STUDIES RELEVANT TO THE BIOSYNTHESIS OF OROTIC ACID

A Thesis presented for the Degree of
Doctor of Philosophy in the University
of London

by

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The biosynthesis of orotic acid is briefly reviewed. This investigation is concerned with elucidating the stereochemical requirements of dihydro-orotic dehydrogenase, the enzyme involved in the final stage of the biosynthesis, with respect to the configuration of 4,5-dihydro-orotic acid (I).

The unambiguous synthesis of cis- and trans-4,5-dihydro-5-methylorotic acids (II and III), by cyclisation of the hitherto unknown N-ethoxycarbonyl-threo- and erythro-β-methylasparagines, is described.

Threo- and erythro-β-methylaspartic acids were prepared and conversion of them into N-ethoxycarbonyl-threo- and erythro-β-methylasparagines by various routes was studied.
The reduction, both chemical and catalytic, of orotic acid and of 5-methylorotic acid was also studied, cis-4,5-dihydro-5-methylorotic acid being obtained from the latter.

The physical properties of the threo and erythro series of derivatives of β-methylaspartic acid have been compared, also those of the cis- and trans-4,5-dihydro-5-methylorotic acids, with a view to correlating the properties with configuration.

Possible methods for the synthesis of 5-fluoro-4,5-dihydro-orotic acid are reviewed. Preliminary experiments on the synthesis of 5-bromo-4,5-dihydro-orotic acid were carried out.

Enzymic studies were carried out with L- and DL-4,5-dihydro-orotic acid and with cis- and trans-4,5-dihydro-5-methylorotic acids as substrates.

Ehrlich ascites cells were used as a source of the enzyme dihydro-orotic dehydrogenase in the first instance. The oxidation of the "dihydro-orotic acids" to the "orotic acids" was measured spectrophotometrically. The cis-isomer of 5-methyl-dihydro-orotic acid was apparently metabolised to some extent, but the enzyme preparation was not sufficiently pure to furnish unambiguous assay results. Subsequent experiments were performed with an enzyme preparation obtained from
Zymobacterium oroticum. This enzyme preparation proved to be more satisfactory and was active towards trans-4,5-dihydro-5-methylorotic acid but not towards the cis-isomer. The results for the oxidation of the substrates, and for the reduction of orotic acid with or without 5-methylorotic acid present do not entirely accord with published observations. It is, however, concluded that the enzyme-catalysed dehydrogenation proceeds by a trans-elimination mechanism.
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SECTION I

INTRODUCTION

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IA THE BIOSYNTHESIS OF OROTIC ACID

Studies on the biosynthesis of pyrimidine mononucleotides have shown that the first compound to be formed with a pyrimidine structure is orotic acid (IV); these investigations have included a certain amount of work on the biosynthesis of orotic acid itself [1]. Thus, the incubation of $[^{15}N]$ ammonium chloride, with rat-liver slices in the presence of orotic acid (unlabelled) led to considerably less $[^{15}N]$ being incorporated into the RNA-pyrimidines than was the case in the absence of orotic acid [2]. Moreover, with rat-liver slices in an appropriate incubation system, $[^{15}N]$, $[1,4-^{13}C]$, or $[2,3-^{14}C]$ L-aspartic acid or sodium $[^{13}C]$ bicarbonate, or $[^{15}N]$ ammonium chloride or carbamoyl-L-aspartic acid, gave labelled orotic acid, the position of the labelled atom(s) depending upon the precursor [3]. Such findings led Reichard to suggest that aspartic acid(I) and carbamoylaspartic acid(II) were true intermediates in the biosynthesis of orotic acid in mammalian liver [4].

It is now accepted that the biosynthesis of orotic acid occurs through the following sequence of reactions:
Each of these steps is briefly considered below, the trivial name of the enzyme involved, together with its I.U.B. code number, being given.

(The numbering of the dihydro-orotic acid ring is discussed later, in Section IIA).
1A Conversion of Aspartic acid into Carbamoylaspartic acid.  
Aspartate-Carbamoyltransferase (2.1.3.2.)

An enzymic system capable of synthesising carbamoylaspartic acid was first detected in 1954, in rat-liver mitochondria [4]. It was later established, with an enzymic preparation from rat-liver mitochondria, that carbamoylaspartic acid was synthesised from aspartic acid, and also that a compound referred to in the literature as the "carbamoyl donor" was synthesised by the enzymic preparation [5]. The work of Lowestein and Cohen [6] substantiated these findings. Carbamoyl phosphate(V) was synthesised by Jones et al. [7], and was shown to be identical with the "carbamoyl donor". In the light of this, Reichard and Hanshoff [8] reinvestigated the formation of carbamoylaspartic acid with purified enzymic preparations obtained not only from mammalian sources, but also from E. Coli. By the use of [13C] carbamoyl phosphate and [14C] L-aspartic acid as substrates, and the examination of the carbamoylaspartic acid thus obtained, they showed that the following reaction did indeed occur:

\[
\begin{align*}
\text{carbamoyl phosphate} & \quad \text{aspartic acid} \\
\text{(V)} & \quad \text{(I)} \\
\text{carbamoylaspartic acid} & \quad \text{(II)}
\end{align*}
\]
Several amino-acids were examined as possible substrates for the enzyme in these preparations, but the enzyme was found to be specific for L-aspartic acid and carbamoyl phosphate, and the equilibrium was found to favour carbamoyl aspartic acid formation. From their investigations Reichard and Hanshoff [8] concluded that the interconversion of aspartic acid and carbamoyl aspartic acid is catalysed by a single enzyme which does not contain a prosthetic group, nor does it require the presence of a co-enzyme.

The presence of the enzyme in other materials has been demonstrated, particularly in human leucocytes and erythrocytes [9], in Ehrlich ascites cells [10], and in Zymobacterium oroticum [11]. Moreover, to date, there have been no reports in the literature which seriously conflict with the conclusions reached by Reichard and Hanshoff [8] on the interconversion of aspartic and carbamoyl aspartic acid.

\[ \text{IA}_2 \text{ Conversion of carbamoyl aspartic acid into Dihydro-orotic acid} \]

\[ \text{Dihydro-orotase (3.5.2.3.)} \]

A consideration of the experimental results obtained with isotopically labelled substrates had led Reichard and Lagerkvist [3] in 1953 to suggest that carbamoyl aspartic acid was a true intermediate in the biosynthesis of orotic acid by rat-liver slices. Two years later, Cooper, Wu and Wilson [12]
studied the incubation of $[^{14}C]$ labelled carbamoylaspartic acid and dihydro-orotic acid with rat-liver slices or with rat-liver homogenates, and they found that dihydro-orotic acid was converted into orotic acid at a faster rate than was carbamoylaspartic acid. Furthermore, they found that incubation of $[^{14}C]$ labelled carbamoylaspartic acid with either system, even in the presence of dihydro-orotic acid (unlabelled), gave $[^{14}C]$ labelled dihydro-orotic acid. From these observations they suggested that in mammalian liver, carbamoylaspartic acid is converted into dihydro-orotic acid, which is then converted into orotic acid.

During the same period the existence of an enzyme which catalysed the interconversion of carbamoylaspartic acid and dihydro-orotic acid was demonstrated in the anaerobic bacterium, Zymbacterium oroticum [13], and in an aerobic bacterium, an unspecified Corynebacterium [14]. The presence of the enzyme has also been detected in other materials, in particular in human leucocytes and erythrocytes [9], Ehrlich ascites cells [10], and E. Coli [15,16].

Lieberman and Kornberg [13], whilst studying the enzymic synthesis and breakdown of orotic acid, investigated the enzymic constitution of Zymbacterium oroticum which had been adaptively grown on a medium containing orotic acid; they
detected the presence of an enzyme which catalysed the interconversion of carbamoylaspartic acid (II) and 5-carboxymethylhydantoin (VI). However, all attempts by them and by several other workers to detect the presence of such an enzyme in the other systems mentioned above, were unsuccessful; furthermore, Lieberman and Kornberg [13] concluded that 5-carboxymethylhydantoin was not an intermediate in the biosynthesis of orotic acid, from aspartic acid. Also, to date, there is no evidence for the view that more than one enzyme or any stable intermediate is involved in the interconversion of carbamoylaspartic acid and dihydoro-orotic acid. Recently, it has been reported [17] that for an enzyme preparation from Zymobacterium oroticum maximum enzyme activity was obtained by the addition of the divalent zinc ion to the enzyme preparation; cobalt was found to have a similar effect but to a lesser extent. However,
until the enzyme has been purified further it is not possible to draw any conclusions from these observations.

IA-3 Conversion of Dihydro-orotic acid into Orotic acid.

Dihydro-orotic dehydrogenase (1.3.3.1)

IA-3a Historical Review.

The enzyme, dihydro-orotic dehydrogenase, which catalyses the interconversion of dihydro-orotic acid and orotic acid was first obtained [18] from an obligate anaerobe isolated by Kornberg, from San Francisco Bay mud by the enrichment culture technique. The organism was characterised by Wachsman and Barker [19], who found it to be an anaerobe having the form of gram-positive, non-sporeforming rods which yielded mainly ethanol and carbon dioxide from the fermentation of glucose. They named the organism *Zymobacterium oroticum*. An enzyme which catalyses the interconversion of dihydro-orotic acid and orotic acid has also been found in aerobic bacteria, *Corynebacteria* [14], *E. coli* [15,16] and in human leucocytes [9], mammalian liver [12] and Ehrlich ascites cells [20]. The level of the enzyme activity in mammalian tissues is very low compared with that found in bacteria which utilise orotic acid as an energy source. Accordingly, most of the work on purification of the enzyme and on its properties and characteristics

* The systematic name is 1-4,5-dihydro-orotate: oxygen reductase; however, oxygen is not an obligatory reactant.
has been carried out on enzyme preparations from bacterial sources, in particular from *Zymobacterium oroticum* [18, 21, 22]. Indeed, Friedmann and Vennesland [22] in 1960 succeeded in obtaining a crystalline specimen of the enzyme from this organism. The isolation and purification of the enzyme from aerobic bacteria [23] as well as from *Zymobacterium oroticum*, an anaerobic bacterium, has also been studied.

**IA 3b Isolation and purification of dihydro-choric dehydrogenase.**

Lieberman and Kornberg [18] grew the organism *Zymobacterium oroticum* under anaerobic conditions in a medium containing orotic acid. After harvesting the cells, and disrupting the cell membranes, they partially purified the crude enzyme preparation by fractional precipitation. Development of the fractional precipitation procedure, by Friedmann and Vennesland [21, 22], enabled the enzyme to be obtained in a crystalline form; furthermore, the enzyme activity of the crystalline enzyme preparation per mg. of protein was approximately a 100-fold greater than that of the partially purified enzyme preparation obtained by Lieberman and Kornberg [18]. Also, when an aqueous solution of the crystalline enzyme was analysed in the ultracentrifuge only one peak was obtained [22]. Further
purification of the enzyme isolated from *Zymobacterium oroticum* was investigated by Miller and Massey [24]; they obtained a preparation of the enzyme which was not only more stable but also had a higher specific activity than that obtained by Friedmann and Vennesland [22]. However, Miller and Massey [24] did not obtain the enzyme in a crystalline form.

A crude preparation of dihydro-orotic dehydrogenase has also been obtained from an unspecified aerobic bacterium isolated from soil samples by Reynolds, Lieberman and Kornberg [14] in 1955; the procedures used by them were similar to those described earlier for the isolation of the enzyme from *Zymobacterium oroticum* [18]. Later, in 1961, Udaka and Vennesland [23] effected a partial purification of the enzyme from an unidentified aerobic bacterium by the use of the fractional precipitation technique.

Attempts to obtain a purified enzyme preparation from a mammalian source, in particular from human leucocytes [9], have been unsuccessful. It was not found possible to obtain a soluble and active preparation of the enzyme.

**Characteristics of dihydro-orotic dehydrogenase.**

Lieberman and Kornberg [18] found that the enzyme from *Zymobacterium oroticum* required pre-activation by incubation
with cysteine. Furthermore, they found that the conversion of orotic acid into dihydro-orotic acid (this being the direction conventionally used for the assay of activity) required the presence of the reduced form of the co-enzyme nicotinamide adenine dinucleotide (NADH: DPNH in older terminology), which could not be replaced by the reduced form of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH: TPNH in older terminology). Several years later, studies [21,22] with a purified sample of the enzyme confirmed these earlier findings. A study of the conversion of orotic acid into dihydro-orotic acid in the presence of $[^2H]NADH$ indicated that the enzyme contained a prosthetic group [25], and spectrophotometric analysis on a purified sample of the enzyme showed that the enzyme contained flavin mononucleotide (FMN) (i.e. riboflavin monophosphate) and flavin adenine dinucleotide (FAD) in approximately equivalent amounts [21,22]. Furthermore, analysis of a crystalline sample of the enzyme, which was obtained as orange-yellow needles, not only confirmed this but showed that for each molecule of flavin present there was one molecule of iron [22]. Paper chromatographic analysis of the enzyme confirmed that the only flavins present were FAD and FMN [26]. A more recent investigation by Miller and Massey [24], with a more highly purified preparation of the enzyme from Zymobacterium...
oroticum, established that the enzyme contains iron, flavin, and "inorganic" sulphide in equivalent amounts, and that the number of free sulphydryl (thiol) groups on the enzyme increased in the presence of substrates. However, the authors were unable to assign a precise function to each of the various groups.

A certain amount of work has also been carried out on the enzyme obtained from an aerobic source; this enzyme differs only in a few respects from that isolated from Zymobacterium oroticum. For the conversion of orotic acid into dihydro-orotic acid by the enzyme from aerobic bacteria the co-enzyme NADPH is required, and this cannot be replaced by NADH [23]; this is in contrast to the enzyme from the anaerobic bacterium Zymobacterium oroticum, which requires NADH and not NADPH. (Although terms such as NAD+ and DPN+ dependent are commonly used in the present context, the conversion of dihydro-orotic acid into orotic acid under aerobic as distinct from anaerobic conditions of incubation, does not call for NAD+ supplementation.) Moreover, Udaka and Vennesland [23] found that for the enzyme from an aerobic bacterium it was not necessary to incubate a freshly prepared preparation of the enzyme with cysteine to obtain an active preparation, although some of the activity lost on keeping the enzyme could be
reclaimed by incubation with cysteine. In other respects the enzyme was very similar to that obtained from *Zymobacterium oroticum*; for example, it contained approximately equivalent amounts of FAD and FMN. However, the iron content of the enzyme was not determined.

For mammalian sources of the enzyme there have been no reports on the possible need to pre-incubate with cysteine to obtain an active enzyme preparation, but there has been no work carried out with a purified preparation of the enzyme from a mammalian source. Also, with mammalian sources of the enzyme it has been established that the addition of NAD$^+$ or NADP$^+$ does not stimulate the oxidation of dihydro-orotic acid to orotic acid [9]. The conversion of orotic acid into dihydro-orotic acid with a mammalian source of the enzyme has not been studied and thus the enzyme requirements for this reaction are not known.

**IA$_3$d Specificity.**

In general, pyrimidines are not active substrates of dihydro-orotic dehydrogenase; however, several pyrimidines have been found to partially inhibit the reduction of orotic acid to dihydro-orotic acid. Friedmann and Vennesland [21], during their studies on the NADH-dependent enzyme (i.e. that from *Zymobacterium oroticum*), found that it oxidised NADH to NAD$^+$ both in the presence and absence of orotic acid, and from
a study of the specificity of the enzyme, they found that uracil-5-carboxylic acid, veronal, barbituric acid and 6-methyluracil each inhibited to a certain degree the conversion of orotic acid into dihydro-orotic acid. However, they found that of these compounds only barbituric acid and 6-methyluracil exhibited any inhibition of the oxidation of NADH to NAD⁺ in the absence of orotic acid. They also reported that 5-fluoroorotic acid was as active a substrate as orotic acid itself. In a later paper [22], it was stated by them that "the most potent inhibitor among various pyrimidine analogues tested was 5-methyl-orotate ... this substance inhibited the overall reaction and the blank by about 50%". Since no experimental details were given, nor was there any further comment, it is concluded by analogy with their previous work [21] that 5-methyl-orotic acid is not an active substrate for the enzyme, and that it inhibits to approximately 50% the conversion of orotic acid into dihydro-orotic acid and the oxidation of NADH to NAD⁺ in the absence of orotic acid.

It has also been reported that the same pyrimidine analogues inhibited the conversion of orotic acid into dihydro-orotic acid by the NADPH-dependent enzyme (i.e. that from the aerobic sources[23]).
For the biosynthesis of orotic acid from L-aspartic acid in rat-liver slices it has been established that the asymmetric carbon atom of L-aspartic acid and of L-carbamoylaspartic acid becomes the C-4 carbon atom of orotic acid [3]. Moreover, Reichard and Hanshoff [8] in 1956 found that the L- but not the D-isomer of aspartic acid was a substrate for aspartate-carbamoyl transferase and that the corresponding carbamoyl derivative was formed during the reaction:

\[
\begin{align*}
H - C^* - NH_2 & \rightleftharpoons H - C^* \cdot NH \cdot C \cdot NH_2 \\
L-aspartic & \text{ acid} & L-carbamoylaspartic & \text{ acid} \\
\end{align*}
\]
No detailed study has been carried out on the stereospecificity of dihydro-orotase, but from a study of the enzymic synthesis and breakdown of orotic acid with a cell free extract from *Zymobacterium oroticum* it was found that although L-carbamoylaspartic acid was converted into orotic acid, the corresponding D isomer was not metabolised [13].

Published work with dihydro-orotic dehydrogenase has involved, mainly a study of the conversion of orotic acid (IV) into dihydro-orotic acid (III), this approach being preferred since the equilibrium between (IV) and (III) is in favour of (III). From this reaction Lieberman and Kornberg [13,18] isolated (+)-dihydro-orotic acid. They tentatively assigned to the isomer the "L configuration" since with their enzyme preparation from *Z. oroticum* carbamoyl-L-aspartic acid, but not carbamoyl-D-aspartic acid, was converted into orotic acid. Moreover, L-5-carboxymethylhydantoin could be isolated from the same system. They also draw attention to the fact that when synthetic dihydro-orotic acid is oxidised with dihydro-orotic dehydrogenase, only 46% of the theoretical yield of orotic acid was obtained.

The pyrimidine requirements of *Lactobacillus bulgarius* cannot be satisfied by any pyrimidine other than orotic acid; however [27,28], the requirement can be satisfied by
carbamoylaspartic acid, or by dihydro-orotic acid. Miller, Gordon and Engelhardt [29] have since shown that L-dihydroorotic acid and DL-dihydro-orotic acid will each support the growth of the mutant and this activity as well as that of orotic acid and carbamoylaspartic acid, is reversibly inhibited by D-dihydro-orotic acid. From the evidence available it would seem that to serve as a substrate for the enzyme dihydro-orotic dehydrogenase, dihydro-orotic acid (III) must have, at the C-4 atom, the configuration corresponding to that of L-aspartic acid.

Although it would appear that dihydro-orotic dehydrogenase is sterospecific with reference to the removal of hydrogen at C-4 of dihydro-orotic acid, no information is available concerning the stereospecificity in connection with the removal of hydrogen from the C-5 carbon atom. Friedmann and Vennesland [21], whilst studying the enzymic conversion of orotic acid into dihydro-orotic acid, showed that 5-fluoro-orotic acid is reduced at a slightly faster rate than is orotic acid itself. However, the reaction was followed spectrophotometrically, and the product was not isolated. Consequently, no evidence is available as to whether the enzyme is stereospecific with reference to the C-5 atom and whether overall cis- or trans- addition of hydrogen occurred.
For the conversion of orotic acid into dihydro-orotic acid by dihydro-orotic dehydrogenase obtained from Zymobacterium oroticum NADH is essential [18], and the enzyme exhibits stereospecificity in the removal of hydrogen from it [25]. In enzymic oxidation-reduction reactions involving the co-enzymes NAD$^+$ and NADP$^+$ there is a transfer of hydrogen between the substrate ($XH_2$) and the pyridine nucleotide, a hydrogen atom attached to the C-4 atom of the nicotinamide ring participating in the reaction [30]:

\[
\begin{align*}
\text{NAD}^+; R, \text{adenosine diphosphoribosyl} & \quad \iff \quad (\text{NADH}) \\
\end{align*}
\]

The two-hydrogen atoms, $H_A$ and $H_B$ are stereochemically distinct, and an enzyme is said to exhibit either "A" or "B" stereospecificity depending upon whether it transfers $H_A$ or $H_B$ respectively [31].

Studies with deuterium labelled NADH and with deuterium oxide have shown that dihydro-orotic dehydrogenase exhibits "A" stereospecificity; moreover, the hydrogen atoms which add to the orotic acid are not directly transferred from the NADH, but are derived from the reaction medium[25].
IB PRESENT INVESTIGATION

It is now accepted that there is a sequence of enzymic reactions through which orotic acid is formed from aspartic acid. The final stage of the sequence involves the conversion of dihydro-orotic acid into orotic acid by the enzyme dihydro-orotic dehydrogenase. This enzyme has been studied in greater detail than the others involved in the biosynthesis of orotic acid. Procedures have been published for obtaining a highly purified preparation of the enzyme from Zymobacterium oroticum, and the enzyme has been crystallised. However, some aspects of the steric requirements of the enzyme still need to be established.

As has been discussed in the section on the stereospecificity of the enzymes (see Section IA), it would appear that for dihydro-orotic dehydrogenase to act a specific configuration of the C-4 atom of dihydro-orotic acid is required, corresponding to that of the L-amino acids [13]. However, there is no evidence as to which of the two hydrogen atoms attached to C-5 of dihydro-orotic acid is removed, that is whether there is cis or trans-elimination of hydrogen from the C-4 and C-5 atoms.
The aim of this investigation is to shed some light on the steric requirements of dihydro-orotic dehydrogenase at the C-5 atom of dihydro-orotic acid.

In general, earlier studies with dihydro-orotic dehydrogenase have been concerned with the reduction of orotic acid to dihydro-orotic acid. These studies have included the enzymic reduction of orotic acid in deuterium oxide, from which was isolated a deuterated dihydro-orotic acid [25], and the reduction of 5-fluoro-orotic acid [21]. No stereochemical studies were made of the deuterated dihydro-orotic acid, which contained 1.5 atoms of deuterium per molecule, and the reduction product of 5-fluoro-orotic acid was not even isolated, the reaction being followed spectrophotometrically. The elucidation of the configuration of the products obtained from these reactions might have provided the answer to the question of steric requirements at the C-5 atom. However, an alternative
approach is to compare the behaviour of cis- and trans-5-mono- substituted dihydro-orotic acids with the enzyme. This latter approach was used in this investigation.

From the practical point, the investigation entailed the preparation of cis and trans-5-mono-substituted dihydro-orotic acids as well as a comparison of their behaviour on treatment with dihydro-orotic dehydrogenase. The dihydro-orotic acids which it was decided to study were those with 5-alkyl substituents since the replacement of a hydrogen by an alkyl group did not involve a major structural change. The methyl analogue was chosen so as to minimise steric effects. As is discussed in subsequent sections the investigation centred around the unequivocal synthesis of the cis- and trans-4,5-dihydro-5- methylorotic acids from the threo- and erythro-3-methylaspartic acids respectively, and a comparison of their behaviour on treatment with dihydro-orotic dehydrogenase preparations obtained from Zymobacterium oroticum and from Ehrlich ascites tumour cells.

It was hoped to extend the investigation to a study of 5-fluoro-4,5-dihydro-orotic acid, and possible routes for the synthesis of this compound are reviewed (see Section VA). In this connection the preparation of 5-bromo-4,5-dihydro-orotic acid by the bromination of 4,5-dihydro-orotic acid was investigated (see Section VB).
SECTION II

THE PREPARATION OF 5-ALKYL-DIHYDRO-OROTIC ACIDS

IIA INTRODUCTION

IIB THE SYNTHESIS OF DIHYDRO-OROTIC ACID, AND OF
   5,6-DIHYDOURACIL AND ITS 5-METHYL ANALOGUE

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IIB₂ Cyclisation reactions.

IIB₃ Résumé,
IIA INTRODUCTION

The first 5-mono-alkyl substituted dihydro-orotic acid reported in the literature was the 5-methyl compound, which was used by Cullambine and Simpson [32] in 1947 for pharmacological experiments being conducted on rabbits; however, there was no indication of the method of preparation of the compound, and no physical constants were given. In 1960, the catalytic hydrogenation of 5-methylorotic acid with 5% rhodium on alumina to give 5-methyldihydro-orotic acid was reported by Smith et al. [33]; again, no physical constants were given for the compound. Also in 1960 Ferguson in a U.S. patent [34] claimed the preparation of the sodium salts of several 5-alkyl-dihydro-orotic acids by the treatment of the sodium salt of dihydro-orotic acid in ethanol with sodium ethoxide, and subsequently with the appropriate alkyl bromide.

No other references to the preparation of 5-monoalkyl dihydro-orotic acids by a chemical reaction can be found in the literature.

In view of the lack of information available on the preparation of 5-monoalkyl dihydro-orotic acids it was thought profitable to review the methods available for the synthesis of 5-alkyl-5,6-dihydrouracils, with a view to the use of analogous methods for the preparation of the related compounds with a
carboxyl group at C-6\textsuperscript{IV}. In general, methods similar to those used for the preparation of dihydrouracil and its analogues, apart from catalytic hydrogenation, are not suitable for the preparation of dihydro-orotic acid and its analogues. The methods available for the preparation of dihydro-orotic acid itself have also been reviewed.

\textsuperscript{3} Footnote

The 4-position of dihydro-orotic acid [I; 4,5-dihydro-orotic acid; 4-carboxy-4,5-dihydro-2,6-dioxopyrimidine] corresponds to the 6-position of dihydrouracil [II; 5,6-dihydrouracil; 5,6-dihydro-2,4-dioxopyrimidine].

\(\text{H} \quad \text{N} \quad \text{H}_2\text{C}_5\text{CH}_3\text{CO}_2\text{H} \quad \begin{array}{c} \text{H} \\ \text{N} \\ \text{H}_2\text{C}_5\text{CH}_3\text{OH} \end{array} \)

\(\text{I} \quad \text{II}\)
IIB THE SYNTHESIS OF DIHYDRO-OROTIC ACID, AND OF 5,6-
DIHYDROURACIL AND ITS 5-METHYL ANALOGUE

The methods reviewed are considered under two headings, namely catalytic hydrogenation, and cyclisation reactions.

IIB₁ Catalytic Hydrogenation

Dihydouracil and dihydro-orotic acid have been prepared by the catalytic reduction of uracil [35-38] and orotic acid [12,39,40] respectively. To obtain the dihydro compound as the major product of the reaction relatively mild conditions are required, and it is probable that under such conditions cis- rather than trans- addition of hydrogen occurs. Thus, the catalytic reduction of a 5-substituted orotic acid would furnish only one diastereoisomeric racemate. For this investigation it is essential to obtain each of the diastereoisomeric racemates, preferably by unambiguous routes; consequently, methods other than those involving catalytic hydrogenation needed to be investigated also. However, the catalytic reduction of 5-methylorotic acid was studied, and this, together with the preparation of 5-methyl-orotic acid, are discussed in Section IV.
Cyclisation Reactions

5,6-Dihydrouracil (II; R = H) and 5,6-dihydro-5-methyluracil (II; R = CH₃) may be prepared by the condensation of urea with acrylic acid and methylacrylic acid respectively [41]. However, the analogous reaction with maleic acid did not furnish dihydro-orotic acid [42]. The compound obtained by Bachstez and Cavallini by this route had an elemental analysis corresponding to that of dihydro-orotic acid; however, it behaved differently from authentic dihydro-orotic acid in biological systems [43]. It was later [39] shown to be fumarylurea, cyclisation not having occurred during the reaction.

The thermal cyclisation of β-ureido fatty acids or esters also yields dihydrouracil or analogues thereof. Thus, Langfield and Stieglitz [44] prepared dihydrouracil (II) from methyl β-ureidopropionate (III)
However, an attempt to prepare dihydro-orotic acid by an analogous route, viz. the cyclisation of ureidosuccinic acid(IV), furnished 5-carboxymethyl-hydantoin(V), the 5-membered ring rather than the 6-membered ring being formed [45].

The cyclisation of N-ethoxycarbonylasparagine(VI) in ethanol with sodium ethoxide as catalyst does, however, furnish dihydro-orotic acid(I) by an unambiguous route [29].
The application of this method to the synthesis of 5-alkyl-dihydro-orotic acids from the corresponding N-ethoxycarbonyl-β-alkylasparagine was particularly attractive since, thereby, it was probable that each of the two diastereoisomeric racemic 5-alkyl-dihydro-orotic acids could be obtained free from each other. The synthesis of the 5-methyl-dihydro-orotic acids by this route would entail the preparation of both the threo- and the erythro-N-ethoxycarbonyl-β-methyelasparagine.

Neither of the two diastereoisomeric racemic N-ethoxycarbonyl-β-methyelasparagines has been reported in the literature, and when this investigation was started neither of the two diastereoisomeric β-methyelasparagines had been reported. However, the threo- and erythro-β-methyelaspartic acids had been reported [46] and configurations assigned to them [47]. Thus, methods for the conversion of each of these into the corresponding N-ethoxycarbonyl-β-methyelasparagine were studied together.
with the cyclisation to give the corresponding cis- or trans-4,5-dihydro-5-methylorotic acid.

Briefly, the routes studied for the preparation of the two diastereoisomeric racemic N-ethoxycarbonyl-β-methylasparagines are as follows:

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{CO}_2\text{H} \\
\text{NH}_2\text{CH} \quad \rightarrow \quad \text{H}_2\text{N} - \text{CH} & \quad \rightarrow \quad \text{C}_2\text{H}_5\text{OC} - \text{NH} - \text{CH} \\
\text{CH(CH}_3) \quad \text{CH(CH}_3) & \quad \text{CH(CH}_3) \\
\text{CO}_2\text{H} & \quad \text{CONH}_2 \\
\text{(VII)} & \quad \text{(VIII)} & \quad \text{(X)}
\end{align*}
\]

These routes are reviewed in detail in Section III.
The methods studied in this investigation for the preparation of the 5-methyldihydro-orotic acids may be classified under three headings, viz.:

(a) The cyclisation of the diastereoisomeric racemic N-ethoxycarbonyl-β-methyasparagines.

(b) Reduction (chemical and catalytic) of 5-methyorotic acid.

(c) Substitution of dihydro-orotic acid at the 5-position (see Section IIA, p. 31).

These methods are described in detail in Section IV.

The preparation of the diastereoisomeric β-methyaspartic acids and of the derivatives required for the cyclisation studies are considered in Section III.
SECTION III

β-METHYLASPARTIC ACID AND ITS DERIVATIVES

IIIA A REVIEW OF SYNTHETIC METHODS

IIIA₁ (†)-Threo- and Erythro-β-Methyaspartic acid.

IIIA₂ (†)-Threo- and Erythro-β-Methyasparagine.

IIIA₂₁ via N-protected β-Methyaspartic anhydride.

IIIA₂₂ via β-Methyl or β-Ethyl ester of β-Methyaspartic acid.

IIIA₂₃ via The reaction of β-methyaspartic acid with dicyclohexylcarbodiimide.

IIIA₃ Synthesis of N-Ethoxycarbonyl-β-Methyasparagine.

IIIA₃₁ From β-Methyasparagine.

IIIA₃₂ From N-Ethoxycarbonyl-β-methyaspartic acid.

IIIA₃₃ Résumé.
SECTION IIIB
STUDIES ON THE SYNTHESIS OF β-METHYLASPARTIC ACID AND ITS
DERIVATIVES

IIIB₁ Preparation of (±)-threo- and erythro-β-methylaspartic acid

IIIB₂ Preparation of (±)-threo- and erythro-β-methylasparagine.

IIIB₂a Via N-phthaloyl-β-methylaspartic acid

IIIB₂b Via N-benzyloxycarbonyl-β-methylaspartic acid

IIIB₂c Via ammonolysis of β-methyl α-hydrogen β-methylaspartate

IIIB₂d Via reaction of β-methylaspartic acid with dicyclohexylcarbodiimide

IIIB₂e Summary

IIIB₃ Preparation of N-ethoxycarbonyl-(±)-threo- and erythro-β-methylasparagine

IIIB₃a From β-Methylasparagine

IIIB₃b From N-ethoxycarbonyl-β-methylaspartic acid

IIIB₃c Summary

IIIB₄ Comparison of the physical properties of the β-methylaspartic acid derivatives

IIIB₄a Solubility

IIIB₄b Melting-points

IIIB₄c Infra-red spectra
In general, the nomenclature used for the amino-acid derivatives is based on the trivial name of the parent amino-acid. This practice has been followed in the present investigation, and listed below are the formulae and trivial names of some of the compounds which are discussed in this Section.

