths of the maxima and minima towards the visible range by 2 to 3 mμ. The same shift was observed when the ultraviolet absorption spectra of UDr and the material suspected to be 5-hydroxymethyldeoxyuridine were compared (Fig. II, 15).

Final confirmation that this material synthesised from UDr and formaldehyde was 5-hydroxymethyl-deoxyuridine was obtained by acid hydrolysis of the material. Paper chromatographic separation of the hydrolysis products showed that all but a trace of the material had been degraded to one product which migrated at the same rate as did 5-hydroxymethyluracil (Fig. II, 16). Further identification of this product from 5-hydroxymethyldeoxyuridine hydrolysis was obtained from the ultra-violet absorption spectra, which agreed exactly with those of authentic 5-hydroxymethyluracil, at pH1 and pH13. On the basis of this evidence it was established that UDr and formaldehyde in the presence of HCl had condensed to give a small yield (1% or less) of 5-hydroxymethyldeoxyuridine.

Although 5-hydroxymethyldeoxyuridine is readily degraded to 5-hydroxymethyluracil by treatment with 0.1 N-HCl at 100° C. for 24 hours, it was found that UDr yielded

Figure II, 15

Ultraviolet absorption spectra of uracil, deoxyuridine and the corresponding 5-hydroxymethyl derivatives in acid and alkali.

Ultraviolet absorption spectra of

(a) UDr (I) and 5-hydroxymethyldeoxyuridine (II) at pH 1;

(b) UDr (I) and 5-hydroxymethyldeoxyuridine (II) at pH 13;

(c) uracil (III) and 5-hydroxymethyluracil (IV) at pH 1;

(d) uracil (III) and 5-hydroxymethyluracil (IV) at pH 13.
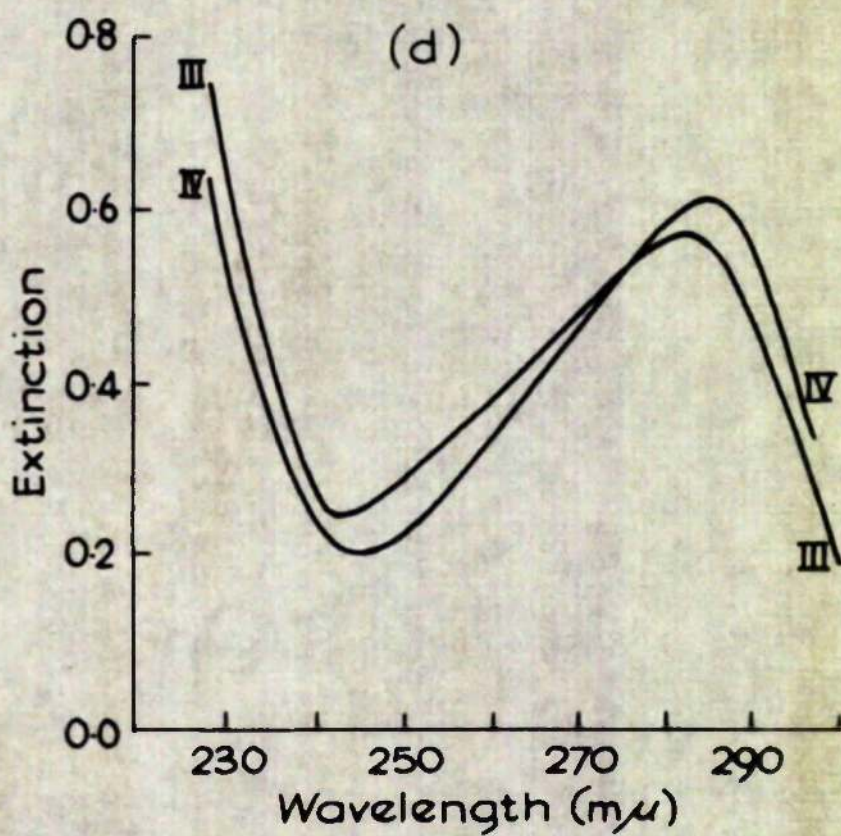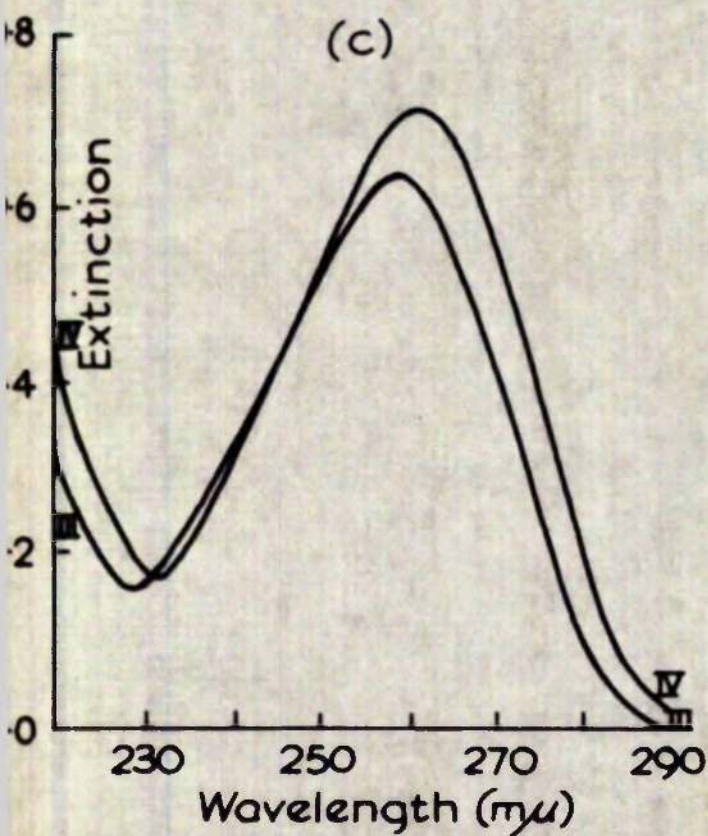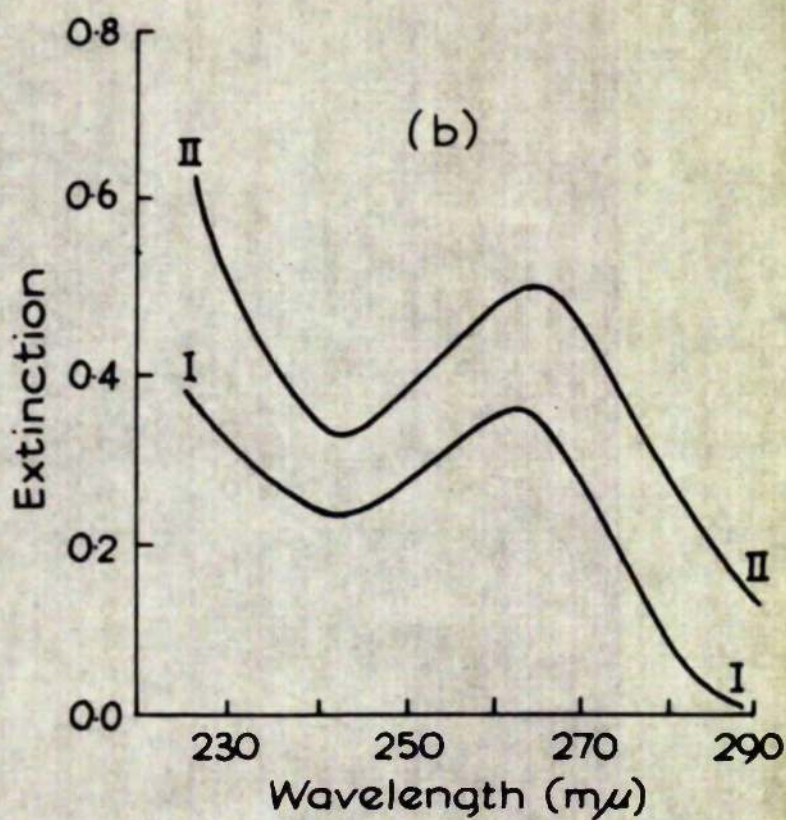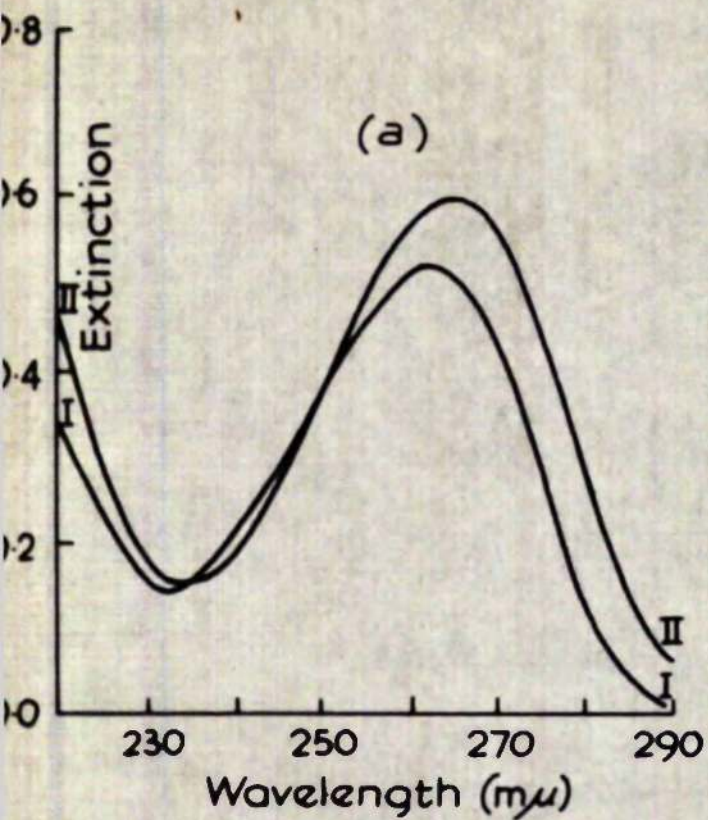
Figure II. 15

Degradation of 5-hydroxymethyldeoxyuridine by HCl

Ultraviolet photograph of paper chromatographic separation of 5-hydroxymethyldeoxyuridine hydrolysis products (1) together with marker 5-hydroxymethyluracil(2).

Hydrolysis system - 5-hydroxymethyldeoxyuridine (about 0.1 μmole) + 0.1 ml. 0.1N-HCl. The mixture was sealed in a capillary tube and heated at 100° C. for 24 hours.

Figure II, 16.

only a trace of uracil when treated under the same
conditions. Since the major product of UDr and
formaldehyde condensation is 5-hydroxymethyluracil,
the condensation must precede the hydrolysis, that is
Scheme 1:
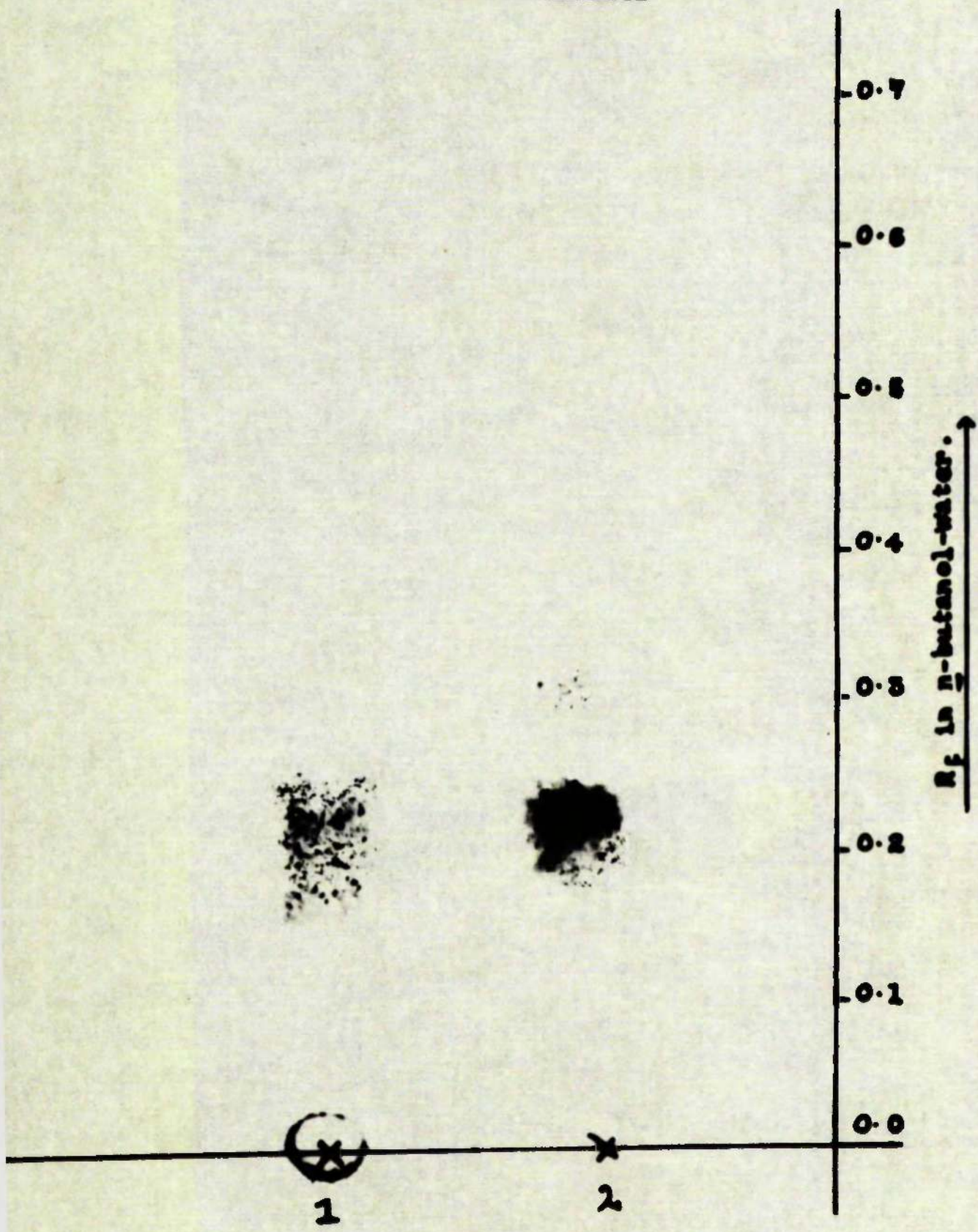
$$UDr + HCHO \xrightarrow{HCl} 5\text{-}CH_2OH\text{-}UDr \xrightarrow{HCl} 5\text{-}CH_2OH\text{-}uracil$$

Scheme 1

will represent the sequence of reactions taking place
and not Scheme 2:

$$UDr \xrightarrow{HCl} uracil \xrightarrow{HCHO/HCl} 5\text{-}CH_2OH\text{-}uracil$$
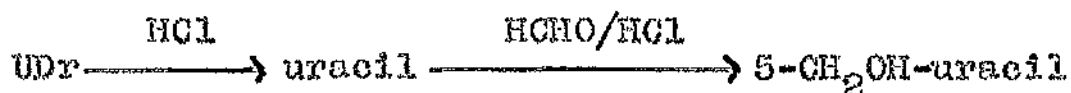
Scheme 2

It follows, therefore, that the hydroxymethyl group on
position 5 of the pyrimidine ring renders the N^3-
glycosidic bond labile to mild acid hydrolysis. Thus
5-hydroxymethyldeoxyuridine was hydrolysed almost as
rapidly as it was formed and the end-product was largely
5-hydroxymethyluracil. Accordingly, attempts were made
to find a set of conditions under which (a) condensation
of UDr and formaldehyde takes place and (b) the N^3-
glycosidic bond is relatively stable. Such a set of
conditions would lead to the synthesis of 5-hydroxymethyl-

deoxyuridine in reasonable yield.

Both (i) a decrease in the concentration of HCl to 0.008 N, heating for 24 hours at 100° C. and (ii) an increase in the concentration of HCl to 0.2 N, heating for 8 hours at 100° C. resulted in the isolation of 5-hydroxymethyluracil and UDr only.   Similar results were obtained when UDr and formaldehyde, in the presence of 0.08 N-HCl, were heated (i) at 80° C. for 24 hours and (ii) at 100° C. for 8 hours.   Thus it appears that the presence of HCl in the reaction mixture is undesirable.   However, acid conditions are essential for condensation to take place, no condensation products being detected when the HCl was omitted.   When alkali (0.08N- or 0.008N-NaOH) was substituted for HCl, heating at 100° C. for 24 hours, the UDr was hydrolysed to uracil and no 5-hydroxymethyl derivatives were isolated. Condensation took place only at elevated temperatures, no reaction being found to have taken place when a mixture of UDr, formaldehyde and HCl was kept at room temperature in a sealed tube for 72 hours.

UDr and a trace of 5-hydroxymethyluracil were isolated when UDr (60 μmoles), hexamethylenetetramine (140 μmoles) and HCl (80 μmoles) were refluxed in dioxane-water (9:1 v/v.) for 6 hours.   Only unchanged UDr was isolated when the same mixture was heated in a

sealed tube at 100° C. for 24 hours. When the
concentration of HCl in this mixture was increased by
a factor of 2.5 complete hydrolysis of the UDr to
uracil took place.

The condensation of uridylic acid (UMP) and
formaldehyde was studied as a preliminary to attempting
the synthesis of 5-hydroxymethyldeoxyuridylic acid.
UMP, formaldehyde and HCl were heated in a sealed tube
at 100° C. for 24 hours and the reaction products were
separated by paper chromatography (in n-butanol-water).
Fig. II, 14 shows that these products included uracil,
a trace of 5-hydroxymethyluracil, UR and nucleotide
material (R_F values 0.37, 0.28, 0.15 and 0.0, respect-
ively). Larger quantities of the nucleotide material
were isolated by chromatography of the products of this
condensation as bands in n-butanol-water. The bands
of R_F 0.0 to 0.05 were eluted with water and
rechromatographed in acetone-water-formic acid. The
nucleotide material migrated as one discrete spot of
UMP together with a trace of ultraviolet absorbing
material of R_F 0.79. Insufficient of this material
(R_F 0.79) to allow investigation of its structure was
obtained. When the condensation was repeated with
dUMP under the same conditions the only products
isolated were uracil, 5-hydroxymethyluracil, UDr and

a trace of dUMP.   It was found that, when dUMP was heated with 0.08N-HCl in a sealed tube at 100° C. for 24 hours, dUMP was hydrolysed almost completely to UDr.   Thus the reactions taking place when dUMP was heated with formaldehyde and HCl are as follows:

$$dUMP \xrightarrow{HCl} UDr \xrightarrow[HCl]{HCHO/} 5\text{-}CH_2OH\text{-}UDr \xrightarrow{HCl} 5\text{-}CH_2OH\text{-}uracil$$
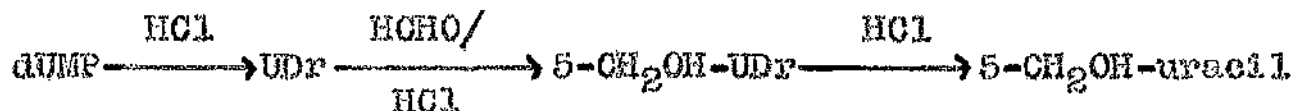
Since the chemical synthesis of 5-hydroxymethyl-deoxyuridine from UDr and formaldehyde, although successful, had given low yields, the possibility of enzymic coupling of 5-hydroxymethyluracil and deoxyribose was investigated.   The hydrolysis of nucleosides by nucleoside phosphorylase activity (Section II, 3) is, in theory, a reversible reaction.   Accordingly, a mixture of a nucleoside and a free pyrimidine base in the presence of nucleoside phosphorylase and inorganic phosphate will attain an "equilibrium" consisting of a mixture of the two nucleosides and the two free bases:

<center>nucleoside</center>

<center>phosphorylase</center>

$$B_1 - R \rightleftharpoons B_1$$
$$B_2 \qquad B_2 - R$$
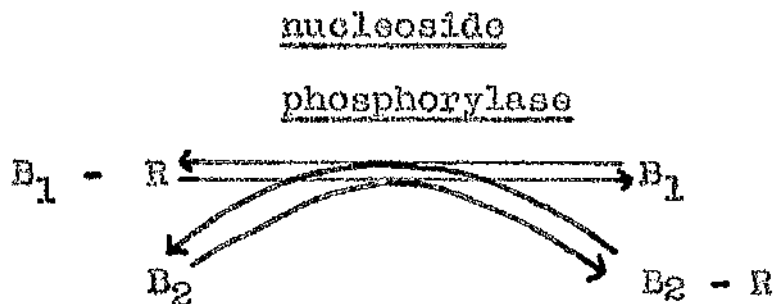
B_1 and B_2 = pyrimidine bases;   R = deoxyribose

The net result is transfer of deoxyribose from one

nucleoside to the other. This "equilibrium" holds for short time intervals only as deoxyribose-1-phosphate (the intermediate form of the deoxyribose) is removed rapidly from the reaction by the action of phosphoribomutase, yielding deoxyribose-5-phosphate which undergoes further metabolism.

The synthesis of UDr from uracil under these conditions was investigated initially as a model system. Uracil and thymidine were incubated at 37° C. for 1 hour with a cell-free extract of Esch. coli PA/15 (containing nucleoside phosphorylase activity) in the presence of inorganic phosphate and the products of the reaction were separated by paper chromatography. Fig. II, 17 shows that an ultraviolet absorbing material with the chromatographic properties of UDr was isolated from the reaction mixture. The ultraviolet absorption spectrum of this material at pH15 coincided with that of authentic UDr. Thus the synthesis of UDr has been effected by transfer of deoxyribose from thymidine to uracil in the presence of nucleoside phosphorylase. However, when 5-hydroxymethyluracil (0.5 μmoles) replaced uracil in the same system, no trace of 5-hydroxymethyldeoxyuridine was isolated from the reaction mixture.

Thus it has been possible to synthesis 5-hydroxymethyldeoxyuridine in very small yield using

Figure II, 17.

Synthesis of deoxyuridine from uracil by nucleoside phosphorylase of Esch. coli PA/15.

Ultraviolet photograph of paper chromatographic separation of thymine, thymidine and UDr mixture (1) from the UDr synthetic system together with marker thymine (2) and UDr (3).

UDr synthetic system - 0.5 μmoles uracil + 0.5 μmoles thymidine + 20 μl. phosphate buffer (0.1M, pH 7.2) + 0.1ml. cell-free extract of Esch. coli PA/15 (0.1 mg. protein) incubated on paper for 1 hour at 37° C. Initial separation was by chromatography with iso-propanol-water-HCl; the area of paper containing thymine, thymidine and UDr was eluted and rechromatographed on paper with n-butanol-water.

Controls - 20 μl. phosphate buffer (0.1M, pH 7.2) + (i) 0.5 μmoles uracil; (ii) 0.5 μmoles UDr; (iii) 0.1 ml. cell-free extract (0.1 mg. protein); and (iv) 0.5 μmoles UDr + 0.1 ml. cell-free extract (0.1 mg. protein).

Figure II, 17.
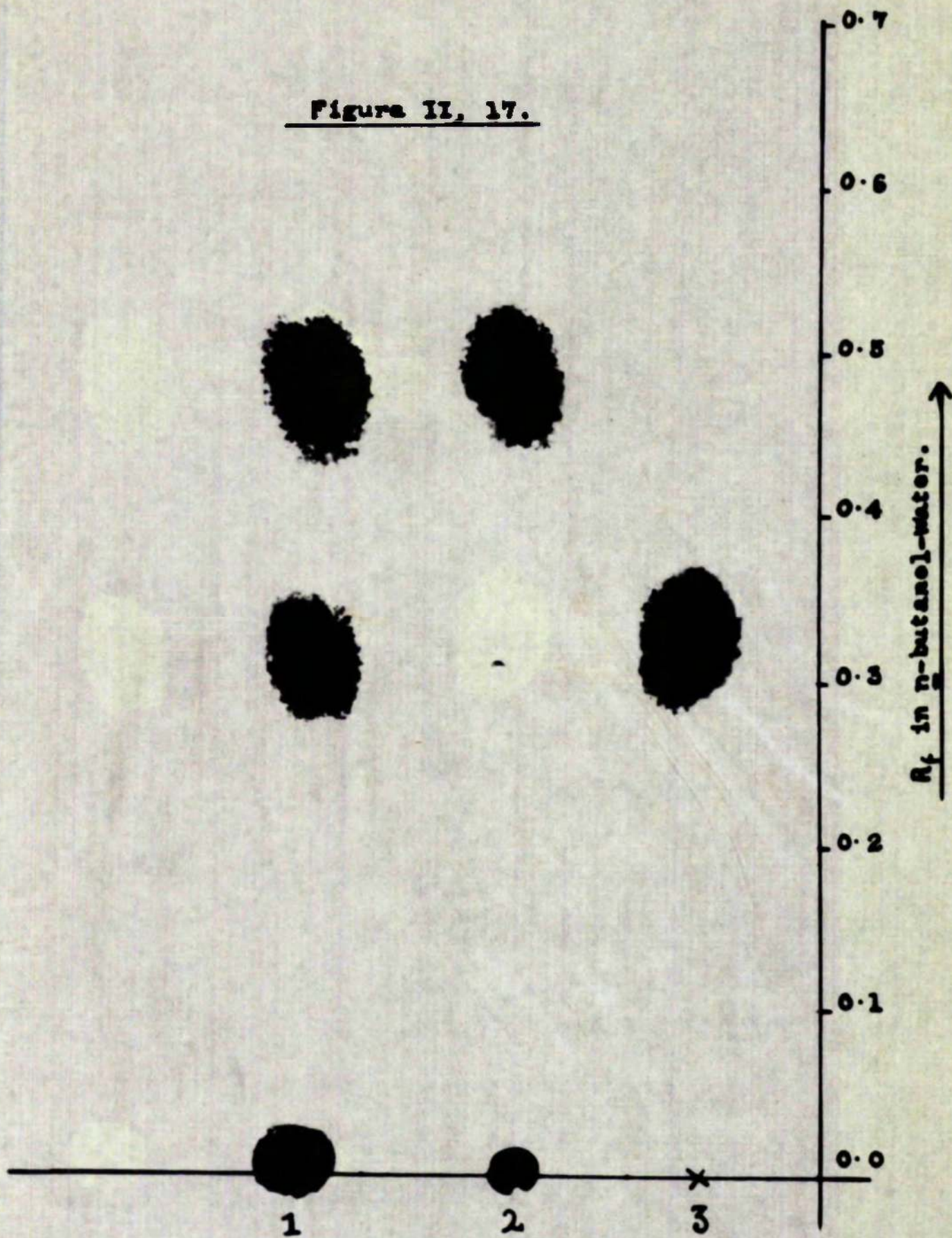
the method of Fink, Cline and Fink (1956).   No
attempted variations in the condensation conditions
resulted in an increased yield of 5-hydroxymethyl-
deoxyuridine, not were attempts to synthesis the
corresponding nucleotide successful.   Enzymic
synthesis of 5-hydroxymethyldeoxyuridine was
similarly unsuccessful.

14.   Degradation of 5-hydroxymethyluracil.

Although it has been reported that
5-hydroxymethyluracil is very labile, readily being
degraded to uracil and formaldehyde by a trace of
acid (Bendich, 1955), attempts to assay 5-hydroxymethyl-
uracil by the strongly acidic chromotropic acid reagent
were unsuccessful.   When 5-hydroxymethyluracil was
treated with H_2SO_4(5N- or 10N-)and steam-distilled, no
trace of formaldehyde was detected in the distillate.
Hydrolysis with 5N-NaOH at 100° C. was necessary before
any detectable amount of formaldehyde was liberated from
5-hydroxymethyluracil.   Fig. II, 18 shows that, after
20 minutes hydrolysis, only 7% of the 5-hydroxymethyluracil
has been degraded.   At longer hydrolysis times some
formaldehyde liberated by this reaction has volatilised.
Although this introduces an error into the estimation of
the degree of 5-hydroxymethyluracil degradation, it is

Figure II, 18

The hydrolysis of 5-hydroxymethyluracil by 5N-NaOH at 100° C.

1 ml. portions (0.7 μmoles each) of
5-hydroxymethyluracil were heated with 1 ml. NaOH
(10N) at 100° C. for periods of time varying from
20 to 120 minutes.   After being cooled each portion
was neutralised and assayed for formaldehyde by the
chromotropic acid reagent, and the free formaldehyde
assayed was plotted against time.
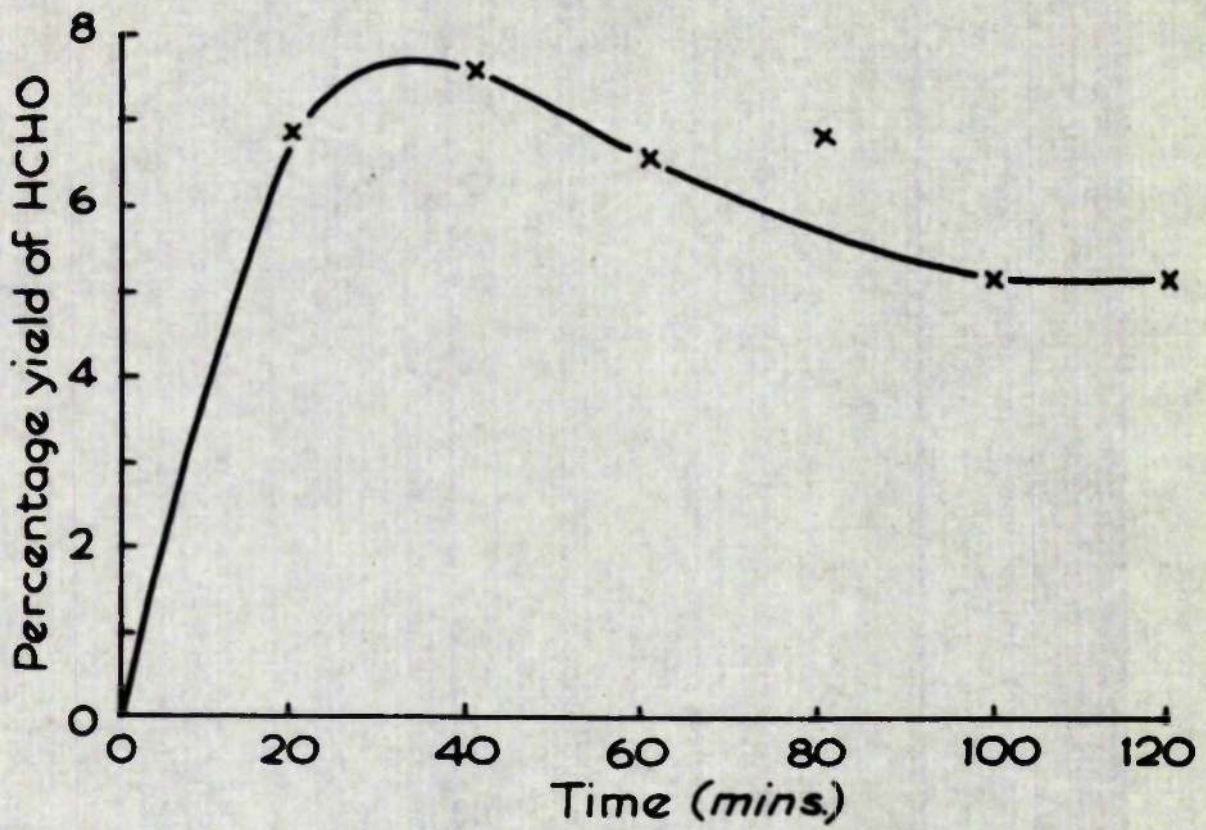
Figure II, 18

seen that 5-hydroxymethyluracil is a relatively stable compound.

15. Degradation of 5-hydroxymethyldeoxyuridine.

Acid hydrolysis of 5-hydroxymethyldeoxyuridine to 5-hydroxymethyluracil in almost 100% yield has been shown already to be effected by treatment with 0.1N-HCl at 100° C. in a sealed tube (Fig. II, 16). Enzymic degradation of 5-hydroxymethyldeoxyuridine was also attempted, making use of the nucleoside phosphorylase activity of Esch. coli PA/15. 5-Hydroxymethyldeoxyuridine was incubated in phosphate buffer with a cell-free extract of Esch. coli PA/15 and the reaction mixture was submitted to chromatography on paper after incubation for one hour. Fig. II, 19 shows that no trace of 5-hydroxymethyluracil was produced from 5-hydroxymethyldeoxyuridine by the action of nucleoside phosphorylase under these conditions.

Thus 5-hydroxymethyldeoxyuridine is readily degraded to 5-hydroxymethyluracil by treatment with dilute acid, but the synthetic compound is not a substrate for nucleoside phosphorylase activity present in Esch. coli PA/15. The resistance of 5-hydroxymethyldeoxyuridine to attack by nucleoside phosphorylase confirms the observation (Section II, 13) that

Degradation of 5-hydroxymethyldeoxyuridine by nucleoside phosphorylase.

Ultraviolet photograph of paper chromatographic separation of a reaction mixture containing 5-hydroxymethyldeoxyuridine and a cell-free extract of Esch. coli PA/15 in the presence of inorganic phosphate (2) together with a marker of 5-hydroxymethyluracil (1).

5-Hydroxymethyldeoxyuridine degradative system - 5-Hydroxymethyldeoxyuridine (about 0.5 μmoles) + 20 μl. phosphate buffer (0.1M, pH 7.2) + 0.1 ml. cell-free extract of Esch. coli PA/15 (0.1 mg. protein) incubated on paper for 1 hour at 37° C.

Figure II, 19.
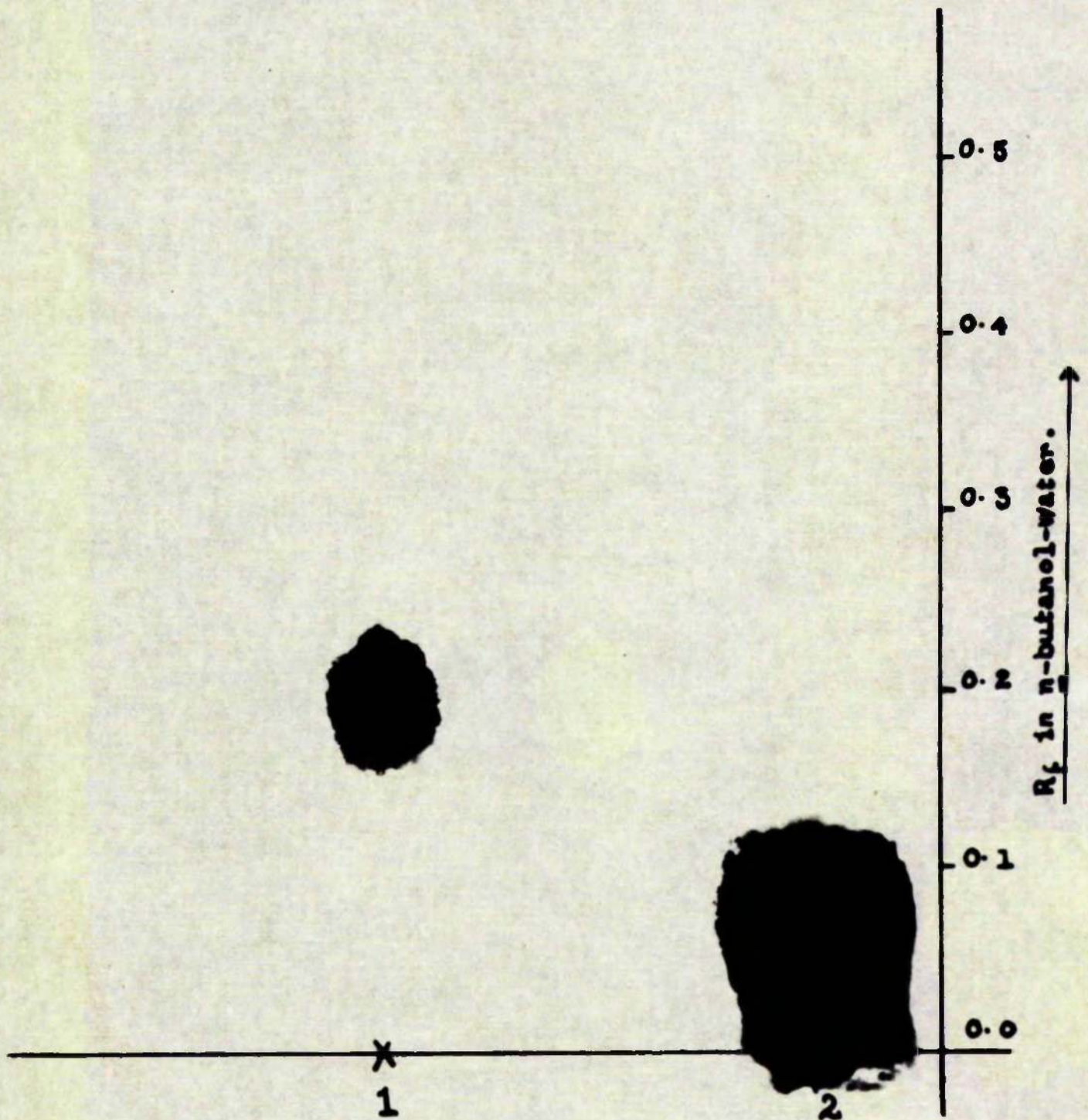
5-hydroxymethyluracil and deoxyribose-1-phosphate
are not coupled to yield 5-hydroxymethyldeoxyuridine by the
action of nucleoside phosphorylase.

16.   Acid hydrolysis of thymidylic acid.

The degradation of TMP to thymine and thymidine
under mild, controlled conditions was of importance in
experiments to confirm the structure of enzymically
synthesised ^{14}C-labelled TMP.   Accordingly, the
hydrolysis of TMP under a number of different conditions
was studied.

TMP (2 μmoles) was heated with 0.1 ml. 0.1N-
HCl in a sealed tube at 100° C. for 6 hours.   Paper
chromatographic separation (solvent, n-butanol-water)
of the reaction products revealed ultra-violet absorbing
material which had migrated at the same rate as did markers
of thymine and thymidine   The ultra-violet absorption
spectra, at pH1 and pH13, of this material in the
thymine-thymidine position corresponded exactly to the
spectra of authentic thymidine.   Thus hydrolysis of
TMP under these conditions degrades the nucleotide to
thymidine (in 10% yield) without causing further breakdown
of the nucleoside to the free base.

The hydrolysis of TMP by treatment with (i) N-
HCl and (ii) 2N-HCl at 100° C. for times ranging from 10

to 90 minutes was investigated also. The hydrolysis
was followed by paper chromatographic separation of the
reaction products followed by spectrophotometric
measurement of the ratio of undegraded TMP to thymine-
thymidine. Fig. II, 20 (a) shows that N-HCl hydrolysis
for 90 minutes degrades 50% of the TMP present whereas
99% is hydrolysed by 2N-HCl in 60 minutes.

The proportions of thymine and thymidine
present in the products of TMP hydrolysis by (i) N-
HCl and (ii) 2N-HCl for times ranging from 10 to 60
minutes were determined by differential spectrophotometry.
Fig. II, 20(b) shows that the product of TMP hydrolysis
by 2N-HCl was 100% thymine after 30 minutes. In the
case of N-HCl hydrolysis only 80% of the product was
thymine after 60 minutes hydrolysis, the other 20%
being thymidine.

Thus relatively mild, controlled conditions
have been found under which TMP is hydrolysed (i) to
thymidine without contamination by thymine and (ii) to
thymine without contamination by thymidine.

The hydrolysis of thymidylic acid at 100° C.
by (i) N-HCl and (ii) 2N-HCl.

(a) TMP (30 μmoles) was heated at 100° C.
with (i) 2 ml. N-HCl and (ii) 2 ml. 2N-HCl. 10 ul.
samples were withdrawn from both (i) and (ii) at
fixed time intervals over a period of 90 minutes.
Each sample was chromatographed on paper with
n-butanol-water (solvent 1), and the ratio of TMP
to thymine + thymidine determined by elution and
spectrophotometric measurements at pH 1.

(b) The ratio of thymine to thymidine in
the hydrolysis products of each sample withdrawn
was determined by differential spectrophotometry
at 260 mμ and 280 mμ.
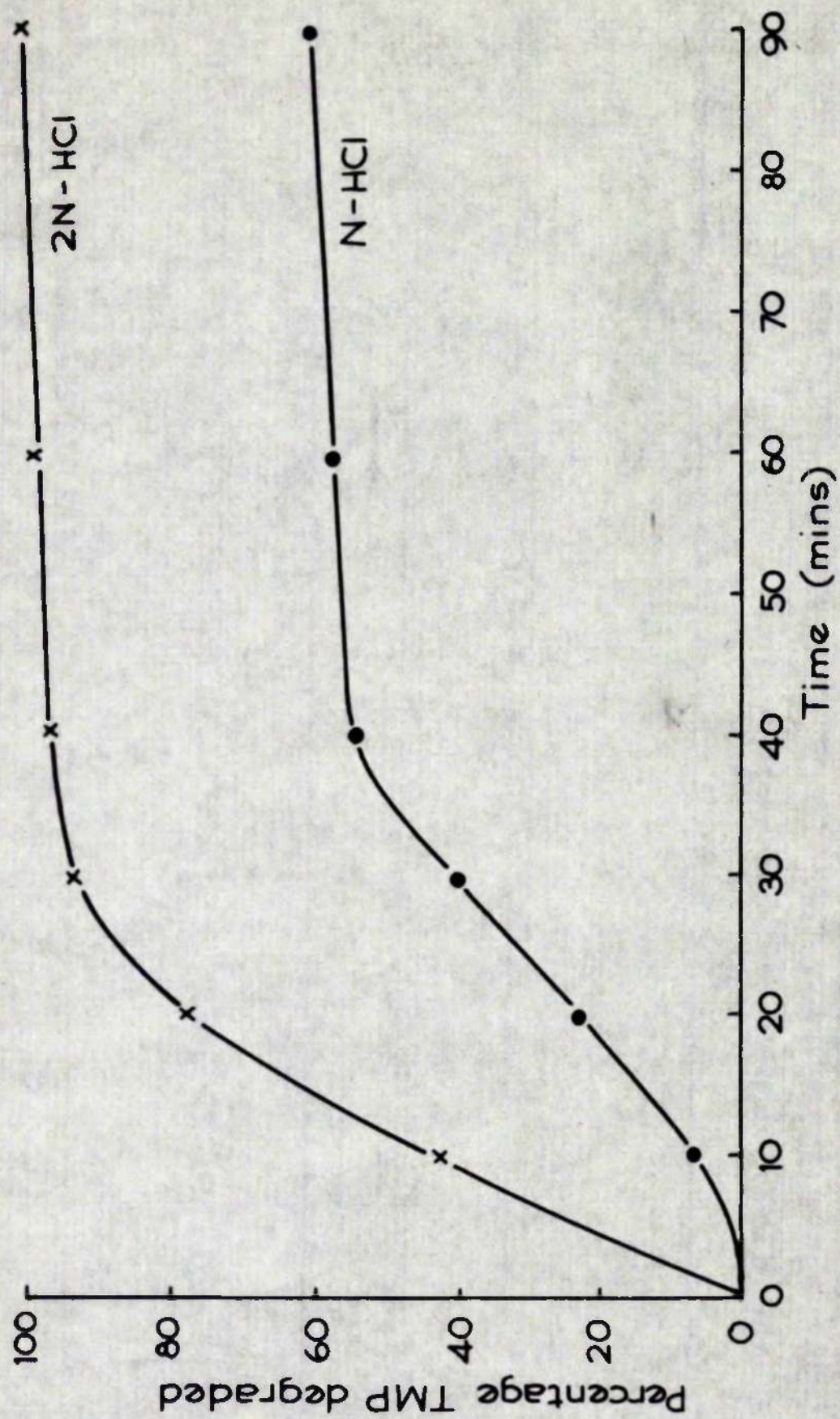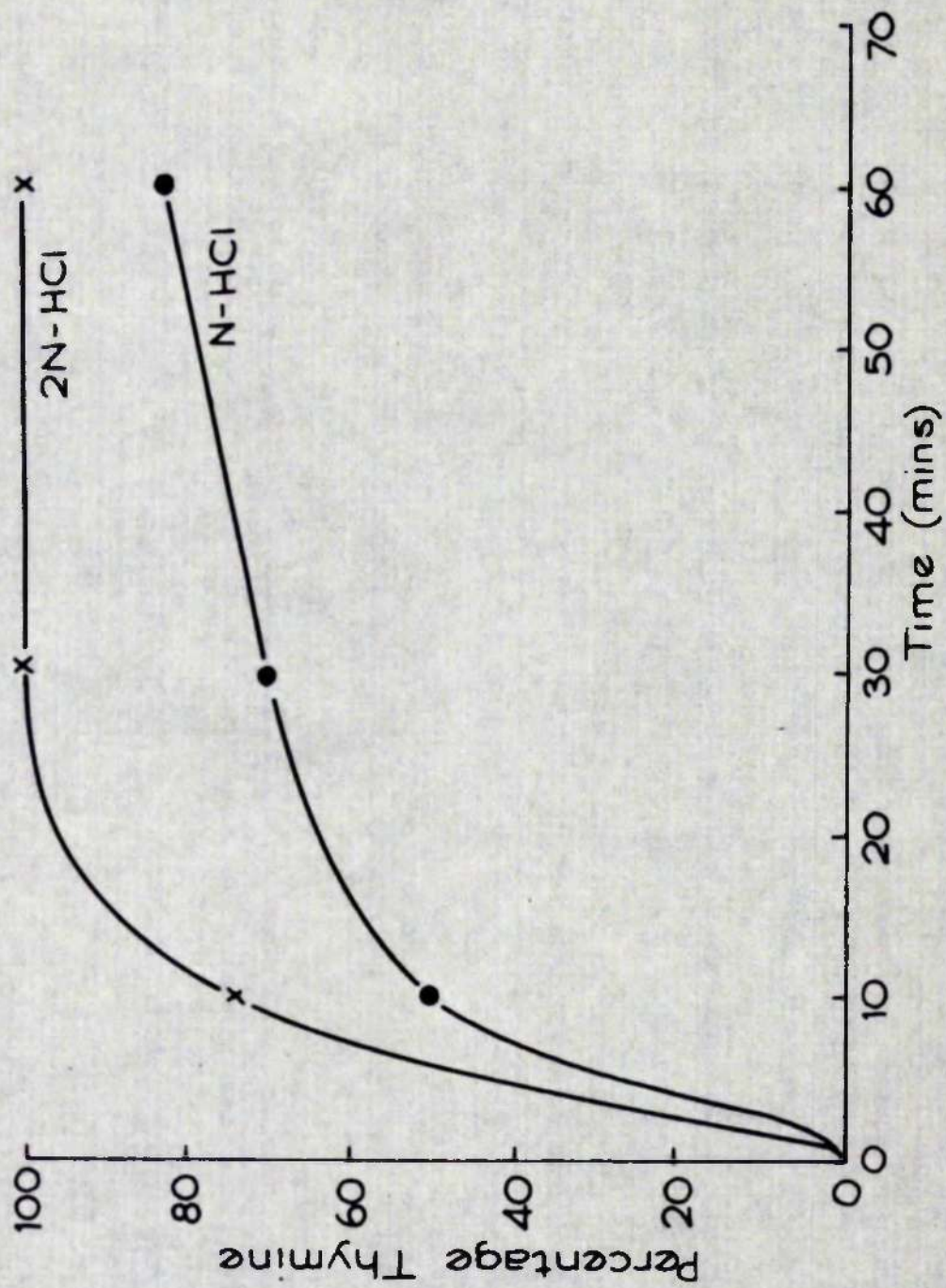
Figure II, 20(a)

Figure II, 20(b)

17. Attempted syntheses of thymine and thymidine by Bacillus subtilis 8059 and Esch. coli PA/15.

It has been stated already (Section II, 12) that the mechanism for the "methylation" of homocysteine to yield methionine is analagous to that postulated for the "methylation" of UDr to yield thymidine. Thus there is a possibility that substitution of UDr for homocysteine in a crude bacterial system containing methionine synthetase activity will lead to the synthesis of thymidine. Also, Rege and Sreenivasan (1954) have reported the synthesis of thymine from uracil in the presence of washed whole-cell suspensions of B. subtilis. These authors found that the yield of thymine was increased by the addition of one-carbon unit precursors such as serine, glycine and methionine. This system is analagous to that in which methionine synthetase activity was demonstrated in Esch. coli PA/15 (Section II, 12).

