AN ABSTRACT OF THE THESIS OF

....SIDNEY JOSEPH HAYES III.......... for the .....Ph. D.....

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Title: .PROPERTIES AND DERIVATIVES OF 5-HYDROXYURIDINE

.A COMPONENT OF RIBONUCLEIC ACID.....................

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Abstract approved:                               ...............

Dr. Adam W. Lis

This investigation involved a physico-chemical character-
ization of 5-hydroxyuridine ( iB), its methylated derivatives, and
related compounds. Dissociation constants, relation of optical
rotation ( cotton effects ) to molecular conformation, the ability
to bind divalent cations, and transformations in alkaline media
and toward various nucleophiles were studied. It was shown
that iB becomes unstable toward nucleophilic attack subsequent
to dissociation of the 5-hydroxyl proton. It was proven that the
instability results from nucleophilic attack rather than molecular
transformation as a consequence of proton removal. Alteration
products formed include 1-(B-D-ribofuranosyl)-2-oxo-4-imid-
azoline-4-carboxylic acid as well as at least four other derivatives
which have not so far been described in the literature.

Isotope dilution analysis for iB in yeast RNA revealed that the compound is unstable toward routine RNA fractionation procedures and cannot be isolated intact. The label in the analyses was associated with a spectrally unique complex which when hydrolyzed yielded cytidine and a labeled derivative which appeared to be dihydrouracil by mass spectroscopic and paper chromatographic analysis. Calculations of isotope dilution, based on a number of assumptions, revealed that four per cent of the nucleosides present in ribosomal RNA are iB.

It was concluded that the monoanion of iB firmly binds $Mg^{2+}$ and that the latter may stabilize it to nucleophilic attack. iB can be modified to dihydro-compounds and imidazoles spectrally resembling dihydrouracil, as well as other derivatives, and exhibits multiple modes of reactivity under RNA isolation, fractionation, and chemical hydrolytic conditions. Quantitative isolation of intact iB from biological material would not be possible above neutral pH or in the presence of a reactive nucleophile. There is a far greater potential for possible loss of iB during isolation than for its formation. Thus iB should be considered a natural component of yeast ribosomal RNA.

Properties and Derivatives of 5-Hydroxyuridine
A Component of Ribonucleic Acid

by

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Typed by Mrs. Aase Austad for Sidney J. Hayes lll

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# INTRODUCTION

This work involves an investigative characterization of I-β-D-ribofuranosyl-5-hydroxyuracil, also called 5-hydroxyuridine or isobarbituridine attempted through a purely chemical approach. Biological considerations were neglected because valid assumptions required for their design have not yet been conclusively substantiated at the chemical level. It is not necessary to make a hypothesis that 5-hydroxyuridine may be a cellular product in studies involved with the molecule's chemistry or in its use as an antimetabolite. To assume such would however provide an a priori rationale for experimental considerations pertaining to the biological raison d' être of such a molecule. A chemical constituent which is isolated from biological material may be adjudged a cell product if the consideration that such a compound could have been formed during the isolation procedure can be ruled out. A cell product formed from the raw material of a metabolic pathway may constitute an intermediate or the endproduct of that pathway. Such an endproduct is dynamically useful to the cell except in the case of a few ancillary pathways which convert a functionally active to an inactive molecule. Intermediates, present under prototrophic conditions at steady state levels, are useful to the cell only in that they are part of a bridge to a component which is structurally or functionally of a unique and dynamic significance to the cell. Thus an assumption

that a molecule has afunctional cellular role does not follow directly

from the fact that it may be isolated from biological material or that it

is a cell product, and may only be valid after its specific structural

and mechanistic involvement have been ascertained.

The above criteria apply to 5-hydroxyuridine. This molecule

was isolated in 1966 from RNA of the yeast Torula utilis by Lis and

Passarge (83). The molecule was first reliably synthesized in 1952 by

Visser and Coworkers (138) who demonstrated its antimetabolic acti-

vity. 5-hydroxyuridine came to be employed physiologically as a

chemotherapeutic lethal synthetic inhibitor in controlling malignancies

and was considered by early workers analogous to the 5-halo analogs

of uracil, and without potential as a natural cellular constituent. Its

isolation from biological material thus came as something of a shock

to workers employing it as an antimetabolite which could inhibit the

growth of certain yeast.

The initial communication of the isolation of 5-hydroxyuridine

from biological material did not establish the level of the component

present in yeast RNA digests, its potential for formation during isola-

tion, whether or not the component was a cell product, i.e. as an

integral component of a nucleic acid chain, or mechanistically the

possible functional relationship of the component to the cell. However,

these studies did suggest that 5-hydroxyuridine was present in RNA of

yeast origin combined in 3'-5'-phosphodiester linkages and implied

that as a natural cellular constituent it possesses interesting functional

possibilities attributible to its unique C-5-hydroxyl group. This work represents a preliminary attempt to examine the biological implications of the chemistry and origin of this molecule.

## 5-Hydroxyuridine as a Potential Dynamic Cellular Metabolite

A hydroxyl group attached to an accessible position of a standard nucleic acid pyrimidine base, which is bound in a nucleic acid molecule, would potentially be biologically useful, e.g., it could serve as a site to bind the nucleic acid to other nucleic acid, protein or carbohydrate. There are a number of considerations which tend to support the assumption that 5-hydroxyuridine represents a potential dynamic cellular metabolite with unique functional importance to the cell. These include:

(1.) In addition to its isolation from yeast RNA, as outlined above, 5-hydroxyuridine may be isolated from commercial preparations of uridine obtained by digestion of yeast RNA (83).It was found inadvertantly during an assay for 1, 5-diribosyl-uracil, which had been previously isolated from the same source. This was accomplished due to the fact that both molecules complex two molecules of tetraborate. On paper electrophoresis employinga solution of the latter each of these complexes possess a net divalent cationic charge. They are thus separated from the standard RNA nucleosides which complex only one molecule of borate.

(2.) 5-hydroxyuridine has been used as an antimetabolite and is generally included with the 5-halo analogs of uracil in discussions of their metabolic characteristics and chemistry (10, 58). General dissimilarities, however, in hydroxyl vs. halogen substituent chemistry admit unique molecular properties. Most obvious is the fact that the halosubstituents are good electron withdrawing groups whereas the dissociated hydroxyl is a good electron donating group. Biologically, this difference is reflected in a considerable lowering in the potential for mispairing of 5-hydroxyuracil with guanine as compared, e.g., with the frequency of mispairing of 5-bromouracil with guanine. These considerations will be discussed in detail later in the text.

(3.) There is some precedent for functional hydroxyl substituents of nucleic acid bases. For example, some DNA phage synthesize an enzyme which hydroxylates deoxycytidinemonophosphate (40). Phage coded glucosyl transfereases then transfer glucosyl groups from uridine diphosphate glucose to the hydroxymethyl groups of hydroxymethyl cytosine residues which had previously been incorporated into the viral DNA. This indicates that the 5-position of pyrimidines, which in models is situated perpendicular to the axis of the strands-- substituents extending distally into the groove(s) created by the helix--is sufficiently exposed to permit enzymatic recognition of the substituent. Different phage glucosyl transferases with unique specificities apparently recognize specific base sequences of clusters as the T-even phages possess different glucosylation patterns along their DNA (40).

(4.) Microbial hydroxylation reaction mechanisms have been studied which could conceivably react with uridine giving 5-hydroxy-uridine. A cytochrome linked hydroxylase isolated from <u>Pseudomonas fluorescens</u> catalyzes:

(a) $-CH=CH- + H_2O \longrightarrow -CH(OH)-CH_2 \longrightarrow -C(OH)=CH- + 2H$
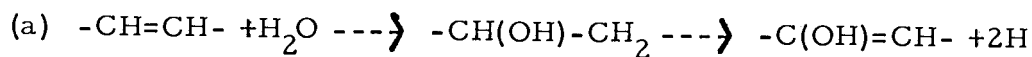
also mono-oxygenases from Pseudomonas species have been shown to catalyze (44).

(b) $-CH=CH(O \cdot OH) + 2H \longrightarrow -CH=CH(OH) + H_2O$ and

(c) $-CH=CH- + O_2 \longrightarrow -\overset{O}{\overset{\diagup \diagdown}{CH-CH}}- + H_2O$

$$-\overset{O}{\overset{\diagup \diagdown}{CH-CH}} \longrightarrow -CH(OH) \cdot CH(OH) \longrightarrow \begin{cases} -C(OH)=C(OH)- \\ -CH = C(OH)- \end{cases}$$
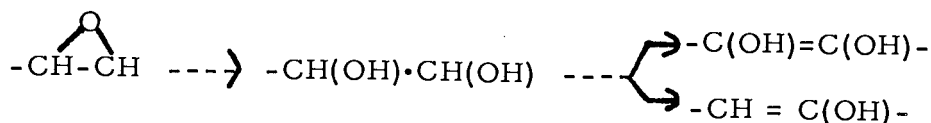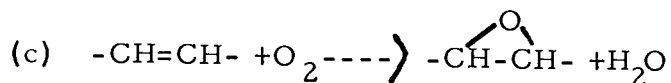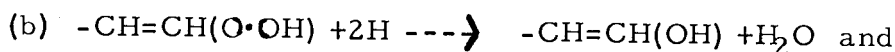
Other pertinent examples of microbial hydroxylation include: a) hydroxylation of 6-mercaptopurine to give corresponding mono- and dihydroxy derivatives at the 2 and 8 positions (27); b) uracil and thymine may be hydroxylated to barbituric and 5-methylbarbituric acid (60): c) enzymes have been shown to be present in liver slices and in bacterial species which catalyze an oxidative attack on the methyl group of thymine (or thymidine) to give 5-hydroxymethyl uracil and uracil-5-carboxylic acid (1); d) and hydroxylation of 4-hydroxy-5-halogeno-pyrimidines has been shown to take place at C-2 of the pyrimidine ring,

giving rise to corresponding 5-halouracils (24), presumably the same

reaction could occur with 4,5-dihydroxypyrimidine giving isobarbituric

acid (5-hydroxyuracil).

(5.) A fundamental question in nucleic acids biochemistry pre-

sently pertains to the biosynthesis and function of the minor compon-

ents of ribonucleic acids. For example, are they involved in mechan-

isms of cellular regulation and control, do they function merely as

sites for enzyme recognition, or are they functionally involved at the

active site level in enzyme catalysis. 5-hydroxyuridine potentially

could function at all three levels. Substituent modification of the

major ribonucleosides produces minor nucleosides which possess in-

dividual chemical and physical properties. More than twenty minor

ribonucleosides and deoxyribonucleosides have been isolated from

various sources (52). Nucleotide-peptides, which may or may not be

considered minor components have also been characterized. A varie-

ty of linkages exist between the nucleotide and the peptide as through

phosphoanhydride bonds, ester linkages (at 2' or 3'), phosphoramide

bonds (between the amino group of an amino acid and phosphate re-

sidue of a nucleotide), and through aminoacyl links to the base itself,

e.g., as in $N^6$-amino-acyl-adenosines (53). There is also one report

of a uracil-5-peptide (C-C bond) (21). Aside from these latter two

components, and possibly nucleotides in which an amino acid is

esterified at the 2' position the bonding in the other nucleotide-pep-

tides is such that the interposition of the modified nucleotide, by 3'-

5'-phosphodiester bonding, into the chain of a nucleic acid molecule

is not possible. As such they could function only at chain termini. This would tend to restrict their involvement in interstrand sites as the codon, or anticodon, and presumably also at enzyme recognition sites.

Excluding the methylated components, inosine, epimerization products, and all but one type of the numerous nucleotide-peptides, the following minor components with functional groups are known: $N^6$-(aminoacyl)-adenosine (53), $N^6$-secondary amines of adenosine in which R is a small unsaturated hydrocarbon (cytokinin) (56,109), pseudouridine (34), dihydrouracil (86), 5-hydroxyuridine (83), 1,5-diribosyluracil (82), ribothymidine (144), $N^6$-acetyl-cytidine (144), hydroxymethylcytosine (142), $N^5$-ribosylguanine (62,118) and the sulfur containing bases (4-thiouridine (80), 2-thiocytosine (25), 5-methylaminomethyl-2-thiouracil (25), 2-thio-5-uridine acetic acid methyl ester (5) and possibly a thioadenosine (85). More than half of these minor components have been isolated from transfer RNA, however, only five --- dihydrouridine, pseudouridine, ribothymidine, $N^6$-acetyl-cytidine, and $N^6 - \Delta^2$-isopentenyl adenosine have been shown to be present in the transfer RNA's which have so far been sequenced (85,41). Eight (of the 15) contain C-5-substituted pyrimidines (61). The question as to whether these compounds are genuine constituents of RNA, or DNA must be assessed on an individual basis (52).

## Reported Antimetabolic Activity of 5-Hydroxyuridine (5-OHUR)

As stated above, 5-hydroxyuridine has been used as an antime-
tabolite. Work of this nature and its implications have been based on
the assumption that 5-hydroxyuridine is not a natural cellular consti-
tuent. In reviewing this work, this assumption, by the investigators
involved, should be kept in mind in following the interpretation of their
results. A reexamination of the expressed implications, by consider-
ing the molecule as a potential dynamic metabolite, might ultimately
necessitate a modification of the presented interpretations.

5-hydroxyuridine has been found to inhibit the growth of proto-
trophic Neurospora (as do several other C5-substituted nucleosides),
and Theiler's GDVII strain of murine encephalomyelitis virus in tissue
culture of one day old mouse brain and on chick embryo tissue (138).
It was noted that the cells do recover from this inhibition (122). In
other experiments in which small amounts of 5-hydroxyuridine were
added to log cultures of E. coli, or the yeast Saccharomyces cerevi-
siae, growth was immediately inhibited. After a while the cells re-
cover from the inhibition and logarithmic growth, without a lag, con-
tinues at the same generation rate as that preceding the addition of the
inhibitor (125). It has been postulated, although without experimental
evidence or the suggestion of a mechanism, that this escape of the
cells from growth inhibition is likely due to metabolism of the 5-hyd-
roxyuridine by the cells to perhaps an "inactive form" (122). 5-hyd-
roxyuridine also has been found to completely inhibit $\beta$-galactosidase

formation in E. coli at concentrations which had no effect on over-all protein synthesis (125,126). Even when the synthesis of this enzyme is actively in progress, the addition of 5-hydroxyuridine to the cells can effect an immediate cessation of the biosynthesis of $\beta$-galactosidase. There is a complete inhibition of the induction of this enzyme when 5-hydroxyuridine is introduced simultaneously with an inducer as n-butyl-beta-D-galactoside. When only a small amount of 5-hydroxyuridine is added, however, the cells recover from the inhibition and enzyme synthesis is reinitiated (126). Constitutive mutants of E. coli for $\beta$-galactosidase synthesis are as sensitive to inhibition by 5-hydroxyuridine as are their inducible counterparts (125). The same results have been obtained in work with the yeast Saccharomyces cerevisiae as were derived from the observed effects of 5-hydroxyuridine on $\beta$-galactosidase synthesis in E. coli (126). Other workers have found that 5-hydroxyuridine competitively inhibits the growth stimulation produced by purines in purine requiring E. coli mutants. The inhibitory effect is completely reversed by uridine or cytidine. In addition, 5-hydroxyuridine inhibits the uptake of phosphate into lipids and into each of the nucleotides of rat hepatoma in vitro (137).

## Characteristics of 5-Hydroxyuridine as an Antimetabolite

In an in vitro study of the concentration dependent inhibition by 5-hydroxyuridine of orotic acid-6-$^{14}$C into RNA and valine-1-$^{14}$C into protein from Ehrlich ascites cells the effect of the analog on RNA

synthesis was found to be much more pronounced than its effect on protein synthesis. Incorporation of the label from orotic acid into RNA was inhibited asymptotically to a maximum of 70 per cent at the highest 5-hydroxyuridine concentration during a 30 minute incubation period. During the same period protein synthesis was inhibited by 25 per cent (123). In contrast, the results obtained with E. coli 15T⁻ show a more proportional decrease in RNA and protein synthesis on inhibition by other analogs known to inhibit RNA formation. Inhibition of protein and RNA synthesis by 5-hydroxyuridine and thiouracil is evident only during initial periods of incubation (9). The more immediate effect of RNA antagonists on protein synthesis in bacteria may be attributed to the short half life of 2-8 minutes ( 40) of their messenger RNA. Thus interference with RNA synthesis would be reflected by an almost immediate decrease in protein synthesis. Messenger RNA of some mammalian cells has a much longer half-life and inhibition of RNA synthesis in these cells has little or no effect on protein synthesis for an hour or more (104).

All known RNA inhibitors in sufficient concentrations inhibit RNA and protein synthesis completely after a short incubation period (47), however, 5-hydroxyuridine and thiouracil are exceptions to this generalization (9). Several derivatives of thiouracil as noted above have recently been obtained as natural constituents of transfer RNA. Inhibition is immediate with 5-OHUR and thiouridine and, at least in the case of the former, complete inhibition of RNA synthesis does not occur. This is illustrated by the above experiment with Ehrlich as-

cites cells in which 30 per cent residual RNA synthesis (70 per cent inhibition) occurred at extremely high 5-hydroxyuridine concentrations (123). The type of RNA inhibited, i.e., messenger, ribosomal, or transfer, has never been examined, indeed most of these studies were attempted before these were defined. Residual RNA synthesis may reflect an inability of 5-hydroxyuridine to inhibit certain types of RNA transcription. The breakdown of inhibition is discussed in the discussion section in relation to the DNA-dependent-RNA polymerase.

Although the physiological action of 5-OHUR may appear to be divergent, its action in relation to the immediate inhibition of an inducible enzyme indicates that its activity does not depend upon requisite incorporation into the genome or into newly formed RNA but is involved in inhibition at the transcriptional or translational level. The evidence that it inhibits the formation of the inducible enzyme β-galactosidase at concentrations which had no effect on over all protein synthesis suggests its activity may be at the transcriptional level. By analogy, its activity is similar to the mode of action postulated by Jacob and Monod (67) for the product of cellular regulator genes. Its possible action in relation to the transcribing enzyme the DNA-dependent-RNA polymerase and its chemical state in the cell are dicussed below.

Metabolism of 5-Hydroxyuridine

5-hydroxyuridine, as has been mentioned, is one of numerous compounds which effect, to some extent, tumor growth (137, 9). The

question of whether or not inhibition of nucleic acid metabolism is indeed a mechanism for tumor inhibition has provided impetus for studies on the action of base analogs. Studies on labeled 5-hydroxyuridine incorporated by Ehrlich's ascites tumor cells have shown that it undergoes most of the reactions of uridine and 5-fluorouracil (32, 59). For example, it is readily converted in vivo to 5 hydroxyuridine 5'-phosphate, 5-hydroxyuridine 5'-diphosphate, 5-hydroxyuridine 5'-triphosphate, 5-hydroxyuridine 5'-diphosphate glucose, and 5-hydroxyuridine 5'-diphosphate glucuronate intermediates (123). These components when tested with ferric chloride (45) give a purple color characteristic of the 5-hydroxyuracil moiety (96).

The inhibitory effect of the nucleoside analog on bacterial, viral, and tumor growth (122,137,102) may possibly be explained by its conversion to phosphorylated derivatives which inhibit nucleic acid metabolism ( 9 ). It has been found that 5-hydroxyuridine 5'-phosphate has only a slight inhibitory effect on the conversion of uridine 5'-phosphate to uridine 5'-triphosphate, whereas it effectively blocks the conversion of orotidine 5'-phosphate to uridine 5'-phosphate by inhibiting the activity of orotidylic acid decarboxylase (123). At a concentration one-tenth that of orotic acid, 5-hydroxyuridine 5'-phosphate inhibits the conversion of orotic acid to uridine nucleotides by 35 per cent. At 5 times the orotic acid concentration, 5-hydroxyuridine 5'-phosphate inhibits conversion to uridine nucleotides by 93 per cent (123). Inhibition of orotidylic acid decarboxylase rather that that of orotidylic acid pyrophosphorylase was considered to occur because inhibition of the

conversion of orotic acid to uridine nucleotides is not accompanied by a significantly lowered conversion of orotic acid to orotidylic acid. Accumulation of orotidylic acid, a product of the pyrophosphorylase reaction, because of a block in the carboxylase reaction would however be expected to retard orotic acid utilization, since the equilibrium constant for the pyrophosphorylase reaction is 0.12 (79). 6-azauridylic acid also inhibits decarboxylation of orotidylic acid under conditions similar to those used in the studies of 5-hydroxyuridine 5'-phosphate inhibition (101). Competitive feedback inhibition of orotidylic acid decarboxylase by uridylic acid has also been shown to occur, although the quantity required is much greater (57,15) than the amount required for inhibition by 6-azauridylic acid or 5-hydroxyuridine 5'-phosphate (123). In these studies the level of orotidylic acid accumulation in the nucleotide pool is very small. Because of the action of certain phosphatases, the level of accumulation of orotidine represents an accurate approximation of the molar level of orotidylic acid (101).