(I)  
\[
\begin{align*}
\text{CO}_2\text{H} & \\
\text{CH(CH}_3) & \\
\text{CH.NH}_2 & \\
\text{CO}_2\text{H} & \\
\end{align*}
\]

(II)  
\[
\begin{align*}
\text{CO}_2\text{H} & \\
\text{CH(CH}_3) & \\
\text{CH.NHC.O.CH}_2\text{C}_6\text{H}_5 & \\
\text{CO}_2\text{H} & \\
\end{align*}
\]

(IIIA)  
\[
\begin{align*}
\text{CH}_3 & \\
\text{CH}_3 & \\
\text{CH.NH.C.O.O.CH}_2\text{C}_6\text{H}_5 & \\
\text{CO}_2\text{H} & \\
\end{align*}
\]

(III)  
\[
\begin{align*}
\text{CO}_2\text{H} & \\
\text{CH(CH}_3) & \\
\text{CH.NHC.O.O.CH}_2\text{C}_6\text{H}_5 & \\
\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5 & \\
\end{align*}
\]

(IIIA)  
\[
\begin{align*}
\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5 & \\
\text{CH(CH}_3) & \\
\text{CH.NHC.O.O.CH}_2\text{C}_6\text{H}_5 & \\
\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5 & \\
\end{align*}
\]

(IV)  
\[
\begin{align*}
\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5 & \\
\text{CH(CH}_3) & \\
\text{CH.NHC.O.O.CH}_2\text{C}_6\text{H}_5 & \\
\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5 & \\
\end{align*}
\]
CO₂H
\[\text{CH(CH₃)}\]
\[\text{NH}_2\]
\[\text{CO₂H}\]
(V)

CO₂H
\[\text{CH(CH₃)}\]
\[\text{NH}_2\]
\[\text{CO₂H}\]
(VA)

CO₂H
\[\text{CH(CH₃)}\]
\[\text{NH}_2\]
\[\text{CO₂H}\]
(VI)

CO₂H
\[\text{CH(CH₃)}\]
\[\text{NH}_2\]
\[\text{CO₂H}\]
(VII)

(I) \(\beta\)-Methlaspartic acid.
(II) \(N\)-benzyloxy carbonyl-\(\beta\)-methylaspartic acid.
(IIA) \(N\)-benzyloxy carbonyl-\(\beta\)-methylaspartic anhydride.
(III) \(\alpha\)-Benzyl \(\beta\)-hydrogen \(N\)-benzyloxy carbonyl-\(\beta\)-methylaspartate.
(IIIA) \(\alpha\)-Hydrogen \(\beta\)-benzyl \(N\)-benzyloxy carbonyl-\(\beta\)-methylaspartate.
(IV) \(\alpha\)-Benzyl \(N\)-benzyloxy carbonyl-\(\beta\)-methylasparagine.
(V) \(\beta\)-Methylasparagine.
(VA) \(\alpha\)-Methylisoasparagine.
(VI) \(N\)-ethoxycarbonyl-\(\beta\)-methylaspartic acid.
(VII) \(N\)-ethoxycarbonyl-\(\beta\)-methylasparagine.
IIIA  A REVIEW OF SYNTHETIC METHODS

Possible routes for the synthesis of (±)-threo- and erythro-β-methylaspartic acids and derivatives thereof are described below; each derivative is considered in a separate sub-section.

IIIA₁ (±)-Threo- and Erythro-β-methylaspartic acid

β-Methylaspartic acid (I) was first prepared by Dakin [48] in 1941 by a method analogous to that used by Redmann and Dunn [49] for the preparation of aspartic acid. Dakin [48] treated a solution of diethyl benzylaminomalonate (VIII; R = PhCH₂, R' = CO₂C₂H₅) in ethanol with sodium ethoxide, and subsequently with ethyl 2-bromopropionate. Hydrolysis of the product with acid furnished only one product, later shown to be the (±)-threo isomer (IX). In an attempt to obtain both diastereoisomeric racemic β-methylaspartic acids, Benoiton and co-workers [50] carried out an analogous reaction with ethyl acetamidocyanocacetate (VIII; R = CH₃CO, R' = CN); however, they isolated the same isomer as that obtained by Dakin.

\[
\begin{align*}
R' & \quad \text{Br} \\
\text{R} - \text{NH} - \text{C} - \text{CO}_2\text{C}_2\text{H}_5 + \text{CH}_3 - \text{CH} & \quad \text{CO}_2\text{H} \\
\text{CO}_2\text{C}_2\text{H}_5 & \quad \text{CH(CH}_3) \text{CH(NH}_2) \\
\text{(VIII)} & \quad \text{CO}_2\text{H} \\
\text{R} & \quad \text{Br} \\
\text{R} - \text{NH} - \text{C} - \text{CO}_2\text{C}_2\text{H}_5 + \text{CH}_3 - \text{CH} & \quad \text{CO}_2\text{H} \\
\text{CO}_2\text{C}_2\text{H}_5 & \quad \text{CH(CH}_3) \text{CH(NH}_2) \\
\text{(I)} & \quad \text{CO}_2\text{H}
\end{align*}
\]
The use of diethyl acetamidomalonate [46] (VIII; \(R = \text{CH}_3\text{CO}, R' = \text{CO}_2\text{C}_2\text{H}_5\)) did, however, furnish both of the diastereoisomeric racemic isomers, which Barker and Wawszkiewicz [46] separated by fractional recrystallisation from water. It was established that the isomer which was less soluble and had the higher decomposition point [m.p. 268-272° (decomp.)] had the threo configuration (IX), and the one which was the more soluble and had the lower decomposition point [m.p. 250-255° (decomp.)], had the erythro configuration (X) [51].

\[
\begin{align*}
\text{Threo-racemate (IX)} \\
\text{Erythro-racemate (X)}
\end{align*}
\]

It has recently been established that the Leuckart reaction with diethyl oxalopropionate, followed by hydrolysis of the products with acid furnished, together with \(\alpha\)-aminobutyric acid (XI), \((\pm)\)-threo-\(\beta\)-methylaspartic acid (IX), none of the erythro-isomer being obtained [52].
(-)-Threo-β-methylaspartic acid has also been obtained by treating β-hydroxy-β-methylaspartic acid with constant boiling hydriodic acid [53].

For the present investigation, the threo- and the erythro-isomer were each required. Thus, the method of Barker and Wawaskiewicz [46], in which ethyl bromopropionate and diethyl acetamidomalonate are condensed, was studied; our results are discussed in Section IIIB1.

IIIA2 (-)-Threo- and Erythro-β-Methylasparagine

IIIA2a Via N-Protected β-methylaspartic anhydride

The preparation of the two diastereoisomeric(±)-β-methylasparagines from the corresponding β-methylaspartic acids, presented similar problems to those encountered in the preparation of asparagine and glutamine from aspartic acid and glutamic acid respectively; namely, the α-amide is obtained in
preference to either the \( \beta \)- or \( \gamma \)-amide. For example, Bergmann and co-workers [54] in 1932 attempted to prepare \( \text{N}-\text{benzyloxycarbonylasparagine}(XIII) \) by the ammonolysis of \( \text{N}-\text{benzyloxycarbonylaspartic anhydride}(XII) \), but the only product isolated was \( \text{N}-\text{benzyloxycarbonyl-isoasparagine}(XIV) \).

![Chemical structures](image)

The problem was to find a suitable blocking group for the \( \alpha \)-carboxyl group which could easily be removed after the formation of the \( \beta \)- or \( \gamma \)-amide without the amide group being attacked. Bergmann and co-workers [55] found that the acid could be obtained from benzyl esters by catalytic hydrogenolysis under conditions to which the amide group is stable. In 1933 they reported the synthesis of \( \text{L}-\text{asparagine} \) from \( \text{L}-\text{aspartic acid} \) and by the following route, making full use of the fact
that the α-benzyl ester and the \( N \)-benzyloxycarbonyl protecting
groups could be removed by catalytic hydrogenolysis \([55]\).

\[
\begin{align*}
\text{CH}_2\text{NH}_2 & \quad \text{CO}_2\text{H} \quad \text{(XV)} \\
\text{CO}_2\text{H} & \quad \text{CO}_2\text{H} \quad \text{(XVI)} \\
\text{CH}_2 & \quad \text{CH}_2 \quad \text{(XII)} \\
\text{HC}_2\text{H}_7 & \quad \text{CO}_2\text{H} \quad \text{(XVII)}
\end{align*}
\]

During the course of the present investigation, Brain\([56]\)
(in 1963) reported the preparation of (1)-erythro-β-methyl-
asparagine from the corresponding β-methylaspartic acid, by
a method analogous to the above.

King and Kidd \([57]\), in 1949, found that by protecting the
amino group of glutamic acid by the formation of the \( N \)-phthaloyl
derivative, it was possible to prepare through the anhydride N-phthaloylglutamine from N-phthaloylglutamic acid without protecting the α-carboxyl group. Furthermore, two years later they prepared asparagine from aspartic acid by an analogous route [58], as outlined schematically below:

Further studies by Tanenbaum [59] on the ammonolysis of N-phthaloylaspartic anhydride (XXI) demonstrated that the reaction medium was important in influencing the course of reaction. Thus, although the reaction with a dry ethereal solution of
ammonia furnished the β-amide, a wet ethereal solution of ammonia gave a mixture of α- and β-amides. Furthermore, the action of a solution of ammonia in aqueous ethanol furnished the α-amide only.

During the course of the present investigation Brain [56] reported the preparation of (†)-threo-β-methylasparagine from the corresponding acid by a method analogous to that described by King and Kidd [58] for the conversion of aspartic acid into asparagine. He also reported that the reaction of phthalic anhydride with the erythro-β-methylaspartic acid gave mainly N-phthaloyl-(†)-threo-β-methylaspartic acid, only a small quantity of the erythro-isomer being obtained. The fact that very little erythro-isomer was isolated made this an impracticable method for the preparation of (†)-erythro-β-methyl-asparagine.

However, in 1960, Nefkens [60] reported a new general synthesis for the preparation of N-phthaloyl derivatives of amino-acids, namely, the reaction of N-ethoxycarbonylphthalimide with an aqueous solution of the sodium salt of the amino acid. Moreover, he claims that under these conditions racemisation of the amino-acid does not occur, a danger which is present when phthalic anhydride itself is used. Thus, N-phthaloyl-L-glutamic acid was obtained from L-glutamic acid,
whereas King and Kidd [57] obtained the racemic N-phthaloyl-
glutamic acid by the reaction of L-glutamic acid with phthalic
anhydride. Therefore, it may be possible to convert erythro-
\( \beta \)-methylaspartic acid into the corresponding N-phthaloyl
derivative by the method described by Nefkens [60] and so pre-
pare the erythro-\( \beta \)-methylasparagine by the route proposed for
the threo-isomer; namely, by the ammonolysis of N-phthaloyl-
erthro-\( \beta \)-methylaspartic anhydride, and the subsequent removal
of the phthaloyl group, as described in Section III.B.2a.

III.A.2b Via \( \beta \)-Methyl or \( \beta \)-Ethyl ester of \( \beta \)-Methyl-
aspartic acid.

One route for converting a carboxyl group into an amide
is ammonolysis of the corresponding methyl or ethyl ester, and
Piutti [61] made use of this fact when he prepared asparagine
by the following route. Reduction of the oxime of ethyl
oxaloacetate with sodium amalgam and dilute acetic acid fur-
nished the two isomeric mono-ethyl aspartates (XXIV), which
were separated from each other by fractional recrystallisation
of their copper salts. The action of alcoholic ammonia on
the \( \beta \)-ethyl aspartate furnished asparagine (XIX)
Crocker [62] in 1940 modified the method; he found that by reducing the oxime of ethyl oxaloacetate with aluminum amalgam in ether he obtained diethyl aspartate which, on hydrolysis with aqueous ammonia under pressure, furnished asparagine. He also reported that the action of boiling water, under pressure, on diethyl aspartate furnished aspartic acid. However, Traynham and Williams [52], in 1962, intended to prepare the two diastereoisomeric β-methyaspartic acids by a similar route, but they were unable to obtain the oxime of ethyl oxalopropionate. Furthermore, they reported that the Leuckart reaction with ethyl oxalopropionate, followed by hydrolysis with acid, gave α-amino-butyric acid and three-β-methyaspartic acid.
but no erythro-isomer. Therefore, if instead of acid hydrolysis the last step was ammonolysis, it is highly probable that only the threo-isomer of β-methasparagine would be obtained, if any β-methasparagine were obtained at all.

The β-methyl and β-ethyl esters of aspartic acid were prepared by Coleman [63] and Haskizume [64] respectively in 1951 by the reaction of the acid with the appropriate alcohol in the presence of one equivalent of hydrogen chloride. Also, in 1957, Schwarz and co-workers [65] described the preparation of β-methyl aspartate hydrochloride by the reaction of thionyl chloride in methanol with aspartic acid. Furthermore, Beecham [66], in 1954, confirmed that although the action of aqueous ammonia on the γ-methyl or ethyl ester of glutamic acid gave pyrrolidone carboxylic acid, the reaction with β-methyl L-aspartate hydrochloride furnished L-asparagine.

The possibility of preparing the two diastereoisomeric β-methasparagines via the ammonolysis of the corresponding β-methyl ester was studied in this present investigation and is described in Section IIIIB2c.

III A 2c Via the reaction of β-methaspartic acid with dicyclohexylcarbodiimide.

In 1958 Chang and Barker [67] claimed in a U.S. patent
the preparation of glutamine by the reaction of the copper salt of glutamic acid with dicyclohexylcarbodiimide, followed by the addition of ammonia. This report followed an earlier one by Sheehan and Hess [68] who, in 1955, described the formation of the amide bond of peptides by the treatment of an N-protected amino-acid with a C-protected amino acid in the presence of dicyclohexylcarbodiimide

\[
\text{RCO}_2\text{H} + \text{NH}_2\text{R}'+ \text{C}_6\text{H}_{11}\text{N}=\text{C}=\text{N}\text{C}_6\text{H}_{11} \rightarrow \text{RCONHR'}
\]

\[
\text{C}_6\text{H}_{11}\text{NHCO} + \text{NHC}_6\text{H}_{11}
\]

For example, the benzyl ester of N-phthaloyl-L-alanyl-L-proline was prepared by the addition of the benzyl ester of L-proline to a solution of N-phthaloyl-L-alanine and dicyclohexylcarbodiimide in tetrahydrofuran.

Therefore, it appeared profitable to study the treatment of the diastereoisomeric β-methlaspartic acids with dicyclohexylcarbodiimide as a means of obtaining the corresponding β-amides. In connection with the application of this method to the synthesis of the β-methlasparagines, it is relevant that Gish and co-workers [69], in 1956, found that dicyclohexylcarbodiimide converted the amide group of asparagine into the nitrile by dehydration. Nevertheless the dicyclohexylcarbodiimide method was studied in this investigation and the results
obtained are described in Section IIIB2a.

III A3 Synthesis of $\text{N}$-Ethoxycarbonyl-$\beta$-Methylasparagine

$\text{N}$-Ethoxycarbonyl-$\beta$-methylasparagine has not hitherto been reported. Possible methods for the preparation are considered under two main headings:

(a) From $\beta$-methylasparagine.

(b) From $\text{N}$-ethoxycarbonyl-$\beta$-methylaspartic acid.

III A3a From $\beta$-Methylasparagine

Koenigs and Mylo [70] in 1908 reported the preparation of $\text{N}$-ethoxycarbonyl-$L$-asparagine by the addition of ethyl chloroformate to an aqueous solution containing the sodium salt of asparagine and an equivalent amount of sodium carbonate. Bergmann and Zervas [54] in 1932 reported a general method for the preparation of $\text{N}$-benzyloxy carbonyl derivatives of optically active amino-acids by the addition of benzyl chloroformate to an aqueous solution of the amino-acid in the presence of excess magnesium oxide. Subsequently, Johnston [71] described the preparation of benzyl oxyglycine by a modification of this method, sodium hydroxide being used instead of magnesium oxide. An analogous procedure was found to be suitable for the preparation of $\text{N}$-ethoxycarbonyl derivatives, Ishai and Kalchaki [72] in 1952...
using it for the N-ethoxycarbonyl as well as N-benzyloxycarbonyl derivatives of DL-alanine. Optically active N-ethoxycarbonyl and N-benzyloxycarbonyl derivatives of amino-acids have been prepared by all of the procedures described above.

In the present investigation each of the two diastereoisomeric N-ethoxycarbonyl-β-methylasparagines were prepared from the appropriate β-methylasparagine by a method analogous to that described by Bergmann and Zervas [54], as described in Section IIIB_{3a}.

III A_{3b} From N-ethoxycarbonyl-β-methylaspartic acid.

Mckay and Albertson [73] in 1957 reported that although the N-benzyloxycarbonyl group could be removed from a molecule by catalytic hydrogenolysis, as described by Bergmann and Zervas [54], N-alkoxycarbonyl groups were stable under these conditions.

As described previously (see Section IIIA_{2a}) the two diastereoisomeric racemic β-methylasparagines may be prepared from the corresponding β-methylaspartic acid by the following sequence of reactions (R = C₆H₅CH₂):
Therefore, it seemed profitable to study a similar series of reactions using the ethoxycarbonyl (R = CH₂CH₂O) instead of the benzoxycarbonyl derivative. Thereby in the final hydrogenolysis stage the amino-blocking group would not be removed. Moreover, this route, using the benzoxycarbonyl derivative, has been shown to be suitable for obtaining the erythro-β-methyasparagine from erythro-β-methyaspartic acid [56].
This route, via the \(N\)-ethoxycarbonyl-\(\beta\)-methylaspartic acid, was investigated extensively in this investigation, as described in Section IIIB, \(3b\).

IIIA. Résumé

The methods studied for the preparation of both the threo- and the erythro-\(N\)-ethoxycarbonyl-(\(\ddagger\))-\(\beta\)-methylasparagines from the corresponding (\(\ddagger\))-\(\beta\)-methylaspartic acids are summarised in the scheme on the following page. The series of reactions are shown for the threo-isomer, but they were also studied with the erythro-isomer.

**Route 1.** An authentic sample of (\(\ddagger\))-threo-\(\beta\)-methylasparagine was obtained by this route. Studies with the erythro-isomer were also made with a view to obtaining (\(\ddagger\))-erythro-\(\beta\)-methylasparagine.

**Routes 2 and 3.** Both of these routes were studied with a view to obtaining a less tedious route for the preparation of each of the diastereoisomeric (\(\ddagger\))-\(\beta\)-methylasparagines, and also to a high yield of conversion of the acid into the amide.

**Route 4.** An authentic specimen of erythro-\(\beta\)-methylasparagine was obtained by this route, and the analogous reactions with the threo-isomer were also studied so that a comparison of the properties of the threo- and erythro-derivatives could be made.
Three-β-Methylaspartic acid

(1) 

(2) 

(3) 

(4) 

(5)
Route 5. Authentic samples of both of the diastereoisomeric N-ethoxycarbonyl-β-methylasparagines were obtained by this route. This route was studied primarily as an alternative route for the preparation of N-ethoxycarbonyl-(-)-erythro-β-methylasparagine in the hope that the yield obtained would be significantly greater than that obtained via Route 2, which is one stage longer. Studies with threo-isomer were carried out as a trial for the experiments with the erythro-isomer, since the (±)-erythro-β-methylaspartic acid is not so readily available as the threo-isomer, and the threo-isomer is apparently less liable to epimerisation. Moreover, a comparison of the properties of the threo- and erythro-derivatives could then be made.

All of the above routes were investigated and the results obtained are discussed later in this chapter (Section IIIB).
Preparation of (±)-threo- and erythro-β-methylaspartic acid

\[
\text{R-NH-CH(CO}_2\text{C}_2\text{H}_5)_2 + \text{CH}_3\text{CO}_2\text{C}_2\text{H}_5 \xrightarrow{(i)\text{NaOC}_2\text{H}_5} \text{CO}_2\text{H} \xrightarrow{(ii)\text{HCl aqueous}} \text{CH(CH}_3)\text{CH.NH}_2
\]

(XXV) (XXVI) (I)

A solution of diethyl acetamidomalonate (XXV; \(R = \text{CH}_3\text{CO}\)) was treated with sodium ethoxide, and subsequently with ethyl 2-bromopropionate (XXVI). The condensation product was hydrolysed and decarboxylated by boiling under reflux with concentrated hydrochloric acid, and the solution was then concentrated under reduced pressure to remove excess hydrochloric acid and water. If the temperature is maintained at 45° or below each of the two racemic diastereoisomeric β-methylaspartic acids can be isolated; the less soluble (±)-threo-isomer, m.p. 268-272° (decomp.), was obtained in yields of 35-40% and the more soluble erythro-isomer, m.p. 250-255° (decomp.), in 20% yield. These results are comparable to those obtained by Brain [56] who by a similar method obtained the threo-isomer, m.p. 270° (decomp.), in 30-35% yield and the erythro-isomer, m.p. 250-255° (decomp.), in 20% yield. Barker and Wawzakiewicz
[46] obtained the threo-isomer in 23% yield, and the erythro-isomer in 6-7% yield, but they did not give the melting-points of the two isomers.

We found that if the temperature of the solution being concentrated was allowed to rise above 45° the yield was considerably diminished particularly that of the erythro-isomer. If the temperature rose to 60° about 50% of the threo-isomer disappeared as compared with apparent complete disappearance of the erythro-isomer; thus, the erythro-isomer is less stable than the threo-isomer under the conditions used to isolate the material.

The use of diethyl benzylaminomalonate (XXV; R = C₆H₅(OH)₂) instead of diethyl acetamidomalonate furnished (+)-threo-β-methylaspartic acid, but none of the erythro-isomer [48]. Therefore, the reaction with diethyl formamidomalonate (XXV; R = HCO), instead of diethyl acetamidomalmonate, was studied to ascertain whether an increased yield of the erythro-isomer could be obtained. However, the use of the formyl analogue furnished the threo-isomer only, and the yield (14%) was lower than that obtained with either the benzyl or acetyl analogue. The main difficulty in isolating erythro-β-methylaspartic acid from aqueous reaction media is that it is very soluble in water even in the presence of excess ethanol.
Attempts were made to convert threo-β-methylaspartic acid into the erythro-isomer by heating it in pyridine or triethylamine, under reflux; however, only threo-β-methylaspartic acid could be isolated, the percentage recovery being 90-95%.

IIIB2 Preparation of (1-threo- and erythro-β-methylasparagine

All the methods reviewed for the conversion of the two racemic diastereoisomeric β-methylaspartic acids into the corresponding β-methylasparagines (see Section IIIA2) were studied in this investigation and they are discussed below in separate subsections.

IIIB2a Via N-phthaloyl-β-methylaspartic acid.

\[ I \quad (XXVII) \quad (XXVIII) \quad (XXIX) \quad (V) \]
The main problem encountered during this sequence of reactions was in the preparation of the N-phthaloyl derivative of β-methylaspartic acid. Brain [56] had reported that the reaction of (±)-threo-β-methylaspartic acid with phthalic anhydride in pyridine furnished N-phthaloyl-(±)-threo-β-methylaspartic acid (m.p. 202-204°) in 42% yield, and the (±)-erythro-isomer (m.p. 189-190°) in 10% yield. However, in this investigation the maximum yield obtained of the N-phthaloyl derivative of the threo-isomer (m.p. 203-204°), was 22%, and of the erythro-isomer (m.p. 188-189°) 4%. Furthermore, 25-35% of threo-β-methylaspartic acid was recovered from each experiment. This difficulty was partially overcome by the use of N-ethoxycarbonylphthalimide for the formation of the N-phthaloyl derivatives, as described by Nefkens [60] (see Section IIIA2a). By this procedure, threo-β-methylaspartic acid furnished N-phthaloyl-threo-β-methylaspartic acid, m.p. 203-204°/65% yield; erythro-β-methylaspartic acid furnished the erythro-isomer, m.p. 188-189° but in yields of 12% only. The very low yield obtained with the erythro-isomer is probably due to the fact that N-phthaloyl (±)-erythro-β-methylaspartic acid is very soluble in water, and consequently difficult to isolate since the reaction is carried out in water. Since such a low yield of the N-phthaloyl derivative of the erythro-isomer was obtained,
it was not considered a practical proposition to pursue this route as a method for obtaining **erythro-β-methyasparagine**.

The conversion of **N-phthaloyl-threo-β-methyasparagic acid (XXVII)** into **N-phthaloyl-threo-β-methyasparagic anhydride (XXVIII)** proceeded without difficulty in good yield and the subsequent ammonolysis of the product (XXVIII) under anhydrous conditions furnished **N-phthaloyl-threo-β-methyasparagine hydrate (XXIX)** in good yield. However, it was found that if the ammonolysis was carried out under conditions which were not anhydrous the yield of the β-amide (XXIX) was low and the presence of the α-amide was detected. This is analogous to the findings of Tanenbaum [59] on the ammonolysis of **N-phthaloylaspartic anhydride** (see Section IIIA2a).

The conversion of **N-phthaloyl-threo-β-methyasparagine into threo-β-methyasparagine** by treatment with hydrazine also proceeded smoothly in reasonable yield.

The overall yield of [(+)-threo-β-methyasparagine, m.p. 240-245° (decomp.) from [(+)-threo-β-methyasparagic acid was 33% if the N-phthaloyl derivative of threo-β-methyasparagic acid was prepared by the N-ethoxycarbonylphthalimide method [60], or 11% if it was prepared by the phthalic anhydride method [58]. By the latter method Brain [56] claimed an overall yield of crude threo-β-methyasparagine of approximately 40%.
Although a route for the synthesis of erythro- rather than from threo- β-methyelasparagine was required, the conversion of each of the racemic diastereoisomeric β-methyelaspartic acids into the corresponding β-methyelasparagine, by the above series
of reactions, was studied. Thus, a direct comparison between the derivatives of the two isomers could be made in order to establish whether interconversion occurs. Such a comparison indicated that interconversion had not occurred. The synthesis, carried out as described by Brain [56] for the preparation of erythro-β-methyasparagine from erythro-β-methyaspartic acid, proceeded smoothly. The results obtained for the two diastereoisomers are tabulated below, together with the results obtained by Brain for the erythro-isomer. As can be seen from the table, there are no real discrepancies between the results obtained in this investigation and those reported by Brain [56]. However, we found that erythro-β-methyasparagine separated from aqueous ethanol as the hemihydrate, whereas Brain reported that erythro-β-methyasparagine separated from aqueous ethanol with one and a half molecules of water of crystallisation.
<table>
<thead>
<tr>
<th>Compound</th>
<th>β-methylaspartic acid</th>
<th>N-benzyl-N-benzyloxycarbonyl-β-methylaspartic acid</th>
<th>β-methylaspartic anhydride</th>
<th>α-benzyl N-benzyloxycarbonyl-β-methylaspartate</th>
<th>α-benzyl N-benzyloxycarbonyl-β-methylasparagine</th>
<th>Overall yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythro (Brain) m.p.</td>
<td>250-255°</td>
<td>122-123°</td>
<td>119-120°</td>
<td>111-112°</td>
<td>158-160°</td>
<td>235°</td>
</tr>
<tr>
<td>% yield</td>
<td>93</td>
<td>69</td>
<td>80</td>
<td>80</td>
<td>93</td>
<td>38</td>
</tr>
<tr>
<td>Erythro m.p.</td>
<td>250-255°</td>
<td>an oil</td>
<td>119-120°</td>
<td>110-112°</td>
<td>158-160°</td>
<td></td>
</tr>
<tr>
<td>% yield</td>
<td>90</td>
<td>91</td>
<td>75</td>
<td>80</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Threo m.p.</td>
<td>268-272°</td>
<td>150°</td>
<td>120°</td>
<td>166°</td>
<td>240-245°</td>
<td></td>
</tr>
<tr>
<td>% yield</td>
<td>91</td>
<td>66</td>
<td>85</td>
<td>86</td>
<td>86</td>
<td>44</td>
</tr>
</tbody>
</table>

Note: The % yield values are approximate and may vary slightly due to experimental conditions.
IIIb.2c Via-ammonolysis of β-methyl α-hydrogen β-methyl-aspartate

At the beginning of this investigation only a very limited quantity of the two racemic β-methylaspartic acids were available, and so preliminary experiments were carried out with aspartic acid as a model compound.

Asparagine (XIX) was prepared from aspartic acid (XI) via the β-methyl ester hydrochloride:

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{CO}_2\text{H} & \quad \text{CO}_2\text{H} \\
\text{NH}_2\text{CH} & \quad \text{NH}_2\text{CH} & \quad \text{CH}(-\text{NH}_2) \\
\text{CH}_2 & \quad \text{CH}(-\text{NH}_3)^+\text{Cl}^- & \quad \text{CONH}_2 \\
\text{CO}_2\text{H} & \quad \text{CO}_2\text{CH}_3 & \\
\text{CH}_2 & \\
\end{align*}
\]

(XV) \hspace{2cm} (XXX) \hspace{2cm} (XIX)

β-Methyl aspartate hydrochloride (XXX) was obtained in a 74% yield by the addition of aspartic acid to a solution of thionyl chloride in methanol [65]. Ammonolysis of the methyl ester with excess 0.880 ammonia furnished asparagine in 70% yield. Attempts were made to prepare and isolate the β-methyl ester hydrochlorides of threo- and erythro-β-methylaspartic acids by an analogous method, but only deliquescent glassy solids were
obtained. The crude product from each isomer was treated with 0.880 ammonia, as in the preparation of asparagine. From the threo-isomer a 10% yield of threo-β-methylasparagine (m.p. 240-245° (decomp.)) was obtained, and another compound was isolated also. The latter compound gave a red colour with ninhydrin. The erythro-isomer furnished one compound only. This gave a correct elemental analysis for the monohydrate of erythro-β-methylasparagine, but had a different melting-point and infra-red spectrum, as compared with those of an authentic sample of erythro-β-methylasparagine. The compound gave a wine-red colour with ninhydrin, whereas an authentic sample of erythro-β-methylasparagine gave a tan colour. Asparagine and isocasparagine give with ninhydrin a yellow-tan, and a red-wine colour respectively [59]. Consequently it would seem that β-methyl erythro-β-methylaspartate gives the α-amide rather than the β-amide, and the second compound isolated from the reaction with the threo-isomer was also an α-amide.

**III B**

Via reaction of β-methylaspartic acid with dicyclohexylcarbodiimide

\[
\begin{align*}
\text{CHNH}_2 & \quad \text{(i) } \text{Cu}^{2+} \quad \text{CHNH}_2 \\
\text{CHCH}_3 & \quad \text{(ii) } \text{C}_6\text{H}_{11}N=\text{C}=\text{NC}_6\text{H}_{11} \\
\text{CO}_2\text{H} & \quad \text{(iii) } \text{NH}_4\text{OH} \\
\text{CO}_2\text{H} & \quad \text{CONH}_2
\end{align*}
\]
An attempt was made to prepare threo-β-methylasparagine by the addition of dicyclohexylcarbodiimide to an aqueous solution of the dicupric salt of threo-β-methylaspartic acid, and the subsequent addition of excess dilute aqueous ammonia to the reaction mixture (see Section IIIA_{20}); but the only compounds isolated were dicyclohexylurea and threo-β-methylaspartic acid. A similar experiment was carried out with the dicupric salt of aspartic acid; again the only compounds isolated were dicyclohexylurea, and the starting amino-acid.

Since these preliminary experiments had been unsuccessful, it was decided to examine more closely the claim that glutamine could be prepared from glutamic acid by a similar reaction [67] (see Section IIIA_{20}). A similar experiment to those described above was carried out with the dicupric salt of glutamic acid, but the only compounds isolated were dicyclohexylurea and glutamic acid. Furthermore, paper chromatographic analysis of the reaction medium with Whatman No.1 paper and n-butanol-acetic acid-water (2 : 1 : 1) as developing phase, confirmed that glutamic acid was the only amino-acid present in the solution.