Accordingly the system described by Rege and Sreenivasan (1954) was reinvestigated. A washed whole-cell suspension of B. subtilis 8059 (17 mg. dry wt.) was incubated with uracil (20 μmoles) in phosphate buffer (0.05M, pH 7.2) in a total volume of 6 ml. After incubation under aerobic conditions at 37° C. for 4 hours the reaction mixture was centrifuged (20,000 g) and

the supernatant assayed for thymine, using Esch.
coli 15T-, a thymine- or thymidine-less mutant, as
assay organism. No trace of thymine was detected
in the reaction supernatant. Extensive paper
chromatography also failed to reveal the presence of
thymine. The incubation mixture was supplemented
with (i) serine (20 μmoles) or (ii) glycine (20 μmoles)
but again no thymine was detected. Thus the
attempted repeat of Rege and Sreenivasan's work with
one strain of B. subtilis was unsuccessful.

This experiment was repeated using a washed
whole-cell suspension of Esch. coli PA/15. A washed
whole-cell suspension of Esch. coli PA/15 (3.2 mg.
dry wt.) was incubated with glucose (40 μmoles), uracil
(20 μmoles), serine (40 μmoles) and vitamin B_{12} (0.1 μg.)
in phosphate buffer (0.1 M, pH 6.9) in a total volume
of 2 ml. After incubation under aerobic conditions
at 37° C. for 5 hours the reaction mixtures were heated
at 100° C. for 20 minutes and centrifuged (20,000 g).
The supernatant was assayed for thymine, using Esch.
coli 15T- as assay organism. No trace of thymine was
detected in the reaction supernatant.

As attempts to carry out the "methylation"
reaction at the level of the free base had proved
unsuccessful the reaction was investigated at the

Table II, 7

Omissions	Thymine assayed mµmoles/hr./mg. cells(
None	6,4
None [1].	8,0
Cell suspension	8,0
Vitamin B_{12}	5,9
Serine	5,6
Deoxyuridine	0,0

1. Incubation mixture heated at 100° C. for 20 minutes before incubation.

Table II, 7

Synthesis of thymine by washed whole-cell suspensions of Esch. coli PA/15.

Tubes contained a washed whole-cell suspension of Esch. coli PA/15 (5.2 mg. dry wt.), glucose (40 μmoles), UDr (30 μmoles), serine (40 μmoles), and vitamin B_{12} (0.1 μg) in phosphate buffer (0.1M, pH 6.9), total volume 2 ml. Incubation was carried out under aerobic conditions at 37° C. for 7 hours. At the end of the incubation period the reaction mixtures were centrifuged (20,000 g) and the supernatants were assayed for thymine using Esch. coli 15T-, a thymine- or thymidine-less mutant, as assay organism.

Table II, 8.

Synthesis of thymine by cell-free extracts of Esch. coli PA/15.

Tubes contained a cell-free extract of Esch. coli PA/15 (3 mg. protein), ATP (120 μmoles), fructose-1:6-diphosphate (120 μmoles), MgSO$_4$ (20 μmoles), UDr (30 μmoles), serine (40 μmoles) and pyridoxal phosphate (0.2 μmoles) in phosphate buffer (0.1M, pH 7.2), total volume 5 ml. Incubation was carried out under aerobic conditions at 37° C. for 7 hours. At the end of the incubation period the reaction mixtures were deproteinised by heating at 100° C. for 10 minutes, centrifuged (5,000 g) and the supernatants were assayed for thymine using Esch. coli 15T-, a thymine or thymidine-less mutant, as assay organism.

Table II, 8

Omissions	Thymine assayed mμmoles/hr./mg. protein
None	33.0
None [1.]	42.0
Cell-free extract	41.0
Pyridoxal phosphate	38.0
Serine	31.0
Deoxyuridine	0.0

1. Incubation mixture heated at 100° C. for 20 minutes before incubation.

nucleoside level, using UDr as the one-carbon unit
acceptor compound.   A washed whole-cell suspension
of Esch. coli PA/15 was incubated with UDr, serine,
glucose and vitamin B_{12} under aerobic conditions and
the incubation supernatant was assayed for thymine,
using Esch. coli 15T- as assay organism.   This assay
revealed the presence of a small amount (0.085 μmoles/
ml.) of thymine in the reaction supernatant.   The
results in Table II, 7 show that the amount of thymine
assayed was decreased when serine or vitamin B_{12} had
been omitted from the incubation mixture.   However,
the presence of thymine was detected also when the
incubation mixture had been heated at 100° C. for 20
minutes before incubation or when the cell suspension
had been omitted from the incubation mixture.
Investigation of the UDr used in these incubations, by
microbiological and paper chromatographic techniques,
showed that the UDr was contaminated with thymidine
(about 1%).

Taking the contamination concentration of
thymidine as the base-level it was found that, when
serine or vitamin B_{12} had been omitted from the
incubation mixture, the concentration of thymine present
after incubation was 70% to 75% of the base-level.   When
the complete system was incubated the concentration of

thymine detected was 80% of the base-level. These
results were confirmed by repetition of the experiment.
In no case was a net synthesis of thymine observed.

Thus it appears that this system is capable
of thymine metabolism and if synthesis is taking place
it is proceeding at a slower rate than is the metabolism.
The metabolism of thymine by washed whole-cell
suspensions of Esch. coli PA/15 was confirmed by
incubating a whole-cell suspension of Esch. coli PA/15
(4 mg. dry wt.) with vitamin B_{12} (0.2 ug) and thymine
(0.1 umole) in phosphate buffer (0.1M, pH 6.9). After
7 hours incubation under aerobic conditions at $37^{\circ}C$.
microbiological assay of the reaction supernatant
revealed that 0.05 umoles (50%) of the thymine remained.

Possible requirements of the synthetic system
for folic acid derivatives were investigated also since
a number of systems involving the transfer of one-carbon
units have been shown by several workers to require
these derivatives (Blakley, 1954; Kisluik and Sakami,
1955; Friedkin, 1957; Woods, 1958 among others). Folic
acid (20 umoles), N^{10}-formylfolic acid (20 umoles) or
N^5-formyl-FAH_4(20 umoles) were used to supplement the
incubation system. In no case did the addition of any
one of these supplementary factors cause a detectable
net synthesis of thymine. Similarly, variations in the

concentrations of glucose, UDr, serine, vitamin B_{12} or cell suspension did not yield any net synthesis of thymine.

The synthesis of thymine by cell-free extracts of Esch. coli PA/15 was investigated as this technique lends itself to a closer study of cofactor requirements. A cell-free extract of Esch. coli PA/15 was incubated with ATP, fructose-1:6-diphosphate, $MgSO_4$, UDr, serine and pyridoxal phosphate in phosphate buffer. After deproteinisation the reaction supernatant was assayed for thymine, using Esch. coli 15T- as assay organism. Table II, 8 shows that, as in the systems using cell-suspensions, thymine was detected in the supernatant from each incubation except when UDr had been omitted. In the absence of serine the concentration of thymine present after incubation was 74% of the base-level concentration whereas, when the complete system had been incubated, this concentration rose to 79% of the base-level.

The addition of supplementary cofactors in the form of folic acid (20 μmoles), N^{10}-formylfolic acid (20 μmoles) or N^{5}-formyl-FAH_4 (20 μmoles) had no effect on the net synthesis of thymine in this system.

Although both systems containing (i) whole-cell suspensions or (ii) cell-free extracts of Esch.

coli PA/15 have been found to metabolise the thymidine
present in the incubation mixtures, the figures quoted
in Tables II, 7 and II, 8 show that the net amount of
thymine present after incubation is greater in the
systems in which synthesis could have taken place than
in the systems in which, presumably, synthesis would
proceed at a slower rate, if at all, that is where
serine, the one-carbon donor, had been omitted from the
incubation mixtures.   Thus the synthesis of thymine
should be unequivocally demonstratable only by the
use of ^{14}C-labelled one-carbon units.

Accordingly, the washed whole-cell suspension
and cell-free extract experiments already described
(Tables II, 7 and LL, 8) were repeated with the serine
(40 μmoles) replaced by 3-^{14}C-serine (40 μmoles, 2 μc).
Microbiological assay of the reaction supernatants for
thymine revealed the same results as were obtained
previously (Tables II, 7 and II, 8).   In addition, the
reaction supernatants, to which carrier thymidine had
been added, were chromatographed on paper in two
dimensions (solvents, iso-propanol-water-HCl and n-
butanol-water).   Autoradiography of these chromatograms
did not show the presence of ^{14}C-labelled material
coincident with the carrier thymidine.   Isolation of
larger quantities of thymidine from the reaction

supernatants by chromatography of the supernatant
as bands (solvent, n-butanol-water) on paper
followed by paper chromatography of the isolated
thymidine in two dimensions (solvents, iso-
propanol-water-HCl and n-butanol-water) and auto-
radiography of these chromatograms also failed to
reveal the presence of ^{14}C-labelled material
coincident with the carrier thymidine.

Thus, although it appeared from the
microbiological assay data that thymine (or thymidine)
may be synthesised to a very small extent in systems
containing UDr, serine and either a washed whole-
cell suspension or a cell-free extract of Esch. coli
PA/15, this synthesis was not confirmed by the
studies using $3-^{14}C$-serine as the one-carbon unit
donor.   Two possible explanations of these observ-
ations are (a) that the "methylation" of the UDr
and the metabolism of the "thymine" takes place at a
level higher than the nucleoside, perhaps at the
nucleotide level, the sequence of reactions in this
case being:

```
                                            Thymidine
                                               |    ATP
Deoxyuridine              Thymidine            |   /
kinase                    kinase               |  /
                          "1-C"                | / --> ADP
                                               |/
UDr ----------> dUMP ------------> TMP ------------> metabolic
                                                      products.
```

or (b) there may be a common intermediate in the "thymine" synthetic and metabolic pathways, the sequence of reactions in this case being:
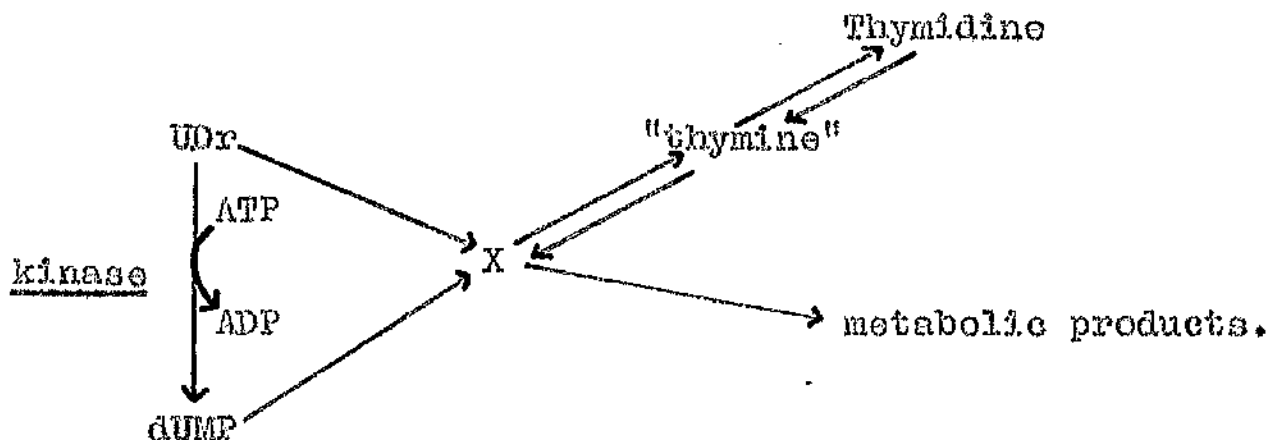
In either of these cases, no ^{14}C-labelled thymine (or thymidine) would be isolated after the incubation since any which was synthesised would be metabolised immediately. The only effect that the synthesis of "thymine" would have in such mechanisms would be to decrease the rate at which the added thymidine (that is, the UDr contaminant) was metabolised— an effect which was observed (Tables II, 7 and II, 8).

18. The synthesis of thymidine by extracts of rabbit thymus glands.

During the course of the work described in Section II, 17, the methylation of deoxyuridine by soluble enzymes of rabbit thymus glands was reported by Blakley (1957). The thymus extracts used by Blakley

were inactivated by treatment with Dowex-1-(chloride)
and extracts so treated could be reactivated by the
addition of ATP, DPNH and FAH_4. This work has been
confirmed by Phear and Greenberg (1957) who used
extracts of rat thymus glands. It must be noted,
however, that whereas Blakley found thymidine to be
the main product of the methylation, Phear and
Greenberg found TMP to be the primary product.

The evidence described in Section II, 17
showed no unequivocal synthesis of thymine or
thymidine. The conclusions drawn from this evidence
indicate that synthesis took place at the nucleotide
level rather than at the nucleoside level whereas
Blakley's work (1957) indicated that the reaction
took place at the nucleoside level (or, if at the
nucleotide level, the phosphorylated products were
dephosphorylated at a rate comparable with that of the
"methylation" reaction). In view of this difference
between the two systems a reinvestigation of the
system studied by Blakley was undertaken.

An extract of rabbit thymus gland was
incubated with UDr (10 μmoles) and 5-^{14}C-serine (10
μmoles, 0.2 μc) in Tris buffer (0.1M, pH 7.2), total
volume 1 ml. Incubation was carried out under
anaerobic conditions at 37° C. for 4 hours. Deprotein-

isation was effected by heating at 100° C. for 10 minutes and centrifuging (5,000 g). Carrier thymidine (2 μmoles) was added to the supernatant and re-isolated by chromatography on paper as bands (solvent, n-butanol-water). The material isolated from the thymidine position on this chromatogram was rechromatographed in two dimensions (solvents, isopropanol-water-HCl and n-butanol-water). The spot of thymidine so separated was eluted with water, plated on to a planchette and counted. No radioactivity was detected in the carrier thymidine. Repetitions of this experiment with extracts of fresh rabbit thymus glands using $3-^{14}C$-serine of higher specific activity ((i) 10 μmoles, 1 μc and (ii) 8 μmoles, 4 μc) and incubating under (a) aerobic and (b) anaerobic conditions also failed to demonstrate the synthesis of any ^{14}C-labelled material which could be isolated with added carrier thymidine by paper chromatography. Similarly, incubation of Dowex-1-(chloride)-treated, extracts of rabbit thymus glands for 4 hours at 37° C. in Tris buffer (0.1M, pH 7.2) under anaerobic conditions with UDr (20 μmoles), DPNH (0.5 μmoles) and (i) $3-^{14}C$-serine (20 μmoles, 0.4μc), ATP (1 μmole) and FAH_4 (0.25 μmoles) or (ii) $3-^{14}C$-serine (40 μmoles, 4 μc), ATP (4 μmoles), FAH_4 (5 μmoles) and $MgSO_4$ (40 μmoles) failed to effect the synthesis of any

detectable quantities of ^{14}C-labelled thymidine.

However, when an untreated extract of rabbit
thymus was supplemented with ATP (4 μmoles), FAH$_4$
(5 μmoles), MgSO$_4$ (40 μmoles) and DPNH (0.3 μmoles) and
incubated in Tris buffer (0.1 M, pH 7.2) with UDr
(40 μmoles) and 3-^{14}C-serine (8 μmoles, 4 μc) for 4
hours at 37° C. under anaerobic conditions, the added
carrier thymidine, isolated as before by paper
chromatography, contained ^{14}C-labelled material.
Aerobic incubation of the same system produced no
^{14}C-labelled thymidine, nor was ^{14}C-labelled thymidine
isolated from systems which had been incubated under
anaerobic conditions but from which the supplementary
ATP or FAH$_4$ had been omitted.

The specific activity of the carrier
thymidine isolated from this system was very low and
was not sufficient to enable successful autoradiographs
to be obtained. However, the material was sufficiently
radioactive to yield a counting rate which was
statistically above background.

Although the methylation reaction appears to
proceed at the nucleoside level in this system, the
addition of ATP has been shown to be necessary for the
reaction to take place (Blakley, 1957). The ATP may
be involved in the reaction in the way shown by the

following scheme:

$$\text{UDr} \xrightarrow{\text{ATP} \quad \text{ADP}} \text{dUMP} \xrightarrow{\text{"1-C units"}} \text{TMP} \longrightarrow \text{thymidine}$$
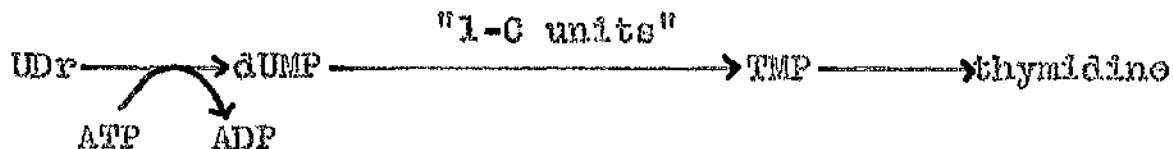
In this scheme the UDr is phosphorylated to dUMP which then undergoes "methylation". The TMP which is formed as the initial product is degraded by thymidylate phosphomonoesterase action yielding thymidine as the main reaction product. This is in accordance with the view that the "methylation" reaction takes place at the nucleotide level. The failure to demonstrate this reaction at the nucleoside level (i) in the presence of cell-free extracts of Esch. coli PA/15 (Section II, 17) and (ii) in the presence of extracts of rat thymus glands (Phear and Greenberg, 1957) may well be due to low thymidylate phosphomonoesterase activity in those systems.

19. Synthesis of thymidylic acid by cell-free extracts of Esch. coli PA/15 and Esch. coli 113/3.

The results of the experiments described in Sections II, 17 and II, 18 suggest that the methylation of UDr may take place at the nucleotide level. Also, while this work was in progress Friedkin (1957) reported the synthesis of TMP from dUMP by cell-free extracts of Esch. coli B. Accordingly the methylation of UDr was

reinvestigated at the nucleotide level.

A cell-free extract of Esch. coli PA/15 was incubated under anaerobic conditions with UDR, 3-^{14}C-serine, ATP, FAH$_4$, MgSO$_4$, pyridoxal phosphate and TPNH in Tris buffer. The deproteinised reaction mixture, to which carrier thymidine and TMP had been added, was submitted to two-dimensional paper chromatography (solvents, iso-propanol-water-HCl and n-butanol-water) followed by autoradiography. Fig. II, 21 shows radioactive material coinciding with the ultraviolet-absorbing spot of carrier TMP whereas there is no ^{14}C-labelled material associated with the spot of carrier thymidine. Fig. II, 21 also shows 3-^{14}C-serine together with two other strongly radioactive spots, material A and material B, which do not coincide with any ultraviolet-absorbing area on the chromatogram.

The nucleotide fraction of the reaction mixture was isolated by paper chromatography of the reaction supernatants as bands (solvent, n-butanol-water). The base-bands (R_F 0.0 to 0.05) were eluted with water and the nucleotide material so obtained was rechromatographed in two dimensions (solvents, iso-propanol-water-HCl and acetone-water-formic acid). This chromatogram was submitted to autoradiography and Fig. II, 22 shows that radioactive material, coincident with the spot of carrier TMP, has been separated from the nucleotide fraction of

Figure II, 21

Synthesis of ^{14}C-labelled "thymidylic acid" by a cell-free extract of Esch. coli PA/15.

Autoradiograph of a two-dimensional paper chromatographic separation of a deproteinised reaction mixture containing 3-^{14}C-serine, UDr, and a cell-free extract of Esch. coli PA/15 together with carrier thymidine and TMP, which had been added after deproteinisation.

Complete incubation mixture - 3-^{14}C-serine (8 μmoles, 4 μc), UDr (40 μmoles), ATP (40 μmoles), FAH$_4$ (5 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.2 μmoles), TPN (0.3 μmoles), glucose-6-phosphate (3 μmoles) and a cell-free extract of Esch. coli PA/15 (5 mg. protein) in Tris buffer (0.1M, pH 7.2), total volume 4 ml. Incubation was carried out under anaerobic conditions at 37° C. for 4 hours. After the period of incubation, the reaction mixtures were deproteinised by heating at 100° C. for 10 minutes.
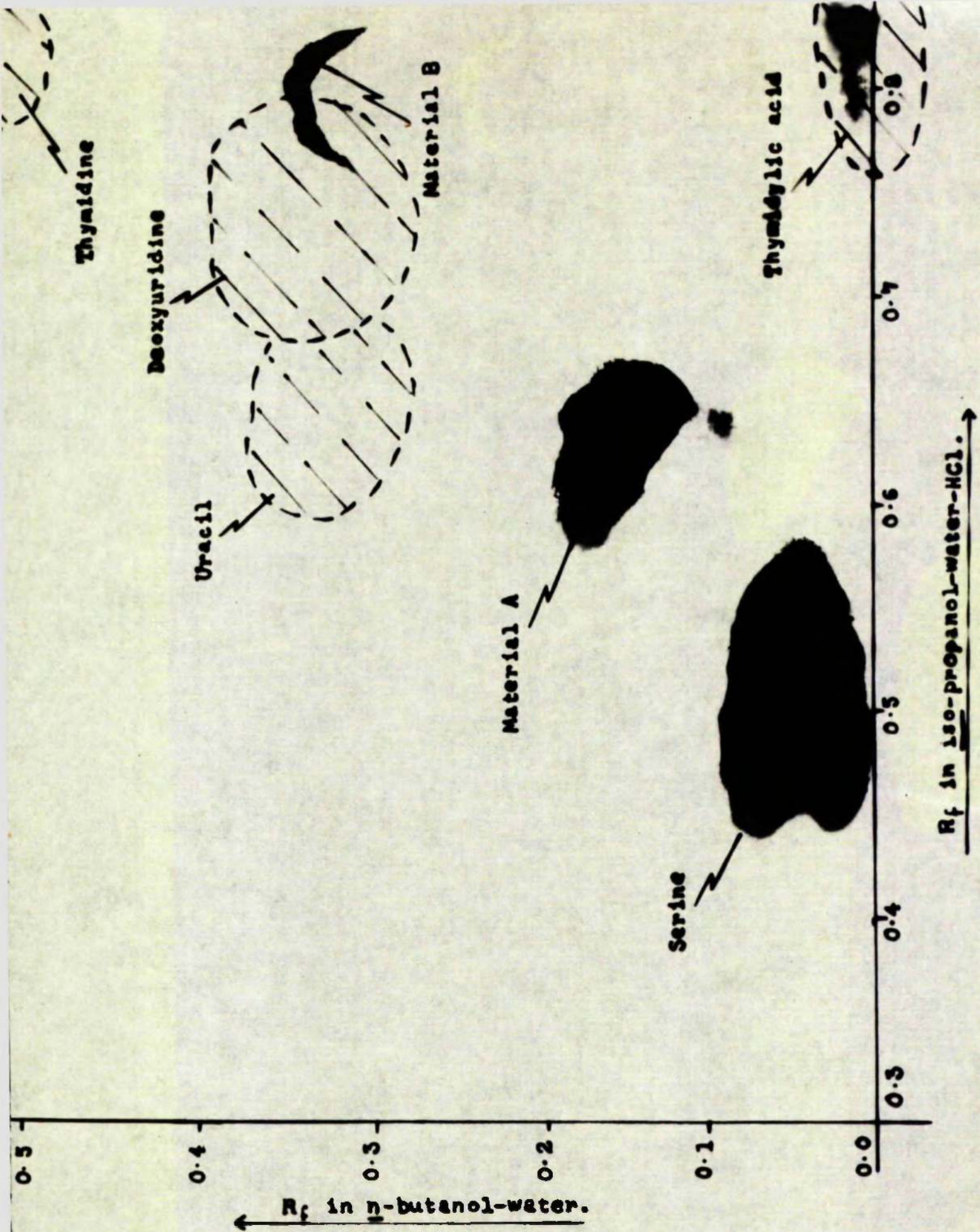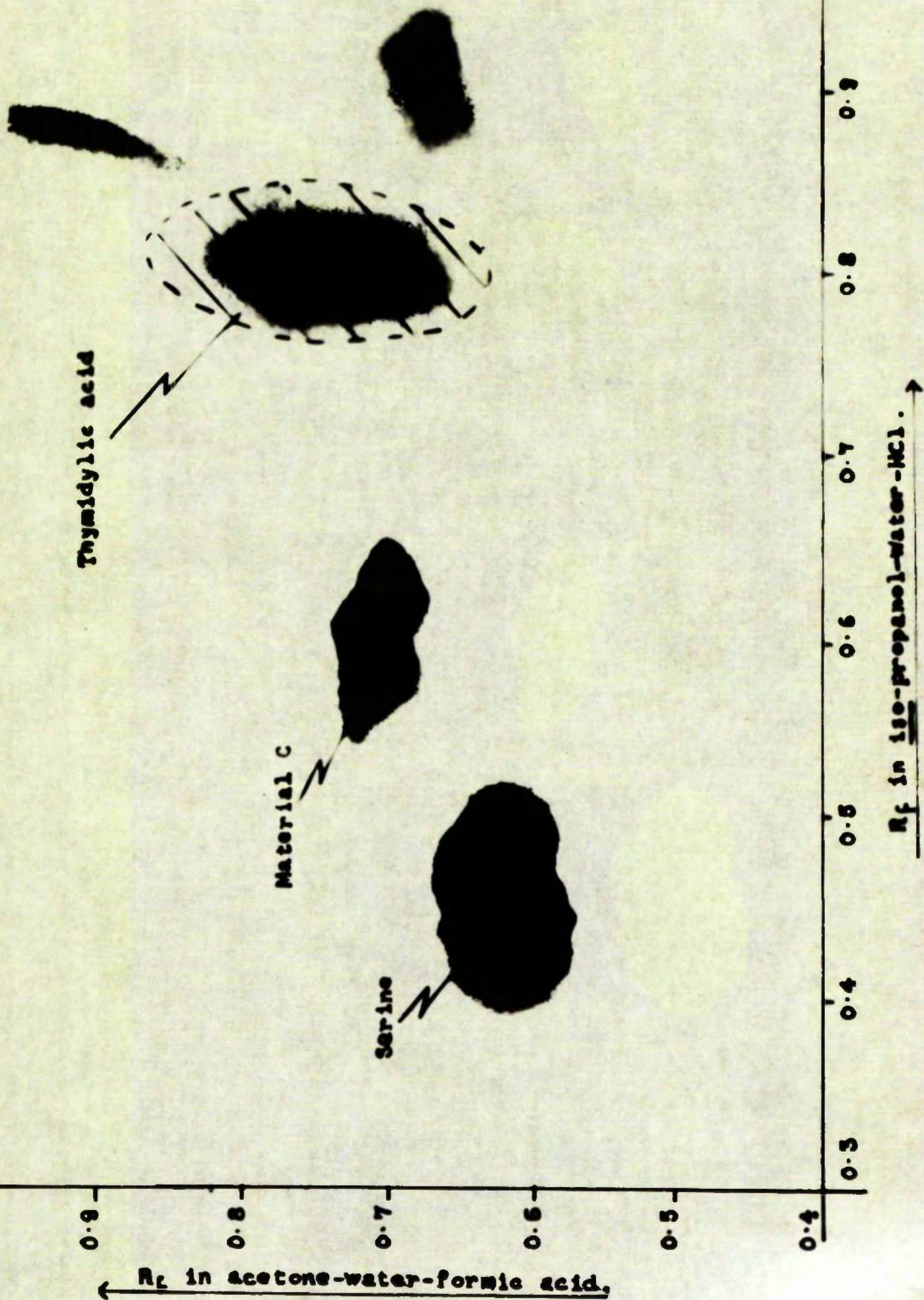
Figure II, 21.

Synthesis of ^{14}C-labelled "thymidylic acid" by a cell-free extract of Esch. coli PA/15

Autoradiograph of a two-dimensional paper chromatographic separation of the base-bands (R_F 0.0 to 0.05) of a n-butanol-water separation of deproteinised reaction mixtures containing $3-^{14}C$-serine, UDr and a cell-free extract of Esch. coli PA/15 together with carrier TMP, which had been added after deproteinisation.

Complete incubation mixture - $3-^{14}C$-serine(8 μmoles, 4 μc), UDr (40 μmoles), ATP (40 μmoles), FAH$_4$ (5 μmoles), MgSO$_4$ (40μmoles), pyridoxal phosphate (0.2 μmoles), TPN (0.3 μmoles), glucose-6-phosphate (3 μmoles) and a cell-free extract of Esch. coli PA/15 (5 mg. protein) in Tris buffer (0.1M, pH 7.2), total volume 4 ml. Incubation was carried out under anaerobic conditions at 37^0 C. for 4 hours. After the period of incubation the reaction mixtures were deproteinised by heating at 100^0 C. for 10 minutes.

Figure II, 22.

Thymidylic acid

Material C

Serine

R_f in iso-propanol-water-HCl.

R_f in acetone-water-formic acid.

of the reaction mixture by this procedure.   Fig. II,
22 also shows 3-^{14}C-serine together with one other
strongly radioactive spot, material C, which was not
coincident with any ultra-violet-absorbing material
on the chromatogram.

Thus, incubation of 3-^{14}C-serine and UDr in
the presence of a cell-free extract of Esch. coli PA/15,
together with a number of cofactors, has produced
^{14}C-labelled material which was not separated from
added carrier TMP by paper chromatography in three
solvents.   On this basis, the ^{14}C-labelled material
may be tentatively identified as ^{14}C-TMP.

These results were confirmed by repetition of
this experiment using (a) UDr or (b) dUMP as the
pyrimidine one-carbon unit acceptor compounds.   The
same results were obtained also when DPNH replaced
TPNH in the incubation mixture.   Paper chromatographic
separation of these reaction mixtures in one-dimension
(solvent, n-butanol-water) followed by autoradiography
showed that there was no ^{14}C-labelled material present
in the position occupied by thymidine on this
chromatogram (Figs. II, 23 (a) and II, 23 (b)).   Fig.
II, 23 (b) shows a radioactive spot corresponding to
material B (Fig. II, 21) together with a radioactive
spot of R$_F$ 0.58, material D.   Elution of material D

Synthesis of ^{14}C-labelled "thymidylic acid" by
cell-free extracts of Esch. coli PA/15.

(a) Ultraviolet photograph and (b)
autoradiograph of paper chromatographic separations
of deproteinised reaction mixtures containing a
cell-free extract of Esch. coli PA/15 and (1) 3-^{14}C-
serine, dUMP and TPNH (2); (ii) ^{14}C-formaldehyde,
UDr and TPNH (3); (iii) 3-^{14}C-serine, UDr and TPNH
(4); (iv) 3-^{14}C-serine, UDr and DPNH (5) together
with markers of thymine (1) and ^{14}C-formaldehyde (6).

Complete incubation mixture - A cell-free extract of
Esch. coli PA/15 (9 mg. protein); ATP (60 μmoles),
FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate
(0.2 μmoles) and (1) 3-^{14}C-serine (40 μmoles, 20 μc),
dUMP (40 μmoles), TPN (4 μmoles) and glucose-6-phosphate
(40 μmoles); (ii) ^{14}C-formaldehyde (8 μmoles, 12 μc),
UDr (40 μmoles), TPN (4 μmoles) and glucose-6-phosphate
(40 μmoles); (iii) 3-^{14}C-serine (40 μmoles, 20 μc),
UDr (40 μmoles), TPN (4 μmoles) and glucose-6-phosphate
(40 μmoles); (iv) 3-^{14}C-serine (40 μmoles, 20 μc),
UDr (40 μmoles) and DPNH (4 μmoles) in Tris buffer
(0.1M, pH 7.2), total volume 4 ml.   Incubation was
carried out under anaerobic conditions at 37°C. for 4
hours.   After the period of incubation, deproteinisation
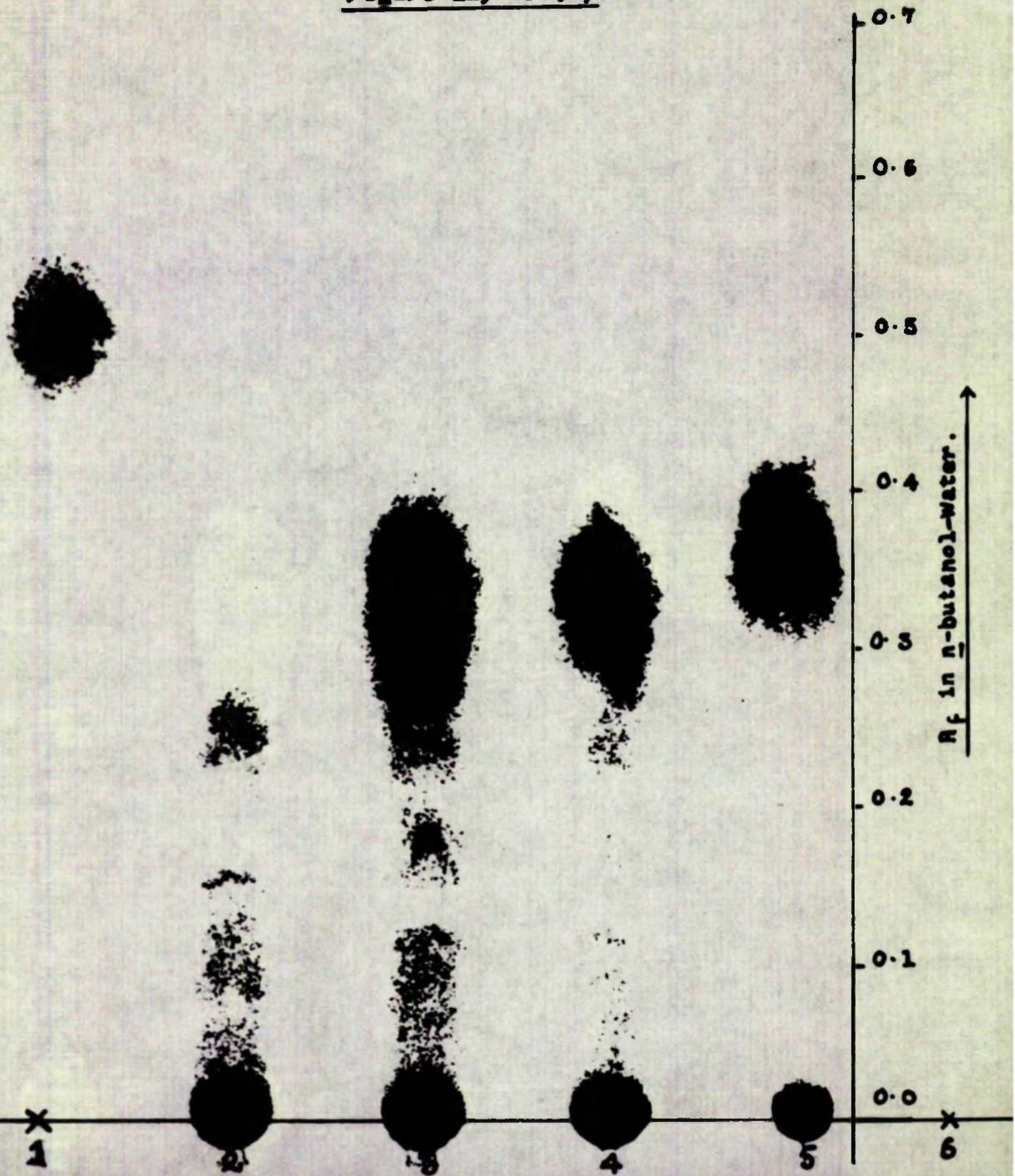was effected by heating at 100° C. for 10 minutes.

Figure II, 25 (a).

Figure II, 28 (b).

and rechromatography in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water) with carrier thymine and thymidine showed that material D was neither thymine nor thymidine.

Further confirmation of the identity of TMP and the ^{14}C-labelled material coincident with carrier TMP in Figs. II, 21 and II, 22 was obtained by an extensive programme of paper chromatography and electrophoresis. The nucleotide fractions of deproteinised reaction mixtures which contained 3-^{14}C-serine and (i) UDr and TPNH; (ii) dUMP and TPNH; or (iii) UDr and DPNH and to which carrier TMP had been added, were isolated by paper chroma-tography (solvent, n-butanol-water). The TMP was isolated from these nucleotide fractions by chromatography (solvent, acetone-water-formic acid) of the nucleotide fractions as bands on paper. The ultraviolet-absorbing area of the carrier TMP was eluted and this material was submitted to chromatography in two dimensions (solvents, iso-propanol-water-HCl and acetone-water-formic acid), followed by autoradiography. Figs. II, 24(a) and II, 24(b) show again the presence of ^{14}C-labelled material coincident with the ultraviolet-absorbing spot of carrier TMP, together with 3-14C-serine and material C which were present in the TMP fraction.

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

(a) Ultraviolet photograph and (b) autoradiograph of a two-dimensional paper chromatographic separation of a mixture of 3-^{14}C-serine, material C and ^{14}C-labelled TMP, together with added carrier TMP.

This material was isolated from an incubation mixture containing 3-^{14}C-serine, UDr and a cell-free extract of Esch. coli PA/15 by paper chromatography (solvent, n-butanol-water) of the deproteinised reaction mixture as bands with added carrier TMP. The base-bands (R_F 0.0 to 0.05) were eluted and the material obtained was rechromatographed (solvent, acetone-water-formic acid) as a band on paper. The carrier TMP was eluted and the material obtained was the mixture of 3-^{14}C-serine, material C and TMP separated on the chromatogram described in this figure.

Complete incubation mixture - the incubation mixture was that described in Fig. II, 23 with 3-^{14}C-serine (40 μmoles, 4 μc), UDr (40 μmoles), TPN (4 μmoles) and glucose-6-phosphate (40 μmoles).

Thymidylic acid

R_f in iso-propanol-water-HCl.

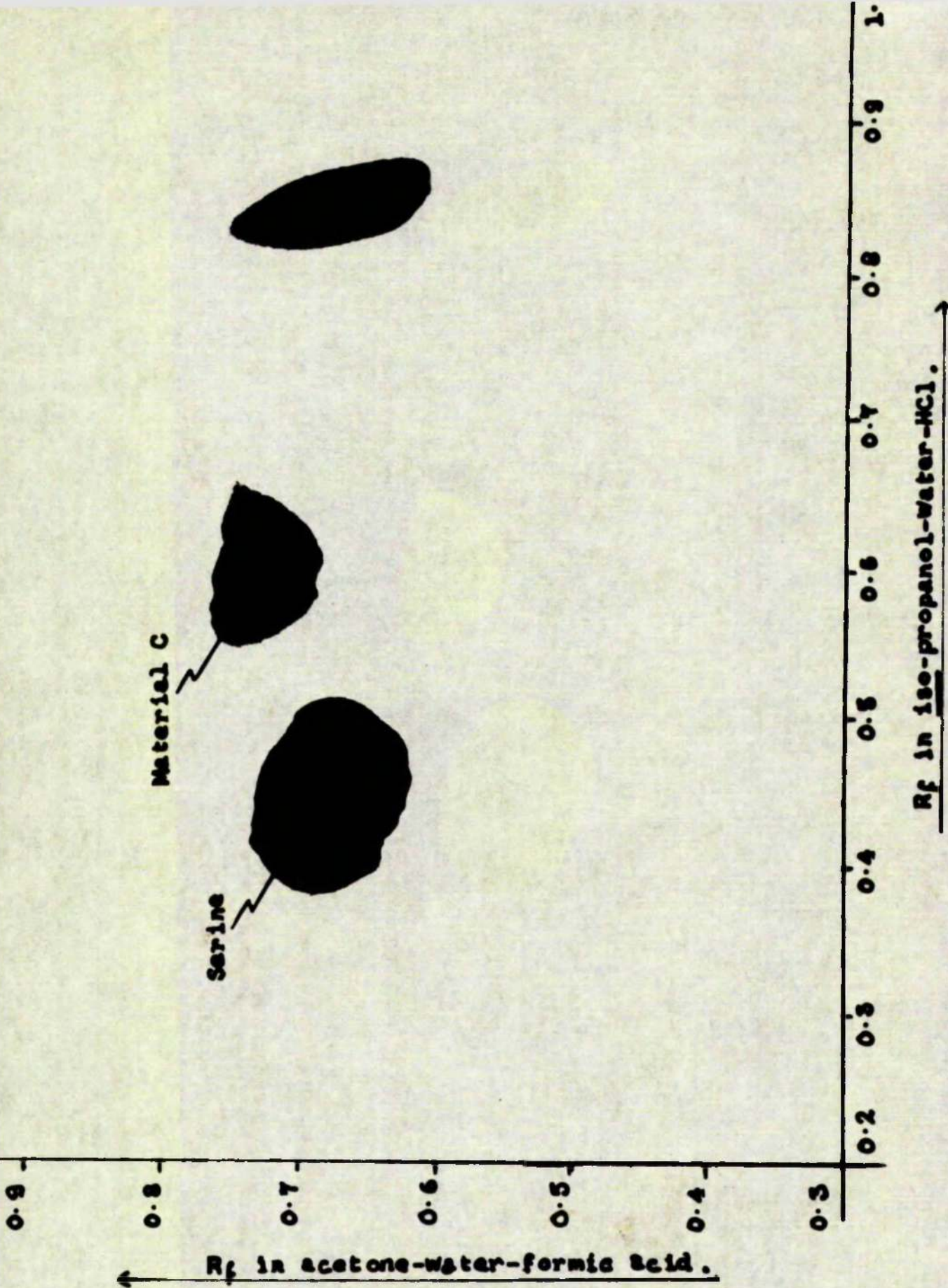R_f in acetone-water-formic acid.

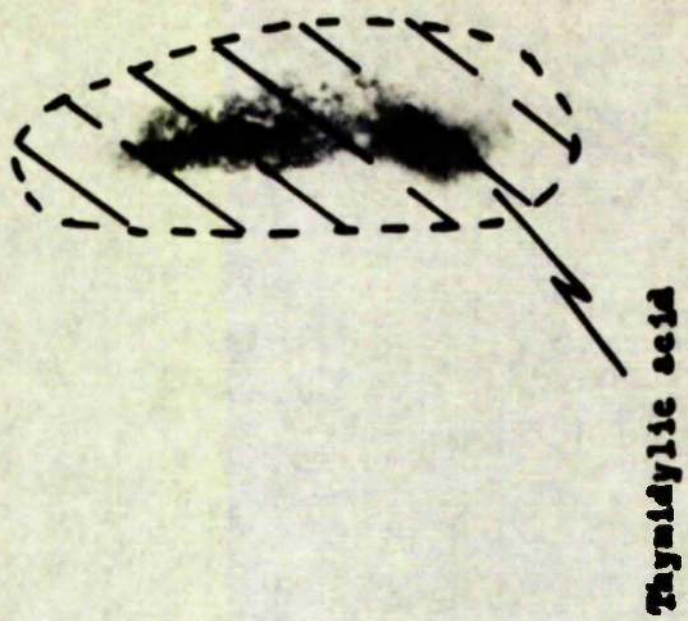Figure II, 24 (a).

Figure II, 24 (b).