The conversion of uridine 5'-triphosphate to cytidine 5'-triphosphate is not affected by 5-hydroxyuridine nucleotides (123). It has been found, however, that 5-hydroxyuridine 5'diphosphate glucose, which is formed from 5-hydroxyuridine 5'-triphosphate and glucose 1-phosphate by uridine diphosphate glucose pyrophosphorylase, inhibits the conversion of uridine diphosphate-glucose to uridine diphosphate glucuronic acid by uridine diphosphate glucose dehydrogenase (112). 5-hydroxyuridine diphosphate glucose inhibited uridine diphosphate glucose oxidation in this study in a manner suggesting that the analog in-

terferes with the $NAD^+$ binding site of the enzyme. The rate of oxidation of 5-hydroxyuridine diphosphate glucose was one-sixth that of uridine diphosphate glucose at pH 8.7 and one-half that of uridine diphosphate glucose at pH 7.0 (112).

The suggestion has been made (121) that the conversion of uridine diphosphate glucose (UDP-glucose) to uridine diphosphate glucuronic acid (UDP-glucurnic acid) involves the two step reaction:

1.) $UDP\text{-}glucose + NAD^+ \relbar\relbar\relbar\relbar XUDP\text{-}glucose + NADH + H^+$

2.) $XUDP\text{-}glucose + NAD^+ \relbar\relbar\relbar\relbar UDP\text{-}glucuronate + NADH + H^+$
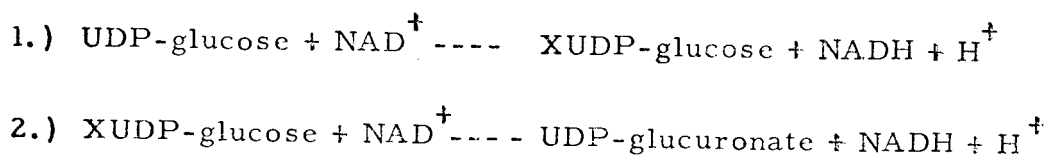
Reaction 2 does not take place until Reaction 1 is essentially complete, and where XUDP-glucose represents a substance at an intermediate level of oxidation in which glucose remains unchanged and the pyrimidine moiety is oxidized. Roy-Burman et al. (112) studied the effectiveness of 5-hydroxyuridine diphosphate glucose, 5,6-dihydrouridine diphosphate glucose and uridine diphosphate glucose as substrates for this reaction. They indicated that the glucose moiety is altered chemically from the very beginning of the reaction and concluded that if there is an initial oxidation of the pyrimidine moiety it is accompanied by a simultaneous change in the glucose residue. The fact that both 5,6-dihydrouridine diphosphate glucose and 5-hydroxyuridine diphosphate glucose serve as substrates for the dehydrogenase and utilize 2 moles of $NAD^+$ per mole of cofactor while producing glucuronic acid derivatives of the unchanged nucleotides was taken as evidence against direct participation of positions C-5 and C-6 uracil in the oxidation reactions.

Preliminary studies with mouse liver glycogen synthetase have shown that both 5,6-dihydrouridine diphosphate glucose and 5-hydroxyuridine diphosphate glucose serve as substrates for the enzyme. Radioactivity from uridine diphosphate glucose -$^{14}$C and its 5,6-dihydroderivative was incorporated into glycogen with equal efficiency, but incorporation of isotope from 5-hydroxyuridine diphosphate glucose-$^{14}$C into glycogen occurred at one-third the efficiency of the other two substrates (112).

## Comparison of Metabolism of 5-hydroxyuridine-2-$^{14}$C and Uridine-2-$^{14}$C.

Quantitative differences in the metabolism of uridine and 5-hydroxyuridine have been studied in Ehrlich ascites cells. The extent of incorporation of each radioactive nucleoside into their respective nucleotides and into nucleic acids was determined. A maximum two-fold difference in concentration was found in the acid-soluble compounds that have been described above, whereas the amount of uridine-2-$^{14}$C incorporated into nucleic acids is 27 times that of 5-hydroxyuridine-2-$^{14}$C (123). The observed differences have been explained on the basis that the synthesis of RNA presumably involves not only enzymatic specificity, but also requires that a base pair be formed with a base from the DNA or RNA primer. 5-hydroxyuridine may not be able to form hydrogen-bonded base pairs with adenosine (123). Consistent with this is the observation that substitution of groups at position 5 of the uracil moiety alters the base pairing characteristics of the molecule (51, 30).

## Effect of Poly-5-hydroxyuridylic Acid on Amino Acid Incorporation

Poly-5-hydroxyuridylic acid has been synthesized from 5-hydroxyuridine 5'-diphosphate with polynucleotide phosphorylase obtained from <u>Micrococcus lysodeikticus</u> by the method of Beers ( 7)., The polymer was tested for its ability to stimulate amino acid incorporation into acid-insoluble material according to the procedure of Nirenberg and Matthaei ( 94). It has been found that poly-5-hydroxyuridylic acid directs phenylalanine polymerization inefficiently (51) or not at all (110). The order of incorporation of activity for phenylalanine is polyuridylic acid = polyiodouridylic acid > polybromouridylic acid > polychlorouridylic acid > polyribothymidylic acid >> poly-5-hydroxyuridylic acid. These polymers, aside from the latter two, also code to some extent for isoleucine, leucine, and serine incorporation. Poly-5-hydroxyuridylic acid and polyribothymidylic acid stimulate rather feeble incorporation of leucine, but are completely inactive with regard to the other two amino acids (51).

It has been suggested by Roy-Burman <u>et al.</u> (113)that because of the inefficient or ineffective ability of poly-5-hydroxyuridylic acid, a homopolymer,to direct phenylalanine incorporation, even a small percentage of the analog present in messenger RNA may have an inhibitory effect on protein synthesis. This is a faulty conclusion, however, as a 5-hydroxyuridylic acid copolymer with another common base has never been tested for incorporation of amino acids. Some dramatic differences exist in the character of incorporation between heteropolymers and

homopolymers. For example, polypseudouridylic acid is inactive, whereas copoly (uridylate, pseudouridylate) with a ratio of uridylate: pseudouridylate = 2.5 is more effective than polyuridylic acid for the incorporation of phenylalanine (113).

## Inhibition of Uridine 5'-triphosphate Incorporation into RNA by 5-hydroxyuridine 5'-triphosphate

The observation (123) that there is a facile conversion of 5-hydroxyuridine to 5-hydroxyuridine 5'-triphosphate in Ehrlich ascites cells as compared to a low level of incorporation into RNA resulted in studies of the effect of 5-hydroxyuridine 5'-triphosphate on DNA-primed RNA polymerase activity. It was shown by Roy-Burman, et al. (113) that the 5-hydroxynucleoside exerted a strong competitive inhibitory effect on the in vitro incorporation of uridine 5'phosphate-$^{14}$C into RNA only when it existed as the triphosphate. The reaction consisted of calf thymus DNA, a DNA dependent RNA polymerase isolated from E. coli W, adenosine 5'-triphosphate, guanosine 5'-triphosphate, cytidine 5'-triphosphate, uridine 5'-triphosphate-2-$^{14}$C, and varying concentrations of 5-hydroxyuridine 5'-triphosphate. 5-Hydroxyuridine 5'-di- and monophosphates inhibited incorporation of uridine 5'-phosphate into RNA only slightly as compared to 5-hydroxyuridine 5'-triphosphate (113). When 5-hydroxyuridine 5'-triphosphate-2-$^{14}$C replaces uridine 5'triphosphate-2-$^{14}$C in this reaction it is incorporated optimally into RNA at pH 7.0 and not at all when the analog exists predominantly in the ionized form at pH 8.6 or 9.0 (113).

5-hydroxyuridine 5'-triphosphate inhibits incorporation of isotope from uridine-5'-triphosphate-2-$^{14}$C into RNA to a greater extent than uridine 5'-triphosphate inhibits utilization of 5-hydroxyuridine 5'-triphosphate-2-$^{14}$C in the polymerase reaction suggesting that 5-hydroxyuridine 5'-triphosphate acts as a competitive inhibitor of uridine 5'-triphosphate. The inhibitory effect is at a maximum at pH 9.0 and is lowest at pH 7.0 (113).

The behavior of 5-hydroxyuridine 5'-triphosphate in the polymerase reaction is distinctly different from other nucleoside triphosphate analogs (113). 6-Azauridine 5'-triphosphate does not permit incorporation of ribonucleotides into RNA when it is substituted for any one of the natural nucleotides, however, in contrast to 5-hydroxyuridine 5'-triphosphate it does not inhibit RNA synthesis when it is added to reaction mixtures containing all four natural substrates (74). Additionally, other nucleoside analogs, as pseudouridine 5'-triphosphate (93,50) ribothymine 5'-triphosphate (110), 5,6-dihydrouridine 5'-triphosphate (70), 5-fluorouridine 5'-triphosphate, and 5-bromouridine 5'-triphosphate (110, 50) are utilized by RNA polymerase with an efficiency of 30% or more when one of these derivatives replaces uridine 5'-triphosphate. Data (113) indicate that 5-hydroxyuridine 5'-triphosphate is incorporated into RNA at 3% of the level of optimal uridine 5'-triphosphate incorporation at pH 8.0 indicating that the analog is a very inefficient substitute for uridine 5'-triphosphate in RNA synthesis. The difference between the inhibitory effect of 5-hydroxyuridine 5'-triphosphate and the above derivatives in the presence of all four natural substrates is best explained

by an ability of the latter derivatives to act as alternate substrates for uridine 5'-triphosphate. The inhibitory effect of ionized (pH9.0) 5-hydroxyuridine 5'-triphosphate on uridine 5'-phosphate incorporation into RNA is twice that for the unionized molecule. The ability of the latter to reduce uridine 5'-phosphate incorporation by 60 per cent indicates the inhibitory effect of 5-hydroxyuridine is not related directly to an inductive effect. It has been suggested that its effect may be related to its affinity for RNA polymerase or a polymerase-DNA complex (113). I will pursue a speculative mechanism for the inhibitor which is not based on an affinity of the analog for enzyme, in relation to some of my findings, in the text of the discussion.

## Considerations of the Chemistry of 5-hydroxypyrimidines

Among the various pyrimidines only a limited number possess 5-hydroxyl substituents (31). These include divicine (2,6-diamino-4-β-5-hydroxypyrimidine) which has been obtained by dilute acid hydrolysis of vicine (2,6-diamino-4-oxypyrimidine-5-β-D-glucopyranoside) a natural constituent of vetch seeds and the first simple pyrimidine derivative found to occur in nature (11), isouramil (4-amino-5-hydroxyuracil) (39),dialuric acid (5,6-dihydroxyuracil)(12,63,106,37) isodialuric acid (structure below) (71),isobarbituric acid (5-hydroxyuracil)(39,37,72) 2-amino-5,6-dihydroxypyrimidine, and 4-amino-2,5-dihydroxypyrimidine (72,71,70),5-hydroxyuridine (isobarbituridine)(122,108) and 5-hydroxydeoxyuridine (122,108) and 5-hydroxycytidine (40). Chemically, 5-hydroxypyrimidines are not capable of lactam-lactim tautomeric

change as are pyrimidines in which a hydroxyl group lies at the 2-, 4-, or 6-positions of the ring. 5-hydroxypyrimidines are therefore comparable with β-hydroxpyridines and they would be expected to exhibit some aromatic characteristics, like phenols. Dialuric acid appears to be a rare example of stable tautomerization in which the enol form is favored (135, p. 29).

## Chemistry of Isobarbituric Acid

Isobarbituric acid (5-hydroxyuracil) has been found to be an exception to general resistance to oxidation shown by both purine and pyrimidine ring systems. Previous investigations have shown that on oxidation isobarbituric acid readily reacts at the double bond. Thus, by bromine water it is converted into isodialuric acid
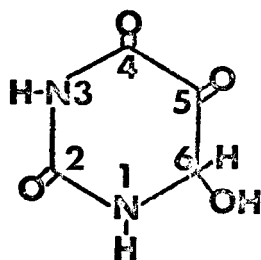
isodialuric acid

while with permanganate the ring is ruptured and formyl-oxalylurea is produced (96).
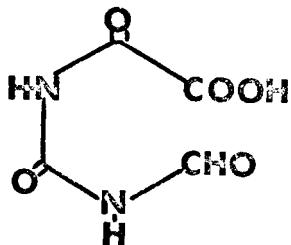
formyl-oxalylurea

By utilizing ferricyanide as the oxidant it has been found possible to control the oxidation so that, in at least a portion of the oxidized material, the double bond remains intact. The reaction has been postulated to proceed with the loss of one hydrogen atom from each molecule and the subsequent coupling (C6-C6) of the two rings (37).



isobarbituric acid    6,6-di-isobarbituric acid (enol form)    6,6-di-isobarbituric acid (keto form)
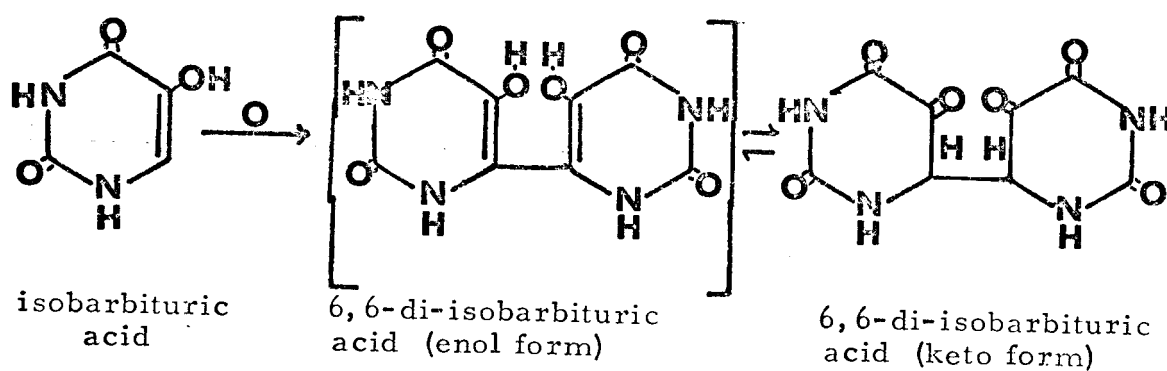
6,6-di-isobarbituric acid is exceedingly insoluble, has a bright yellow color, forms red alkali metal salts in dilute alkali, and dissolves in excess of alkali to give solutions possessing a green fluorescence. With ammonia it gives a deep purple-red color characteristic of a positive murexide test. A small quantity of the red sodium salt of di-isobarbituric acid can also be obtained by evaporating a solution of isobarbituric acid in sodium bicarbonate in an open dish on a water bath, or by passing oxygen into a warm solution of sodium isobarbiturate. Unlike isobarbituric acid and other 5-hydroxypyrimidines, di-isobarbituric acid does not yield a blue salt with ferric chloride and has been postulated to exist in the keto form (37). The above reaction resembles that of aromatic phenols which, on oxidation yield ortho-di-phenols, with the difference, however, that di-isobarbituric acid does not remain in the enol form, but appears in the keto form. On continued oxidation with nitric acid or bromine water, 6,6-di-isobarbituric acid is converted into

an unsaturated scarlet-colored compound urindigo (diuracil-6,6-indigo) with the loss of two hydrogen atoms (37).

6,6-di-isobarbituric
acid

$\xrightarrow{\quad\text{O}\quad}$
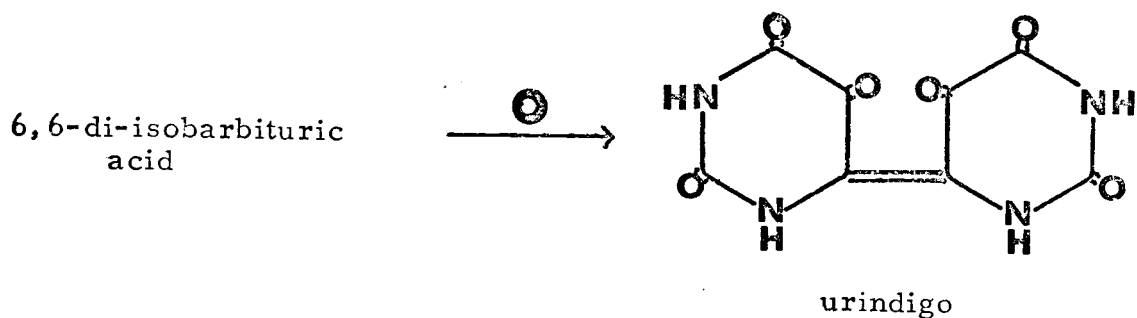
urindigo

Urindigo is distinct from the isomeric colorless compound dehydrohydurilic acid which has been described by Biltz and Heyn (13).

dehydrohydurilic
acid
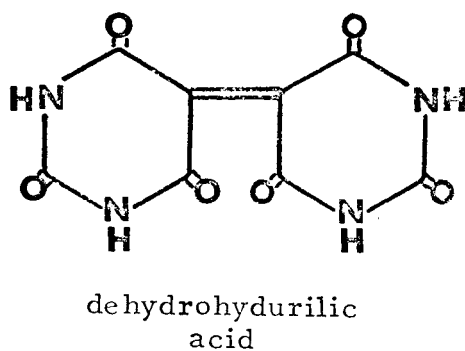
Urindigo is insoluble in common organic solvents, it dissolves in concentrated sulfuric acid and, like di-isobarbituric acid, reprecipitates on dilution. It is gradually oxidized in concentrated nitric acid or in bromine water giving products which form a purple precipitate with excess barium hydroxide which is presumed to be barium dialurate. Urindigo is immediately decomposed by alkali; on neutralizing the

solution and adding ferric chloride a deep blue ferric salt characteristic of 5-hydroxy-derivatives of uracil is obtained. It has been suggested (37) that the solution probably contains 5-hydroxyuracil-6-aldehyde.