Presumably the mechanism of the required reaction would be the same as for the formation of peptide bonds by this route, the proposed mechanism of which is as follows [74]:

-71-
From the very limited experimental details given in the patent [27] it would appear that the dicupric salt of glutamic acid was used, but since the initial step is probably the addition of a proton to dicyclohexylcarbodiimide, it was decided to carry out similar experiments with the mono cupric salt. However, again the only compounds isolated were dicyclohexylurea and glutamic acid. The main practical difficulty was that dicyclohexylcarbodiimide is not very soluble in water, and that the cupric salts of the amino-acids are not soluble in the organic solvents in which dicyclohexylcarbodiimide is soluble. In an attempt to obtain a homogenous reaction medium experiments were carried out with aqueous ethanol as solvent; again the only compounds isolated were dicyclohexylurea and unchanged amino-acid. Furthermore, if sufficient alcohol were added to the reaction medium to dissolve all of the dicyclohexylcarbodiimide, the cupric salt of the amino acid was precipitated from solution.
Summary

Three-β-methylasparagine could suitably be prepared from N-benzylloxycarbonyl-threeo-β-methylaspartic acid and from N-phthaloyl-threeo-β-methylaspartic acid. Although the former route gave a slightly better yield of product, the latter had the advantage that it was experimentally simpler and quicker, and it was subsequently used as the main synthetic route for obtaining threeo-β-methylasparagine. The route via β-methyl-threeo-β-methylaspartate was less suitable.

The route via N-benzylloxycarbonyl-erythro-β-methylaspartic acid was the only one by which erythro-β-methylasparagine could be satisfactorily prepared.

Preparation of N-ethoxycarbonyl-(+)-threeo- and erythro-β-methylasparagine

The preparation of each of the diastereoisomeric N-ethoxycarbonyl-β-methylasparagines from the corresponding β-methylasparagines and from the corresponding N-ethoxycarbonyl β-methylaspartic acids is described below under the appropriate heading.

From β-methylasparagine

In the first instance the preparation of N-ethoxycarbo-
asparagine was studied since asparagine was readily available whereas the two racemic diastereoisomeric-β-methylasparagines were not. Moreover, N-ethoxycarbonyl-L-asparagine was required for further studies in the investigation.

N-ethoxycarbonylasparagine, m.p. 161-162°, was obtained in 71% yield by the interaction of asparagine and ethyl chloroformate in the presence of excess magnesium oxide. Koenigs and Mylo[70] reported m.p. 169-170°.

The two diastereoisomeric N-ethoxycarbonyl-β-methyl-asparagines were similarly prepared from the corresponding β-methylasparagine. N-ethoxycarbonyl-threo-β-methylasparagine, m.p. 144-146°, was obtained thus in 74% yield. The preparation of N-ethoxycarbonyl-erythro-β-methylasparagine by this route gave rise to some anomalous results. When erythro-β-methyl-asparagine, m.p. 230° (decomp.), prepared by catalytic hydrogenolysis of α-benzyl N-benzzyloxycarbonyl-erythro-β-methylasparagine (Route No.4; see Section IIIA[30]) was used without further purification, a compound designated (A), m.p. 188°, was obtained in 70% yield. A similar experiment with erythro-β-methylasparagine, m.p. 230-233° (decomp.), obtained similarly but recrystallised from aqueous ethanol, furnished a compound designated (B), m.p. 168°, in 65% yield. Both of the compounds (A) and (B) had the correct elemental analysis for
N-ethoxycarbonyl-β-methylasparagine. The infra-red spectra of the two specimens were identical and differed from that of the threo-isomer only slightly (see Appendix I). Furthermore, an admixture of (A), m.p. 188°, and (B), m.p. 168°, had m.p. 168°.

It would appear that (A) and (B) were different crystalline forms of the same compound, and if this is so it should be possible to interconvert them. However, attempts to do so were unsuccessful. Thus, when a hot saturated solution of (A), m.p. 188°, in ethanol was cooled and seeded with a crystal of (B), m.p. 168°, the only compound isolated had an m.p. 188°. From a similar experiment in which a solution of (B) was seeded with (A) the only product isolated had m.p. 168°.

IIIB_3b From N-ethoxycarbonyl-β-methylaspartic acid

The same sequence of reactions as described in Section IIIA_3b, except that N-ethoxycarbonyl-β-methylaspartic acid was used instead of the N-benzylxycarbonyl derivative, were studied. This had the advantage that during the final hydrogenolysis stage the blocking group is not removed from the amino group. Briefly the reaction sequence is:
No serious experimental difficulties were encountered with this route; however, it was not found possible to purify N-ethoxycarbonyl-erythro-3-methylaspartic anhydride, since it was readily hydrolysed to the corresponding acid, even under conditions in which the threo-isomer was stable.

The results obtained with each racemic diastereoisomer are summarised in the table below:
<table>
<thead>
<tr>
<th>Compound</th>
<th>Threo-isomer</th>
<th>Erythro-isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% yield m.p.</td>
<td>% yield m.p.</td>
</tr>
<tr>
<td>β-methylaspartic acid</td>
<td>240-245°</td>
<td>233°</td>
</tr>
<tr>
<td>N-ethoxycarbonyl-β-methylaspartic</td>
<td>90 154-156°</td>
<td>80 110-112°</td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-ethoxycarbonyl-β-methylaspartic</td>
<td>90 127-129°</td>
<td>not purified</td>
</tr>
<tr>
<td>anhydride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-benzyl β-hydrogen N-ethoxycarbonyl</td>
<td>80 80°</td>
<td>80 oil</td>
</tr>
<tr>
<td>carbonyl-β-methylaspartate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-benzyl N-ethoxycarbonyl-β-</td>
<td>70 135-137°</td>
<td>85 125-127°</td>
</tr>
<tr>
<td>methylasparagaine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-ethoxycarbonyl-β-methylasparagine</td>
<td>67 146°</td>
<td>75 188°</td>
</tr>
</tbody>
</table>

The overall yield of N-ethoxycarbonyl-threo-β-methylasparagine from threo-β-methylaspartic acid was 30% compared with the 39% yield obtained with the erythro-isomer.

The threo-isomer obtained by this route had the same melting-point and infra-red spectrum as the product obtained from threo-β-methylasparagine. The erythro-isomer, m.p.188°, had the identical infra-red spectrum to the compounds having m.p. 188° and 168°, prepared from erythro-β-methylasparagine, and it gave the correct elemental analysis for N-ethoxycarbonyl-β-methylasparagine.
Summary

Of the two routes investigated for the synthesis of N-ethoxycarbonyl-threo-β-methylasparagine neither route offered any real advantage over the other. Thus, N-ethoxycarbonyl-threo-β-methylasparagine was obtained from N-ethoxycarbonyl-threo-ô-methylaspartic acid and from threo-β-methylasparagine in yields of 38 and 74% respectively. The overall yield from threo-β-methylaspartic acid was 30% and 28% respectively.

However, the preparation of N-ethoxycarbonyl-erythro-β-methylasparagine from N-ethoxycarbonyl-erythro-β-methylaspartic acid was more satisfactory than from erythro-β-methylasparagine, since the former method was quicker and gave an improved yield. This method gave N-ethoxycarbonyl-erythro-β-methylasparagine in 44% yield as compared with 70% from erythro-β-methylasparagine. The overall yield from erythro-β-methylaspartic acid was 39% and 27% respectively.
Comparison of the physical properties of the β-methylaspartic acid derivatives

Solubility.

In general, the derivatives of threo-β-methylaspartic acid are less soluble in a given solvent than are those of the corresponding erythro-isomer; for example, N-phthaloyl-(±)-threo-β-methylaspartic acid is less soluble in water than is the erythro-isomer and similarly threo-β-methylasparagine is less soluble in aqueous ethanol than the erythro-compound. However, one exception to this generalisation has so far been found, namely the N-ethoxycarbonyl-β-methylasparagines. In this case the threo-isomer is considerably more soluble in ethanol, in water, or in ethyl acetate, than is the erythro-compound.

It is of interest that Y. Liwschitz et. al. [105] reported that erythro-β-hydroxyaspartic acid was more soluble in water than the corresponding threo-isomer. Singerman and Liwschitz [106] reported that the N-alkyl or N-aralkyl erythro-β-hydroxy-DL-aspartic acid(C) are more soluble in aqueous ethanol than the corresponding threo-isomers.
A literature search has shown that few erythro and threo pairs of compounds of comparable structure have been prepared; therefore, no generalisations can be made.

The observed differences in the solubility of the derivatives of erythro and threo-β-methyaspartic acid indicate that the solute-solvent intermolecular bonds are formed more readily with the compounds with the erythro configuration than with compounds with the threo configuration. Possible explanations for the differences in solubility are discussed in Section IIIB.4b.

Two other interesting points arising from the solubility studies are discussed below:

The internal anhydrides of the threo derivatives were less susceptible to hydrolysis by traces of water in solvents than were the erythro isomers. For example, N-ethoxycarbonyl-threo-β-methyaspartic anhydride could be recrystallised from benzene; however, the erythro-isomer with the same batch of
solvent gave the acid. The difference in the stability of the anhydride ring of the erythro and threo compounds, with respect to hydrolysis, is probably due to differences in the sum of the intramolecular interactions in the racemic diastereoisomers. The racemic diastereoisomeric internal anhydrides will exist in the eclipsed conformation, one enantiomorph of each is shown in (A) and (B):

In the erythro molecule there is steric interaction between RNH- and CH₃- and between two hydrogen atoms. Whereas in the threo molecule there is steric interaction between RNH- and H, and between H and CH₃-. The total steric interactions will be greater for the former than the latter [102], and consequently it is expected that the internal anhydride with the erythro configuration will be under greater strain.
than the corresponding threo molecule. Hence, the anhydrides with the erythro configuration will be more susceptible to hydrolysis than the corresponding threo isomers.

Although threo- and erythro-\(\beta\)-methylaspartic acids can be separated from each other by fractional recrystallisation attempts to separate them on a paper chromatogram, with Whatman No.1 paper and butanol-acetic acid-water (2:1:1) or phenol-water as the eluting phase, were unsuccessful. Similarly, the threo- and erythro-\(\beta\)-methylasparagines may be separated from each other by fractional recrystallisation from aqueous ethanol, but attempts to separate them on a paper chromatogram, under conditions similar to those described for the \(\beta\)-methylaspartic acids, were unsuccessful. This is very interesting in view of the fact that each of the threo compounds is considerably less soluble in aqueous solvents than the corresponding erythro-isomer.

IIIb Melting-points.

The melting-points of the derivatives of threo-\(\beta\)-methylaspartic acid are, in general, higher than those of the corresponding erythro-isomer, as shown in the table below.
Comparison of melting-points of derivatives of threo and erythro-6-methylespartic acid

<table>
<thead>
<tr>
<th>RR¹N-CHCOR²</th>
<th>CH₃-CHOOR³</th>
<th>Melting-points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythro</td>
<td>Three</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₂H₅OCO</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₂H₅OCO</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₂H₅OCO</td>
<td>H</td>
<td>OCH₂C₆H₅ OH</td>
</tr>
<tr>
<td>C₂H₅OCO</td>
<td>H</td>
<td>OCH₂C₆H₅ NH₂</td>
</tr>
<tr>
<td>C₂H₅OCO</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₆H₅CH₂OCO</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₆H₅CH₂OCO</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>C₆H₅CH₂OCO</td>
<td>H</td>
<td>OCH₂C₆H₅ OH</td>
</tr>
<tr>
<td>C₆H₅CH₂OCO</td>
<td>H</td>
<td>OCH₂C₆H₅ NH₂</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH₂</td>
</tr>
</tbody>
</table>
A similar trend in melting-points can be seen in the work of Singerman and Liwschitz [106]. They found that, in general, the N-alkyl or N-aralkyl-erythro-β-hydroxyaspartic acids had higher melting-points than the corresponding threo-compounds; whereas the converse is true for the corresponding β-hydroxyaspartic acids.

A comparison of the melting-points of the derivatives of threo- and erythro-β-methyelaspartic acid, and the solubility studies on these derivatives indicates that, in general, compounds with the threo configuration form stronger intermolecular bonds than the corresponding erythro-isomer. The reasons for this difference in intermolecular bond strength is probably due to differences in polar interactions such as hydrogen bonding, rather than to differences in direct steric interactions. The different ways by which polar interactions could affect the intermolecular bonding energies are discussed below.

The spatial arrangement within the crystal lattice for molecules with the threo configuration may favour stronger intermolecular bonding than for the erythro molecules. However, there is no evidence to date to support this hypothesis.

A more probable explanation is that the difference in intermolecular bonding energy is due to a difference in the degree of intramolecular bonding, in particular hydrogen
bonding. That is, the stronger the intramolecular hydrogen bonding the weaker the intermolecular hydrogen bonding since the participating groups will be less readily available to enter into intermolecular bond formation. On this hypothesis the intramolecular hydrogen bonding should be strongest for the derivatives with the erythro configuration.

\[ R'\text{CO} \cdot \text{CH(NHCOR'')} \cdot \text{CH(CH}_3\text{)COR''} \]

For the compounds prepared in the present investigation the groups capable of entering into hydrogen bonding are the two carboxyl groups \((R' = R''' = \text{OH})\), the \(\alpha\)-benzyl ester \((R' = \text{OCH}_2\text{C}_6\text{H}_5)\), the \(\beta\)-amide \((R''' = \text{NH}_2)\) and the carbamate group \((R'' = \text{OCH}_2\text{C}_6\text{H}_5 \text{ or } R'' = \text{OC}_2\text{H}_5)\). If it is assumed that intramolecular hydrogen bonding occurs between the two carboxyl groups or their derivatives, then from a consideration of steric interaction the compound with the threo configuration would be more stable since the steric interactions will be minimised[102].
However, if the intramolecular hydrogen bonding occurs between the carbamate group and the \( \beta \)-carboxyl or \( \beta \)-amide group, compounds with the \textit{erythro} configuration will be favoured on the grounds that steric interactions will be minimised with this configuration.

Similarly, considering the free amino acids the important intramolecular polar interaction will be between the amino group and the \( \beta \)-carboxyl group, possible after internal salt formation, as was found with aspartic acid [107].
Again, intramolecular steric interactions will be less with the erythro configuration rather than with the threo-isomer. Hence, the erythro-isomer will form the stronger internal salt, but possibly the weaker intermolecular bond. Therefore, the racemic acid with the threo configuration would be expected to have the higher melting-point and to be less soluble in polar solvents, as was observed.

The fact that N-ethoxycarbonyl-threo-β-methy Lasparagine has a lower melting-point and is more soluble in polar solvents than the corresponding erythro compound cannot be explained by assuming that intramolecular hydrogen bonding between the carbamate group and the β-amide is the important polar interaction. However, for this compound it is likely that strong intermolecular hydrogen bonding will occur between the α-carboxyl and the β-amide and for this system the threo-isomer would be expected to form the stronger intramolecular hydrogen bond since steric interaction is minimised and hence the weaker intermolecular hydrogen bond. Therefore, on this basis, N-ethoxycarbonyl-threo-β-methy Lasparagine might be expected to have a lower melting-point and a greater solubility in polar solvents than the corresponding erythro-isomer.
It is also interesting to note that as the two diastereoisomeric β-methylaspartic acids become more heavily substituted, so the difference in melting-point of the erythro- and threo-isomers decreased. This indicates that as the molecule became more heavily substituted so the intra and intermolecular polar interactions become less significant and the steric interactions become more significant. Moreover, the difference in intramolecular steric interactions for the two diastereoisomers will decrease as the molecule becomes more heavily substituted. Therefore, the difference in the melting-points of the two diastereoisomers might be expected to decrease, as was indeed observed. Similarly, the differences in the solubility might be expected to decrease; however, from qualitative observations this would not appear to be the case, the threo-isomers being considerably less soluble in a
given solvent than the corresponding erythro compound. However, as substitution increased so the polarity of the solvent required decreased.

Further information on the relative structures of the two diastereoisomeric series could be obtained from crystallographic studies, but this would be a difficult and tedious process. A more expedient method may be to study the infra-red spectra (high resolution) of the compounds, both in solution and in a mull; thereby it may be possible to determine the nature of the hydrogen bonding, if present.

IIIB.4c Infra-red spectra

The infra-red spectra of the threo- and erythro-β-methylaspartic acid derivatives (see Appendix I)* were studied with a view to correlating a band or bands with the threo or erythro configuration, and/or to find a general trend in the spectra which was characteristic of a particular configuration. For example, were the carbonyl bands of the threo-isomers consistently higher or lower than those for the corresponding erythro-isomers? However, although there were pronounced

* Only representative infra-red spectra are given in the Appendix. These are not the originals which unfortunately disappeared.
differences in the spectra of the two diastereoisomeric series of compounds, it was not possible to make any generalisations about them. Similarly, Singerman and Liwschitz were unable to correlate any bands with configuration from a study of the spectra of \( \text{N-substituted threo- and erythro-}\beta\text{-hydroxyaspartic acids.} \) The one generalisation they were able to make was only applicable within the series of compounds studied and could not be extended to different functional groups.

Although little or no information concerning the configuration of the diastereoisomeric \( \beta\)-methylaspartic acid derivations could be obtained from a study of the spectra, it was possible to assign some of the absorption bands to various functional groups. For example, \( \text{threo- and erythro-}\beta\text{-methylaspartic acid absorbed at } 3100 \text{ cm}^{-1} \) which is characteristic of the \( \text{NH}_3^+ \)-group arising from internal salt formation. Like other amino acids they did not absorb at \( 3500-3200 \text{ cm}^{-1} \), which is characteristic of the amino group (\( \text{NH}_2^- \)) [103,104]. As expected the absorption in the region \( 3120-3020 \text{ cm}^{-1} \), characteristic of internal salt formation (\( \text{NH}_3^+ \)) [103], was absent in all those compounds which had \( \text{N-substituents} \).
The \( \text{N-ethoxycarbonyl} \) and \( \text{N-benzyloxycarbonyl} \) derivatives have an absorption band between 1720-1700 cm\(^{-1}\) which can be assigned to the carbonyl stretch of the carbamate group. Also those compounds having a free unionised carboxyl group absorb at 1700 cm\(^{-1}\) approx.

However, the only general comment that can be made from a study of the infra-red spectra is that we were unable to correlate any of the differences observed between the spectra of two diastereoisomers with differences in physical properties. Moreover, the spectra were too complex to observe hydrogen bonding.
SECTION IV

SYNTHESIS AND PROPERTIES OF $\pm$-CIS- AND TRANS-
4,5-DIHYDRO-5-METHYLOROTIC ACID

IVA Cyclisation of N-Ethoxycarbonyl-$\pm$-threo- and erythro-$\pm$-
methylasparagine

IVB Conversion of 5-Methylorotic Acid into 4,5-Dihydro-5-
methylorotic acid

IVB Survey of methods available

IVB Chemical reduction of 5-methylorotic acid

IVB Catalytic hydrogenation of 5-methylorotic acid

IVB2 Present investigation

IVB2a Chemical reduction of 5-methylorotic acid

IVB2b Catalytic hydrogenation of 5-methylorotic acid

IVB2c Preparation of 5-methylorotic acid

IVC Attempted Methylation of 4,5-Dihydro-orotic Acid

IVD Studies on the Interconversion of Cis- and Trans-
4,5-dihydro-5-methylorotic Acids

IVE Paper Chromatographic Analysis of "4,5-Dihydro-orotic Acids"

IVF Comparison of the Physical Properties of $\pm$-Cis and

Trans-4,5-dihydro-5-methylorotic Acids

IVF Absorption spectra
SYNTHESIS OF (±)-CIS AND TRANS-4,5-DIHYDRO-5-METHYLOROTIC ACID

The routes studied for the preparation of each of the two racemic diastereoisomeric 4,5-dihydro-5-methylorotic acids are considered below under the following headings:

(i) Cyclisation of N-ethoxycarbonyl-(±)-threo- and erythro-β-methylasparagine.

(ii) Conversion of 5-methylorotic acid into 4,5-dihydro-5-methylorotic acid.

(iii) Substitution of 4,5-dihydro-orotic acid.

IVA CYCLISATION OF N-ETHOXYCARBONYL-(±)-THREO- AND ERYTHRO-
β-METHYLASPARAGINE

The preparation of dihydro-orotic acid by the cyclisation of N-ethoxycarbonylasparagine in ethanol with sodium ethoxide as catalyst, has already been discussed (see Section IIIB2).

\[
\begin{align*}
\text{CONH}_2 \\
\text{CH}_2 \\
\text{CHNHCO.C}_2\text{H}_5 \quad \text{(i) NaOC}_2\text{H}_5 \\
\text{CO}_2\text{H}
\end{align*}
\]

\[
\begin{align*}
\text{H}^+ \quad \text{(ii) H}^+ \\
\text{H}_2\text{C} \\
\text{CH} \\
\text{CO}_2\text{H}
\end{align*}
\]

If N-ethoxycarbonyl-\(L\)-asparagine is used \(L\)-dihydro-orotic acid is obtained [29], racemisation not occurring. A method
analogous to this would be attractive for the synthesis of the 4,5-dihydro-5-methylorotic acid, since thereby an authentic specimen of each of the four 4,5-dihydro-5-methylorotic acids might be obtained from the appropriate N-ethoxycarbonyl-β-methylasparagine. The results obtained from the study of the cyclisation are discussed herewith.

In the first instance preliminary studies were carried out with N-ethoxycarbonyl-DL-asparagine rather than with the β-methyl analogue for two reasons:

(a) N-ethoxycarbonylasparagine is readily available, whereas the two diastereoisomeric N-ethoxycarbonyl-β-methylasparagines are obtained in relatively poor yields from multi-stage syntheses. Thus, it was essential that the β-methylasparagine derivatives were conserved and not sacrificed on establishing suitable conditions for the reaction.

(b) A quantity of dihydro-orotic acid was required for a study of its substitution reactions (see Section IVD), and also as a substrate for kinetic studies with the enzyme dihydro-orotic dehydrogenase.

The interaction of N-ethoxycarbonylasparagine and sodium ethoxide in boiling absolute ethanol, gave a white gelatinous precipitate. Addition of a slight excess of hydrochloric acid to a solution of the gelatinous precipitate in water furnished
4,5-dihydro-orotic acid, m.p. 265-267° (decomp.), in yields of 60-65%. Miller, Gordon, and Engelhardt [29] reported that they obtained dihydro-orotic acid in a 70% yield by this method. We found that if the gelatinous precipitate was added to water containing a slight excess of hydrochloric acid, rather than acid being added to an aqueous solution of the precipitate, the yield of dihydro-orotic acid increased to 80-82%.

Similar experiments with N-ethoxycarbonyl-threo-β-methylasparagine (I) gave cis-4,5-dihydro-5-methylorotic acid (II), m.p. 252-254° (decomp.), in 39% yield.

![Chemical structure](image)

Analogous experiments with the erythro isomer in some cases furnished, as well as the expected trans isomer, the cis isomer. Thus, when acid was added to an aqueous solution of the gelatinous product obtained from the interaction of N-ethoxycarbonyl-erythro-β-methylasparagine (m.p. 168-188°); see
Section IIIB\textsubscript{3a}) (III) and sodium ethoxide, a mixture of trans-4,5-dihydro-5-methylorotic acid (IV), m.p. 220° (decomp.), in 20-25% yield, and the cis-isomer, m.p. 252-254° (decomp.), in 10-15% yield, was obtained. However, if the gelatinous product was added to water containing a slight excess of hydrochloric acid trans-4,5-dihydro-5-methylorotic acid (IV) was obtained in yields of up to 34%, the amount of the cis-isomer being reduced to between 0-4%.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {CONH$_2$};
\node at (1.2,0) {H-C - CH$_3$};
\node at (2.4,0) {H-C - NHCO.C$_2$H$_5$};
\node at (3.6,0) {CO$_2$H};
\node at (4.8,0) {(III)};
\node at (5.6,0.5) {(i) NaOC$_2$H$_5$};
\node at (6.8,0.5) {(ii) H$^+$};
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node at (0,0) {CO$_2$H};
\node at (1.2,0) {C = C - N = C - C = O};
\node at (2.4,0) {CH$_3$ - C - H - H};
\node at (3.6,0) {C - C - C - C};
\node at (4.8,0) {H - H - N - H};
\node at (6,0) {CO$_2$H};
\node at (7.2,0) {(IV)};
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node at (0,0) {CO$_2$H};
\node at (1.2,0) {C = C - C - C = O};
\node at (2.4,0) {CH$_3$ - C - H - H};
\node at (3.6,0) {C - C - C - C};
\node at (4.8,0) {H - H - N - H};
\node at (6,0) {CO$_2$H};
\node at (7.2,0) {(II)};
\end{tikzpicture}
\end{center}
CONVERSION OF 5-METHYLOROTIC ACID INTO 4,5-DIHYDRO-5-
METHYLOROTIC ACID

IVB1 Survey of methods available

Possible methods for the conversion of 5-methylorotic acid into the 4,5-dihydro derivative are considered below under the following headings: (a) Chemical reduction of 5-methylorotic acid; (b) Catalytic hydrogenation of 5-methylorotic acid.

IVB1a Chemical reduction of 5-methylorotic acid

The chemical reduction of simple pyrimidines has been studied [76] and it has been found that, if reaction occurs, the tetrahydro derivative is generally obtained. For example, 5-ethyl-1,4,5,6-tetrahydro-2-hydroxy-5-phenylpyrimidine (VI) was obtained by the reduction of 5-ethyl-5-phenyl-barbituric acid (V) with lithium aluminum hydride [76]. However, in 1955,

\[
\begin{align*}
\text{V} & \quad \rightarrow \quad \text{VI}
\end{align*}
\]
Burk [77] found that the pyrimidine units of nucleosides were reduced to the dihydro derivatives by the action of sodium with ethanol in liquid ammonia. Moreover, in 1965, the reduction of orotic acid to dihydro-orotic acid with sodium amalgam in aqueous ammonia was reported in a Japanese patent [75].

In the present investigation the reduction of 5-methyl-orotic acid with sodium amalgam in aqueous ammonia was studied, and the results obtained are discussed in Section IVB2a.

IVB1b Catalytic hydrogenation of 5-methylorotic acid

The main problem encountered in the hydrogenation of pyrimidines to dihydropyrimidines is that the reaction often proceeds one stage further to yield the tetrahydro derivative. However, in 1912, Levene and La Forge [78] reduced the uracil portion of uridine to the dihydrouracil derivative with colloidal palladium as catalyst; later, Brown and Johnson [35], in 1923, reported that uracil itself was reduced to dihydrouracil with colloidal palladium or platinum as catalyst. Moreover, Adams catalyst [36], 5% rhodium on alumina [37,79], and Raney nickel [38] have each been used as catalysts for the conversion of uracil into dihydrouracil. Adams catalyst [12,39], and 5% rhodium on alumina [40] have also been used as catalysts for the conversion of orotic acid into dihydro-orotic acid.
Furthermore, the reduction of 5-methylorotic acid to the corresponding dihydro-derivative with 5% rhodium on alumina as catalyst has been reported [9], but no physical constants were reported for the compound obtained.

Therefore, from a survey of the literature it would appear that there are three catalysts - Adams catalyst, Raney nickel, and 5% rhodium on alumina - which might be suitable to effect the catalytic hydrogenation of 5-methylorotic acid. However, the choice of catalyst was limited because of the following experimental difficulties:

(i) 5-Methylorotic acid is only sparingly soluble in ethanol or in glacial acetic acid, two solvents commonly used as reaction media for catalytic reduction with Adams catalyst or Raney nickel. 5-Methylorotic acid is, however, more soluble in water, the medium which is generally used with 5% rhodium on alumina, but which is unsuitable for other catalysts.

(ii) Since a relatively large quantity of the dihydro-derivative was required, it was desirable to use as concentrated a solution as possible - thus water was the most suitable reaction medium.

(iii) The reduction of orotic acid with Adams catalyst required a reaction pressure of 3 to 4 atmospheres of hydrogen or a reaction temperature of 80°, depending on whether ethanol
or glacial acetic acid was used as the reaction medium. It was not possible to achieve either of these conditions with the apparatus available, which was designed for use at room temperature and pressure.

Therefore, experiments were carried out with 5% rhodium on alumina as catalyst, the conditions being essentially as described by Green and Cohen [37], and the results obtained are discussed in Section IVB2b.

IVB2  Present investigation

In the first instance the conversion of orotic acid into dihydro-orotic was studied. The extension of this work to 5-methylorotic acid entailed the preparation of this compound which is described in Section IVB2c.

IVB2a  Chemical reduction of 5-methylorotic acid

The chemical reduction of 5-methylorotic acid was studied in an attempt to obtain the cis- and/or the trans 4,5-dihydro-5-methylorotic acid, since it was found that the catalytic hydrogenation of 5-methylorotic acid gave the cis-isomer only.

From the attempted reduction of orotic acid with 3% sodium amalgam in aqueous ammonia at room temperature, the only
product isolated was carbamoylaspartic acid, which had m.p. 178° and was obtained in a 65% yield. Thus, it is possible that under these conditions dihydro-orotic acid is formed, but is unstable, as reported by Janion and Shugar [40]. A similar reaction, carried out at -10°, furnished a mixture of dihydro-orotic acid, orotic acid, and carbamoylaspartic acid, as shown by infra-red, ultra-violet, and paper chromatographic analysis (see Section IVB). All attempts to isolate pure dihydro-orotic acid from the reaction mixture by fractional recrystallisation from water or glacial acetic acid were unsuccessful. An analogous reaction, at -10°, with 5-methylorotic acid furnished cis-4,5-dihydro-5-methylorotic acid, m.p. 252-254° (decomp.), in 45% yield. Paper chromatographic analysis (see Section IVB) of the reaction medium showed that neither trans-4,5-dihydro-5-methylorotic acid nor N-carbamoyl-β-methylaspartic acid were present. The indication is that the cis-4,5-dihydro-5-methylorotic acid is rather more stable to strong alkali than is 4,5-dihydro-orotic acid itself.

The reduction of orotic acid was also attempted under acid conditions so as to avoid the exposure of the dihydro-orotic acid formed to strong alkali. 3% Sodium amalgam was added to a solution of dihydro-orotic acid in glacial acetic acid at room temperature. However, only orotic acid (90% recovery) was isolated.
Catalytic hydrogenation of 5-methylorotic acid

Preliminary experiments were carried out with orotic acid, which is commercially available to establish reaction conditions for the less readily available 5-methylorotic acid. Dihydro-orotic acid, m.p. 265-267° (decomp.), was obtained in 62% yield by the hydrogenation of orotic acid, with 5% rhodium on alumina as catalyst (see Section IVB1b). Spectrophotometric analysis of the reaction medium at 282 m\(\mu\), after hydrogen uptake had ceased, confirmed that the reaction had gone to completion. Furthermore, it was found that the rate of reduction was approximately the same whether an aqueous solution of orotic acid, or an aqueous suspension of it, was used. This was particularly useful since orotic acid is relatively insoluble in water.

Similar experiments with 5-methylorotic acid furnished cis-4,5-dihydro-5-methylorotic acid, m.p. 252-254° (decomp.), in 59% yield. The rate of uptake of hydrogen with 5-methylorotic acid was approximately 70% of that with orotic acid under similar conditions. Furthermore, spectrophotometric analysis of the reaction medium, after hydrogen uptake had ceased, showed that approximately 90% of the 5-methylorotic acid had been reduced to the dihydro derivative. Paper chromatographic analysis (see Section IVB) of the reaction medium established
that the trans isomer was not present.

IVB₂c Preparation of 5-methylorotic acid

The synthesis of 5-methylorotic acid (IX) was first accomplished by Johnson [80] in 1907, by the condensation of S-methyl-thiourea hydrogen iodide (VII) with ethyl oxaloproponate (VIII) in the presence of potassium hydroxide, and subsequent treatment of the product with hydrochloric acid.

\[
\begin{align*}
\text{NH}_2 & \quad \text{C-S-CH}_3 \quad \text{I}^- \\
\text{NH}_2 & \quad \text{OH} \quad \text{OH},
\end{align*}
\]

(VII) (VIII) (IX)

In 1947 Nyc and Mitchell [45] found that the acid-catalysed condensation of urea (X) with ethyl oxaloacetate (XI) and subsequent hydrolysis with potassium hydroxide, furnished orotic acid (XII). Two years later Mentzer and Billet [81] reported the preparation of 5-methylorotic acid by a similar procedure, ethyl oxalopropionate (VIII) being used instead of ethyl oxaloacetate.
This method was used by Laursen and co-workers [82] in 1957, and also in the present investigation.