The TMP isolated by these chromatographic separations (Fig. II, 24) was eluted and submitted to further chromatography and to electrophoresis in an attempt to separate the ^{14}C-labelled material from the carried TMP.   Part of the TMP was chromatographed in two dimensions (solvents, 12% Na_2HPO_4 and acetone-25% aqueous trichloroacetic acid) and, as Fig. II, 25 shows, the radioactive material was coincident with the ultraviolet-absorbing spot of carrier TMP.   The other part of the TMP was submitted to electrophoresis on paper and again, as Fig. II, 26 shows, the ^{14}C-labelled material coincided with the ultraviolet-absorbing spot of carrier TMP.   The thymidine seen in Fig. II, 26(a) was produced by the partial degradation of the carrier TMP which takes place when TMP is chromatographed using iso-propanol-water-HCl as solvent.

When ^{14}C-formaldehyde (8 μmoles, 12 μc) replaced $3-^{14}C$-serine in this system, paper chromatography of the reaction products (solvent, n-butanol-water) followed by autoradiography revealed radioactive materials with R_F values 0.58, 0.30 and 0.0 corresponding to material D, material B and nucleotide material respectively (Fig. II, 23).   Autoradiography of a two-dimensional paper chromatographic separation (solvents,

Figure II, 25

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

      Autoradiograph of two-dimensional paper chromatogram of enzymically synthesised ^{14}C-labelled TMP together with carrier TMP. The TMP on this chromatogram was obtained by elution of the TMP separated by the paper chromatography described in Fig. II, 24.

Thymidylic acid

Rf in 15% NH₄HPO₄ .

Rf in acetone-25% aqueous trichloroacetic acid.

Figure II, 28.

Figure II, 26.

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

(a) Ultraviolet photograph and (b) auto-radiograph of a paper electrophoretic separation of enzymically synthesised ^{14}C-labelled TMP together with carrier thymidine and TMP. The TMP on this electrophoretogram was obtained by elution of the TMP separated by the paper chromatography described in Fig. II, 24.

Electrophoresis was carried out on paper soaked in citrate buffer (0.05M, pH 3.5). Separation was achieved by applying a potential difference of 13 volts/cm. for $6\frac{1}{2}$ hours.

Thymidine

Serine

Figure II, 26 (a).

Figure II, 26 (b).

15

distance from origin (cm.).

18

20

Thymidylic acid

25

iso-propanol-water-HCl and acetone-water-formic acid)
of this nucleotide material with carrier TMP did not
show any ^{14}C-labelled material associated with the
carrier TMP. However, when the TMP was eluted and
counted it was found to be weakly radioactive. No
further evidence of formaldehyde acting as a source
of one-carbon units in the synthesis of TMP was
obtained.

When UR replaced UDr in this system, paper
chromatographic separation of the reaction products
(solvents, n-butanol-water; iso-propanol-water- HCl;
acetone-water-formic acid) followed by autoradiography
revealed ^{14}C-labelled material coincident with the
ultraviolet-absorbing spots of carried TMP. Electro-
phoresis of the TMP on paper confirmed the coincidence
of the ^{14}C-labelled material with the carrier TMP.
However, this failure to separate the ^{14}C-labelled
material from carrier TMP does not preclude the
possibility that the "methylation" product, which was
obtained when UR replaced UDr in the incubation mixture,
was in fact the ribose analogue of TMP. Ribonucleotides
and their deoxyribonucleotide analogues can be separated
by paper chromatography using ethanol-M-NH$_4$ acetate-
ethylenediaminetetraacetic acid (EDTA)-borate as solvent.
In this solvent the ribose derivatives form a borate

complex of slow mobility (Figs. II, 27(a) and II, 27(b)). The mixture of carrier TMP and its associated ^{14}C-labelled material isolated from incubation mixtures which contained UR in place of UDr was submitted to paper chromatography using this solvent. Autoradiography of this chromatogram showed that, whereas some of the ^{14}C-labelled material had migrated with the carrier TMP, a proportion of it had not migrated from the origin. However, the same effect was observed with ^{14}C-TMP isolated from incubation mixtures which contained UDr. Accordingly, the results obtained by using this technique were inconclusive.

The synthesis of ^{14}C-TMP was demonstrated also using cell-free extracts of Esch. coli 115/3, a methionine- or vitamin B_{12}-less mutant. This mutant was used in a study of the effect, if any, of vitamin B_{12} on the synthesis of TMP from (i) UDr and (ii) UR. ^{14}C-TMP was isolated from reaction mixtures containing a cell-free extract of this mutant and (i) UDr or (ii) UR, both in the presence and the absence of vitamin B_{12}. Quantitative results of this experiment will be discussed later (Section II, 21).

Thus it has been shown that the incubation of cell-free extracts of (a) Esch. coli PA/15 and (b)

Separation of ribonucleotides and their deoxyribo-
nucleotide analogues by paper chromatography.

Ultraviolet photographs of paper chromato-
graphic separations of (a) adenosine-5'-phosphate
(AMP-5') and deoxyadenosine-5'-phosphate (dAMP-5')
using (i) ethanol-M-NH_4acetate-EDTA and (ii) ethanol-
M-NH_4acetate-EDTA-borate as solvents; (b) uridine-
5'-phosphate (UMP-5') and deoxyuridine-5'-phosphate
(dUMP-5') using (i) ethanol-M-NH_4 acetate-EDTA and
(ii)ethanol-M-NH_4acetate-EDTA-borate as solvents.

Figure IX, 27 (a).
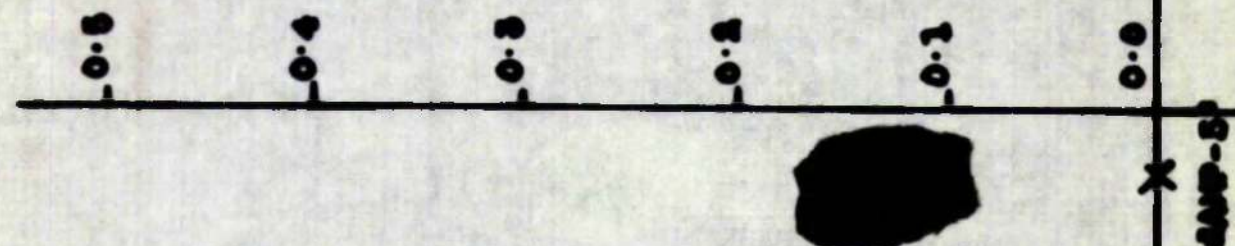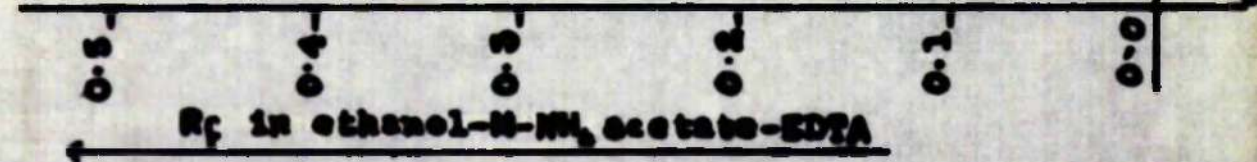
Figure II, 27.(b).

Esch. coli 113/3 with (i) UDr, (ii) dUMP or (iii) UR
and 5-^{14}C-serine in the presence of the cofactors
ATP, MgSO$_4$, FAH$_4$, pyridoxal phosphate and TPNH (or
DPNH) resulted in the synthesis of ^{14}C-labelled material
which has been identified as ^{14}C-TMP by virtue of the
fact that the material was not separated from authentic
carrier TMP by paper chromatography in five solvents
or by paper electrophoresis at pH 3.5.

20. Investigation of the thymidylic acid synthetic
system for the presence of intermediates.

The structures of the ^{14}C-labelled compounds
materials A, B, C, and D (Figs. II, 21, II, 22 and II,
23 (b)), which were isolated from incubation supernatants
containing ^{14}C-TMP, were of interest as these materials
may have been intermediates on the synthetic pathway to
^{14}C-TMP. Materials A and C were of special interest
since the migration rates of these materials in the
solvents used agreed with those known or predicted for
5-hydroxymethyldeoxyuridine and 5-hydroxymethyldeoxy-
uridylic acid respectively.

Observations made during attempts to synthesise
5-hydroxymethyldeoxyuridine and 5-hydroxymethyldeoxyuridylic
acid (Section II, 13) indicated that both of these
compounds were extremely sensitive to mild acid
hydrolysis, readily yielding 5-hydroxymethyluracil.

Accordingly, samples of material A and material C were treated with 0.1 ml. 0.1N-HCl in a sealed tube at 100° C. for 24 hours. The reaction products were submitted to paper chromatography in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water) with carrier 5-hydroxymethyluracil, followed by autoradiography. No ^{14}C-labelled material coincident with the ultraviolet-absorbing spot of carrier 5-hydroxymethyluracil was detected. The R_F values of both materials A and C were unchanged and it appeared that the compounds had been unaffected by this treatment with 0.1 N-HCl. Repetition of this treatment using N-HCl similarly had no effect upon materials A and C.

There are a number of compounds which could have been synthesised from 3-^{14}C-serine in these incubation mixtures and which, therefore, may have appeared as materials A and C when the constituents of the reaction mixtures were separated by paper chromatography. The chromatographic behaviour of these compounds was investigated in the solvent systems used to isolate materials A and C. Figs. II, 28(a) and II, 28(b) are diagrams of the separations observed with serine acting as a reference compound. In addition to those compounds shown in these diagrams ^{14}C-formaldehyde, ^{14}C-formic acid and

Paper chromatographic behaviour of compounds
investigated for identity with materials A and C.

Diagrams of two-dimensional paper
chromatographic separations of some compounds
which could have been synthesised from $3-^{14}C-$
serine in the incubation mixtures in which the
synthesis of $^{14}C-TMP$ was studied (Figs. II, 21 and
II, 22.).

Thymine, Thymidine

5-Hydroxymethyluracil

Methionine

5-Hydroxymethyldeoxyuridine

Material A

Alanine, Glutamic acid

Serine

Phosphoserine

Hypoxanthine

Inosinic acid

R_F in *iso*-propanol - water - HCl

R_F in *n*-butanol - water
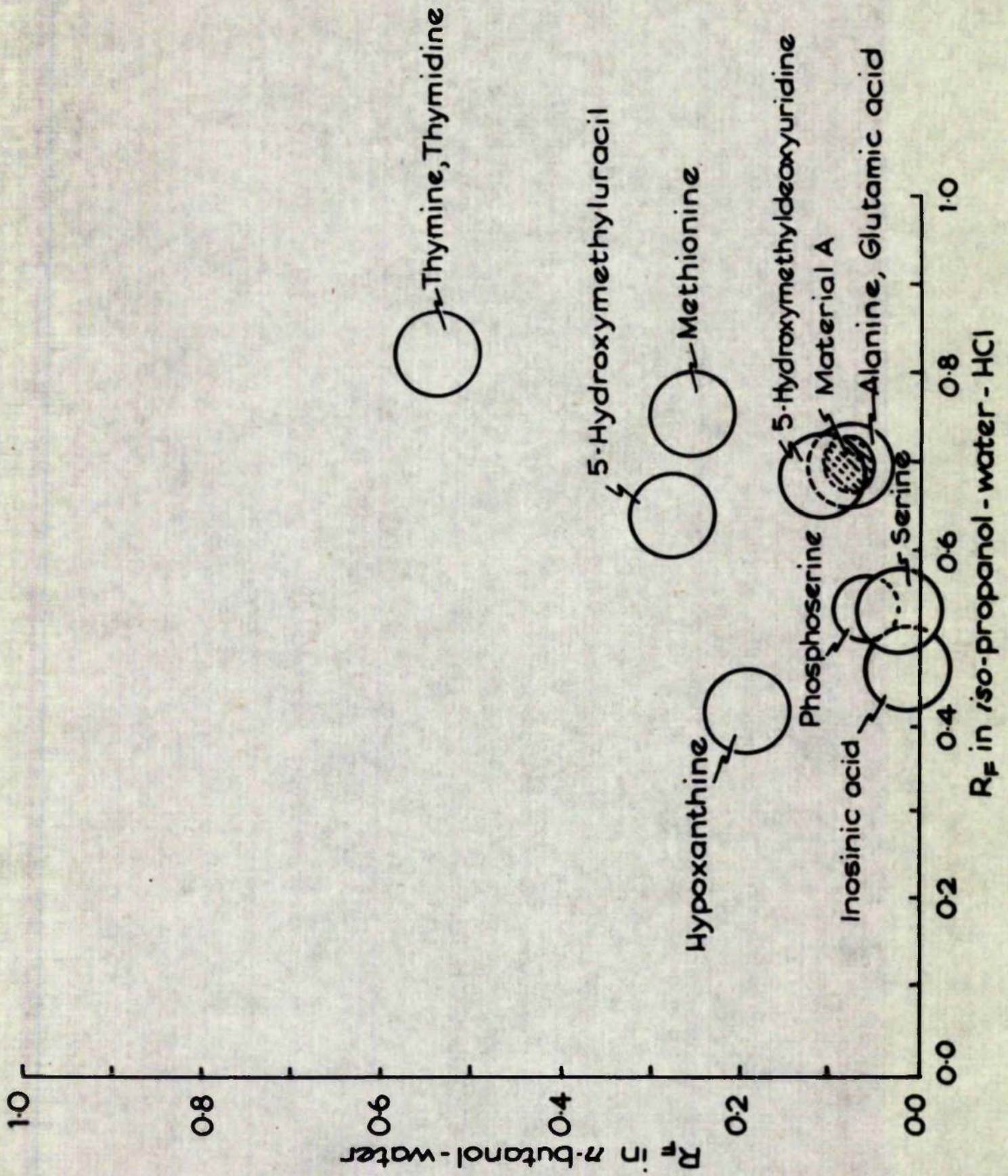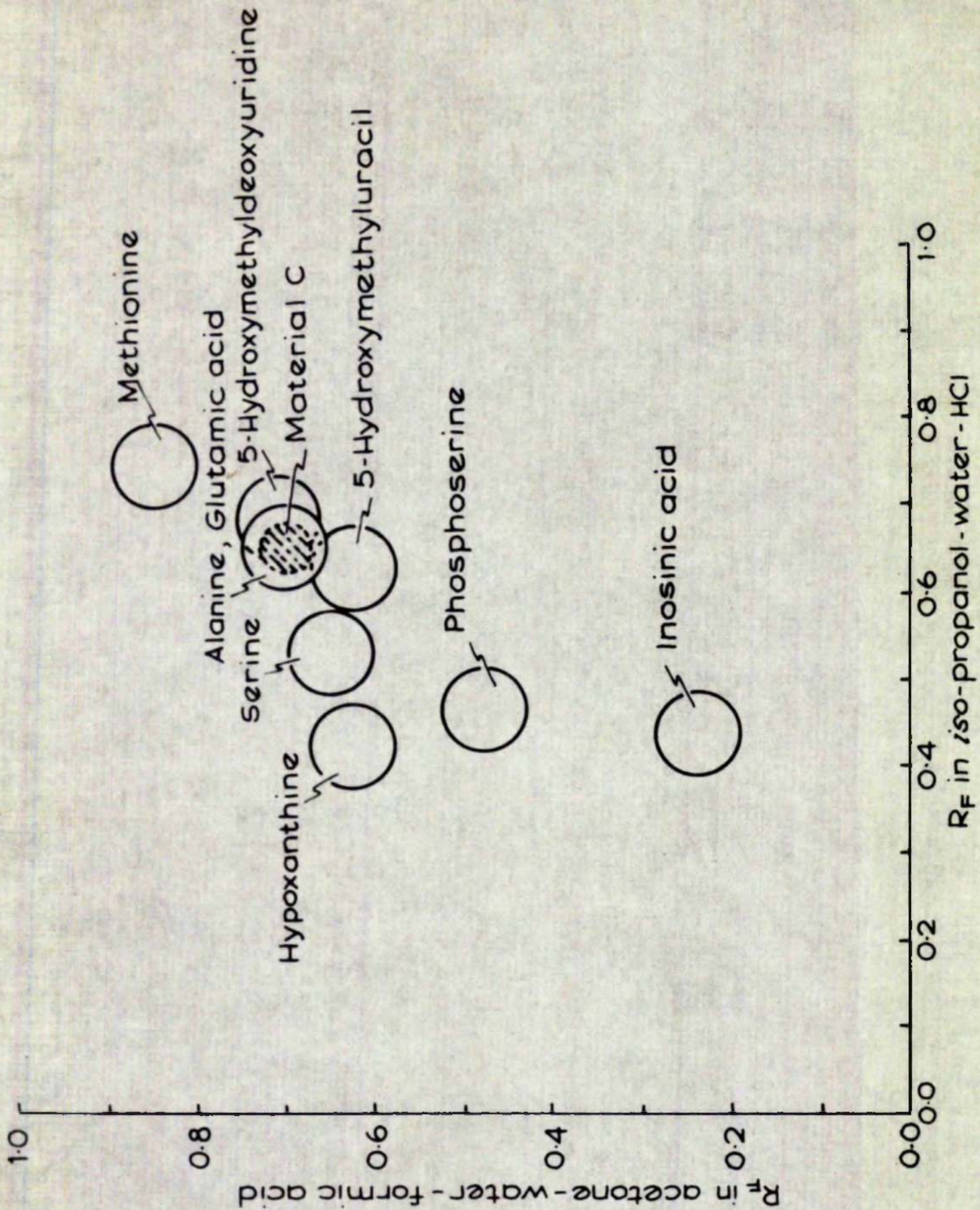
Figure II. 28(b)

[14]C-pyruvate were chromatographed in these systems, but no trace of radioactivity due to these materials remained on the chromatograms after drying.

Fig. II, 28(a) shows that the compounds corresponding most closely to material A are alanine, glutamic acid and 5-hydroxymethyldeoxyuridine. Accordingly material A was mixed with carrier alanine and 5-hydroxymethyldeoxyuridine and submitted to paper chromatography in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water) followed by autoradiography. Figs. II, 29(a) and II, 29(b) show that the ^{14}C-labelled material coincided in position with the carrier alanine. The mixture of material A, alanine and 5-hydroxymethyldeoxyuridine was treated with 0.1 ml. 0.1N-HCl in a sealed tube at 100° C. for 24 hours, conditions which have been shown to degrade 5-hydroxymethyldeoxyuridine to 5-hydroxymethyluracil (Section II, 13). Two-dimensional paper chromatography (solvents, iso-propanol-water-HCl and n-butanol-water) followed by autoradiography showed that there was no ^{14}C-labelled material associated with the ultraviolet-absorbing spot of 5-hydroxymethyluracil. All the radio activity present on this chromatogram was coincident with the carrier alanine.

Fig. II, 28(b) shows that the compounds most

Elucidation of the structures of materials A
and C (Figs. II, 21 and II, 22).

(a) Photograph and (b) autoradiograph
of a two-dimensional paper chromatographic
separation of a mixture of alanine, 5-hydroxy-
methyldeoxyuridine and material A.   Material A
was isolated from incubation mixtures by the
procedure described in Fig. II, 21.   The alanine
spot was developed by spraying with ninhydrin.

Figure II, 29 (a).

Figure XI, 20 (b).

Rg in iso-propanol-water-HCl.

Rg in n-butanol-water.

closely corresponding to material C are again alanine, glutamic acid and 5-hydroxymethyldeoxyuridine. A comparison of Fig. II, 30 with Fig. II, 22 also shows the resemblance in chromatographic properties between material C and alanine, glutamic acid and 5-hydroxymethyl-deoxyuridine. Accordingly, a mixture of material A and alanine was submitted to paper chromatography in two dimensions (solvents, iso-propanol-water-HCl and acetone-water-formic acid) followed by autoradiography. Figs. II, 31(a) and II, 31(b) show that the ^{14}C-labelled material coincided with the carrier alanine.

Thus both material A and material C have been identified tentatively as alanine or glutamic acid (or a mixture of both). The conclusion that materials A and C were identical compounds was confirmed by submitting a mixture of alanine, 5-hydroxymethyldeoxyuridine and material C to the same treatment as undergone by the corresponding mixture of alanine, 5-hydroxymethyldeoxy-uridine and material A. The ^{14}C-labelled material coincided exactly with the spot of alanine after each chromatographic separation. No ^{14}C-labelled material was found to be associated with the ultraviolet-absorbing spots of 5-hydroxymethyldeoxyuridine or 5-hydroxymethyluracil after acid hydrolysis. Similarly, the ^{14}C-labelled material coincided exactly with the

Elucidation of the structures of materials A and
C (Figs. II, 21 and II, 22).

Photograph of a two-dimensional paper
chromatographic separation of alanine, glutamic
acid, 5-hydroxymethyldeoxyuridine and serine.
The aminoacid spots were developed by spraying
with ninhydrin.
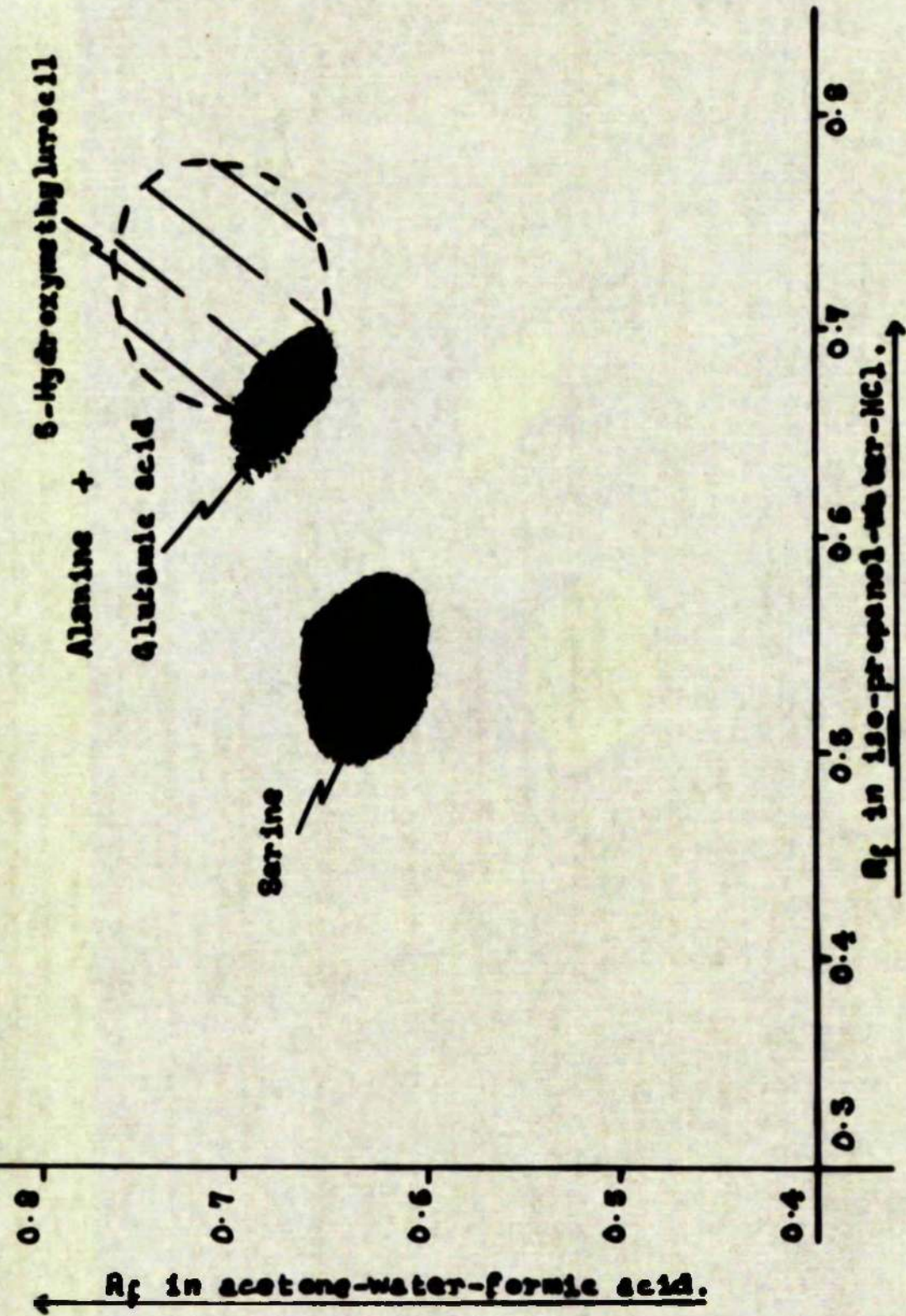
Figure II, 30.

Elucidation of the structures of materials A and
C (Figs. II, 21 and II, 22).

      (a) Photograph and (b) autoradiograph
of a two-dimensional paper chromatographic
separation of a mixture of alanine and material
C.   Material C was isolated from incubation
mixtures by the procedure described in Fig. II,
22.   The alanine spot was developed by spraying
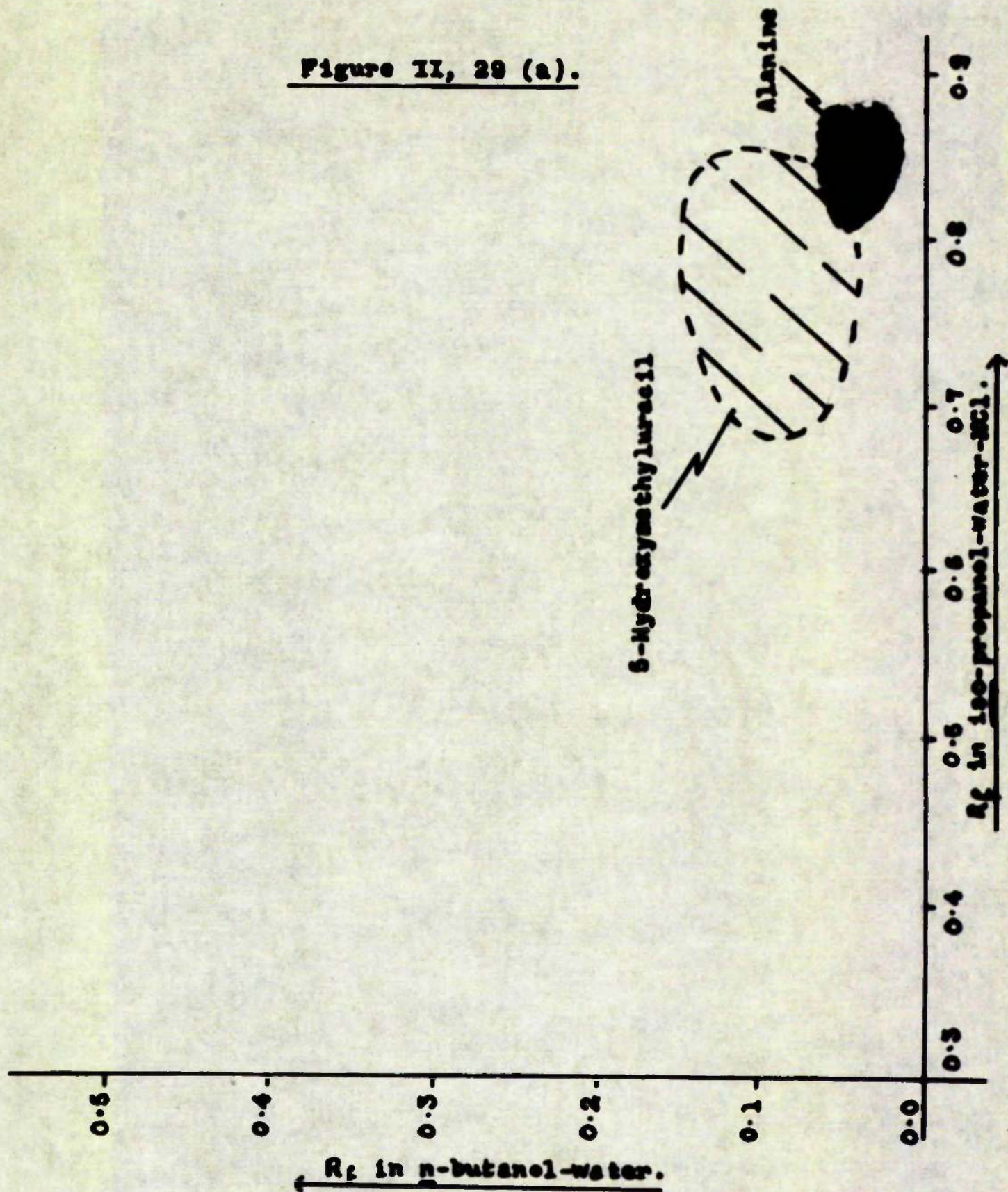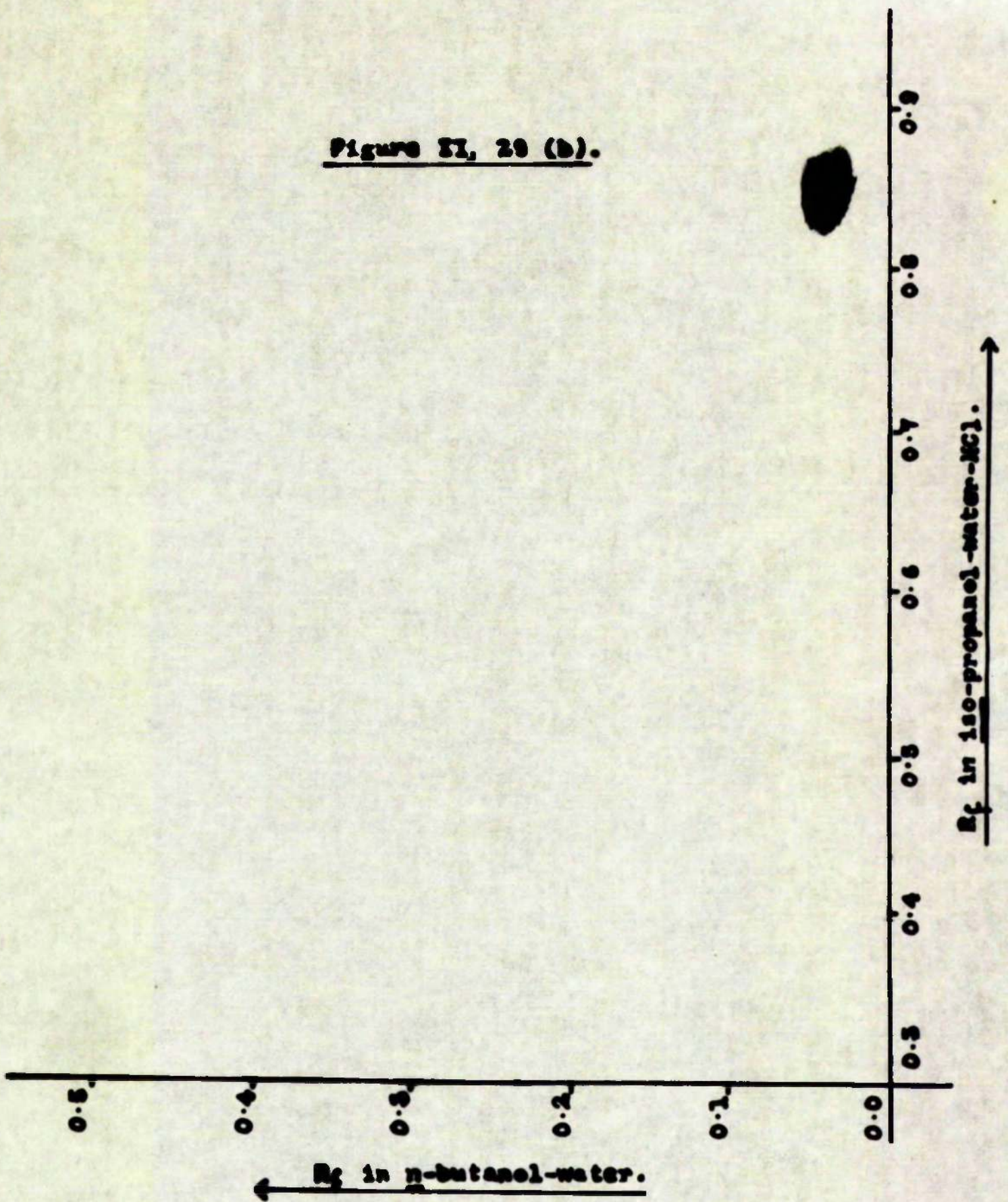with ninhydrin.

Figure II, 31 (a).
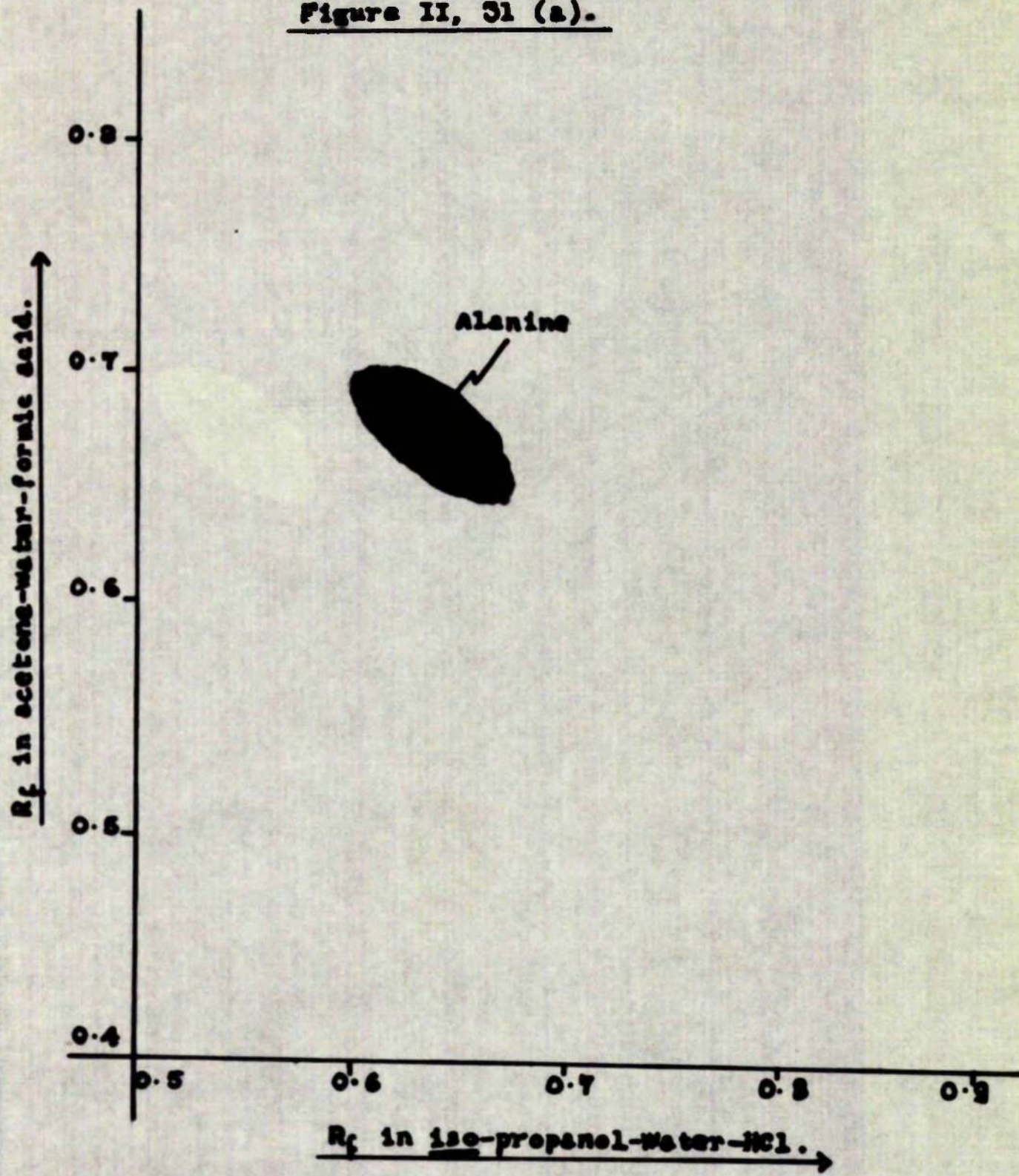
Figure II, 31 (b).
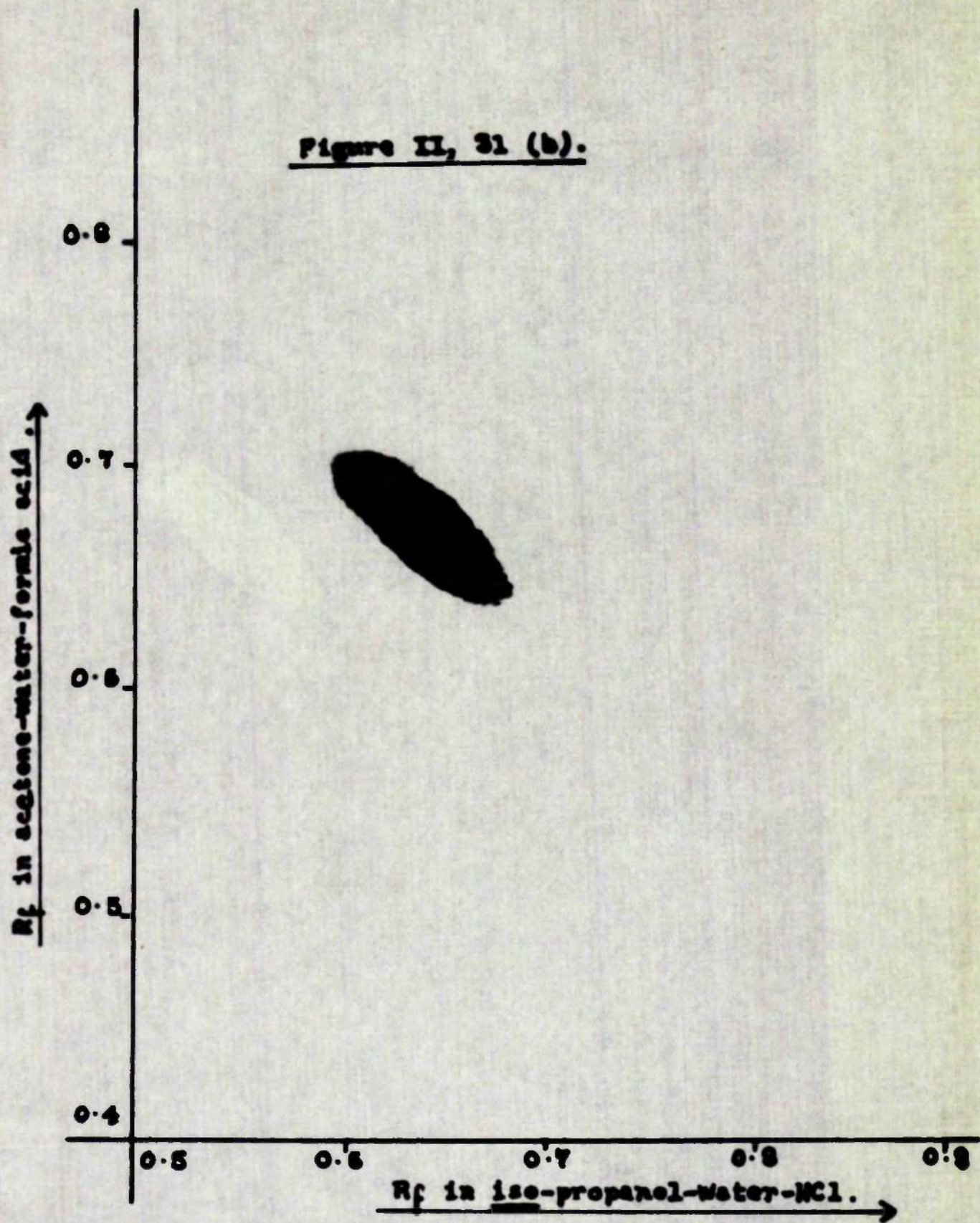
spot of carrier alanine when a mixture of alanine and material A was submitted to paper chromatography in two dimensions (solvents, iso-propanol-water-HCl and acetone-water-formic acid) followed by autoradiography.

Final confirmation of the structures of materials A and C was obtained by treatment of the compounds in the presence of carrier alanine with chloramine-T and by paper chromatography of the materials with carrier alanine (solvent, phenol-water). The acetaldehyde produced by degradation of the alanine by chloramine-T was trapped in a solution of 2:4-dinitrophenylhydrazine in $2N-H_2SO_4$. The acetaldehyde-2:4-dinitrophenylhydrazone was extracted with CCl_4, plated on to planchettes and counted. The derivative from both material A and material C was found to be radioactive. Treatment of $3-^{14}C$-serine under the same conditions did not yield a radioactive derivative. The paper chromatography of materials A and C confirmed that most of the radioactivity was associated with the carrier alanine. A small proportion of ^{14}C-labelled material was in the position occupied by glutamic acid.

Thus both materials A and C have been found to consist of alanine together with a small proportion of glutamic acid. This result is in agreement with observations made during a study of the synthesis of

alanine from serine by washed whole-cell suspensions of Esch. coli PA/15 (Section II, 7). In the original fractionations of deproteinised reaction mixtures by paper chromatography (solvent, n -butanol-water) material A has been included in the nucleotide fraction (R_F 0.0 to 0.05) presumably because of the presence of a high concentration of salts which had the effect of retarding the migration of the material. Thus material A has reappeared as material C on further paper chromatographic separation of the nucleotide fractions.

The chromatographic properties of material B (Figs. II, 21 and II, 23) resembled those of 5-hydroxymethyluracil. Accordingly a mixture of material B and 5-hydroxymethyluracil was submitted to chromatography in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water) followed by autoradiography. No trace of ^{14}C-labelled material was found to be coincident with the ultraviolet-absorbing spot of carrier 5-hydroxy-methyluracil. The chromatographic behaviour of material D (Fig. II, 23) did not resemble that of a 5-hydroxy-methyl-pyrimidine. As has been shown previously (Section II, 19), material D was not identical with thymine or thymidine.

Deproteinised reaction mixtures from which ^{14}C-TMP had been isolated were chromatographed on paper with carrier 5-hydroxymethyluracil and 5-hydroxymethyl-

deoxyuridine (solvent, n-butanol-water). The carrier 5-hydroxymethyluracil and 5-hydroxymethyldeoxyuridine were isolated and rechromatographed on paper in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water). No ^{14}C-labelled material was found to be coincident with the ultraviolet-absorbing spots of carrier 5-hydroxymethyluracil and 5-hydroxymethyldeoxy-uridine. The 5-hydroxymethyldeoxyuridine fraction was hydrolysed to 5-hydroxymethyluracil by heating with 0.1N-HCl in a sealed tube at 100° C. for 24 hours and rechromatographed on paper, but again no ^{14}C-labelled material was found to be associated with the ultraviolet-absorbing spot of carrier 5-hydroxymethyluracil. The nucleotide fractions of these reaction supernatants were mixed with carrier 5-hydroxymethyluracil and hydrolysed by (a) heating with 2N-HCl at 100° C. for one hour or (b) heating with 0.1N-HCl in a sealed tube at 100° C. for 24 hours. The hydrolysis products were chromato-graphed on paper in two dimensions (solvents, iso-propanol-water-HCl and n-butanol-water). No ^{14}C-labelled material was found to be coincident with the ultraviolet-absorbing spot of carrier 5-hydroxymethyl-uracil in either case.

Thus no 5-hydroxymethyl intermediates have been isolated from the incubation mixtures in which ^{14}C-TMP

was synthesised. Since the chemical synthesis of
5-hydroxymethyldeoxyuridylic acid was not successful
(Section II, 13) and therefore the nucleotide material
isolated from the incubation mixtures could not be
hydrolysed after admixture with authentic 5-hydroxy-
methyldeoxyuridylic acid, it is still possible that
a small quantity of ^{14}C-labelled 5-hydroxymethyl-
deoxyuridylic acid has been present in these incubation
mixtures. The 5-hydroxymethyl group of 5-hydroxymethyl-
deoxycytidylic acid present in bacteriophage DNA has been
shown to be much more acid-labile (towards certain acids)
than the 5-hydroxymethyl group of 5-hydroxymethyldeoxy-
cytosine (Wyatt and Cohen, 1952). Thus, although
5-hydroxymethyluracil is stable to acid hydrolysis,
the 5-hydroxymethyl group of 5-hydroxymethyldeoxy-
uridylic acid may be hydrolysed more readily. If this
was the case, acid hydrolysis of 5-hydroxymethyldeoxy-
uridylic acid would yield unlabelled uracil rather than
^{14}C-labelled 5-hydroxymethyluracil.

21. Comparison of deoxyuridine, uridine and deoxyuridylic
     acid as one-carbon unit acceptors in the synthesis
     of 14C-TMP.

In preliminary experiments to estimate the
relative efficiencies of UDr, UR and dUMP as one-carbon

unit acceptors in the synthesis of ^{14}C-TMP under a variety of conditions, the deproteinised reaction mixtures, to which carrier TMP had been added, were submitted to paper chromatography as bands (solvent, n-butanol-water). The nucleotide fractions (R_F 0.0 to 0.05) were rechromatographed on paper in two dimensions (solvents, iso-propanol-water-HCl and acetone-water-formic acid). The radioactive materials were located by autoradiography and, in each case, the distribution of the ^{14}C-labelled material was the same as that observed previously (Fig. II, 22). The ultraviolet-absorbing spot of carrier TMP was eluted and counted, together with the other ^{14}C-labelled materials revealed by autoradiography. The amount of ^{14}C-TMP synthesised was calculated as a percentage of the total counts isolated. In later experiments, the same amount of carrier TMP was added to each incubation mixture (a) before incubation or (b) after incubation but before deproteinisation. The carrier TMP was isolated from the incubation mixtures, after deproteinisation had been effected by treatment with HCl (final concentration, normal) and heating at 100°C. for 10 minutes, by the paper chromatographic separations outlined above. The TMP was eluted, plated on to

planchettes and counted.   The specific activity
(counts/min./μmole) of the TMP was estimated by
eluting the planchettes with 4 ml. 0.1 N-HCl and
observing the extinction of the solution so obtained
at 260 mμ.