6,6-di-iso-barbituric acid $\xrightarrow{\text{H}_2\text{O}}$ 5-hydroxyuracil-6-aldehyde + [structures]
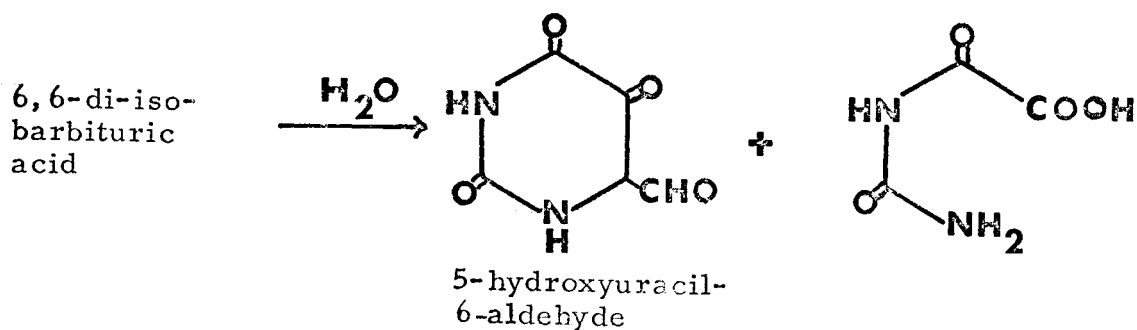
## Synthesis and Stability of Isobarbituric Acid and Its N-1 Substituted Derivatives

Although, in 1912, Levene and LaForge (78) reported the preparation of 5-hydroxyuridine, via a 5-bromouracil intermediate, by a bromine and lead oxide method; subsequent workers have found their method difficult and variable (108,139). Various indirect methods (72, 71, 69,130, 65) for the synthesis of 5-hydroxypyrimidines, which do not involve a bromo-pyrimidine as an intermediate were developed. It was felt that a 5-bromo atom in pyrimidines which were additionally substituted at C-4 or C-6 with amino or hydroxyl groups, would be extremely stable toward acid or alkali, thereby making syntheses involving such intermediates unfavorable. Wang, however, has stated (139) that

for the 5-bromo atom in pyrimidines to be stable, the pyrimidine nucleus would have to be aromatic. Other workers (88,19,18) have suggested that in aqueous solution C-2 or C-4-hydroxypyrimidine exists predominantly in the lactam or ketonic form. As such, Wang felt the 5-bromo atom should be fairly reactive because it is the $\alpha$-bromo atom in an $\alpha$, $\beta$-unsaturated ketone. He postulated (139) it would be possible to hydrolyze the 5-bromo derivatives to hydroxy derivatives by means of relatively weak bases. He has been able to demonstrate that 5-bromo-6-hydroxy-5,6-dihydrouracil derivatives are the intermediates which react with lead oxide to form 5-hydroxy derivatives, rather than 5-bromo derivatives as previously suggested by Levene and LaForge (78). This intermediate has been crystallized and characterized (140); methods for the preparation of 5-hydroxyuracil derivatives by bromine are summarized below (139):R = H, methyl, ribose, or ribose 5'-phosphate:
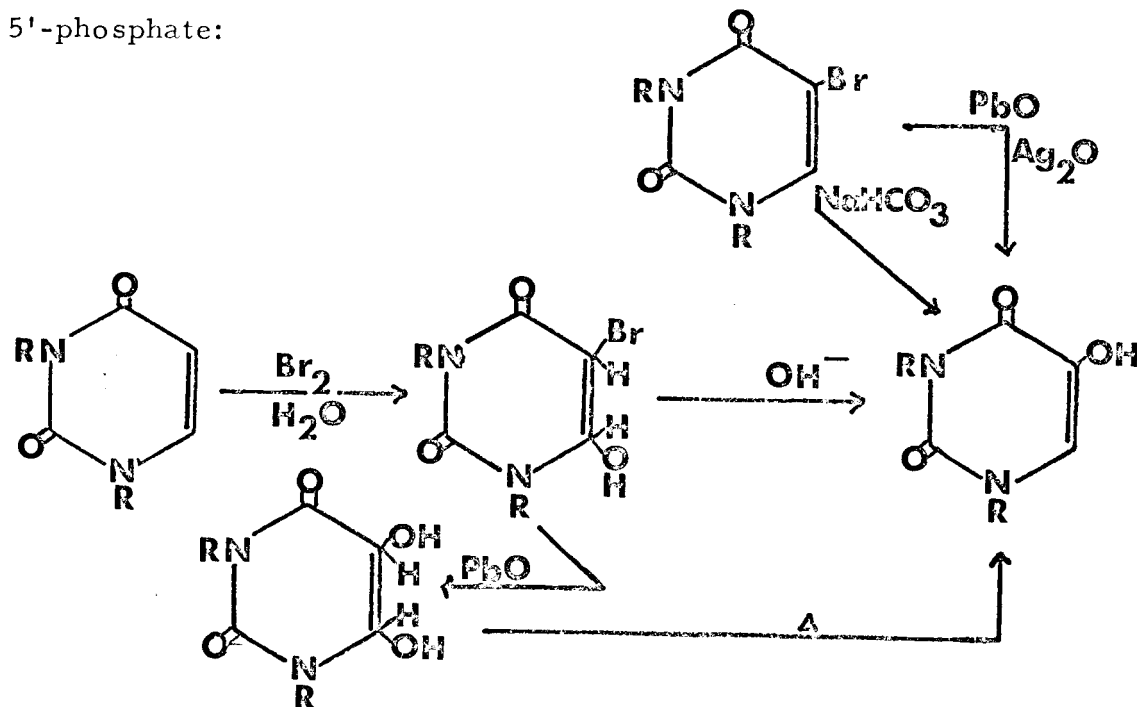
Levene (77) has used the difference in reactivity of uracil and 1-substituted uracils treated with two equivalents of bromine and subsequently with phenylhydrazine as an indication that in uridine the ribose is substituted at position one of the pyrimidine. He postulated that when uracil reacts under these conditions the precursor of 5-phenylhydrazinouracil is isobarbituric acid because the latter gives this derivative directly on treatment with phenylhydrazine without previous treatment with bromine. 5-bromouracil does not give 5-phenylhydrazinouracil directly without prior treatment with bromine-water. When uridine and 1-substituted uracils are treated as above a 5,6-bisphenylhydrazino derivative is formed. The intermediate was postulated to be 1-substituted isodialuric acid. The spectra and stability of these products have not been determined. 5-bromouridine reacts in a manner analogous to that of 5-bromouracil, but forms the bis derivative.

Wang in 1959 (139) was the first to observe the effect of alkali on 5-hydroxy-pyrimidines. He observed that the ultraviolet absorbance of 5-hydroxyuracil at 305 nm decreased with increasing time on refluxing in sodium carbonate. He suggested that the instability of 5-hydroxyuracil in strong bases was probably the reason for the failure by a number of workers to isolate compounds of this type when strong bases were used for their preparation. Later, Smith and Visser (123) noted that two labeled peaks, one containing up to 80 per cent of the total radioactivity, could be obtained from an alkaline digest of RNA which had been obtained from Ehrlich ascites cells that were labeled with 5-hydroxyuridine-2 $^{14}$C. They concluded that the peak which contained the

most label was a breakdown product of 2',3'-5-hydroxyuridylic acid formed during the alkaline hydrolysis of RNA because they found that both 5-hydroxyuridine and 2',3'-5-hydroxyuridylic acid each slowly lost their characteristic spectra upon treatment with alkali at conditions comparable to those used in the hydrolysis of RNA. They found that this material gave a positive test (48) for carbamyl groups and speculated that it was most likely an analog of β-N-ribosyl-carbamyl-B-alamine 2',3'-phosphate which is formed during hydrolysis of dihydrouridylic acid (28). Lis and Passarge (83) in isolating 5-hydroxyuridine from yeast RNA have also noted that alkaline conditions contribute to the loss of ultraviolet absorption of 5-hydroxyuridine. They found that yields of 5-hydroxyuridine obtained by alkaline hydrolysis of RNA were considerably lower than those obtained by enzymatic hydrolysis. Modifications of 5-hydroxyuridine under standard RNA chemical hydrolytic procedures has been investigated by Hayes and Lis (61) and will be treated more fully in the text.

It may be concluded that although a very limited, and in most cases sketchy amount of work has been done on the chemistry of 5-hydroxyuridine, its reactivity, generally parallels that of isobarbituric acid.

# EXPERIMENTAL METHODS AND MATERIALS

## Paper Chromatography

Preparation of paper: Whatman No. 1 filter paper was employed for paper chromatography and electrophoresis. This paper contains significant quantities of ultraviolet absorbing impurities. These may be removed by heating about twenty 28 x 46 cm sheets of this paper in 6 liters of 2M acetic acid at $95^{\circ}$ C for two hours (84). The acid is decanted and the paper rinsed four to six times in distilled water until the rinse is no longer acidic. The paper then is dehydrated in 95 per cent ethanol for two hours; it hardens, and the sheets are suspended, and permitted to dry in air, the papers are then flattened under weights.

Photography of chromatograms: Ultraviolet light photographs of paper chromatograms are useful for recording the extent of migration of absorbing substances. They are made by covering Ansco Scona Reflex, type C, semi-matte photographic print paper with the paper chromatogram and exposing the paper for several seconds with an 8-w mineral lamp[1] with peak emission at 2537 $\overset{\text{o}}{\text{A}}$. The print is developed in Kodak D-11 developer, stopped in water, bathed in Kodak Fixer, washed and dried. The U.V. absorbing areas on the chromatogram are not exposed and appear as white spots on a grey-black background.

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[1] UVS·11 Mineralight. Ultra-violet Product Inc. San Gabriel, California.

Solvents used in paper chromatography: Substances to be chromatographed on acid washed filter paper were applied one and one-fourth inches from an edge. This side was introduced into one of the following solvent systems contained in a glass covered 31 x 31 x 53 cm jar. The chromotograms were developed in ascending solvents about 16 hours. Solvent systems employed:

1. 1-Butanol-glacial acetic acid-$H_2O$ (50:25:25) or (5:2:3)

2. 1-Butanol-$H_2O$-concentrated $NH_3$ (86:14:5)

3. 1-Butanol-$H_2O$ (86:14) in $NH_3$ gas

4. 2-Propanol-1 per cent aqueous $(NH_4)_2SO_4$ (2:1)

5. 1-Butanol-$H_2O$ (86:14)

6. Upper phase ethyl acetate-1-propanol-$H_2O$ (4:1:2)

7. 1-Butanol-$H_2O$-absolute ethanol (50:20:30)

8. 2-Propanol-$H_2O$-concentrated HCL (680:144:170)

9. 2-Propanol-$H_2O$-concentrated $NH_3$ (7:2:1)

Elution of samples from chromatograms: The ultraviolet absorbing areas, or bands containing radioactivity, of compounds which had been separated by migration in a particular solvent system were removed from paper chromatograms for further investigation. This was accomplished by cutting the band from the paper chromatogram and fashioning from it paper wicks. The base of each wick is moistened with distilled water and the sample contained thereon migrates to the tip by capillary action. The tips containing the concentrated sample

are cut off and eluted with a small volume of water. The aqueous sample solutions are concentrated and dried over $P_2O_5$.

## Electrophoresis

Washed Whatman No. 1 filter paper, prepared as above, was moistened in buffer and stretched across a glass electrophoresis plate; both ends of the paper were emersed in buffer. Buffers employed were:

1. N/20 potassium borate solution, pH 9.2
2. N/100 ammonium formate buffers at pH 3.5 and 9.3

Subsequent to equilibration of the buffer the samples were applied in micro quantities directly to the moist paper. Electrophoresis was carried out at a constant voltage of 19V/cm on tables cooled with circulating tap water at about 8 C. The electrophoretograms were dried on the glass electrophoresis plate with a hair dryer and were subsequently photographed by the procedure used for the paper chromatograms.

## Reagents

| | | |
|---|---|---|
| 1. | 5-Hydroxyuridine | Nutritional Biochemical Corporation and Calbiochem, A grade |
| 2. | 5-Hydroxy-2'-deoxyuridine | Calbiochem |
| 3. | Uridine | Chromatographically pure, Dr. A. W. Lis |

| | | |
|---|---|---|
| 4. | Uridine-6-3H | New England Nuclear |
| 5. | Uridine-2-14C | Nuclear Chicago |
| 6. | Orotidine | Calbiochem |
| 7. | Cytidine | Pabst Laboratories |
| 8. | Cytidine-5-3H | New England Nuclear |
| 9. | Guanosine | British Drug House |
| 10. | Adenosine | Cyclo Chemical Corporation |
| 11. | 5-Bromouridine | Calbiochem, A grade |
| 12. | Dihydrouridine | Calbiochem, B grade |
| 13. | 5-Methyluridine | Mann Research Laboratories |
| 14. | 5-Hydroxymethyl-deoxyuridine | Calbiochem, B grade |
| 15. | Uracil | Schwartz Laboratories |
| 16. | Dihydrouracil | California Foundation for Biochem. Res. |
| 17. | 5-Hydroxymethyl-uracil | California Foundation for Biochem. Res. |
| 18. | Isobarbituric acid | Cyclo Chemical Corp., Grade III |
| 19. | Dialuric acid | Calbiochem |
| 20. | Alloxan | General Biochemicals |
| 21. | Alloxantin· $2H_2O$ | Calbiochem |
| 22. | Barbituric acid | General Biochemicals |
| 23. | β-alanine | Nutritional Biochemicals |
| 24. | β-aminoisobutyric acid | Nutritional Biochemicals |

| | | |
|---|---|---|
| 25. | N-carbamyl-β-alanine | Sigma Chemical Company |
| 26. | Parabanic acid | Nutritional Biochemical Corporation |
| 27. | Uridylic acid | Schwartz Laboratories |
| 28. | Cytidylic acid | Schwartz Laboratories |
| 29. | Adenylic acid | Schwartz Laboratories |
| 30. | Guanylic acid | Nutritional Biochemical Corporation |
| 31. | D-ribose | California Found. for Biochemical Res. |
| 32. | Aniline hydrogen phthalate | K & K Laboratories |
| 33. | p-Dimethylamino-benzaldehyde | "certified," Fisher Scientific Co. |
| 34. | Trishydroxy methyl amino methane | "certified," Fisher Scientific Co. |
| 35. | 2-Mercaptoethanol | "chromatoquality," Matheson Coleman & Bell |
| 36. | Piperidine | "chromatoquality," Matheson Coleman & Bell |
| 37. | Phenylhydrazine | reagent grade, Eastman Organic Chemicals |
| 38. | Phenylhydrazine hydrochloride | reagent grade, Eastman Organic Chemicals |
| 39. | Hydroxylamine hydrochloride | reagent grade, Mathesom Coleman & Bell |

40. p-Toluenesulfonic  
acid  
reagent grade, Matheson Coleman  
& Bell

41. Urea  
reagent grade, Matheson Coleman  
& Bell

42. Phenol, liq. 88%  
Mallinckrodt Chemical Works

43. Ninhydrin  
chromatography grade, Pierce  
Chemical Co.

44. Additional reagents and solvents used in preparing routine  
solutions were reagent or special quality grades.

Column Chromatography-Fractionation Techniques

Chromatography on Dowex: The commercially available[2] anion
exchange resin Dowex-1-chloride x 2 per cent crosslinking, 200-400
mesh may be converted to the formate form, which is the most useful
for separation of nucleosides, by the following procedure:

1. Resin washed with 2 M ammonium formate salt in 6 M formic
(2 liters per half-pound resin) acid

2. Wash each half-pound with 3-4 liters of distilled water

3. Wash each half-pound with 2-liters 20 M formic acid
(technical grade)

4. Wash each half-pound with 1 liter 2 M formic acid (analytical
grade)

5. Wash with distilled water until eluant is pH 6.0.

Aliquots of approximately 500 micro-liters of concentrated samples were fractionated on Dowex-1-formate, 2 per cent, 200-400 mesh, 50 or 100 ml columns. The elution was initiated with 1 1/2 column volumes of water followed by 1 M formic acid gradient via 200 ml water mixer (84). Elution was continued with 4 M formic acid, 0.4 M ammonium formate in 4 M formic acid and 2 M hydrochloric acid. The absorbance of the eluant was recorded automatically at 254 nm with a Gilson Recording Fraction Collector[3]. Individual fractions were measured manually at the desired wavelength with a Cary Model 15 Recording Spectrophotometer[4]. Fractions of interest were pooled, concentrated with a rotary evaporator, and desiccated over $P_2O_5$.

Celite partition chromatography: Nucleosides are more amenable to mild separation techniques, such as partition chromatography, than either the nucleotides or free bases. Resolving RNA constituents at the nucleoside level also facilitates differentiation of the sugar portion of the constituent (55). The partition chromatography method of Hall (55) represent the mildest and the most successful and quantitative procedure for separating major and minor nucleosides that has thus far been devised. In this procedure, nucleoside mixtures are resolved into six major fractions, corresponding to the major ribonucleosides, with neutral solvents. These major fractions are resolved into a number of subfractions by further column partition chromatography using

[2] Bio-Rad Laboratories, Richmond, California
[3] Gilson Medical Electronics, Middelton, Wisconsin
[4] Varian Instruments Inc., Monrovia, California

different solvent systems. The minor nucleosides contained in each of the subfractions are then isolated and purified by means of paper chromatography. This technique was employed to fractionate ribosomal yeast RNA. The details are as follows:

1. General. All solvents used were reagent grade and were re-distilled before use. Celite 545 and Microcel E[5] were washed successively with 6 M and 3 M hydrochloric acid, respectively, with distilled water, and absolute ethanol; and dried at $100^{o}$C for 16 hours, and again before use (55).

2. Two-Phase solvent systems. Solvent (F) ethylacetate-2-ethoxyethanol-water (4:1:2); (G) ethylacetate-1-butanol-ligroin (bp66-75$^{o}$C)-water (1:2:1:1); (I) ethylacetate-glacial acetic acid-water (5:1:2).

3. Hydrolysis. Yeast RNA, prepared as described below was degraded to its constituent nucleosides by means of crude snake venom[6] and bacterial alkaline phosphatase[7]. The procedure employed was scaled down to 1/22 of that given by Hall (53). Each gram of RNA was dissolved in 66 ml of a $5 \times 10^{-3}$ M solution of $MgCL_2$. The covered solution was mixed at a slow magnetic stirrer speed in an incubator at

[5]Trade names of the Johns-Manville Co.

[6]Ross Allen Reptile Institute, Silver Springs Florida 32688

[7]Worthington Biochemical Corp., Freehold, New Yersey

$37^{\circ}$C. Seventy-five milligrams of venom and 1.66 milligrams

of phosphatase were added to the solution along with several

drops of toluene and the pH was adjusted and maintained at

8.6 with N/2 NaOH. After seven hours of incubation an addi-

tional 33 milligrams of venom and 7.9 milligrams of $MgCL_2$

were added. The solution was allowed to incubate for 24-27

hours or until the pH remained constant at 8.6. The solution

was then placed at $3^{\circ}$C for 3 hours and subsequently centrifuged

at $4^{\circ}$C at $13 \times 10^3$ rpm for 30 minutes. The clear supernatant

was lyophilized.

4.  Primary fractionation. The lyophilized digest was fraction-

ated by means of column partition chromatography (55). The

column was charged with 690 grams of 9:1 mixture of Celite

545 and Microcel E which had been thoroughly mixed with

308 ml of the lower phase of solvent F. The Celite mixture

was dry packed in 25-gram increments with a close-fitting

plunger machined from a Kel-F rod. The sample was dis-

solved in 35 ml of the lower phase of solvent F. The pH

tested with pH paper was always found to be approximately

7.0. The solution was filtered to remove insolubles through

Whatman No. 1 filter paper. Labeled spikes of 5-hydroxyuri-

dine-2-14 C        were then added to the 35 ml sample solu-

tion. The resulting solution would become cloudy on shaking

however, phase separation did not occur. This spiked solu-

tion was mixed with 80 grams of the Celite-Microcel mixture

and tamped on top of the column. The column was eluted

successively with the upper phases of solvents F and G. App-

roximately 17 ml fractions were collected and their absorban-

ce at 260,270, and 280 nm was measured manually in cells

with a pathlength of 2 millimeters on a Cary Model 15 spec-

trophotometer. The blank contained the upper phase of the

eluant employed. The separation required more than 30

hours. Hall (52) has stated that the column may be operated

on three consecutive days without diminishing its resolving

power.

5. <u>Subfractionation columns</u>. Uridine preparations may be puri-

fied on a smaller partition column (2.54 x 86 cm) employing

solvent system I. One hundred and fifty grams of Celite 545

is mixed with 60 ml of lower phase solvent I and packed into

the column in small increments with a close fitting plunger.

The sample is dissolved in 10 ml of lower phase, mixed with

25 gram of Celite 545 and tamped on top of the column. Elu-

tion is carried out with the upper phase of solvent I (52).

Columns 1/16 to 1/55 the size of the 770 gram celite

column have been made up by proportionally decreasing

the quantity of materials required.

Collected fractions obtained from the partition columns

were evaporated <u>in vacuo</u> to near dryness in a rotating eva-

porator. Distilled water was added and the mixture was re-

evaporated. This procedure was repeated three times in order to volatilize and remove the organic solvents.

## Ultraviolet Absorption Measurements

Five hundred microliters of an aqueous sample solution near neutral pH was used to obtain the ultraviolet absorbance spectrum of each sample. Two hundred and fifty microliters were placed in a quartz microcuvette with a 10 mm path length and the absorbance spectrum was determined between 350 and 210 nm with a Cary Model 15 recording spectrophotometer against a water blank. Five microliters of 4 M hydrochloric acid was added directly to the sample cell and blank and mixed with a glass capillary tube. The absorbance spectrum was recorded. To the remaining 250 microliters, five microliters of 4 M sodium hydroxide was added to both the sample and the blank and the absorbance spectrum was recorded. Spectral reversibility was tested by the addition of ten microliters of 12 M hydrochloric acid to the alkaline sample and blank and the absorbance measured.