The condensation of urea and ethyl oxalopropionate in glacial acetic acid with dry hydrogen chloride furnished ethyl hydantoidene-2-propionate, which when heated with 1N-potassium hydroxide gave 5-methylorotic acid. We found that the length of time of heating ethyl hydantoidene-2-propionate with potassium hydroxide solution was critical; prolonged heating gave a product, m.p. 318° (decomp.), which contained 18.2% N as compared with 16.45% in 5-methylorotic acid. Apparently decarboxylation had occurred, since the impurity could be removed from an alkaline solution of the product, by extraction with ether. Addition of acid to the alkaline solution furnished 5-methylorotic acid, m.p. 326-327° (decomp.), in 28% yield, which may be compared with the 16% yield claimed by Laursen and co-workers [82], and 34% reported by Mentzer and Billet [81].

The 5-methylorotic acid separated from water in the anhydrous form, as shown by equivalent weight determinations, and
infra-red spectra of samples before and after drying under reduced pressure. However, Mentzer and Billet [81] reported that they obtained the monohydrate, m.p. 325-327°, and Johnston [80] reported that he obtained both the anhydrous and the monohydrated compounds, m.p. 328-330°, from water. Laursen and co-workers [82] did not comment as to whether the compound they isolated from water was hydrated or not; they gave the melting-point as 327.5-328.5° (decomp.).

The variation in the ultra-violet spectra of 5-methyl-orotic acid with variation in pH was studied and the results obtained are given in Appendix I. The observed variations in the spectra were similar to those observed with orotic acid under similar conditions [100].

From a study of the variation of the ultra-violet absorption spectra of uracil and its derivatives, with change in pH, Shugar and Fox [100] concluded that in acid or neutral conditions uracil existed in the keto form (XIII), but in alkaline conditions in the keto-enol (XIV) or enol (XV) form depending on the pH.
It would also appear from their work that thymine and orotic acid behave similarly. Therefore, it is probable that the observed variation in ultra-violet spectrum of 5-methylorotic acid with change in pH is also due to keto-enol tautomerism.

\[
\begin{align*}
\text{H} & \quad \text{O} \quad \text{N} \\
\text{C} & \quad \text{O} \quad \text{C} \quad \text{O} \\
\text{N} & \quad \text{C} \quad \text{O} \\
\text{CH}_3 & \quad \text{CO}_2^- \\
\text{OH}^- & \quad \text{H}^+ \\
\end{align*}
\]

**IVC ATTEMPTED METHYLATION OF 4,5-DIHYDRO-OROTIC ACID**

The preparation of the sodium salts of several 5-alkyl-dihydro-orotic acids by the reaction of the sodium salt of dihydro-orotic acid with sodium ethoxide, and subsequent addition of the alkyl bromide has been reported [34] (see Section IIIA). However, no other reactions of this type with dihydro-orotic acid have been reported, nor have any similar reactions with 5,6-dihydouracil. This method was investigated as a possible route for the preparation of at least one of the two racemic diastereoisomeric 4,5-dihydro-5-methylorotic acids, and the results obtained are discussed herewith.

Dihydro-orotic acid was treated with two equivalents of
sodium ethoxide in ethanol, followed by the addition of methyl iodide. A compound, m.p. 265-267° (decomp.) was isolated, which was shown to be dihydro-orotic acid, from its infra-red spectrum and by paper chromatographic analysis (see Section IV). The experiment was carried out five times under similar conditions, with the same batch of dihydro-orotic acid, of methyl iodide, and of solvent (ethanol). However, paper chromatographic analysis of the reaction products (see Section IV) showed that with only two of the experiments a trace of material was present which had the same $R_p$ (0.55) as cis-4,5-dihydro-5-methylorotic acid; from the other three experiments only dihydro-orotic acid was detected.

In view of the extremely low yield of cis-4,5-dihydro-5-methylorotic acid obtained this method was not further investigated.

**IVD STUDIES ON THE INTERCONVERSION OF CIS- AND TRANS-4,5-DIHYDRO-5-METHYLOROTIC ACIDS**

The mother liquors from the recrystallisation of crude trans-4,5-dihydro-5-methylorotic acid, obtained by the cyclisation of N-ethoxycarbonyl-erythro-β-methylasparagine (see Section IVA), were analysed paper chromatographically. The solution contained mainly the trans-isomer, only a trace of the
cis-isomer being present. The solution from one experiment was kept in a desiccator over concentrated sulphuric acid for one week. Analysis of the solution showed that the proportion of the cis-isomer to trans-isomer had increased. After a further three weeks the solution contained mainly the cis-isomer. All attempts to repeat this experiment, with mother liquors obtained from the recrystallisation of other samples of crude trans-4,5-dihydro-5-methylorotic acid containing a trace of the cis-isomer were unsuccessful.

Further experiments were carried out in which small amounts of the cis-isomer were added to an aqueous solution of the trans-isomer, but again no interconversion was observed. Moreover, there was no evidence for interconversion when either isomer was dissolved in aqueous sodium carbonate, sodium bicarbonate, or in 0.2M tris-(hydroxymethyl)-aminomethane (tris) buffer, at pH 8.0, and stored at 0°, 18°, or 37.4°C.

Although cis- and trans-4,5-dihydro-5-methylorotic acid are both hydrolysed to carbamoyl-β-methyaspartic acid in aqueous sodium hydroxide, it would appear that in the presence of hydroxyl ions the trans-isomer may be converted into the cis-isomer. Thus, in the preparation of trans-4,5-dihydro-5-methylorotic acid from the cyclisation of N-ethoxycarbonyl-erythro-β-methyasparagine with sodium ethoxide as catalyst
(see Section IVA), the product is isolated as the insoluble gelatinous sodium salt, which contains traces of sodium ethoxide; if this salt is dissolved in water and the free acid is then liberated by the addition of hydrochloric acid, a mixture of the cis- and trans-isomers is obtained. On the other hand, if the sodium salt is added to water containing a slight excess of hydrochloric acid, the only compound isolated is the trans-isomer; moreover, the yield obtained is approximately the same as the combined yield of cis- and trans-isomers obtained as described above.

**Interconversion of cis- and trans-4,5-dihydro-5-methylorotic acids.**

Interconversion of cis- and trans-4,5-dihydro-5-methylorotic acids could occur because of lability of either the hydrogen attached to the C-4 atom or that attached to the C-5 atom; in both cases the reaction would be expected to be base catalysed.

The structure at the C-4 atom can be compared to that of an α-amino-acid, or more correctly an α-imino-acid. α-Amino-acids racemise in the presence of nucleophilic reagents owing to loss of a proton from the α-carbon atom. With reference to 5-methyldihydro-orotic acid, removal of a proton gives the carbanion (A).
Subsequent addition of a proton to (A) could give either the trans- or cis-isomer. Under thermodynamic control the product will contain a predominance of the more stable isomer. Thus, there would be conversion of the trans-isomer into the cis-isomer. The hydrogen attached to the C-5 atom is α- to a carbonyl group and thus may be labile. Hence under thermodynamic control the less stable isomer will be converted into the more stable.
Graves and Vennesland [25] from enzymic studies with dihydro-orotic dehydrogenase in deuterated water found that a hydrogen atom attached to the C-4 or C-5 atom of 4,5-dihydro-orotic acid was labile. They concluded, without positive evidence, that it was the hydrogen attached to the C-5 atom that was labile. However, until further evidence is obtained the mechanism of interconversion must remain a matter of conjecture.

PIE PAPER CHROMATOGRAPHIC ANALYSIS OF "4,5-DIHYDRO-OROTIC ACIDS"

Fink [83] in 1956 described a method for detecting dihydrouracils on paper chromatograms; the paper was sprayed with sodium hydroxide solution and then with p-dimethylaminobenzaldehye. The sodium hydroxide solution hydrolyses the dihydrouracil to the corresponding carbamoyl derivative which gives a coloured reaction product with p-dimethylaminobenzaldehyde hydrochloride. Dihydro-orotic acid is also hydrolysed to the corresponding carbamoyl derivative by the action of sodium hydroxide [40] and may be detected by this method which should therefore be applicable to the detection of 5-alkyl-dihydro-orotic acids.

In general, the solvent systems used for developing amino-acids on paper chromatograms have been used for the paper
chromatographic analysis of dihydropyrimidines [83,40]. In this investigation one such solvent, n-butanol : acetic acid : water (2 : 1 : 1), was used as the developing phase. For this system, with Whatman No.1 paper, $R_F$ values of between 0.40 and 0.42 were obtained for dihydro-orotic acid, compared with 0.43 reported by Janion and Shugar [40]. With this system not only is it possible to separate dihydro-orotic acid from the 4,5-dihydro-5-methylorotic acids, but also to separate cis- and trans 4,5-dihydro-5-methylorotic acids. The trans-isomer was found to run slightly faster than the cis-isomer; for example, the trans-isomer travelled 23 cm. and the cis-isomer 22 cm. for a solvent front development of 40 cm., giving $R_F$ values of approximately 0.57 and 0.55 respectively. The $R_F$ values varied slightly from one experiment to another, since the temperature was not maintained constant throughout the development of the chromatogram. Both the cis- and the trans-isomer gave the same colour spot, a deep yellow, when the chromatograph was treated with sodium hydroxide and p-dimethylaminobenzaldehyde.

IVF COMPARISON OF THE PHYSICAL PROPERTIES OF (±)-CIS- AND TRANS-4,5-DIHYDRO-5-METHYLOROTIC ACIDS

IVF$_1$ Absorption spectra

Infra-red spectra

The infra-red spectra of the cis- and trans-4,5-dihydro-5-methylorotic acids (see Appendix I) were studied and compared
with that of dihydro-orotic acid [39]. The spectra are very complex and it is difficult to account for any of the minor differences between the cis- and trans-isomers. It is interesting to note that the spectrum of the cis-isomer more closely resembles that of dihydro-orotic acid than does that of the trans-isomer, the main difference being in 3,200 cm.\(^{-1}\) to 3,400 cm.\(^{-1}\) range as shown in the table below.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>dihydro-orotic acid cm.(^{-1})</th>
<th>cis-4,5-dihydro-5-methylorotic acid cm.(^{-1})</th>
<th>trans-4,5-dihydro-5-methylorotic acid cm.(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-NH-) stretch</td>
<td>3260</td>
<td>3200-3250</td>
<td>3300-3375</td>
</tr>
<tr>
<td>(-CO--; ring) stretch</td>
<td>1720</td>
<td>1720-1730</td>
<td>1720-1740</td>
</tr>
<tr>
<td>-CO- carboxylic acid</td>
<td>1660</td>
<td>1660</td>
<td>1660</td>
</tr>
</tbody>
</table>

It is also of interest to note that dihydro-orotic acid and cis-4,5-dihydro-5-methylorotic acid absorb at 2600 cm.\(^{-1}\) whereas the trans-isomer does not. This absorption is characteristic of salt formation (-NH\(^+\)). Moreover, from a consideration of the NH stretching frequencies it would appear that the NH group of the trans-isomer was subjected to less polar interactions, such as hydrogen bonding or salt formation, than was
the cis-isomer or dihydro-orotic acid itself. However, from steric considerations it is difficult to see why internal salt formation should not occur with the trans-isomer if indeed it occurs with the cis-isomer or dihydro-orotic acid. Moreover, it is possible that due to steric hindrance the carboxyl group of the trans-isomer was unable to enter into intramolecular polar interactions, whereas the carboxyl group of the cis-isomer and of dihydro-orotic acid, were. If this were so, then one would expect a difference in the carbonyl stretch frequency of the carboxylic acid group; however, all of the compounds absorb at the same wavelength 1660 cm.\(^{-1}\); thus indicating that the carboxyl group of all three compounds are in very similar environments. Neither dihydro-orotic acid [39] nor the two diaster-eoisomeric 5-methyl-dihydro-orotic acids exhibit any absorption at 3500-3400 cm.\(^{-1}\) which is characteristic of enolic -C\(=\)O.

Nujol mulls of the compounds were used to determine the infra-red spectra of the 4,5-dihydro-orotic acids since they are not soluble in the organic solvents (bromoform, carbon disulphide and carbon tetrachloride) normally used for determining infra-red spectra in solution. Therefore, it was not possible to obtain information about hydrogen bonding in the molecules by comparing the spectrum of a solution of the compound, in which hydrogen bonding would be absent, and the spectrum of a nujol mu!
of the compound, in which hydrogen bonding may take place. Also it is not possible to compare the absorptions of the CH$_3$-group in the two diastereoisomeric 5-methyl-dihydro-orotic acids.

**Ultra-violet spectra**

As expected, neither of the two diastereoisomeric 4,5-dihydro-5-methylorotic acids exhibited any characteristic absorption in the ultra-violet. However, just as dihydrouracil, dihydrothymine, and dihydro-orotic acid absorb at 230 m$\mu$ in the presence of hydroxyl ions [39,37], so do the two diastereoisomeric 4,5-dihydro-5-methylorotic acids. In neutral solution dihydrouracil, dihydrothymine and dihydro-orotic acid (B) exist in the keto form [39,37], but in the presence of hydroxyl ions enolisation can occur [39], a compound such as that shown in (C) being formed [40]. Therefore a conjugated double bond system is formed which gives rise to absorption at 230 m$\mu$.

![Diagram](image)
The absorption at 230 μ is transient because in the presence of hydroxyl ions dihydrouracil and substituted dihydrouracils are hydrolysed to the corresponding ureido-derivative. The mechanism of hydrolysis is unknown;

However, it is of interest to note that the rate of hydrolysis of dihydrouracil is approximately twice that of 5-methyl dihydrouracil (dihydrothymine) [39,37]. The fact that a slower rate of hydrolysis was observed for dihydrothymine was probably due to steric hindrance, and/or the inductive effect of the methyl group. From qualitative data obtained from the present investigation, it would appear that the relative rates of hydrolysis were cis-4,5-dihydro-5-methylorotic acid < 4,5-dihydro-orotic acid < trans-4,5-dihydro-5-methylorotic acid. Since the
trans-isomer is hydrolysed at a faster rate than the cis-isomer, the difference in rate of hydrolysis would appear to be due to a steric effect, since the inductive effect will be the same for both isomers.

The mechanism of hydrolysis is unknown; however, it is possible that hydrolysis occurs by the mechanism shown below, initial attack of hydroxyl ions at the C-6 atom of the dihydroorotic acid being the first step in the reaction.
SECTION V

5-FLUORO-4,5-DIHYDRO-CROTIC ACID

VA REVIEW OF POSSIBLE METHODS OF PREPARATION

VA1 Introduction

VA2 Hydrogenation of 5-fluoro-crotic acid

VA3 Cyclisation of N-ethoxycarbonyl-β-fluoro-asparagino

VA4 Halogenation of dihydro-crotic acid

VA5 Resume

VB ATTEMPTED PREPARATION OF 5-BROMO-DIHYDRO-CROTIC ACID
VA REVIEW OF POSSIBLE METHODS OF PREPARATION

VA 1 Introduction

5-Fluoro-4,5-dihydro-orotic acid has not been described in the literature, neither have the corresponding chloro- or bromo- compounds. However, the enzymic reduction of 5-fluoro-orotic acid has been reported [21]; but no attempt was made to isolate or characterise the product which was, presumably, the corresponding 4,5-dihydro-derivative.

Since there are no published methods for the preparation of 5-fluoro-dihydro-orotic acid, the methods studied for the preparation of the diastereoisomeric racemic 5-methyl-dihydro-orotic acids were considered with a view to adapting them for the preparation of the 5-fluoro derivative. Possible methods for the preparation are discussed under the three headings:

(1) Hydrogenation of 5-fluoro-orotic acid.
(2) Cyclisation of N-ethoxycarbonyl-ß-fluoro-asparagine.
(3) Halogenation of dihydro-orotic acid.

VA 2 Hydrogenation of 5-fluoro-orotic acid

5-Fluoro-orotic acid (III) was prepared by Duschinsky, Pleven and Heidelberger [34], in 1957, by the interaction of diethyl fluorooxalacetate (I) and S-ethyl thio urea (II).
The catalytic hydrogenation of 5-fluoro-orotic acid (III) has not been studied; however, the hydrogenation of 5-fluoro-uracil has been studied [84]. With palladium on charcoal as catalyst, only uracil was isolated; but with 5% rhodium on alumina as catalyst a mixture of products was obtained from which 5-fluoro-5,6-dihydouracil, in 6.5% yield, was isolated.

VA₃ Cyclisation of N-ethoxycarbonyl-β-fluoro-asparagine

Another possible route for the preparation of 5-fluorodihydro-orotic acid is the cyclisation of N-ethoxycarbonyl-β-fluoro-asparagine (IV) with sodium ethoxide as catalyst, by a method analogous to that described for the synthesis of 5-methylidihydro-orotic acid (see Section IVA₁).
Moreover, since it has been shown that the fluorine atoms of 5-trifluoromethyl-5-hydroxy-5,6-dihydrouracil [85] are stable to both methoxide ions and hydrochloric acid, the above route might profitably be investigated. Suitable derivatives of \( \beta \)-fluoro-aspartic acid are not known, neither is \( \beta \)-fluoro-aspartic acid itself. However, McBee et al. [86] have reported the synthesis of ethyl bromo-fluoro-acetate, from which \( \beta \)-fluoro-aspartic acid might be obtained by a method similar to that described for the synthesis of \( \beta \)-methyllaspartic acid (see Section IIIB_1).

**VA_4 Halogenation of dihydro-orotic acid**

A general method for the preparation of alkyl and aryl fluorides is by a halogen exchange reaction with the corresponding bromide or chloride. Therefore, it may be possible to prepare 5-fluoro-dihydro-orotic acid by a halogen exchange reaction with
either 5-bromo- or 5-chloro-dihydro-orotic acid. Although there have been no reports of the preparation of fluoro-dihydropyrimidines by a halogen exchange reaction, such reactions have been reported with non-hydrogenated pyrimidines [83,84]. For example, 2,4,6-trifluoropyrimididine was prepared by the reaction of silver fluoride with the corresponding trichloro-compound [83]. The application of this method to 5-fluoro-dihydro-orotic acid would require the synthesis of 5-bromo-dihydro-orotic acid. As mentioned earlier, 5-bromo-dihydro-orotic acid is unknown; however, 5-bromo-dihydrouracil has been obtained by the reaction of dihydrouracil with bromine [41,89,90], although the reaction with dihydrothymine furnished thymine only [91]. Therefore, it may be possible to obtain 5-bromo-dihydro-orotic acid by the direct bromination of dihydro-orotic acid and subsequently obtain from it by a halogen exchange reaction the corresponding fluoro compound. However, the product would most probably be a mixture of the two possible diastereoisomeric racemates.

Resume

Although the routes via the hydrogenation of 5-fluoro-orotic acid, and the cyclisation of N-ethoxycarbonyl-β-fluoro-asparagine were attractive because they would probably furnish 5-fluoro-4,5-dihydro-orotic acids of known configuration, they suffered from the disadvantage that the overall yields would
most probably be very low, and the cost of the starting materials was prohibitive. Therefore, the only route studied in this investigation was via the 5-bromo-dihydro-orotic acid, and the results obtained are described below.

**VB ATTEMPTED PREPARATION OF 5-BROMO-DIHYDRO-OROTIC ACID (V, VI)**

The treatment of dihydro-orotic acid with bromine and with N-bromosuccinimide was studied.

In preliminary experiments either a suspension or a solution of dihydro-orotic acid (V) in refluxing glacial acetic acid was treated with bromine; the only compounds isolated from the reaction mixture were orotic acid and dihydro-orotic acid, no brominated compounds being obtained.

![Chemical Structures](attachment:image.png)

The formation of orotic acid was probably due to the elimination of hydrogen bromide immediately after bromination had occurred. This would be analogous to the reaction of bromine with
dihydrothymine [91] from which the only product isolated was thymine. Therefore, attempts were made to carry out the reaction under milder conditions. No reaction occurred at room temperature, either with or without the addition of iron wire. Reaction did occur at 40-50° as shown by the discharge of the bromine colour from the solution; but again dihydroorotic acid and orotic acid were the only compounds isolated.

The treatment of dihydro-orotic acid with N-bromo-succinimide (VII) was also briefly studied. There was no apparent reaction when a solution of dihydro-orotic acid in glacial acetic acid containing a small quantity of acetic anhydride was treated with N-bromo-succinimide under conditions analogous to those described for the preparation of 5-bromo-4-methylaminopyrimidine from 4-methylaminopyrimidine [92], dihydroorotic acid being the only compound isolated from the reaction mixture.

No further work was carried out on the preparation of 5-bromo-dihydro-orotic acid.
SECTION VI

EXPERIMENTAL

VIA (±)-Threo- and Erythro-β-Methylaspartic acid
VIB (±)-Threo- and Erythro-β-Methylasparagine
VIC N-Ethoxycarbonyl-(±)-Threo- and Erythro-β-
Methylasparagine
VID Attempted Preparation of Glutamine from Glutamic acid
VIE (±)-Cis and Trans-4,5-Dihydro-5-methylorotic acid
VIF 4,5-Dihydro-orotic acid
VIG Attempted Preparation of 5-Bromo-4,5-dihydroorotic acid
GENERAL NOTES

(i) Microanalyses were carried out by Dr. A. Bernhardt, Max-Planck Institut, Mulheim, Germany.

(ii) Melting-points are corrected.
SECTION VIA

(±)-Threo- and Erythro-β-Methyaspartic acid

VIA₁ Preparation

VIA₂ Attempted conversion of (±)-threo-β-methyllaspartic acid into the (±)-erythro-isomer
VIA Preparation of (\textsuperscript{1})-Threo- and Erythro-\textbeta-Methylaspartic acid

A solution of sodium ethoxide (0.52 mole) in absolute ethanol (225 ml.) was added dropwise to a stirred, cooled solution of diethyl acetimidomalonate (99 g., 0.46 mole) in absolute ethanol (240 ml.). Ethyl-2-bromopropionate (120 g., 0.66 mole) was slowly added to the stirred solution and, after addition was complete, the mixture was stirred and boiled under reflux for 7 hours. The ethanol was distilled off under reduced pressure, the residue was dissolved in water (150 ml.) and the aqueous solution was extracted with ether (2 x 150 ml.). The combined ethereal extracts were washed with water (2 x 20 ml.) and the ether was distilled off under reduced pressure. Water (150 ml.) and concentrated hydrochloric acid (450 ml.) were added to the residue, and the mixture was boiled under reflux for 8 hours. The resulting solution was concentrated, under reduced pressure, to approximately 150 ml. Water (150 ml.) was added to the residue and the solution was again concentrated to 150 ml.; this operation was twice repeated. The residue was dissolved in ethanol (400 ml.), and aniline was added to the stirred solution until Congo Red paper was not turned blue when treated with the solution. The solution was kept at 2° for 24 hours; the solid (40 g., 59%) which separated was
filtered off, washed with ethanol, and with ether, and air-dried. Recrystallisation of the product from water yielded (+)-threo-β-methylaspartic acid (23 g.), m.p. 268-272° (decomp.). The mother liquor from the recrystallisation, after it had been concentrated under reduced pressure, furnished a further 3 g. of the (+)-threo-amino-acid. The addition of three volumes of ethanol to the mother liquor, after the removal of the threo-isomer, gave (+)-erythro-β-methylaspartic acid (13 g.), m.p. 250-255° (decomp.). An admixture of the (+)-erythro- and the threo-β-methylaspartic acid had m.p. 240° (decomp.).

A similar experiment was performed in which diethyl formamidomalonate (19 g., 0.09 mole) was used instead of the acetamidomalonate; the threo-amino-acid (1.9 g., 15%), m.p. 268-272° (decomp.) was isolated.

VIA2 Attempted conversion of (+)-threo-β-methylaspartic acid into the (+)-erythro isomer

A suspension of (+)-threo-β-methylaspartic acid (1.0 g.), m.p. 268-272° (decomp.) in pyridine (20 ml.) was boiled under reflux for 2 hours. After being kept at room temperature for 16 hours, the reaction mixture was filtered, and the solid (0.95 g.), m.p. 268-272° (decomp.), was washed with ether and
dried.

In a similar experiment when triethylamine was used instead of pyridine the amino-acid (0.93 g.) had m.p. 268-272° (decomp.).

No erythro-amino-acid was isolated from either experiment.
SECTION VIB

(±)-Threo- and Erythro-β-Methyasparagine

VIB₁ Preparation via N-phthaloyl-β-methyaspartic acid

VIB₂ Preparation via N-benzyloxycarbonyl-β-methyllaspartic acid.

VIB₃ Preparation via β-methyl α-hydrogen β-methyllaspartate
SECTION VIB

(±)-Threo- and Erythro-β-methylasparagine

Preparation via N-phthaloyl-β-methylaspartic acid

VIB 1a N-Phthaloyl-(±)-threo- and erythro-β-methylaspartic acid.

VIB 1a(i) Reaction of β-methylaspartic acid with phthalic anhydride

VIB 1a(ii) Reaction of β-methylaspartic acid with N-ethoxycarbonylphthalimide.

VIB 1b N-Phthaloyl-(±)-threo-β-methylaspartic anhydride

VIB 1c N-Phthaloyl-(±)-threo-β-methylasparagine hydrate

VIB 1d (±)-Threo-β-methylasparagine
VIIBla  \( N \)-Phthaloyl-(\( \dagger \))-threo- and erythro-\( \beta \)-methylaspartic acid

(i) Reaction of the \( \beta \)-methylaspartic acid with phthalic anhydride

Pyridine (30 ml.) was added to a finely powdered mixture of (\( \dagger \))-three-\( \beta \)-methylaspartic acid (1.5 g., 0.01 mole) and phthalic anhydride (1.5 g., 0.01 mole), and the mixture was boiled under reflux for 2 hours. Some insoluble material (A) was filtered off before the filtrate was concentrated under reduced pressure. The sticky residue was dissolved in the minimum quantity of hot water, and the resulting solution concentrated under reduced pressure. The residue was dissolved in hot water, and solution was cooled and 6\( N \)-hydrochloric acid was added to it until the solution was acid to Congo Red. The solution was kept at room temperature overnight and the crude \( N \)-phthaloyl-(\( \dagger \))-three-\( \beta \)-methylaspartic acid was filtered off. On being kept in a refrigerator for a further 24 hours the mother liquor yielded crystals of the (\( \dagger \))-eryth tho isomer. The \( N \)-phthaloyl-(\( \dagger \))-three-\( \beta \)-methylaspartic acid crystallised from water in prisms (0.67 g., 22%); it had m.p. 203-204°, softening at 199°. The more soluble \( N \)-phthaloyl-(\( \dagger \))-erythro-\( \beta \)-methylaspartic acid crystallised from water in needles (0.12 g., 4%); it had m.p. 188-189°.
The insoluble material (A; 0.45 g.), m.p. 268-272° (decomp.) did not depress the melting-point of an authentic sample of (±)-threo-β-methylaspartic acid, m.p. 268-272° (decomp.), and the infra-red spectra were identical; the quantity of the amino-acid recovered varied between 25-35% on several experiments.

(ii) Reaction of β-methylaspartic acid with N-ethoxy-carbonylphthalimide

To a stirred solution of (±)-threo-β-methylaspartic acid (3.675 g., 0.025 mole) and sodium carbonate (5.3 g., 0.05 mole) in water (35 ml.), which was cooled to 0°, was added N-ethoxy-carbonylphthalimide (5.625 g., 0.026 mole). The reaction mixture was stirred for 10 minutes at 0°, and for a further 60 minutes at room temperature. The solution was filtered, the filtrate was cooled in ice-water and was acidified (to pH 2) with cold concentrated hydrochloric acid. When the solution was kept in the refrigerator overnight crude N-phthaloyl-(±)-threo-β-methylaspartic acid (5.6 g.) separated; crystallisation from water gave prisms (4.8 g., 65%), m.p. 203-204°.

From a similar experiment in which the erythro isomer (1.2 g., 0.008 mole) was used N-phthaloyl-(±)-erythro-β-methylaspartic acid (0.3 g., 12.5%) was obtained; it separated from
water in needles, m.p. 189-190°.

\[ VIB_{1b} \quad \text{N-Phthaloyl-}(^+)-\text{threo-}\beta\text{-methylaspartic anhydride} \]

N-phthaloyl-\((^+)-\text{threo-}\beta\text{-methylaspartic acid (4.8 g., 0.027 mole) and acetic anhydride (10 ml.) were heated together on a steam-bath for 10 minutes. Removal of the acetic anhydride under reduced pressure, and recrystallisation of the residue from glacial acetic acid furnished N-phthaloyl-\((^+)-\text{threo-}\beta\text{-methylaspartic anhydride (4.2 g., 83%), m.p. 197-198°.} \]

\[ VIB_{1c} \quad \text{N-Phthaloyl-}(^+)-\text{threo-}\beta\text{-methylasparagine hydrate} \]

A cold saturated solution of ammonia in dry ether (600 ml.) was added to a cooled solution of N-phthaloyl-\((^+)-\text{threo-}\beta\text{-methylaspartic anhydride (4.2 g., 0.026 mole) in dry dioxan (80 ml.). The copious precipitate was filtered off and dissolved in the minimum quantity of water. The cooled solution was acidified with 6N-hydrochloric acid, and the precipitate was collected and recrystallised from aqueous ethanol. N-phthaloyl-\((^+)-\text{threo-}\beta\text{-methylasparagine hydrate (3.5 g., 73%), m.p. 142-145° (sintering at 132°) was obtained.} \]
VIB_1d (±)-Threo-β-methyasparagine

N-Phthaloyl-(±)-threo-β-methyasparagine hydrate (3.5 g, 0.019 mole) was dissolved in N sodium carbonate (16 ml.), and treated with hydrazine hydrate (1.0 g, 0.02 mole). The resulting solution was kept at room temperature overnight and then cold concentrated hydrochloric acid was added until the solution was acid to Congo Red. The solid was filtered off and washed with water; the mother liquor and washings were combined and the pH was adjusted to 7 by the addition of dilute aqueous ammonia. The solution was concentrated under reduced pressure, the temperature being kept below 45° to approximately 10 ml. Ethanol (30 ml.) was added to the residue and the solution was stored at 2° overnight. Recrystallisation of the crude solid from aqueous ethanol gave (±)-threo-β-methyasparagine (1.32 g, 28%); m.p. 240-245° (decomp.).
SECTION VIB₂

(±)-Threo- and Erythro-β-methylasparagine

Preparation via N-benzyloxy carbonyl-β-methylaspartic acid

VIB₂ᵃ  N-Benzyloxy carbonyl-(±)-threo- and erythro-β-methylaspartic acid

VIB₂ᵇ  α-Benzyl β-hydrogen N-benzyloxy carbonyl-(±)-threo- and erythro-β-methylaspartate

VIB₂ᶜ  α-Benzyl N-benzyloxy carbonyl-(±)-threo- and erythro-β-methylasparagine

VIB₂ᵈ  (±)-Threo- and erythro-β-methylasparagine
VI

Bga 2-Benzyloxyoai>l)onyl~(±)"toeo- and e r y t i i r o - - -( 3 -
methylaspartic acid

(±)-Threo-β-methylaspartic acid (4.4 g., 0.03 mole) was added to a suspension of magnesium oxide (2.5 g., 0.064 mole) in water (25 ml.) and treated with benzyl chloroformate (7.0 g., 0.041 mole). The benzyl chloroformate was added in small portions and the reaction mixture was shaken until all of the benzyl chloroformate had reacted. The reaction mixture was acidified to Congo Red with cold concentrated hydrochloric acid and the oil which separated was extracted with ethyl acetate (2 x 175 ml.). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate, filtered and concentrated to dryness under reduced pressure at room temperature. Recrystallisation, to constant melting-point, of the residue (8.3 g.), m.p. 138°, from ethyl acetate-light petroleum (b.p. 60-80°), gave N-benzyloxy carbonyl-(±)-threo-β-methylaspartic acid (8.0 g., 91%) m.p. 146-148°. It was soluble in hot water, but gave an oil on cooling, which solidified on standing in ice-water, m.p. 150°. (Found: C, 55.5; H, 5.15; N, 4.9. C_{13}H_{15}NO_{6} requires C, 55.5; H, 5.4; N, 5.0%).