In the preliminary experiments, a cell-
free extract of Esch. coli PA/15 was incubated under
anaerobic conditions with 3-^{14}C-serine and (a) UDr,
(b) UR or (c) dUMP in the presence of the cofactors
ATP, FAH$_4$, TPNH and pyridoxal phosphate.   Table II,
9 shows that there was a slightly lower proportion of
radioactivity associated with the carrier TMP when
UR replaced UDr as the one-carbon unit acceptor in
the incubation mixture.   The extremely small difference
obtained when dUMP replaced UDr in this system is of
doubtful significance.

These experiments were extended to a study of
(a) the effect of vitamin B$_{12}$ and (b) the effect of
replacing TPNH with DPNH by using cell-free extracts of
Esch. coli 113/3, a methionine- or vitamin B$_{12}$- less
mutant.   A cell-free extract of Esch. coli 113/3 was
incubated under anaerobic conditions with 3-^{14}C-serine
and (i) UDr and TPNH, (ii) UR and TPNH or (iii) UR
and DPNH in the presence or absence of vitamin B$_{12}$.   The
cofactors present in the incubation mixtures were ATP,

Table II, 9

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

Tubes contained a cell-free extract of Esch. coli PA/15 (20 mg. protein), ATP (120 μmoles), FAH$_4$ (12 μmoles), MgSO$_4$ (50 μmoles), TPN (4 μmoles), glucose-6-phosphate (40 μmoles), pyridoxal phosphate (0.3 μmoles), 3-^{14}C-serine (60 μmoles, 30 μc) and (i) UDr (60 μmoles), (ii) UR (60 μmoles) or (iii) dUMP (60 μmoles) in Tris buffer (0.1M, pH 7.2), total volume 6 ml. Incubation was carried out under anaerobic conditions at 37° C. for 4 hours. At the end of the incubation period the reaction mixtures were deproteinised by heating at 100° C. for 10 minutes, centrifuged (5,000 g) and the supernatants were chromatographed on paper with n-butanol water. The bands of R$_F$ 0.0 to 0.05 were eluted and the nucleotide material so obtained was rechromatographed on paper with carrier TMP in two dimensions (isopropanol-water-HCl and acetone-water-formic acid). The radioactive materials were located by autoradiography, eluted and counted. The amount of ^{14}C-labelled material associated with the carrier TMP was estimated as a percentage of the total counts isolated.

Table II, 9

One-carbon unit acceptor pyrimidine	Total counts/min. isolated	Counts/min. in TMP: percentage of total isolated
Deoxyuridine	82,675	7.2
Uridine	98,265	6.0
Deoxyuridylic acid	108,880	6.8

FAH_4, $MgSO_4$ and pyridoxal phosphate. Table II, 10
shows that the proportion of radioactivity
associated with the carrier TMP was decreased
significantly when (a) UR replaced UDr in the system
containing Vitamin B_{12} (by 8%); (b) vitamin B_{12} was
omitted from incubation mixtures containing UDr and
TPNH (by 10%); (c) DPNH replaced TPNH in the system
containing UR and vitamin B_{12} (by 30%).

The results obtained from these preliminary
experiments show, by a comparison of Tables II, 9 and
II, 10, that there is a considerable difference between
the thymidylate synthetase activity present in Esch.
coli PA/15 and that present in Esch. coli 113/3. The
results indicate that UDr functions more efficiently
as a one-carbon unit acceptor than does UR. They
also show that the rate at which ^{14}C-TMP was synthesised
from UDr in the presence of cell-free extracts of Esch.
coli 113/3 was increased slightly by the addition of
vitamin B_{12}. The decrease in ^{14}C-TMP synthesised when
DPNH was replaced by TPNH in incubation systems
containing UR may be due to (i) the reduction of the
ribose to deoxyribose being TPNH-dependent; (ii) the
lower concentration of $N-CH_2OH-FAH_4$ (or in effect, of
one-carbon units) observed in systems containing DPNH
as compared with those containing TPNH (Table II, 10);

Table II, 10

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch coli 113/3.   Effect of vitamin B_{12} on thymidylic acid synthesis.

Tubes contained a cell-free extract of Esch. coli 113/3 (12 mg. protein), ATP (60 μmoles), FAH_4 (5 μmoles), $MgSO_4$ (40 μmoles), pyridoxal phosphate (0.1 μmoles), 3-^{14}C-serine (30 μmoles, 15 μc), vitamin B_{12} (2 μg) and (i) UDr (20 μmoles), TPN (2 μmoles) and glucose-6-phosphate (20 μmoles); (ii) UR (20 μmoles), TPN (2 μmoles) and glucose-6-phosphate (20 μmoles); or (iii) UR (20 μmoles) and DPNH (2 μmoles) in Tris buffer (0.1M, pH 7.2), total volume 2 ml.   Incubation was carried out under anaerobic conditions at 37° C. for 4 hours.   At the end of the incubation period the reaction mixtures were treated with 0.25 ml. 10N-HCl, heated at 100° C. for 10 minutes and centrifuged (5,000 g).   The ^{14}C-labelled TMP was isolated with added carrier TMP by paper chromatography as in Table II, 9 and estimated as a percentage of the total counts isolated.

Table II, 10

No.	System containing			Omissions	Counts/min in TMP:percentage of total isolated
1.	Deoxyuridine	+	TPNH	None	1.37
2.	Deoxyuridine	+	TPNH	Vitamin B_{12}	1.22
3.	Uridine	+	TPNH	None	1.27
4.	Uridine	+	TPNH	Vitamin B_{12}	1.19
5.	Uridine	+	DPNH	None	0.89

Statistical analysis - Student's "t" test

Comparison	"t" test value	Significance level (P)
1. v 2.	4.671	>0.01
1. v 3.	2.513	$>0.05 <0.02$
2. v 4.	0.413	<0.05
3. v 4.	0.946	<0.05
3. v 5.	6.67	>0.01

or (iii) a combination of (i) and (ii).

In the later experiments, a cell-free extract of Esch. coli PA/15 was incubated under anaerobic conditions with $3-^{14}C$-serine and (a) UDr or (b) dUMP in the presence of the cofactors ATP, FAH_4, $MgSO_4$, DPNH, TPNH and pyridoxal phosphate. After incubation for one hour, 1.0ml. of each incubation mixture was withdrawn. After incubation for $2\frac{1}{2}$hours, a further 1.0 ml. of each incubation mixture was withdrawn. Carrier TMP was added to each 1 ml. sample before deproteinisation was effected. The carrier TMP was re-isolated by paper chromatography as described in Fig. II, 22 and the specific activity was estimated. Although duplication is not good, the results in Table II, 11 do show that most of the ^{14}C-TMP was synthesised in the first hour of incubation. The results show that the addition of a pyrimidine one-carbon unit acceptor has a very marked effect on the specific activity of the carrier TMP isolated from the incubation mixtures. However, the scatter in the results makes it impossible to draw any conclusions as to the relative efficiencies of UDr and dUMP as one-carbon unit acceptors.

If all the ^{14}C-material isolated with the carrier TMP from these reaction mixtures is composed

Table II, 11

Synthesis of 14C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

Tubes contained a cell-free extract of Esch. coli PA/15 (3.6 mg. protein), ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), 3-14C-serine (30 μmoles, 15 μc) and (a) UDr (30 μmoles) or (b) dUMP (30 μmoles) in Tris buffer (0.1M, pH 7.2), total volume 3 ml. Incubation was carried out under anaerobic conditions at 37° C. for (i) 1 hour; (ii) 2 hours; or (iii) 2½ hours. Carrier TMP (5 μmoles) was added to 1.0 ml. samples of each incubation mixture before deproteinisation was effected by adding 0.3 ml. 5N-HCl, heating at 100° C. for 10 minutes and centrifuging (5,000 g).

The TMP was isolated from the supernatants by paper chromatography (solvents, n-butanol-water, iso-propanol-water-HCl and acetone-water-formic acid). The specific activity (counts/min./μmoles) was estimated in the conventional manner.

The TMP isolated was hydrolysed to thymine by heating with 2N-HCl at 100° C. for one hour. The thymine was isolated by paper chromatography (solvents, n-butanol-water and acetone-water-formic acid). The

specific activity (counts/min./μmole) was
estimated in the conventional manner.

Table II. 11

One-carbon unit acceptor pyrimidine	Time of incubation hrs.	Specific Activity (counts/min./μmole)	
		of TMP	of thymine from TMP
None	2	8,300	43
Deoxyuridine	1	22,100	112
Deoxyuridine	$2\frac{1}{2}$	36,300	261
Deoxyuridine	1	21,200	108
Deoxyuridine	$2\frac{1}{2}$	28,400	127
Deoxyuridylic acid	1	16,000	76
Deoxyuridylic acid	$2\frac{1}{2}$	25,200	152
Deoxyuridylic acid	1	20,500	106
Deoxyuridylic acid	$2\frac{1}{2}$	27,400	124

entirely of ^{14}C-TMP, degradation of the TMP isolated
to thymine will yield ^{14}C-thymine of the same specific
activity as that of the parent TMP. Accordingly, in
an attempt to obtain final confirmation that the ^{14}C-
labelled material associated with the carrier TMP was
^{14}C-TMP, the isolated TMP was hydrolysed with 2N-HCl
at 100° C. (Section II, 16). The thymine produced
was isolated by two-dimensional paper chromatography
(solvents, n-butanol-water and acetone-water-formic
acid) and the specific activity was determined. Table
II, 11 shows that the specific activity of this
thymine was very low compared with that of the parent
TMP. Thus only a very small proportion of the
radioactivity associated with the carrier TMP was,
in fact, present in ^{14}C-TMP. However, Table II, 11
shows that the specific activities of the thymine
samples are proportional to those of the parent TMP
samples and therefore the conclusions drawn from a
consideration of the TMP specific activities are still
valid.

Fink et al. (1956b) have shown that methyl-
^{14}C-thymine is metabolised by rat liver slices to yield
5-hydroxymethyluracil, uracil-5-carboxylic acid,
4:5-dihydrothymine (DHT), β-ureido-iso-butyric acid
(BUIB), β-amino-iso-butyric acid (BAIB), urea, alanine,

glucose and thymine glycol. Fig. II, 32 shows that
these metabolic products can be separated from each
other and from thymine, thymidine and TMP by two-
dimensional paper chromatography (solvents, tert-
butanol-methylethylketone-water-NH_4OH and tert-
butanol-methylethylketone-water-formic acid).
Accordingly, the TMP isolated from the incubation
mixtures was hydrolysed (2N-HCl at 100° C.) and the
hydrolysis products were submitted to two-dimensional
paper chromatography in this solvent system (Fig. II,
32), followed by autoradiography.   As the specific
activity of the thymine produced by the hydrolysis of
the carrier TMP was very low, it was difficult to
obtain satisfactory autoradiographs.   However, Fig.
II, 33 does show the presence of radioactive material
which is coincident with the ultraviolet-absorbing
spot of carrier thymine.   No ^{14}C-labelled material was
found to be coincident with the spots of carrier DHT
and BUIB, but a small amount was found to coincide with
the spot of carrier BAIB.   The natures of the other
radioactive materials shown in Fig. II, 33(b) were not
determined.

The effect of pre-incubation addition of
carrier TMP to incubation systems synthesising ^{14}C-TMP
was determined by incubating a cell-free extract of
Esch. coli PA/15 under anaerobic conditions with

Figure II, 32

Paper chromatographic separation of some metabolic products of thymine.

       Two-dimensional paper chromatographic separation of thymine, thymidine and TMP together with some products of the metabolism of these compounds. R_F values are those quoted by Fink et al. (1956b).
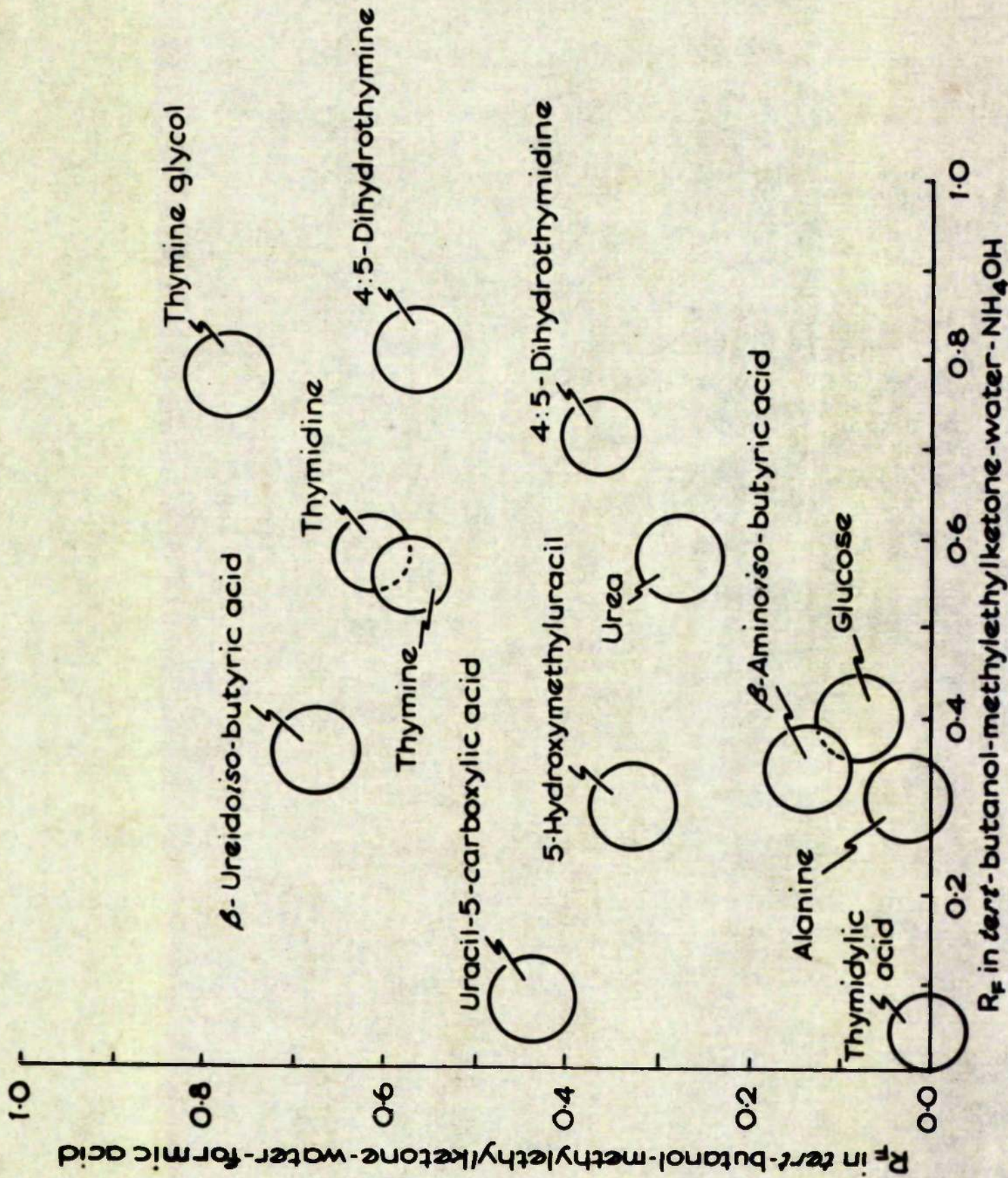
Figure II, 32

Hydrolysis of ^{14}C-labelled "thymidylic acid" isolated from reaction mixtures containing a cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and (a) UDr or (b) dUMP.

(a) Ultraviolet photograph and (b) autoradiograph of a two-dimensional paper chromatographic separation of the hydrolysis products of TMP isolated from incubation mixtures containing a cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and (a) UDr or (b) dUMP, together with carrier BAIB, BUIB, DHT and uracil-5-carboxylic acid. The TMP was isolated with carrier TMP by the paper chromatographic techniques described in Fig. II, 22. The TMP was hydrolysed by heating with 2N-HCl at 100° C. for 30 minutes.

Complete TMP-synthetic incubation mixture - A cell-free extract of Esch. coli PA/15 (3.6 mg. protein), ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), 3-^{14}C-serine (30 μmoles, 15 μc) and (a) UDr (30 μmoles) or (b) dUMP (30 μmoles) in Tris buffer (0.1M, pH 7.2), total volume 3 ml. Incubation was carried out under anaerobic conditions for (i) 1 hour or (ii) 2½ hours at 37° C. Carrier TMP (5 μmoles) was added to 1.0 ml. samples of each incubation mixture before deproteinisation.
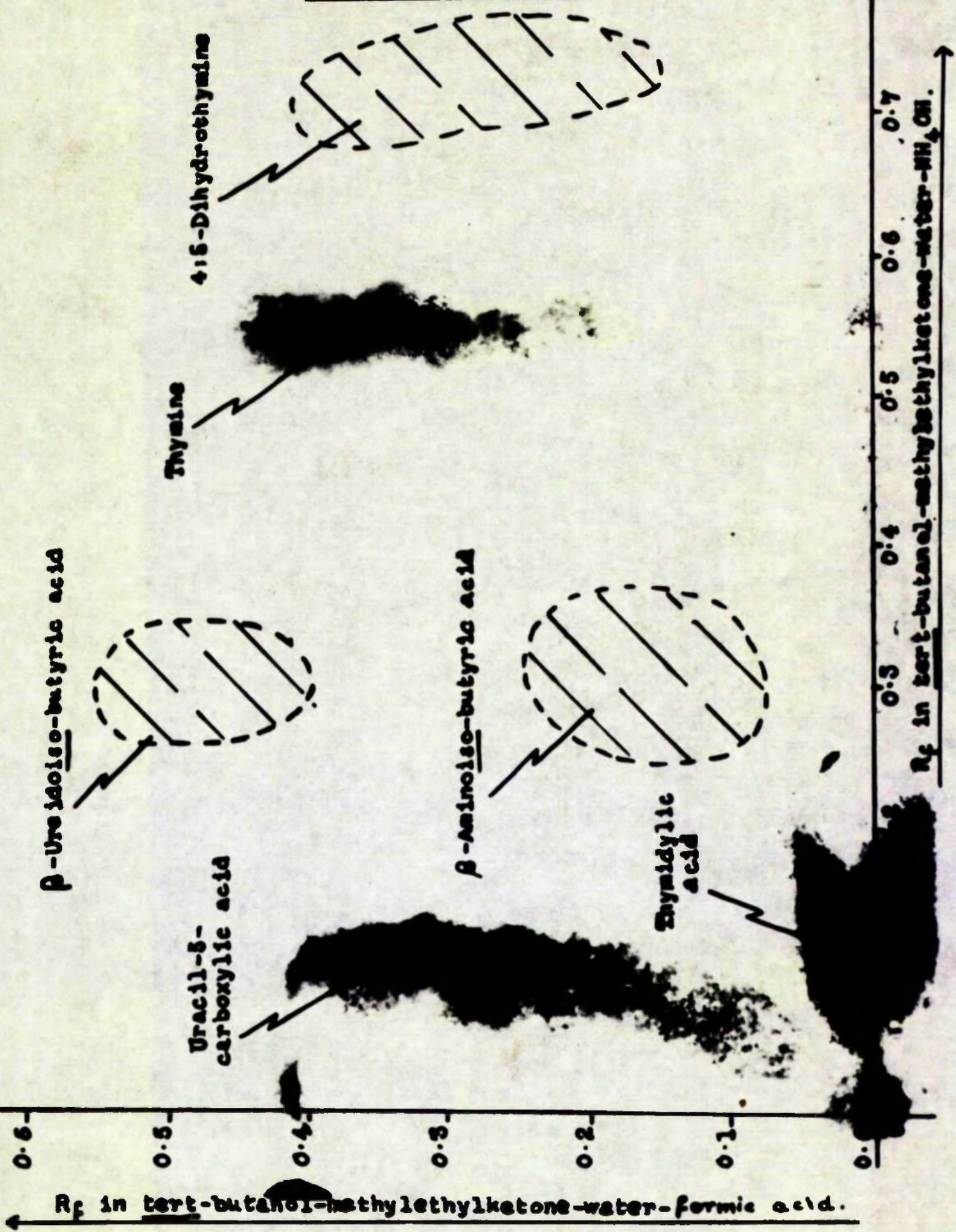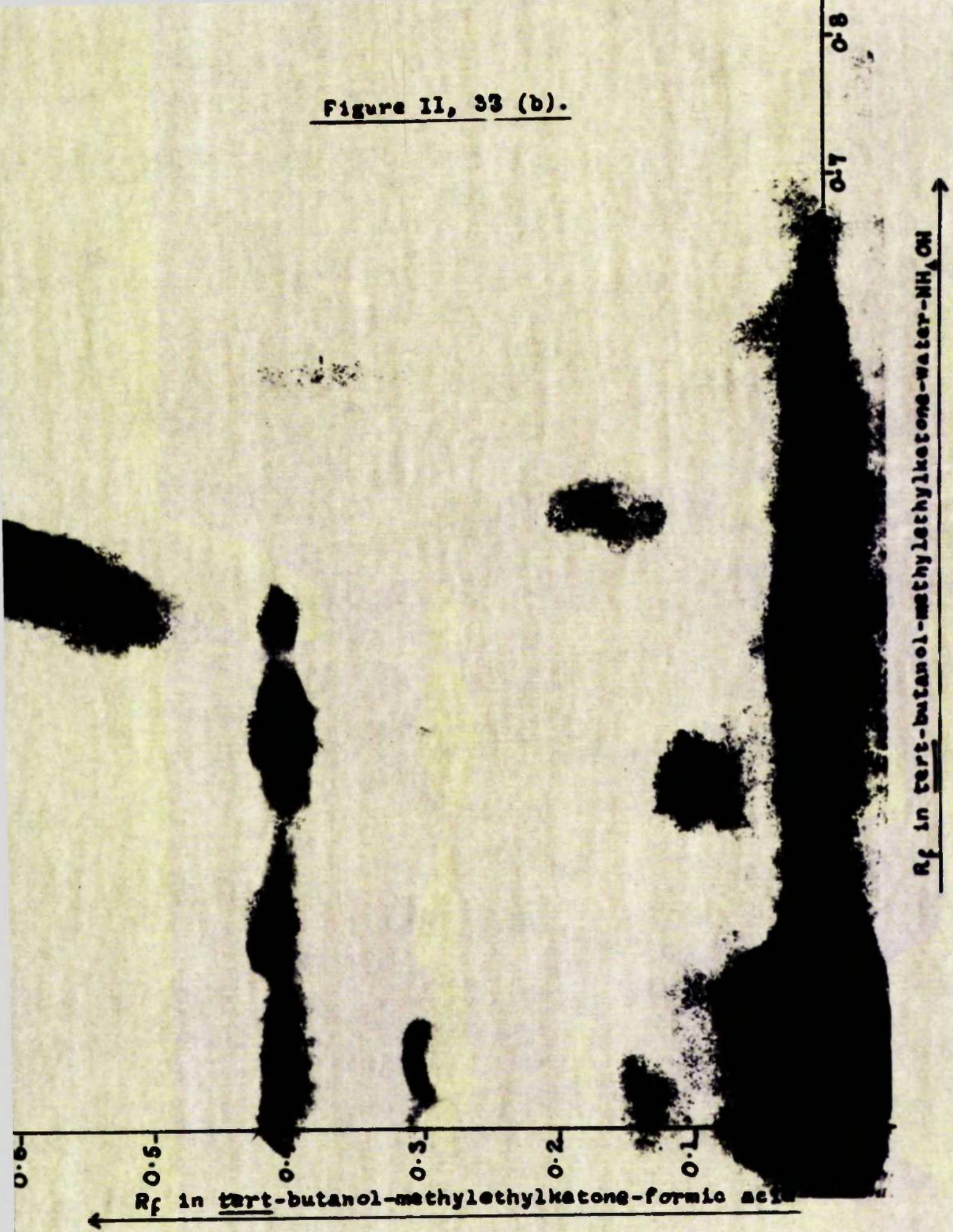
Figure II, 85 (a).

Figure II, 33 (b).

R_f in tert-butanol-methylethylketone-water-NH_4OH

R_f in tert-butanol-methylethylketone-formic acid

$3-^{14}C$-serine and (a) UDr or (b)UR in the presence
or absence of TMP.   The cofactors present were
ATP, FAH$_4$, MgSO$_4$, pyridoxal phosphate, DPNH and
TPNH.   Carrier TMP was added to each incubation
mixture which did not contain carrier TMP after
incubation but before deproteinisation was effected.
The carrier TMP was reisolated from each reaction
mixture by the paper chromatography described in
Fig. II, 22 and the specific activity was estimated.
The results in Table II, 12 show again that the
specific activity of the TMP isolated was increased
greatly when a pyrimidine one-carbon unit acceptor
was included in the incubation mixture.   The specific
activity of the carrier TMP isolated was higher when
carrier TMP had been added before incubation.   This
difference was small (7%) when UDr was the one-
carbon unit acceptor, large (44%) when UR was the
one-carbon unit acceptor.

The TMP isolated from these incubation
mixtures was   hydrolysed (2N-HCl at 100°C.) and the
thymine produced was isolated by two-dimensional paper
chromatography (solvents, tert-butanol-methylethylketone-
water-NH$_4$OH and tert-butanol-methylethylketone-water-
formic acid).   The specific activity of each thymine
sample was again much lower than that of the parent TMP

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15.

Tubes contained a cell-free extract of Esch. coli PA/15 (19 mg. protein), ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), 3-^{14}C-serine (30 μmoles, 15 μc), TMP (7 μmoles) and (i) UDr (30 μmoles) or (ii) UR (30 μmoles) in Tris buffer (0.1M, pH 7.2), total volume 3 ml. Incubation was carried out under anaerobic conditions at 37° C. for 3 hours. Deproteinisation was effected by adding 0.35 ml. 10N-HCl, heating at 100° C. for 10 minutes and centrifuging (5,000 g).

The specific activity (counts/min./μmole) (a) of the TMP and (b) of the thymine from this TMP was determined as in Table II, 11.

Table II, 12

		Specific Activity (counts/min./μmole)	
One-carbon unit acceptor pyrimidine	Omissions	of TMP	of thymine from TMP
None	TMP	12,800	182
Deoxyuridine	None	46,200	218
Deoxyuridine	TMP	43,300	610
Uridine	None	72,100	149
Uridine	TMP	49,400	259

(Table II, 12). Moreover, the thymine specific activities were not proportional to those of the parent TMP. The thymine specific activities show (i) that the addition of carrier TMP before incubation decreased the synthesis of ^{14}C-TMP; (ii) that more ^{14}C-thymine (from ^{14}C-TMP) was isolated from incubations containing UDr than from those containing UR; and (iii) that the specific activity of the thymine isolated was increased by the presence of UDr or UR in the incubation mixture.

This quantitative study of the synthesis of ^{14}C-TMP from 3-^{14}C-serine and (a) UDr, (b) UR or (c) dUMP in the presence of cell-free extracts of Esch. coli PA/15 has shown (i) that the ^{14}C-labelled material which was synthesised by these systems and which was not separated from added carrier TMP by extensive paper chromatography (Section II, 19) was a mixture of compounds; (ii) that a small proportion (about 0.01%) of the ^{14}C-labelled material associated with the carrier TMP was in fact ^{14}C-TMP (as shown by acid hydrolysis to ^{14}C-thymine); (iii) that a large proportion(at least 70%) of the ^{14}C-labelled material associated with the carrier TMP was a pyrimidine derivative other than TMP (the increase in TMP specific activity observed when (a) UDr, (b) UR or (c) dUMP was present in the incubation mixture indicates this since

there was little ^{14}C-thymine isolated as compared with
the other products - Tables II, 11 and II, 12); (iv) that
the metabolism of TMP synthesised in incubation mixtures
was not spared by the presence of carrier TMP; (v) that
the amount of ^{14}C-thymine isolated was decreased when
carrier TMP was present in the incubation mixture;
(vi) that UDr and dUMP were more efficient one-carbon
unit acceptors than was UR; (vii) that there was no
significant difference between UDr and dUMP in the rôle
of one-carbon unit acceptors.

22. Comparison of 3-^{14}C-serine, ^{14}C-formaldehyde and [14]C-
formate as one-carbon unit donors in the synthesis of
[14]C-TMP.

The replacement of 3-^{14}C-serine with [14]C-
formaldehyde in the incubation system used to study the
synthesis of ^{14}C-TMP has been described previously
(Section II, 19). The specific activity of the TMP
isolated from incubation mixtures containing [14]C-
formaldehyde in place of 3-^{14}C-serine was not determined.
However, it was concluded that 3-^{14}C-serine functions
much more efficiently as a one-carbon unit donor than
does ^{14}C-formaldehyde in this system since the specific
activity of the ^{14}C-TMP isolated from systems containing
[14]C-formaldehyde was too low to register on an X-ray
film.

When ^{14}C-formate replaced 3-^{14}C-serine in
the incubation mixture, carrier TMP was added to the
incubation mixture before deproteinisation was
effected. The carrier TMP was reisolated by paper
chromatography as described previously (Fig. II, 22)
and the specific activity was determined. Although
the ^{14}C-formate had the same activity (0.5 µc/µmole)
as the 3-^{14}C-serine it replaced, the results in Table
II, 13 show that the ^{14}C-TMP isolated had a much lower
specific activity when ^{14}C-formate was the one-carbon
donor than when 3-^{14}C-serine provided the one-carbon
units. Hydrolysis of the TMP yielded ^{14}C-thymine
which again was of much lower specific activity when
the source of the one-carbon units was ^{14}C-formate
than when it was 3-^{14}C-serine.

Thus serine was by far the most efficient of
the three one-carbon unit donors investigated. The
reason for formaldehyde being very inefficient is not
clear although it may be that the concentrations used
were toxic to the cell-free extracts. The very low
activity of the formate was expected since it has
been shown already that the synthesis of N-CH$_2$OH-FAH$_4$
from formate takes place at a very slow rate (Section
II, 11). Moreover, ^{14}C-formate has been shown not to
label the methyl group of DNA-thymine in growing Esch.

Synthesis of ^{14}C-labelled "thymidylic acid" by cell-free extracts of Esch. coli PA/15 and Esch. coli 15T-.

Tubes contained a cell-free extract of (a) Esch. coli PA/15 (3.6 mg. protein) or (b) Esch. coli 15T- (5 mg. protein), ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), UDr (30 μmoles) and (i) 3-^{14}C-serine (30 μmoles, 15 μc) or (ii) ^{14}C-formate (30 μmoles, 15 μc) in Tris buffer (0.1M, pH 7.2), total volume 3 ml. Incubation was carried out under anaerobic conditions at 37° C. for (1) 1 hour, (2) 2 hours, (3) 2½ hours or (4) 5 hours. Carrier TMP (5 μmoles) was added to 1.0 ml. samples of each incubation mixture before deproteinisation was effected by adding 0.3 ml. 5N-HCl, heating at 100° C. for 10 minutes and centrifuging (5,000 g).

The specific activity (counts/min./umole) (a) of the TMP and (b) of the thymine from this TMP was determined as in Table II, 11.

Table II, 13

One-carbon unit acceptor pyrimidine	One-carbon unit donor	Esch. coli mutant	Time of incubation hrs.	Specific Activity (counts/min/μmole).	
				of TMP	of thymine from TMP
None	$3-^{14}C$-serine	PA/15	2	8,300	43
Deoxyuridine	$3-^{14}C$-serine	PA/15	1	21,200	108
Deoxyuridine	$3-^{14}C$-serine	PA/15	$2\frac{1}{2}$	28,400	127
Deoxyuridine	^{14}C-formate	PA/15	2	584	42
Deoxyuridine	^{14}C-formate	PA/15	5	640	74
Deoxyuridine	$3-^{14}C$-serine	15T-	2	14,900	50
Deoxyuridine	$3-^{14}C$-serine	15T-	5	15,700	49

coli cells although it is freely utilised in
labelling the 2 and 8 positions of the purine
nucleus in the same system (Crosbie, 1958). The
synthesis of ^{14}C-TMP from UDr and ^{14}C-formate by
cell-free extracts of Esch. coli PA/15 may be by
(i) a slow reduction of formate to formaldehyde
or (ii) a slow reduction of N^5-formyl-FAH$_4$ to
N-CH$_2$OH-FAH$_4$. Neither of these processes takes
place in growing Esch. coli cells since, in these
systems, formate is (a) utilised rapidly in the
synthesis of purine and (b) oxidised rapidly to
CO$_2$. The reactions of formate in these two
systems (that is, in (i) growing Esch. coli cells
and (ii) cell-free extracts of Esch. coli) may be
represented by the scheme:

$$
\begin{array}{ccc}
& \text{Reduction} & \text{oxidation} \\
\text{HCHO} \longleftarrow\text{ -- -- -- -- --}\text{ Formate} & \longrightarrow & \text{CO}_2 \\
\text{FAH}_4 & N^{10}\text{-formyl-FAH}_4 \longrightarrow & \text{Carbon atoms 2} \\
& & \text{and 8 in purines.} \\
& \text{TPNH} \\
& \text{TPN} \\
& N\text{-CH}_2\text{OH - FAH}_4 \\
& | \quad \text{-UDr} \\
& \text{TMP-methyl}
\end{array}
$$
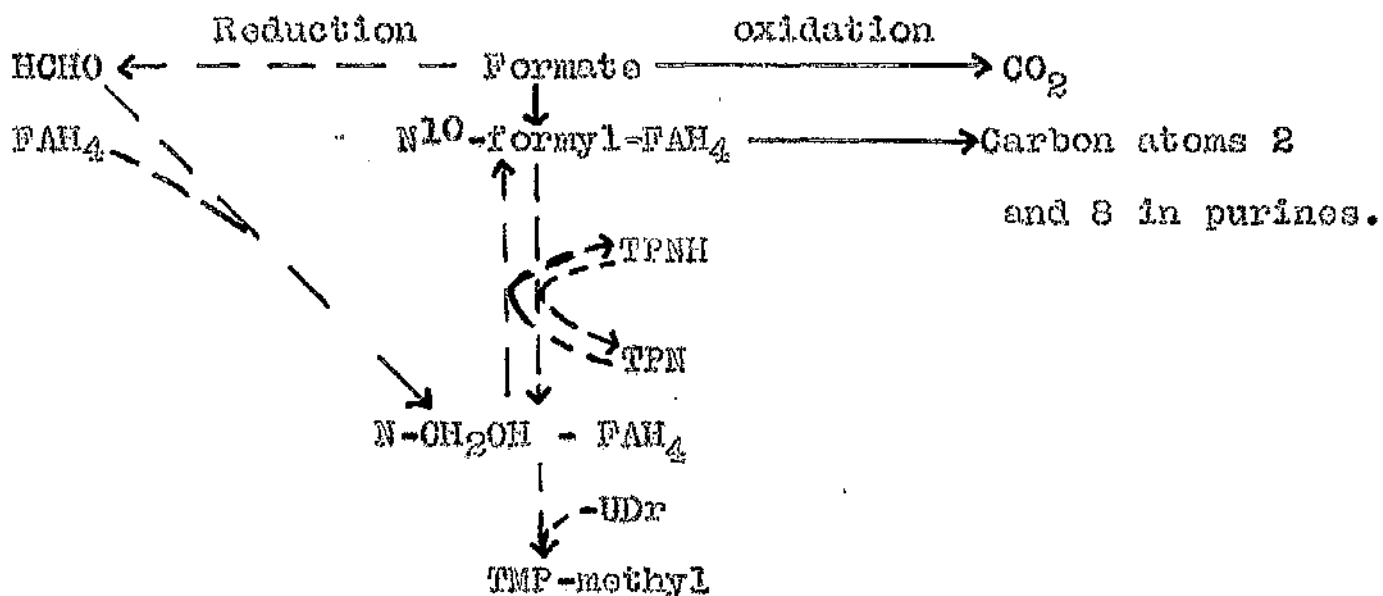
———— pathways in growing cells.

-------- pathways in cell-free extracts of resting cells.

23. Synthesis of ^{14}C-TMP by cell-free extracts of

Esch. coli 15T-.

As Esch. coli 15T- is a mutant which requires
either thymine or thymidine for growth, cell-free extracts
of this organism were not expected to catalyse the
synthesis of ^{14}C-TMP from UDr and 3-^{14}C-serine. The
enzyme block in this organism is thought to be at a
stage immediately before thymine (or TMP) on the synthetic
pathway. Therefore, a pool of the thymine precursor
immediately before the block may accumulate when cell-
free extracts of the mutant are incubated in the system
in which ^{14}C-TMP is synthesised by Esch. coli PA/15.

Accordingly, a cell-free extract of Esch. coli
15T-was incubated under anaerobic conditions with
3-^{14}C-serine and UDr in presence of the cofactors ATP,
FAH$_4$, MgSO$_4$, DPNH, TPNH and pyridoxal phosphate. Carrier
TMP was added before deproteinisation was effected. Two-
dimensional paper chromatographic separations of the
reaction supernatant followed by autoradiography revealed
the same pattern of distribution of radioactive materials
as had been obtained from reaction mixtures containing
UDr, 3-^{14}C-serine and a cell-free extract of Esch. coli
PA/15 (Figs. II, 21 and II, 22), with ^{14}C-labelled
material coincident with the ultraviolet-absorbing spots
of carrier TMP.

The specific activity of the carrier TMP, isolated as described previously (Fig. II, 22), was determined. The TMP was hydrolysed also to thymine (2N-HCl at 100° C.) and the specific activity of the isolated thymine was determined. The results in Table II, 13 show that (i) there was no significant difference between the specific activities of TMP isolated from reaction mixtures which had been incubated for 2 hours or for 5 hours; (ii) the specific activity of the carrier TMP was about 50% of that of the carrier TMP isolated from a corresponding incubation mixture containing a cell-free extract of Esch. coli PA/15; and (iii) the specific activity of the thymine isolated from the ^{14}C-TMP was very low.

Thus it has been shown that cell-free extracts of Esch. coli 15T- synthesise ^{14}C-labelled material, which is not separated from carrier TMP by paper chromatography, from UDP and 3-^{14}C-serine. A minute proportion (less than 0.5%) of this ^{14}C-labelled material proved to be ^{14}C-TMP. The remainder consisted most probably of compounds which are intermediates in the synthesis of TMP rather than metabolic products of ^{14}C-TMP.

This system has not as yet been investigated further, but it should provide a promising approach to

the study of structures of intermediates on the TMP
synthetic pathway.

24. Synthesis of ^{14}C-thymidine-5'-triphosphate and
DNA-thymine by cell free extracts of Esch. coli PA/15.

It was observed that when the nucleotide
fractions of systems from which ^{14}C-TMP was isolated were
submitted to paper electrophoresis, ^{14}C-labelled material
migrated at a rate faster than that of the carrier TMP.
Under an applied potential difference of 13 volts/cm. at
pH 3.5 this ^{14}C-labelled material had a mobility 1.4
times greater than that of TMP. The ^{14}C-labelled
material was found to occupy approximately the position
to which thymidine-5'-triphosphate (TTP) migrates under
the same conditions (Keir, 1958).

Accordingly, a reaction mixture containing a
cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and
dUMP, together with the cofactors ATP, FAH$_4$, MgSO4,
TPNH and pyridoxal phosphate, was chromatographed on
paper as a band (solvent, n-butanol-water) before
deproteinisation was effected. The nucleotide fraction
(R$_F$ 0.0 to 0.05) was isolated and submitted to
electrophoresis on paper in citrate buffer, pH 3.5, with
carrier TMP and TTP, under a potential difference of
6.5 volts/cm. for 17 hours. Fig. II, 34 shows that
autoradiography of this electrophoretogram revealed

Figure II, 34.

Synthesis of ^{14}C-labelled thymidine-5'-triphosphate by cell-free extracts of Esch. coli PA/15.

Autoradiograph of a paper electrophoretic separation of the nucleotide fraction isolated from an incubation mixture containing 3-^{14}C-serine, dUMP and a cell-free extract of Esch. coli PA/15 together with carrier TMP and TTP. The nucleotide fraction was isolated by paper chromatography (solvent, n-butanol-water). Electrophoresis was carried out on paper soaked in citrate buffer (0.05M, pH 3.5). Separation was achieved by applying a potential difference of 6.5 volts/cm. for 17 hours.

Complete incubation mixture - A cell-free extract of Esch. coli PA/15 (20 mg. protein), ATP (120 μmoles), FAH$_4$ (12 μmoles), MgSO$_4$ (50 μmoles), TPN (4 μmoles), glucose-6-phosphate (40 μmoles), pyridoxal phosphate (0.3 μmoles), 3-^{14}C-serine (60 μmoles, 30 μc) and dUMP (60 pmoles) in Tris buffer (0.1M, pH 7.2), total volume 6 ml. Incubation was carried out under anaerobic conditions for 4 hours at 37° C.

Figure II, 34.
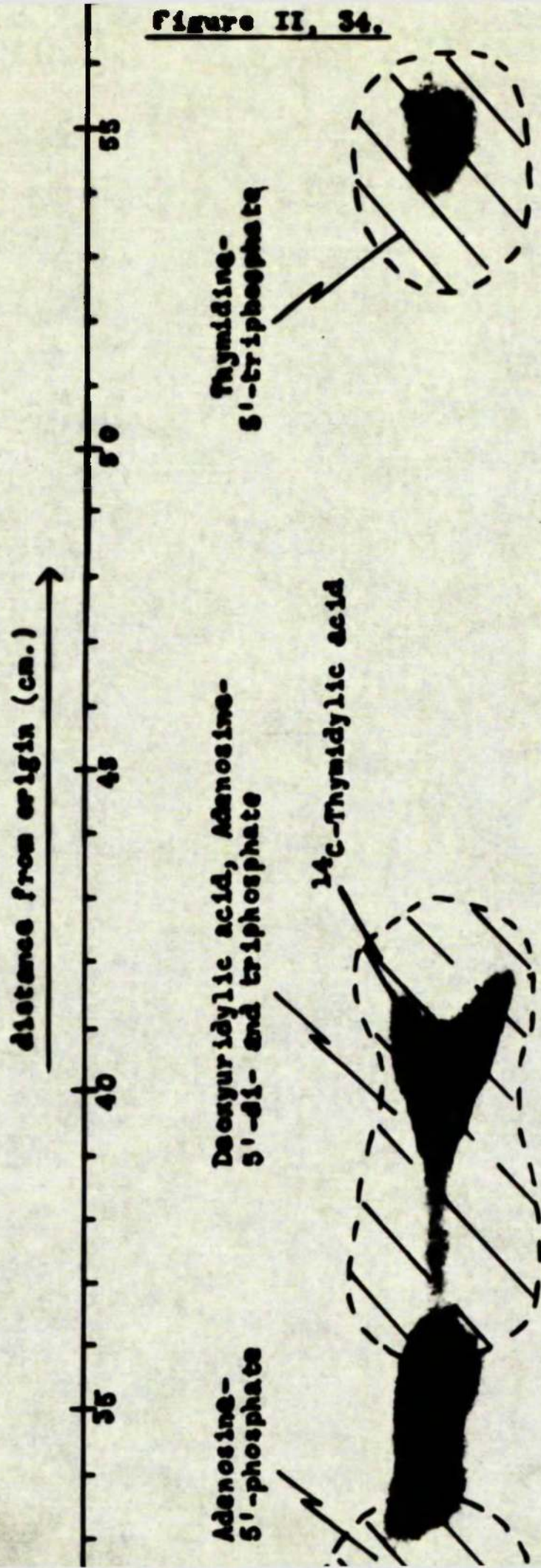
^{14}C-labelled material coincident with the ultraviolet-absorbing spot of carrier TTP.

This separation of the nucleotide materials from the incubation mixture was carried out before deproteinisation of the reaction mixture was effected as TTP is both acid- and heat-labile. Accordingly, the protein present in the incubation was precipitated by heating for 10 minutes at 100° C. This protein was isolated, washed and hydrolysed. The hydrolysis products were submitted to two-dimensional paper chromatography (solvents, phenol-water and n-butanol-water-acetic acid) followed by autoradiography. No ^{14}C-labelled material was found to be associated with the amino-acids of the protein thus precluding the unlikely possibility that the ^{14}C-labelled material coincident with the carrier TTP on the electrophoreto-gram (Fig. II, 34) was ^{14}C-labelled protein.

The synthesis of ^{14}C-TTP in incubation mixtures from which ^{14}C-TMP had been isolated was not investigated further. However, the evidence obtained does indicate that the fast-moving ^{14}C-labelled material, observed on electrophoretic separations of the nucleotide fractions of those incubation mixtures, is probably ^{14}C-TTP.

Lehman et al.(1958) have described a cell-free preparation of bacterial origin which effects the

transphosphorylations TMP $\xrightarrow{\text{ATP}}$ TDP $\xrightarrow{\text{ATP}}$ TTP and
they have shown also that TTP is involved in DNA
synthesis in vitro in the same enzyme system.   As
one product isolated from incubation mixtures in
which ^{14}C-TMP was synthesised has been tentatively
identified as ^{14}C-TTP, the possibility that DNA is
synthesised in these systems was investigated.   A
cell-free extract of Esch. coli PA/15 (12.8 mg.
protein) was incubated in Tris buffer (0.1M, pH 7.2)
with 3-^{14}C-serine (40 μmoles, 20 μc) and UDr (40
μmoles), in the presence of the cofactors ATP (80 μmoles),
FAH$_4$ (5 μmoles), MgSO$_4$ (40 μmoles), DPNH (2 μmoles),
TPN (2 μmoles), glucose-6-phosphate (20 μmoles) and
pyridoxal phosphate (0.1 μmoles).   Incubation was
carried out in a total volume of 4 ml. under anaerobic
conditions at 37° C. for 4 hours.   The DNA was
isolated from the incubation mixture and hydrolysed
(Section IV, 20).   The DNA in incubation mixtures
containing cell-free extracts of Esch. coli 113/3
(Section II, 21) was isolated also and hydrolysed.