## Spectral Titrations

One hundred ml of a distilled water solution of each sample titrated was made up to an absorbance of approximately $0.9A_{280}$ units/ml and measured in a cell with a 10 mm path length. This solution was placed in a 150 ml beaker along with a teflon coated magnetic stirring bar, mixed rapidly, and adjusted to approximately pH 3 with a drop of 2 M hydrochloric acid. The pH of the swirling solution was constantly monitered with a Radiometer Model 25 pH meter. Five microliter ali-

quots of 2M, 0.2 M, 0.4 M, 2M, and 10 M sodium hydroxide were added in varying amounts in this order to the titrated solution. After the addition of alkali a three ml aliquot of the solution was removed, placed in a four cc quartz cell with a path length of 10 mm, and its absorbance recorded between 350-210 nm against a water blank with a Cary Model 15 spectrophotometer. This aliquot was returned to the mixing solution and the titration continued to approximately pH 13. The cells were dried with air between each measured interval. In cases where only a small amount of sample was available two ml solutions, similar to those made up above, were titrated in four ml nalgene capped vials. Stirring was accomplised with a magnetic bar, and the pH was monitored before each sample was withdrawn. The absorbance of a 250 microliter aliquot was returned to the capped mixing sample and the titration continued to pH 13. Generally it was necessary to add 100 microliters of alkali to bring the pH from approximately 3 to above 11. The initial pH was obtained by the addition of one microliter of hydrochloric acid to the solution. In the intermediate pH range alkali was added at one-tenth the molarity of that employed above.

## Calculation of pKa

Ionization constants were calculated from the absorbancies at different pH values according to the relation

$$pKa = pH + \log \frac{A(pHI) - A(pHX)}{A(pHX) - A(pHUI)}$$

where A equals absorbance at a given wavelength, pHI equals the pH of the ionized species, pHUI the pH of the unionized species, and pHX an intermediate pH (95). The absorbancy values were obtained from spectral titration curves with dilution changes of less than one per cent. Careful inspection of spectral titration reveals distinct isosbestic points which characterize the ionization of UI to I for each dissociating proton. This information is essential for defining the limits of the pH values of UI and I.

## Ultraviolet Absorption Measurements for Chemical Stability Tests

All ultraviolet spectral measurements were made at room temperature with a Cary Model 15 recording spectrophotometer employing 10 mm quartz cells with a volume of 4 ml. Contribution of the absorbance of the reagent to the sample absorbance was eliminated by balancing the blank and sample cell, each containing only the employed concentration of reagent, at zero absorbance between 205 and 400 nm. This procedure eliminated any contribution of the solvent or reagent to the sample's absorbance. Sample absorbance was recorded for the full spectra between 350 and 210 nm without additional balancing. Acid spectra of the sample were obtained by the addition of 12 N HCL directly to the cells; mixing was with a glass capillary tube. The pH was tested with pHydrion indicator paper. The acid spectra of N/10 alkaline samples were diluted 35/36, and those of N alkaline samples 14/15.

## Solutions and Incubation Procedure for Chemical Stability Tests

Double strength (2X) solutions of the following reagent grade ma-
terials were used: N/20, N/10, and N solutions of KOH; 1 per cent and
10 per cent solutions of piperidine; N/10 and N solutions of $NH_3$; 0.17 M
trishydroxymethyl-amino-methane (Tris); and a $5.86 \times 10^{-2}$ M solution
of hydroxylamine. A solution (2X) of A grade 5-hydroxyuridine obtained
from Calbiochem was made up to 1.80 $A_{280}$ units/ml. Fifty ml of this
solution (5-hydroxyuridine) was mixed with an equal amount of each of
the double strength (2X) solutions (2 x N/20, N/10 KOH, etc.). The
zero time spectra were immediately recorded on 3.5 ml aliquots of the
above diluted solutions. Solubility factors required that 2M solutions of
$(NH_4)_2 CO_3$ be made up by addition of the reagent directly to the diluted
solution of 5-hydroxyuridine. The samples were then placed in a New
Brunswick incubator at $37^{\circ}$ C, or in a water bath at $80^{\circ}$ and $100^{\circ}$C. The
latter samples were held at constant volume and atmospheric pressure
by a needle stuffed with cotton which was pushed through the stopper of
the sample flask. The 100 ml mixtures of 5-hydroxyuridine:hydroxyl-
amine (1:1v/v) were titrated to pH 7.0, 8.0, 8.7, 9.6, 10.5, and 11.6
with 4N or 10 N NaOH. A Radiometer Model 25 pH meter was used for
the pH measurements.

## Cultures, Culture Medium and Incubation Procedure

Cadida utilis (Henneberg), NRRL Y-600 (Torulopsis utilis) was ob-
tained from the American Type Culture Collection, No. 8205, Rockville,
Maryland. Growth medium was employed which would provide oxidatively

competent yeast cells. The medium had the following composition: 20 gm Difco-peptone; 10 gm dextrose; 10 gm Difco-yeast extract, made up to one liter with distilled water. The pH of this solution was adjusted to 7.0 before sterilization in an autoclave for 15 minutes at 15 pounds pressure and $121^{\circ}$ C. Cultures were stored on slants containing 1.5 per cent Difco-agar in addition to the nutrients. Cultures were incubated at $30^{\circ}$ C for 24-48 hours with vigorous aeration. Larger cultures were grown employing the same medium and incubation temperature with vigorous aeration in 35 liter batches using a New Brunswick Fermacel. These culture suspensions were maintained at a constant pH 7.0 by the automatic addition of 1N HCl or 1N Na OH. Foaming was prevented by the addition of Dow Corning Antifoam A spray or solution. Cells were harvested employing a Sharples superspeed centrifuge or a Sorvall Szent-Gyorgyi -Blum continuous flow system.

## Extraction of Ribonucleic Acid from Yeast

Procedures which have been employed for extracting ribosomal RNA from yeast were reviewed ( 3, 23, 34, 50a, 73 ). Consideration was paid to the proportion of RNA, in relation to the total cellular RNA which could be obtained: the type and extent of labilization of RNA-protein binding sites; and to factors which might introduce alteration of 5-hydroxyuridine present in situ. Methods for isolation of ribosomal RNA can be broadly subdivided into those that involve a preliminary separation of the ribosomes and those in which total RNA is extracted from whole cells (23). Problems of the latter approach include: separation of RNA from other high molecular weight molecules; removal of

DNA (by specific enzymes) which is usually liberated with RNA; and
the presence of polysaccharide and lipid material. With all methods
available purication of ribosomal RNA involves separation of RNA from
bound and associated protein. The phenol method is probably more
drastic in its denaturing action on protein than other methods (23). Aside
from mild acid or alkaline hydrolysis, methods employing guanidinium
chloride, lithium chloride, mixtures of phenol with sulfones or surfa-
cants, and methods employing sulfones by themselves e.g. sodium
dodecyl sulfate (SDS) are some which have been employed. Separation
of RNA and nucleoprotein is generally accomplished by their inherent
difference in solubility. The action of many of these agents on the RNA-
protein bond is not clearly understood. 5-Hydroxyuridine participation
at these sites must be considered a possibility.

The procedure of Crestfield et al. (1955) (36) was selected. It
appears to be the gentlest procedure for separating the nucleoprotein
from RNA and is probably the best procedure for use with yeast, in
that yeast DNA content in proportion to cellular RNA is less than two
per cent and represents only a minor contaminant of the RNA extracted.
Enzymatic degradation is also eliminated. Ribonucleic acid is extracted
from yeast by lysing the cells in a solution containing 2 per cent puri-
fied sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$), 4.5 per cent
ethanol, $1.25 \times 10^{-2}$M $NaH_2PO_4$, and $1.25 \times 10^{-2}$M $Na_2HPO_4$. The
solution is brought to boiling, the yeast is added, and the mixture
heated at $92\text{-}94^\circ$ C for 3 minutes. It is then poured into a beaker
immersed in a dry ice-cellosolve freezing mixture and on cooling to

$4\,^{\circ}$C is centrifuged at 2000 rpm for 30 minutes at $0\,^{\circ}$C. The crude ribo-

nucleate with denatured protein is precipitated by addition of two volumes

of cold absolute ethanol. Pure ribonucleic acid settles out from an

aqueous solution of the precipitate on addition of sodium chloride to

make the solution one molar. Contaminating proteins and DNA remain

soluble in one molar sodium chloride. The solution is centrifuged at

2000 rpm for one hour and the supernatant liquid decanted. The pre-

cipatant gel is washed with 80 per cent ethanol containing a small amount

of sodium chloride, and is then dissolved in water and dialyzed for 36

hours at $4\,^{\circ}$C against frequent changes of distilled water. The clear

solution on lyophilization yields a white fibrous solid which represents

60 to 70 per cent of the ribonucleic acid originally present in the yeast.

It has been reported to contain less than 0.5 per cent DNA and less than

2 per cent contamination with protein ( 36 ). I could regularly obtain

almost three grams of white, light fibrous RNA from 300 grams of

wet packed yeast.

Methods of Analysis of Radioactivity

Paper chromatograms and electrophoretograms: Chromatograms

were cut in strips about one centimeter wide along the path of migra-

tion of the sample. Each strip was sectioned at centimeter or smaller

intervals along the strip and each section was placed in a scintillation

vial. Ten ml of fluor were added to each vial. The vials were counted

at gains of 9 per cent or 52 per cent for 14C or ^{3}H respectively, at a

window width of 50-1000 on a Packard Model 3002 Tri Carb - Scintilla-

tion Spectrometer. The fluor consisted of 4.0 grams 2,5-diphenyloxa-

zole (PPO), and 0.2 grams of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-

benzene (POPOP) per liter of toluene.

Column fractions: One-half ml was withdrawn from each fraction of

collected eluant from both Dowex-1-formate and celite partition columns.

Each aliquot was transferred to a scintillation vial and counted in 15 ml

of Bray's counting fluor (17). This fluor contains 60 grams naphthalene,

4.0 gram PPO, 0.2 gram POPOP, 100 ml absolute ethanol, 20 ml

ethylene glycol, and enough reagent grade p-dioxane to make up to a

liter. The extent of quenching in each counting vial was determined by

the channels ratio method. For this procedure a quench correction cur-

ve was constructed for 0.5 ml aqueous samples in 15 ml of Bray's solu-

tion. Two hundred microliters of naphthalene $-^{14}C$, in a solution of p-

dioxane, standarized at 641 dps/0.2 ml, 0.5 ml $H_2O$, and varying

amounts of pyridine quencher were added to ten counting vials contain-

ing 15 ml of Bray's solution. The vials were counted at 9 per cent gain

at window widths of 50 - 100 and 50 - 1000. The ratio of the counts ob-

tained in each channel, 50-100/50-1000, was plotted against the percent

counting efficiency at 50-1000 for each of the variously quenched vials.

When unknown samples are counted in 15 ml of Bray's solution at each

of the window settings, at 9 per cent gain, it is possible to determine

the counting efficiency from this curve, and hence calculate the actual

disintegration rate of the radioactive sample. Similar curves were con-

structed for $^3H_2O$ counted at 52 per cent gain in 15 ml of Bray's solu-

tion. In an analogous manner other correction curves were established

for 15 ml of Bray's solution for determining the activity of an isotope

in a sample doubly labeled with 3H and 14C, as follows: a) counting

efficiency at 52 per cent, 50 - 1000 for 14C vs. channels ratio at 9 per

cent, 240-260/240-1000; b) counting efficiency at 9 per cent, 240-1000

for 14C vs. channels ratio at 9 per cent, 240-260/240-1000; and c)

counting efficiency at 52 per cent, 50-1000 for $^3$H vs. channels ratio at

9 per cent, 240-260/240-1000.

Synthesis of Labeled 5-Hydroxyuridine

5-Hydroxyuridine-2-$^{14}$C and 5-hydroxyuridine-6-$^3$H were synthe-

sized to facilitate separation of the nucleoside from mixtures of nucleic

acid degration products, cell pools, and control mixtures. Synthesis

was carried out primarily by the method of Ueda (1960) (133), modified

according to the suggestion of Smith and Visser (1965) (123) and reincor-

porating some of the features of the original method of Roberts and

Visser (1952) (108). Sixty ml of bromine-water, prepared by adding a

saturating amount of bromine to distilled water, was added to a gram

of chromatographically pure uridine containing label plus carrier. Air

was bubbled through the solution for about 2 hours until it became color-

less. Five ml of pyridine was added to maintain a pH of about 6, and

the solution was heated for 6 hours on a boiling water bath. The solu-

tion was evaporated to dryness under reduced pressure. Seventy-five

ml absolute ethanol were added and the solution placed at -12° C for

four days. Labeled 5-hydroxyuridine separated out by the second day

as fine needle crystals which were removed by filtration and air dried.

The product was purified by $H_2O$ elution through a Dowex-1-formate

column and recrystalization. Initial yields range from 10 to 40 per cent.

Products characteristically gave positive phosphomolybdic acid

(11 ) and ferric chloride (37) color tests characteristic only of pyrimi-

dines possessing an enolic hydroxyl at the C-5 postition. A positive

reaction to both these tests is the production of a deep-blue color on

addition of 2 per cent phosphomolybdic acid or 1 per cent ferric chloride

(27) to a small amount of an aqueous alkaline or neutral, solution of 5-

hydroxyuridine respectively. Analysis of synthetically prepared 5-hyd-

roxyuridine has always been negative for the presence of an isodialuric

acid contaminant, a possible synthetic intermediate suggested by Ueda

(1960) (133). Isodialuric acid, on condensation with urea, by heating

easily forms uric acid. This latter compound, on heating with $HNO_3$,

cooling, and adding $NH_3$ gives murexide, a purple colored substance

(11 ).

The specific activity of the chromatographically purified labeled

substance was calculated in terms of dpm per absorbance at a specified

wavelength in a microcuvette with a 10 mm pathlength per 250 micro-

liters of aqueous sample. The specific activity has also been calculated

in terms of dpm/micromole.

Qualitative Chemical Spray Tests

Sugar test: Aniline hydrogen phthalate was used as a spraying re-

agent for chromatography of sugars. This reagent is valuable as a con-

firmation test for pentoses and hexoses. It is much more selective for

sugars than ammonical silver nitrate and is extremely sensitive for

aldo-pentoses and aldo - hexoses. It detects as little as one microgram

of either (99). A $10^{-1}$ molar solution of the reagent is made up with water-saturated butanol. After spraying, the chromatogram is heated for five minutes at $105^{\circ}$C to develop the color. The aldo-pentoses give a bright red color, while the aldo-hexoses, deoxy-sugars and uronic acids give various shades of green and brown.

Pyrimidine reduction test: Dihydropyrimidines were estimated by the method of Fink et al., (1955) (45). Dihydropyrimidines were converted to the corresponding β-ureido acids by spraying the paper chromatograms with aqueous N/2 NaOH and allowing them to dry for 30 minutes or more. The chromatograms were then sprayed with a solution containing 100 ml of ethanol, 10 ml of concentrated HCl , and one gram of p-dimethylaminobenzaldehyde, and were allowed to dry in a hood. This last step initiated development of a typical yellow color (E max 440nm) with the ureido acids formed by the NaOH spray, and with other compounds of the general formula $RNHCONH_2$, including citrulline, urea, and allantoin. Maximal yellow color development is obtained within two to six hours and is rather variable. It is generally possible, however, to detect dihydropyrimidines and β-ureido acids in amounts as small as 1 millimicromole per square centimeter.

Ninhydrin test: Paper chromatograms were sprayed with ninhydrin for detection and estimation of β-amino acids and their amides. Above pH 2 a blue color forms with a variety of compounds containing $NH_2$-groups, including amino acids, peptides, primary amines and ammonia. (91). Ninhydrin-positive materials may be satisfactorily detected on chromatograms by spraying the papers with 0.2 or 0.4 per cent ninhyd-

rin in water saturated butanol or 95 per cent ethanol followed by drying at $110^{\circ}$C for five minutes. Because of its convenience the ethanol based spray solution was employed.

Melting points: The melting points of structurally unidentified components along with those of controls were obtained on a Fischer-Jones Melting point apparatus.[8] A microgram quantity of the sample was placed between two glass cover slips, the temperature was slowly raised and changes in the physical character of the sample were observed with a 40-power disecting microscope. In cases of refractory materials, temperatures were recorded to $330^{\circ}$C, the instrumental limit. All samples were run in duplicate and in the purest form that could be obtained.

## Methods of Spectrometric Organic Analysis

Mass spectroscopy: A mass spectrum was obtained on an Atlas single focusing mass spectrometer. My sample was run by Dr. L. M. Libby, Associate Professor in Food Science and Technology, Oregon State University. The sample, 0.2 milligram was volatilized in a crucible at $145^{\circ}$C; inlet lines to the ionization chamber were held at and below $175^{\circ}$C.

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[8]Fisher Scientific Co. Pittsburgh, Pa.

Additional mass spectra were obtained on a Consolidated Electro-dynamics Corporation Mass Spectrometer 21-110 at the Oregon Graduate Center, Beaverton, Oregon, through the courtesy of Dr. Doyle Daves, Associate Professor of Chemistry. Sample introduction was by direct inlet with an ionization voltage of 70 electron volts. The source tempe-rature was varied. The molecular ions were recorded on an Ilford Type Q20.040 cm glass photographic plate. The spectra on the plate were examined with a Consolidated Electrodynamics Corporation Auto-matic Mass Spectrum Data System No. 21-087 coupled to a Grant Instru-ments Comparator-Microphotometer. High resolution mass measure-ments of sample ions were calculated with a Wang Electronic Calculator Model 370 relative to fragments of perfluorotri-n-butylamine. To fa-cilitate sample volatilization within the mass spectrometer trimethyl-silyl derivatives were prepared with Pierce 'Tri-Sil Z' which is N-tri-methylsilylimidazole. This reagent only exchanges hydroxyl protons; it is reacted in a stoppered vial under vacuum at a concentration of 10-15 mg of sample to one ml Tri-Sil Z at 60-70° C until the sample is dissolved. An aliquot of this solution is transferred to a one centimeter capillary tube, of which one end has been sealed, and the solution dried under vacuum at 60° C. The sample may then be introduced into the mass spectrometer.

Infrared spectroscopy: Infrared spectra of structurally unidentified components along with spectra of controls were obtained on a Beckman IR-9 infrared spectromenter. Through the courtesy of Dr. B. Kittinger, Professor of Biochemistry, Oregon Regional Primate Center,

Beaverton, Oregon, these were obtained by grinding 25 micrograms of sample with 3 milligrams of dry KBR in an agate mill, and compressing the mixture into a small rectangular glass pellet 1 mm x 5 mm x 0.3 mm.

Nuclear magnetic resonance spectroscopy: Nuclear magnetic resonance spectra were obtained through the courtesy of Dr. D. Daves at the Oregon Graduate Center, Portland, Oregon. Samples of approximately 100 micrograms in 25 microliters of deuterated water, dimethylsulfoxide, or trifluoroacetic acid were run by Mr. W. Anderson on a Varian H-100 spectrometer coupled to a Varian C1024 time averaging computer, which accumulates the sample signal and averages out instrument noise.

RESULTS

Characterization of Absorbance and Ionization Characteristics of
5-Hydroxyuridine

Some of the prominant spectral characteristics of an aqueous solu-
tion of 5-hydroxyuridine may be seen from Figure 1. Acidic solutions
of 5-hydroxyuridine between pH 1.5 and 5.4 give identical spectra.
They exhibit a maximum absorbance at 280 nm with a molar extinction
of 8,200 at this wavelength, and a minimum at 243 nm. On increasing
the pH of the solution from around pH 6.0 to 9.5 there is a progressive
loss in extinction at 280 and gains in absorbance at both 243 and 307 nm.
A comparison of the spectra at pH 5.4 and 9.5 reveals that on addition
of alkali a bathochromic shift occurs with the formation of new maxima
at 307 and 243 nm. Increasing the pH of the solution above 11 produces
a gradual hypsochromic shift in the maximum. The spectrum shifts
from 307 to 302.5 nm, and in addition above pH 12.5 there is a loss of
the 243 nm peak. It may be noted that at isosbestic point c at 294 nm
the extinction for acidic though alkaline solutions is constant at 5610.
Thus measurement of the absorption of a pure solution of 5-hydroxyuri-
dine at 294 nm at any pH permits calculation of the molarity of that
solution.