From a similar experiment in which the (±)-erythro-isomer
(4.0 g., 0.027 mole) was used, an oil (6.9 g., 90%) was obtained which would not solidify even after prolonged drying under reduced pressure.

\[ \text{VIB}_{2b} \quad \alpha\text{-Benzyl } \beta\text{-hydrogen } N\text{-benzyloxy carbonyl-}(\ddagger)\text{-threo- and erythro- } \beta\text{-methylaspartate} \]

\[ N\text{-Benzyloxy carbonyl-}(\ddagger)\text{-threo- } \beta\text{-methylaspartic acid} \]
(8.0 g., 0.029 mole) and acetic anhydride (15 ml.) were heated together on a steam-bath for 15 minutes, the solution cooled and concentrated to dryness under reduced pressure. Benzyl alcohol (5.1 g., 0.047 mole) was added to the residue and the mixture heated on a steam-bath for 3½ hours. The solution was cooled, dissolved in ether and the product extracted with excess aqueous sodium carbonate. The aqueous extract was acidified to Congo Red by the addition of cold concentrated hydrochloric acid, and the product, which separated as an oil, extracted with ether (2 x 100 ml.). The combined ethereal extracts were dried over anhydrous sodium sulphate, filtered, and the ether removed from the filtrate by distillation. The oily residue eventually solidified after the addition of ethyl acetate (5 ml.), and the product was recrystallised from aqueous ethanol; yield (7.0 g., 66%), m.p. 120° (Found: C, 65.1; H, 5.5; N, 3.8. \( C_{29}H_{21}NO_6 \) requires C, 64.8; H, 5.7; N, 3.8%).
A similar experiment was carried out with the crude 
N-benzylxoxycarbonyl-(\(\pm\))-erythro-\(\beta\)-methyaspartic acid (6.9 g., 0.024 mole), except that the corresponding anhydride was isolated. Recrystallisation of the crude anhydride from dry chloroform, gave N-benzylxoxycarbonyl-(\(\pm\))-erythro-\(\beta\)-methyaspartic anhydride (6.5 g., 91%), m.p. 110-112°. The anhydride was converted into thea-benzyl ester as described for the corresponding threo-isomer, and recrystallisation of the product from ethyl acetate-light petroleum (b.p. 60-80°) gave a-benzyl-\(\beta\)-hydrogen N-benzylxoxycarbonyl-(\(\pm\))-erythro-\(\beta\)-methyaspartate (4.9 g., 75%), m.p. 110-112°.

VIB\(_2\)C a-Benzyl N-benzylxoxycarbonyl-(\(\pm\))-threo- and erythro-\(\beta\)-methyasparagine

Finely powdered phosphorus pentachloride (5.2 g., 0.025 mole) was shaken with an ice-cold solution of a-benzyl \(\beta\)-hydrogen N-benzylxoxycarbonyl-(\(\pm\))-threo-\(\beta\)-methyaspartate (7.0 g., 0.019 mole) in ether (70 ml.) until virtually all of it had dissolved. The ethereal solution was poured slowly into a stirred, ice-cold, 6N-aqueous ammonia solution (125 ml.), and the product was filtered off, washed with water and then dried in a vacuum over calcium chloride. Recrystallisation of the product from methanol gave a-benzyl N-benzylxoxycarbonyl-(\(\pm\))-
threo-β-methyasparagine (6.0 g., 85%), m.p. 166°. (Found; C, 64.5; H, 6.2; N, 7.4. C_{20}H_{22}N_{2}O_{5} requires C, 64.5; H, 6.0; N, 7.55%).

From a similar experiment in which the (+)-erythro isomer (4.9 g., 0.013 mole) was used, α-benzyl N-benzyloxy carbonyl-(+)-erythro-β-methyasparagine (3.9 g., 80%), m.p. 158-160° was obtained after recrystallisation from aqueous methanol.

VIB_{2d} (+)-threo- and erythro-β-methyasparagine

Palladium black (3.0 g., 0.028 mole) was added to a solution of α-benzyl N-benzyloxy carbonyl-(+)-threo-β-methyasparagine (6.0 g., 0.0162 mole) in methanol (150 ml.) and water (75 ml.) and the reactants were shaken in an atmosphere of hydrogen for 3 hours at atmospheric pressure, with occasional warming to dissolve the product. The solution was filtered and the residue washed with hot water (2 x 20 ml.). The filtrate and washings were combined and concentrated under reduced pressure to a final volume of approximately 15 ml. (the temperature being kept below 45°) and ethanol (50 ml.) was then added to the solution with stirring and the solution kept at 2° overnight. The crude product was filtered off and recrystallised
from aqueous ethanol; (+)-threo-3-methylasparagine (2.0 g., 85%), m.p. 240-245° (decomp.), was obtained (Found: C, 41.3; H, 7.0; N, 19.0 calculated for C_{5}H_{10}N_{2}O_{3}, C, 41.1; H, 6.85; N, 19.15%).

A similar experiment with the erythro isomer (3.9 g., 0.0106 mole) yielded (+)-erythro-3-methylasparagine hydrate (1.28 g., 80%), m.p. 233° (decomp.) (Found: C, 38.7; H, 7.3; N, 17.85. C_{5}H_{10}N_{2}O_{3} \cdot \frac{1}{2}H_{2}O requires C, 38.70; H, 7.15; N, 18.05%).
SECTION VIB

(±)-Threo- and Erythro-β-methylasparagine

Preparation via β-methyl α-hydrogen β-methylaspartate

(Preparation of (±)-threo-, and attempted preparation of (±)-erythro- β-methylasparagine)
Preparation of \((\pm)\)-threo-, and attempted preparation of \((\pm)\)-erythro-, \(\beta\)-methy lasparagine via \(\beta\)-methyl \(\alpha\)-hydrogen
\(\beta\)-methyl aspartate

Finely ground \((\pm)\)-threo-\(\beta\)-methyl aspartic acid (1.0 g., 0.0068 mole) was added to a solution of thionyl chloride (0.8 ml., 0.0108 mole) in methanol (5.0 ml.) at \(-10^\circ\), and the suspension shaken until all the \((\pm)\)-threo-\(\beta\)-methyl aspartic acid had dissolved. The solution was kept at room-temperature for 30 minutes, dry ether (25 ml.) was added and an oil separated which would not solidify. The ethereal solution was concentrated to dryness under reduced pressure, and 0.880 ammonia solution (10 ml.) was added to the residue, which was very deliquescent. The solution was kept at 2\(^\circ\) for 24 hours and then concentrated under reduced pressure to a final volume of approximately 4 ml. Ethanol (20 ml.) was added to the solution with stirring and the solution stored in a refrigerator overnight. The product was filtered off and recrystallisation from ethanol gave \((\pm)\)-threo-\(\beta\)-methy lasparagine (0.1 g., 10\%), m.p. 240-245\(^\circ\) (decomp.). The infra-red spectrum of a nujol mull of the compound was identical to that of an authentic sample of \((\pm)\)-threo-\(\beta\)-methy lasparagine. The compound was indistinguishable from authentic \((\pm)\)-threo-\(\beta\)-methy lasparagine when run on a whatman No.1 paper chromatogram with
n-butanol:acetic acid: water (2:1:1) as solvent, the chromatogram being developed with ninhydrin.

From a similar experiment with the erythro isomer (1.0 g., 0.0068 mole) a product (0.60 g., 60%) m.p. 235-240° (decomp.) was obtained. (Found: C, 36.45; H, 7.5; N, 16.95. C₅H₁₀N₂O₃.H₂O requires C, 36.6; H, 7.35; N, 17.05%). The infra-red spectrum of a nujol mull of the compound was different to that of an authentic sample of (1)-erythro-β-methyasparagine, or of the threo-isomer. The compound ran slightly faster than (1)-erythro-β-methyasparagine on a paper chromatogram, whatman No.1 paper, N-butanol:acetic acid:water as solvent, and gave a red-wine colour with ninhydrin as compared with the tan colour given by asparagine and the diastereomeric β-methyasparagines.
SECTION VIC

N-Ethoxycarbonyl-(\pm)-threo- and erythro-\beta-methyllasparagines

VIC₁ Preparation from (\pm)-threo- and erythro-\beta-methyl-

asparagines

VIC₃ Preparation via N-ethoxycarbonyl-(\pm)-threo- and erythro-

\beta-methyllaspartic acid.

(VIC₃a Attempted interconversion of low and high melting
specimens of N-ethoxycarbonyl-(\pm)-erythro-\beta-

methyllasparagines)
Preparation from (±)-threo- and erythro-β-methylasparagine

Ethyl chloroformate (0.82 g, 0.0076 mole) was added in small portions to a suspension of (±)-threo-β-methylasparagine (1.0 g, 0.0069 mole) and magnesium oxide (0.59 g, 0.015 mole) in water (10 ml.), and the mixture was shaken until all the ethyl chloroformate had reacted. The solution was acidified (to pH 3) by the addition of cold hydrochloric acid and then stored at 2° overnight. The product was filtered off, and recrystallisation from absolute ethanol gave N-ethoxycarbonyl-(±)-threo-β-methylasparagine (1.1 g, 74%), m.p. 144-146°. (Found: C, 44.1; H, 6.35; N, 13.05; C₈H₁₄N₂O₅ requires C, 44.05; H, 6.5; N, 12.8%).

From a similar experiment with (±)-erythro-β-methylasparagine, m.p. 233° (decomp.), (1.0 g, 0.0067 mole), N-ethoxycarbonyl-(±)-erythro-β-methylasparagine (1.04 g, 70%), m.p. 166-168°, was obtained. (Found: C, 44.1; H, 6.55; N, 12.8. C₈H₁₄N₂O₅ requires C, 44.05; H, 6.5; N, 12.85%).

A similar experiment was carried out with crude (±)-erythro-β-methylasparagine (1.0 g, 0.0067 mole), m.p. 230-233° (decomp.), obtained from hydrogenolysis of α-benzyl N-benzyloxycarbonyl(±)-erythro-β-methylasparagine, and used without
recrystallisation after its isolation. Recrystallisation of
the crude N-ethoxycarbonyl-(\textsuperscript{\textpm})-erythro-\textbeta-methylasparagine from
ethanol gave N-ethoxycarbonyl-(\textsuperscript{\textpm})-erythro-\textbeta-methylasparagine
(0.96 g., 65%), having m.p. 186-188°. (Found: C, 44.1; H, 6.6;
N, 12.9 \textsubscript{C}\textsubscript{8}H\textsubscript{14}N\textsubscript{2}O\textsubscript{5} requires C, 44.05; H, 6.5; N, 12.85%).

In the following sub-section (VIC\textsubscript{1a}) N-ethoxycarbonyl-
(\textsuperscript{\textpm})-erythro-\textbeta-methylasparagine having m.p. 166-168° is desig­
nated 'A', and that having m.p. 186-188° is designated 'B'.

VIC\textsubscript{1a} Attempted interconversion of the two N-ethoxycarbonyl-
(\textsuperscript{\textpm})-erythro-\textbeta-methylasparagines having m.p. 166-168°(A)
and 186-188°(B) respectively

Attempted conversion of 'A' into 'B'

(i) A hot saturated solution of 'A' in 96% ethanol was
cooled and seeded with a crystal of 'B'. The solution was kept
at 2° overnight and the crystals were filtered off; they had
m.p. 168°. Concentration of the mother liquor gave a crop of
crystals having m.p. 168°.

(ii) A small amount of activated palladium black in
aqueous methanol was added to a saturated solution of 'A' in
ethanol and the solution boiled under reflux for 30 minutes.
The solution was filtered, cooled and the crystals isolated; they had m.p. 168°.

An admixture of 'A' and 'B' had m.p. 168°.

**Attempted conversion of 'B' into 'A'**

From a similar experiment with a saturated solution of 'B' which was seeded with a crystal of 'A', crystals having m.p. 186-188° were isolated.
SECTION VIC₂

N-Ethoxycarbonyl-(±)-threo- and erythro-β-methyasparagine

Preparation via N-ethoxycarbonyl-(±)-threo- and erythro-β-methyaspartic acid

VIC₂ᵃ N-Ethoxycarbonyl-(±)-threo- and erythro-β-methyaspartic acid.

VIC₂ᵇ N-Ethoxycarbonyl-(±)-threo- and erythro-β-methyaspartic anhydride

VIC₂ᶜ α-Benzyl β-hydrogen N-ethoxycarbonyl-(±)-threo- and erythro-β-methyaspartate

VIC₂ᵈ α-Benzyl N-ethoxycarbonyl-(±)-threo- and erythro-β-methyasparagine

VIC₂ᵉ N-Ethoxycarbonyl-(±)-threo- and erythro-β-methyasparagine
VIC$_{2a}$ N-Ethoxycarbonyl-(+)-threo- and erythro-$\beta$-methylaspartic acid

Finely powdered (+)-threo-$\beta$-methylaspartic acid (4.0 g., 0.027 mole) was added to a suspension of magnesium oxide (2.1 g., 0.054 mole) in water (25 ml.), and ethyl chloroformate (3.8 g., 0.035 mole) was then added in small portions with shaking until all the ethyl chloroformate had reacted. The solution, after acidification to Congo Red with cold concentrated hydrochloric acid, was stored in a refrigerator overnight. The crude N-ethoxycarbonyl-(+)-threo-$\beta$-methylaspartic acid (5.5 g., 90%) was filtered off and recrystallised from water; it had m.p. 154-156°, (softening 152°) (Found: C, 44.0; H, 5.8; N, 6.3. $C_{8}H_{13}O_{4}N$ requires C, 43.85; H, 5.95; N, 6.4%).

A similar experiment was carried out with the erythro-isomer (4.0 g., 0.027 mole) but in this case the product was isolated from the acidic solution by extracting it with ethyl acetate (2 x 100 ml.). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate, filtered, and the ethyl acetate distilled off under reduced pressure. Recrystallisation of the product from ethyl acetate-light petroleum (b.p. 60-80°) gave N-ethoxycarbonyl-(+)-erythro-$\beta$-methylaspartic acid (5.0 g., 80%), m.p. 110-112° (Found: C, 43.85; H, 5.95;
N, 6.4. C₈H₁₃NO₆ requires C, 43.85; H, 5.95; N, 6.4%).

VIC₂b N-Ethoxycarbonyl-(-)-threo- and erythro-β-
methylaspartic anhydride

N-Ethoxycarbonyl-(-)-threo-β-methylaspartic acid
(5.5 g., 0.025 mole) and acetic anhydride (10 ml.) were heated
together on a steam-bath for 15 minutes. The reaction mixture
was cooled, and the excess acetic anhydride removed by distilla-
tion under reduced pressure. After being chilled and
scratched, the oily product solidified; recrystallisation
from dry chloroform gave N-ethoxycarbonyl-(-)-threo-β-methyl-
aspartic anhydride (4.9 g., 90%), m.p. 127-129° (Found:
C, 47.45; H, 5.85; N, 6.8. C₈H₁₁O₅N requires C, 47.45;
H, 5.50; N, 6.95%).

A similar experiment in which the (±)-erythro isomer
(4.9 g., 0.023 mole) was used furnished a crude material (4.5 g.),
the infra-red spectrum of which indicated that the product
was mainly the anhydride. No suitable solvent for recrystall-
sation was found.
VIC$_2$c α-Benzyl β-hydrogen N-ethoxycarbonyl-(-)-threo- and erythro-β-methylaspartate

N-ethoxycarbonyl-(-)-threo-β-methylaspartic anhydride (4.9 g., 0.0245 mole) and benzyl alcohol (5.0 g., 0.046 mole) were heated together on a steam-bath for 3½ hours. The reaction mixture was cooled, dissolved in ether (20 ml.) and the product extracted with aqueous sodium bicarbonate. Concentrated hydrochloric acid was added to the aqueous extract until it was acid to Congo Red, and the oil which separated was extracted with ether (2 x 100 ml.). The combined ethereal extracts were dried over anhydrous sodium sulphate, filtered, and the ether removed from the filtrate by distillation. Recrystallisation of the crude material from ethyl acetate-light petroleum (b.p. 60-80°) gave α-benzyl β-hydrogen N-ethoxycarbonyl-(-)-threo-β-methylaspartate (6.0 g., 80%), m.p. 80° (Found: C, 58.2; H, 6.15; N, 4.7. C$_{15}$H$_{19}$NO$_6$ requires C, 58.25; H, 6.2; N, 4.55%).

A similar experiment, in which the crude erythro-isomer (4.5 g., 0.022 mole) was used, furnished an oil (5.5 g., 80%) which would not solidify. A comparison of the infra-red spectrum of this oil with that of the corresponding threo-compound indicated that the oil was mainly the α-benzyl ester of the erythro-isomer, very little erythro acid being present.
VIC.2d α-Benzyl N-ethoxycarbonyl-(\(\ddagger\))-threo- and erythro-β-methylasparagine

Finely powdered phosphorus pentachloride (6.0 g., 0.029 mole) was added to an ice-cold solution of α-benzyl β-hydrogen N-ethoxycarbonyl-(\(\ddagger\))-threo-β-methylaspartate (6.0 g., 0.02 mole) in dry ether (75 ml.) with shaking until no more would dissolve. The ethereal solution was decanted into stirred ice-cold 6N-aqueous ammonia (150 ml.). The precipitate was filtered off, washed with cold water, and dried in vacuo over anhydrous calcium chloride. Recrystallisation from aqueous methanol gave α-benzyl N-ethoxycarbonyl-(\(\ddagger\))-threo-β-methylasparagine (4.2 g., 70%), m.p. 135-137° (Found: C, 58.15; H, 6.7; N, 8.95. \(\text{C}_{15}\text{H}_{20}\text{N}_{2}\text{O}_{5}\) requires C, 58.4; H, 6.55; N, 9.1%).

In a similar experiment in which the crude α-benzyl β-hydrogen N-ethoxycarbonyl-(\(\ddagger\))-erythro-β-methylaspartate (5.5 g., 0.018 mole) was used, recrystallisation of the product, from chloroform-light petroleum (b.p. 60-80°), gave α-benzyl N-ethoxycarbonyl-(\(\ddagger\))-erythro-β-methylasparagine (4.7 g., 85%), m.p. 125-127° (Found: C, 58.1; H, 6.75; N, 9.0. \(\text{C}_{15}\text{H}_{20}\text{N}_{2}\text{O}_{5}\) requires C, 58.4; H, 6.55; N, 9.1%).
VIC$_2$e N-Ethoxycarbonyl-(±)-threo- and erythro-β-
.methylasparagine

Palladium black (2.0 g., 0.019 mole) was added to a
solution of α-benzyl N-ethoxycarbonyl-(±)-threo-β-
methylasparagine (4.2 g., 0.014 mole) in methanol (120 ml.) and water (30 ml.);
and the suspension was shaken in an atmosphere of hydrogen, at
atmospheric pressure, for 3 hours. The suspension was filtered
and the filtrate concentrated to dryness under reduced pressure
at room temperature. Recrystallisation of the residue from
absolute ethanol gave N-ethoxycarbonyl-(±)-threo-β-
methylasparagine (2.0 g., 67%), m.p. 146° (Found: C, 43.9; H, 6.7;
N, 12.9. C$_8$H$_{14}$O$_5$N$_2$ requires C, 44.05; H, 6.45; N, 12.85%).

From a similar experiment in which the erythro-isomer
(4.7 g., 0.015 mole) was used, N-ethoxycarbonyl-(±)-erythro-β-
methylasparagine (2.5 g., 75%), m.p. 188°, was obtained
(Found: C, 43.8; H, 6.75; N, 12.8. C$_8$H$_{14}$N$_2$O$_5$ requires
C, 44.05; H, 6.45; N, 12.85%).
SECTION VID

Attempted Preparation of Glutamine from Glutamic acid
Attem-ded preparation of Glutamine from Glutamic acid

Experiment 1

A hot solution of glutamic acid (0.2 g., 0.0014 mole) in water (10 ml.) was shaken with an excess of cupric hydroxide. The solution was filtered and dicyclohexylcarbodiimide (0.8 g., 0.0039 mole) was added to the filtrate. The reaction mixture was shaken for 10 minutes, 3N-aqueous ammonia (10 ml.) was added, and the solution was stored at room-temperature overnight. The solution was filtered and the filtrate saturated with hydrogen-sulphide until all of the cupric sulphide had been precipitated. The suspension was filtered and the filtrate was concentrated under reduced pressure to a final volume of approximately 3 ml. Paper chromatographic analysis of the solution, with whatman No.1 paper and phenol: water or n-butanol: acetic acid: water as solvent, showed that the only amino-acid present was glutamic acid.

The above conditions were modified, as described below.

Experiment 2

A solution of dicyclohexylcarbodiimide (0.8 g., 0.0039 mole) in ethanol (10 ml.) was used in an experiment which was otherwise similar to Experiment 1.
Experiment 3

Excess cupric hydroxide was added to a hot solution of glutamic acid (0.1 g., 0.0007 mole) in water (5 ml.), and after 10 minutes the suspension was filtered. The residue was washed with hot water (5 ml.), and glutamic acid (0.1 g., 0.0007 mole) was added to the combined filtrate and washings. Dicyclohexylcarbodiimide (0.8 g., 0.0039 mole) was added to the solution, and the experiment was completed as described in Experiment 1.

Experiment 4

Experiment 4 was similar to Experiment 3 except that a solution of dicyclohexylcarbodiimide (0.8 g., 0.0039 mole) in ethanol (10 ml.) was added instead of solid dicyclohexylcarbodiimide.

Paper chromatographic analysis of the reaction medium of Experiments 2, 3 or 4 (see Experiment 1) showed that the only amino-acid present was glutamic acid.
SECTION VIE

(±)-Gis and Trans-4,5-Dihydro-5-methylorotic Acid

VIE₁ Cyclisation of \text{N-ethoxycarbonyl-(±)-threo- and erythro-} \beta-methylasparagine

VIE₂ Reduction of 5-methylorotic acid

VIE₂ₐ Preparation of 5-methylorotic acid
VIE₂ₐ Reduction of 5-methylorotic acid
VIE₂ₕ Chemical reduction of 5-methylorotic acid

VIE₃ Attempted conversion of dihydro-orotic acid into
4,5-dihydro-5-methylorotic acid
Cyclisation of $\text{N}$-Ethoxycarbonyl-$(\ddagger)$-Threo- and Erythro-$\beta$-Methylasparagine

A solution of sodium ethoxide (0.0092 mole) in ethanol (15 ml.) was added to a solution of $\text{N}$-ethoxycarbonyl-$(\ddagger)$-threo-$\beta$-methylasparagine (1.0 g., 0.0046 mole) in ethanol (30 ml.), and the reactants were boiled under reflux for 4 hours. The reaction mixture was cooled and filtered. The precipitate was dissolved in the minimum quantity of cold water, and cold concentrated hydrochloric acid was added to the solution until it was acid to Congo Red. The solid was filtered off, after the solution had been kept at 2° overnight, and was washed with a small quantity of cold water. Recrystallisation of the product from water gave cis-4,5-dihydro-5-methylorotic acid (0.31 g., 3%), m.p. 252-254° (decomp.) (Found: C, 41.65; H, 4.85; N, 16.15. C$_6$H$_8$N$_2$O$_4$ requires C, 41.85; H, 4.7; N, 16.25%).

Cyclisation of $\text{N}$-ethoxycarbonyl-$(\ddagger)$-erythro-$\beta$-methylasparagine (1.55 g., 0.0071 mole), m.p. 188 or 168°, under similar conditions gave trans-4,5-dihydro-5-methylorotic acid (0.4 g., 34%), m.p. 220° (decomp.) (Found: C, 41.95; H, 4.7; N, 16.3. C$_6$H$_8$N$_2$O$_4$ requires C, 41.85; H, 4.7; N, 16.25%). Cis-4,5-dihydro-5-methylorotic acid (0.05 g., 4%), m.p. 252-254° (decomp.), was isolated from the mother liquor.
VIE2a. Preparation of 5-methylorotic acid

Urea (9.0 g., 0.15 mole) and diethyl α-oxalylpropionate (30 g., 0.15 mole) were dissolved in glacial acetic acid (15 ml.), and a stream of dry hydrogen chloride was passed through the solution, whilst it was being heated on a steam-bath, for one hour. The crude ethyl hydantoidene-2-propionate was filtered off, washed with cold water, and recrystallised from water. The ethyl hydantoidene-2-propionate (10.4 g., 0.052 mole), m.p. 181-182°, was dissolved in a large excess of aqueous 1N-potassium hydroxide (500 ml.), and the solution, in an evaporating basin, was heated on a steam-bath until the final volume was approximately 50 ml. The solution was cooled, and cold concentrated hydrochloric acid was added until the solution was acid to Congo Red. The reaction mixture was stored at 2° overnight, and the crystals which separated were filtered off, washed with water, and recrystallised from water to give 5-methylorotic acid (7.2 g., 29%), m.p. 326-327° (decomp.) (Found: C, 42.05; H, 4.05; N, 16.35. Calculated for C₆H₆N₂O₄; C, 42.35; H, 3.55; N, 16.45%).

VIE2b. Catalytic hydrogenation of 5-methylorotic acid

5% Rhodium on alumina (0.50 g.) was added to a suspension of 5-methylorotic acid (0.50 g., 0.003 mole) in water
(200 ml.), and the reaction mixture was shaken in an atmosphere of hydrogen at room temperature (15-17°) and pressure until the uptake of hydrogen ceased. The suspension was filtered, the catalyst was washed with hot water, and the combined filtrate and washings were concentrated under reduced pressure to a final volume of approximately 20 ml. The solution was stored at 2° overnight, and the crystals which separated were filtered off, and recrystallised from water. Cis-4,5-dihydro-5-methylorotic acid (0.3 g., 59%), m.p. 252-254° (decomp.), was obtained (Found: C, 41.8; H, 4.75; N, 16.35; C6H8N2O4 requires C, 41.85; H, 4.7; N, 16.25%).

VIE2c Chemical reduction of 5-methylorotic acid

3% Sodium amalgam (20 g.) was added to a solution of 5-methylorotic acid (2.0 g., 0.012 mole) in 0.880 ammonia solution (15 ml.) and water (25 ml.) at -10°, and the reaction mixture was stirred for 1 hour at -10°. The solution was acidified (to pH 2) with cold concentrated hydrochloric acid and the product, m.p. 250-252° (decomp.), was filtered off and washed with cold water. Recrystallisation of the product from water gave cis-4,5-dihydro-5-methylorotic acid (0.9 g., 45%), m.p. 252-254° (decomp.).

The ultra-violet absorption spectrum of the compound in
water showed that the product was not contaminated with 5-methylorotic acid. The infra-red spectrum of a nujol mull of the compound was identical to that for the compound prepared by the catalytic reduction of 5-methylorotic acid.

VIE3 Attempted conversion of dihydro-orotic acid into 4,5-dihydro-5-methylorotic acid

Sodium ethoxide (0.013 mole) in ethanol (25 ml.) was added to a suspension of dihydro-orotic acid (1.0 g., 0.0063 mole) in ethanol (25 ml.) and the mixture was stirred and boiled under reflux for 1 hour. To the gelatinous suspension, cooled in ice-water, methyl iodide (0.91 g., 0.0063 mole) was added, and stirring was continued for a further 10 minutes. The gelatinous suspension was filtered and the filtrate (A) and residue examined separately. Gold concentrated hydrochloric acid was added to a solution of the residue in water until the solution was acid to Congo Red, and the solution was chilled in an ice-bath. Recrystallisation from water of the crystals, which had separated, gave dihydro-orotic acid (0.9 g., 90%), m.p. 266-268° (decomp.). No other product was isolated, but paper chromatographic analysis (Whatman No.1 paper) n-butanol-acetic acid-water (2:1:1 by vol.) of the mother liquor showed the presence of a trace of a compound which behaved similarly.
to an authentic sample of cis-4,5-dihydro-5-methylorotic acid.

The ethanolic filtrate (A) was evaporated to dryness under reduced pressure. Cold concentrated hydrochloric acid was added to a solution of the residue in cold water until it was acid to Congo Red. Dihydro-orotic acid (0.05 g., 5%), m.p. 267-268° (decomp.), was filtered off; paper chromatographic analysis of the compound, as described above, showed that there was no 4,5-dihydro-5-methylorotic acid present.

A similar experiment was carried out with a saturated solution of dihydro-orotic acid (0.3 g., 0.0019 mole) in ethanol (85 ml.); the only compound isolated was dihydro-orotic acid (0.24 g., 80%), m.p. 266-268° (decomp.).
SECTION VIF

$(\pm)-4,5$-Dihydro-orotic acid

VIF$_1$ Cyclisation of $\text{N}$-ethoxycarbonyl-$\text{L}$-asparagine

VIF$_{1a}$ $\beta$-Methyl-$\text{L}$-aspartate hydrochloride (6-methyl $\alpha$-hydrogen-$\text{L}$-aspartate hydrochloride)

VIF$_{1b}$ $\text{L}$-Asparagine

VIF$_{1c}$ $(\pm)-4,5$-Dihydro-orotic acid

VIF$_2$ Reduction of orotic acid

VIF$_{2a}$ Catalytic hydrogenation of orotic acid

VIF$_{2b}$ Chemical reduction of orotic acid
Cyclisation of N-ethoxycarbonyl-L-asparagine

β-Methyl-L-aspartate hydrochloride (β-methyl α-hydrogen aspartate hydrochloride)

Finely ground L-aspartic acid (1.0 g., 0.0075 mole) was added to a solution of thionyl chloride (0.77 ml., 0.0104 mole) in methanol (5 ml.), at -15° and the reaction mixture was shaken until all of the L-aspartic acid had dissolved. The solution was kept at room temperature, with occasional shaking, for 35 minutes, and then dry ether (15 ml.) was added. The solution was cooled in an ice-bath and the product (1.02 g., 74%), m.p. 182°. was filtered off. Recrystallisation of the product from methanol-ether gave β-methyl-L-aspartate hydrochloride (0.79 g., 57%), m.p. 189-191°.

L-Asparagine

Ammonia solution (S.g.0.880; 10 ml.) was added to a solution of β-methyl-L-aspartate hydrochloride (0.64 g., 0.035 mole) in water (5 ml.) and the solution was stored at room temperature for 24 hours. The solution was concentrated, under reduced pressure, to a final volume of approximately 4 ml., and then ethanol (20 ml.) was added with stirring and the solution stored at 2° overnight. Recrystallisation of the
solid, from aqueous ethanol, gave L-asparagine (0.33 g., 77%), m.p. 280-290° (decomp.).

\[ \text{VIF}_{1\alpha} \] \((\dagger)\)-4,5-Dihydro-orotic acid

N-Ethoxycarbonyl-L-asparagine (2.9 g., 71%), m.p. 160-161°, was prepared from L-asparagine hydrate (3 g., 0.02 mole) by a procedure similar to that described for the preparation of N-ethoxycarbonyl-(\dagger)-threo-\(\beta\)-methylasparagine from (\dagger)-threo-\(\beta\)-methylasparagine (see Section VIF\(_\alpha\)). Cyclisation of N-ethoxycarbonyl-L-asparagine (4.1 g., 0.02 mole) by a method similar to that previously described for the preparation of cis-4,5-dihydro-5-methylorotic acid from N-ethoxycarbonyl-(\dagger)-threo-\(\beta\)-methylasparagine (see Section VIF\(_\alpha\)), furnished (\dagger)-4,5-dihydro-orotic acid (2.55 g., 82%), m.p. 265-267° (decomp.).

\[ \text{VIF}_2 \] Reduction of orotic acid

\[ \text{VIF}_{2a} \] Catalytic hydrogenation of orotic acid

Orotic acid (0.45 g., 0.0029 mole) was treated with 5%-rhodium on alumina (0.45 g.) and hydrogen by a method similar to that previously described for the preparation of cis-4,5-dihydro-5-methylorotic acid from 5-methylorotic acid (see Section VIF\(_{2b}\)). (\dagger)-Dihydro-orotic acid, m.p. 265-267° (decomp.), was obtained in a 62% yield.
VIF$_{2b}$ Chemical reduction of orotic acid

Orotic acid (2.0 g., 0.013 mole) was treated with 3% sodium amalgam (20 g.), by a method similar to that described for the preparation of cis-4,5-dihydro-5-methylorotic acid from 5-methylorotic acid (see Section VIF$_{2c}$). Analysis of the material isolated, in the ultra-violet and infra-red regions of the spectrum showed that it was a mixture of orotic acid, dihydro-orotic acid, and carbamoylaspartic acid. Attempts to separate the components by fractional recrystallisation from water, or glacial acetic acid were unsuccessful.