The DNA hydrolysates were submitted to two-
dimensional paper chromatography (solvents, iso-
propanol-water-HCl and n-butanol-water-NH$_4$OH) followed
by autoradiography.   No ^{14}C-material was found to be in
the thymine position on these chromatograms.   The

hydrolysates were fractionated into the four bases
(adenine, guanine, cytosine and thymine) by paper
chromatography as bands (solvents, n-butanol-water).
Each of the four bases were rechromatographed on
paper (solvents, iso-propanol-water-HCl and n-butanol-
water-NH$_4$OH). Autoradiography again failed to reveal
the presence of any ^{14}C-labelled material which was
coincident with the ultraviolet-absorbing spots of the
bases.

This investigation has shown that, although the
TMP synthetic systems also synthesis TTP, the ^{14}C-TMP
is not incorporated into the DNA. Thus the DNA synthetic
pathway is not one of the pathways by which TMP (or
thymine, Section II, 17) is metabolised by cell-free
extracts of Esch. coli PA/15 under the conditions used
in this work.

25. The catabolism of thymine, thymidine and thymidylic
acid by cell-free extracts of Esch. coli PA/15.

The catabolism of thymidine was observed first
when it was found that the thymidine present in UDr as
a contaminant partially disappeared when incubated with
washed whole-cell suspensions or cell-free extracts of
Esch. coli PA/15 (Section II, 17). The experiments
described in Section II, 24 have shown that the
thymidine was not incorporated into DNA. Moreover,

the thymidine was rapidly hydrolysed to thymine by the powerful nucleoside phosphorylase activity present in Esch. coli PA/15 and it seems unlikely that a mechanism for the synthesis of TMP by coupling thymine and a phosphorylated deoxyriboside exists in these systems. In addition, washed whole-cell suspensions of Esch. coli PA/15 have been shown to metabolise thymine (0.05 μmoles in 7 hours) in a system in which the only source of deoxyribose was by de novo synthesis or by degradation of DNA (Section II, 17). Thus it was concluded that it was not an anabolic pathway by which thymine or thymidine is metabolised by Esch. coli PA/15 under these conditions.

Fink et al. (1956b) reported that rat liver slices catalyse the catabolism of thymine by a number of pathways including one in which the initial attack is by the reduction of the 4:5-double bond of thymine to yield 4:5-dihydrothymine (DHT) which is further degraded to β-ureido-iso-butyric acid (BUIB) and β-amino-iso-butyric acid (BAIB). Accordingly, cell-free extacts of Esch. coli PA/15 were investigated for the presence of this catabolic pathway using thymine, thymidine and TMP as substrates.

The reduction of thymine, thymidine and TMP was followed by a spectrophotometric method based on

that used to confirm the presence of pyridine nucleotide
transhydrogenase activity in Esch. coli PA/15 (Section
II, 8). A solution of (a) thymine, (b) thymidine or
(c) TMP was added to a reaction mixture containing a
cell-free extract of Esch. coli PA/15 and TPNH which
had been synthesised in situ from TPN and glucose-6-
phosphate by the action of glucose-6-phosphate
dehydrogenase. Conditions chosen were those under
which a limited amount of TPNH had been synthesised by
including less than a molar proportion of glucose-6-
phosphate in the incubation mixture.. The reaction
was followed spectrophotometrically by making use of
the relatively high extinction of TPNH at 240 mμ
compared with that of a mixture of TPN, thymine and
the enzyme solution at the same wavelength. Fig. II,
35 shows that the extinctions of the solutions to
which thymine or TMP had been added decreased more
rapidly than the extinction of the control to which
water had been added. This is the same effect,
although much less marked, that was obtained when DPN
was added to a solution of TPNH under the same
conditions (Fig. II, 8) and therefore it was concluded
that these observations are consistent with a very slow
reduction of TMP and thymine by TPNH. Fig. II, 35
also shows that the reduction of TMP proceeded at a

Figure II, 35

The hydrogenation of thymine, thymidine and thymidylic acid.

A solution of TPNH was prepared by mixing 200 μmoles $MgSO_4$, 1 μmole glucose-6-phosphate, 2.6 μmoles TPN and a cell-free extract of Esch. coli PA/15 (7 mg. protein) in 15 ml. Tris buffer (0.1M, pH 7.2) - a system in which glucose-6-phosphate is the limiting factor in TPNH synthesis. Tests - 3 ml. "TPNH solution" +

      (a) 0.5 ml. thymine solution (5 μmoles);

      (b) 0.5 ml. thymidine solution

                             (5 μmoles);

      (c) 0.5 ml. thymidylic acid solution

                             (5 μmoles).

Control - 3 ml. "TPNH solution" + 0.5 ml. glass-distilled water

The extinction of each test and the control was determined at 240 mμ at fixed time intervals over a period of 5 hours and plotted against time.
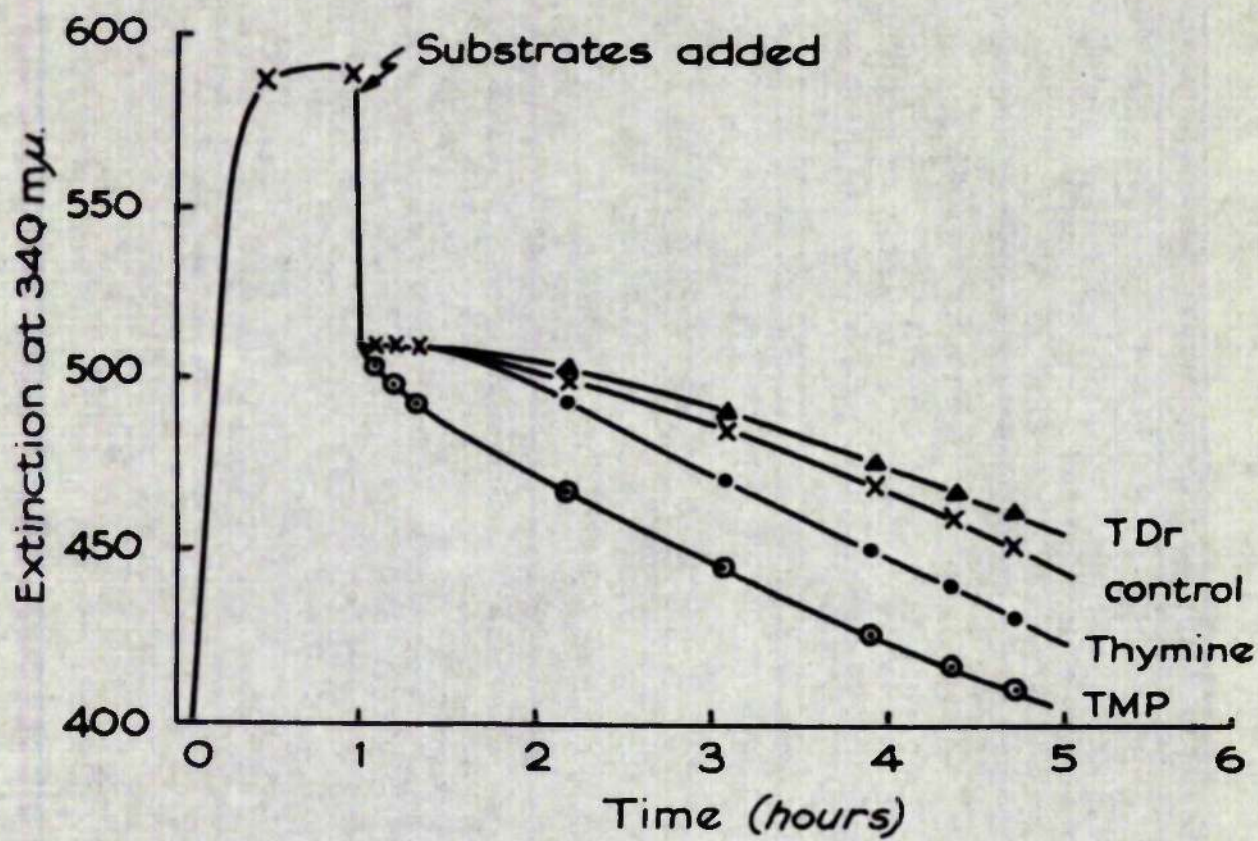
Figure II. 35

faster rather than the reduction of thymine, whereas thymidine was not reduced under these conditions. However, the catabolism of thymidine by Esch. coli PA/15 in the presence of phosphate buffer is explained readily by the action of nucleoside phosphorylase on the thymidine to form thymine which, as is shown in Fig. II, 35, undergoes reduction.   In the present experiments there is no source of inorganic phosphate and accordingly the thymidine is not hydrolysed.

On the basis of the assumption that 1 mole of TPNH will reduce 1 mole of TMP it was calculated that a total of 0.025 μmoles of TMP had been reduced during 4 hours incubation at 25° C.   In the case of the thymine, about 0.012 μmoles were reduced during the same period.   This result is of the same order of magnitude as that obtained previously when it was found that 0.05 μmoles of thymine were catabolised on incubation with a washed whole-cell suspension of Esch. coli PA/15 at 37° C. for 7 hours.

The nature of the products of TMP catabolism in this system was investigated by incubating a cell-free extract of Esch. coli PA/15 (19 mg. protein) in Tris buffer (0.1M, pH 7.2) with TMP(7 μmoles) in the presence of the cofactors TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), $MgSO_4$(40 μmoles)

and pyridoxal phosphate (0.6 μmoles). Incubation
was carried out in a total volume of 5 ml. under
anaerobic conditions at 37° C. for (a) 3 hours and
(b) 5 hours. Deproteinisation was effected by
adding 0.4 ml. 10N-HCl and heating at 100° C. for
10 minutes before the reaction mixtures were
submitted to extensive paper chromatography.

When the supernatant from the reaction
mixture which had been incubated for 3 hours was
submitted to chromatography on paper (solvent,
n-butanol-water), a trace of material which had
migrated at the same rate as authentic BAIB was
revealed by spraying the chromatogram with ninhydrin.
This material was present also in the supernatant of
the reaction mixture which had been incubated for 5
hours, together with a trace of a compound which gave
a positive reaction with p-dimethylaminobenzaldehyde
(PDAB) and which had migrated to the position
occupied by authentic BUIB.

The presence of nucleotide products of TMP
catabolism was confirmed by paper chromatographic
separation (solvent, n-butanol-water) of the reaction
mixture which had been incubated for 5 hours. The
nucleotide fraction (R_F 0.0 to 0.05) was hydrolysed by
heating with 2N-HCl at 100° C. for 1 hour and the

hydrolysis products were rechromatographed on paper
(solvent, tert-butanol-methylethylketone-water-
formic acid).   A small spot of material in the
position of BAIB was developed by spraying with
ninhydrin.

Since TMP is hydrolysed to thymine by
treatment with 2N-HCl at 100° C. for 1 hour (Section
II, 16) and DHT is hydrolysed to BAIB under the same
conditions (Fig. 36), it is a reasonable conclusion
that compounds such as 4:5-dihydrothymidylic acid are
hydrolysed to BAIB by this treatment.   Thus the
appearance of BAIB after the hydrolysis of the
nucleotide fraction of incubation mixtures containing
TMP, TPNH and a cell-free extract of Esch. coli PA/15
is consistent with the occurrence of compounds such
as 4:5-dihydrothymidylic acid in the reaction mixtures.
In addition, when the deproteinised reaction mixture
was chromatographed on paper (solvent, iso-propanol-
water- HCl), two spots (R_F values 0.40 and 0.50,
respectively) of material, which gave a positive spray
reaction with PDAB, were revealed.   The nature of the
material appearing as these spots was not determined,
but it was noted that their mobilities did not
correspond with those of DHT, BUIB, BAIB or 4:5-
dihydrothymidine (R_F values 0.81, 0.95, 0.70 and 0.77

Hydrolysis of 4:5-dihydrothymine by (a) N-HCl
and (b) 2N-HCl.

Photograph of a paper chromatographic
separation of the products of DHT hydrolysis by
(a) heating with N-HCl at 100° C. for 10 minutes
(2) and (b) heating with 2N-HCl at 100° C. for
1 hour (1).

Figure II, 36.

4:5-Dihydrothymine

β-Aminoiso-butyric acid
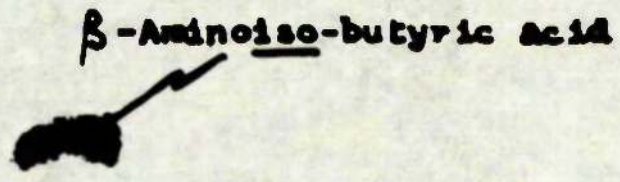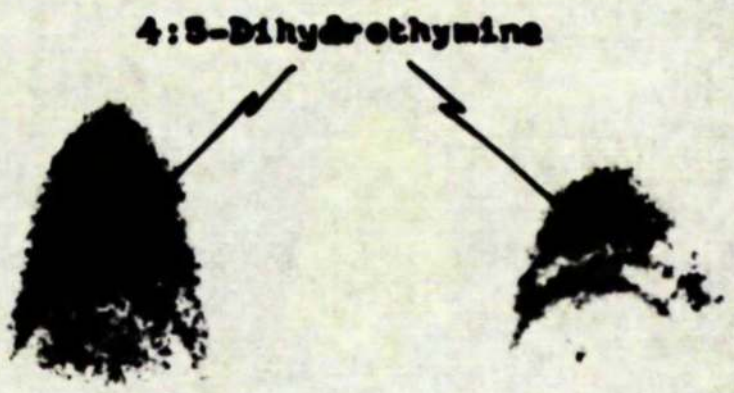
R_f in n-butanol-water.

respectively, in this solvent). This material may
have been the nucleotide material which gave rise to
BAIB on hydrolysis.

Thus it has been shown that TPNH will reduce
thymine and TMP in the presence of a cell-free extract
of Esch. coli PA/15. BUIB and BAIB have been isolated
from the reaction mixtures together with a nucleotide
material which yields BAIB on hydrolysis with 2N-HCl.
The structure of this nucleotide material has not as
yet been more fully determined but it is suggested
that it may be 4:5-dihydrothymidylic acid.

26. ^{14}C-labelled materials produced by the catabolism
    of ^{14}C-TMP synthesised in vitro by cell-free extracts
    of Esch. coli PA/15.

The quantitative investigation of ^{14}C-TMP
synthesis described in Section II, 21 revealed, among
other things, that (i) the ^{14}C-labelled material
associated with carrier TMP after extensive paper
chromatography contained very little ^{14}C-TMP and (ii)
a large proportion of this ^{14}C-labelled material,
although not ^{14}C-TMP, was still a derivative of uracil.
The results discussed in Section II, 25, show that TMP
is catabolised by cell-free extracts of Esch. coli PA/15,
the evidence available indicating that this catabolism

proceeds by way of a reduction of the 4:5-double bond
in the pyrimidine ring. Accordingly, the nature of
the ^{14}C-labelled material which was synthesised from
3-^{14}C-serine and (a)UDr; (b) UR or (c) dUMP and which
had chromatographic properties very similar to those
of TMP was investigated with particular reference to
the possibility that it was composed of ^{14}C-TMP
catabolic products.

The first evidence for the presence of ^{14}C-
labelled catabolic products of TMP in incubation
mixtures from which ^{14}C-TMP was isolated was obtained
from the paper chromatographic separations of these
incubation mixtures (solvents, (i) iso-propanol-water-
HCl and n-butanol-water; (ii) n-butanol-water).
Autoradiography revealed the presence of radioactive
materials which migrated (i) at the same rate as
authentic BUIB (Material B, Figs. II, 21 and II, 23)
and (ii) at the same rate as authentic DHT (Material
D, Fig. II, 23).

Some evidence for the presence of 4:5-
dihydrothymidylic acid in incubation mixtures from
which ^{14}C-TMP was isolated was obtained when a cell-
free extract of Esch. coli PA/15 was incubated with
3-^{14}C-serine and (a) UDr; (b) UDr and TMP; (c)UR; or
(d) UR and TMP in the presence of the usual cofactors
(Table II, 12). The nucleotide fractions of these

reaction mixtures were isolated by paper chromatography (solvent, n-butanol-water) and hydrolysed by treatment with 2N-HCl at 100° C. for one hour. The hydrolysis products were submitted to paper chromatography (solvent, tert-butanol-methylethylketone-water- formic acid) followed by autoradiography. Figs. II, 37 (a) and II, 37 (b) show, in each case, the presence of ^{14}C-labelled material which has migrated to the same position as a marker of authentic BAIB. The ^{14}C-material is coincident with material which developed the characteristic amino-acid colour when the chromatogram was sprayed with ninhydrin. In addition, Figs. II, 37 (a) and II, 37 (b) show small "double" spots of radioactive material which has migrated to the thymine-DHT position (R_F 0.60) on this chromatogram. It is probable that these "double" spots are composed of thymine and DHT.

A number of other ^{14}C-labelled compounds were revealed by autoradiography of a two-dimensional paper chromatographic separation (solvents, tert-butanol-methylethylketone-water-NH$_4$OH and tert-butanol-methylethylketone-water-formic acid) of the hydrolysis products of these nucleotide fractions together with carrier BAIB, BUIB and DHT (Fig. II, 38). The autoradiographs showed that 3-^{14}C-serine ("X"), ^{14}C-

Figure II. 37

^{14}C-labelled catabolic products from incubation mixtures from which ^{14}C-TMP has been isolated.

(a) Photograph and (b) autoradiograph of a paper chromatographic separation of the hydrolysis products from nucleotide material isolated by paper chromatography (solvent, n-butanol-water) from incubation mixtures containing a cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and (a) UDr (1); (b) UDr and TMP (2); (c)UR (3); and (d) UR and TMP (4), together with markers of BAIB, BUIB and DHT, (5).

Complete reaction mixture - A cell-free extract of Esch. coli PA/15 (19 mg. protein), ATP (60 μmoles), FAH$_4$ (3 μmoles), MgSO$_4$ (40 μmoles), pyridoxal phosphate (0.6 μmoles), TPN (2 μmoles), glucose-6-phosphate (20 μmoles), DPNH (2 μmoles), 3-^{14}C-serine (30 μmoles, 15 μc) and (a) UDr (30 μmoles); (b) UDr (30 μmoles) and TMP (7 μmoles); (c) UR (30 μmoles); or (d) UR (30 μmoles) and TMP (7μmoles) in Tris buffer (0.1M, pH 7.2), total volume 3 ml. Incubation was carried out under anaerobic conditions at 37° C. for 3 hours. Deproteinisation was effected by adding 0.35 ml. 10N-HCl, heating at 100° C. for 10 minutes and centrifuging (5,000g.).
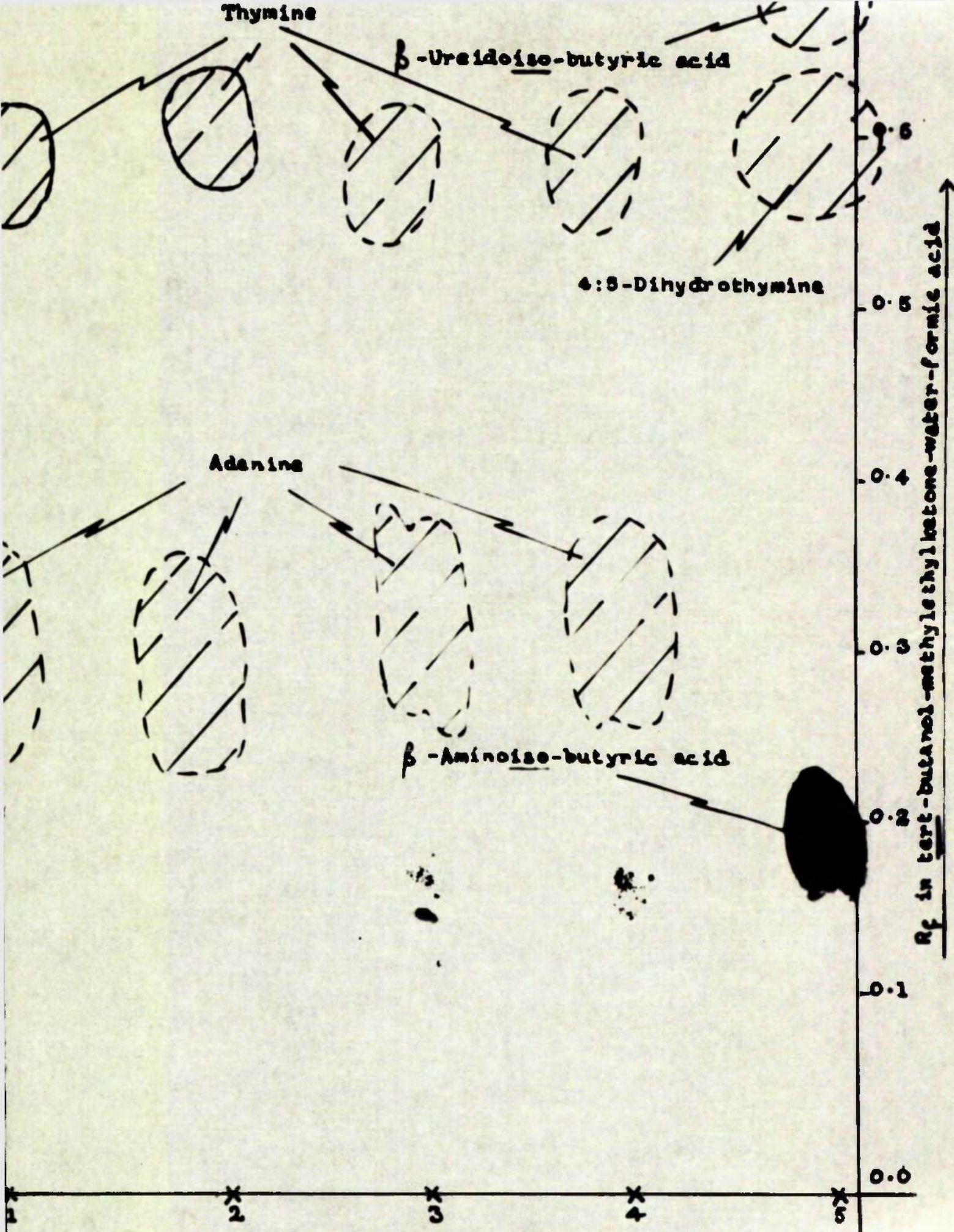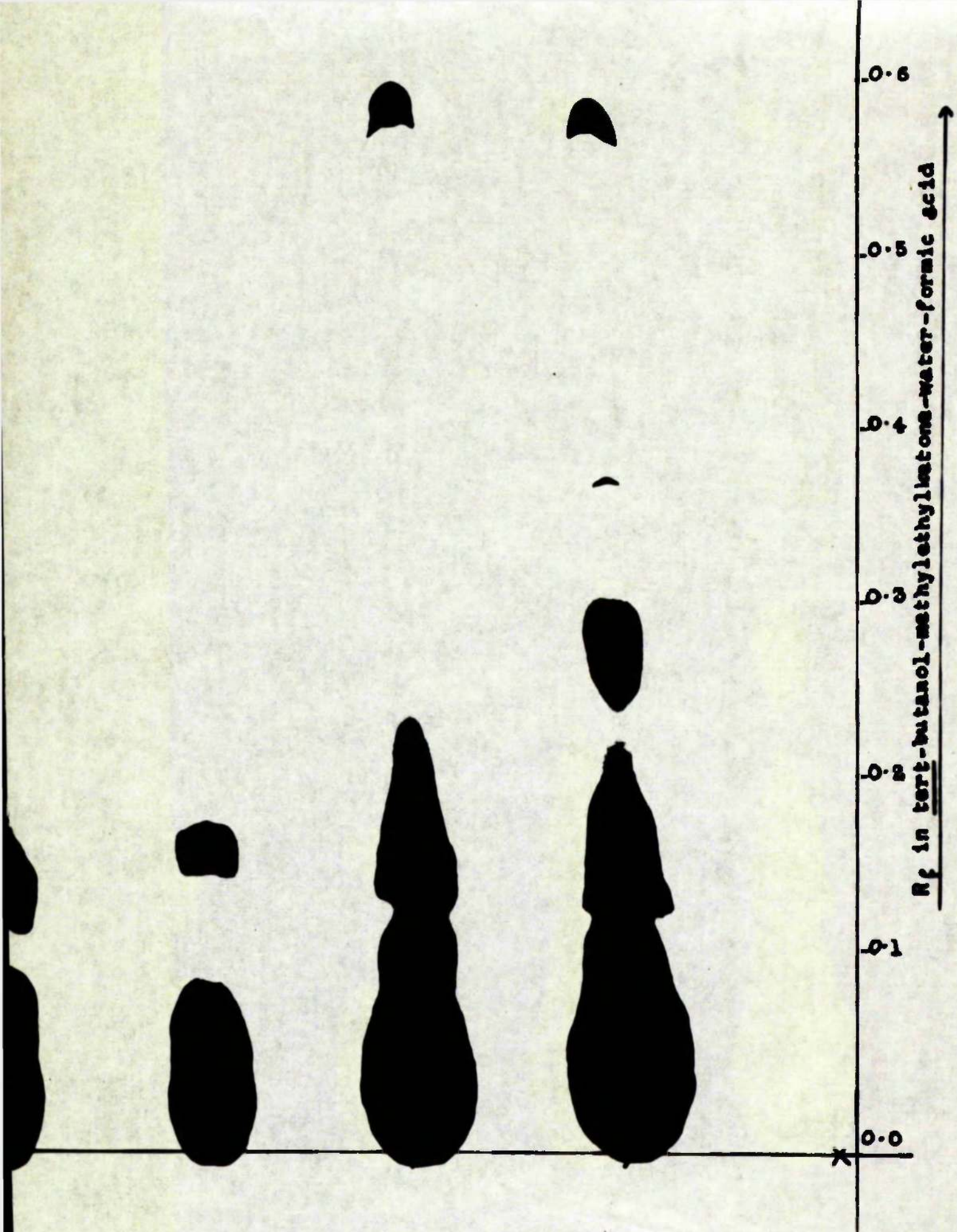
Figure II, 37 (a).

Figure II, 37 (b).

R_f in tert-butanol-methylethylketone-water-formic acid

14C-labelled catabolic products from incubation
mixtures from which 14C-TMP has been isolated.

Autoradiograph of a two-dimensional
paper chromatographic separation of the hydrolysis
products from nucleotide material isolated by
paper chromatography (solvent, n-butanol-water)
from incubation mixtures containing a cell-free
extract of Esch. coli PA/15, 3-14C-serine and
(a) UDr or (b) UDr and TMP, together with markers
of BAIB, BUIB, and DHT.

Complete incubation mixture - The incubation
mixtures were those described in Fig. II, 37.

Figure II, 38
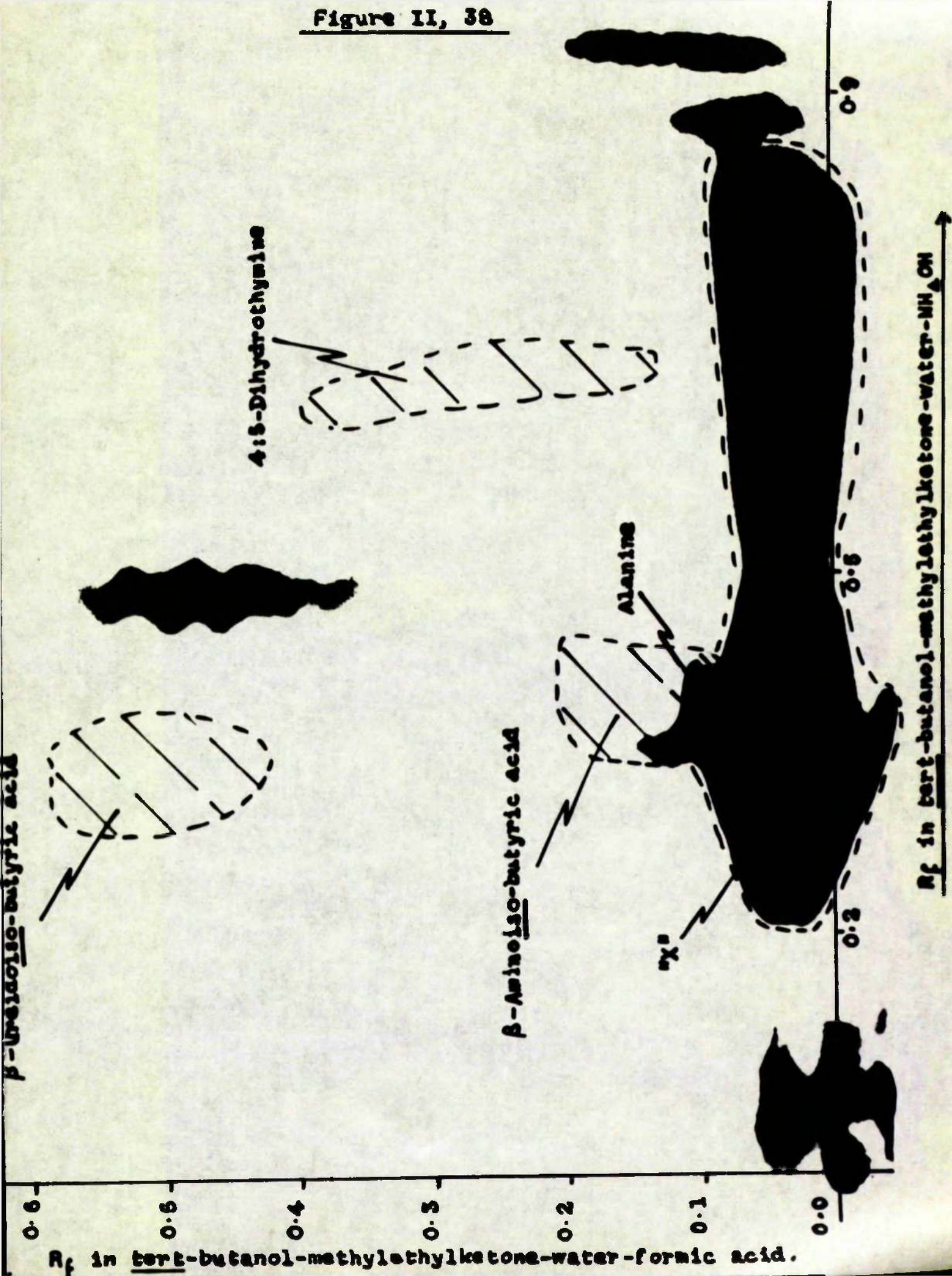
alanine and a trace of ^{14}C-labelled material coincident with the carrier BAIB were present. The nature of the other ^{14}C-labelled materials shown in Fig. II, 38 was not determined.

The incubation mixtures containing a cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and (a) UDr or (b) UDr and TMP (Table II, 12) were chromatographed as bands on paper (solvent, iso-propanol-water-HCl). The products separated in this way were divided into three fractions - fraction A(R_{F} 0.60-0.75), fraction B which included the carrier TMP (R_{F} 0.75-0.85) and fraction C (R_{F} 0.85 - 1.0). After the addition of carrier BAIB, BUIB and DHT, fractions A and C were submitted to two-dimensional paper chromatography (solvents, tert-butanol-methylethylketone-water-NH$_4$OH and tert-butanol-methyl-ethylketone-water-formic acid) followed by autoradiography. Fractions A, B and C were hydrolysed (2N-HCl at 100° C. for one hour) and, after the addition of carrier BAIB, BUIB, DHT and uracil-5-carboxylic acid, the hydrolysis products were submitted to two-dimensional paper chromatography in the same solvent system, followed again by autoradiography.

Autoradiography of the fraction A chromatograms showed the presence of ^{14}C-alanine and

a trace of ^{14}C-labelled material coincident with the carrier BAIB (Fig. II, 39(a)). No radioactivity was found to be associated with the carrier BUIB or carrier DHT but there was ^{14}C-labelled material (R_F values 0.70, 0.08) which was in the position to which the amide of BAIB migrates in this solvent system (R_F values 0.70, 0.06; Fink et al, 1956b). Autoradiography of the fraction A hydrolysate chromatograms gave the same result (Fig. II, 39(b)). There was no ^{14}C-labelled material associated with the carrier uracil-5-carboxylic acid.

Autoradiography of the fraction B hydrolysate chromatograms showed ^{14}C-labelled materials coincident with (i) the carrier TMP and (ii) the carrier thymine (Fig. II, 33). Of the other radioactive spots shown in Fig. II, 33, one was in the position to which alanine migrates in this solvent system but no ^{14}C-labelled material coincident with the spots of carrier BAIB, BUIB, DHT or uracil-5-carboxylic acid was detected.

Autoradiography of the fraction C chromatograms revealed the presence of a trace of ^{14}C-labelled material which was coincident with the carrier BUIB (Fig. II, 40(a)). No radioactivity coincident with the carrier BAIB or DHT was detected but two

Figure II, 39

^{14}C-labelled catabolic products from incubation
mixtures from which ^{14}C-TMP has been isolated.

Autoradiographs of two-dimensional paper
chromatographic separations of fraction A material
(a) before hydrolysis and (b) after hydrolysis by
2N-HCl at 100° C. for one hour. Fraction A was
the material of R_F 0.60 to 0.75 separated by paper
chromatography (solvent, iso-propanol-water-HCl) of
incubation mixtures containing a cell-free extract
of Esch. coli PA/15, 3-^{14}C-serine and (i) UDr or
(ii) UDr and TMP.

Complete incubation mixture - The incubation mixtures
were those described in Fig. II, 37.

Figure II. 39 (a).

Figure II, 39 (b).
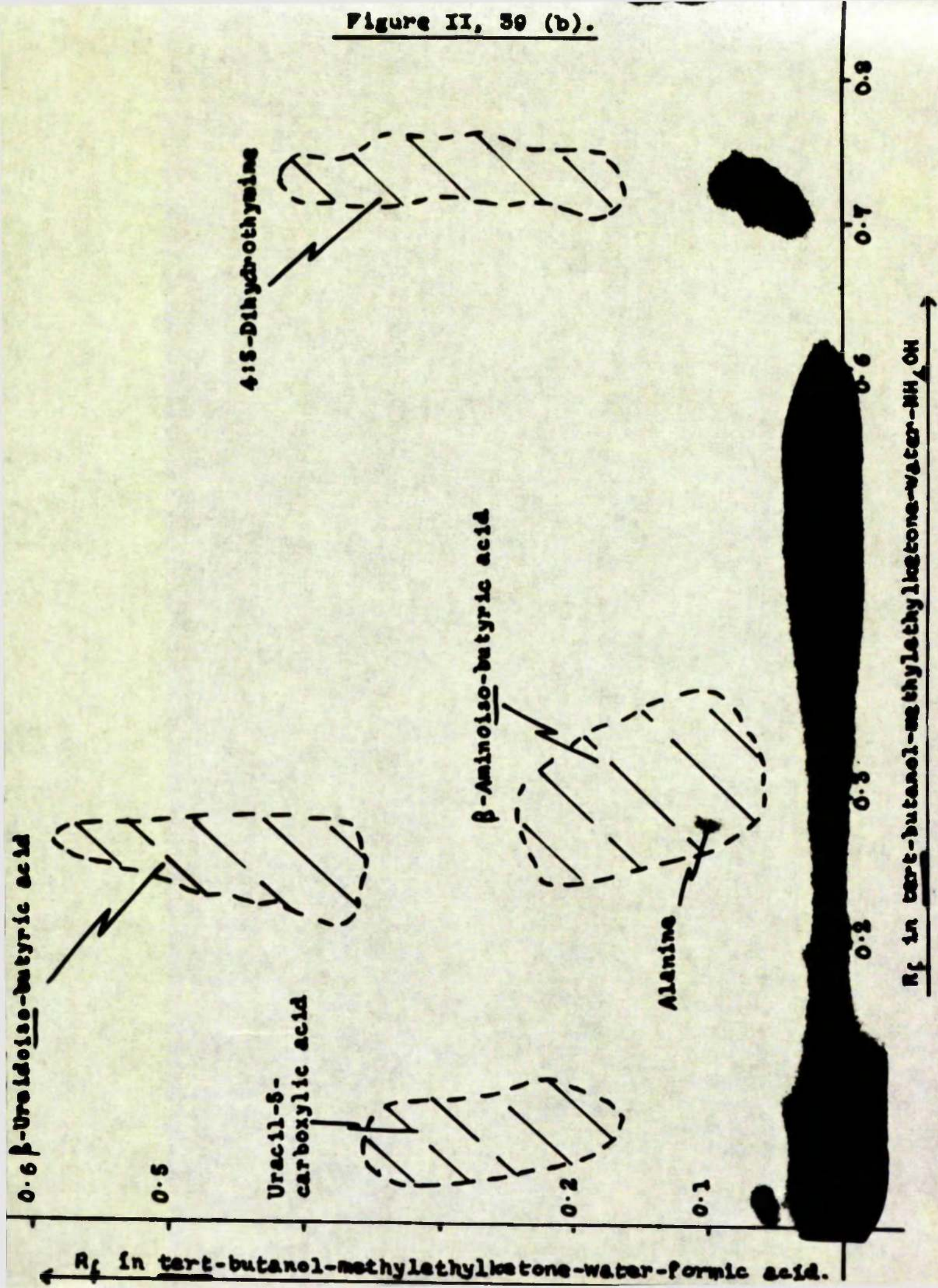
strongly radioactive spots (R_F values 0.32, 0.85 and 0.0, 0.0) were visible. After fraction C had been hydrolysed, a trace of ^{14}C-labelled material which was coincident with the carrier BAIB was detected but no radioactivity was found to be associated with the spots of carrier BUIB, DHT or uracil-5-carboxylic acid. The radioactive spot of R_F values 0.32, 0.85 was no longer present and the spot of R_F values 0.0, 0.0 was greatly decreased in intensity while a radioactive spot (R_F values 0.58, 0.0), which was not present before hydrolysis, had made its appearance.

The results obtained from this investigation are not as clear-cut as desired. The ^{14}C-labelled compounds which are synthesised in incubation mixtures from which ^{14}C-TMP was isolated were not all identified but some evidence for the presence of ^{14}C-BAIB and ^{14}C-BUIB was obtained. The appearance of ^{14}C-BAIB after hydrolysis of the nucleotide fractions of these incubation mixtures strongly suggests the presence of a compound such as 4:5-dihydrothymidylic acid. No evidence for the presence in the reaction mixtures of 5-hydroxymethyl derivatives, either as precursors or as catabolic products of ^{14}C-TMP, was obtained nor was ^{14}C-uracil-5-
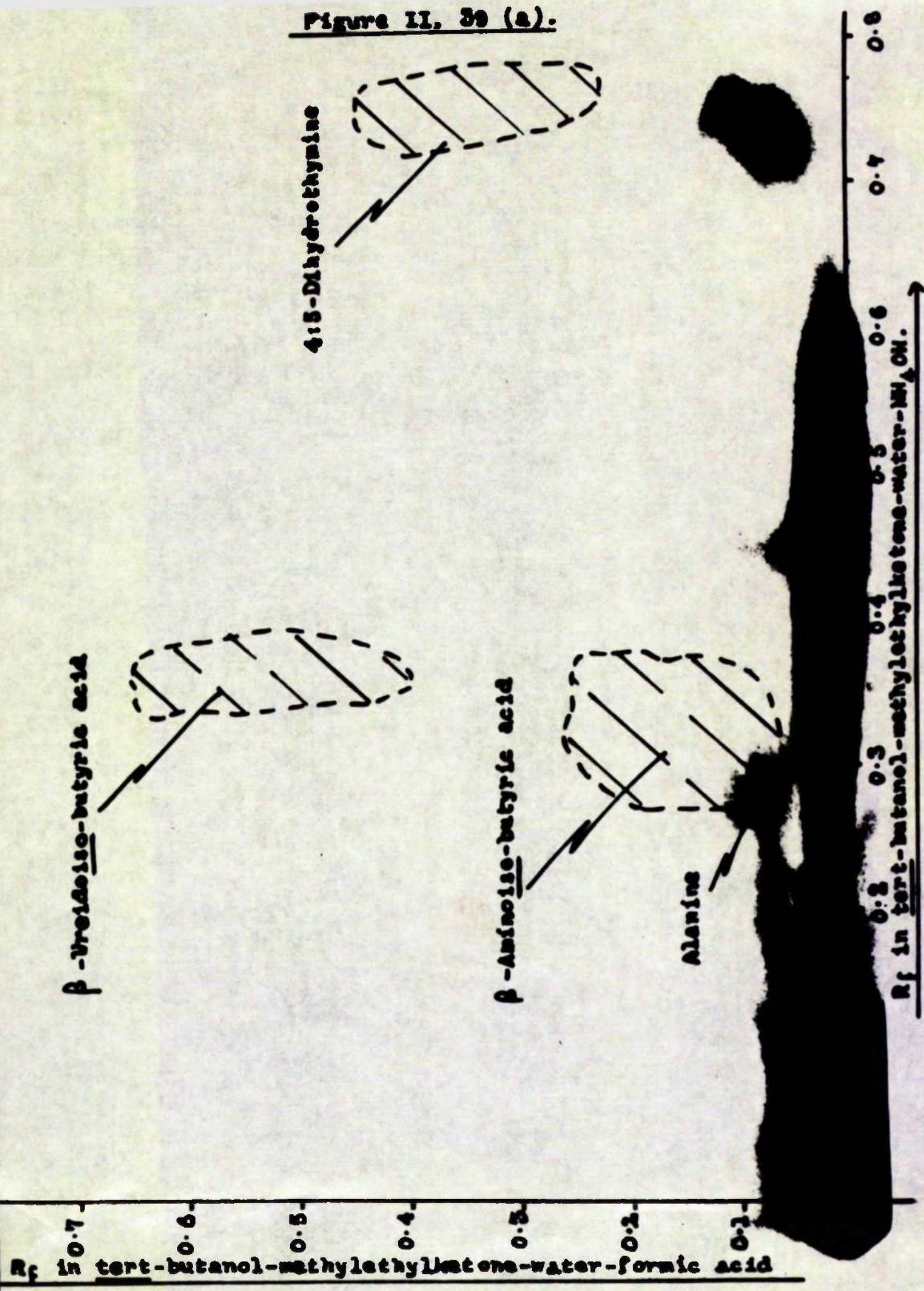
Figure II, 40

^{14}C-labelled catabolic products from incubation mixtures from which ^{14}C-TMP has been isolated.

Autoradiographs of two-dimensional paper chromatographic separations of fraction C material (a) before hydrolysis and (b) after hydrolysis by 2N-HCl at 100° C. for one hour. Fraction C was the material of R_F 0.85 to 1.0 separated by paper chromatography (solvent, iso-propanol-water-HCl) of incubation mixtures containing a cell-free extract of Esch. coli PA/15, 3-^{14}C-serine and (i) UDr or (ii) UDr and TMP.

Complete incubation mixture - The incubation mixtures were those described in Fig. II, 37.

Figure II, 40 (a).

4:5-Dihydrothymine

β-Ureidoiso-butyric acid

β-Aminoiso-butyric acid

Rf in tert-butanol-methylethylketone-water-formic acid.

Rf in tert-butanol-methylethylketone-water-NH$_4$OH

Figure II, 40 (b).