Figure 2 shows a more detailed absorption spectra for solutions of
5-hydroxyuridine between pH 2.9 and 9.5. These spectra clearly define
three isosbestic points: a at 229, b at 258, and c at 294 nm (293.5).
The gradual loss of extinction at 280 nm, the increase at 243 nm, and

the bathochromic shift on addition of alkali are easily recognizable. The spectral changes observed are due to the ionization of one proton. Two protons on the pyrimidine ring, the N-3 proton and the 5-hydroxyl proton are capable of dissociation. There are no fundamental criteria, except for analogues, for deciding which proton ionizes to produce the spectral shifts observed in Figure 2. The approach and solution of this problem required methylation studies, which are reported below. Although at this point the dissociating proton cannot be specified the pKa can be calculated from the curves of Figure 2. Table 1 shows the re-sults of 56 individual calculations of the $pKa_1$ of 5-hydroxyuridine. The average value was found to be 7.83. This value agrees well with a value of 7.8 which was obtained by electrometric titration with a Radio-meter autotitrator by Roy-Burman et al. (1966)(113)which is the only published pKa value for 5-hydroxyuridine. These workers did not spe-cify which proton dissociated at this pKa.

The absorbance spectra of 5-hydroxyuridine between pH 9.5 and 11.0 are indicated in Figure 3. A spectral curve for pH 8.4, which in-tersects the pH 9.5 spectrum at three points a, b, and c, has been added to define these isobestic points. The spectras of solutions above pH 9.5 pass through isosbestic point c at 293.5 nm but do not pass through points a or b. Examination of the spectra between 220 and 228 nm reveals a progressive gain in extinction above pH 9.5. The spectra of solutions at pH 9.7 through 10.7 do not pass through isosbestic point a, but do appear to pass through point d at 246-247 nm. Note that the spectrum of the pH 11.0 solution does not pass through point d. The

detail at higher pH values is presented in Figure 4. The spectra of so-lutions at pH 10.7 through 12.275 all pass through point $\underline{c}$ at 241 nm. There is no spectral change above pH 12.5. These solutions do not form isosbestic points at $\underline{a}$ or $\underline{b}$, or at $\underline{d}$, or $\underline{e}$, but do approximate isosbestic point $\underline{c}$ at 293.5 nm.

The second and third ionization constants of 5-hydroxyuridine have been calculated from the above spectrophotometric titration and the data is presented in Tables 2 and 3. Twenty values have been calculated for the intermediate ionization constant, $pKa_2$, and are shown in Table 2. The average value is 10.57. There is much less agreement among these values than there was for $pKa_1$. The value of 11.70 for $pKa_3$ has been obtained by averaging ten individual calculations as shown in Table 3. This value presumably represents the dissociation of the 2'-hydroxyl of the ribose. As with $pKa_2$, the value for $pKa_3$ should only be considered a rough average. Spectral titration data as these, however, may be the only type obtainable for high pKa values as potentiometric titration becomes of less value at higher alkaline pH's.

## Preparation, Absorbance, and Ionization Characteristics of Methylated Derivatives of 5-Hydroxyuridine

The spectral and chemical characteristics of methylated derivatives of 5-hydroxyuridine have not been reported in the literature. These derivatives were prepared in order to determine the effect of methylation on the ionization constants of 5-hydroxyuridine and its chemical

stability. The method of Scannel et. al. (1959)(114) was employed.

Fifty micromoles of 5-hydroxyuridine were dissolved in two ml of

distilled water and adjusted and maintained at pH 9.4 with a microsyrin-

ge containing 5 per cent (w/w) potassium hydroxide. Two hundred mic-

romoles of dimethyl sulfate were added at the start of the experiment,

after one hour, and thereafter when the pH of the solution dropped to

2.0. At various intervals 20 microliter aliquots were withdrawn,

applied to paper chromatograms, and submitted to ascending chromato-

graphy butanol-$H_2O$ (86:14). Preparation of sufficient quantity of the

methylated derivatives for chemical characterization was accomplished

by the same procedure only larger amounts were submitted to chroma-

tography. Chromatography of the methylated products of 5-hydroxyuri-

dine is shown in Figure 5 along with migration of a 5-hydroxyuridine

control. Derivative a migrates parallel to that of 5-hydroxyuridine

and was thought to represent the unmethylated substrate until the spec-

trum of this material was obtained, Figure 6. A comparison of this

spectra with that of 5-hydroxyuridine, Figure 1, reveals several dif-

ferences. The dissociation of a at pH 7.1, as shown at its maximum at

278 nm, is not as extensive as that of 5-hydroxyuridine at 7.0 at its

maximum at 280 nm; the pH 8.1 spectrum of a does not exhibit as ex-

tensive a bathochromic shift as the pH 8.0 spectrum of 5-hydroxyuridi-

ne; and, the absorbance of derivative a between 225-250 nm is consider-

ably different from that of 5-hydroxyuridine, in particular isosbestic

points a, d, and e are missing, without a characteristic hump or peak at

243 nm. The average of several $pKa_1$ calculations gave a value of 8.18

for this derivative. Although its migration is the same as that of the control, it has been definitely modified, but not by methylation.

Figure 5 shows that three methylated derivatives of 5-hydroxyuridine, b, c, and d are produced by 40 minutes after the initial addition of dimethyl sulfate. An additional derivative, e, is produced after extensive methylation for 13 hours. It may be observed that as the reaction time increased there is an increasing formation of derivatives d and e, and a disappearance of a and b. There is a slight increase with time in the amount of c. The extent of migration and the changing concentrations of these latter three species indicates that they are less fully methylated than d. Derivative e does not appear until there is an extensive concentration of d, in proportion to the other derivatives, and because of its faster migration, it appears to be a methylated product of d; and the following methylation scheme may be advanced:

$$5\text{-hydroxyuridine} \dashrightarrow \text{"}\underline{a}\text{"} \begin{cases} \longrightarrow \underline{b} \\ \longrightarrow \underline{c} \end{cases} \longrightarrow \underline{d} \longrightarrow \underline{e}$$
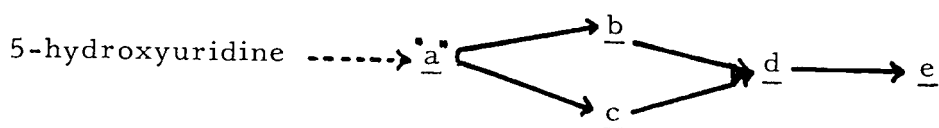
The spectral titration of b is indicated in Figure 7. The methylated derivative possesses an acid absorbance maximum at 277.5 and minimum at 245 nm; and an alkaline maximum at 274 nm. The characteristic bathochromic shift which is produced on addition of alkali to 5-hydroxyuridine is not observed. There is, however, a decrease in extinction on addition of alkali. The calculated dissociation values for derivative b are presented in Table 4. The $pKa_1$ and $pKa_2$ values for

b each represent the average of nine, calculations and are respectively

9.33 and 11.42. The higher pKa represents the dissociation of the sugar

moiety (2'-hydroxyl). Thus b represents a methylated derivative of

5-hydroxyuridine in which one of the two dissociable base protons has

been methylated.

The results of the spectral titration of c are indicated in Figure

8. This methylated derivative possesses an acid absorbance maximum

at 277.5 and a minimum at 246.5 nm; and an alkaline maximum and

minimum at 306 and 270 nm respectively. These spectra resemble

those of 5-hydroxyuridine, however, there are several distinct differen-

ces: There is no hypsochromic shift in the alkaline absorbance maxi-

mum on raising the pH from 9.5 to 13.1, as the 307 to 302.5 nm shift

which is characteristic of 5-hydroxyuridine and derivative a. The acid

maximum has shifted from 280 to 277.5 nm and the minimum from 243

to 246.5 nm. The peak or hump at 243 nm exists through pH 13.1

whereas with 5-hydroxyuridine it disappears above pH 11.0; and, the

isosbestic points a, d, and c which are associated with this peak in 5-

hydroxyuridine are not present in derivative c. The average pKa,

value of c of 8.33 was determined from 40 separate calculations as

shown in Table 5. The value for $pKa_2$ of 12.67 could only be calculated

at 235 nm for two ionized solutions, nevertheless the calculated values

closely agree. The value for the $pKa_2$ of c represents the dissociation

of a sugar moiety (2'-hydroxyl). C may be considered to be a deriva-

tive of 5-hydroxyuridine in which one of the two dissociable base pro-

tons has been methylated.

Figure 9 shows the spectral titration of d. The derivative poss-
esses an absorbance maximum between pH 1.0 and 10.7 at 276.5 nm,
which undergoes a slight bathochromic shift to 278.5 nm at and above
pH 11.4. The minimum is constant at 245 nm. The shift is presumably
due to the dissociation of the 2'-hydroxyl; both dissociable base protons
appear to have been methylated.

The spectral titration of derivative e is presented in Figure 10.
The absorbance maximum occurs at 276 nm and the minimum at 246.5
nm. There is no shift in these values or significant changes in the ex-
tinction between pH 1.0 and 12.9. In this derivative presumably the 2'-
hydroxyl, as well as the two dissociable base protons have been methy-
lated. Thus e is probably 3-methyl-5-methoxy-(2'-o-methyl)-uridine.
Properties of the methylated or modified derivatives of 5-hydroxyuri-
dine are summarized below.

| Compound | Ionization Constants | | | No. of Methyl Groups | Ferric Chloride Test |
|---|---|---|---|---|---|
| | $pKa_1$ | $pKa_2$ | $pKa_3$ | | |
| 5-hydroxy-uridine | 7.83 | 10.57 | 11.70 | 0 | (+) |
| a | 8.18 | | | 0 | (+) |
| b | 9.33 | 11.42 | | 1 | (-) |
| c | 8.33 | 12.67 | | 1 | (+) |
| d | approx. 11 | | | 2 | (-) |
| e | none | | | 3 | (-) |

The ferric chloride test was carried out in neutral solution accor-
ding to the procedure of Davidson and Baudisch (1925) (37). The test
does not work in alkaline solution according to the procedure of
Bendich and Clements (1953) (11). It was run on approximately $10^{-2}$ - $10^{-3}$

molar solutions of the derivatives; giving excellent results on $10^{-4}$-$10^{-5}$ molar solutions of a 5-hydroxyuridine control. The phosphomolybdic acid test (11) gave questionable results with $10^{-3}$-$10^{-2}$ molar control solutions and thus was not employed. The above results indicate that a, c, and 5-hydroxyuridine each possess an enolic hydroxyl at the C-5 position. On the basis of this data the structures of these derivatives can be assigned:

"a"   *conformationally altered* 5-hydroxyuridine (?)

b     5-methoxyuridine; pKa$_1$ 9.33 for N3-proton

c     3-methyl-5-hydroxyuridine pKa$_1$ = 8.33 for 5-hydroxyl proton

d     3-methyl-5-methoxyuridine

e     3-methyl-5-methoxy-(2'-o-methyl)-uridine

The spectra of derivative c, Figure 8, clearly shows the pronounced bathochromic shift in maxima on addition of alkali to an acid or neutral solution, which is characteristic of 5-hydroxyuridine. This indicates that the spectral shift in 5-hydroxyuridine is due to the dissociation of the 5-hydroxyl proton. This shift is complete by pH 9.3 and must have a dissociation constant of pKa$_1$ 7.83 as shown in Table 1. Thus the 5-hydroxyl proton of 5-hydroxyuridine dissociates with a pKa$_1$ of 7.83 and the N3-proton of the base possess the pKa$_2$ of 10.57. Summary of the ionization constants of 5-hydroxyuridine is presented in Figure 11.

## Characterization of Absorbance and Ionization Characteristics
## of Isobarbituric Acid (5-Hydroxyuracil)[8]

Some of the spectral curves for the titration of isobarbituric acid are presented in Figure 12. Three ionization constants for isobarbituric acid have been calculated from the spectral titration and the data are presented in Table 6. The values for $pKa_1$ through $pKa_3$ are respectively 8.0, 10.8, and 12.3. Spectral curves which characterize the first dissociation constant, presumably for the ionization of the 5-hydroxyl proton, are defined by isosbestic points a, c, and d, at 226, 259, and 291 nm, as shown in Figure 12. The spectra for pH values between 8.7 and 10.2 are closely identical; those below pH 6.4 are similarly identical. The second dissociation, $pKa_2$ for the N1 proton is defined by spectral curves which pass through isosbestic point b at 229 nm. The third disso- ciation constant, $pKa_3$ may be calculated from spectral curves for pH values above the upper limit for $pKa_2$ which possess ab- sorption maxima at 302 nm rather than 304 nm characteristic of the curves which reflect the ionization of the N-1 proton. The cal- culated values presented in Table 6 agree fairly well considering both the general difficulty of determining accurate dissociation values above pH 12 and the instability of isobarbituric acid in alkali. It is assumed in the calculation that the significant in- crease in instability of isobarbituric acid in this pH region is related to dissociation of the

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[8] Compound listed as isobarbituric acid in Chemical Abstracts.

N-3 proton and that each curve obtained in this region is a reflection of the inherent immediate instability of the molecule at that pH, as only a few minutes at ambient temperature elapsed during the measurement of these spectra. Several literature values for the dissociation of isobarbituric acid protons are available which may be compared with obtained data:

| $pKa_1$ | Method of Measurement | References |
|---|---|---|
| 8.09 | spectrophotometric | 97 |
| 8.11 | potentiometric | 2 |
| $pKa_2$ | | |
| 11.48 | potentiometric | 2 |
| $pKa_1$ - 8.0 | spectrophotometric | these studies |
| $pKa_2$ - 10.8 | " | " |
| $pKa_3$ - 12.3 | " | " |

## Characterization of Absorbance and Ionization Characteristics of 5-Hydroxy 2'-deoxyuridine

The absorbance spectrum of 5-hydroxy 2'-deoxyuridine is presented in Figures 13 and 14. The striking feature of the spectrum is the absence of isobestic points which describe the orderly progression of changes in maximum and minimum for the spectral lines associated with the dissociation of each proton. Thus it is not possible to calculate the dissociation constants for 5-hydroxy 2'-deoxyuridine spectrophotometrically. It is possible, however, to estimate the range for

nm beginning about pH 8.600 which drops in absorbance between pH 9.300 and 10.200, and then raises through pH 10 to 11 becomming ob-viated above pH 11. It is similar to 5-hydroxyuridine a in this respect. It should also be noted that an hypsochromic shift occurs in the absorption spectrum of 5-hydroxy 2'-deoxyuridine when the pH of the solution is raised between pH 10.425 and 11.500. Such a shift also occurs in the spectra of both uracil and cytosine ribosides and deoxyribosides (46).

## Optical Rotatory Dispersion Curves for 5-Hydroxyuridine

The optical rotatory dispersion curves of 5-hydroxyuridine at pH 7.0 in distilled water and in $5 \times 10^{-3}$M Mg $Cl_2$ are shown in Figure 15. These curves were kindly run for me by Dr. T.E. King, Professor of Chemistry, Science Research Institute, Oregon State University, Corvallis, Oregon. Measurements of the optical rotatory dispersion of pyrimidine and purine nucleosides provides an extremely simple and sensitive method for determining the anomeric configuration of these substances (43). The optical rotatory dispersion curves of pyrimidine $\alpha$-nucleosides give negative Cotton effects whereas the pyrimidine $\beta$-nucleosides give positive Cotton effects. The signs of the Cotton effects of purine nucleosides are reversed(42). Distilled water and $MgCl_2$ solutions of 5-hydroxyuridine at pH 7.0 give positive Cotton effects in the first extremum, indicating that the sample predominantly exists as the $\beta$ anomer. This appears to be the first report of the optical rotatory dispersion spectrum of this compound. Both curves were obtained on a synthetically prepared sample of 5-hydroxyuridine-2-[14] C.

This sample has been used in an isotope dilution analysis experiment which will be considered in detail in sections below. It should be pointed out, however, that the optical dispersion curve does not discriminate whether or not the sample is in a bridged β-conformation. It is a broad generality that bridged derivatives exhibit much enhanced molecular rotation due to the rigidity of their chromophore; thus the curves shown, because of the rather weak character ot the Cotton effect, probably represent those for an unbridged compound. The optical rotatory dispersion curve presented in Figure 15 for 5-hydroxyuridine in distilled water is very similar to that of β-thymidine given in reference 43.

"The variation in both size and magnitude of the Cotton effect is due to the changing conformation of the planar pyrimidine ring with reference to the sugar ring, i.e., the position of the chromophore relative to the asymmetric centers in the sugar." Emerson et. al. (1967) (44) additionally states that rotation about the glycosidic (Nl-Cl') bond is theoretically possible but in practice is restricted by steric factors rather than by hydrogen bonding. "In uracil, thymine, and cytosine nucleosides the proximity of the carbonyl group in position two of the pyrimidine ring to the groups on C-2' and C-5' of the sugar interferes with free rotation about the glycosidic bond; hence, the pyrimidine ring, although not rigidly held; has a preferred conformation such that the oxygen atom on C-2 is directed away from the furanose ring" (44). I have drawn the structure of 5-hydroxyuridine in Figure 11. according to this approach. It is assumed that a line drawn between C2 and C4 of

the base passes through the plane of the furanose ring and that rotation about the glycosidic bond is somewhat restricted. These two criteria have been suggested by Emerson et. al. (1967) (44) to produce a positive cotton effect in uracil, thymine, and cytosine pentofuranosides.

## Binding of Magnesium Cations with 5-Hydroxyuridine

The spectral titration of 5-hydroxyuridine in $5 \times 10^{-3}$M $MgCl_2$ is shown in Figure 16. The spectra between pH 4.5 and 8.0 only approximate the isosbestic points a, b, and c of distilled water solutions of 5-hydroxyuridine. These occur at 229, 250, and 294 nm and are formed by spectra of acid solutions through pH 9.5. The absorbance maximum of artifact $MgCl_2$ solutions occurs at 280 nm with minimum at 243 nm. These values are identical to those of distilled water solutions of 5-hydroxyuridine. On addition of alkali there is a pronounced bathochromic shift to a new maximum at 304, rather than to 307 nm; and on continued addition of alkali there is a small hypsochromic shift to 300 nm rather than to 302.5 nm which is characteristic of distilled water solutions of 5-hydroxyuridine. The gain in extinction of the pH 11.5 $MgCl_2$ solution may be due in part to the formation of magnesium hydroxide, however, turbidity cannot be visibly detected at this pH. The initial ionization constant, $pKa_1$ could not be calculated accurately due to the distortion of the spectras and the lack of correspondence of isosbestic points. Those values which were calculated indicated a $pKa_1$ which was approximately equal or somewhat lower than that for distilled water solutions of 5-hydroxyuridine. An average value for $pKa_1$ of 7.6 was obtained.

The spectrum of the $MgCl_2$ solution at pH 8.7 resembles that of a pH 11.0 solution of 5-hydroxyuridine in distilled water. A comparison of the spectra of equimolar solutions of 5-hydroxyuridine at exactly pH 8.70 in $5X10^{-3}$ M $MgCl_2$ and in distilled water is shown in Figure 17. There is pronounced difference in the spectra, especially in the area of the hump or peak at 243 nm. The influence of magnesium cations on the spectrum of 5-hydroxyuridine is interpreted as strong ionic bonding between the two which alters normal resonance properties of the molecule at this pH. The magnesium binding spectrum indicates that the hump or peak at 243 may also be attributed to ionization of the 5-hydroxyl proton as well as the general bathochromic shift which has been considered above.