From an analogous experiment carried out at room temperature (19°), the only compound isolated was carbamoylaspartic acid (1.46 g., 65%), m.p. 178°.

**Attempted Chemical Reduction of Orotic Acid in Glacial Acetic Acid**

3% Sodium amalgam (5.0 g.) was added to a solution of orotic acid (0.5 g., 0.0032 mole) in glacial acetic acid (500 ml.) at room temperature (16°), and the solution stirred for 2 hours. The solution was filtered, and concentration of the filtrate, under reduced pressure, gave orotic acid (0.45 g., 90%), m.p. 340-345° (decomp.); no other compound was isolated.
At attempted preparation of 5-bromo-4,5-dihydro-orotic acid

\[ \begin{align*}
\text{VIG}_1 & \quad \text{Treatment of dihydro-orotic acid with bromine} \\
\text{VIG}_2 & \quad \text{Treatment of dihydro-orotic acid with N-bromosuccinimide}
\end{align*} \]
Vig Treatment of dihydro-orotic acid with bromine

Bromine (0.3 g., 0.0019 mole) was added to a solution of dihydro-orotic acid (0.3 g., 0.0019 mole) in glacial acetic acid (50 ml.) at 80°, and the solution kept at this temperature until the bromine-colour had disappeared (approximately 1 hour). The solution was freeze-dried. The residue had m.p. 260-290° (decomp.) which varied slightly with the rate of heating. The ultra-violet absorption spectra of the residue in water and in glacial acetic acid showed the presence of orotic acid. The bands in the infra-red spectrum of a nujol mull of the residue could all arise from dihydro-orotic acid and orotic acid; there were no bands in the region 750-400 cm.\(^{-1}\) which could be assigned to a C-Br vibration. A Lassaigne sodium fusion on the product confirmed that there was no bromine present. Recrystallisation of the residue from water gave orotic acid (0.09 g., 30%), m.p. 345° (decomp.). Concentration of the mother liquor gave dihydro-orotic acid (0.17 g., 56%), m.p. 255-260° (decomp.).

Only orotic acid and dihydro-orotic acid were obtained from similar experiments carried out at 50°, or in boiling glacial acetic acid, or by the dropwise addition of bromine to a solution of dihydro-orotic acid in acetic acid, the bromine colour being discharged from the solution before each addition of bromine.
When the experiment was carried out at room temperature dihydro-orotic acid was the only compound isolated.

Treatment of dihydro-orotic acid with N-bromosuccinimide

N-Bromosuccinimide (0.3 g., 0.0017 mole) was added to a solution of dihydro-orotic acid (0.2 g., 0.0013 mole) in glacial acetic acid (40 ml.) and acetic anhydride (1 ml.) at room temperature. The reaction mixture was stirred at 50-55° for 1 hour, and then for a further 2 hours at room temperature. The solution was freeze-dried. The residue was washed with ethanol, and the residue (0.15 g., 75%), m.p. 265-267° (decomp.), gave a negative test for bromine (Lassaigne). The infra-red spectrum of a nujol mull of the compound was identical with that of an authentic sample of dihydro-orotic acid, and the ultra-violet absorption spectrum of the compound in water had no bands characteristic of orotic acid.
SECTION VII

ENZYMIC STUDIES

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VIIB  ISOLATION AND PURIFICATION OF DIHYDRO-OROTIC DEHYDROGENASE

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/Contd...
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VIIA INTRODUCTION

A survey of the literature (see Section I) showed that for the purpose of this investigation, *Zymobacterium oroticum* was the most suitable source of the enzyme dihydro-orotic dehydrogenase, mainly because methods for the isolation and purification of the enzyme from this source have been published. Although the enzyme dihydro-orotic dehydrogenase has been isolated from aerobic bacteria (see Section I), very little work has been carried out on it as compared with that on the enzyme from *Z. oroticum*. Most of the enzymic studies in this investigation were carried out with a preparation from *Z. oroticum*. However, because of an unforeseen difficulty in obtaining an active culture of the organism, an alternative source of the enzyme was sought so that preliminary studies with the 5-methyl-dihydro-orotic acids could be carried out. It was decided to investigate the possibility of obtaining a preparation of the enzyme from a mammalian source. The main problem was to obtain an enzyme preparation not only of high specific activity, but also free from interfering enzymes, in particular dihydro-orotase.

Smith and Baker [9] in 1959 investigated pyrimidine biosynthesis in man. They reported that human leucocytes contained both dihydro-orotase and dihydro-orotic dehydrogenase,
but that it was not possible to obtain a soluble preparation of either of them by the standard techniques such as freezing and thawing. However, they found that if a suspension of the leucocytes was treated ultrasonically, a soluble preparation of dihydro-orotase was obtained which contained approximately 50% of the total dihydro-orotase activity in the cell. The rest of the dihydro-orotase and the dihydro-orotic dehydrogenase remained firmly attached to the cell debris. Attempts to obtain a soluble preparation of dihydro-orotic dehydrogenase from the insoluble cell debris by the freeze-thawing technique in isotonic saline, or by freeze-drying, or by digitonin treatment, were all unsuccessful. They found, however, that it was possible to use the insoluble fraction as a source of dihydro-orotic dehydrogenase.

Two years later Bresnick and Hitchings [10] reported that they had obtained a soluble preparation of dihydro-orotase from Ehrlich ascites tumour cells. They found that if a suspension of the cells was treated ultrasonically and the resulting suspension centrifuged, dihydro-orotase activity was found in the supernatant. Later, Bresnick in a personal communication to Dr. E. Reid [20] stated that the ultrasonic treatment left the dihydro-orotic dehydrogenase in the insoluble cell debris. Because of this separation, and because cancer cells may surpass
normal mammalian cells in respect of capacity for pyrimidine biosynthesis [93], it was decided to investigate Ehrlich ascites tumour cells as a possible source of dihydro-orotic dehydrogenase.

VIIB. ISOLATION AND PURIFICATION OF DIHYDRO-OROTIC DEHYDROGENASE

VIIB1 From Ehrlich ascites tumour cells

The methods developed were based on those described by Bresnick and Hitchings [10].

Mice were inoculated with Ehrlich ascites tumour cells and after 9-10 days the animals were killed and the tumour cells aspirated from the stomach by means of a hypodermic syringe. The cells were spun down in a centrifuge at 600 g, the supernatant was removed, and the cells were washed three times with isotonic saline. The packed wet cells were resuspended in an equal volume of isotonic saline, and subjected to ultrasonic oscillations at 10,000 Kc/sec. for 15 seconds (preliminary experiments had established that the cells were completely disrupted after 15 seconds at 10,000 Kc/sec.). The resulting suspension was centrifuged at 600 g for 30 minutes. The supernatant was removed, and the cell debris washed with isotonic saline. The supernatant and washings were combined and kept for analysis. The cell debris was dispersed in twice its
own volume of isotonic saline to give a suspension which served as a preparation, admittedly crude, of dihydro-orotic dehydrogenase.

The suspension of cell debris, the supernatant, and the original suspension were analysed by the method of Reid [93] for total capacity to convert carboxyglutamic acid into orotic acid, through the successive reactions of dihydro-orotase and dihydro-orotic dehydrogenase. Although a partial separation of the enzymes had been achieved, the activity was disappointingly low. Preliminary studies showed that the supernatant could not be used as a preparation of dihydro-orotic dehydrogenase, because of its low activity and of the very high optical density of the solution in the ultraviolet. This high absorption was a serious difficulty since the only convenient method available for studying the conversion of dihydro-orotic acid and its 5-methyl analogues into the corresponding "orotic acid" is by measuring the change in optical density of the solution at an appropriate wavelength in the ultraviolet spectrum.

Attempts were made to obtain a soluble preparation of dihydro-orotic dehydrogenase from the cell debris by the following methods, none of which were successful:
(1) Freeze-thawing in isotonic saline.

(2) Treatment with n-butanol.

(3) Fractionation by centrifugation at various speeds.

(4) Deoxycholate treatment.

Thus:

(1) Repeated freeze-thawing of a suspension of the cell debris in isotonic saline did not yield a soluble preparation of the enzyme. Furthermore, the activity in the insoluble fraction was eventually reduced to below detectable limits. The enzyme activity was determined as described in Section VIIC.

(2) Treatment of the insoluble debris with n-butanol, by a method analogous to that described by Morton [94] for obtaining a soluble preparation of succinic dehydrogenase from a mitochondrial preparation from horse heart, gave a solution with no detectable enzymic activity (see Section VIIID).

(3) Attempts to fractionate the insoluble cell debris by centrifugation at various speeds were unsuccessful since 90-95% of the insoluble material sedimented even at very low speeds. The experiments were carried out in both isotonic saline and aqueous sucrose.

(4) Addition of an aqueous solution of the detergent, deoxycholate, to the cell debris, with or without magnesium ions
present, yielded an unworkable sticky mass.

As previously stated, a suspension of the insoluble cell debris was used as a preparation of dihydro-orotic dehydrogenase for substrate studies.

VIIB2 From Zymobacterium oroticum

The method was based on that described by Friedmann and Vennesland [22].

Attempts to obtain an active culture of Z. oroticum from freeze-dried samples of the organism, obtained from both the British (Mill Hill) and U.S. Type Culture Collections, were unsuccessful. We are indebted to Dr. H. C. Friedmann, Department of Biochemistry, University of Chicago, Dr. V. Aleman, Medical Center, Duke University, and Dr. R. W. Miller, New England Institute of Medical Research, for supplying unfrozen cultures from their own collections. The culture supplied by Dr. Miller was used as the parent culture for this investigation.

Zymobacterium oroticum grown on a medium containing orotic acid, such as Friedmann and Vennesland [21,22] have described, adaptively produces the enzyme dihydro-orotic dehydrogenase. In this investigation the organism was grown for 48 hours at 30°C, compared with 15-20 hours at 30°C as reported in the literature. This
difference in culture times is discussed later, in Section VII D. The cells were harvested by centrifugation (at 0°C) for 30 minutes, 6,000 g, a high speed of centrifugation being found necessary to achieve packing of the cells. From three litres of culture 8 g. of wet packed cells were obtained. Friedmann and Vennesland [22] reported a yield of 4-5 g./litre.

**Extraction of the Enzyme**

Preliminary experiments showed that the cells could be disrupted by stirring a paste of the cells with acid-washed glass beads (B.D.H., 100 mesh) and 0.05M sodium phosphate buffer, pH 6.5.

A fine paste of the cells (8 g.) in 0.05M sodium phosphate buffer (8 ml.), pH 6.5, was added to acid washed glass beads (50 g.), the mixture being cooled in an ice-bath and stirred mechanically for 20 minutes. A further aliquot of buffer (8 ml.) was added to the suspension, the mixture was stirred for a few minutes and then the supernatant was decanted off. Centrifugation of the supernatant gave 12 ml. of a clear pale yellow solution. The glass beads were washed with buffer (2 x 8 ml.), and a further 14 ml. of clear supernatant was obtained. The first and second cell-free extracts were not combined, but were worked up separately.
Purification of the first cell-free extract

Sodium chloride (0.28 g.) was dissolved in the 0.05M sodium phosphate extract (12 ml.) and 1% protamine sulphate (Elanico Products Company, Batch No. 2379) solution (6 ml., 0.5 vol.) in 0.4M sodium chloride was added slowly with vigorous stirring. After 10 minutes, the white precipitate of inert protein was removed by centrifugation. (Experiments with the second phosphate extract showed that little or no further precipitation took place on the addition of 1% protamine sulphate solution in excess of 0.3 volume and the enzyme activity of the supernatant was the same whether 0.3 or 0.5 volumes of protamine solution was added. However, if the protamine solution (0.3 or 0.5 volumes) was added to the cell-free extract without adequate mixing, the enzyme was precipitated together with the inert protein, as judged by the absence of enzyme activity in the supernatant). To the supernatant (17 ml.) ammonium sulphate (5 g.) was added with stirring and the solution was kept for 30 minutes. The precipitate which separated on standing was removed by centrifugation; a further aliquot of ammonium sulphate (1.5 g.) was added to the supernatant (19 ml.), and the solution was kept overnight. The precipitate was collected by centrifugation, and the enzyme extracted from it with 0.2M sodium phosphate
buffer, pH 5.8 (2 x 0.75 ml.). The deep yellow extracts were combined, diluted with an equal volume of 0.2M tris buffer, pH 8.0. This solution served as the preparation of the enzyme dihydro-orotic dehydrogenase for the substrate studies.

All the steps involved in the purification of the enzyme were carried out in a cold room at 0°. The enzyme preparation was found to be relatively stable at 0-4° at least for ten days, although once inactivation of the enzyme starts, the rate of inactivation increases rapidly. For the first of the two preparations used below (Section VIID₂b), about 5% of the enzyme activity, as measured by following the conversion of orotic acid into dihydro-orotic acid, was lost during the first 10 days at 0°, but about 80-90% of the activity was lost in the following 2 or 3 days. These results, together with other results on the stability of the enzyme preparation are given and discussed in Section VIIIE₁. (From one observation made in this investigation it would appear that the enzyme is irreversibly deactivated when stored at -25° for 3 days). After each step in the isolation and purification of the enzyme, the enzymic activity of each fraction was determined spectrophotometrically (see Section VIIIC₂a) by following the rate of decrease of the optical density at 340 μm which is associated with the oxidation of NADH to NAD⁺ in the presence of excess orotic acid.
ASSAY PROCEDURES

The assay procedures used for the two different preparations of dihydro-orotic dehydrogenase are discussed separately under the following headings:

(1) Activity of enzyme preparation from Ehrlich ascites tumour cells.
(2) Activity of enzyme preparation from Zymobacterium oroticum

Activity of enzyme preparation from Ehrlich ascites tumour cells

The enzyme activity was determined by following the rate of conversion of L-dihydro-orotic acid into orotic acid, by measuring the increase in optical density at 282μ. This increase is associated with orotic acid formation, since L-dihydro-orotic acid, and 4,5-dihydro-orotic acids in general do not absorb at 282μ. However, the optical density change to be measured was relatively small compared with the high optical density of the protein-containing blank, and it was therefore necessary to deproteinize the solutions before optical density measurements could be made. This, of course, made it impossible to follow the time course of the reaction with a single sample.
Materials

0.2M Tris-hydroxymethyl-amino-methane [Tris] buffer, pH 8.0, perchloric acid solution 1C% W/V.

All solutions were made up with water distilled from an all glass apparatus.

Procedure

The volume of enzyme preparation required for the assay procedure is found by trial and error; the quantities used in this investigation, together with the results obtained, are given in Section VIID. However, the general assay procedure is described below.

To a suspension of the enzyme preparation in isotonic saline at 0° was added an equal volume of 0.2M tris buffer at 0°, followed by a solution of the substrate in 0.1M tris buffer also at 0°, the overall substrate concentration being adjusted to 1 μmole/ml. The assay mixture was kept in a bath at 37.4° for a known time, the reaction mixture being shaken continuously throughout the incubation period. The reaction was quenched by cooling the assay mixture in an ice-bath, and to the cold suspension was added an equal volume of cold perchloric acid. The suspension was centrifuged, an aliquot of the supernatant was diluted to a known volume with distilled water until a suitable optical density reading, compared with water as the instrument blank, was obtained. An 'experimental blank', that
is the assay system without substrate, was also run and the optical density of this compared with water. The difference between the optical densities of the experimental and the experimental blank gives a measure of the orotic acid present. The enzyme and buffer concentrations in the experimental blank were the same as in the experimental.

Similar studies were carried out with DL-dihydro-orotic acid and with cis- and trans-4,5-dihydro-5-methylorotic acid as substrates.

VIIC2  Activity of enzyme preparation from Zymobacterium oroticum

The conversion of orotic acid into dihydro-orotic acid, as well as the reverse reaction, was studied in this investigation. The assay procedures depended upon which reaction was being followed, and are described below under the following headings:

(a) Conversion of orotic acid into dihydro-orotic acid, as followed by NADH oxidation.
(b) Conversion of dihydro-orotic acid into orotic acid, as followed by orotic acid formation.

In the literature, as in this investigation, the term "enzyme activity" as applied to a preparation of dihydro-orotic
dehydrogenase from *Zymobacterium oroticum* refers to the capacity of the enzyme preparation to oxidise NADH in the presence of orotic acid. A unit of enzyme activity is defined as the amount of enzyme required to oxidise 1 μ mole of NADH/min. [21].

VIIC2a Conversion of orotic acid into dihydro-orotic acid as followed by NADH oxidation

As indicated in Section VIIb2, the purification of dihydro-orotic dehydrogenase was followed quantitatively essentially by the method of Friedmann and Vennesland [21]. The assay procedure is based on the fact that the conversion of orotic acid into dihydro-orotic acid, catalysed by dihydro-orotic dehydrogenase obtained from *Zymobacterium oroticum* requires the presence of the co-enzyme NADH which is oxidised to NAD$. One molecule of NADH is oxidised to NAD$ for each molecule of orotic acid reduced to dihydro-orotic acid, and the reaction was followed by measuring the decrease in optical density at 340nm which is associated with the oxidation. The assay is carried out in the presence of excess orotic acid so that it is the enzyme concentration and not the substrate concentration that is rate-determining.
Materials

All solutions were made up with water redistilled from all glass apparatus.
0.5M sodium phosphate buffer, pH 6.2.
NADH solution (approximately 0.0035M).
0.1M cysteine hydrochloride solution. A fresh solution was prepared for each experiment.
0.02M sodium orotate solution.

Procedure

As found by earlier workers [18,21] (see Section IA), to obtain an active preparation, it was necessary to "pre-incubate" the enzyme preparation with cysteine at room-temperature before addition of the NADH solution. A standard incubation time of 10 minutes was used, since it was found that further incubation with cysteine did not enhance the enzyme activity.

Phosphate buffer (0.4 ml.), cysteine hydrochloride solution (0.2 ml.), sodium orotate solution (0.3 ml.), enzyme solution (0.2 ml.), and water (1.7 ml.) were added in that order to a 1 cm.² silica cuvette. After 10 minutes incubation at room temperature, NADH solution (0.2 ml.) was added, and the rate of change in optical density at 340mμ was measured over several minutes at half-minute intervals. Some blanks were set up with
the sodium orotate omitted, but a blank was not taken each time since it was found that any correction for changes in the blank was well within experimental error. The concentration of the enzyme solution used was adjusted by trial and error until a suitable rate of change of optical density was obtained.

VII C2b  Conversion of dihydro-orotic acid into orotic acid as followed by orotic acid formation

The rate of conversion of dihydro-orotic acid and of the two diastereoisomeric 4,5-dihydro-5-methylorotic acids into the corresponding "orotic acids" was determined spectrophotometrically by measuring the rate of increase in optical density at 282µm due to formation of orotic acid, and or its 5-methyl analogue. For an assay system, hypothetically occupying only 1 ml., a change in optical density at 282µm (1 cm. light path) of 7.5 would represent, and would correspond to, the formation of 1 µmole of orotic acid. Similarly a change of 6.87 optical density units would correspond to the formation of 1 µmole of 5-methylorotic acid (see Appendix 1B).

For the purpose of comparing substrates it should be the substrate concentration and not the enzyme concentration that is kept limiting in the assay.
Materials

0.2M tris buffer, pH 8.0.

0.1M L-cystein hydrochloride solution.

Solutions of substrate in 0.1M tris buffer, pH 8.0 (the solutions of substrates were made up on the day of the assay).

Procedure

Tris buffer (1.25 ml.), cystein hydrochloride solution (0.2 ml.), enzyme preparation (0.02 ml.) and water (1 ml.) were added, in that order, to a silica cuvette. After incubation for 10 minutes at room temperature the substrate solution (0.5 ml.) was added, and the rate of change in optical density at 282μ compared with that for an experimental blank. The latter consisted of tris buffer (1.5 ml.), cystein hydrochloride solution (0.2 ml.), the enzyme preparation (0.02 ml.), and water (1.25 ml.)

It was necessary to run a blank in each experiment since it was found that consistency of the blank could not be relied on.

VII C2c Assay for dihydro-orotase activity

The above procedure was also used in an attempt to detect dihydro-orotase activity, which might have led to disappearance of dihydro-orotic acid through conversion into carbamoylaspartic acid. The substrate was DL-carbamoylaspartic
acid, and the rate of change in optical density at 282μ was measured; it was assumed that any dihydro-orotic acid formed would be further converted into orotic acid and thereby give a measurable end-product.

RESULTS

The results obtained for the two different enzyme preparations are described below under the following headings:

1. Enzyme preparation from Ehrlich ascites tumour cells.
2. Enzyme preparation from Zymobacterium oroticum.

Enzyme preparation from Ehrlich ascites tumour cells

The enzyme preparation was obtained from Ehrlich ascites tumour cells as described in Section VII B 1.

Preliminary experiments were carried out with L-dihydro-orotic acid as substrate and an enzyme preparation from one batch of cells. For the studies with other substrates, another preparation of the enzyme was used.

Studies with L-dihydro-orotic acid as substrate

From 9.75 ml. of wet packed Ehrlich ascites tumour cells, 11 ml. of cell debris suspension was obtained (see Section VII B 1); this served as a preparation of the enzyme dihydro-orotic dehydrogenase.
Assay system

<table>
<thead>
<tr>
<th>Components</th>
<th>Experimental ml.</th>
<th>Experimental blank ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme preparation</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.2M Tris buffer, pH 8.0</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>L-Dihydro-orotic acid</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>0.25</td>
</tr>
</tbody>
</table>

L-dihydro-orotic acid (0.5 μ mole/ml.) in 0.1M tris buffer, pH 8.0.

The reaction was quenched with 10% (W/V) perchloric acid (2.5 ml.); 1 ml. of the resultant solution was diluted to 5 ml. and the optical density measured at 282μ against water as an instrument blank.

The possible need for NAD⁺ was investigated, a solution of NAD⁺ (3.4 x 10⁻³M) and nicotinamide (2 x 10⁻²M) in 0.2M tris pH 8.0, being used in the assay system.
**Assay system**

<table>
<thead>
<tr>
<th>Components</th>
<th>Experimental ml.</th>
<th>Experimental blank ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme preparation</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.2M Tris buffer, pH 8.0</td>
<td>0.8</td>
<td>1.05</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>NAD⁺ solution</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Dihydro-orotic acid*</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

* L-Dihydro-orotic acid (0.5 μ mole/ml) in 0.1M tris buffer, pH 8.0.

The assay was completed as described above, and the results obtained are given herewith.

<table>
<thead>
<tr>
<th>time (t) min.</th>
<th>O.D.ₘₛ</th>
<th>O.D.ₘₜ</th>
<th>O.D.ₘₜₑₘₑ</th>
<th>O.D.ₘₜₑ₇ₜ</th>
<th>ΔO.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.670</td>
<td>0.480</td>
<td>0.672</td>
<td>0.486</td>
<td>0.186</td>
</tr>
<tr>
<td>30</td>
<td>0.680</td>
<td>0.490</td>
<td>0.717</td>
<td>0.510</td>
<td>0.207</td>
</tr>
<tr>
<td>40</td>
<td>0.753</td>
<td>0.522</td>
<td>0.786</td>
<td>0.535</td>
<td>0.251</td>
</tr>
<tr>
<td>60</td>
<td>0.784</td>
<td>0.532</td>
<td>0.931</td>
<td>0.585</td>
<td>0.346</td>
</tr>
<tr>
<td>60*</td>
<td>0.978</td>
<td>0.592</td>
<td>1.091</td>
<td>0.735</td>
<td>0.346</td>
</tr>
</tbody>
</table>

* indicates that NAD⁺ was present in the assay system (apparently without effect on the activity).
The term optical density (O.D.) here connotes the absorbance of the assay solution at 282mμ.

O.D.₀ and O.D.₀ are the optical densities actually determined for the "experimental" (i.e. with L-dihydro-orotic acid as substrate) and "experimental blank" respectively.

O.D.ₘ is the average of the O.D.₀ values for a given time point. For the above results O.D.ₘ is the average of one experiment in which NAD⁺ was absence and one in which it was present.

O.D.₀ is the value of the blank obtained from a graph of O.D.₀ against time (t).

ΔO.D. is the difference between O.D.ₘ and O.D.₀.

So that the final result for the rate of formation of orotic acid could be expressed in a conventional biochemical form, i.e. μ moles of orotic acid/min./ml. of wet packed cells, the rate of change of optical density obtained from the graph of ΔO.D. against time (t) had to be corrected as follows:

(1) The optical density measurements were carried out on a diluted reaction medium; therefore it was necessary to correct for dilution, converting to a hypothetical reaction volume of 1 ml. For the above system this dilution factor was 25.
(2) To convert the corrected optical density reading to moles of orotic acid, it was necessary to multiply the optical density reading by the corresponding extinction coefficient for orotic acid. Its molar extinction coefficient measured at 282 nm is $7.5 \times 10^3$. Therefore for a hypothetical reaction volume of 1 ml., the correction factor is:

$$\frac{1}{7.5 \times 10^3} \times \frac{1}{10^3}$$

(3) The rate of formation of orotic acid is corrected as follows to refer to 1 ml. of wet packed cells. The incubation mixture contained 1 ml. of cell debris, and 9.75 ml. of wet packed cells furnished 11 ml. of cell debris suspension; therefore a correction factor of $\frac{11}{9.75}$ must be applied.

Thus, the composite correction factor is

$$25 \times \frac{11}{9.75} \times \frac{1}{7.5 \times 10^3} \times \frac{1}{10^3}$$

for a hypothetical reaction volume of 1 ml. containing 1 ml. of wet packed cells.

From a graph of ΔO.D. against time (Fig. I), the rate of change of optical density was calculated to be 0.00496 O.D. units/min.; there was no marked departure from linearity during the first 20-60 minutes of incubation. Therefore, the rate of
formation of orotic acid from \( \text{L-dihydro-orotic acid} \) was

\[
25 \times \frac{11}{9.75} \times \frac{1}{7.5 \times 10^3} \times \frac{1}{4.3} \times 0.00496
\]

= \( 1.87 \times 10^{-8} \) mole/min./ml. of wet packed cells

= \( 1.87 \times 10^{-2} \) \( \mu \) mole/min./ml. of wet packed cells.

Results for \( \text{DL-dihydro-orotic acid} \) and for the \( \text{cis} \) and \( \text{trans-5-methyl-dihydro-orotic acids} \) as substrates

For this work a cell debris suspension (40 ml.) obtained from Ehrlich Ascites tumour cells (30 ml.) was used as a preparation of the enzyme dihydro-orotic dehydrogenase. A few time points with \( \text{L-dihydro-orotic acid} \) as substrate were also studied so that a direct comparison between the two preparations of the enzyme from Ehrlich Ascites tumour cells could be made.

**Assay system**

The substrate concentration in the incubation mixture was 1.0 \( \mu \) mole/ml.

<table>
<thead>
<tr>
<th>Component</th>
<th>Experimental ml.</th>
<th>Experimental blank ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme preparation</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>0.2M tris buffer, pH 8.0</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Substrate(^+)</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^+\) Substrate (5 \( \mu \) mole/ml.) in 0.1M tris buffer, pH 8.0.
The reaction mixture was quenched with 10% perchloric acid (1 ml) and 1 ml. of the resulting solution was diluted to 4 ml. with distilled water. The optical density (O.D.) of the solution was then measured at 282mu against water as an instrument blank. Several points were redetermined, as indicated, and all the results obtained are given below.

**Results for DL-dihydro-orotic acid as substrate**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>O.D.&lt;sub&gt;S&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;B&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;GS&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;GB&lt;/sub&gt;</th>
<th>Δ O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.418</td>
<td>0.300</td>
<td>0.435</td>
<td>0.303</td>
<td>0.132</td>
</tr>
<tr>
<td>10</td>
<td>0.542</td>
<td>0.350</td>
<td>0.469</td>
<td>0.323</td>
<td>0.146</td>
</tr>
<tr>
<td>10&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.431</td>
<td>0.315</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.620</td>
<td>0.316</td>
<td>0.529</td>
<td>0.362</td>
<td>0.167</td>
</tr>
<tr>
<td>20&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.454</td>
<td>0.363</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.621</td>
<td>0.436</td>
<td>0.652</td>
<td>0.439</td>
<td>0.213</td>
</tr>
<tr>
<td>50&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.773</td>
<td>0.473</td>
<td>0.720</td>
<td>0.476</td>
<td>0.244</td>
</tr>
<tr>
<td>60</td>
<td>0.803</td>
<td>0.510</td>
<td>0.776</td>
<td>0.514</td>
<td>0.262</td>
</tr>
<tr>
<td>80</td>
<td>0.899</td>
<td>0.590</td>
<td>0.900</td>
<td>0.587</td>
<td>0.313</td>
</tr>
</tbody>
</table>

The above results were obtained from two separate experiments and <sup>+</sup> indicates the points obtained in the second experiment.

O.D.<sub>S</sub> and O.D.<sub>B</sub> are the optical densities actually determined for the "experimental" (i.e. with DL-dihydro-orotic acid as substrate)
and "experimental blank" respectively.

O.D.\textsubscript{GS} and O.D.\textsubscript{GB} are the values obtained from a smoothed graphical plot of O.D.\textsubscript{G} and O.D.\textsubscript{B} against time (t in min.).

From a graph of ΔO.D. against time (Fig.1), the rate of change of optical density = 0.0024 O.D. units/min.; this rate was fairly constant during the first 80 minutes of incubation.

After correction to a hypothetical reaction volume of 1 ml. containing 1 ml. of wet packed cells, as described for the results with L-dihydro-orotic acid as substrate, the observed rate of formation of orotic acid was 0.87 x 10\textsuperscript{-2} μ mole/min./ml. of wet packed cells.

### Results for L-dihydro-orotic acid as substrate

<table>
<thead>
<tr>
<th>time (min.)</th>
<th>O.D.\textsubscript{G}</th>
<th>O.D.\textsubscript{B}</th>
<th>O.D.\textsubscript{GB}</th>
<th>ΔO.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.690</td>
<td>0.351</td>
<td>0.352</td>
<td>0.338</td>
</tr>
<tr>
<td>40</td>
<td>0.865</td>
<td>0.442</td>
<td>0.435</td>
<td>0.430</td>
</tr>
<tr>
<td>60</td>
<td>1.056</td>
<td>0.520</td>
<td>0.525</td>
<td>0.531</td>
</tr>
</tbody>
</table>

From a graph of ΔO.D. against time (t in min.) the rate of change of optical density ΔO.D./t was calculated to be 0.005 O.D. units/min. Therefore the rate of formation of orotic acid was 1.78 x 10\textsuperscript{-2} μ mole/min./ml. of wet packed cells. This result is very similar to that obtained with the other preparation of the enzyme from Ehrlich Ascites tumour cells.
Results for cis-4,5-dihydro-5-methylorotic acid as substrate

<table>
<thead>
<tr>
<th>time (min.)</th>
<th>O.D. S</th>
<th>O.D. B</th>
<th>O.D. MS</th>
<th>O.D. GB</th>
<th>ΔO.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.276</td>
<td>0.274</td>
<td>0.276</td>
<td>0.274</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.397</td>
<td>0.297</td>
<td>0.397</td>
<td>0.307</td>
<td>0.090</td>
</tr>
<tr>
<td>13+</td>
<td>0.398</td>
<td>0.330</td>
<td>0.398</td>
<td>0.323</td>
<td>0.075</td>
</tr>
<tr>
<td>20φ</td>
<td>0.351</td>
<td>0.325</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20+</td>
<td>0.425</td>
<td>0.317</td>
<td>0.424</td>
<td>0.352</td>
<td>0.072</td>
</tr>
<tr>
<td>30</td>
<td>0.495</td>
<td>0.335</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.442</td>
<td>0.412</td>
<td>0.439</td>
<td>0.397</td>
<td>0.042</td>
</tr>
<tr>
<td>60</td>
<td>0.493</td>
<td>0.508</td>
<td>0.474</td>
<td>0.525</td>
<td>0.051</td>
</tr>
<tr>
<td>60+</td>
<td>0.458</td>
<td>0.512</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The above results were obtained from two separate experiments. *

+ indicates the results obtained from the second experiment, and

φ indicates a duplicate point taken in the first experiment.
Results for trans-4,5-dihydro-5-methylorotic acid as substrate

<table>
<thead>
<tr>
<th>time (min.)</th>
<th>O.D.&lt;sub&gt;S&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;B&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;MS&lt;/sub&gt;</th>
<th>O.D.&lt;sub&gt;MB&lt;/sub&gt;</th>
<th>ΔO.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.414</td>
<td>0.461</td>
<td>0.448</td>
<td>0.420</td>
<td>0.028</td>
</tr>
<tr>
<td>20&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.481</td>
<td>0.359</td>
<td>0.544</td>
<td>0.544</td>
<td>-0.066</td>
</tr>
<tr>
<td>40</td>
<td>0.478</td>
<td>0.544</td>
<td>0.478</td>
<td>0.506</td>
<td>0.023</td>
</tr>
<tr>
<td>60</td>
<td>0.529</td>
<td>0.495</td>
<td>0.529</td>
<td>0.608</td>
<td>0.127</td>
</tr>
<tr>
<td>60&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.528</td>
<td>0.517</td>
<td>0.528</td>
<td>0.657</td>
<td>0.149</td>
</tr>
<tr>
<td>70</td>
<td>0.735</td>
<td>0.608</td>
<td>0.753</td>
<td>0.698</td>
<td>0.178</td>
</tr>
<tr>
<td>80</td>
<td>0.805</td>
<td>0.685</td>
<td>0.806</td>
<td>0.698</td>
<td>0.178</td>
</tr>
<tr>
<td>80&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.806</td>
<td>0.629</td>
<td>0.806</td>
<td>0.698</td>
<td>0.178</td>
</tr>
<tr>
<td>90</td>
<td>0.860</td>
<td>0.698</td>
<td>0.860</td>
<td>0.698</td>
<td>0.178</td>
</tr>
<tr>
<td>100</td>
<td>0.940</td>
<td>0.738</td>
<td>0.943</td>
<td>0.736</td>
<td>0.207</td>
</tr>
<tr>
<td>100&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.945</td>
<td>0.735</td>
<td>0.943</td>
<td>0.736</td>
<td>0.207</td>
</tr>
</tbody>
</table>

<sup>+</sup> indicates results obtained from a second experiment.