4:5-Dihydrothymine
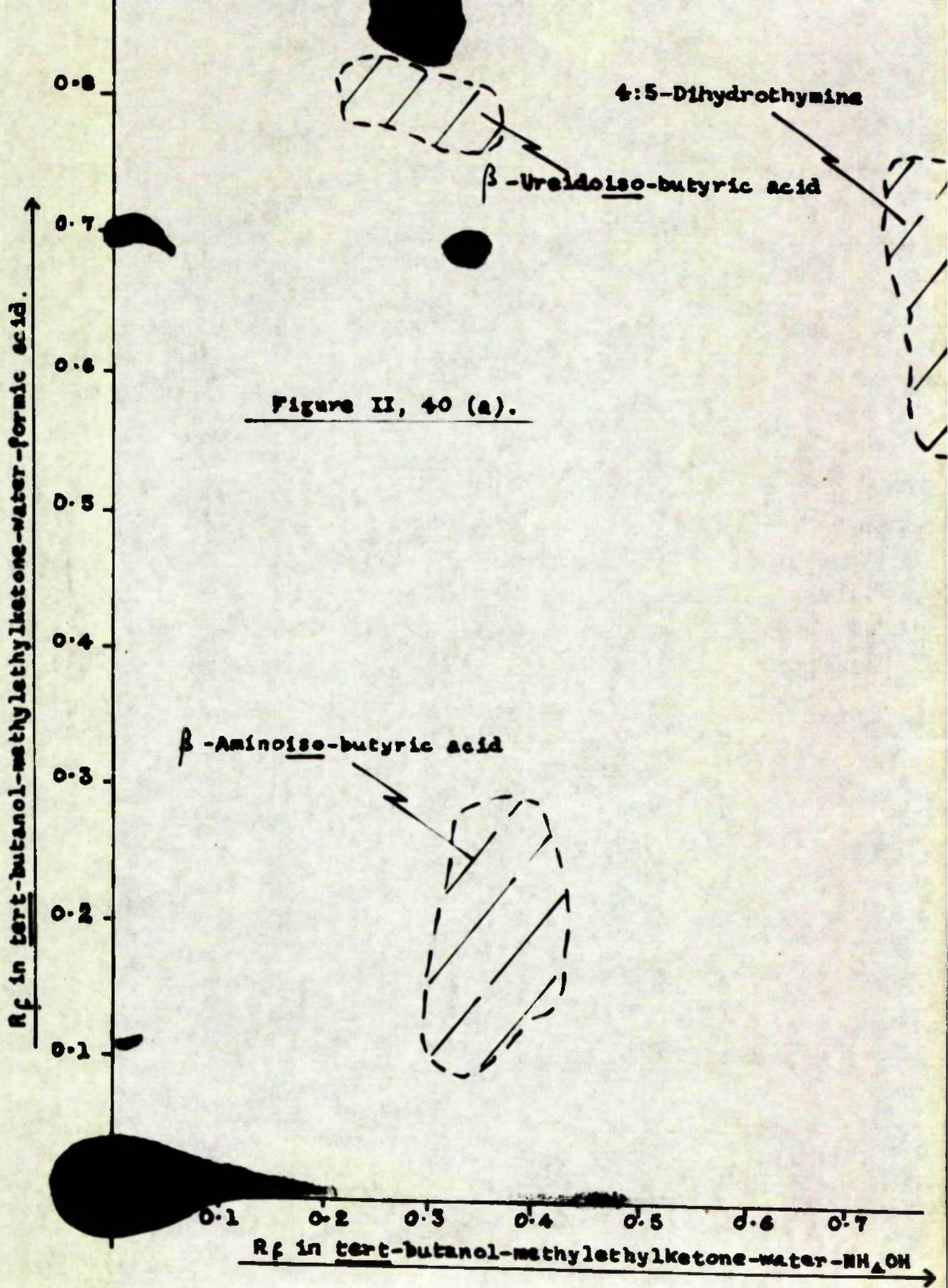
β-Ureido-butyric acid
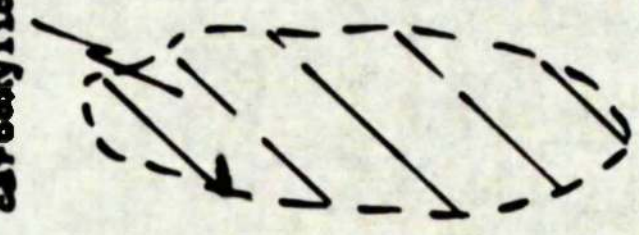
β-Amino-butyric acid

Uracil-5-carboxylic acid

R_f in tert-butanol-methylethylketone-water-NH_4OH

R_f in tert-butanol-methylethylketone-water-formic acid

carboxylic acid isolated. The other ^{14}C-labelled
materials were not identified.

A comprehensive study of the catabolism
of ^{14}C-TMP by cell-free extracts of Esch. coli
PA/15 has not as yet been carried out. The
projected synthesis of authentic samples of
possible catabolic products of TMP should offer
a promising approach to the problem of the mechanism
of authentic ^{14}C-methyl-TMP catabolism by Esch. coli
PA/15.

Section III : Discussion.

Contents.

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2. The "5-methylation" of the pyrimidine ring.

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    (v) Pyrimidine derivative intermediates in the biosynthesis of thymidylic acid.

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1. Introduction.

The purpose of this study was to develop a cell-free system in which the biosynthesis of thymine, thymidine or thymidylic acid could be studied. As has been explained in Section I, 1, the section devoted to a description of the observations made during this study (Section II) also incorporates a discussion of the results of each experiment as they were obtained. Since each experiment was planned on the basis of information derived from previous experiments, it was found necessary to adopt this somewhat unusual format in order that the logical sequence of the experiments could be properly understood. Section III, therefore, consists of a short general discussion of the overall significance of the experimental results described in Section II, together with a correlation of these results with the observations reported by other investigators in this field. Section III also takes the form of a summary of the results obtained during this study.

2. The "5-methylation" of the pyrimidine ring.

(1) Choice of the enzyme source.

The choice of the source of the enzymes used in the study of the "5-methylation" of the pyrimidine ring fell on Esch. coli mutants as a result of the work of Woods and his colleagues (1952 and 1954) on the biosynthesis of methionine. Gibson and Woods (1952) and Cross and Woods (1954) have shown that whole-cell suspensions and cell-free extracts of Esch. coli PA/15, a glycine- or serine-less mutant, catalyse the synthesis of methionine from homocysteine and a one-carbon unit produced from serine. This reaction is formally analagous to the 5-hydroxymethylation of deoxyuridine (or deoxyuridylic acid) and, as the 5-hydroxymethyl derivative of deoxyuridine (or deoxyuridylic acid) is a postulated intermediate in the synthesis of thymidine (or thymidylic acid) (Cohen et al.,1956), Esch. coli PA/15 was chosen as the primary source of the enzymes used in this study of thymine, thymidine and thymidylic acid biosynthesis.

A preliminary investigation of the effect of vitamin B_{12} on thymidylic acid biosynthesis was carried out using cell-free extracts of Esch. coli 113/3, a methionine- or vitamin B_{12}-less mutant. However, no

effect of vitamin B_{12} on the biosynthesis of TMP from UDr was demonstrated with this mutant and a more detailed study of the vitamin B_{12}-dependence of the methylation reaction is planned using Lactobacillus leichmanii, an organism requiring deoxynucleoside or vitamin B_{12} for growth.

A promising approach to the study of the structures of intermediates on the thymidylic acid biosynthetic pathway should be provided by employing a mutant requiring thymine for growth as the enzyme source. Esch. coli 15T-, a thymine- or thymidine-less mutant, is thought to have an enzyme block at a stage immediately proceeding thymidylic acid on the biosynthetic pathway and, therefore, it was thought possible that a pool of the immediate precursor may accumulate when a cell-free extract of Esch. coli 15T- is used in a system in which a cell-free extract of Esch. coli PA/15 catalyses the synthesis of thymidylic acid. Unfortunately, no identifiable thymidylic acid precursors were isolated from such systems containing Esch. coli 15T- extracts. However, this approach will bear further investigation.

(ii) Composition of the "5-methylating" system.

As has been stated previously, the synthesis
of methionine from homocysteine and a one-carbon unit
is formally analagous to the synthesis of thymine from
a pyrimidine one-carbon unit acceptor and a one-
carbon unit.   Accordingly, when it had been verified
that both whole-cell suspensions and cell-free
extracts of Esch. coli PA/15 will catalyse the
formation of methionine from homocysteine and serine
(Section II,12) attempts were made to study the
synthesis of thymine and thymidine in the same systems
and under the same conditions with uracil or deoxy-
uridine replacing homocysteine as the one-carbon unit
acceptor molecule.   A microbiological assay system,
using Esch. coli 15T- as the assay organism, was
developed to enable the thymine and/or thymidine
synthesised in these systems to be estimated.
However, no detectable net synthesis of thymine or
thymidine was found in these systems.   Repetition of
these experiments using $3\text{-}^{14}C\text{-serine}$ (0.05 μc./μmole)
as the one-carbon unit donor and employing the
techniques of paper chromatography and autoradiography
to detect $^{14}C\text{-thymine}$, $^{14}C\text{-thymidine}$ and $^{14}C\text{-thymidylic}$
acid synthesised also failed to demonstrate the
synthesis of thymine or its derivatives.   Similarly,

the addition of cofactors known to be involved in one-carbon unit transfer (folic acid, N^{10}-formylfolic acid and N^5-formyltetrahydrofolic acid) failed to induce detectable net synthesis of thymine or thymidine.

Initial attempts to synthesise thymine, thymidine or thymidylic acid using whole-cell suspensions or cell-free extracts of Esch. coli PA/15 having failed, a detailed examination of the enzyme activities present in Esch. coli PA/15 extracts was undertaken. A thymine biosynthetic system consists of three separate systems, namely (a) a one-carbon unit donor system; (b) a one-carbon unit acceptor system; and (c) a hydrogen donor (or reducing) system and, accordingly, a separate investigation of each of these three systems was undertaken.

(a) The one-carbon unit donor system.

The production of one-carbon units from serine is dependent on the presence of serine hydroxymethylase in the Esch. coli PA/15 extracts. Blakley (1954) and Kisluik and Sakami (1955) very neatly confirmed the presence of this enzyme in pigeon liver extracts by demonstrating the synthesis of $2\text{-}^{14}C$-serine from $2\text{-}^{14}C$-glycine and (1) formaldehyde

or (ii) a one-carbon unit derived from L-serine.
The presence of serine hydroxymethylase activity
in Esch. coli PA/15 extracts was confirmed in the
same way by the demonstration of ^{14}C-glycine
synthesis from $^{14}C_3$-serine and of ^{14}C-serine
synthesis from glycine and ^{14}C-formaldehyde
(Section II, 10).

Having established that serine hydroxy-
methylase activity was present in Esch. coli PA/15
extracts, the conditions for optimum activity of
the enzyme were studied by determining the amount
of ^{14}C-labelled one-carbon units (in the form of
^{14}C-formaldehyde) liberated from $3-^{14}C$-serine
under a variety of conditions.   By this means,
it was established that the reaction was 5:6:7:8-
tetrahydrofolic acid-dependent, thus confirming
an observation made during the study of serine-
glycine interconversion when it was shown that
the addition of tetrahydrofolic acid accelerated
the rate at which $^{14}C_3$-serine disappeared from
incubation mixtures containing Esch. coli PA/15
extracts (Table II, 2).   In addition, it was
found that incubation under anaerobic conditions,
which obviates air-oxidation of tetrahydrofolic
acid, was necessary for one-carbon unit production.

The nature of the complex forming the one-carbon unit pool produced from serine was investigated by paper chromatographic analysis of the products of serine hydroxymethylase action on $3-^{14}C$-serine in the presence of tetrahydrofolic acid. Weakly radioactive spots of the same mobility were separated both from the products of this reaction and from the products obtained from the non-enzymic interaction of tetrahydrofolic acid and ^{14}C-formaldehyde. Since tetrahydrofolic acid and formaldehyde are known to undergo non-enzymic condensation to form N-hydroxymethyltetrahydrofolic acid (Jaenicke, 1956; Kisluik, 1957; and Blakley, 1958), the evidence presented by the autoradiograph reproduced in Fig. II, 12 strongly suggests that N-hydroxymethyltetrahydrofolic acid is one product of the action of serine hydroxymethylase on serine.

On the basis of this information, the primary one-carbon unit donor system was composed of $3-^{14}C$-serine, tetrahydrofolic acid and pyridoxal phosphate in the presence of serine hydroxymethylase. Pyridoxal phosphate was added to the system as a result of the demonstrated pyridoxal phosphate-dependence of serine-glycine interconversion

(Blakley, 1955).

Although the results obtained during the study of one-carbon unit production from serine under the influence of Esch. coli PA/15 extracts (Table II, 2) indicated that the crude cell-free extracts employed contained pyridoxal phosphate, in view of the evidence presented by Blakley (1955) it was decided to supplement the pyridoxal phosphate content of the extracts to ensure that an adequate pool of this cofactor was present.

Two other one-carbon unit donor systems were investigated. The first consisted of ^{14}C-formaldehyde in the presence of tetrahydrofolic acid and depends on the previously discussed non-enzymic condensation of formaldehyde and tetrahydrofolic acid to form N-hydroxymethyltetrahydrofolic acid. The second consisted of ^{14}C-formate in the presence of tetrahydrofolic acid, ATP and TPNH. This system depends on the formation of N-hydroxymethyltetrahydrofolic acid from formate and tetrahydrofolic acid via N^{10}-formyltetrahydrofolic acid and the N^5, N^{10}-methenyl- and methylene-bridge derivatives of tetrahydrofolic acid by a pathway which has been discussed already at some length (Section I, 4). The operation of this

pathway in extracts of resting cells of Esch. coli
PA/15 was confirmed by the isolation of ^{14}C-labelled
one-carbon units (in the form of ^{14}C-formaldehyde)
from this system.

Comparison of the rates of one-carbon
unit production from serine and formate showed
that, as expected, serine is a much more prolific
source of one-carbon units in the form of
N-hydroxymethyltetrahydrofolic acid than is formate.
No comparable comparison of serine and formaldehyde
was obtained.

(b) The one-carbon unit acceptor system.

In all, four compounds (uracil, uridine,
deoxyuridine and deoxyuridylic acid) were
investigated for ability to act as one-carbon unit
acceptors. Although Rege and Sreenivasan (1954)
reported that uracil was converted to thymine by
resting Bacillus subtilis cultures, repetition of
their work failed to show that uracil could act
as a one-carbon unit acceptor in thymine biosynthesis
under conditions which followed those described by
the authors as closely as possible. A similar
system using Esch. coli PA/15 in place of B. subtilis
also failed to demonstrate the synthesis of thymine
from uracil. This result was not unexpected in

view of the evidence obtained from a variety of
incorporation studies which have clearly implicated
deoxyuridine (or a nucleotide derivative thereof)
as the primary one-carbon unit acceptor, (Section
I, 4).

The use of deoxyuridine (and uridine) as
the one-carbon unit acceptor was complicated by the
presence of nucleoside phosphorylase activity in
Esch. coli.   However, it was found this enzyme was
dependent on an external supply of inorganic phosphate
and, therefore, could be inhibited by replacement
of the phosphate buffer of the system by Tris buffer
(Section II, 3).   In addition, the inorganic phosphate
produced from ATP present by the adenosine-5'-
triphosphatase activity of Esch. coli was found to be
insufficient to cause hydrolysis of more than 50%
of the nucleoside present thus ensuring that an
adequate pool of the nucleoside remained in the
system.

The evidence presented by Friedkin (1957)
and Phear and Greenberg (1958) indicates that the
primary one-carbon unit acceptor in thymidylic acid
biosynthesis was a nucleotide derivative of
deoxyuridine whereas Blakley (1957), on the other
hand, found that the nucleoside acted as the primary

one-carbon unit acceptor. However, no evidence
pointing to deoxyuridylic acid as the primary
one-carbon unit acceptor in preference to deoxy-
uridine, or _vice versa_, was obtained from the
present experiments. _Esch. coli_ PA/15 extracts
were found to contain both deoxyuridylic acid
phosphomonoesterase and deoxyuridine-5'-phospho-
kinase activities with the effect that the
addition of either deoxyuridylic acid or deoxy-
uridine to an _Esch. coli_ PA/15 extract quickly
resulted in a mixture of both nucleoside and
nucleotide being formed (Sections II, 5 and II, 9).

(c) The hydrogen donor system.

Since the "5-methylation" of a pyrimidine
ring obviously involves the reduction of a one-
carbon unit at the oxidation level of formaldehyde,
the provision of a hydrogen donor (or reducing)
system is necessary. Both TPNH and DPNH were
utilised in this rôle.

TPNH was synthesised _in situ_ from TPN and
glucose-6-phosphate by the action of the glucose-6-
phosphate dehydrogenase present in _Esch. coli_
extracts, the presence of this enzyme activity in
the extracts readily being confirmed by a spectro-
photometric method (Section II, 8). Moreover, the

presence of this enzyme activity in Esch. coli extracts enabled a rapidly self-regenerating hydrogen donor system to be established by the use of TPN and glucose-6-phosphate in the molar ratio of 1:10.

The use of DPNH as the hydrogen donor was complicated by the presence of DPNH oxidase activity in the Esch. coli extracts. However, the use of anaerobic conditions inhibits the action of this enzyme and, moreover, a rapidly self-regenerating system for DPNH formation in situ can be established by using DPNH, TPN and glucose-6-phosphate in the molar ratio of 1:1:10 by virtue of the observed pyridine nucleotide transhydrogenase activity of Esch. coli extracts (Section II, 8).

(iii) The biosynthesis of thymidylic acid.

When the presence of the necessary accessory enzyme activities in Esch. coli PA/15 had been confirmed and the optimum conditions for the operation of each of the three systems (one-carbon unit donor, one-carbon unit acceptor and hydrogen donor) necessary for the "5-methylation" of the pyrimidine ring had been established, the biosynthesis of thymine was reinvestigated.

However, although the synthesis of thymidine from
deoxyuridine by rabbit thymus extracts was demonstrated
under the same conditions as used by Blakley (1957),
no evidence of thymine or thymidine biosynthesis by
Esch. coli PA/15 extracts under the same conditions
was obtained.   Accordingly, in view of the evidence
that the methylation reaction takes place at the
nucleotide level (Friedkin, 1957), the reaction was
reinvestigated at this level.

It was found that cell-free extracts of
Esch. coli PA/15 or Esch. coli 113/3 will catalyse
the formation of ^{14}C-thymidylic acid from deoxyuridine,
uridine or deoxyuridylic acid and a one-carbon unit
derived from $3-^{14}C$-serine, ^{14}C-formate or ^{14}C-formalde-
hyde in the presence of the cofactors ATP, Mg^{2+},
tetrahydrofolic acid, pyridoxal phosphate and TPNH
(or DPNH) when incubation is carried out under anaerobic
conditions (Sections II, 19; II, 21; II, 22 and II, 24).
A preliminary report of the biosynthesis of ^{14}C-
thymidylic acid in this system has been made already
in a paper read to the Biochemical Society (Birnie and
Crosbie, 1958).   A copy of the published abstract of
this paper is appended.

The identification of the ^{14}C-thymidylic acid
synthesised in these systems depended upon the failure

to separate radioactivity from added carrier thymidylic acid by paper chromatography in five different solvents and by paper electrophoresis at pH 3.5. Although this method of identifying compounds is not wholly satisfactory, its use in this case was unavoidable. The defect in the method was clearly illustrated when it was found that the ^{14}C-thymidylic acid, on acid hydrolysis, yielded ^{14}C-thymine of much lower specific activity. However, the fact that the ^{14}C-thymine isolated from the hydrolysis products of the parent "^{14}C-thymidylic acid" proved to be chromatographically homogeneous confirmed that a proportion, at least, of the parent "^{14}C-thymidylic acid" was indeed authentic ^{14}C-thymidylic acid (Section II, 21). Moreover, the very large decrease in the yield of the parent "^{14}C-thymidylic acid" produced by the exclusion of pyrimidine one-carbon unit acceptors from the thymidylic acid biosynthetic system suggests that the parent "^{14}C-thymidylic acid" is composed of pyrimidine derivatives. This, together with the observation that the specific activities of the ^{14}C-thymine samples isolated were proportional to those of the parent "^{14}C-thymidylic acid" samples, suggests that the conclusions drawn from considerations of the parent "^{14}C-thymidylic acid" specific activities

are not invalidated by the recognition of the heterogeneous nature of the parent "^{14}C-thymidylic acid".

The recognition of the heterogeneous nature of the "^{14}C-thymidylic acid" isolated immediately posed the question of the nature of the ^{14}C-labelled material which was not separated from authentic thymidylic acid by extensive paper chromatography and electrophoresis. It has been pointed out that evidence to suggest that the radioactivity is associated with a pyrimidine derivative has been obtained. Although "^{14}C-thymidylic acid" was isolated from systems to which no pyrimidine compound had been added, this "^{14}C-thymidylic acid" was shown to contain authentic ^{14}C-thymidylic acid in the same proportion as in "^{14}C-thymidylic acid" isolated from systems to which deoxyuridine or deoxyuridylic acid had been added (Tables II, 11 and II, 12). This observation suggests that the "^{14}C- thymidylic acid" synthesised in "pyrimidine-less" systems is a pyrimidine derivative and not a serine metabolite. The presence of a small pool of deoxynucleoside or deoxynucleotide in the Esch. coli extracts would account for the synthesis of "^{14}C-thymidylic acid" in "pyrimidine-less" systems.

However, the possibility that the parent "^{14}C-thymidylic acid" is mainly composed of material which is not a pyrimidine derivative must be admitted.   It is conceivable that the material is total unrelated to thymidylic acid and that it may be a compound such as a phosphorylated ^{14}C-labelled sugar derivative derived from 3-^{14}C-serine and deoxyribose (or ribose) liberated from deoxyuridine (or uridine) by the feeble nucleoside phosphorylase activity of the enzyme system used. Indeed, the observation that cell-free extracts of Esch. coli 15T-, a thymine- or thymidine-less mutant, would catalyse the formation of "^{14}C-thymidylic acid" from 3-^{14}C-serine and deoxyuridine provides evidence in support of this suggestion.

(iv)   The effect of a pool of unlabelled thymidylic acid on ^{14}C-thymidylic acid biosynthesis.

The presence of a pool of unlabelled thymidylic acid was found to decrease the yield of authentic ^{14}C-thymidylic acid synthesised from deoxyuridine or deoxyuridylic acid and 3-^{14}C-serine. This observation indicates that a pool of thymidylic acid tends to inhibit the synthesis of ^{14}C-thymidylic acid.   On the other hand, the amount of the parent "^{14}C-thymidylic acid" synthesised was not decreased

by a pool of unlabelled thymidylic acid. This observation again suggests that the bulk of the parent "[14]C-thymidylic acid" is unrelated to thymidylic acid. However, the significance of this observation will remain obscure until the structure of the parent "[14]C-thymidylic acid" is elucidated.

(v) Pyrimidine derivative intermediates in the biosynthesis of thymidylic acid.

It was suggested previously (Birnie and Crosbie, 1958) that two ^{14}C-labelled materials isolated from systems in which ^{14}C-thymidylic acid was synthesised were intermediates on the thymidylic acid biosynthetic pathway and that these materials were, in fact, the 5-hydroxymethyl derivatives of deoxyuridine and deoxyuridylic acid. Investigation subsequent to this report has shown that these two materials were both a mixture of alanine and glutamic acid produced by the action of the serine deaminase activity of Esch. coli extracts on 3-^{14}C-serine followed by further metabolism (Section II, 20).

Despite extensive investigation, no trace of the 5-hydroxymethyl derivatives of uracil,

deoxyuridine or deoxyuridylic acid were found to be present after incubation of the thymidylic acid biosynthetic systems. The evidence obtained appears to rule out the possibility of the presence of a detectable trace of 5-hydroxymethyluracil or 5-hydroxymethyldeoxyuridine. However, by analogy with the known acid-lability of the hydroxymethyl group of 5-hydroxymethyldeoxycytidylic acid (Wyatt and Cohen, 1952), the possibility that 5-hydroxymethyldeoxyuridylic acid may give rise to uracil rather than 5-hydroxymethyluracil on acid hydrolysis must be admitted. No authentic 5-hydroxymethyldeoxyuridylic acid being available, the behaviour of the nucleotide on acid hydrolysis could not be investigated.

No recognisable pyrimidine derivative intermediates were isolated from the systems in which Esch. coli 15T- extracts replaced Esch. coli PA/15 extracts. ^{14}C-labelled material with the same chromatographic properties as thymidylic acid was isolated from these systems but, unfortunately, attempts to identify this substance were unsuccessful. However, the similarity between this ^{14}C-labelled material and the parent "[14]C-thymidylic acid" isolated from the Esch. coli PA/15 systems indicates that the

possibility that it is not a pyrimidine derivative
warrants careful consideration.

The difficulties inherent in synthesising
authentic samples of possible intermediates such
as 5-hydroxymethyldeoxyuridylic acid, the 4:5-
dihydro-5-hydroxymethyl derivatives of uracil,
deoxyuridine and deoxyuridylic acid and the
condensation products of uracil, deoxyuridine and
deoxyuridylic acid with N-hydroxymethyltetrahydro-
folic acid (I, Fig. I, 5) precluded the investigation
of these compounds as intermediates on the thymidylic
acid biosynthetic pathway.

(vi)   The effect of vitamin B_{12} on thymidylic acid
        biosynthesis.

The mutant used in these experiments,
Esch. coli 113/3, was not wholly suited to the study
attempted since the organism is a methionine- or
vitamin B_{12}-less mutant and, therefore, it is possible
that vitamin B_{12}-deficient cells of Esch. coli 113/3
contain, or can synthesise, sufficient vitamin B_{12} to
effect thymidylic acid biosynthesis while being unable
to synthesise methionine.   Repetition of this work
using Lactobacillus leichmannii, which requires
vitamin B_{12} or deoxynucleoside for growth and which,
therefore, is more suitable for this study than is

Esch. coli 113/3.

However, it was found that the addition of
vitamin B_{12} to a system containing deoxyuridine,
3-^{14}C-serine and an extract of vitamin B_{12}-deficient
cells of Esch. coli 113/3 increased the amount of
"^{14}C-thymidylic acid" synthesised, indicating that
vitamin B_{12} was involved in the "methylation"
reaction.   No effect of vitamin B_{12} on uridine-
ribose reduction to thymidylic acid-deoxyribose was
found although Downing and Schweigert (1956) have
shown that vitamin B_{12} is involved in DNA-deoxyribose
synthesis in Lactobacillus leichmannii (Section II, 19).

It must be pointed out, however, that the
validity of these results is placed in doubt by the
subsequently discovered heterogeneous nature of the
"^{14}C-thymidylic acid".

3.   The catabolism of thymidylic acid.

It was observed that a small proportion
of any thymine or thymidine present disappeared
from incubation mixtures containing either washed
whole-cell suspensions or cell-free extracts of
Esch. coli PA/15 in phosphate buffer.   The failure
to detect any ^{14}C-thymine in the DNA isolated from
systems in which ^{14}C-thymidylic acid was synthesised
ruled out the possibility of thymine incorporation

into the DNA of the organism, although it was found that ^{14}C-thymidine-5'-triphosphate was synthesised in these systems. Alternatively, the thymine may undergo an Esch. coli-catalysed degradation by the pathways observed by Fink et al. (1956b) using rat liver slices.

The reductive pathway of thymine catabolism was investigated by a spectrophotometric technique. It was found that TPNH slowly disappeared from incubation mixtures containing thymine or thymidylic acid and Esch. coli PA/15 extract in Tris buffer, indicating that Esch. coli extracts catalyse the reduction of the pyrimidine 4:5-double bond in thymine and thymidylic acid. Confirmation was obtained with the isolation of small amounts of β-amino-iso-butyric acid and β-ureido-iso-butyric acid from among the products of thymidylic acid catabolism. Moreover, β-amino-iso-butyric acid was isolated after acid hydrolysis of the nucleotide fraction of the thymidylic acid degradation products, suggesting the presence of 4:5-dihydrothymidylic acid among these products (Section II, 25).

It was found that thymidine did not undergo reduction in a phosphate-free buffer,

indicating that the previously noted catabolism of thymidine proceeds by way of a nucleoside phosphorylase-catalysed hydrolysis to thymine. However, the observation that thymidylic acid is reduced at a rate twice that of thymine indicates that the reduction of thymidylic acid does not follow a similar hydrolysis to thymine.

The study of the ^{14}C-labelled materials produced by the catabolism of ^{14}C-thymidylic acid synthesised in situ by Esch. coli PA/15 extracts (Section II, 26) yielded no further information as to the structures of the thymidylic acid catabolic products. Tentative identification of ^{14}C-labelled 4:5-dihydrothymine, β-ureido-iso-butyric acid and β-amino-iso-butyric acid was made by paper chromatographic analysis of the reaction products. No trace of ^{14}C-5-hydroxymethyl or ^{14}C-5-carboxylic acid derivatives was found and thus no evidence for the operation of an oxidative catabolic pathway was obtained.

4. The mechanisms of thymidylic acid biosynthesis and catabolism.

The evidence discussed in Section I, 4 has clearly implicated N-hydroxymethyltetrahydrofolic

acid as the immediate one-carbon unit donor involved in the synthesis of the thymidylic acid-methyl group. Evidence in corroboration of this conclusion was obtained during the present study on the biosynthesis of thymidylic acid when the presence of a pool of N-hydroxymethyltetrahydro-folic acid was detected in the thymidylic acid biosynthesis system used (Section II, 11). No evidence as to the nature of the primary one-carbon unit acceptor was obtained. However, it has been shown that deoxyuridine, uridine and deoxyuridylic acid all act as one-carbon unit acceptors in the biosynthesis of thymidylic acid, but no evidence as to whether deoxyuridine or deoxyuridylic acid is the primary one-carbon unit acceptor was obtained although it was found that the product of the methylation reaction was a nucleotide derivative.

The further details of the biosynthetic mechanism were not elucidated. No identifiable intermediates were isolated and no reliable evidence as to the precise mechanism of thymidylic acid biosynthesis was obtained.

The catabolism of thymidylic acid by Esch. coli PA/15 has been shown to proceed by way of an

initial reduction of the 4:5-double bond of the
pyrimidine ring. The pathway of further catabolism
of thymidylic acid was not elucidated, but the
detection of β-amino-iso- butyric acid and
β-ureido-iso-butyric acid among the catabolic
products strongly suggests that the catabolic
pathway is similar to that proposed by Fink et al.
(1956 b) for the reductive catabolism of thymine
by rat liver slices.

Section IV   :   Experimental

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26. Assay of radioactivity.

27. General enzymic incubation techniques.

1. Cleaning and sterilizing glassware.

Tubes and flasks used for microbiological work were cleaned by heating with boiling 10% nitric acid for one hour, washed thoroughly with tap-water and finally rinsed three times in glass-distilled water. Pipettes were steeped overnight in a mixture of methanol, water and 60% KOH (15.7: 3.3: 1) and rinsed successively with water, dilute HCl, tap-water (several times) and glass-distilled water (three times). All other glassware used - centrifuge bottles for harvesting cells, centrifuge tubes, incubation tubes ("Quickfit" stoppered test-tubes), pipettes etc. - was steeped in concentrated chromic acid at least overnight, washed thoroughly in tap-water and finally in glass-distilled water (three times). All glassware was dried in an oven at 100° C.

Solutions were sterilized in flasks or tubes plugged with non-absorbent cotton wool. Nutrient agar slopes for bacterial cultures were sterilized by heating in an autoclave at 15 lbs./s.in. for 15 minutes. Solutions were sterilized in the same way or by boiling. Tubes and pipettes for microbiological assays were dry-sterilized by heating at 180° C. for one hour.

2. Culture and harvesting of micro-organisms.

The organisms used in this work were
(i) Escherischia coli PA/15, a glycine-less, serine-less mutant which was kindly provided by Professor D.D. Woods; (ii) Esch. coli CW 194, a methionine-less mutant kindly supplied by Dr. Ceitham; (iii) Esch. coli 15^T-, a thymine-less mutant kindly provided by Dr. S.S. Cohen; (iv) Esch. coli 113/3, a methionine- or vitamin B_{12}-less mutant kindly provided by Dr. B.D. Davis and (v) Bacillus subtilis N.C.I.B. 8059, a wild-type organism capable of growth on a simple glucose-ammonium salt synthetic medium. The micro-organisms were maintained on nutrient agar slopes (prepared from "Oxoid" brand tablets, code No. CM4) at 4° C. with monthly transfer.

The mutants of Esch. coli, when required for an experiment, were grown on a liquid basal medium supplemented according to the needs of the particular organism. The basal medium consisted of KH_2PO_4 (5.4 g.), $(NH_4)_2SO_4$ (1.2 g.), $MgSO_4$ (0.4 g.) and glucose (12 g.) dissolved in one litre of glass distilled water and adjusted to pH 7.2 with 5N-NaOH (approx. 6.2 ml.). Supplementary growth factors, in excess to that necessary for full growth of the organisms, were added as follows:

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(i) DL-serine (final concentration, 280 mg./litre) and
DL-alanine (final concentration, 170 mg./litre) for
Esch. coli PA/15 (Davis and Maas (1949) have shown
that D-serine exerts an inhibitory effect on the growth
of Esch. coli PA/15,giving rise to an initial lag-
phase of up to 48 hours; the addition of DL-alanine
overcomes this inhibition);

(ii) thymine (final concentration, 1.2 mg./litre) for
Esch. coli 15T-;

(iii) L-methionine (final concentration, 50 mg./litre)
for Esch. coli 113/3.

Bacillus subtilis was grown in Roux bottles
on a basal medium consisting of $(NH_4)_2$ HPO_4 (1.0 g.),
KCl (0.2 g.), $MgSO_4$ (0.2 g.), glucose (10 g.) and agar-
agar (200 g.) dissolved in one litre of glass-distilled
water.   This organism would not grow in liquid basal
medium, even with aeration, but grew well on the
surface of the semi-solid medium employed.   For
harvesting, the clusters of cells were washed off the
surface of the agar with a stream of water.

In each case, with the exception of Esch.
coli 113/3, the basal medium was inoculated from a
slope, either directly or from a suspension of cells
in sterile water which had been inoculated directly
from a slope.   In the case of Esch. coli 113/3 it was

necessary to deplete the cells of vitamin B_{12}.
The inoculum of Esch. coli 113/3 was taken from
the second of two serial sub-cultures in basal
medium supplemented with L-methionine (50 ug./ml.).

The inoculated medium was incubated at
(i) $37^{\circ}C$. for Esch. coli or (ii) 30° C. for B.
subtilis for 18 to 24 hours, the cell suspensions
so obtained being harvested by centrifuging
(2,000 g) at 0° C.   The thick paste of cells
collected was washed three times with chilled glass-
distilled water and stored in a refrigerator.

3.   Microbiological assays.

L-methionine was assayed using Esch. coli
CW194, a mutant which is blocked between homocysteine
and methionine.   The growth response curve of this
organism (Fig. IV, 1) was constructed as follows:

To each of a series of sterile tubes (6" x
1" rimless "pyrex") was added 8.3 ml. basal medium and
1.0 ml. of a sterile solution of L-methionine of
various known concentrations.   A blank tube, to which
1.0 ml. sterile water was added, was included in the
series.   Each tube was inoculated with 0.1 ml. of a
cell-suspension of Esch. coli CW194 which had been
grown previously in basal medium supplemented with a

Figures IV, 1 and IV, 2

Growth response curves of (i) Esch. coli CW194 to L-methionine and (ii) Esch. coli 15T- to thymine.

Assay system : 8.3 ml. basal medium + 1.0 ml. of a sterile solution of (i) L-methionine or (ii) thymine inoculated with 0.1 ml. of a suspension of (i) Esch. coli CW194 or (ii) Esch. coli 15T-. Incubation was carried out at 37° C. to stationary phase (18 to 24 hours). The cell suspensions were treated with one drop of formalin (40% formaldehyde) and allowed to stand at room temperature for one hour before the extinctions at 450mμ were determined.
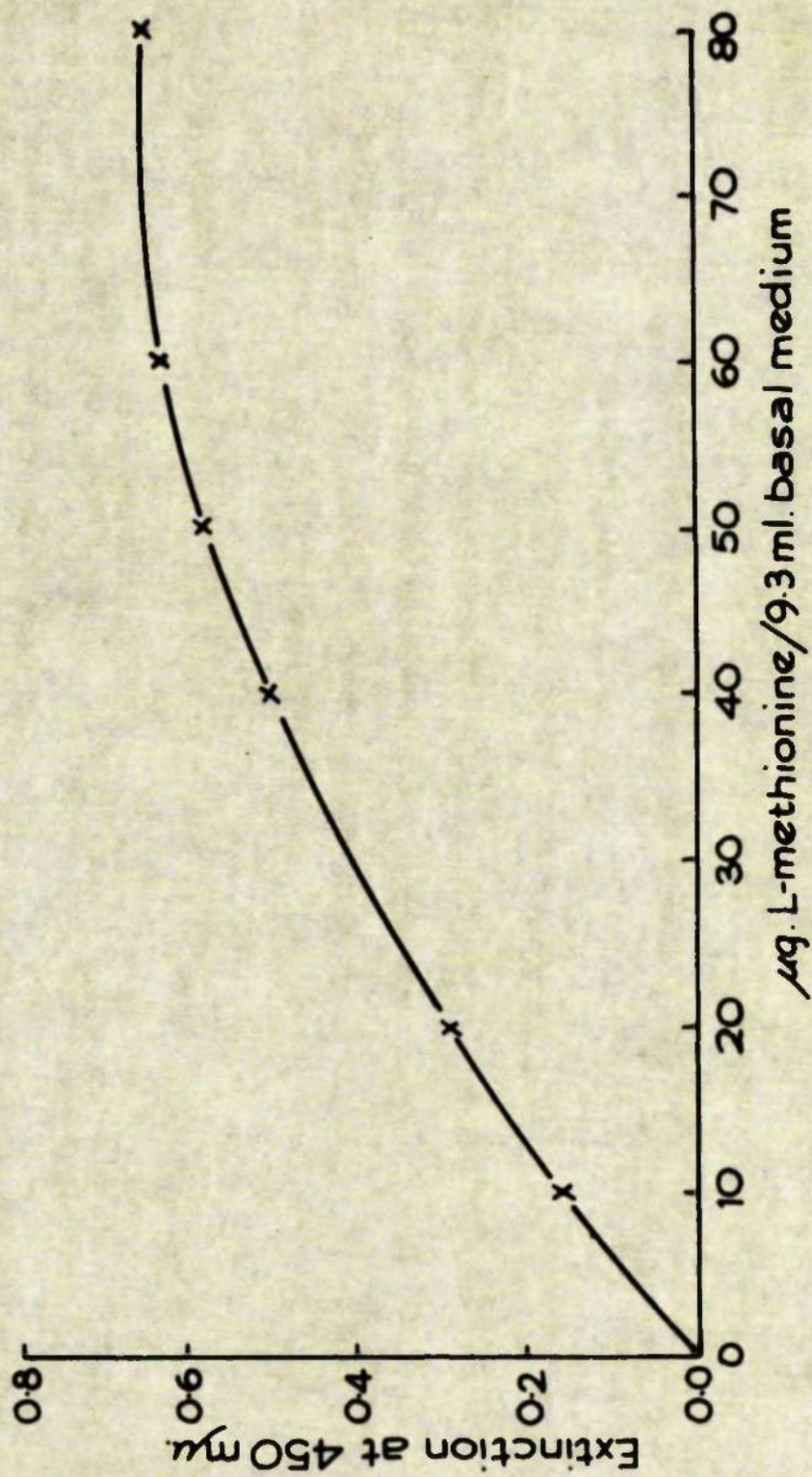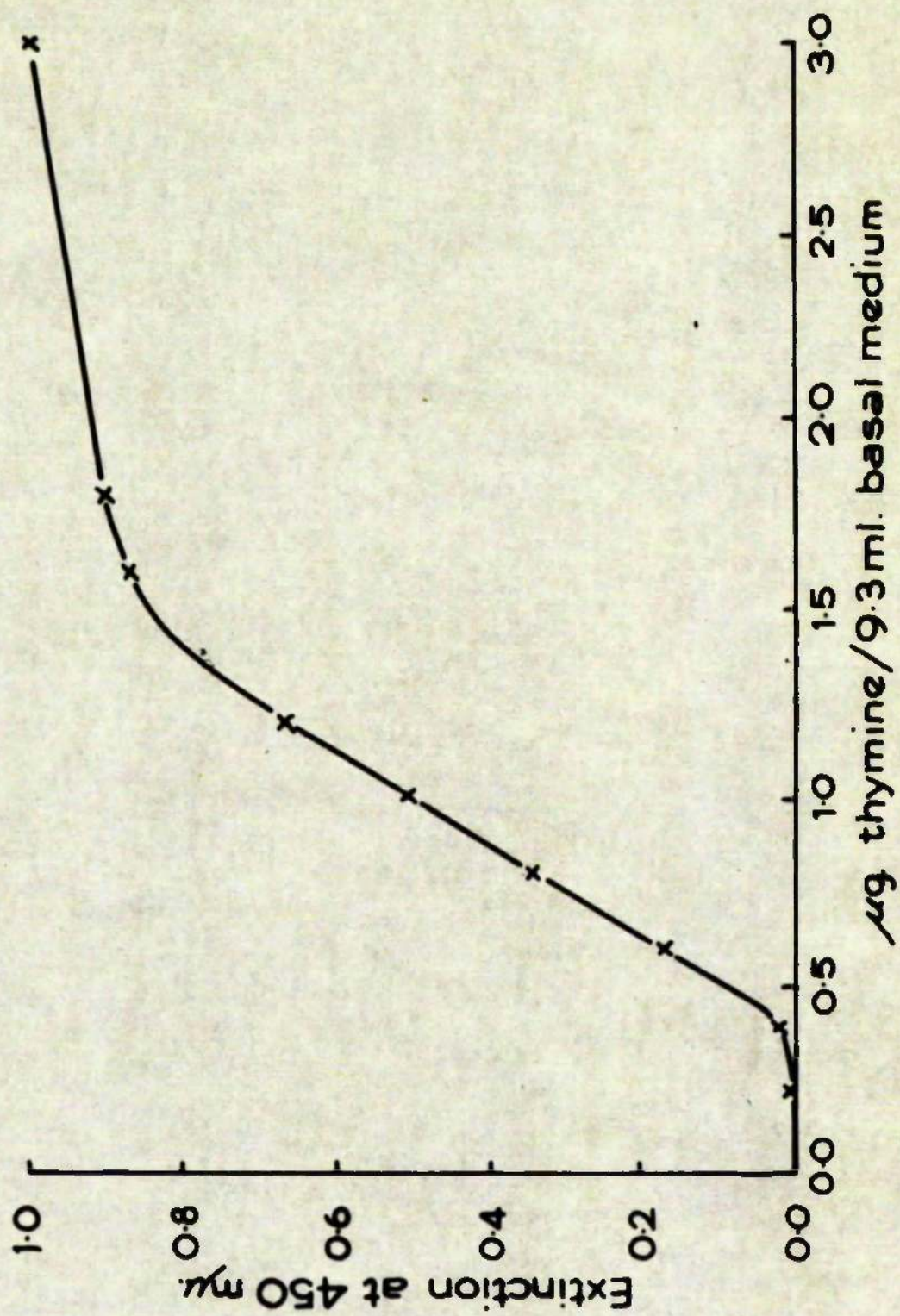
Figure IV, 1

Figure IV, 2

sub-optimal amount of L-methionine.   By this means, errors due to carry-over of excess L-methionine in the inoculum were avoided.   The inoculated media were incubated at 37° C. for 24 hours.   When growth was complete the cells were killed by the addition of one drop of formalin (40% formaldehyde) to each tube.   After one hour the optical density of each cell suspension was determined in a spectrophotometer (Unicam SP600) at 450 mμ.

The assay tubes were prepared and incubated in duplicate and the growth response curve confirmed by repetition.   Excellent duplication of results was obtained under standard conditions.

Thymine and thymidine were assayed using Esch. coli 15T-, and the growth response surve of this organism (Fig. IV, 2) was constructed in precisely the same way as was the growth response curve of Esch. coli CW194, using thymine in place of L-methionine.   Duplication was not achieved as readily in this case, especially at low concentrations of thymine (of the order of 0.1 to 0.4 mμmoles/ml.) due to the occasional tendency of the organism to form long filamentous cells and undergo clumping.

Solutions of methionine and thymine or thymidine of unknown concentration were assayed in

the same way as the growth response curves were constructed. 1.0 ml. of each sterile test solution being added to the system in place of the standard L-methionine or thymine solutions. Assays were carried out in duplicate and a number of tubes to which were added standard L-methionine or thymine solutions were included in each assay. The concentration of each test solution was adjusted by dilution to ensure that it lay on the straight portion of the growth response curve.

4.   Preparation of cell-free extracts of Esch. coli.

Cell-free extracts of Esch. coli mutants were prepared by a slightly modified version of the method described by McIlwain (1948). The preparation of these extracts was carried out in the cold-room (4^0 C.).

The thick paste of washed cells, drained of excess water, was mixed intimately with alumina powder (Griffin and Tatlock 'Microid Polishing Alumina' grade 3/50-slow cutting) in a chilled agate mortar, sufficient alumina being added to produce a dry, crumbling powder. The mixture was ground with an agate pestle with maximum hand pressure for 30 seconds during which time the mixture became

moist. The mixture was allowed to stand for one minute to cool, more alumina was mixed with the paste until a dry powder was obtained and the grinding was repeated. The process was repeated four times in all and the paste obtained was introduced into a chilled centrifuge tube. The alumina was extracted with (i) 5 ml. phosphate buffer (0.1M, pH 7.2) or (ii) 5 ml. glass-distilled water. The alumina and cell debris were spun down by refrigerated centrifugation (20,000 g) for 30 minutes and the protein-laden supernatant was transferred to a chilled stoppered test-tube. The extract was diluted to the required volume and stored in ice in a refrigerator. Extracts prepared and stored in this way remained active for a week or more.

5. <u>Preparation of extracts of rabbit thymus glands.</u>

Young rabbits of body weight about 1500 g. were killed by cervical dislocation. The thymus gland was removed rapidly, chilled on ice, blotted dry with filter paper and weighed. The gland (2.5 to 3.0 g.) was shredded with scissors and homogenised in a Potter blender in 6 ml. Tris-(hydroxymethyl-) aminomethane-HCl (Tris) buffer (0.1M, pH 7.2). The

homogenate was centrifuged (5,000 g) at 0° C. for 75 minutes and the supernatant removed and stored at 0° C.

Cofactors were removed from extracts prepared in this way by passage through a short column of Dowex-1-(chloride) ion exchange resin at pH 7-8. The first 10 ml. eluted from this column was collected and stored at 0° C.

6. Estimation of protein concentration in cell-free extracts.

Protein in cell-free extracts was estimated by a modification of the method described by Gornall, Bardawell and David (1949). The Biuret reagent used consisted of $CuSO_4.5H_2O$ (1.5 g.) and sodium potassium tartrate (6.0 g) dissolved in 500 ml. distilled water. To this solution 300 ml. NaOH (10% w./v.) was added with stirring and the mixture was diluted to one litre with distilled water. The Biuret reagent was stored in a polythene container in the refrigerator.

The solution of protein (0.5 ml.) was pipetted into a 15 ml. graduated conical centrifuge tube and mixed with 0.2 ml. 5N-perchloric acid (PCA). The mixture was centrifuged (5,000 g) and

the supernatant fluid discarded.   The protein
residue was washed with 2 ml. 2N-PCA, centrifuged
(5,000 g) and the supernatant discarded.   Biuret
reagent was added to the protein residue to a
total volume of 8.0 ml. and the mixture was
incubated at 37° C. for 30 minutes.   The
extinction of the characteristic colour was
determined in a spectrophotometer (Unicam SP600)
at 540 mμ against a blank of Biuret reagent.