## Analysis of Ribonucleic Acids

5-Hydroxyuridine has been isolated by this laboratory (83) from yeast hydrolysates and from commercial uridine which had been obtained from crude RNA of the yeast Torula utilis. The 5-hydroxyurdine isolated from the commercial preparation of uridine is presumed to be a natural component of yeast RNA, however, it could have perhaps also been chemically produced in the course of commercial alkaline hydrolysis. In addition, the isolation of minor amounts of 5-hydroxyuridine directly from yeast RNA appears to indicate the molecule is a normal minor component, however, the possibility that it could have been formed during the isolation procedure by some unknown mechanism can not be entirely ruled out. One of the steps in examining this

question required an attempt to quantitate the amount of the nucleo-

side present in preparations of enzymatically undegraded RNA from

this yeast.

Yeast Nucleotide Pool Analysis

The following analyses were carried out in order to ascertain

whether 5-hydroxyuridine was significantly present in leachings from

washed Candida utilis cells, in the dialysates of ground cells, or in

crude RNA preparation dialysates: (A) C. utilis cells were harvested

in the stationary phase, washed with distilled water and leached using

the method of Lis and Passarge (83). The leachings from 48 grams of

lyophylized yeast were dried, dissolved in water, neutralized, and ly-

ophylized. This was subjected to paper chromatographic analysis. (B)

One hundred-twelve grams of lyophylized C. utilis cells were ground

in a ball mill, mixed into solution with distilled water, and dialyzed at

$4^{o}C$ against 6 liters of distilled water. The dialysate was vacuum dried

to a light grey, fatty mass and subjected to paper chromatographic an-

alysis. (C) Forty grams of crude Torula RNA[9] were dissolved in dis-

tilled water and dialyzed at $4^{o}C$ against 6 liters of distilled water. The

dialysate was concentrated to dryness in vacuo to fine mica-like, brown

flakes and submitted to paper chromatographic and electrophoretic an-

alysis. The undialyzable material was run on a DEAE-cellulose column

with a linear sodium chloride gradient and was shown to contain five

[9.] General Biochemicals, Inc.

peaks, one of which represented approximately 95 per cent of the sample.

The above chromatographic and electrophoretic analyses revealed the presence of: (a) uridine, adenosine, uracil, and a small amount of guanosine; (b) nucleotides were present in approximately the same amount as the nucleosides; (c) no bases, except uracil, could be demonstrated; (d) an ultraviolet absorbing band of material from the crude Torula RNA preparation was purified and exhibited an unidentifiable spectrum, as shown in Figure 18. This material was not altered by incubation with snake venom and alkaline phosphatase. Athough interest was given primarily to those areas on chromatograms parallel to 5-hydroxyuridine control migration, ultraviolet spectral identification of this compound from the above samples was not obtained due to insufficient quantities. It will be pointed out later that the ability of 5-hydroxyuridine to form complexes may mask its recognition by this precedure. It was concluded from this work that 5-hydroxyuridine is not appreciably present in C. utilis pool leachings or in crude RNA preparation dialysates. In addition, it was decided that a quantitative column method was needed to facilitate separation of this minor component from mixtures of the standard RNA and pool components, at very high concentration, before an examination of ribonucleic acids could be begun.

## Column Fractionation Procedures and Results

Our initial objective was to find a column chromatographic system which could be used to separate 5-hydroxyuridine from other nucleic acid components, such that cellular RNA fractions could be quantitavely assayed for the presence of the former. Dowex anion exchange resins and Celite partition column chromatographic procedures were examined for their utility in separating labeled 5-hydroxyuridine from mixtures of major and minor nucleosides:

Dowex columns: 5-hydroxyuridine-2-14C was not eluted with water through Dowex-1-chloride. With Dowex-1-formate 5-hydroxyuridine came off as a wide peak with water elution primarily between one, and one and a half column volumes; however, the label trails through five column volumes. In this area, cytidine, uridine, uracil, 5-ribosyluracil, orotate, xanthine, adenosine, etc. were eluted. Separation could be improved by increasing the length of the column relative to its diameter; however, large samples of nucleoside mixtures could not practically be separated. Separation was not good enough to permit meaningful chase experiments for quantitating the randomization of a labeled precursor metabolite.

Celite columns: The partition chromatography method of Hall (1965) (52) was explored to sidestep disavantages of Dowex-1. In this procedure nucleoside mixtures are resolved into 6 major fractions corresponding to the major ribonucleosides, with neutral solvents. These major fractions are resolved into a number of subfractions by further column par-

tition chromatography using different solvent systems. The minor nucleosides contained in each of the subfractions are isolated and purified by means of paper chromatography.

Quantitative chromatography on Celite represented a technique with which our laboratory had had no experience and required a considerable expenditure of time before it was operational. It was found that the column size and procedure employed by Hall must be precisely duplicated in order to approximately reproduce similar separation. Smaller columns than those used by Hall, i. e. than the 770 gm Celite column he employed, progressively separate the major ribonucleosides less effectively. Columns 1/55 to 1/10 this size yielded three large peaks and one small one when mixtures of nucleosides were applied. 5-Hydroxyuridine was found in the final peak together with guanosine and cytosine as shown in Figure 19. The components which are separable by the small partition columns are eluted by 7 column volumes with the upper phase of solvent F, ethyl acetate-2-ethoxyethanol-water (4:1:2), and nothing additional is brought down with the second solvent system, G, consisting of the upper phase of ethyl-acetate-1-butanol-ligroin (bp 66-75°)-water, (1:2:1:1), Figure 19.

The four principal nucleotides, orotidine, and significant amounts of cytidine, guanosine and 5-hydroxyuridine are held up and can only be washed off these small columns by eluting them with water. It was assumed this situation would significantly improve when fractionation was attempted on the much larger 770 gram partition column. It was felt that a simple method for separating

5-hydroxyuridine from the major nucleosides might arise if the label was not eluted by the normal solvents but could be obtained from a water wash of the column.  Dr. Hall (54) has stated that the amount of nucleosides eluted in the water wash is variable and he believes it is due to some sort of complexing, perhaps with metal ions or between nucleosides themselves.

## Celite Fractionation of Commercial Uridine

A commercial preparation of uridine[10] was spiked with 5-hydroxyuridine-2-$^{14}$C and was run on a 178 gram (2.54x86 cm) Celite subfractionation column.  This was eluted with the upper phase of ethyl-acetate-glacial acetic acid-water (5:1:2) according to the procedure of Hall (52).  Two columns, each with different concentrations of uridine applied, were run and in each case, as the recorded absorbance of the eluted uridine peak dropped to less than 1/4 that maximally observed the C14-label began appearing and was eluted in an equal column volume as the uridine peak, Figure 20. It should be noted that Hall's data (52) do   not show any peak after that of uridine, indicating that perhaps 5-hydroxyuridine had complexed on his column and had not been eluted, or that it had been chemically altered, or was simply not present in his sample.  In the area of label elution, the 280/260 ratio is greater than that for fractions containing eluted uridine.  Only a small fraction of the administered label was eluted from the column.  Most was found in the upper organic phase of the solution collected subsequent to wash-

[10] Cyclo lot No. UN

ing the column with water.  In an earlier experiment uridine and

a small spike of 5-hydroxyuridine-2-$^{14}$C had been layered on the

same size column as above and elution carried out with the upper

phase of solvents F and G.  In this instance uridine was obtained

as a peak; however, no trace of label was eluted.  This column

was not washed with water.

Fractionation of Yeast Ribosomal  RNA

A flow sheet of the procedure used to fractionate Candida

utilis  RNA is given in Figure 21.  The procedures for extracting

RNA from yeast and digestion with Crotalus  adamanteus venom

and bacterial alkaline phosphatase have been described in the

Methods section.  The lyophylized hydrolysate was dissolved in

the lower phase of solvent F and filtered through Whatman No. 1

paper in a small Buchner funnel; the pH, tested with paper, was

approximately 7.  Chromatographically purified 5-hydroxyuridine-

2-$^{14}$C, which contained no uridine or bromouridine, was then added

to a concentration of 0.7 per cent w/w of the RNA digest and shaken

into solution in a test tube.  Although the filtrate of the lyophylizate

solution was fairly clear, on shaking it became cloudy; however,

no phase separation resulted.  This material was allowed to sit in

a screw capped tube while the column was made up.  It was then

placed on a 770 gram 5x105 cm Celite partition column and eluted

with 6.7 liters of upper phase solvent F and 5.7 liters of upper phase

solvent G.  The elution profile is presented in Figure 22.  The label

was not obtained throughtout the full elution schedule but 89 per cent

was recovered as a sharp peak in the first 425 ml. of water wash. This fraction will be referred to as the labeled "water-wash peak" from column HC #13. It was concentrated by a procedure described in the methods section and was accordingly termed the "wash concentrate" from column HC #13.

## Methods of Examination of the Celite Column Labeled Water-Wash Peak

The column wash peak was pooled and concentrated and contaminating celite removed by centrifugation. It was assumed that the labeled material could be rigorously purified and analyzed for isotopic dilution. Such an isotope dilution analysis would permit some conclusion concerning the quantity of cold 5-hydroxyuridine present in the yeast RNA. Some of the subfractionation procedures used to analyze the content of the "wash peak" and to purify the label are presented in Figures 23 and 24. In general, the labeled "wash concentrate" was either submitted initially to column chromatography on Dowex-1-formate or was directly analyzed by ascending paper chromatography employing several solvent systems. Fractions were resubmitted to paper chromatography employing differential solvents and to paper electrophoresis. Identification of fraction components was made on the basis of their ultraviolet absorption spectra measured at pH 1.7, 7, and 12, and by various chemical spray tests.

## Paper Chromatographic Analysis of the Labeled Wash Concentrate

The migration of the wash concentrate on paper chromatography after five submissions to butanol-$H_2O$ (86:14) is shown in Figure 25 along with the migration of 5-hydroxyuridine control. It can be seen

that the label runs slower than either the front running band consisting primarily of cytidine or a second band containing guanosine; and it does not parallel the migration of the control. At least two labeled peaks are evident, one of which is associated with the origin. This was the first indication that the label was not behaving as a 5-hydroxyuridine. In order to determine if 5-hydroxyuridine, which normally runs faster than cytidine or guanosine in this solvent, falls behind them when present in a mixture of an equal concentration of each of the nucleosides and nucleotides, such a mixture was made and chromatographed. The results are presented in Figure 26. 5-Hydroxyuridine did not complex with these constituents and migrated in the mixture as the control, i. e. ahead of cytidine and guanosine. Similar results were obtained when it was chromatographed in a mixture of all the nucleosides. In both experiments, 5-hydroxy-uridine-2-$^{14}$C migrated homogeneously as one peak through the other components and did not appear to be held back or to complex with them. 5-Hydroxyuridine-2-$^{14}$C was then added to another aliquot of the labeled wash concentrate and submitted to the same chromatographic conditions. The results are presented in Figure 27. The label migrated as two bands, one containing about twice the activity of the other and neither paralleled the control. It can be seen that the label migration is similar to that from the wash concentrate, Figure 25, indicating that the 5-hydroxyuridine-2-$^{14}$C, which was added to the labeled wash concentrate, is either forming one or more molecular complexes or is undergoing multiple chemical alteration or both.

Analysis of the Labeled Wash Concentrate From Partition
Chromatography

An aliquot of the labeled wash concentrate was applied to a
103 cm$^3$ Dowex-1-formate column; Figure 28 shows the elution
profiles. Labeled controls were run and their position of elution
is indicated. The total C-14 label of the wash elutes in the same
peak as that of cytidine. At least three materials peak in the area
in which guanosine is eluted. The material eluting after one N
formic acid probably represents mono- and oligonucleotides resis-
tant to snake venom diesterase and alkaline phosphatase. Peaks
I through IX may contain components which have not yet been pre-
viously described. Figure 29 shows the spectra of an unknown
compound which has been obtained from peak II. Its acid spectrum
somewhat resembles that of guanosine, but the compound does not
dissociate between pH 1.7 and 12.3, as does guanosine. This un-
known appears to be a nucleoside and is not changed by incubation
with snake venom or alkaline phosphatase.

Smaller aliquots of the labeled wash concentrate were applied
to 54 cm$^3$ columns of Dowex-1-formate along with labeled controls;
and the profile of the first peaks which are eluted with water is in-
dicated in Figure 30. The top graph shows the position of elution
of 5-hydroxyuridine-2-$^{14}$C and cytidine-6-3H; the central graph
represents the C-14 label and ultraviolet absorption profile of an
aliquot of the labeled wash concentrate; and the bottom graph rep-
resents a double isotope dilution analysis of the wash to which
cytidine-6-3H was added. All columns and elution schedule were

kept exactly identical. The C-14 label present in the wash concen-
trate does not elute at the same position as does 5-hydroxyuridine-
$2-^{14}C$ in relation to cytidine. Its point of elution is roughly identical
to that of cytidine, which is the same as for the data presented in
Figure 28. The dissimilarities in the elution of the wash label and
labeled 5-hydroxyuridine, agrees with the observation of their di-
vergent migration on paper chromatography seen in Figure 25.
This constituted preliminary evidence that the 5-hydroxyuridine-
$2-^{14}C$ spike which had been added to the solution of the lyophylized
RNA hydrolysate as well as any which might have been present in
the RNA, had been altered.

## Purification of Labeled Constituents from the Labeled Wash Con-
centrate

Examples of electrophoretic as well as column and paper
chromatographic techniques which have been employed to examine
the character of the C-14 label in the peak from the water wash of
Celite column HC #13 are presented on Flow Sheets 1 and 2 in
Figures 23 and 24. Flow Sheet 1 shows that the label from a
small aliquot of the wash can be separated into three different
labeled components by various paper chromatographic solvent sys-
tems. These are:

(1.) C73-74Aa, is a labeled band with an Rf of 0.169 in butanol-
H2O-ethanol (5-2-3) which migrates below 5-hydroxyuridine,
cytidine, and guanosine controls. The absorbance spectrum
of this component is shown in Figure 32. It exhibits an ultra-
violet absorbance maximum at 272-274 nm in dilute HCl at

pH 1.7 and in distilled water, however, the material is unstable and flocculates on the addition of alkali. This microflocculant cannot be sedimented by centrifugation at 18,000x g for one hour; and it will redissolve on acidification of the sample solution, with restoration of the origional acidic absorbance spectrum.

(2.) C73-74Ab is a labeled band with an Rf of 0.434 in butanol-H2O-ethanol (5:2:3) which migrates above 5-hydroxyuridine, cytidine, and guanosine controls. This labeled derivative of 5-hydroxyuridine-2-14C does not absorb ultraviolet light from a mineral lamp and is only located on the chromatogram by ascertaining the position of label migration. An ultraviolet absorbance spectrum of the component could not be obtained.

(3.) C21b (Compound A) was obtained as a single labeled band from paper chromatography after five submissions in butanol-g. acetic acid-H2O (5:2:3), shown in Flow Sheet 1. This compound was examined by paper chromatography in nine solvent systems, paper electrophoresis in three buffers, by acid hydrolysis, and by various chemical spray tests.

The results are presented in sections below.

Compound A has also been obtained in much larger quantity by submitting the concentrated labeled water wash peak from Column HC #13 to anion exchange chromatography on Dowex-1-formate, by taking advantage of its cathodic migration in borate buffer and by differential ascending paper chromatography of the labeled peak

eluted from the Dowex column. Figure 31 shows the spectra of

two highly purified C-14 labeled preparations of Compound A ob-

tained by the procedures elaborated on Flow Sheets 1 and 2. The

spectra of the Dowex purified material, prepared as on Flow Sheet

2, is shown on the right half of Figure 31, and is identical in all

respects to C21b which was previously characterized.

## Acid Hydrolysis of Compound A

Compound A was diluted to 0.8 $A_{256}$ units/ml with distilled

water. Three hundred microliters of this solution was adjusted to

N/10 HCl with 7 microliters of 4N HCl and sealed in a glass ampule.

In addition, 250 microliters of a distilled water solution of Compound

A was added to an ampule and sealed for use as a control to determine

the effect of heat on the material. The ampules were heated in an

oven at $105^{\circ}C$ for one hour. They were then allowed to cool, and were

broken open; 250 microliters were withdrawn and the spectrum of

this material taken against a 250 microliter water blank to which

7 microliters of 4N HCl had been added. Ten microliters of 4N NaOH

was then added to the sample and blank and the spectrum recorded

on the approximately neutral solution. An additional 5 microliters

of 4N NaOH were then added and the spectrum recorded for the al-

kaline solution. The results of acid hydrolysis of Compound A,

(sample C21b) are summarized in Table 9. Compound A is stable to

heat but appears to be labile in the acid. The spectrum of the acid

treated product is indicated in Figure 33. The spectrum is similar

to that of guanine, but shows significant differences as indicated below.

|  | Guanosine | Compound A before Hydrolysis | Guanine | A after Hydroly- sis |
|---|---|---|---|---|
| neutral UV absorbance max | 252 | 253 | --- | --- |
| neutral UV absorbance min | 223 | 229 | --- | --- |
| acid UV absorbance max | 256 | 256 | 249 | 247 |
| acid UV absorbance min | 228 | 232 | 224 | 230 |
| alkaline UV absorbance max | 258 | 257 | 274 | 272 |
| alkaline UV absorbance min | 230 | 238 | 239 | 257 |

## Electrophoresis of Compound A

Paper electrophoresis was carried out at a constant voltage of 19v/cm in 0.05M potassium borate solution at pH 9.2, and in 0.01M ammonium formate buffers at pH 3.5 and 9.3. The migration of Compound A, which has been termed an artifact of 5-hydroxyuridine, and that of control compounds are presented in Table 10. In borate the nucleosides migrate to the anode (+) due to the formation of a negatively charged complex of borate with the vicinal hydroxyls of the ribose. Free bases are not complexed; however, isobarbituric acid, with its adjacent 4 keto 5 enol group does complex borate and migrates toward the anode. Migration in ammonium formate buffer depends mainly on the pKa of the compound. It is of note that Compound A does not complex borate and does not migrate as a nucleoside or as isobarbituric acid or 5-hydroxyuridine. Compound A resembles uracil in its migrating character and possesses a positive charge between pH 3.5 and 9.3. The inability of Compound A to complex borate suggests the lack of vicinal hydroxyls in the molecule. This indicates that the 2',3'-hydroxyls of the ribose of the spike have been either altered or the sugar has been entirely split from the base. In addition, the 5-hydroxyl group must be altered or

missing such that a borate complex cannot form between the C4-C5 oxygens or between any C5-C6 hydroxyls; the latter has been considered as a potential hydrolysis product of isobarbituric acid. One might account for this observation if the 5-hydroxyl was modified. The basicity of Compound A appears to increase as the pH is raised from 3.5 to 9.3. Dihydropyrimidines do not behave in this manner; because of a reduction in resonance energy dihydropyrimidines have much reduced pKa's for the N-3 proton as compared to the standard unsaturated bases. There is no specific information, however, that even the ring itself remains intact in Compound A.

## Paper Chromatography of Compound A

The migration of Compound A in nine paper chromatographic solvent systems in ascending paper chromatography was examined and is presented in Table 11. Papers were photographed under ultraviolet light and the ultraviolet absorbing materials noted. They were also cut into narrow strips and counted in a toluene fluor to determine the C-14 activity. Both the migration of the absorbing material and label are indicated in the table; and the label and absorbing spot for Compound A migrated homogeneously in six out of nine solvent systems. Compound A migrated the same as 5-hydroxyuridine in only one solvent system, butanol-glacial acetic acid-$H_2O$ (50:25:25). In the butanol-$H_2O$-absolute ethanol (50:20:30) solvent there was some separation of the migration of the label from the ultraviolet absorbing spot. Two labeled spots and one ultraviolet absorbing spot, which corres-

ponded to the slower migrating labeled spot, were observed.

In two other solvents, one of which was strongly acidic (2-propanol-$H_2O$-conc. HCl-680:144:170) and the other strongly

basic (2-propanol-H2O-conc. $NH_3$-7:2:1) the migration of label

differed significantly from that of the ultraviolet absorbing band.

Thus in three of the nine solvents Compound A broke down into

a labeled component which did not absorb ultraviolet light, Com-

pound B, and a strongly absorbing material, Compound C. This

probably occurred through the potential hydrolytic action of these

solvents.

The above results showed that labeled Compound A was not

5-hydroxyuridine, uridine, or uracil. The characteristic mi-

gration of controls on paper chromatography indicated that it was

also not isobarbituric acid, guanosine, cytosine or adenosine.