O.D.<sub>S</sub>, O.D.<sub>B</sub>, O.D.<sub>MS</sub> and O.D.<sub>GB</sub> are as defined earlier in this section.

O.D.<sub>MB</sub> is the average value of O.D.<sub>B</sub> for a given time point.

The results obtained in the substrate studies are summarized in Fig.I, and are discussed in Section VIIE. It will be evident from the above results, and from Fig.I, that with cis- and trans-4,5-dihydro-5-methylorotic acid as substrates "O.D.<sub>GS</sub>" values cannot validly be used in place of "O.D.<sub>MS</sub>" values for determining ΔO.D. values.
obtained with the second preparation of the enzyme.

The results shown for \( \alpha \)-d-glucopyranose and \( \alpha \)-d-mannopyranose, and the results
obtained with the first preparation of the enzyme, and the results
obtained with the second preparation were

The results shown for \( \alpha \)-d-glucopyranose and \( \alpha \)-d-mannopyranose, and the results
obtained with the first preparation of the enzyme, and the results
obtained with the second preparation were
Moreover, the initial rates of conversion of the two diastereoisomeric 5-methyl-dihydro-orotic acids into 5-methyl-orotic acid cannot be calculated.

**VIID**<sub>2</sub> Enzyme preparation from *Zymabacterium oroticum*

The results obtained with the preparation of dihydro-orotic dehydrogenase from *Zymabacterium oroticum* are described below under the following headings:

(a) Course of purification of the enzyme.
(b) Conversion of dihydro-orotic acid and the 5-methyl analogues into the corresponding "orotic acid".
(c) Analysis for dihydro-orotase activity.
(d) Conversion of orotic acid and its 5-methyl analogue into the corresponding dihydro-derivative.

**VIID**<sub>2a</sub> Course of purification of the enzyme

A unit of enzymic activity has been defined by Friedmann and Vennesland as the amount of enzyme which causes the oxidation of 1μ mole of NADH per minute, in the assay system described in Section **VIIC**<sub>2a</sub>, at 20° [21].

In this investigation the temperature at which the assay was carried out varied between 22 and 24° on different days.
The *Zymobacterium oroticum* was grown under anaerobic conditions at 30°, as described in the literature [18,22], see Section VII B2. Earlier workers [18,21] reported that the growth of the organism *Z. oroticum* (cf. Section VII B2) at 30° reached a plateau within 24 hours and that the cells should be harvested within 15–20 hours of inoculation of the medium, because with longer times the yield of enzyme per litre of medium actually decreased. However, in this investigation it was found that very little growth had occurred in 24 hours at 30°, and the cells were in fact harvested after 48 hours. Analysis of the cell-free extract showed that approximately 2.7x10^4 units of enzyme/g of wet packed cells were obtained, which is similar to the value given by Friedmann and Vennesland [22] for cells harvested within 24 hours of the inoculation of the medium, although the yield of cells was lower than published yields (Section VII B2). Furthermore, examination of the growth medium under the microscope after 48 hours incubation at 30° showed the presence of strings of elongated cells, as Waschman and Barker [19] described for the organism. These were not seen 24 hours after the inoculation of the growth medium. The fact that the organism required a longer growth time in this investigation than was found necessary by earlier workers was
probably due to a difference in the size of the innoculums used to initiate growth. However, further work is warranted on the relationship between the enzyme activity and the time of growth of the organism.

During the course of the purification of the enzyme preparation several interesting points arose. Friedmann and Vennesland [22] reported that the nucleic acids and some proteins were precipitated out from the cell-free extract, in 0.4M saline, on the addition of 0.4 to 0.5 volumes of 1% protamine sulphate in 0.4M saline. However, in this investigation it was found that no further precipitation took place upon addition of protamine solution in excess of 0.3 volumes. Furthermore, it was found that if the protamine was not added slowly with adequate mixing co-precipitation of the enzyme also occurred and this was independent of the total volume of protamine solution added.

**Assay results**

The first extract of cell-free material (12 ml.) (see Section VIIB, p181) contained 1.5 x 10^5 units of enzyme; the second extract (14 ml.) contained 6.4 x 10^4 units. Altogether 2.14 x 10^5 units were obtained from 8 g. of wet packed cells, i.e. approximately 2.7 x 10^4 units per g. of wet packed cells, which is the same yield as reported by
Friedmann and Vennesland [22]. For the purification process, carried out on the first cell-free extract, previously described (see Section VIIB2), the following results were obtained:

**Purification of dihydro-orotic dehydrogenase**

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Volume (ml)</th>
<th>Total number of enzyme units</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>12</td>
<td>$1.5 \times 10^5$</td>
<td>(100)</td>
</tr>
<tr>
<td>Supernatant after protamine treatment</td>
<td>17</td>
<td>$6.4 \times 10^4$</td>
<td>43</td>
</tr>
<tr>
<td>Supernatant after 1st $(\text{NH}_4)_2\text{SO}_4$ addition</td>
<td>19</td>
<td>$6.1 \times 10^4$</td>
<td>41</td>
</tr>
<tr>
<td>0.2M Sodium phosphate, pH 5.8, extract</td>
<td>1.5</td>
<td>$3.0 \times 10^4$</td>
<td>20</td>
</tr>
</tbody>
</table>

The overall yield was 20% as compared with 57% reported by Friedmann and Vennesland [22]. The large difference in the yields could possibly be due to the fact that we were necessarily working on a much smaller scale than they did and hence normal experimental losses were more significant in this investigation. However, the explanation lies mainly in the fact that we were comparatively inexperienced in the techniques of enzyme purification, whereas they had been
working on the isolation and purification of the enzyme for several years; this point is born out by comparison of their earlier [21] and later [22] papers with respect to the recovery in the purified product of the activity present at the outset. In the first instance [21] they report an overall yield of 14% with a specific activity of 4,350 units/mg. of protein, whereas later [22] they report an overall yield of 19% with a specific activity of 17,700 units/mg. of protein.

The enzyme preparation used for studying the conversion of dihydro-orotic acid into orotic acid was obtained by diluting an aliquot (1.3 ml.) of the 0.2M, pH 5.8, phosphate buffer extract, with 0.2M, pH 8.0, tris buffer (1.3 ml.). Therefore, this diluted preparation contained $1 \times 10^4$ units/ml., as was confirmed by assay for NADH oxidation by the procedure outlined in Section VIIIC2a.

The protein content of the diluted enzyme preparation was determined by the method of Lowry et al. [95] with bovine albumin as standard, and values of 21–22 mg. of protein/ml. were obtained compared with approximately 12 mg./ml. as reported by Friedmann and Vennesland [22] at the same stage in the purification process. Thus, the specific activity of the enzyme preparation used in the present investigation was 480 units/mg. of protein as compared with 1,920 units per mg.
of protein obtained by Friedmann and Vennesland at the same stage in the purification process.

VIID$_2$ Conversion of dihydro-orotic acid and the 5-methyl analogues into the corresponding "orotic acid"

For such comparisons of different substrates it is important that the substrate rather than the enzyme should be limiting. Therefore, pilot experiments were carried out with D$_4$-dihydro-orotic acid as substrate. The initial rate of reaction over the first 5 minutes, for a substrate concentration of 21.3 $\mu$ mole/ml., was 0.048 optical density units/min., and that for a substrate concentration of 2.13 $\mu$ mole/ml. was 0.035 optical density units/min. These experiments were carried out on the same day (first) as the final diluted enzyme preparation was obtained, and subsequent experiments were carried out 3-4 days later. As discussed in Section VII$_2$ there was a significant difference between the initial rates of conversion of dihydro-orotic acid into orotic acid observed on the first and fourth days, under similar conditions.

The results obtained on the third and fourth days are tabulated below. The values represent $\Delta$O,D., viz.,
the difference in optical density at 282μ between the "experimental" (i.e. with substrate) and the "experimental Blank". A value of 7.5 optical density units would correspond to the formation of 1 μ mole of orotic acid per ml., and a value of 6.87 units would correspond to the formation of 1 μ mole of 5-methylorotic acid per ml.
**DL-Dihydro-orotic acid as substrate (A.O.D. values)**

<table>
<thead>
<tr>
<th>time (min.)</th>
<th>42.6</th>
<th>21.3</th>
<th>1.065</th>
<th>0.532</th>
<th>0.266</th>
<th>0.213</th>
<th>0.160</th>
<th>0.1065</th>
<th>0.080</th>
<th>0.0532</th>
<th>0.026</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.026</td>
<td>0.028</td>
<td>0.029</td>
<td>0.032</td>
<td>0.037</td>
<td>0.037</td>
<td>0.040</td>
<td>0.034</td>
<td>0.025</td>
<td>0.021</td>
<td>0.013</td>
</tr>
<tr>
<td>2</td>
<td>0.061</td>
<td>0.065</td>
<td>0.071</td>
<td>0.074</td>
<td>0.081</td>
<td>0.090</td>
<td>0.077</td>
<td>0.083</td>
<td>0.072</td>
<td>0.064</td>
<td>0.063</td>
</tr>
<tr>
<td>3</td>
<td>0.097</td>
<td>0.101</td>
<td>0.110</td>
<td>0.108</td>
<td>0.123</td>
<td>0.135</td>
<td>0.126</td>
<td>0.127</td>
<td>0.116</td>
<td>0.105</td>
<td>0.095</td>
</tr>
<tr>
<td>4</td>
<td>0.128</td>
<td>0.127</td>
<td>0.141</td>
<td>0.144</td>
<td>0.156</td>
<td>0.167</td>
<td>0.158</td>
<td>0.165</td>
<td>0.141</td>
<td>0.141</td>
<td>0.127</td>
</tr>
<tr>
<td>5</td>
<td>0.156</td>
<td>0.155</td>
<td>0.167</td>
<td>0.169</td>
<td>0.187</td>
<td>0.197</td>
<td>0.198</td>
<td>0.198</td>
<td>0.168</td>
<td>0.174</td>
<td>0.153</td>
</tr>
<tr>
<td>6</td>
<td>0.186</td>
<td>0.179</td>
<td>0.194</td>
<td>0.192</td>
<td>0.210</td>
<td>0.214</td>
<td>0.221</td>
<td>0.225</td>
<td>0.192</td>
<td>0.186</td>
<td>0.172</td>
</tr>
<tr>
<td>7</td>
<td>0.214</td>
<td>0.205</td>
<td>0.219</td>
<td>0.212</td>
<td>0.236</td>
<td>0.243</td>
<td>0.245</td>
<td>0.248</td>
<td>0.212</td>
<td>0.217</td>
<td>0.199</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>0.242</td>
<td>0.232</td>
<td>0.258</td>
<td>0.266</td>
<td>0.263</td>
<td>0.268</td>
<td>0.227</td>
<td>0.236</td>
<td>0.201</td>
</tr>
<tr>
<td>9</td>
<td>0.255</td>
<td>0.246</td>
<td>0.261</td>
<td>0.252</td>
<td>0.276</td>
<td>0.282</td>
<td>0.291</td>
<td>0.292</td>
<td>0.244</td>
<td>0.252</td>
<td>0.215</td>
</tr>
<tr>
<td>10</td>
<td>0.276</td>
<td>0.266</td>
<td>0.282</td>
<td>0.272</td>
<td>0.296</td>
<td>0.299</td>
<td>0.308</td>
<td>0.304</td>
<td>0.263</td>
<td>0.265</td>
<td>0.224</td>
</tr>
<tr>
<td>15</td>
<td>0.376</td>
<td>0.363</td>
<td>0.366</td>
<td>0.358</td>
<td>0.377</td>
<td>0.376</td>
<td>0.381</td>
<td>-</td>
<td>0.319</td>
<td>0.316</td>
<td>0.243</td>
</tr>
<tr>
<td>20</td>
<td>0.461</td>
<td>0.445</td>
<td>0.438</td>
<td>0.422</td>
<td>0.447</td>
<td>0.435</td>
<td>0.440</td>
<td>0.415</td>
<td>0.358</td>
<td>0.342</td>
<td>0.245</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>-</td>
<td>0.558</td>
<td>-</td>
<td>0.555</td>
<td>0.518</td>
<td>0.520</td>
<td>-</td>
<td>0.402</td>
<td>-</td>
<td>0.248</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td>0.735</td>
<td>-</td>
<td>0.660</td>
<td>0.563</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>-</td>
<td>-</td>
<td>0.800</td>
<td>-</td>
<td>0.685</td>
<td>0.580</td>
<td>0.605</td>
<td>-</td>
<td>0.435</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
**L-Dihydro-orotic acid as substrate (Δ O.D. values)**

<table>
<thead>
<tr>
<th>time (t min.)</th>
<th>Initial concentration of L-dihydro-orotic acid (μ mole/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.13</td>
</tr>
<tr>
<td>1</td>
<td>0.027</td>
</tr>
<tr>
<td>2</td>
<td>0.074</td>
</tr>
<tr>
<td>3</td>
<td>0.115</td>
</tr>
<tr>
<td>4</td>
<td>0.147</td>
</tr>
<tr>
<td>5</td>
<td>0.170</td>
</tr>
<tr>
<td>6</td>
<td>0.193</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.232</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.269</td>
</tr>
<tr>
<td>15</td>
<td>0.346</td>
</tr>
</tbody>
</table>
Trans-4,5-dihydro-5-methylorotic acid as substrate (O.D.values)

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Initial concentration of substrate (µ mole/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.13</td>
<td>1.065 0.532 0.266 0.213 0.160 0.1065 0.060 0.0532 0.0266</td>
</tr>
<tr>
<td>1</td>
<td>0.026 0.012 0.030 0.028 - 0.030 0.020 0.014 0.018 0.004</td>
</tr>
<tr>
<td>2</td>
<td>0.063 0.062 0.086 0.077 0.069 0.068 0.048 0.039 0.040 0.016</td>
</tr>
<tr>
<td>3</td>
<td>0.100 0.102 0.127 0.113 0.124 0.110 0.090 0.064 0.063 0.026</td>
</tr>
<tr>
<td>4</td>
<td>0.130 0.136 0.159 0.143 0.166 0.144 0.128 0.083 0.076 0.032</td>
</tr>
<tr>
<td>5</td>
<td>0.153 0.163 0.192 0.171 0.198 0.166 0.153 0.095 0.088 0.036</td>
</tr>
<tr>
<td>6</td>
<td>0.174 0.187 0.217 0.197 0.224 0.188 0.174 0.104 0.093 0.040</td>
</tr>
<tr>
<td>7</td>
<td>0.196 0.209 0.244 0.216 0.245 0.207 0.190 0.112 0.098 0.041</td>
</tr>
<tr>
<td>8</td>
<td>0.218 0.232 0.261 0.233 0.262 0.223 0.204 0.118 0.102 0.043</td>
</tr>
<tr>
<td>9</td>
<td>0.243 0.268 0.287 0.250 0.283 0.233 0.217 0.122 0.102 0.044</td>
</tr>
<tr>
<td>10</td>
<td>0.264 0.277 0.301 0.266 0.298 0.248 0.226 0.123 0.102 -</td>
</tr>
<tr>
<td>15</td>
<td>- - 0.396 0.333 0.368 0.282 0.251 0.128 0.102 0.044</td>
</tr>
<tr>
<td>20</td>
<td>0.408 - 0.468 0.432 0.419 0.296 0.258 0.128 0.102 0.044</td>
</tr>
</tbody>
</table>
### Cis-4,5-dihydro-5-methylorotic acid as substrate

**A O.D. values**

<table>
<thead>
<tr>
<th>time (t min.)</th>
<th>Initial concentration of substrate (μ mole/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.65</td>
</tr>
<tr>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td>0.007</td>
</tr>
<tr>
<td>3</td>
<td>0.007</td>
</tr>
<tr>
<td>4</td>
<td>0.012</td>
</tr>
<tr>
<td>5</td>
<td>0.017</td>
</tr>
<tr>
<td>6</td>
<td>0.017</td>
</tr>
<tr>
<td>7</td>
<td>0.018</td>
</tr>
<tr>
<td>8</td>
<td>0.020</td>
</tr>
<tr>
<td>9</td>
<td>0.022</td>
</tr>
<tr>
<td>10</td>
<td>0.024</td>
</tr>
<tr>
<td>15</td>
<td>0.024</td>
</tr>
<tr>
<td>20</td>
<td>0.024</td>
</tr>
<tr>
<td>30</td>
<td>0.024</td>
</tr>
<tr>
<td>60</td>
<td>0.030</td>
</tr>
</tbody>
</table>
The results are expressed graphically as a plot of the observed change in absorbance of the xanthine (t) minutes after addition of the substrate oxidase.
The initial rates of reaction, measured over the first 5 minutes of reaction at each substrate concentration studied, were obtained graphically and are tabulated below for each of the substrates.

<table>
<thead>
<tr>
<th>Substrate concentration (S)</th>
<th>Rate of change of optical density per min. for the first 5 mins. (v; ΔO.D./t.)</th>
<th>DL</th>
<th>L</th>
<th>trans</th>
<th>cis</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ mole/ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42.6</td>
<td></td>
<td>0.031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21.3</td>
<td></td>
<td>0.031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10.065</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0015</td>
</tr>
<tr>
<td>2.13</td>
<td></td>
<td>0.035</td>
<td>0.035</td>
<td>0.031</td>
<td>-</td>
</tr>
<tr>
<td>1.065</td>
<td></td>
<td>0.035</td>
<td>0.035</td>
<td>0.032</td>
<td>&lt;0.0015</td>
</tr>
<tr>
<td>0.532</td>
<td></td>
<td>0.038</td>
<td>-</td>
<td>0.039</td>
<td>-</td>
</tr>
<tr>
<td>0.266</td>
<td></td>
<td>0.040</td>
<td>-</td>
<td>0.036</td>
<td>-</td>
</tr>
<tr>
<td>0.213</td>
<td></td>
<td>0.040</td>
<td>-</td>
<td>0.039</td>
<td>&lt;0.0015</td>
</tr>
<tr>
<td>0.160</td>
<td></td>
<td>0.040</td>
<td>-</td>
<td>0.034</td>
<td>-</td>
</tr>
<tr>
<td>0.1065</td>
<td></td>
<td>0.035</td>
<td>-</td>
<td>0.032</td>
<td>&lt;0.0015</td>
</tr>
<tr>
<td>0.080</td>
<td></td>
<td>0.034</td>
<td>-</td>
<td>0.020</td>
<td>-</td>
</tr>
<tr>
<td>0.0532</td>
<td></td>
<td>0.031</td>
<td>0.031</td>
<td>0.019</td>
<td>&lt;0.0015</td>
</tr>
<tr>
<td>0.0266</td>
<td></td>
<td>0.026</td>
<td>0.027</td>
<td>0.0075</td>
<td>-</td>
</tr>
</tbody>
</table>

DL denotes DL-dihydro-orotic acid.
L denotes L-dihydro-orotic acid.
trans denotes trans-4,5-dihydro-5-methylorotic acid.
cis denotes cis-4,5-dihydro-5-methylorotic acid.
The reciprocal values of concentration (S) and rate (v) as used for the Lineweaver-Burk [101] plots are given below; for L-dihydro-orotic acid as substrate too few results were obtained to allow a meaningful plot to be made.

<table>
<thead>
<tr>
<th>(μ mole/ml.)⁻¹(1/S)</th>
<th>rate⁻¹(1/v)</th>
<th>DL</th>
<th>Trans</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0235</td>
<td>32.2</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.0470</td>
<td>32.2</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.47</td>
<td>28.6</td>
<td></td>
<td>32.2</td>
</tr>
<tr>
<td>0.94</td>
<td>28.6</td>
<td></td>
<td>31.3</td>
</tr>
<tr>
<td>1.88</td>
<td>26.4</td>
<td></td>
<td>25.7</td>
</tr>
<tr>
<td>3.76</td>
<td>25.0</td>
<td></td>
<td>27.8</td>
</tr>
<tr>
<td>4.70</td>
<td>25.0</td>
<td></td>
<td>25.7</td>
</tr>
<tr>
<td>6.25</td>
<td>24.4</td>
<td></td>
<td>29.5</td>
</tr>
<tr>
<td>9.40</td>
<td>28.6</td>
<td></td>
<td>31.3</td>
</tr>
<tr>
<td>12.50</td>
<td>29.4</td>
<td></td>
<td>50.0</td>
</tr>
<tr>
<td>18.80</td>
<td>32.2</td>
<td></td>
<td>52.8</td>
</tr>
<tr>
<td>37.60</td>
<td>38.5</td>
<td></td>
<td>133.5</td>
</tr>
</tbody>
</table>

From the Lineweaver-Burk plot (Fig.3) an apparent Michaelis constant, \( K_M \), of \( 1.87 \times 10^{-5} \)M at pH 8.0 was obtained for DL-dihydro-orotic acid as substrate. The significance of the result is discussed in Section VIIIE2.
Fig. 3. A plot of $\frac{1}{A}$ against $\frac{1}{d}$ according to the method of Lineweaver and Burk. The Hill's constant ($H$) was obtained from the above plot and the equation $A + \frac{4}{A} = \frac{4}{H}$. The trans - 4,5-dihydro-5-methylarotic acid and dihydro-arotic acid are represented by $\square$ and $\bigcirc$ respectively.
The reciprocal values of concentration \( S \) and rate \( v \) as used for the Lineweaver-Burk [101] plots are given below; for \( \text{L-} \)dihydro-orotic acid as substrate too few results were obtained to allow a meaningful plot to be made.

<table>
<thead>
<tr>
<th>(( \mu \text{ mole/ml.}))^{-1}(\frac{1}{S})</th>
<th>rate^{-1}(\frac{1}{v})</th>
<th>DL</th>
<th>Trans</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0235</td>
<td>32.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.0470</td>
<td>32.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.47</td>
<td>28.6</td>
<td>32.2</td>
<td></td>
</tr>
<tr>
<td>0.94</td>
<td>28.6</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>1.88</td>
<td>26.4</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>3.76</td>
<td>25.0</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>4.70</td>
<td>25.0</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>24.4</td>
<td>29.5</td>
<td></td>
</tr>
<tr>
<td>9.40</td>
<td>28.6</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>12.50</td>
<td>29.4</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>18.80</td>
<td>32.2</td>
<td>52.8</td>
<td></td>
</tr>
<tr>
<td>37.60</td>
<td>38.5</td>
<td>133.5</td>
<td></td>
</tr>
</tbody>
</table>

From the Lineweaver-Burk plot (Fig. 3) an apparent Michaelis constant, \( K_M^{\text{x}} \), of \( 1.87 \times 10^{-5} \text{M} \) at pH 8.0 was obtained for \( \text{DL-} \)dihydro-orotic acid as substrate. The significance of the result is discussed in Section VIIIE2.
The Michaelis constant ($K_M$) is defined as the value of $[S]$ when $v = V/2$, where $v$ is the velocity of appearance or disappearance of substrate at a given substrate concentration $[S]$ and $V$ is the velocity when the enzyme is saturated with substrate. In the Lineweaver-Burk plot the rate equation is expressed in the form

$$\frac{1}{V} = \frac{K_M}{V} \cdot \frac{1}{S} + \frac{1}{V}$$

Therefore, from a plot of $\frac{1}{V}$ against $\frac{1}{S}$ a straight line is obtained, and from the intercept with the base line (i.e. when $\frac{1}{V} = 0$) $\frac{1}{S} = -\frac{1}{K_M}$. $K_M$ may also be obtained from the intercept with the vertical axis (i.e. when $\frac{1}{S} = 0$) and the gradient of the line ($\frac{K_M}{V}$).

Conversion of dihydro-orotic acid into orotic acid in the presence of NAD$^+$

The effect of NAD$^+$ on the rate of conversion of dihydro-orotic acid into orotic acid was studied. For substrate concentrations of 21.3 µ mole/ml and 2.13 µ mole/ml, the initial rate of formation of orotic acid was unaffected by the addition of (NAD$^+$) ($2.0 \times 10^{-4}M$ or $1 \times 10^{-5}M$).
A study of the effect of cis-4,5-dihydro-5-methylorotic acid on the rate of conversion of DL-dihydro-orotic acid into orotic acid.

The effect of the presence of cis-4,5-dihydro-5-methylorotic acid on the rate of conversion of DL-dihydro-orotic acid into orotic acid was also studied. A system analogous to that described for following the conversion of dihydro-orotic acid into orotic acid was used, except that the cis-isomer was added to both the blank and the experimental. For a given series of experiments the concentration of DL-dihydro-orotic acid was kept constant and the concentration of the cis-isomer varied. The experiments were carried out with DL-dihydro-orotic acid at concentrations of 2.13 μ mole/ml. (I) and 0.213 μ mole/ml. (II), and the results obtained are given herewith. The values represent the differences between the optical density at 282μ of the "experimental" (i.e. with DL-dihydro-orotic acid) and "experimental blank".
The corresponding rates of change of optical density/ min. over the first five minutes of reaction (v; ΔO.D./t) are:

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Concentration of cis-4,5-dihydro-5-methylorotic acid/μ mole/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.65</td>
</tr>
<tr>
<td>1</td>
<td>0.029</td>
</tr>
<tr>
<td>2</td>
<td>0.070</td>
</tr>
<tr>
<td>3</td>
<td>0.110</td>
</tr>
<tr>
<td>4</td>
<td>0.140</td>
</tr>
<tr>
<td>5</td>
<td>0.166</td>
</tr>
<tr>
<td>6</td>
<td>0.194</td>
</tr>
<tr>
<td>7</td>
<td>0.218</td>
</tr>
<tr>
<td>8</td>
<td>0.242</td>
</tr>
<tr>
<td>9</td>
<td>0.260</td>
</tr>
<tr>
<td>10</td>
<td>0.282</td>
</tr>
<tr>
<td>15</td>
<td>0.368</td>
</tr>
<tr>
<td>20</td>
<td>0.436</td>
</tr>
</tbody>
</table>
Further substrate studies were carried out with a preparation of dihydro-orotic acid dehydrogenase obtained from a different batch of *E. oroticum* cells, grown and isolated as before (see Section VIIB2). The final enzyme preparation (i.e. the 0.2M phosphate, pH 5.8, extract) contained approximately $4 \times 10^3$ units of enzyme per ml. All experiments were carried out with an initial substrate concentration of 2.13 μmole/ml. of assay system. Assays were performed with each of the substrates, 3 - 4 days after the enzyme became available, and the following results were obtained:

<table>
<thead>
<tr>
<th>Concentration of cis isomer μmole/ml.</th>
<th>I 2.13 μmole/ml.</th>
<th>II 0.213 μmole/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.65</td>
<td>(V) 0.34</td>
<td>(V) 0.40</td>
</tr>
<tr>
<td>1.065</td>
<td>0.34</td>
<td>0.40</td>
</tr>
<tr>
<td>0.1065</td>
<td>0.35</td>
<td>0.40</td>
</tr>
<tr>
<td>0.0532</td>
<td>0.34</td>
<td>0.40</td>
</tr>
<tr>
<td>0.000</td>
<td>0.35</td>
<td>0.40</td>
</tr>
</tbody>
</table>
L denotes L-dihydro-orotic acid.

DL denotes DL-dihydro-orotic acid.

cis denotes cis-4,5-dihydro-5-methylorotic acid.

trans denotes trans-4,5-dihydro-5-methylorotic acid.

The values represent the difference in optical density at 282 μm between the "experimental" (i.e., with substrate) and the "experimental blank". A value of 7.5 optical density units would correspond to the formation of 10⁻⁶ mole of orotic acid per ml., and a value of 6.87 units would correspond to the formation of 10⁻⁶ mole of 5-methylorotic acid/ml.

<table>
<thead>
<tr>
<th>time (t min.)</th>
<th>L</th>
<th>DL</th>
<th>cis</th>
<th>trans</th>
<th>DL + cis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.011</td>
<td>0.010</td>
<td>0.002</td>
<td>0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>2</td>
<td>0.033</td>
<td>0.031</td>
<td>0.004</td>
<td>0.033</td>
<td>0.032</td>
</tr>
<tr>
<td>3</td>
<td>0.047</td>
<td>0.049</td>
<td>0.007</td>
<td>0.050</td>
<td>0.050</td>
</tr>
<tr>
<td>4</td>
<td>0.068</td>
<td>0.067</td>
<td>-</td>
<td>0.068</td>
<td>0.068</td>
</tr>
<tr>
<td>5</td>
<td>0.085</td>
<td>0.083</td>
<td>0.008</td>
<td>0.082</td>
<td>0.084</td>
</tr>
<tr>
<td>6</td>
<td>0.099</td>
<td>0.098</td>
<td>0.009</td>
<td>0.097</td>
<td>0.098</td>
</tr>
<tr>
<td>7</td>
<td>0.110</td>
<td>0.112</td>
<td>0.009</td>
<td>0.115</td>
<td>0.114</td>
</tr>
<tr>
<td>8</td>
<td>0.123</td>
<td>0.124</td>
<td>0.010</td>
<td>0.122</td>
<td>0.121</td>
</tr>
<tr>
<td>9</td>
<td>0.138</td>
<td>0.139</td>
<td>0.011</td>
<td>0.133</td>
<td>0.140</td>
</tr>
<tr>
<td>10</td>
<td>0.152</td>
<td>0.151</td>
<td>0.012</td>
<td>0.148</td>
<td>0.150</td>
</tr>
<tr>
<td>15</td>
<td>0.204</td>
<td>0.203</td>
<td>0.013</td>
<td>0.207</td>
<td>0.203</td>
</tr>
<tr>
<td>20</td>
<td>0.250</td>
<td>0.251</td>
<td>0.014</td>
<td>0.251</td>
<td>0.250</td>
</tr>
<tr>
<td>40</td>
<td>0.386</td>
<td>0.383</td>
<td>0.013</td>
<td>0.410</td>
<td>0.385</td>
</tr>
<tr>
<td>60</td>
<td>0.433</td>
<td>0.432</td>
<td>0.013</td>
<td>0.458</td>
<td>0.437</td>
</tr>
</tbody>
</table>
The corresponding rates of change of optical density/min. for the first five minutes of reaction are:

\[ \Delta O.D./t = 0.017 \ 0.017 \ 0.0016 \ 0.017 \ 0.017 \]

\( \Delta O.D./t \) = rate of change of optical density/min. (determined graphically) for the first five minutes of the reaction.

However, on the day the enzyme became available, with DL dihydro-orotic acid as substrate at the same concentration as above, a value of 0.031 was obtained for \( \Delta O.D./t \). This apparent decrease in enzyme activity over the first three or four days of storage at 0°C accords with the findings for the other preparation of dihydro-orotic dehydrogenase from \( Z. \ oroticum \) used in this investigation (see Section VIID2b, p. 206).