A calibration curve (Fig. IV, 3) was
constructed using solutions of casein as
standards.

7.   Sources of materials.

(1) Pyrimidines, purines and their derivatives.

Uracil, 5-hydroxymethyluracil, thymine,
dihydrothymine, β-amino-iso-butyric acid, uridine,
deoxyuridine, deoxycytidine, thymidine, uridine-3'-
phosphate, thymidine-5'-phosphate (Ca^{2+} salt) and
deoxycytidine-5'-phosphate were purchased from the
California Foundation for Biochemical Research, Los
Angeles, U.S.A. and were all of C$_p$P grade (that is,
highest purity with analyses supplied).   Adenosine-
5'-triphosphate (crystalline tetrasodium salt) was
purchased from the Sigma Chemical Company.

Figure IV, 3

Calibration curve for the estimation of protein concentration in cell-free extracts of Esch. coli by Biuret reagent.

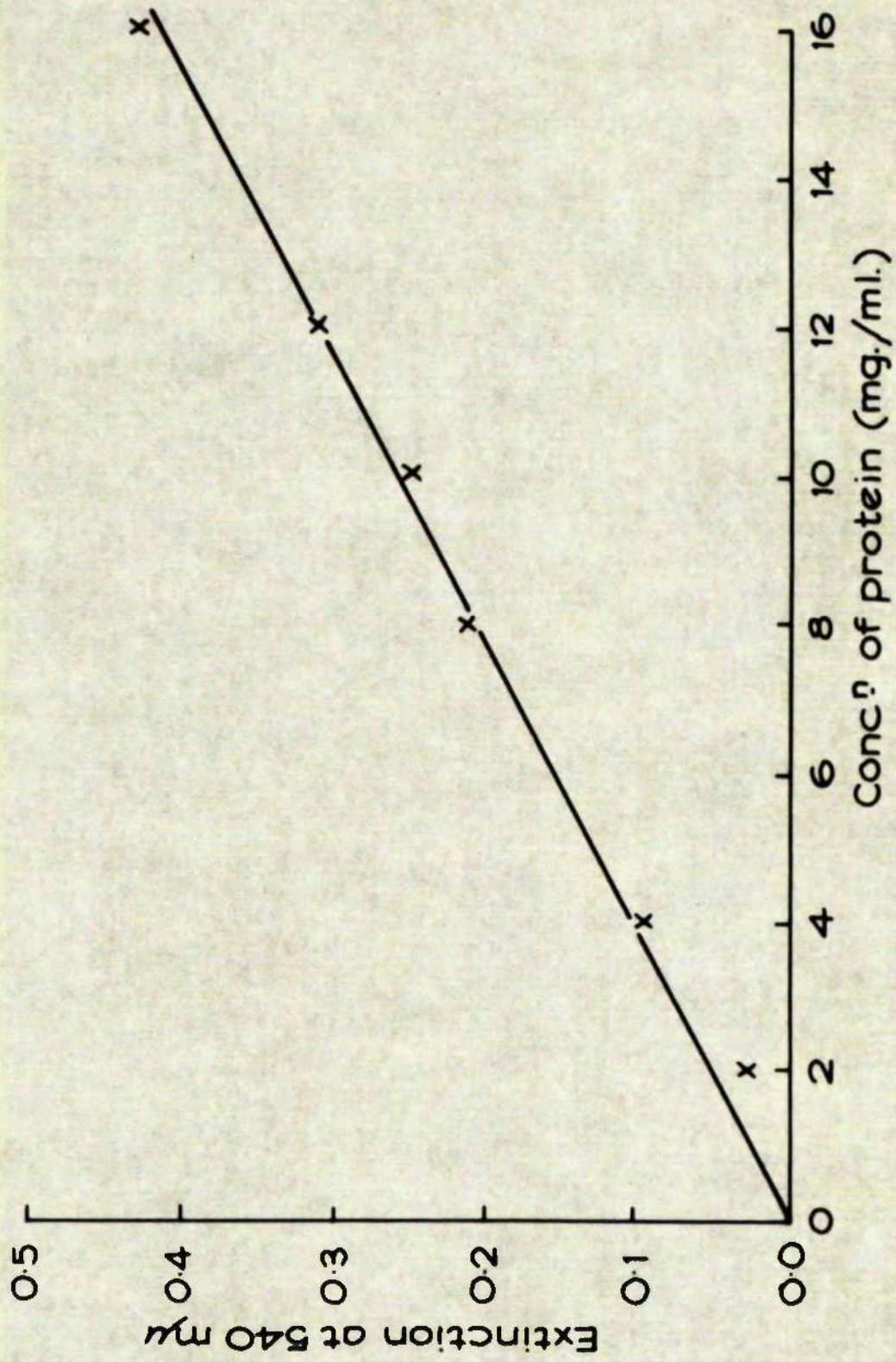Standard protein solutions were prepared by dissolving casein in distilled water.

Figure IV. 3

(ii) Amino-acids.

DL-serine, L-methionine and DL-alanine
were purchased from British Drug Houses Ltd.
DL-serine and DL-homocysteine were purchased from
L. Light and Company Ltd.

(iii) Cofactors.

Fructose-1:6-diphosphate, glucose-6-
phosphate and reduced diphosphopyridine
nucleotide were purchased from C.F. Boehringer
und Soehne GMBH. Triphosphopyridine nucleotide
and glucose-6-phosphate were supplied by the
Sigma Chemical Company. Roche Products Ltd.
supplied folic acid and the research department
of this firm donated the pyridoxal phosphate used
in these studies. Leucovorin (N^5-formyl-5:6:7:8-
tetrahydrofolic acid) (Ca^{2+} salt) was the gift of
Lederle Laboratories Ltd. Cytamen "20" (a
solution of 20 ug. vitamin B_{12}/ml.) was purchased
from Glaxo Laboratories Ltd.

(iv) Radiochemicals.

$3-^{14}C$-serine, $^{14}C_3$-serine, $2-^{14}C$-glycine
and ^{14}C-formaldehyde were purchased from the
Radiochemical Centre, Amersham.

(v) Reagents

p-Dimethylaminobenzaldehyde, ninhydrin
and 2:4-dinitrophenylhydrazine were of Analar grade,
purchased from British Drug Houses Ltd.   1-Fluoro-
2:4-dinitrobenzene was supplied by L. Light and
Company Ltd.

(vi) General materials.

Agar-agar and nutrient agar (Code No.
CM4) were purchased from Oxo Ltd.   Grinding
alumina was purchased from Griffin and Tatlock
Ltd.   Alumina (for chromatography), glucose,
α-oxoglutaric acid, salts solvents and other
reagents were supplied by British Drug Houses Ltd.
Tris-(hydroxymethyl-) aminomethane was purchased
from L. Light and Company Ltd.

8. Preparation of folic acid and some folic acid
   derivatives.

(i) The folic acid purchased was not
chromatographically homogeneous but no solvent
system suitable for recrystallisation of folic
acid was found.   Accordingly, the grosser
impurities were removed by dissolving the folic
acid in dilute NaOH and reprecipitating with
dilute HCl.   The flocculent precipitate was

separated by filtration, washed with glass-distilled water and dried in the air-oven. The ultraviolet-absorption spectrum of folic acid purified in this way is shown in Fig. IV, 5(a).

(ii) Solutions of N^5-formyl-5:6:7:8-tetrahydrofolic acid (N^5-formyl-FAH$_4$) free of Ca^{2+} ions were prepared by dissolving the Ca^{2+} salt of N^5-formyl-FAH$_4$ in a minimum volume of glass-distilled water and adding one equivalent of phosphate buffer (pH 7.2). The precipitated $Ca_3(PO_4)_2$ was removed by centrifugation (5,000 g). The ultraviolet-absorption spectrum of N^5-formyl-FAH$_4$ prepared in this way is shown in Fig. IV, 5(a).

(iii) N^{10}-formylfolic acid was prepared by the method described by Flynn et al. (1959). A mixture of folic acid (10 g.), 100% formic acid (20 ml.) and acetic anhydride (5 ml.) was heated under reflux for one hour at 50° C. The reaction mixture was evaporated to dryness and the residue was dissolved in dilute NaOH. N^{10}-formylfolic acid was reprecipitated with dilute HCl, separated by filtration, washed with glass-distilled water and dried in air. The ultraviolet absorption spectrum of N^{10}-formylfolic acid prepared in this way is shown in Fig. IV, 5(a).

9. Synthesis of 5:6:7:8-tetrahydrofolic acid and
N-hydroxymethyl-5:6:7:8-tetrahydrofolic acid.

(1) The hydrogenation of folic acid to yield
5:6:7:8-tetrahydrofolic acid (FAH$_4$) was carried out
by the method described by O'Dell et al. (1947)
with slight modification.

Folic acid (150 mg.) was dissolved by
shaking in glacial acetic acid (100 ml.) Adam's
catalyst, hydrated platinum oxide (110 mg.), was
added to this solution and the hydrogenation was
carried out under an atmosphere of hydrogen in the
usual manner. Hydrogen uptake was smooth and
rapid, 42 ml. (at room temperature and 760 mm.
pressure) being absorbed by the mixture in under
three hours. No further uptake of hydrogen was
observed in a further 18 hours. The theoretical
uptake of hydrogen for 110 mg. Adam's catalyst and
150 mg. folic acid is 43 ml.

After hydrogenation was complete the
apparatus was flushed out with oxygen-free nitrogen
and the acetic acid solution of FAH$_4$ was separated
from the reduced catalyst by means of the arrange-
ment in Fig IV, 4. This apparatus (Fig. IV, 4)
was flushed out with a stream of nitrogen for 15
minutes during which time the reduced catalyst

Figure IV, 4

Apparatus used for the separation of acetic acid
solutions of 5:6:7:8-tetrahydrofolic acid from
reduced Adam's catalyst under anaerobic conditions.
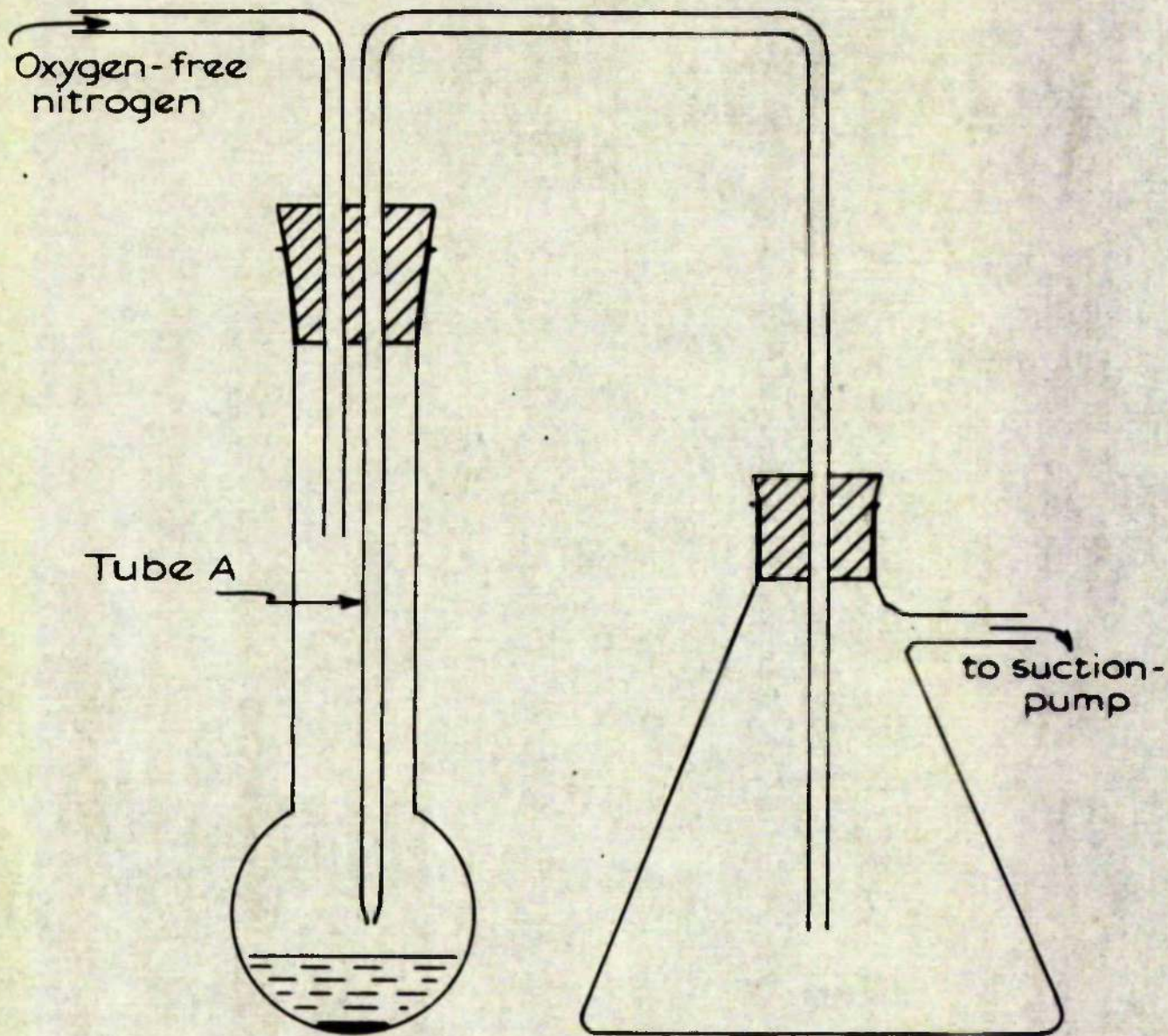
Oxygen-free
nitrogen

Tube A

to suction-
pump

Figure IV, 4

settled completely.   The tip of tube A was dipped
beneath the surface of the liquid and the supernatant
siphoned over smoothly into the second flask.   The
solution was transferred rapidly from this flask to
a round-bottomed flask, which had been flushed with
nitrogen, and immediately freeze-dried.   The
solution yielded a pale pink, powdery solid which
was stored under an atmosphere of nitrogen in a sealed
flask.

Confirmation that the product of this
hydrogenation of folic acid is FAH_4 was obtained from
the following evidence:

(a)   hydrogen uptake was in agreement with the
theoretical hydrogen uptake for the synthesis of
FAH_4;

(b)   the ultraviolet-absorption spectrum of the product
agreed with that of FAH_4 published by O'Dell et al.
(1947) - Fig. IV, 5(b);

(c)   the ultraviolet-absorption spectrum of the product
after reoxidation by aeration did not agree with
that of the oxidation product of 7:8-dihydrofolic
acid (Figs. IV, 5(c) and IV, 5(d); O'Dell et al.
1947).

(ii)   N-hydroxymethyl-5:6:7:8-tetrahydrofolic acid
($N-CH_2OH-FAH_4$) was prepared readily by the non-enzymic

Ultraviolet absorption spectra of folic acid and some derivatives of folic acid.

Ultraviolet absorption spectra of

(a) folic acid (I), N^{10}-formylfolic acid (II) and N^5-formyl-FAH$_4$ (III) in 0.1 N-NaOH;

(b) 5:6:7:8-tetrahydrofolic acid (i) synthesised (I) and (ii) published by O'Dell et al. (1947) (II) in 0.1 N-NaOH and N-hydroxymethyl-5:6:7:8-tetrahydrofolic acid (III) at pH 11;

(c) xanthopterin published by O'Dell et al. (1947) (I) and 5:6:7:8-tetrahydrofolic acid after aeration for 2 hours (II) in 0.1 N-NaOH.

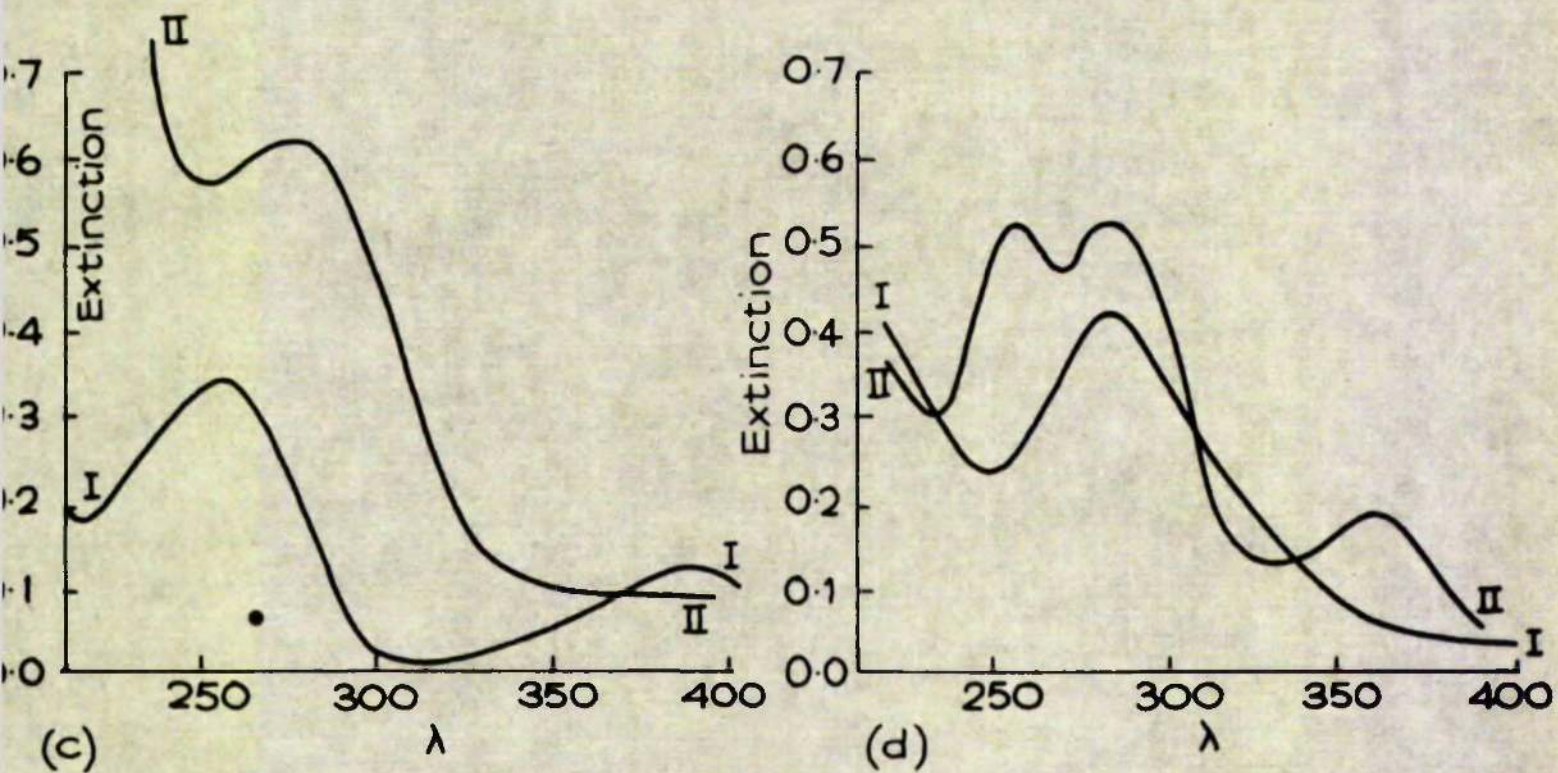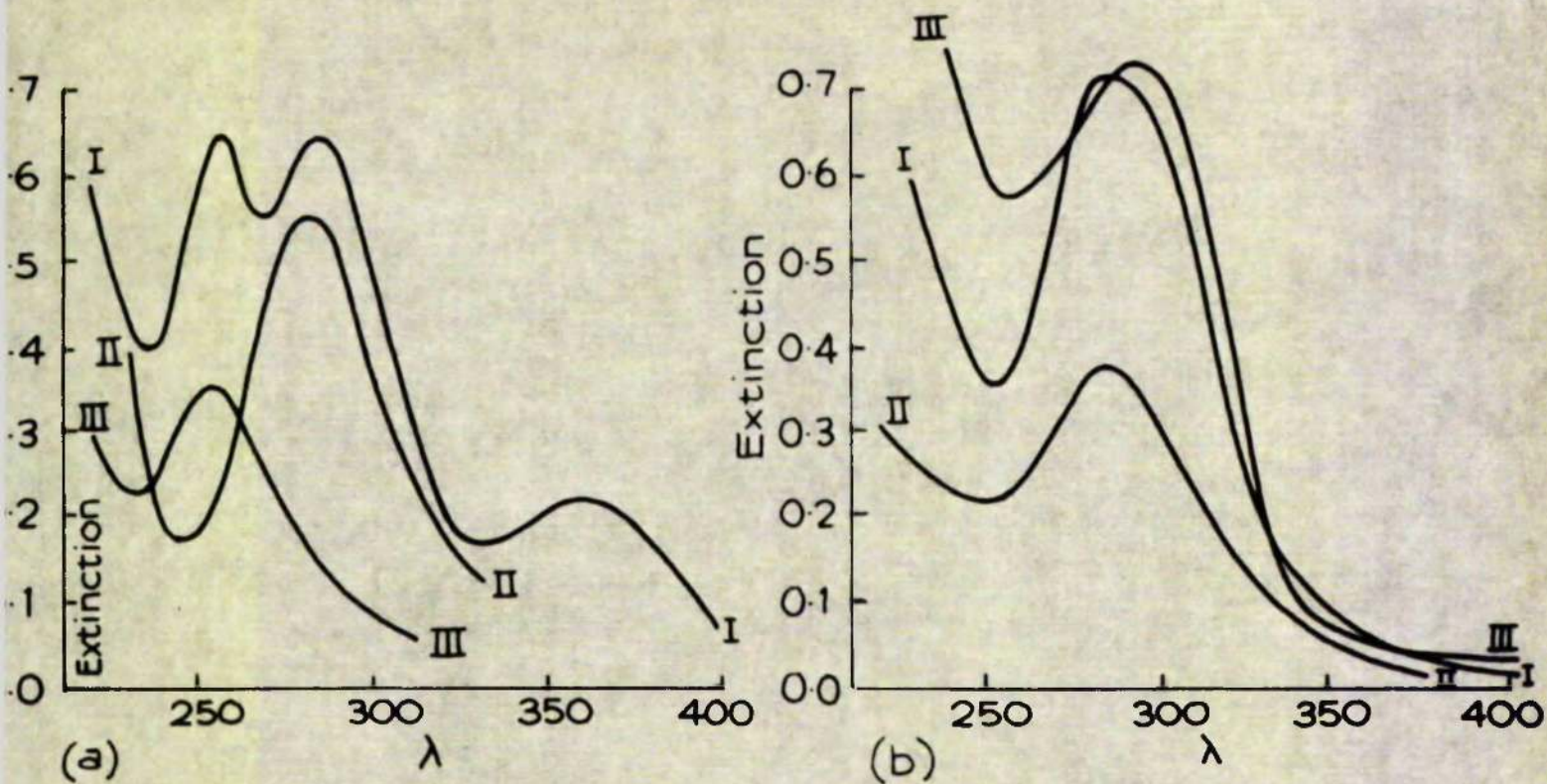(d) 7:8-dihydrofolic acid (I) and reoxidised 7:8-dihydrofolic acid (II) published by O'Dell et al. (1947) at pH 11.

Figure IV. 5

condensation of FAH_4 and formaldehyde.(Blakley, 1958). $N-CH_2OH-FAH_4$ was formed when molar equivalents of formaldehyde and FAH_4 (in solution in dilute alkali) were mixed in the cold. The product of this condensation was not isolated but was used in the solution in which it was prepared. The ultraviolet absorption spectrum of a mixture of formaldehyde and FAH_4 in equimolar amounts is shown in Fig. IV, 5(b).

10. 6-diphosphate and thymidylic acid free of heavy

6-diphosphate and thymidylic acid free of heavy metal ions.

The barium salts of glucose-6-phosphate and fructose-1:6-diphosphate were converted to the corresponding sodium salts by dissolving the barium salts in a minimum volume of $N-HCl$ and adding one equivalent of Na_2SO_4. The precipitated $BaSO_4$ was removed by centrifugation (5,000 g) and the supernatant was adjusted to pH 7 with NaOH. The sodium salts were not isolated from solution. The calcium salt of thymidylic acid was converted to the corresponding sodium salt by passage through a short column of Dowex-50(Na^+ form) ion exchange resin (diameter 1cm.; height 5cm.) at pH 7. Sodium thymidylate was isolated from the eluate of this column by freeze-drying.

11. Synthesis of deoxyuridine-5'-phosphate (deoxy-uridylic acid).

Deoxyuridylic acid (dUMP) was synthesised by the deamination of deoxycytidylic acid, using a method which was adapted from the one described by Wyatt and Cohen (1953) for the deamination of 5-hydroxymethylcytosine.

Glacial acetic acid (0.2 ml.) was added to a solution of deoxycytidylic acid (40 mg.) in 2M-NaNO$_2$ (1 ml.). After being allowed to stand for 24 hours at 27° C., the mixture was taken to dryness in vacuo. The residue was dissolved in distilled water (1.2 ml.) and 0.2 ml. BaCl$_2$ solution (350 mg./ml.) was added to the solution. The mixture was chilled in ice and the barium salt of dUMP was precipitated by adding 7 ml. ethanol. The mixture was centrifuged (5,000 g), the supernatant discarded and the precipitate washed five times with 10 ml. portions of a 5:1 mixture of ethanol and water. Finally the salt was washed with ethanol and ether and dried at 37° C.

Preliminary investigation showed that BaCl$_2$, Ba(NO$_2$)$_2$, Ba(NO$_3$)$_2$ and Ba^{2+} acetate were not precipitated by a 10:1 ethanol-water mixture. Paper chromatography (solvent, iso-propanol-water-HCl)

showed that there was no detectable trace of
deoxycytidylic acid contaminating the product.
The ultraviolet absorption spectra of the product
dissolved in acid and alkali corresponded with
those predicted for dUMP (Fig. IV, 6).

The sodium salt of dUMP was prepared by
dissolving the barium salt in a minimum volume of
0.1N-HCl and adding one equivalent of Na_2SO_4. The
precipitated $BaSO_4$ was removed by centrifugation
(5,000 g) and the supernatant was adjusted to pH 7
with NaOH.

12.   Synthesis of β-ureido-iso-butyric acid.

β-Ureido-iso-butyric acid (BUIB) was
synthesised from 4:5-dihydrothymine (DHT) by the
method of Fink et al. (1956a).   DHT (1.28 g.) was
dissolved in 7.5 N-NaOH (2 ml.) and the solution
was brought to the boil.   After cooling slowly
to room temperature, the solution was acidified with
concentrated HCl and refrigerated overnight.   The
precipitated BUIB was filtered off, washed with water
and redissolved in glass-distilled water.   The
solution of BUIB was stored at 0° C.

The R_F values of the BUIB prepared in this
way (0.35, using tert-butanol-methylethylketone-

Figure IV. 6

Ultraviolet absorption spectra of deoxyuridylic acid prepared by the deamination of deoxycytidylic acid.

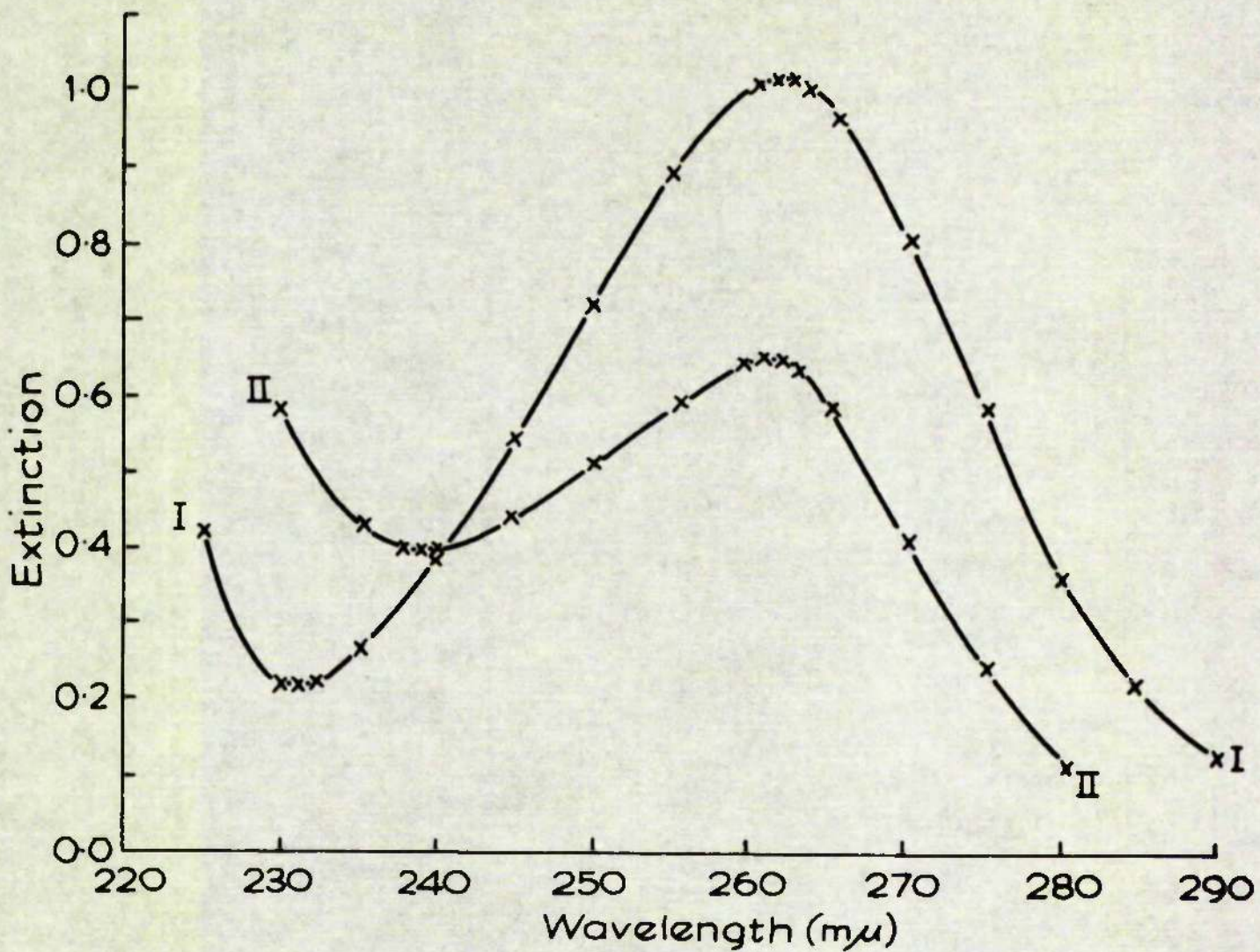Ultraviolet absorption spectrum of dUMP (i) at pH 1 (I) and (ii) at pH 13 (II).

Figure IV. 6

water-NH_4OH; 0.70, using tert-butanol-methylethyl-ketone-water-formic acid) agreed with the R_F values for BUIB quoted by Fink et al. (1956a) (0.34 and 0.68, respectively). This preparation of BUIB was not chromatographically homogeneous, traces of DHT and β-amino-iso-butyric acid (BAIB) being detected on paper chromatographic separations of the reaction product. However, as the BUIB was required only for carrier or marker material on paper chromatograms, no attempt was made to purify the preparation by recrystallisation.

13.   Synthesis of 5-hydroxymethyl derivatives of uracil, uracil nucleosides and uracil nucleotides.

The methods used in attempted syntheses of the 5-hydroxymethyl derivatives of uracil, uridine, deoxyuridine, uridylic acid and deoxyuridylic acid were based on the method described by Fink, Cline and Fink (1956). The published method consists of heating a mixture of the pyrimidine (60 μmoles) and a solution of 37% formaldehyde (1.3 mmoles), which had been acidified with HCl to a final concentration of 0.08 N-HCl, in a sealed capillary tube at 100° C. for 24 hours. The reaction products were separated by paper chromatography as bands (solvents, n-butanol-

water) and characterised by further paper
chromatography and by ultraviolet-absorption spectra.

The results obtained from the attempted
condensations, together with a description of the
wide variety of condensation conditions investigated,
are discussed in Section II, 13.

14.   Estimation of ammonia by Nessler's reagent.

Ammonia, in aqueous solution as ammonium
salts, was estimated by an adaptation of the method
described by Johnson (1941). The Nessler's reagent
used was prepared initially as two separate solutions.
Solution A consisted of KI (4 g.) and HgI_2 (4 g.)
dissolved in 25 ml. distilled water; solution B, of
gum acacia (3.5 g.) in 750 ml. distilled water. The
two solutions were mixed and diluted to one litre
with distilled water to form the Nessler's reagent.

Nessler's reagent (2 ml.), 6N-NaOH (1ml.)
and the solution of NH_4^+ to be assayed (2 ml.) were
mixed in a test-tube and allowed to stand at room
temperature for 15 minutes to develop the colour.
The extinction of the solution was determined using
a spectrophotometer (Unicam SP600) at (i) 420 mµ in
the case of low concentrations of NH_4^+ (1 to 10 µg.
NH_3/ml.) or (ii) 500 mµ in the case of high

concentrations of NH_4^+ (10 to 50 µg. NH_3/ml.) against a reagent blank. A calibration curve (Figs. IV, 7(a) and IV, 7(b) for each range of NH_4^+ concentrations was constructed using $(NH_4)_2SO_4$ as standard. Duplication of results using this method was excellent.

15. Estimation of keto-acids by 2:4-dinitrophenylhydrazine.

Keto-acids were estimated by a slight modification of the method described by Friedemann and Haugen (1943). The 2:4-dinitrophenylhydrazone reagent was prepared by dissolving 2:4-dinitrophenylhydrazine (100 mg.) in 2N-HCl (100 ml.) with gentle heating.

A test-tube containing 3 ml. of the keto-acid solution to be assayed was allowed to stand in a water-bath at 25° C. for 10 minutes before 0.5 ml. of the 2:4-dinitrophenylhydrazine reagent was added. After the mixture had been maintained at 25° C. for 5 minutes the reaction was stopped by the addition of 4N-NaOH (1.5 ml). The colour was allowed to develop at 25° C. for a further 10 minutes and the extinction of the solution was determined using a spectrophotometer (Unicam SP600) at 435 mµ against a reagent blank.

Figure IV, 7

Calibration curves for the estimation of ammonia concentration.

(a) Calibration curve for low concentrations of
   ammonia; and

(b) calibration curve for high concentrations of
   ammonia.

Assay system - 2 ml. NH_4^+ solution, 2ml. Nessler's reagent and 1 ml. 6N-NaOH. The extinctions of the solutions were determined at (a) 420 mμ for low concentrations and (b) 500 mμ for high concentrations of ammonia.

Standard solutions were prepared by dissolving $(NH_4)_2SO_4$ in distilled water.
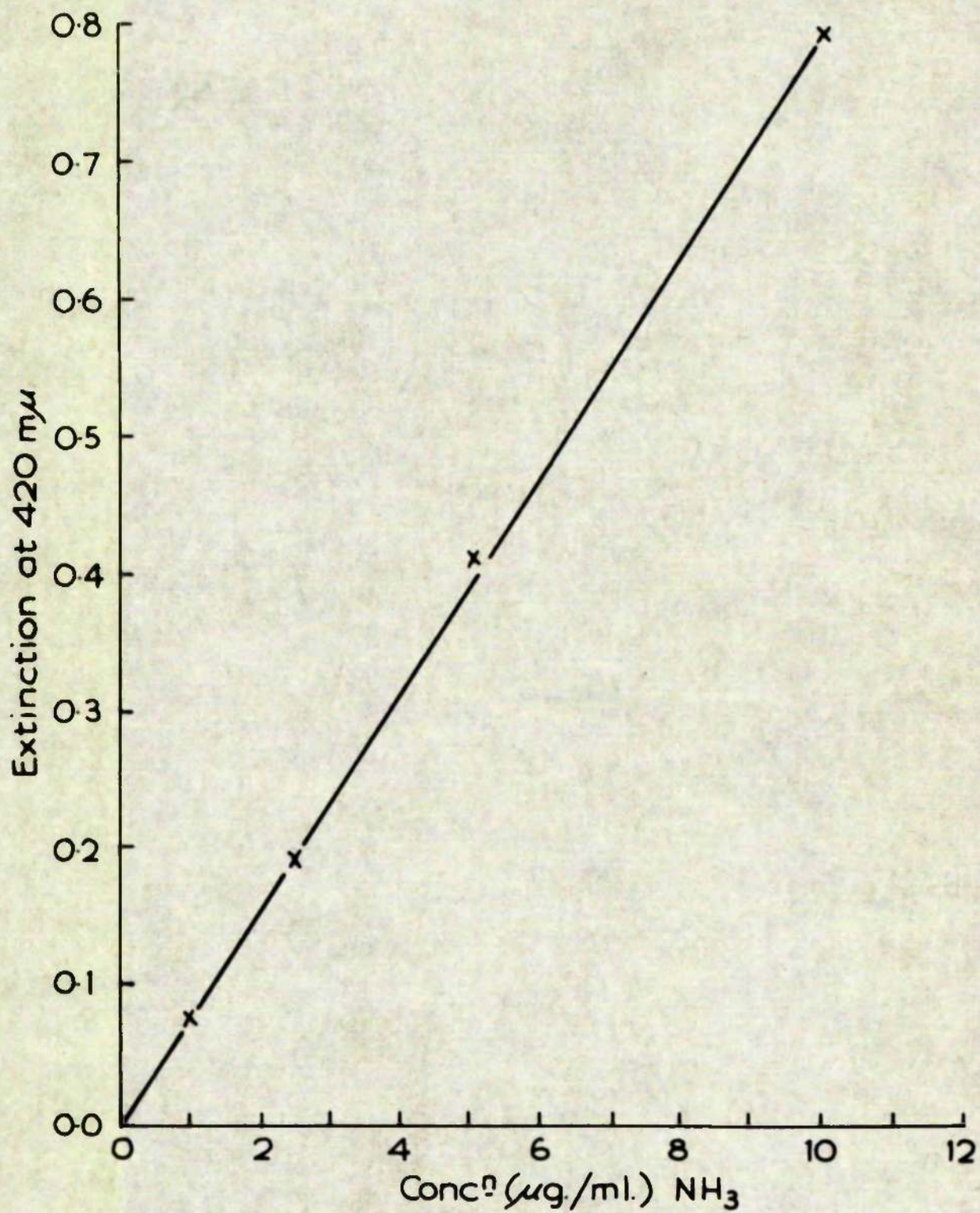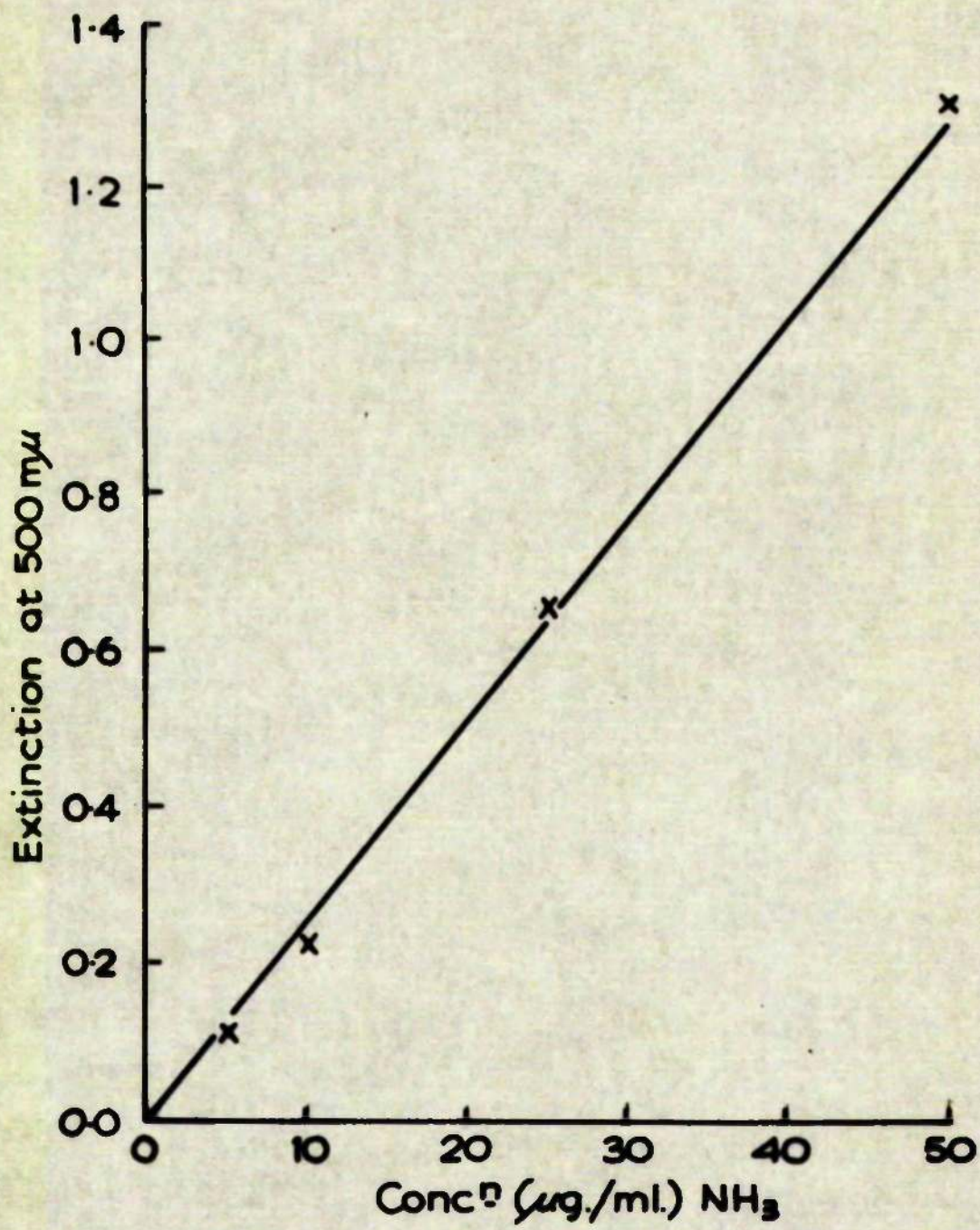
Figure IV, 7(a)

Figure IV, 7(b)

A calibration curve (Fig. IV, 8) was
constructed using α-oxoglutaric acid as standard.
Duplication of results using this method was good.

16.   Estimation of inorganic phosphate in the
       presence of labile organic phosphates.

Inorganic phosphate in the presence of
adenosine-5'-triphosphate was estimated by the
method of Berenblum and Chain (1938).

The sample to be assayed was shaken
vigorously for 10 seconds in a separating funnel
with a mixture of 5% NaCl (2ml.), $10N-H_2SO_4$ (0.5 ml.),
iso-butanol (10 ml.) and 5% NH_4^+ molybdate (2.5 ml.).
The lower layer of this mixture was discarded and the
upper layer washed twice with 10 ml. portions of
$N-H_2SO_4$.   7.5 ml of a solution of $SnCl_2$ (10 g.
$SnCl_2$ dissolved in 25 ml. conc. HCl and diluted
1:200 with $N-H_2SO_4$) was added and the mixture shaken
for one minute.   The lower layer was discarded and
the iso-butanol layer diluted to 25 ml. with a 1:1
mixture of iso-butanol and ethanol.   The extinction
of the solution was determined in a spectrophotometer
(Unicam SP600) at 640 mμ against a reagent blank.

A calibration curve (Fig. IV, 9) was
constructed using KH_2PO_4 as standard.   Duplication

Figure IV. 8

Calibration curve for the estimation of ketoacids.

Assay system - 3 ml. ketoacid solution, 0.5 ml.
2:4-dinitrophenylhydrazine reagent (1 mg./ml. in
2N-HCl) and 1.5 ml. 4N-NaOH.   The extinction of
the solutions was determined at 435 mμ.