On the basis of these data and the above observations we con-

cluded Compound A was a molecular complex or artifact of

5-hydroxyuridine-2-$^{14}$C.

Cleavage Products of Compound A

Figure 34 shows the formation and migration of two bands

of material which are produced by submitting Compound A to

chromatography in one of the hydrolytic solvent systems that

had been tested above, 2-propanol-$H_2O$-conc HCl (680:144:170),

along with the migration of 5-hydroxyuridine-2-$^{14}$C control. The

labeled band, Compound B, migrates ahead of the ultraviolet

absorbing band, Compound C, and has no absorption under a min-

eral light.

The absorbing material C, which contains no label, has a

spectrum identical with that of cytidine; however cytidine and

cytidine-5'-monophosphate controls run in the same solvent have

Rf's of 0.47 and 0.57, whereas the Rf of this compound is 0.26.

In addition, cytidine melts between 212-215$^{\circ}$C, and 5-hydroxyuri-

dine between 230-232$^{\circ}$C, whereas Compound C begins gradually

decomposing above 138$^{\circ}$C and does not exhibit a definite melting

point.

It was assumed that the unlabeled Compound C was a com-

posite of cytidine and another substance in lieu of the fact that

though its absorption spectrum was identical with cytidine its

melting point and Rf were considerably different. Compound C

was submitted to ascending paper chromatography in butanol-

$H_2O$-ethanol (5:2:3) and two bands were observed. The forward

running band, Compound D, possessed the spectrum shown in

Figure 35. This material was eluted from the paper chromato-

gram, concentrated and dried; subsequently a trimethylsilyl

derivative was prepared and mass spectrometric analysis

attempted. Delayed submissions of Compound C, which had

been held a month on the paper chromatograms from which it

had been initially identified, i.e. the 2-propanol-$H_2O$-conc HCl

(680:144:170) solvent system, yielded very little Compound D.

This observation indicates Compound D was an unstable mater-

ial which readily losses its ultraviolet absorbance. The slower

migrating band from the chromatography of Compound C in bu-

tanol-$H_2O$-ethanol (5:2:3) possessed an absorbance spectrum

identical to cytidine. This cytidine-like (CR-Like) substance was

resubmitted to ascending paper chromatography in butanol-$H_2O$ (86:14) and butanol-$H_2O$-ethanol (5:2:3) both individually and with a spike of cytidine-5-3H. The migration of the CR-Like compound and of the CR-Like compound spiked with cytidine-5-3H was compared with the migration of the label of cytidine-5-3H, and the results are shown in Figure 36. It may be seen that the absorbance of the CR-Like compound parallels the absorbance and label of the CR-Like spiked spot and the migration of cytidine-5-3H on both chromatograms. A trimethylsilyl derivative of the CR-Like substance was also prepared and submitted to mass spectrometric analysis. It was concluded that the CR-Like compound was indeed cytidine. It is presumable either a contaminant of Compound C, or a more intimately associated part of the complex between Compound D and C-14-labeled Compound B which make up the artifact Compound A possessing the label from 5-hydroxy-uridine-2-14C.

The spectra of a concentrated solution of Compound B are shown in Figure 37. The alkaline curve only possesses a slightly higher extinction that that of the acidic and neutral spectra. When the alkaline spectrum is acidified the typical acidic spectrum is reestablished except that a new peak is formed at 225 nm with an extinction roughly twice that of the absorbance at 270 nm. The absorbance of this peak drops quickly to zero at 217 nm. Highly purified preparations of Compound B possess activities of 453 dpm/100 micrograms and 0.25 $A_{270}$ units/ml in cells with 10 mm pathlengths at a concentration of 333 micrograms per ml. The

specific activity of the highly pure 5-hydroxyuridine-2-14C spike added to the RNA digest was 31 dpm/microgram or 8060 dpm/micromole. The equivalent amount of isobarbituric acid-2-14C would possess a calculated specific activity of 63 dpm/microgram or 8064 dpm/micromole since on a weight basis 2.031 times the weight of a micromole of isobarbituric acid-2-14C equals the weight of a micromole of 5-hydrosyuridine-2-14C. If one assumes that the molecular weight of Compound B is approximately the same as 5-hydroxyuridine-2-14C then

4.53 dpm/microgram Compound Bx260 micrograms/micromole

=1178 dpm/micromole = specific activity of Compound B

based on a molecular weight similar to that of 5-hydroxy-

uridine-2-14C.

Assuming that the molecular weight of Compound B is approximately the same as isobarbituric acid-2-14C then

4.53 dpm/microgram Compound Bx128 micrograms/micro-

mole

=580 dpm/micromole = specific activity of Compound B

based on a molecular weight similar to that of isobarbi-

turic acid-2-14C.

Rf values from the paper chromatography of Compound B, shown in Table 13 indicate that it migrates faster than known nucleosides in the solvents employed. Both infrared spectroscopy of Compound B and mass spectrometric data on its trimethylsilyl derivative do not suggest the presence of a ribose or attached sugar. Thus we might anticipate that the molecular weight of Compound B more closely approximates that of isobarbituric

acid than 5-hydroxyuridine. If, however, one assumes that even a preparation of the highest purity Compound B that could be obtained is 50 per cent contaminated then the specific activity of the preparation based on a molecular weight similar to that of isobarbituric acid would be:

4.53 dpm/0.5 microgram Compound B x 128 micrograms/ micromole

=1160 dpm/micromole,

or about the same as the specific activity for Compound B based on a molecular weight similar to that of 5-hydroxyuridine. The isotopic dilution of the origional 5-hydroxyuridine-2-14C spike into Compound B is a little more than an eighth.

$$\frac{1178 \text{ dpm/micromole Compound B-14C}}{8060 \text{ dpm/micromole 5-hydroxyuridine-2-14C}} = 0.1461$$

The mass of carrier 5-hydroxyuridine in the RNA sample which diluted the spike of 5-hydroxyuridine-2-14C may be calculated from the following formula:

$$W_o \left( \frac{S_o}{S_1} - 1 \right) = W_u = \text{mass of carrier 5-Hydroxy-uridine in sample}$$

where $W_o$ equals the weight of 5-hydroxyuridine-2-14C added, $S_o$ is the specific activity of the spike and $S_1$ is the specific activity of Compound B. The assumptions are made that:

a) the total activity before dilution equaled the total activity after dilution,

b) the specific activity of the spike 5-hydroxyuridine-2-14C was rigorously established,

c) the spike was completely equilibrated in the mixture,

       d) Compound B was rigorously purified.

Thus,

$$7.46mg\left(\frac{8060 \text{ dpm/micromole 5-hydroxyuridine-2-14C}}{1178 \text{ dpm/micromole Compound B-14C}} - 1\right) = W_u$$

$7.46mg \ (6.842 - 1) = W_u$

$7.46mg \times 5.842 \quad = W_u = 43.58$ milligrams

These calculations suggest that 43.58 milligrams of a 1000 milli-
gram sample of ribosomal RNA nucleosides, or about four per
cent of ribosomal RNA bases are 5-hydroxyuridine. This value
represents a crude estimate at best, but nevertheless positively
suggest that 5-hydroxyuridine must be considered a minor con-
stituent of ribosomal RNA.

      Figure 34 shows that Compound B gives a positive test for
the presence of a dihydropyrimidine of $\beta$-ureido acid (45); but
it does not form a colored derivative with ninhydrin (91), in-
dicating it is not a $\beta$-amino acid degradation product of a di-
hydropyrimidine. It is of note that 5-hydroxyuridine also gives
a positive test for a dihydropyrimidine and for a reducing sugar.
Most of the dihydro-positive spot is directly over the 5-hydroxy-
uridine absorbance spot and is assumed to result from break-
down of the latter by the N/2 NaOH spray used in the test. Some
dihydro-positive material from the control does, however, migrate
ahead of the band of absorbance of 5-hydroxyuridine and parallel
to Compound B. This raises the probability that Compound B
could also be present, albeit in minor amounts in control prep-
arations of 5-hydroxyuridine. Chromatographically pure Com-

pound B exhibits a sharp melting point at 124-126°C with decomposition above 132°C. It was felt this material might be dialuric acid, however, this substance reddens on heating and melts at 217-220°C. The infrared spectrum of Compound B showed strong or medium sized peaks at the following wave numbers in cm$^{-1}$: 3420, 2940, 1650, 1405, 1385, 1070. There was little fine structure in the fingerprint area. The nuclear magnetic resonance spectrum gave non-integrable singlets at 2.1 and 6.4 ppm and a broad peak at 10.7 ppm in $D_2O$ and dimethylsulfoxide-D6.

Mass spectroscopic analysis was initially attempted on Compound B by either introducing the sample directly into the ion source or by prior volatilization of the sample. Difficulty in obtaining sufficiently high vapor pressure to produce even weak spectra were encountered in both procedures, due primarily to the very low volatility of the sample. Peaks at mass 71 and 112 were the only ones which could be unambiguously assigned. Much stronger spectra were obtained when a trimethylsilyl (TMS) derivative of Compound B was prepared, as described in Methods, and introduced directly into the ion source. A large number of peaks were present in these spectra, however, not all could be unambiguously designated. The conclusions drawn from the data include:

(1.) Accurate mass peaks for $C_4H_6N_2O_2$ (114), $C_2H_3N_2O$ (70), and $C_2H_3O$ (43) reflect the presence of dihydrouracil in the sample. Related peaks are also present at $C_4H_7N_2O_2$ (115). Strong peaks at 114, 71, and 43 have been shown by Rice <u>et al.</u> (Journal of the American Chemical Society 87:4569-4576. 1965.)

to be present in the spectrum of dihydrouracil run at 70

electron volts.

(2.) Peaks for $C_4H_6N_2O_3$ (130), $C_2H_3N_2O_2$ (71) and $C_2H_4O_2$

(43) could represent the spectrum of 5-hydroxydihydrouracil:
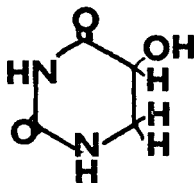


The lack of a TMS derivative for this isomer of $C_4H_6N_2O_3$ or

of peaks for $C_4H_5N_2O_3$, or $C_4H_7N_2O_2$ tend to suggest that the

assignment of $C_4H_6N_2O_3$ as a molecular ion is somewhat untenable.

(3.) The presence of peaks for N-carbamyl-$\beta$-alanine in the

spectrum is not completely unambiguous. Evidence for such a

component is : a TMS peak at 204 ($M^+$); peaks for $M+ - CH_3$(189),

$M^+ - NH_2CONH$(145), $M^+ - TMS-CO_2$(87), and strong $TMS-CO_2$(117),

and $TMS-CO_2-C$(129).      The ion for $C_3H_7N_2O$ (87) probably

represents $H_2N-CO-NH-CH_2-CH_2-$ and could also serve as evi-

dence for the presence of dihydrouracil as well as for N-carbamyl-

B-alanine. The lack of peaks for $TMS-CO_2-CH_2-$, $TMS-CO_2-$

$CH_2-CH_2-$, $TMS-CO_2-CH_2-CH_2-NH-$ or $TMS-CO_2-CH_2-CH_2-NH-$

CO- essentially preclude the presence of N-carbamyl-$\beta$-alanine.

The infrared spectrum of N-carbamyl-$\beta$-alanine is unlike that

of Compound B, suggesting that the former compound, if present

in the mass spectrum, may have been formed during the silation

of Compound B. In addition, the mass spectrum of a TMS deriv-

ative of N-carbamyl-$\beta$-alanine, though showing strong peaks at

117 and 129, does not possess a 204 peak. Significant quantities

of dihydrouracil also show up in the mass spectrum of a TMS

derivative of a chromatographically pure preparation of the
latter compound; due perhaps because during silation, cycliza-
tion takes place in lieu of the fact that O-TMS is a good leaving
group. The compound
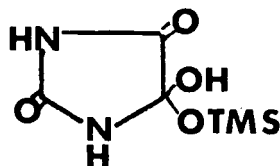


which has a molecular weight of 204 can also be excluded from
the mass spectrum of Compound B.

(4.) The presence of a peak at 219 ($M^+$) cannot be explained. It
has a mass within the probability of being TMS-$CO_2$-CH-OTMS,
however it has no $M\overset{+}{-}CH_3$, $M\overset{+}{+}CH_2$, or $M\overset{+}{+}CH$ peaks and is thus
at least not a parent ion. There are peaks for $M\overset{+}{-}H$ (218), $M\overset{+}{+}C$
(231), and $M+C_2H_2N$ (259), however that is as far as assignments
can be made. It is possible to exclude structures as TMS-$CO_2$-
CH(OTMS)-C-, TMS-$CO_2$-CH(OTMS)-N-, and TMS-$CO_2$-C(OH)
(OTMS)- or any derivatives thereof, including

 and 
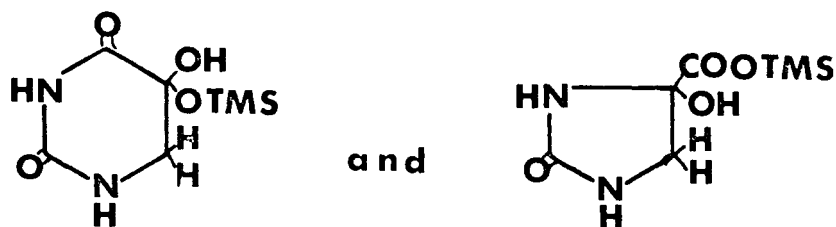
which both have a molecular weight of 218. Also the 219 and 204
peaks are not related.

(5.) The mass spectra of Compound B do not reveal the presence
of a sugar, sugar fragments, a TMS-sugar, TMS-sugar fragments,
or combinations of the above with a pyrimidine base or a TMS-base.

Figure 38 shows the migration of two artifact preparations,

one of which contains two labeled peaks, on ascending paper

chromatography in a nonhydrolytic solvent butanol-$H_2O$ (86:14).

Of importance is the fact that Compound A, which is present at

the origin in both the central and bottom graphs gives negative

tests for a dihydropyrimidine or β-ureido acid and for the

presence of a reducing sugar. It may be seen that the 5-hydroxy-

uridine control gives a positive test for the presence of associated

dihydropyrimidine-type materials but not a positive sugar test.

An outline of the isolated derivatives as well as a few of their

physical properties are presented below:

   (1.)  5-hydroxyuridine-2-14C------> I-14C (perhaps either
                                 C73-74Aa or C73-74Ab)

   (2.)  I-14C + cytidine + N(?)-------> Compound A-14C (Neg-
                                  ative tests for dihydro-
                                  pyrimidine or β-ureido
                                  acid, β-amino acid, and
                                  for a reducing sugar.)

   (3.)  Compound A-14C     -------> Compound B-14C (Posi-
                                  tive tests for dihydro-
                                  pyrimidine or β-ureido
                                  acid; negative tests for
                                  β-amino acid, for pres-
                                  ence of vicinal glycol,
                                  <u>i.e.</u> Siegelson Test ( 6 ),
                                  and ferric chloride test
                                  for phenolic hydroxyl.)
                                        +
                                Compound C
                                        +
                              a reducing sugar

   (4.)  Compound C        --------> CR-Like = cytidine
                                        +
                              Compound D

    The Rf values of the 5-hydroxyuridine artifacts along with

those of a number of pyrimidine related controls are presented

in Table 13. Unknown components and contaminants of controls

have been tentatively identified by comparing the extent of their migration against the known controls. The data suggests that Compound B is dihydrouracil and the CR-Like material is cytidine. It may be noted that commercial preparations of dialuric acid and alloxantin-$2H_2O$ are mixtures of a number of components, no one of which can unambiguously be considered the principal substance; thus these old names may describe mixtures rather than discrete compounds.

## Alteration of 5-Hydroxyuridine and the Formation of Compounds A and B

Subsequent to the above chromatographic results in which the characteristics of the label were found to differ from those of the control spike, I was faced with abandoning the technique of column partition chromatography without knowing if it was indeed responsible for production of the altered species.

The production of an artifact by some type of residual enzymatic activity was considered a possibility. Experiments were undertaken in which 5-hydroxyuridine and standard nucleic acid components were incubated with snake venom and alkaline phosphatase; the reaction products were submitted to paper chromatography and compared with the migration of controls. It was assumed that we might find a natural compound which could bind the label and behave chromatographically like the artifact. The results of 15 such experiments are listed in Table 12. The data does not prove conclusively that the artifact is not formed between 5-hydroxyuridine and standard RNA components in the presence

of these enzymes; however, most of the likely possibilities have
been eliminated.

A new compound was discovered as a contaminant in a com -
mercial preparation of guanylic acid[11] in the course of this investi-
gation. The spectrum of this compound which I have termed "M"
is given in Figure 39. Compound M is stable in alkali and under
acid hydrolytic conditions, but its absorbance is lost in the pres-
ence of bromine, indicating that it is not a derivative of adeno-
sine. The spectra of Compound M resembled that of 3-methyl-
5-methoxyuridine, both in the wavelength of its maximum and
minimum at 278 and 248 nm, and in its ionization characteristics.
The only difference is a slight hump at 248 nm in acidic and
neutral solutions and a depression in alkali at this wavelength.
To my knowledge the spectrum of this compound has not been
previously published. The Rf values of several controls may be
compared with that for Compound M in ascending paper chroma-
tography in 2-propanol-conc HCl-$H_2O$ (680:170:144).

| Compound | Rf | Compound | Rf |
|---|---|---|---|
| Compound M | .193 | uridine | .609 |
| uracil | .663 | adenylic acid | .367 |
| adenosine | .306 | cytidylic acid | .566 |
| cytidine | .425 | guanylic acid | .423 |
| guanosine | .285 | uridylic acid | .767 |
| 5-hydroxyuridine | .556 | | |

The stability of 5-hydroxyuridine in the yeast RNA extraction
procedure of Crestfield et al. (1955) (36) was also tested. This
technique requires temperatures of 92-94°C for three minutes.
A buffered solution, as described in the Methods, which contained

[11]Nutritional Biochemicals Lot 2211

only 5-hydroxyuridine at 0.55 $A_{294}$ units/ml measured in 10 mm cells, was heated and rapidly cooled in an ice bath. The spectrum recorded subsequent to heating showed a small loss in absorbancy and a shift in the maximum from 280 to 277 nm. The absorbance could be measured directly, as aqueous solutions of two per cent sodium dodecyl sulfate do not absorb ultraviolet light. After 10 hours at room temperature the absorbance maximum of the solution shifted back to 280 nm. This slight hypsochromic shift in the maximun has also been noted in a heated 50:50 (v/v) phenol: aqueous solution of 5-hydroxyuridine at final concentration of 0.55 $A_{294}$ units/ml. The spectra of phenol solutions were obtained by centrifuging the mixture and extracting the aqueous layer three times with 3x the volume of anhydrous ether, and then with 3x its volume of carbon tetrachloride. Both of these solutions possess typical alkaline ultraviolet absorbance spectra. Heated alkaline phenol solutions were found to reduce the absorbance of 5-hydroxyuridine whereas standard phenol solutions, e. g. at pH 4.7, showed little if any loss in absorbancy.

The influence of the partition column chromatographic solvents employed were examined and the results are presented in Figure 40. This graph shows that upper solvent G, which is the second eluting solvent employed in Celite partition chromatography reduced the ultraviolet absorbance of 5-hydroxyuridine at 293.5 nm; the absorbance was quite stable, however, in the primary solvent, i. e. the upper phase of solvent F. This observation is important in that with Hall's partition column method

(52) one can successfully recover 22 minor and major components
from transfer RNA and one of three components known to re-
arrange.

Stability of 5-Hydroxyuridine

A general survey of the stability of 5-hydroxyuridine to common laboratory reagents: The above results indicated that 5-hydroxyuridine was not as stable as had been believed and suggested that future work with the compound would be facilitated by a better understanding of the molecules' potential for chemical modification. The stability of 5-hydroxyuridine in solution at 23° C to common laboratory reagents is shown in Table 7. Weakly oxidizing, reducing, acidic, and alkaline conditions were surveyed and trends in stability may be observed. The absorbance of capped solutions of 5-hydroxyuridine was measured daily as:

$$\text{percent change or loss in solution absorbance} = \frac{\text{absorbance at 2935 Å at time x}}{\text{absorbance at 2935 Å at zero time}} \times 100$$

5-Hydroxyuridine appears to be quite unstable under reducing conditions and in alkali; it is only slightly unstable in acid and toward oxidizing media. Many of these reactions will be considered in detail below.