VIID2c Analysis for dihydro-orotase activity

An analogous system to that described for following the conversion of dihydro-orotic acid into orotic acid was used except that DL-carbamoylaspartic acid was used as substrate. The following results were obtained. The values represent the difference in optical density at 282\( \mu \) between the "experimental" (i.e. with DL carbamoylaspartic acid) and the "experimental blank". A change of 7.5 optical density
units would correspond to the formation of 1 μ mole per ml. of orotic acid.

Results of assay for dihydro-orotase activity

<table>
<thead>
<tr>
<th>time (t mins)</th>
<th>Concentration of DL-carbamoylaspartic acid (mole/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 x 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>△ O.D.</td>
</tr>
<tr>
<td>1</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>0.007</td>
</tr>
<tr>
<td>4</td>
<td>0.008</td>
</tr>
<tr>
<td>5</td>
<td>0.008</td>
</tr>
<tr>
<td>10</td>
<td>0.009</td>
</tr>
<tr>
<td>20</td>
<td>0.011</td>
</tr>
<tr>
<td>30</td>
<td>0.012</td>
</tr>
</tbody>
</table>

The significance of these results, together with suggestions for further work on this aspect of the investigation are discussed in Sections VIIID_{2a} and VIE_{3b}.
VIID\textsubscript{2d} Conversion of orotic acid and its 5-methyl analogue into the corresponding dihydro derivative

Preliminary studies were carried out on the effect of the presence of 5-methylorotic acid on the conversion of orotic acid into dihydro-orotic acid. An aliquot of the diluted enzyme preparation (0.03 ml., 1 x 10\textsuperscript{4} units/ml.) was diluted with glass distilled water (5 ml.), and assayed in a system similar to that described for determining dihydro-orotic dehydrogenase activity (see Section VIIC\textsubscript{2e}).

The following systems were set up: (1) A blank (B), i.e. in the absence of orotic acid or the 5-methyl analogue; (2) Orotic acid (0.\text{A}.M) as substrate, in excess; (3) 5-Methylorotic acid (5-Me) as substrate, in excess; (4) Orotic acid and 5-methylorotic acid (5-Me + 0.\text{A}.M) as conjoint substrates.

Each experiment was carried out in duplicate and the results obtained are given below. The values represent the observed change in optical density at 340\textmu m (see Section VIIC\textsubscript{2}) for each system described above. A value of 0.00207 optical density units corresponds to the oxidation of 1 \textmu mole of NADH to NAD\textsuperscript{+}. 
The initial substrate concentration was 0.02M.

The initial rate of reaction (Δ O.D.₃₄₀/min) was calculated for the first three minutes of the reaction and the results are given below.

<table>
<thead>
<tr>
<th>time (min.)</th>
<th>B</th>
<th>B</th>
<th>O.A.</th>
<th>O.A.</th>
<th>5-Me</th>
<th>5-Me</th>
<th>5-Me + O.A.</th>
<th>5-Me + O.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.611</td>
<td>0.607</td>
<td>0.596</td>
<td></td>
<td>0.643</td>
<td>0.628</td>
<td>0.670</td>
<td>0.648</td>
</tr>
<tr>
<td>1.0</td>
<td>0.610</td>
<td>0.606</td>
<td>0.563</td>
<td>0.600</td>
<td>0.639</td>
<td>0.623</td>
<td>0.657</td>
<td>0.639</td>
</tr>
<tr>
<td>1.5</td>
<td>0.610</td>
<td>0.605</td>
<td>0.540</td>
<td>0.577</td>
<td>0.632</td>
<td>0.620</td>
<td>0.649</td>
<td>0.630</td>
</tr>
<tr>
<td>2.0</td>
<td>0.610</td>
<td>0.604</td>
<td>0.517</td>
<td>0.558</td>
<td>0.630</td>
<td>0.618</td>
<td>0.637</td>
<td>0.623</td>
</tr>
<tr>
<td>2.5</td>
<td>0.609</td>
<td>0.604</td>
<td>0.495</td>
<td>0.538</td>
<td>0.626</td>
<td>-</td>
<td>0.633</td>
<td>0.613</td>
</tr>
<tr>
<td>3.0</td>
<td>0.609</td>
<td>0.603</td>
<td>0.469</td>
<td>0.520</td>
<td>0.623</td>
<td>0.617</td>
<td>0.623</td>
<td>0.603</td>
</tr>
<tr>
<td>3.5</td>
<td>0.608</td>
<td>0.602</td>
<td>0.452</td>
<td>0.507</td>
<td>0.620</td>
<td>0.610</td>
<td>0.615</td>
<td>0.597</td>
</tr>
<tr>
<td>4.0</td>
<td>0.608</td>
<td>0.602</td>
<td>0.434</td>
<td>0.490</td>
<td>0.618</td>
<td>0.607</td>
<td>0.608</td>
<td>0.589</td>
</tr>
<tr>
<td>4.5</td>
<td>0.607</td>
<td>0.601</td>
<td>0.418</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.602</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>0.606</td>
<td>0.600</td>
<td>0.400</td>
<td>0.460</td>
<td>0.616</td>
<td>0.600</td>
<td>0.597</td>
<td>0.576</td>
</tr>
<tr>
<td>5.5</td>
<td>0.605</td>
<td>0.600</td>
<td>0.384</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.590</td>
<td>0.569</td>
</tr>
<tr>
<td>6.0</td>
<td>0.604</td>
<td>0.599</td>
<td>0.370</td>
<td>0.437</td>
<td>0.613</td>
<td>0.596</td>
<td>0.582</td>
<td>0.562</td>
</tr>
</tbody>
</table>

The initial substrate concentration was 0.02M.

The initial rate of reaction (Δ O.D.₃₄₀/min) was calculated for the first three minutes of the reaction and the results are given below.
VIIE. DISCUSSION OF RESULTS

VIIE. Studies with an enzyme preparation from Ehrlich ascites tumour cells

Substrate studies with the crude enzyme preparation from Ehrlich ascites tumour cells were severely handicapped because of difficulties in obtaining accurate and reproducible results. A particular difficulty was the very high optical density of the enzyme preparation; this increased during incubation, both in the presence and absence of substrate. As far as can be judged from the limited range of 'experimental blank' values - those from the several kinetic runs (with the same batch of enzyme) being considered together - it appears that the 'blank' results are reproducible, and that the increase in optical density of the blank is approximately linear with time. Nevertheless, because of the high optical density of the perchloric acid-soluble fraction, after deproteinization of the assay system, only semi-quantitative results are achievable for the substrate studies with this assay procedure. However, it was established that L-dihydro-orotic acid was converted into orotic acid at approximately twice the rate of the racemic compound tested at a concentration which was the same if no distinction is made between D and L and which was below the
saturating concentration; this accords with the view that the D isomer is not attacked (see Section IA). Moreover, it was found that the addition of NAD\(^+\) to the assay system did not affect the rate of conversion of dihydro-orotic acid into orotic acid. This latter finding agrees with that of Smith et al. [9] with a preparation of the enzyme from human leucocytes.

The substrate studies with the cis- and trans-4,5-dihydro-5-methylorotic acids furnished some interesting but puzzling results. For the cis-isomer, there is an indication that the compound is initially converted into 5-methylorotic acid (see Fig. I) but later time points give no indication of an increase in the 5-methylorotic acid concentration; on the contrary, the concentration appeared to fall. This fall could be due to the presence of another enzyme for which 5-methylorotic acid is a substrate, but this seems unlikely since similar results were not observed in the studies with dihydro-orotic acid as substrate and the same enzyme preparation. However, for dihydro-orotic acid (L or DL) or for the cis-isomer the linear portion of the plot of the change of optical density against time, on back-extrapolation, did not pass through the origin (see Fig. I). Therefore, it is possible that the initial abrupt increase in optical density is due to some "substrate-enzyme"
interaction, other than actual conversion into "orotic acid". This must remain a guess, since it is difficult to obtain reproducible results with this assay system for time points under five minutes.

For trans-5-methyldihydro-orotic acid little or no conversion into 5-methylorotic acid was detected for the first hour of incubation, but marked activity was detected over the subsequent 40 minutes. Pre-incubation of the trans-isomer in tris buffer, pH 8, at 37.4° for one hour immediately before the assay did not alter the pattern of activity. This negative result at least shows that the delay in the appearance of activity is due rather to some property of the enzyme preparation than to a chemical reaction such as isomerisation of the trans-4,5-dihydro-5-methylorotic acid to the corresponding cis-isomer.

Unfortunately, the substrate studies with the enzyme preparation from Ehrlich ascites tumour cells did not completely provide an answer to the original purpose of the investigation, namely delineation of the steric requirements of the enzyme at the C-5 atom of dihydro-orotic acid. As already stated, no clear-cut results were obtained from the substrate studies with the cis- and trans-4,5-dihydro-5-methylorotic acids, although the results indicated that the trans- but not the cis-isomer was converted into 5-methylorotic acid. Until more work has
been carried out in this connection, particularly to ensure that the assay procedure used is fully valid (which would entail isolation and identification of the reaction products), it is not possible to draw any firm conclusions from the results.

VIIE. Studies with an enzyme preparation from *Zymobacterium oroticum*

As will be discussed below, the substrate studies with a preparation of dihydro-orotic dehydrogenase from *Zymobacterium oroticum* did throw some light on the central topic of this investigation, namely the sterochemical requirements of the enzyme at the C-5 position of dihydro-orotic acid. However, this investigation also raised several queries concerning the properties of the enzyme and the accuracy of the assay methods used.

During the course of the substrate studies the activity of the enzyme preparation, as measured by the rate of conversion of orotic acid into dihydro-orotic acid, was frequently checked, as previously described (see Section VIIC2a). The results, tabulated below, indicate that the activity remained fairly constant over a long period (10 days). It was found, however, that once the enzyme activity started to decline, the rate of loss of activity increased rapidly so much so that after a
further 2 or 3 days the level of activity was almost below the limits of detection.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>1st</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>8th</th>
<th>9th</th>
<th>10th</th>
<th>11th</th>
<th>12th</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/ml. x 10^4</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.99</td>
<td>0.98</td>
<td>0.95</td>
<td>0.42</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* 1st day was the day the enzyme preparation was obtained.

The initial rate of conversion of dihydro-orotic acid into orotic acid was also determined on the same days as the enzyme activity was measured. The results given in the table below indicate a sharp decrease in the capacity to convert dihydro-orotic acid into orotic acid over the first four days.

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Substrate Concentration (μ mole/ml.)</th>
<th>Initial rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>21.3</td>
<td>0.048</td>
</tr>
<tr>
<td>1st</td>
<td>2.13</td>
<td>0.035</td>
</tr>
<tr>
<td>2nd*</td>
<td>2.13</td>
<td>0.031</td>
</tr>
</tbody>
</table>

* Initial rate for the conversion of dihydro-orotic acid into orotic acid, expressed as rate of change of optical density per minute, measured over the first five minutes.

+ The second enzyme preparation contained 4.0 x 10^3 units of enzyme per ml. on the 1st and 4th days.
The results obtained for the subsequent six days (see Section VIID$_2$b), not tabulated, do not indicate any further loss in the capacity to form orotic acid. This pattern of enzyme activity was observed with preparations of the enzyme obtained from two different growths of the organism.

For the conversion of orotic acid into dihydro-orotic acid at substrate concentrations well above enzyme saturation level, the rate of reduction of orotic acid was $10 \mu$ mole/min./ml. of enzyme preparation, whereas for the conversion of dihydro-orotic acid into orotic acid at substrate concentrations approaching enzyme saturation, the rate of formation of orotic acid was $0.7 \mu$ mole/min./ml. of enzyme preparation.

Such a low capacity for the conversion of dihydro-orotic acid into orotic acid, relative to that for the reverse reaction, was probably due to the fact that optimum conditions for the reaction had not been obtained. Previously, other workers [21,24] had reported that although the conversion of dihydro-orotic acid into orotic acid proceeded without added NAD$^+$, addition of NAD$^+$ increased the rate of formation of orotic acid. However, preliminary experiments in the present investigation (see Section VIID$_2$b) indicated that the addition of NAD$^+$ did not affect the rate of conversion of dihydro-orotic acid into orotic acid, although it was later found that the experiments
were carried out at substrate concentrations above enzyme saturation level. Therefore, it would be profitable to study the effect of NAD$^+$ on the rate of conversion of dihydro-orotic acid into orotic acid at substrate concentrations below enzyme saturation level.

The results on the stability of the enzyme preparation indicate that there is a fall in the enzyme activity with respect to the conversion of dihydro-orotic acid into orotic acid, whereas the activity for the reverse reaction, i.e. conversion of orotic acid into dihydro-orotic acid, appears to remain constant. However, in a recent publication on the properties of dihydro-orotic dehydrogenase, Miller and Massey [24] stated that if the assay for the enzyme activity with orotic acid or dihydro-orotic acid as substrate is carried out by following the change in optical density at 340 μm associated with NADH formation or removal, the assay should be carried out under strict anaerobic conditions for valid accurate results. Under aerobic conditions NADH oxidase activity is also measured. Miller and Massey [24] claim that it is not valid to subtract NADH oxidase activity, found in the absence of orotic acid, from the total activity. However, Friedmann and Vennesland [21] considered that such a correction be valid. In the present investigation the conversion of dihydro-orotic acid into orotic
acid was followed at 282 m\textmu, and not at 340 m\textmu; hence the accuracy of the assay is not affected by the presence of NADH oxidase activity. However, the conversion of orotic acid into dihydro-orotic, as followed by NADH oxidation at 340 m\textmu, was not carried out under strictly anaerobic conditions and hence the accuracy of the results may have been affected by the presence of NADH oxidase activity.

A comparison of the results for the conversion of L and DL-dihydro-orotic acids into orotic acid (see Section VIID\textsubscript{2b}), brings out the interesting point that the rates are quite similar over the range of substrate concentrations studied. However, because of the unnoticed decrease in the capacity of the enzyme preparation to convert dihydro-orotic acid into orotic acid, most of the substrate studies were carried out with a saturating amount of substrate; hence, our conclusions have to be based on a few points at low substrate concentrations. These results contradict our findings with the enzyme preparation from Ehrlich ascites tumour cells (see Section VIID\textsubscript{1}), in which L-dihydro-orotic acid was converted into orotic acid at approximately twice the rate at which DL-dihydro-orotic acid was. Moreover, they were unexpected since Liebermann and Kornberg [13] had found that with their preparation of dihydro-orotic dehydrogenase obtained from \textit{Zymobacterium oroticum}, orotic acid was
converted into optically active dihydro-orotic acid, and the L-isomer of carbamoylaspartic acid was also formed. Also, they found that with DL-dihydro-orotic acid as substrate only a 46% yield of orotic acid was obtained. Since only one isomer (L or D) of dihydro-orotic acid was converted into orotic acid, and since orotic acid was converted into L-carbamoylaspartic acid, they concluded that the dihydro-orotic acid formed in the reaction had the L-configuration.

From a Lineweaver-Burk plot of the results obtained for the conversion of DL-dihydro-orotic acid into orotic acid (followed at 282 μm), a Michaelis constant (K_m) of approximately 1.9 x 10^{-5} M, at pH 8, was obtained, as compared with the value of 1.8 x 10^{-5} M reported by Miller and Massay [24] with L-dihydro-orotic acid as substrate. They also reported that if the conversion of dihydro-orotic acid into orotic acid (followed at 340 μm) was carried out under anaerobic conditions a K_m of 1.7 x 10^{-4} M was obtained, a finding perhaps related to suppression of NADH oxidase activity but not lucidly discussed. Furthermore, under anaerobic conditions they did not observe any substrate inhibition, but under aerobic conditions strong substrate inhibition was observed at dihydro-orotic acid concentrations in excess of 1 x 10^{-4} M. The results obtained in the present investigation (see Section VIID_2b) also showed
strong substrate inhibition at substrate concentrations in excess of $5 \times 10^{-4} \text{M}$. Therefore, it would appear that the results obtained in the present investigation with L or DL-dihydro-orotic acid as substrate do not disagree with those obtained with L-dihydro-orotic acid by Miller and Massey [24]; but the close agreement in respect of $K_m$ may be fortuitous.

From the results with the cis- and trans-$(\pm)-4,5$-dihydro-5-methylorotic acids it would appear that the trans-isomer is converted into 5-methylorotic acid whereas the cis-isomer is not. It was also shown that the cis-isomer did not inhibit the conversion of DL-dihydro-orotic acid into orotic acid, over the concentration range studied. Moreover, it was found that over the substrate concentration range used in this investigation DL-dihydro-orotic acid was metabolised at a faster rate than was $(\pm)$-trans-$4,5$-dihydro-5-methylorotic acid (see Section VIID$_2$D$_2$). Substrate inhibition occurred with substrate concentrations in excess of $5 \times 10^{-4} \text{M}$ with the trans-isomer, as with DL-dihydro-orotic acid. However, at low substrate concentrations, i.e. between $1 \times 10^{-4} \text{M}$ and $2.6 \times 10^{-5} \text{M}$, lowering of the concentration caused a relatively greater depression in the observed initial rate with the trans-isomer than with DL-dihydro-orotic acid.

Insufficient results were obtained with the trans-isomer at low substrate concentrations to furnish an accurate value for the
Michaelis constant \( (K_m) \).

The conversion of dihydro-orotic acid into orotic acid in the presence of trans-4,5-dihydro-5-methylorotic acid was not studied in detail, since preliminary experiments with substrate concentrations below saturation level gave no reason to doubt that the activity is then additive. Moreover, at substrate concentrations above saturation level competitive inhibition was observed which is not surprising since trans-4,5-dihydro-5-methylorotic acid is dehydrogenated at a slower rate than dihydro-orotic acid itself.

From our studies with cis and trans-5-methylidihydro-orotic acids it would appear that dihydro-orotic dehydrogenase, isolated from Zymobacterium oroticum, catalyses the removal of hydrogen by a trans elimination, i.e. the hydrogen atom removed from the C-5 atom is trans to the hydrogen atom attached to the C-4 atom. Observations made by Lieberman and Kornberg [13] indicate that the enzyme is stereospecific with respect to the configuration at the C-4 atom of dihydro-orotic acid, L-dihydro-orotic acid being acted upon. These workers had found that when dihydro-orotic acid, obtained by catalytic reduction of orotic acid, was oxidised, under the action of dihydro-orotic
dehydrogenase, 46% of the theoretical yield of orotic acid was obtained. The result suggests that the chemically synthesized dihydro-orotic acid is a racemic mixture of which only one isomer can be enzymatically utilised. Also, they demonstrated that the enzyme converts orotic acid into L-dihydro-orotic acid. However, our results do not accord with a claim that the reaction is stereo-specific with respect to the configuration at the C-4 atom.

Chemically, the biological dehydrogenation which most resembles the conversion of dihydro-orotic acid into orotic acid is the conversion of succinic acid into fumaric acid. Both dihydro-orotic dehydrogenase and succinic dehydrogenase are flavoproteins, containing iron and free sulphydryl groups, but dihydro-orotic dehydrogenase contains both FAD and FMN [26], whereas succinic acid contains FAD only. In containing FAD and FMN dihydro-orotic dehydrogenase is unique amongst flavoproteins. Elimination of hydrogen
from succinic acid under the action of succinic dehydrogenase proceeds by a trans-mechanism; unequivocal evidence for this was obtained by Tchen and Milligan [97], in 1960. An earlier attempt to elucidate the stereochemistry of the reaction had been made by England and Colowick [96]. Deuterated succinic acid, obtained from succinic acid by an anaerobic exchange reaction with $[^2H]_2O$ catalysed by a preparation of succinic dehydrogenase, was treated aerobically with the same enzyme preparation. Since the fumaric acid isolated retained about one-half of the deuterium content of the deuterated succinic acid, it was concluded that either trans or random elimination of hydrogen had occurred; the authors considered that the equivalence of the two methylene groups of succinic acid made unequivocal interpretation of the results impossible. In 1960, Tchen and van Milligan [97] showed that trans-elimination of hydrogen did indeed occur. Meso- and $\pm\alpha-\alpha'$-dideutosuccinic acid were prepared by the catalytic reduction, with deuterium, of maleic
acid and fumaric acid respectively. Each of the dideuterosuccinic acids was treated with succinic dehydrogenase and the fumaric acid formed was isolated. The percentage of dideuterated fumaric acid in the specimen was determined by catalytic hydrogenation of the fumaric acid, with hydrogen, conversion of the "succinic acid" formed into the dimethyl ester and examination of the product by mass spectrometry. Little dideuterated fumaric acid was obtained from (±)-dideuterosuccinic acid, but the "fumaric acid" obtained from meso-dideuterosuccinic acid consisted of 50% dideuterofumaric acid and 50% unlabelled fumaric acid. Thus, trans-elimination had been demonstrated.

\[
\begin{align*}
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H}
\end{align*}
\]

\[
\begin{align*}
\text{succinic dehydrogenase} & \quad \rightarrow \\
\text{C}^2\text{HCO}_2\text{H} & \quad \text{CHCO}_2\text{H}
\end{align*}
\]

(±)-dideuterosuccinic acid

\[
\begin{align*}
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H}
\end{align*}
\]

\[
\begin{align*}
\text{succinic dehydrogenase} & \quad \rightarrow \\
\text{C}^2\text{HCO}_2\text{H} & \quad \text{CHCO}_2\text{H} \\
\text{C}^2\text{HCO}_2\text{H} & \quad \text{CHCO}_2\text{H}
\end{align*}
\]

meso-dideuterosuccinic acid

\[
\begin{align*}
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H} \\
\text{H} & \quad \text{CO}_2\text{H}
\end{align*}
\]

dideuterofumaric acid

fumaric acid
because if cis-elimination had occurred (±)-dideuterosuccinic acid would have given dideutero-fumaric acid and fumaric acid, whereas the meso-dideuterosuccinic acid would have given mono-deutero-fumaric acid only. Moreover, if random elimination had occurred a mixture of the three "fumaric acids" would have been obtained from either of the dideuterated succinic acids.

It is useful to reconsider the findings of England and Colowick [96]. Thus, the deuterated succinic acid, obtained from succinic acid by an anaerobic exchange reaction with [²H]₂O catalysed by a preparation of succinic dehydrogenase, would be meso-dideuterosuccinic acid, which when treated aerobically with the same enzyme preparation would furnish a mixture of dideutero-fumaric acid and fumaric acid, although not necessarily in equal proportions. England and Colowick [96] determined only total deuterium content and did not differentiate between the mono- and di-deutero-fumaric acids. It is important to remember that the ratio of the dideuterated and unlabelled fumaric acids need not necessarily be 50 : 50; thus, Thorn [99] found that α,α'-dideuterosuccinic acid (A) and α,α,α',α'-tetradeuterosuccinic acid (B) were oxidised at approximately 70% and 40% respectively of the rate that succinic acid was.
Further light was shed on the stereochemistry of the succinic dehydrogenase reaction, in 1961, [98] when it was shown that succinic dehydrogenase converted L-chlorosuccinic acid and L-methylsuccinic acid into chlorofumaric acid and methylfumaric acid respectively. Moreover, it was found that whilst D-chlorosuccinic acid was a very poor substrate for succinic dehydrogenase, D-methylsuccinic acid was a competitive inhibitor. Also, it was reported that L-methylsuccinic acid was dehydrogenated at a slower rate than was succinic acid itself, although L-chlorosuccinic acid is converted into the chloro analogue at the same rate. It would seem that in this respect dihydro-orotic dehydrogenase behaves similarly. 4,5-Dihydro-orotic acid substituted at C-5 might be expected to be converted into the substituted orotic acid (if reaction occurs at all) at a slower rate than 4,5-dihydro-orotic acid is converted into orotic acid, provided that steric inhibition is the major factor in determining the relative rates. Thus, the fact that trans-4,5-dihydro-5-methylorotic acid was shown to be dehydrogenated at a slower
rate than dihydro-orotic acid itself, is not unexpected; indeed, it is gratifying that activity was demonstrated, in view of the possibility that 5-methylorotic acid would inhibit the reaction [22]. Friedmann and Vennesland [22] had already claimed that 5-methylorotic acid inhibited the conversion of orotic acid into dihydro-orotic acid by 50% and, furthermore, they claimed that 5-methylorotic acid was an inactive substrate for the system. However, all their work with 5-methylorotic acid as substrate was carried out at substrate concentrations which were above enzyme saturation level, and as discussed in Section VII Eb, further substrate studies at low substrate concentrations are required. Moreover, in this investigation (see Section VII C 2a) it was found that even at substrate concentrations above enzyme saturation level 5-methylorotic acid was an active substrate but much less active than orotic acid itself.

VII Eb. Suggestions for further work

VII Eb 3a Problems related to the isolation and purification of dihydro-orotic dehydrogenase from *Zymobacterium oroticum*

(a) As already suggested, further experiments should be carried out to establish what growth time for the *Zymobacterium oroticum* gives maximum enzyme activity per gram of wet packed
cells. Also, it is desirable to check whether inoculum size effects the growth rate of the bacterium since in the present investigation the rate was slower than that found by earlier workers [18,21].

(b) A comparison of the values for the conversion of orotic acid into dihydro-orotic acid determined under anaerobic and aerobic conditions, as followed by NADH oxidation at 340 m\u00b4.

(c) Another point worthy of investigation is the question of the stability of the enzyme upon storage. Friedmann and Venessland [22] reported that storage of the crystalline enzyme stored in its own mother liquor at 4° for over a month did not result in significant loss in activity. However, as reported in Section VIIB, the enzyme preparation used in this investigation was only relatively stable for 10 days when stored at 0-4°. Moreover, it was found that the storage of the enzyme at -25° for three days irreversibly deactivated the enzyme. There is no comment in the literature on the stability of a frozen preparation of the enzyme except for the comment by Miller and Massey [24] that some activity, though not all, was lost upon storage (conditions not specified) followed by several freeze-thawings. One possible line of approach to this problem of the effect of storage, on enzyme stability, would be to store samples of the enzyme at 0° and -25° for known periods of time and then
determine the enzyme activity (rate of conversion of orotic acid into dihydro-orotic acid) and the rate of conversion of dihydro-orotic acid into orotic acid. These results could then be compared with the results for the fresh enzyme preparation. Particular attention should be paid to the first 3 or 4 days after the preparation of the enzyme, because during this period the decrease in activity for the dihydro-orotic acid into orotic acid reaction was not accompanied by a decrease in activity for the reverse reaction.

(d) In an attempt to assess whether dihydro-orotase was present in the enzyme preparation used for studying the rate of conversion of dihydro-orotic acid into orotic acid, preliminary experiments were carried out under similar conditions but with DL-carbamoylaspartic acid as substrate (see Section VII C2c). The results (see Section VII D2c) indicated the absence of any strong dihydro-orotase activity. However, since the need is for a measure of the capacity of the assay system to convert dihydro-orotic acid into carbamoylaspartic acid, rather than of the reverse reaction, a more suitable line of approach would be to isolate and estimate the amount of carbamoylaspartic acid finally present in the reaction mixture with dihydro-orotic acid as substrate. This problem is probably best tackled by the use of $^{14}$C-dihydro-orotic acid as substrate, followed by estimation
of any $[^{14}C]$-carbamoylaspartic acid formed. The carbamoyl-
aspartic acid can be separated from the accompanying dihydro-
orotic acid and orotic acid by ion-exchange chromatography [12].

VIE. Further substrate studies

The substrate studies carried out in this investigation left several unanswered questions, which might be answerable through the approach suggested below:

(i) In order to clarify whether the $L$- and the $DL$-dihydro-
orotic acids are indeed converted into orotic acid at the same rate, substrate studies with $L$, $D$, and $DL$ dihydro-orotic acids should be carried out over a wide range of concentrations, up to saturating concentration.

(ii) To obtain further information concerning the steric requirements of the enzyme at the C-5 atom of dihydro-orotic acid the resolution of the racemic trans-isomer is required, together with a study on the rate of conversion of each optically active isomer into 5-methylorotic acid. Moreover, to obtain further evidence that the trans-isomer is indeed being converted into 5-methylorotic acid, attempts should be made to isolate 5-methylorotic acid from the reaction medium.

Since no apparent dehydrogenation of cis-4,5-dihydro-5-
methylorotic acid occurred under the action of dihydro-orotic
dehyd.rogenase, and since it has been found that this compound does not inhibit the conversion of DL-dihydro-orotic acid into orotic acid, no further work with this isomer is suggested.

(iii) Two interesting points connected with the question of the stereochemical requirements of the enzyme dihydro-orotic dehydrogenase arose from the work of Friedmann and Vennesland [21,22].

5-Fluro-orotic acid was shown to be metabolised at a slightly faster rate than was orotic acid [21]. Therefore, if it can be shown that 5-fluoro-orotic acid is indeed reduced to 5-fluoro-4,5-dihydro-orotic acid, and this can be isolated, a comparison of its nuclear magnetic resonance spectrum with that of each of the two diastereoisomeric 5-methyldihydro-orotic acids might give an indication as to whether the cis- or trans-addition of hydrogen occurs. Thereby, supporting evidence may be obtained as to the stereochemical requirements of the enzyme at the C-5 atom of dihydro-orotic acid. Another approach to this problem would be to study the rate of conversion of the two diastereoisomeric racemic 5-fluoro-dihydro-orotic acids into 5-fluoro-orotic acid. Unfortunately, as previously discussed in Section V, these substrates are not readily synthesised, and would be expensive. Thus, in this case it may be more expedient to study the conversion of 5-fluoro-orotic acid into the dihydro
(iv) From studies on the conversion of orotic acid into dihydro-orotic acid, Friedmann and Vennesland [22] made the vague statement that "5-methylorotic acid inhibited the overall reaction and the blank by 50\%", at a substrate concentration of $1 \times 10^{-3}\text{M}$. No experimental details were given, but it may be assumed that they carried out the experiments in the presence of a large excess of substrate, as in their determination of enzyme activity (see Section VII C$_{2a}$). It may be guessed that the "50\% inhibition of the blank" was a 50\% inhibition of the NADH oxidase reaction. In an attempt to confirm the findings of Friedmann and Vennesland [22], preliminary experiments have been carried out with 5-methylorotic acid as substrate (see Section VIID$_{2d}$). The results obtained (see Section VIID$_{2d}$) did not agree with their findings. It, in fact, appeared that 5-methylorotic acid does not inhibit the NADH oxidase reaction and is a substrate for dihydro-orotic dehydrogenase, although the rate was significantly lower than that for orotic acid. Moreover, if orotic acid and its 5-methyl analogue are metabolised at different rates, and if they are both present at enzyme saturation levels, it is not unexpected that there should have been indications of competitive inhibition. It must be remembered that in the present investigation only preliminary
experiments, carried out at enzyme saturation level, were studied; the activity being followed by measuring the rate of change of optical density at 340 μm associated with the oxidation of NADH to NAD⁺.

A more detailed study is required over a wide range of substrate concentration, below enzyme saturation level, the reaction being followed by the change in optical density at 282 μm associated with the reduction of 5-methylenorotic acid. Isolation and identification of the reaction product, and a comparison with the two diastereoisomeric 5-methyl-dihydro-orotic acids would give further evidence as to the stereochemical requirements of the enzyme.
APPENDIX I

IA  INFRA-RED ABSORPTION SPECTRA

IB  ULTRA-VIOLET ABSORPTION SPECTRA
The infra-red absorption spectra were determined with a Unicam S.P. 200 spectrophotometer, and the spectra obtained are on the subsequent pages.
ULTRA-VIOLET ABSORPTION SPECTRA

The ultra-violet absorption spectra were determined with a Unicam S.P.800 spectrophotometer.

The results obtained for 5-methylorotic acid are tabulated below and the spectra are given on subsequent pages. The spectra of 5-methylorotic acid was determined at several different values of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>( \lambda ) max.</th>
<th>( \varepsilon \lambda ) max. ( \times 10^{-3} )</th>
<th>( \varepsilon_{282} ) ( \times 10^{-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>281</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>( N^# )</td>
<td>278</td>
<td>7.73</td>
<td>7.55</td>
</tr>
<tr>
<td>8</td>
<td>282</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>11</td>
<td>285</td>
<td>6.09</td>
<td>6.0</td>
</tr>
</tbody>
</table>

\( N^\# \) a solution of 5-methylorotic acid in distilled water was used.

\( \varepsilon \) = molar extinction coefficient.
5- Methylfolic acid

\[ \text{Methylfolic acid} \]

[Chemical structure image]
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