Standard solutions were prepared by
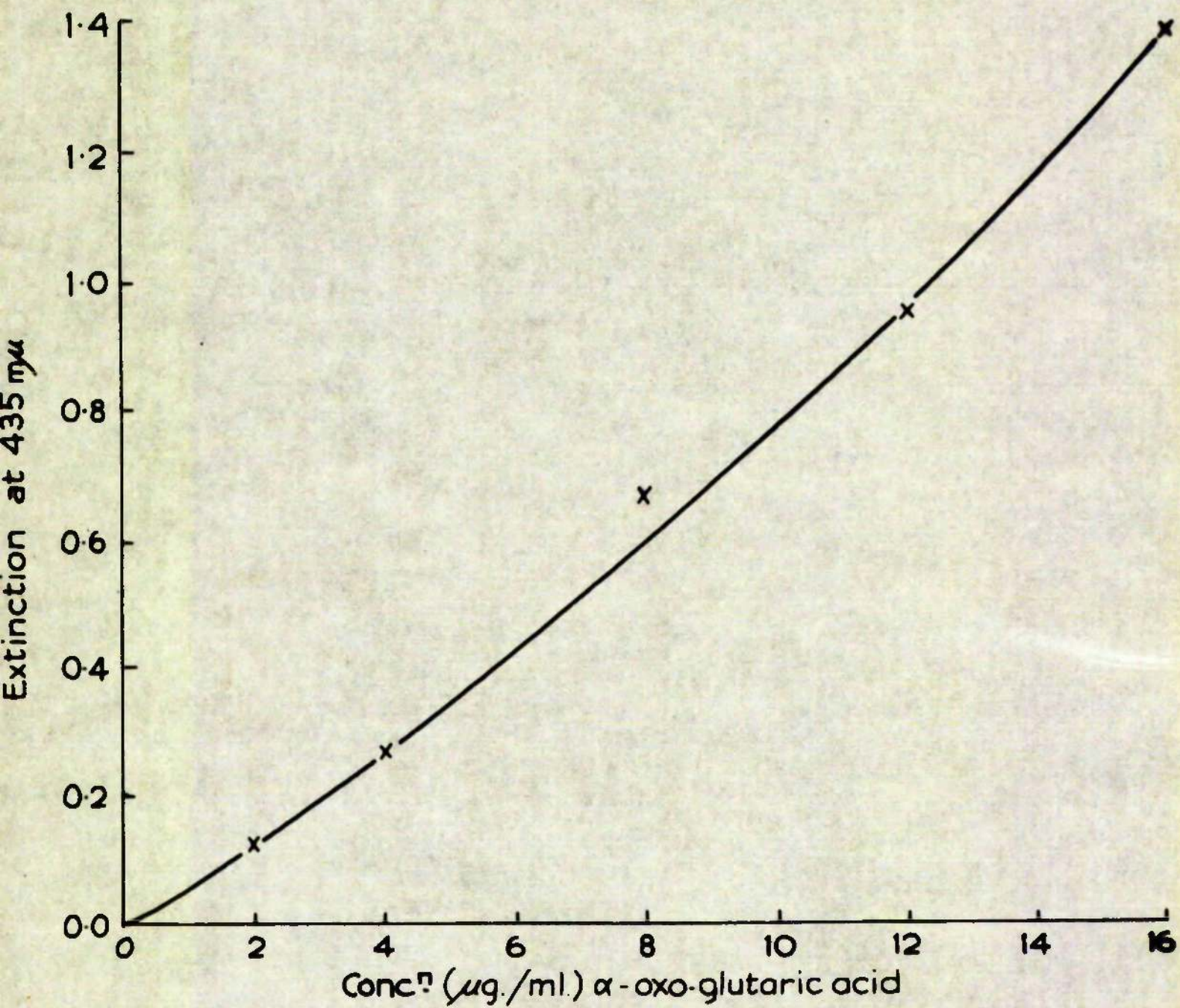dissolving α-oxo-glutaric acid in distilled water.

Figure IV. 8

Calibration curve for the estimation of inorganic

phosphate in the presence of labile organic phosphates.

Assay system - The method used was that described by

Berenblum and Chain (1938). The extinction of the

solutions was determined at 640 mμ.

Standard solutions were prepared by

dissolving KH_2PO_4 in distilled water.

of results using this method was reasonably good.

17. Estimation of formaldehyde by chromotropic acid reagent.

The method used to estimate formaldehyde in solution was adapted from the method for the estimation of mannitol described by Corcoran and Page (1947). The chromotropic acid reagent used was prepared by dissolving 0.2 g. chromotropic acid (4:5-dihydroxy-2:7-naphthalene-disulphonic acid) in distilled water (4 ml.) and diluting this solution to 100 ml. with $15N$-H_2SO_4.

The chromotropic acid reagent (2 ml.) and concentrated H_2SO_4 (2 ml.) were added to 2.0 ml. of the solution to be assayed. The tube was stoppered firmly and shaken to mix the contents thoroughly. After being heated in a water-bath at 100° C. for 45 minutes, the mixture was cooled in crushed ice and the extinction of the colour which had developed was determined in a spectrophotometer (Unicam SP600) at 570 mμ.

A calibration curve (Fig. IV, 10) was constructed using aqueous formaldehyde solutions as standards. The duplication of results using this method was excellent.
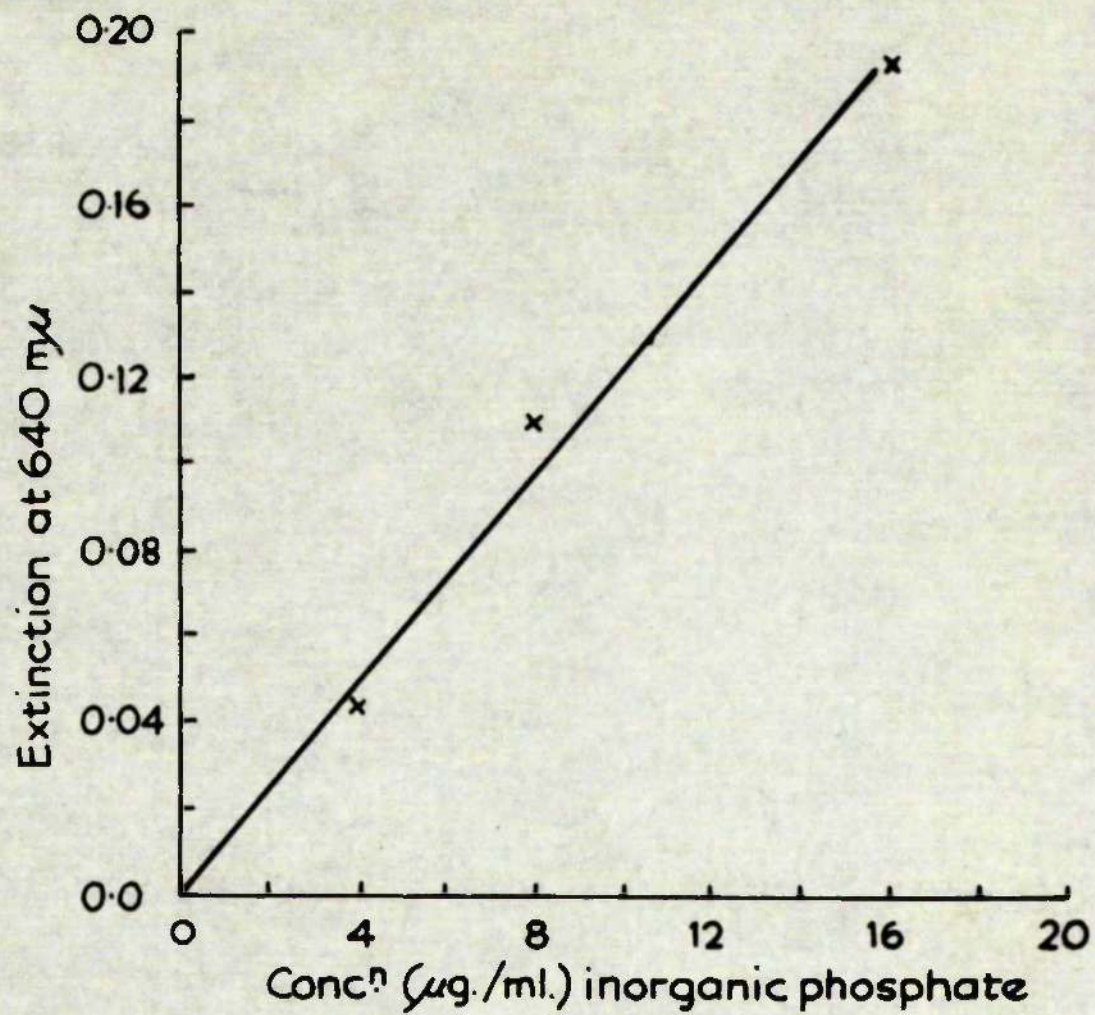
Figure IV. 9.

Figure IV. 10

Calibration curve for the estimation of formaldehyde.

Assay system - 2 ml. formaldehyde solution, 2 ml. chromotropic acid reagent and 2 ml. concentrated H_2SO_4. The extinction of the solutions was determined at 570 mμ.

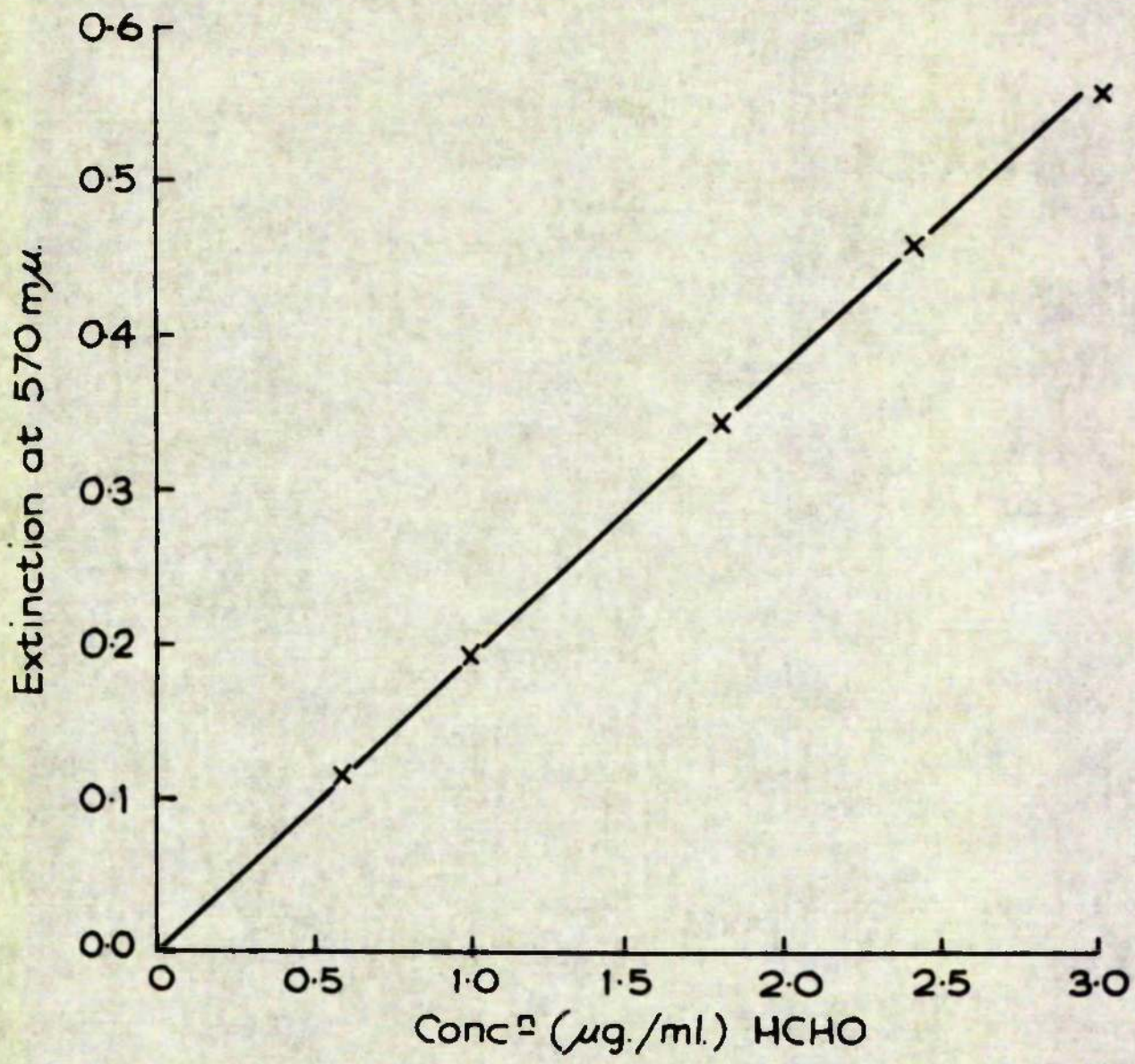Standard solutions were prepared by dilution of a stock 40% formaldehyde solution.

Figure IV. 10

18. Micro-determination of nucleoside phosphorylase

activity of Esch. coli PA/15.

Syntheses and degradations catalysed by the nucleoside phosphorylase activity of cell-free extracts of Esch. coli PA/15 were studied on a microgram scale by carrying out the reactions on spots on chromatography paper prepared for development as paper chromatograms. 5 μl. phosphate buffer (0.1M, pH 7.2) was dried on to the paper as a small discrete spot. The substrate or substrates (0.5 μmoles) dissolved in glass-distilled water were dried on to the spot, covering the same area as did the buffer. The paper was placed in an incubator with the spot arranged over the bottom half of a Petri dish half-filled with water. The top of the dish was placed to cover the spot and held firmly in position with a heavy weight (Fig. IV, 11). After the paper was saturated with water-vapour by incubation at 37° C. for 30 minutes, a cell-free extract of Esch. coli PA/15 (0.1 ml.) was placed on the spot, the top of the Petri dish was replaced and the chromatogram incubated for the required time (1 to 2 hours) at 37° C. If the spot showed any sign of drying out during the incubation it was remoistened with glass-distilled water.

Figure IV, 11

Arrangement for the use of the nucleoside phosphorylase
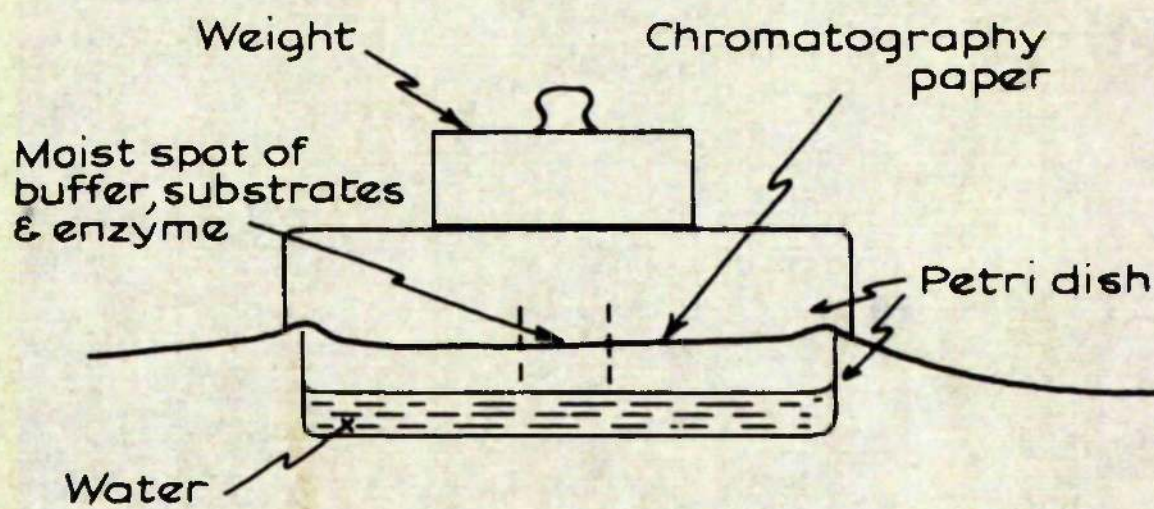
activity of Esch. coli PA/15 on a microgram scale.

Figure IV. 11

After the period of incubation the reaction was stopped by drying the spot with a stream of hot air. The reaction products were separated by developing the chromatogram in a suitable solvent system and identified by their R_F values and by their absorption spectra. A spot of enzyme solution and buffer was incubated and chromatographed in parallel with the test spot as a control.

19. Estimation of $N-(^{14}C$-hydroxymethyl)-5:6:7:8-tetrahydrofolic acid by degradation to ^{14}C-formaldehyde.

^{14}C-labelled $N-CH_2OH-FAH_4$ was estimated by degradation to ^{14}C-formaldehyde by a method based on the acid lability of the N-hydroxymethyl linkage.

1.0 ml. of the solution containing $N-^{14}CH_2OH-FAH_4$ was pipetted directly into 10 ml. $5N-H_2SO_4$ contained in a large tube fitted with a bubbler. The ^{14}C-formaldehyde formed by the acid degradation of $N-^{14}CH_2OH-FAH_4$ was steam-distilled from this mixture into a second tube where it was trapped in 10 ml. of a solution of 2:4-dinitrophenylhydrazine (1 mg./ml.) in $5N-H_2SO_4$. A third tube was interposed between the reaction tube and the trapping tube to preclude the possibility of any mechanical carry-over of non-volatile ^{14}C-labelled material from the reaction tube.

Distillation was continued until about 10 ml. distillate had been collected in the trapping tube.

The 2:4-dinitrophenylhydrazone of formaldehyde was extracted from the trapping tube with three 10 ml. portions of CCl_4 in which the derivative is soluble but the reagent only sparingly so. The combined CCl_4 extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The mixture of formaldehyde 2:4-dinitrophenylhydrazone and 2:4-dinitrophenylhydrazine was dissolved in 1.0 ml. benzene and 0.2 ml. of this solution was plated on to a planchette and counted.

For specific activity determinations carrier formaldehyde (100 µg.) was added to the mixture before steam-distillation was carried out. The mixture of formaldehyde 2:4-dinitrophenyl-hydrazone and 2:4-dinitrophenylhydrazine isolated as described above was dissolved in 1 ml. of a 1:1 benzene-cyclohexane mixture and applied to a column of alumina (diameter, 1 cm.; height 15-20cms.) which had been washed with cyclohexane. The column was eluted with 5 ml. cyclohexane followed by benzene. The fractions containing the formaldehyde

2:4-dinitrophenylhydrazine were combined, evaporated
to dryness under reduced pressure and the residue
was dissolved in benzene, plated on to a planchette
and counted.   The planchette was eluted with
benzene (4.0 ml.) and the extinction of the solution
was determined using an ultraviolet spectrophotometer
(Beckman) at 400 mμ and 450 mμ.

The formaldehyde 2:4-dinitrophenylhydrazone
isolated by this procedure is contaminated with an
unlabelled artefact.   The formaldehyde derivative
may be estimated by differential spectrophotometry
based on the difference in absorption spectra of the
2:4-dinitrophenylhydrazone and the artefact (Fig. IV,
12).   The concentration of the hydrazone is given
by the relationship:

$$H = \frac{R\varepsilon_{450} - \varepsilon_{400}}{R\varepsilon_{450}^{\text{B}} - \varepsilon_{400}^{\text{B}}}$$

where H = concentration of formaldehyde 2:4-
dinitrophenylhydrazone (μmoles/ml.);

R = $\varepsilon_{400}/\varepsilon_{450}$ of the artefact; and
$\varepsilon_{400}^{\text{B}}$ and $\varepsilon_{450}^{\text{B}}$ = the millimolar extinction
coefficients of formaldehyde 2:4-dinitrophenylhydrazone
at 400 mμ and 450 mμ respectively.

The amount of $N\text{-}^{14}CH_2OH\text{-}FAH_4$ present in
incubation mixtures is directly proportional to (i) the

Figure IV, 12

Absorption spectrum of (a) formaldehyde 2:4-
dinitrophenylhydrazone and (b) the inactive
artefact of formaldehyde 2:4-dinitrophenyl-
hydrazone in benzene.

● — ● — ● formaldehyde 2:4-dinitrophenylhydrazine.

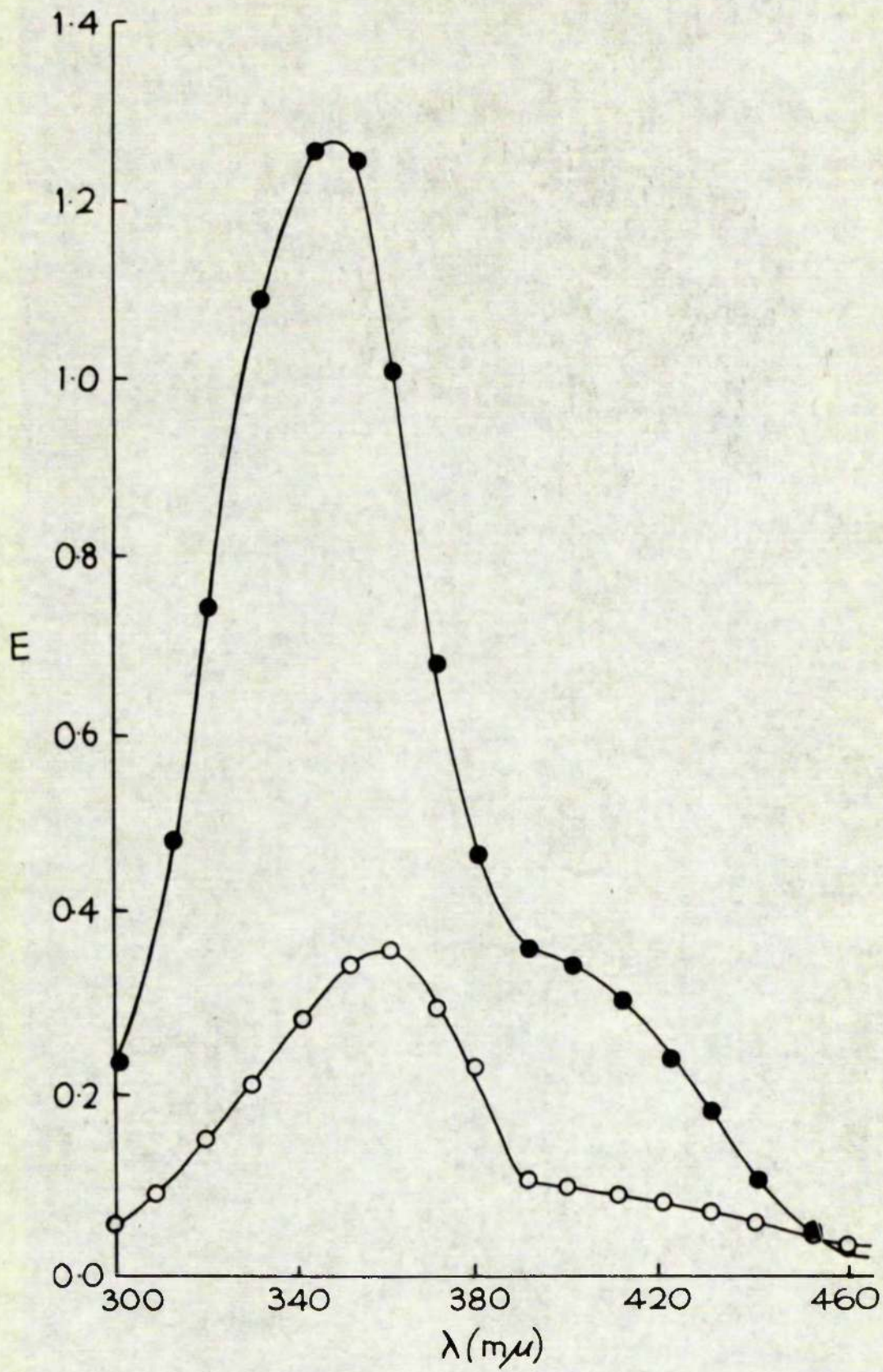o — o — o artefact of formaldehyde 2:4-dinitro-
phenylhydrazine.

Figure IV, 12

number of counts distilled and (ii) the specific
activity of the formaldehyde 2:4-dinitrophenyl-
hydrazone isolated from the incubation mixtures.

20. Isolation and hydrolysis of deoxyribonucleic acid.

The method used for isolating and hydrolysing
the deoxyribonucleic acid (DNA) present in incubation
mixtures which contained cell-free extracts of Esch.
coli PA/15 or Esch. coli 113/3 was taken from Smellie,
Thomson and Davidson (1958).

A mixture of DNA, ribonucleic acid and protein,
present in incubation mixtures containing a cell-free
extract of Esch. coli PA/15 or Esch. coli 113/3, was
precipitated by the addition of one-third of a volume
of 2.1N-PCA. After thorough mixing the suspension
was centrifuged (5,000 g), the supernatant discarded
and the precipitate washed twice with 1 ml. portions
of 0.7N-PCA and with 5 ml. portions of acetone (once),
ethanol (once), 1:1 ethanol-chloroform (once), 1:1
ethanol-ether (twice) and finally ether (once). The
residue was digested with 2 ml. 0.3N-KOH for 18 hours
at 37° C. and the solution was adjusted to pH 1 with
60% PCA after being cooled in ice. The mixture was
centrifuged (5,000 g) and the supernatant discarded.
The precipitate was washed twice with 2 ml. portions

of cold 0.5N-PCA and extracted twice at 70°C. for 20 minutes with 0.5 ml. portions of 0.5N-PCA to yield the DNA components.

The combined extracts were dried *in vacuo* and 50 µl. 72% PCA was added to the residue. This mixture was heated at 100°C. for one hour to complete the hydrolysis of the DNA to the constituent free bases. After cooling in ice the solution was adjusted to pH 7 by the addition of KOH and the precipitated KClO$_4$ removed by centrifugation. The bases of DNA were separated from the supernatant solution by paper chromatography.

21. Preparation and separation of the 2:4-dinitrophenyl derivatives of glycine and serine.

The 2:4-dinitrophenyl (DNP-) derivatives of glycine and serine were prepared and separated by the methods described by Campbell and Work (1952) for the separation of the constituents of a protein hydrolysate. The reaction mixture containing glycine and serine was treated with 1-fluoro-2:4-dinitrobenzene and the mixture of DNP-aminoacids was isolated in the manner described by Campbell and Work (1952). The separation and purification of DNP-glycine and DNP-serine was carried out according to the scheme shown in Chart IV, 1.

Columns of Type I were prepared from celite buffered with phosphate, pH 5.2; of Type II from celite buffered with phosphate, pH 5.9.   The solvents used for developing the columns were (i) a mixture of chloroform and n-butanol (93:7) saturated with water (solvent CB) and (ii) dry methylethylketone (solvent MEK).

The specific activities of the DNP-glycine and DNP-serine were determined by plating the derivatives on to planchettes and counting.   The planchettes were eluted with 4 ml. 1% $NaHCO_3$ and the extinction of each solution was determined in an ultraviolet spectrophotometer (Beckman) at 400 mμ and 450 mμ.   The ratio of the extinctions at 400 mμ and 450 mμ was determined as a check on the purity of the DNP-derivative.

Mixture of DNP-glycine and DNP-serine

Dissolved in 1 ml. CB and applied to

Celite column of Type I; Eluted with CB.

DNP-glycine in CB

Concentrated in vacuo and
applied to Celite column of
Type I; Eluted with CB.

DNP-glycine in CB

Evaporated to dryness in
vacuo, redissolved in ether
and applied to Celite column
of Type I; Eluted with ether.

Pure DNP-glycine in ether

Concentrated in vacuo, plated
on to planchette and counted.
Planchette eluted with 4 ml.
1% NaHCO$_3$ and extinction of
solution at 450mμ determined.

DNP-serine on column

Eluted with acetone

DNP-serine in acetone

Evaporated to dryness in vacuo,
redissolved in MEK and applied
to Celite column of Type II;
Eluted with MEK

DNP-serine in MEK

Concentrated in vacuo and
applied to Celite column of
Type II; Eluted with MEK

Pure DNP-serine in MEK

Concentrated in vacuo, plated
on to planchette and counted.
Planchette eluted with 4 ml.
1% NaHCO$_3$ and extinction of
solution at 450 mμ
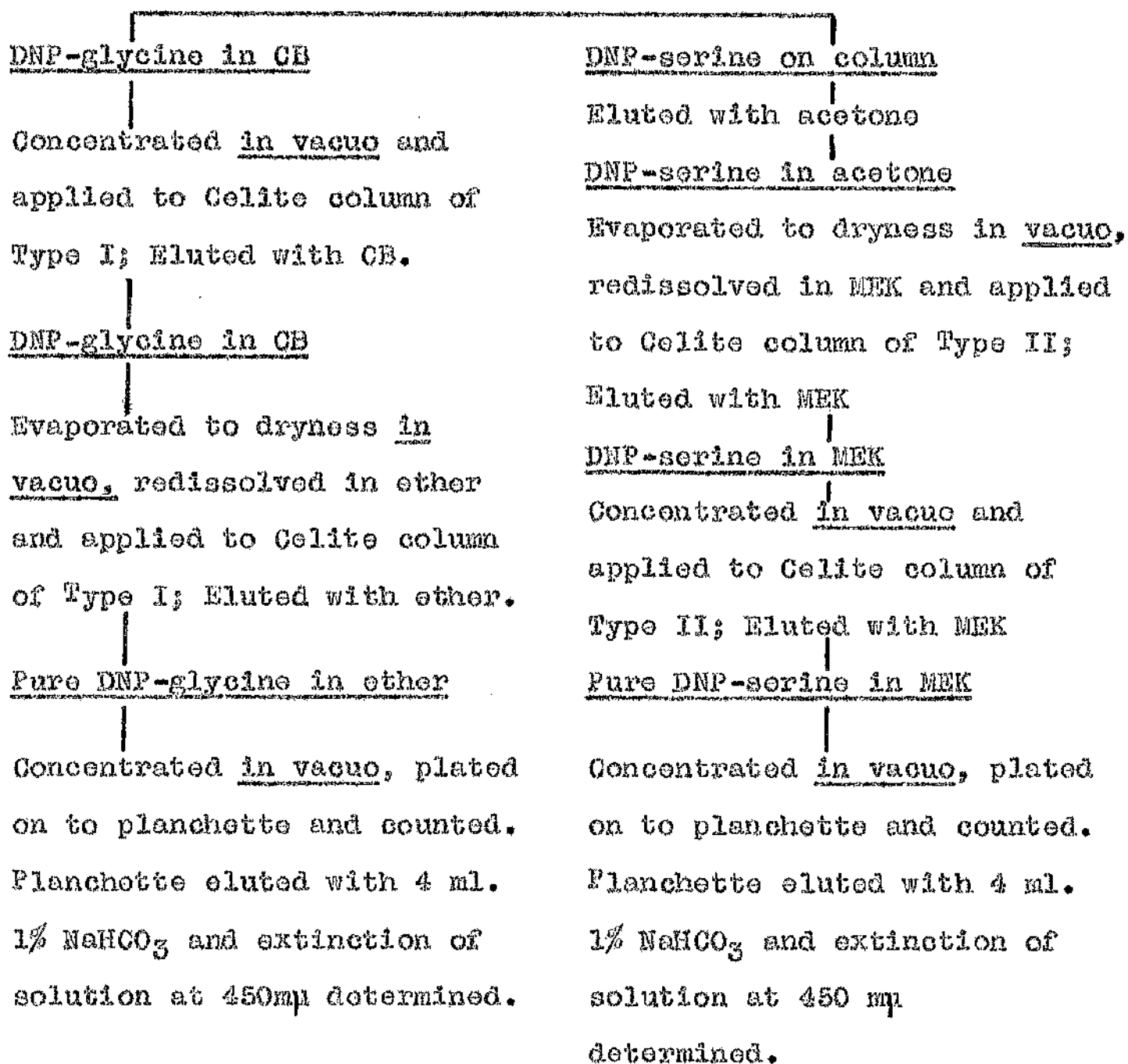determined.

Chart IV, 1.   Scheme for the separation and purification of
             DNP-glycine and DNP serine by column
             chromatography.

22.   Paper chromatography.

Paper chromatography was carried out using Whatman No. 1 chromatography paper.   Paper chromatograms were developed by both the ascending and descending techniques in sealed tanks and materials were separated (i) as spots, development taking place in one direction (one-dimensional paper chromatograms) or in two directions at right angles to each other (two-dimensional paper chromatograms) and (ii) as "streaks" or bands (for larger quantities).

The composition of the various solvents used is as follows:

1. n-butanol-water (86:14 v./v.), ascending; Markham and Smith (1949);

2. n-butanol-water-NH_4OH (Solvent 1 with 5% v./v. 0.880 NH_4OH added), ascending; Markham and Smith (1949);

3. n-butanol-water-acetic acid (4:5:1 by vol.), ascending; Partridge (1948);

4. iso-propanol-water-conc. HCl (39.4:11.2:10 by vol.), descending; Wyatt (1951);

5. acetone-water-100% formic acid (30:13:7 by vol.) ascending; Burrows et al. (1952);

6. acetone-25% w./v. trichloroacetic acid (3:1 v./v.), ascending; Burrows et al. (1952).

7. phenol-water (4:1 w./w.) with 0.880 NH_4OH and KCN in the tank, ascending; adapted from Decker et al. (1951);

8. aqueous phosphate buffer (0.1M, pH 6.9), ascending;

9. aqueous 12% Na_2HPO_4 containing 0.8% w./v. ethylenediamine-tetraacetic acid (EDTA), ascending; Jaenicke (1955);

10. iso-amyl alcohol-5% w./v. aqueous KH_2PO_4 (1:2 v./v.), ascending; Carter (1950);

11. ethanol-M-NH_4 acetate (pH9) (70:30 v./v.) containing 0.2% w./v. EDTA, ascending; Klenow and Lichtler (1957);

12. ethanol-M-NH_4 acetate (pH 9)-Na^+ tetraborate-EDTA (solvent 11 with NH_4 acetate saturated with Na^+ tetraborate); ascending; Klenow and Lichtler (1957);

13. 0.5N-formic acid, ascending; Miller and Waelsch (1957);

14. tert-butanol-methylethylketone-water-NH_4OH (40:30:20:10 by vol.), ascending; Fink et al. (1956b).

15. tert-butanol-methylethylketone-water-formic acid (44:44:11:0.264 by vol.), ascending; Fink et al. (1956b).

16. n-butanol-chloroform (1:99 v./v.), descending on paper impregnated with biphthalate buffer, pH 6.0; Blackburn and Lowther (1951);

17. n-butanol-water-ethanol (5:4:1 by vol.), ascending; Cavallini et al. (1949).

23. Paper electrophoresis.

Paper electrophoretic separations were effected on 72 cm. strips of Whatman No. 1 filter paper. The material was applied to the paper as a spot about 7 cm. from one end of the strip which then was soaked in citrate buffer (0.05M, pH 3.5) and allowed to drain. The strip was suspended over a glass rod in a cabinet and the two ends were dipped into troughs of citrate buffer. The cabinet was sealed to cut down evaporation losses and a potential gradient of 13 volts/cm. was applied between the ends for 5 to 8 hours.

24. Detection of materials on paper chromatograms and electrophoretograms.

(i) Materials which absorbed ultraviolet light or which fluoresced in ultraviolet light (pyrimidines, purines and their derivatives and folic acid and its derivatives, respectively) were located by viewing the paper chromatogram or electrophoretogram in ultraviolet light ("Hanovia" ultraviolet lamp).

(ii) The DNP-derivatives of aminoacids and the 2:4-dinitrophenylhydrazones of ketoacids were visible in ordinary light by virtue of their yellow colour. This colour was enhanced by viewing the chromatogram in

ultraviolet light.

(iii) Aminoacids were located by spraying the
chromatogram or electrophoretogram with a 2% solution
of ninhydrin in ethanol-water-glacial acetic acid
(5:4:1 by vol.).   In the case of chromatograms which
had been developed with strongly acid solvents, the
addition of pyridine (10% v./v.) to the spray was
necessary.   The purple colour which developed by
interaction of an aminoacid and ninhydrin faded
rapidly over a period of a day or so and therefore
the spots were made permanent by spraying with a
solution of $ZnSO_4$.

(iv) Dihydropyrimidines and BUIB were located
by the method described by Fink et al. (1956a).   The
chromatogram was sprayed with 10% KOH in ethanol-
water (1:1) and allowed to dry for 30 minutes.   The
spots were developed by spraying with a 1% solution of
p-dimethylaminobenzaldehyde in ethanol-concentrated
HCl (10:1 v./v.).

(v) The precise location of radioactive materials
on paper chromatograms or electrophoretograms was
determined by the technique of autoradiography.

When materials were eluted from a paper
chromatogram or electrophoretogram for further
identification by ultraviolet absorption spectra, etc.,

an appropriate blank was cut from clean paper near
the spots of material under investigation.

25. Autoradiography of paper chromatograms and electrophoretograms.

The dried paper chromatogram or electro-
phoretogram was clipped to a sheet of X-ray paper
(Ilford) or X-ray film (Kodak Industrex "D") with
metal staples, the whole was wrapped in black paper,
pressed between sheets of hardboard and kept in the
dark for a period of time ranging from two weeks to
two months according to the activity of the ^{14}C-
labelled materials on the paper.   At the end of
this period the X-ray paper or film was separated from
the chromatogram, developed for 15 minutes (Kodak
19B developer) and fixed.

26. Assay of radioactivity.

For the assay of radioactivity by the counting
technique, nickel planchettes, one cm. in diameter,
were used.   For ordinary counting purposes, the
planchettes were scrubbed with "Ajax" cleaner, washed
with hot water, glass-distilled water and absolute
alcohol.   When the material was to be re-eluted from
the planchettes after counting, the planchettes were
scrubbed with "Ajax", washed with running hot water for

15 minutes, rinsed 5 times with tap water, 3 times with
glass-distilled water and allowed to stand for 30
minutes in N-HCl (when elution was to be carried out
with 0.1N-HCl) or 5% NaHCO$_3$ (when elution was to be
carried out with 1% NaHCO$_3$).   The planchettes were
washed again with running hot water for 15 minutes,
rinsed with tap water (5 times), glass-distilled water
(5 times) and ethanol (3 times).

Materials were counted at infinite thinness
with a conventional mica end-window counter or with
a window-less gas-flow counter (Nuclear Chicago
Ultrascalar, model 192).   Planchettes were eluted
with (i) 4 ml. 0.1N-HCl or (ii) 4 ml. 1% NaHCO$_3$ at
37° C. for one hour when the plated material was required
for extinction measurements.

27.   General enzymic incubation techniques.

Enzymic incubations were carried out in
stoppered "Quickfit" test-tubes, total capacity 15 ml.,
at 37°C. in a thermostatically-controlled water-bath
with occasional shaking.   Aerobic incubations were
carried out under an atmosphere of air, anaerobic
incubations under oxygen-free nitrogen.   Oxygen was
removed from solutions required for anaerobic
incubations by a stream of oxygen-free nitrogen which

was passed before and after the addition of FAH_4 and the cell-free extract (or cell suspension) to the reaction mixture.

Enzymic reactions were stopped in one of four ways, namely (i) by centrifugation (20,000 g) for cell suspensions; (ii) by plunging the tubes into a bath of boiling water and heating them for 10 minutes; (iii) by adding 5N- or 10N-HCl until the reaction mixture was normal with respect to HCl; and (iv) by adding one-third of a volume of 2.1N-PCA. Method (iv) was used only when DNA was to be isolated from the incubation mixture. In each case the mixture was centrifuged and the precipitated protein (together with DNA and ribonucleic acid in the case of method (iv)) removed before the supernatant solution was worked up for the reaction products.

Samples taken for the isolation and degradation of $N-CH_2OH-FAH_4$ were removed from incubation mixtures immediately before the reaction was stopped by one of the above methods.

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

COMMUNICATIONS

Pyrimidine Biosynthesis in *Escherichia coli.* By G. W. CROSBIE. *(Department of Biochemistry, University of Glasgow)*

Information has been sought, with the aid of labelled precursors, as to the mechanism of thymine ring biosynthesis in cells of *Escherichia coli* growing exponentially in a simple synthetic medium.

A study of the incorporation of [^{14}C]bicarbonate, [^{14}C]formate, [β-^{14}C]serine and [2-^{14}C]glycine into the nucleic acids and proteins of a wild-type strain, *Esch. coli* N.C.T.C. 5928 and of a uracil-less mutant, *Esch. coli* M 63-86 (kindly provided by Dr B. D. Davis), has indicated:

(*a*) That formate does not function as a thymine methyl group precursor although incorporation into positions 2 and 8 of the purine ring occurs. These findings have been confirmed by the following degradation studies: (i) guanine to glycine and 4-guanidinoglyoxaline (Hunter, 1936); (ii) adenine to glycine and 4-amino-5-glyoxaline carboxamidine (Cavalieri, Tinker & Brown, 1949); and (iii) thymine to iodoform (Elwyn & Sprinson, 1954).

(*b*) That [2-^{14}C]glycine and [β-^{14}C]serine function as thymine methyl group precursors.

(*c*) That in the absence of extracellular supplements, the thymine methyl group is quantitatively derived from the β-carbon of serine by a pathway which does not involve glycine.

(*d*) That the extent of utilization of [2-^{14}C]-glycine for 1-C unit synthesis is dependent on its extracellular concentration (cf. Koch, 1955).

(*e*) That the pathway of incorporation of [2-^{14}C]-glycine into the thymine methyl group does not involve serine.

The incorporation of [β-^{14}C]serine into the DNA-thymine of a methionine-less mutant of *Esch. coli*, CW 194 (kindly provided by Dr J. Ceithaml), has indicated in confirmation of the work of Green & Cohen (1957) that the methionine methyl group is not a thymine precursor.

The results of this investigation are consistent with the role of hydroxymethyltetrahydrofolic acid as the more immediate 1-carbon donor in thymine ring biosynthesis.

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Biosynthesis of Thymidylic Acid. By G. D. BIRNIE and G. W. CROSBIE. *(Department of Biochemistry, University of Glasgow)*

Blakley (1957) and Phear & Greenberg (1957) have recently reported the synthesis by soluble mammalian enzymes of thymidine from deoxyuridine and [β-^{14}C]serine and of thymidylic acid from deoxyuridine and [^{14}C]formaldehyde. Friedkin & Kornberg (1956) and Flaks & Cohen (1957) have also reported the synthesis of thymidylic acid from deoxyuridylic acid by cell-free extracts of *Escherichia coli*. The latter authors have, in addition, prepared 5-hydroxymethyldeoxycytidylic acid enzymically from deoxycytidylic acid.

In the present work a cell-free extract of a serine-less mutant of *Esch. coli*, PA/15, was prepared by

alumina grinding followed by extraction with Tris buffer (0·1 M, pH 7·4). The extract, which effects a tetrahydrofolic acid-dependent serine–glycine interconversion with simultaneous formation of hydroxymethyltetrahydrofolic acid, was incubated under N_2 with deoxyuridine (0·01 M), [β-^{14}C]serine (0·002 M, 1 μc/ml.), $MgSO_4$ (0·01 M), and substrate amounts of ATP, TPNH and tetrahydrofolic acid. Thymidylic acid and thymidine together with a trace of thymine have been detected by chromatographic examination of the incubation mixture. The thymine derivatives have been isolated with the aid of carriers and the identity of the labelled

a

products confirmed by autoradiography following extensive chromatographic and ionophoretic separations.

The structures of two labelled products with chromatographic properties similar to 5-hydroxymethyldeoxyuridylic acid and the corresponding nucleoside are under investigation.

Replacement of TPNH by DPNH greatly reduced the yield of thymine derivatives. No labelled pyrimidine derivatives were formed in the presence of phosphate buffer due to the powerful nucleoside phosphorylase activity of the enzyme preparations used.

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Summary

1. The purpose of this study was to develop a cell-free system in which the biosynthesis of thymine, thymidine or thymidylic acid could be studied, with particular reference to the mechanism by which the methylation of the pyrimidine ring is achieved.

2. The organisms chosen for this study were Escherischia coli PA/15, Esch. coli 113/3, Esch. coli 15T- and Bacillus subtilis N.C.I.B. 8059.

3. The ability of Esch. coli PA/15 to catalyse the synthesis of methionine from homocysteine and serine, a reaction formally analagous to the methylation of uracil, was confirmed.

4. Unsuccessful attempts were made to synthesise thymine or thymidine from uracil or deoxyuridine using Esch. coli PA/15 and B. subtilis under the same conditions as those involved in methionine synthesis.   The cofactors folic acid, N^{10}-formylfolic acid and N^5-formyltetrahydrofolic acid had no detectable effect.

5. Accordingly, a detailed examination of the enzyme activities present in Esch. coli PA/15 was undertaken.   A study of serine-glycine

interconversion confirmed the presence of
serine hydroxymethylase and evidence for the
formation of N-hydroxymethyltetrahydrofolic
acid as the one-carbon pool complex was
obtained. The conversion of serine to
pyruvic acid and alanine was also detected.
The presence of nucleoside phosphorylase and
adenosine-5'-triphosphatase was also confirmed
and conditions were established under which
the hydrolysis of nucleosides by the nucleoside
phosphorylase and inorganic phosphate(produced
from ATP by adenosine-5'-triphosphatase) was
held to a minimum. Deoxyuridylic acid
phosphomonoesterase, thymidylic acid phosphomono-
esterase and deoxyuridine kinase were among the
enzyme activities also found in extracts of Esch.
coli. Conditions were established under which
regenerative TPNH and DPNH systems operated under
the influence of glucose-6-phosphate and
pyridine nucleotide transhydrogenase activities.
6. A system in which thymidylic acid was
synthesised by cell-free extracts of Esch. coli
PA/15 and Esch. coli 113/3 was constructed on
the basis of information derived from the above
investigation.

7. A comparison of serine, formaldehyde and formate as one-carbon unit donors revealed that serine was a much more prolific source of one-carbon units than either formaldehyde or formate. A similar comparison of deoxyuridine, deoxyuridylic acid and uridine as one-carbon unit acceptors revealed that uridine was a less efficient acceptor than either deoxyuridine or deoxyuridylic acid. No significant difference between deoxyuridine and deoxyuridylic acid was detected.

8. Evidence was obtained that thymidine-5'-triphosphate was formed in systems in which thymidylic acid was synthesised. No evidence for the in vitro synthesis of DNA-thymine was obtained.

9. The effect of vitamin B_{12} on thymidylic acid biosynthesis was investigated, using Esch. coli 113/3. No unequivocal evidence for a vitamin B_{12} effect was obtained.

10. No identifiable intermediates on the biosynthetic pathway to thymidylic acid were isolated, even when Esch. coli 15T- was used as the enzyme source. Successful chemical syntheses of 5-hydroxymethyluracil and 5-hydroxy-

methyldeoxyuridine were achieved but attempts to synthesis 5-hydroxymethyldeoxyuridylic acid were unsuccessful.

11.   It was found that a large proportion of the ^{14}C-thymidylic acid isolated from the systems described above was not, in fact, authentic thymidylic acid.   Attempts to elucidate the structure of the contaminant were not successful.

12.   Evidence that thymine and thymidylic acid undergo reduction under the influence of extracts of Esch. coli PA/15 was obtained.   β-Amino-iso-butyric acid and β-ureido-iso-butyric acid were among the catabolic products of thymine and thymidylic acid which were tentatively identified.

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