The effect of heat, pH, and cations on the stability of 5-hydroxyuridine: The absorbance changes of 5-hydroxyuridine at 293.5 nm in solutions employed in RNA fractionation procedures, at various pH's, at 37° C in (A) $5 \times 10^{-2}$M Mg Cl$_2$, (B) in distilled water, and (C) in $2.10^{-2}$ M Tris, 3.5M in urea is shown in Figure 41. 5-Hydroxyuridine loses absorbance at a faster rate in urea and magnesium chloride than it does at comparable pH's below 9.0 in distilled water. The insert, Figure 41, shows the percent change in absorbance with time at 60° C of 5-hydroxyuridine in distilled water at pH's 1.6, 6.2 and 11.9.

At alkaline pH the loss in absorbance is considerably accelerated when heat is employed. All the solutions of 5-hydroxyuridine appear to be stable below pH 6.0, however, in the range 6.0-7.0 stability seems to depend on the solvent, compare e.g., the loss in absorbance in urea at pH 6.9 at 37° C with the refractory nature of a distilled water solution at pH 6.2 at 60° C. These data suggest that the stability of the molecule which is measured in terms of loss of absorbance depends upon whether or not the 5-hydroxyl proton has dissociated. The increase in instability of 5-hydroxyuridine in urea as compared to distilled water or magnesium chloride suggests that instability may be related to additional factors than merely pH. This is especially emphasized by comparing the stability in $2 \times 10^{-2}$M Tris at pH 10.1 and the same solution, pH 10.1, 3.5 molar in urea in Figure 41. The significant stability difference of 5-hydroxyuridine in these two solutions may be perhaps explained by the reactivity of cyanate, which has been reported to be present as a contaminant of aqueous urea solutions (127)with ionized 5-hydroxyuridine.

Figure 41 also shows that in magnesium chloride solutions at 37° C, above pH 10.5 there is a loss in absorbancy with time followed by a rapid gain. The effect of heat and pH on solutions of 5-hydroxyuridine in $5 \times 10^{-3}$M MgCl was examined more closely. The results of auto-claving at 121° C for 15 minutes at 15 pounds pressure is shown in Figure 42. Controls in distilled water at pH values above 8.0 did not absorb indicating a total loss of 5-hydroxyuridine in solution above this pH. Even weakly acidic solutions lost absorbance. A magnesium chloride solution at pH 4.5 dropped in absorbance at 294 nm from 0.55 to 0.24.

Although this solution increased in pH on autoclaving to 7.5, its maximum shifted to 304 nm. The normal maximum for a pH 7.5 solution of 5-hydroxyuridine is 282 in distilled water and approximately 285 nm in $5 \times 10^{-3}$ M MgCl . These results indicate that a derivative of 5-hydroxyuridine with a maximum at 304 nm is formed on autoclaving. This derivative is formed above pH 8.0 in larger amounts. The spectra of the reaction at pH 10.5 whoch dropped on autoclaving to pH 8.4 closely resembles the pH 8.7 solution of 5-hydroxyuridine in $5 \times 10^{-3}$ M MgCl$_2$ which was shown in Figure 17. Either the formation of the 5-hydroxyuridine derivative is greatly accelerated above pH 9.5, or the stabilizing effect of magnesium cations on the molecule does not come into play below this pH. Thus there may be a requirement for dissociation of the N-3 proton. The spectra of the solution at and below an initial reaction pH of 9.5 are those of a newly formed 5-hydroxyuridine derivative. The spectra of solutions above this pH may represent this derivative, or they may be those of unaltered 5-hydroxyuridine. In the latter case the magnesium cations may stabilize 5-hydroxyuridine to heat and alkali by forming, a complex which makes the molecule resistant to such treatment. Such a complex could be a magnesium bridged dimer between two ionized 5-hydroxyl groups.

Stability of 5-Hydroxyuridine in Acid

Solutions of 5-hydroxyuridine and isobarbituric acid were each made up to 0.8 $A_{280}$ units/ml with distilled water. 250 microliters of the distilled water samples were added to ampules and sealed for use

as controls to determine the effect of heat alone. Triplicate samples of 300 microliters of these solutions were adjusted to 0.1 N HCl with 7 microliters of 4 N HCl and sealed into glass ampules. A uridine control was treated similary. The ampules were heated in an oven at $105^\circ$ C for one hour, and then allowed to cool. Each was broken open; 250 microliters was withdrawn and transferred to a microcuvette 10 mm pathlength. The spectrum of this material was recorded against an identical 250 microliter water blank to which 7 microliters of the 4 N HCl had been added. Ten microliters of 4 N NaOH were then added to the sample and blank and the spectrum recorded.

It was not previously known that 5-hydroxyuridine, and isobarbituric acid were stable under these conditions. The spectra obtained subsequent to hydrolysis or heating, Table 8, were identical in absorption at the isosbestic points and at maxima and minima to the spectra obtained prior to heat-acid treatment. In addition, the samples were applied to ascending paper chromatography in butanol:$H_2O$ (86:14) and migration of the treated samples was observed to be identical with those of untreated controls.

Stability of 5-Hydroxyuridine in Alkali

Gentle alkaline hydrolysis is a reproducible preparative procedure generally employed in ribonucleotide analysis for converting RNA(16) and oligonucleotides(144) to a mixture of 2'-,3'-mononucleotides. In general, nucleosides (89) and mononucleotides (20) are stable to alka-

li. Figure 43, curve E, shows the time study of loss of ultraviolet absorbance of 5-hydroxyuridine in 0.3 N KOH at 37 C. This is a widely employed(89,29) alkaline hydrolytic procedure. The two control curves, A and B, demonstrate that loss of absorbance is dependent both on temperature and presence of hydroxide ion. Solutions of 5-hydroxyuridine are moderately stable to 0.3 N KOH at 4°C, curve B.

Figure 44 demonstrates the instability of 5-hydroxyuridine to three accepted(16)RNA alkaline hydrolytic procedures: 1N KOH for one hour at 80°C, N/10 KOH for 20 minutes at 100°C, and N/20 KOH for 40 minutes at 100°C. It can be seen that at 100°C there is a very rapid formation of a non-ultraviolet-absorbing product. Loss of absorption is more rapid in N/10 KOH than in N/20, and a reduction in absorbance of more than 50 percent occurs in less than 5 minutes. At 80°C, ultraviolet absorption is rapidly lost, presumably forming a non-ultraviolet-absorbing intermediate product. Subsequently, an ultraviolet-absorbing derivative begins forming. The spectral characterization of this reaction is shown in Figure 45. The alkaline spectrum of 5-hydroxyuridine at time zero exhibits a maximum at 303 nm with an absorbance of 0.735 spectrum A. By 20 minutes the alkaline peak, Figure 45, curve B, shifted to a new maximum at 307 nm and dropped in absorbance to 0.275. The spectrum of this material acidified to pH 1.0, Figure 45, curve B', exhibits two peaks. One at 276-277 nm, the other at 319-320 nm. The major peak at 276 nm is reduced in absorbance and exhibits a slight hypsochromic shift when compared with the zero time

absorbance, maximum at 280 nm, of an acidified solution of 5-hydro-

xyuridine. In additon, a smaller acid peak has formed with an absor-

bance maximum at 319-320 nm. After 60 minutes of heating, i.e. the

standard length of hydrolysis at this temperature, an increase in ab-

sorbance at 307 and 319 nm may be observed in curves C and C', Figure

45, along with a proportional decrease in absorbance at 276 nm of the

major peak of the acidified solution, curve C'. Curves D and D' of

Figure 45, show a continued formation of the ultraviolet-absorbing de-

rivative, tamed Compound R, at 307 and 319 nm after 3 additional hours

of heating. This newly formed derivative is stable in acid and alkali,

as shown by the reversibility of these spectra, which suggests it poss-

esses a new covalent character.

## Stability of 5-Hydroxyuridine in $NH_3$ and Amines

Stability of 5-hydroxyuridine in $NH_3$: Figure 43, curves D and F,

shows the rapid loss of absorbance of a solution of 5-hydroxyuridine in

N and N/10 $NH_3$ at 37° C and at room termperature 23° C. At the higher

temperature a nonabsorbing product is formed; whereas at room term-

perature, after initial loss of absorbance, an ultraviolet-absorbing de-

rivative begins forming by 24 hours. By 120 hours, the latter obtains

an absorbance at 294 nm of 200 percent of the substrate at time zero,

and appears to be formed from the nonabsorbing intermediary product,

rather than from a direct modification of 5-hydroxyuridine. From these

results it may be anticipated that nonabsorbing derivatives of 5-hydro-

xyuridine would be formed on paper chromatography employing solvent

systems containing $NH_3$, or in desalting procedures in which nucleic acids are eluted from an activated charcoal column with a solution of ammoniated ethanol.

Two molar ammonium carbonate is generally used to elute oligonucleotides from DEAE-cellulose and mononucleotides from Dowex-1 columns in a procedure for the removal of urea and salt from these fractions (131). It can be seen from Figure 43, curve G, that a non-ultraviolet-absorbing derivative of 5-hydroxyuridine would be rapidly form formed by this procedure at pH 9.0. It can also be seen from this figure, curve C, that 5-hydroxyuridine is relatively stable in a pH 8.8 solution of 0.17 M Tris at 37°C. Incubation under these conditions is used to uncharge or hydrolyze amino acids from their transfer RNA(143).

Stability of 5-hydroxyuridine in piperidine: Figure 46 shows that in two standard procedures (16) for hydrolysis of RNA in piperidine more than a 50 percent reduction in the ultraviolet absorbance of 5-hydroxyuridine occurs in less than 10 minutes. An ultraviolet-absorbing derivative, Compound R, begins forming rapidly from the non-absorbing hydrolytic product. The kinetics of the formation of this derivative in a one percent solution of piperidine are illustrated in Figure 47. Curves A and A' show the spectral absorbance of 5-hydroxyuridine at zero time. By one hour, Figure 47, curves B and B', a new compound has formed with an alkaline absorbance maximum at 307-308 nm and an acid maximum at 320 nm. The formation of Compound R increases with time, Figure 47, curves C, D, and E, on an additional 4 hours of

heating. Acidification of an alkaline solution of this compound results in a faint opalescene, indicating a lower solubility in acid. The acid and alkaline spectra of this material are shown to be reversible. The existence of two additional peaks in the acid spectrum at 258 and 268 nm appear to be characteristic of the one percent piperidine hydrolytic conditions. Difference spectra of the 10 percent hydrolysis solution do not reveal detail below 270 nm.

Synthesis of Compound $H_{28}$: In an attempt to prepare the novel Compound R in sufficient quantity for organic analysis two additional derivatives of 5-hydroxyuridine, Compound $H_{28}$ and Compound P were sythesized. The spectra of these substances are shown in Figures 48 and 49. Compound $H_{28}$ was prepared by refluxing two liters of a dilute 0.9 $A_{280}$ units/ml solution of 5-hydroxyuridine in 5 per cent piperidine at 100° C for 40 minutes. One liter of the reaction was stopped by immediately freezing the solution in a dry ice-acetone bath. It was then concentrated to dryness under vacuum in a rotory evaporator similar to the procedure given in Methods for evaporation of celite partition column fractions. The pH of Compound $H_{28}$ taken up in distilled water was 6.2. Compound $H_{28}$ was purified from a minor ultraviolet absorbing compound, two dihydropyrimidine-type compounds, and an aldo-pentose, as shown in Figure 50, by ascending paper chromatography in Butanol-$H_2O$ (86:14), which were present in reaction mixture-28. Values of $pKa_1$ of 2.5 and $pKa_2$ of 11.9 were calculated, as shown in Table 14 from the spectra of Compound $H_{28}$ in Figure 48 . Compound $H_{28}$

with an ultraviolet spectral absorbance maximum at 230 nm, has not previously been described and this is the first report of its preparation.

Compound $H_{28}$ could not be recognized in the absorbance spectrum of the reaction mixture due to the absorbance of the 5 per cent piperidine. All that could be detected in the spectra of the reaction mixture was a drop in the alkaline absorbance maximum at 305 nm from 0.70 to 0.19 by 40 minutes, which continued to drop to 0.06 by 4 1/2 hours; whereas by 65 minutes it was no longer possible to detect ultraviolet absorbance for acidified solutions of the reaction mixture. The nature of the peak with an alkaline absorbance maximum at 305 nm will be considered below.

<u>Synthesis of Compound P:</u>  Compound P was obtained in the course of preparation of Compound R by reacting a concentrated, one milligram per ml, solution of 5-hydroxyuridine in one or ten per cent piperidine in a water bath at 98° C by the procedure given in Methods for the chemical stability tests. Compound P, an ultraviolet absorbing component which migrates very slowly from the point of origin in ascending paper chromatography in butanol-H O (86:14), as shown in Figure 50, can be separated from an aldo-pentose, a small amount of unreacted 5-hydroxyuridine, and two very minor dihydropyrimidine-type compounds not indicated, which are present in reaction mixture-$RI_{26}$. The extent of its migration in several other solvent systems is given in Table 13 and the spectral titration of the purified compound is presented in Figure 49. The spectra of Compound P possess two isosbestic points, a and b, at 254 and 263 nm which define respectively its initial dissociation, $pKa_1$

of 3.1 and a second dissociation pKa$_2$ at 11.9, calculated from the data presented in Table 15.

The course of the formation of Compound P in one per cent piperidine is shown in Figure 51. This figure shows the spectra of a 100th dilution of the reaction mixture into distilled water at: zero time, one hour and three hours. The alkaline absorbance spectrum at zero time at about 9.5 shows the two characteristic 5-hydroxyuridine peaks at 240 and 307 nm. By three hours the peak at 240 shifts to 250 nm with an increase in extinction, wheras the extinction of the 307 nm peak drops from 0.77 to 0.25. The acid absorption maximum in the same time undergoes a hypsochromic shift from 279 to 265 nm with an initial decrease and then an increase in extinction.

The chromatographic migration of derivatives of 5-hydroxyuridine present in reaction mixtures 22, 26, and 28, may be seen in Table 16. These derivatives of 5-hydroxyuridine were produced in the reaction by heating in aqueous one or ten per cent piperidine. Compound P has been isolated from reaction mixtures 22 and 26 and Compound H$_{28}$ was obtained from reaction mixture 28. On the basis of comparing the Rf values of the derivatives with those for controls given in Table 13 it may tentatively be concluded that:

a) In addition to the residual level of unreacted 5-hydroxyuridine and the predominant reaction 22 and 26 product, Compound P, dihydrouracil, dihydrouridine, and an aldo-pentose positive, non-ribose sugar, are also formed.

b) Dihydrouracil, and an unknown dihydroderivative of 5-hyd-
   roxyuridine, and a sugar similar to the one obtained above,
   as well as unreacted 5-hydroxyuridine are present in the
   reaction mixture in which Compound $H_{28}$ is the principal
   product.

c) The small amount of sugar detected in reactions 26 and 28
   probably results from alkaline labilization of the sugar from
   a reduced form of 5-hydroxyuridine, with consequent prod-
   uction of dihydrouracil.

Stability of 5-Hydroxyuridine in Hydroxylamine

The instability of 5-hydroxyuridine in solution at $37^{\circ}$C to
$5.86 \times 10^{-2}$ M hydroxylamine hydrochloride at various pH's is indic-
ated in Figure 52. In acid solution through pH 7.0 5-hydroxyuridine
is stable. A dramatic change in stability is evident on increasing
the pH to 8 or above. The kinetics of loss of absorption of a solution
at pH 8 is indicated in Figure 53. With time, there is a loss in abs-
orbance, as well as a hypsochromic shift in the wavelength of max-
imum absorbance to 277-278 nm. This derivative in solution at pH
7.9 is unstable on addition of acid to pH 1 or alkali to pH 13. Fig-
ure 54 shows the reaction at pH 10.5. By 19 hours a derivative with
a maximum at 277 nm is formed which slowly loses absorption and
shifts to 276 nm. The appearance of the 277 nm peak is much quick-
er at pH 10.5 than at pH 8.0. On addition of alkali an increase in
extinction occurs and the absorbance maximum shifts to 273 nm.

The behavior of 5-hydroxyuridine at pH 11.6 toward hydroxyl-

amine may be seen in Figure 55. By 19 hours there is nearly a two-fold increase in the amount of the derivative which possesses a maximum at 277 nm as compared to the level formed at pH 10.5. With time the absorbance increases and shifts to a maximum at 272 nm. This derivative of hydroxylamine is unstable in acid, pH 1, with consequent loss of ultraviolet absorbance.

The loss of absorption of 5-hydroxyuridine under alkaline conditions may be due to its reaction with a nucleophile present in the solution, as for example a hydroxyl ion or piperidine. 5-Hydroxyuridine could also be unstable when ionized, rearranging with consequent loss of ultraviolet absorbance. The results of a comparison of the stability of $0.9A_{280}$ units/ml (10 mm cells) solutions of 5-hydroxyuridine refluxed at $78^{o}C$ in N/2 sodium hydroxide, i. e. about a five per cent solution, and in five per cent aqueous triethylamine, and five per cent triethylamine in anhydrous diglyme (bis(2-methoxyethyl)ether) are presented in Figure 56. Triethylamine is a very strong base but is not a nucleophile and thus 5-hydroxyuridine should be stable in a strongly basic solution of this reagent if its loss of absorption is indeed due to a nucleophilic reaction. A spectrally clear anhydrous diglyme solution was prepared by absorbing water present in commercial reagent grade diglyme with sodium and filtering the resulting cloudy solution through a thick layer of aluminum oxide which had been previously moistened with the same solution and packed tightly in a scintered glass filter.

The reactivity of 5-hydroxyuridine in aqueous five per cent trithylamine is much slower than its reaction in N/2 sodium hydroxide, nevertheless ultraviolet absorbance is lost; in addition the formation of Compound R may be detected in both reactions by two hours or one-half hour respectively, indicated by the arrows in Figure 56. In contrast there is very little loss in absorption of 5-hydroxyuridine in five per cent triethylamine in anhydrous diglyme.

The result is clear: in an aprotic solvent in which hydroxyl ions
cannot be formed 5-hydroxyuridine is stable under basic conditions
at 78°C. In an aqueous solution of triethylamine hydroxyl ions
are formed due to interaction of the strong amine base with water.
The loss in absorbance of 5-hydroxyuridine in basic solution is
a consequence of its interaction with a nucleophile, e.g. as with
hydroxyl ions, present in the solution. The possible mechanism(s)
and consequence of such a reaction will be considered in the dis-
cussion.

## Reaction of Ammonia and Cytidine with Derivatives of 5-Hydroxy-uridine and with Related Compounds

One of the objectives of examining the stability of 5-hydroxy-
uridine was to obtain a clue as to what can modify the molecule
under biological conditions, especially with regard to isolation.
It has been shown in thses studies that 5-hydroxyuridine is un-
stable in solution to attack by nucleophilic agents when the hy-
droxyl proton is dissociated. Labeled spikes or native 5-hydroxy-
uridine may be modified by biological isolation techniques to an
unstable substance which may further react to form additional
derivatives and complexes. Some derivatives of 5-hydroxyuridine,
as Compound P and $H_{28}$ retain their ultraviolet absorbance and
are stable under harsh alkaline conditions; for example, Figure
51 indicates the continued formation of Compound P in one per
cent piperidine between six and eight hours at 100°C. The stabil-
ity of reaction intermediates and side products, however, whose
formation on treating 5-hydroxyuridine with alkali may be detected
both spectrally and chromatographically, is not as easily ascer-

Changes in the spectra of 5-hydroxyuridine in one per cent piperidine at $100^\circ$C during the first three hours of the formation of Compound P are shown in Figure 50 and have already been discussed. The absorbance spectra of the sixth through eight hour of the same reaction is shown in Figure 51. The absorbance of the 307 nm peak during the course of the reaction drops to a minimum by six hours and remains constant during an additional 2 1/4th hours of heating at $100^\circ$C. The absorbance at 307 nm may be considered to be due to 5-hydroxyuridine and to reaction derivatives which also possess ultraviolet absorbance maxima at this wavelength. Since loss of absorbance at 307 nm between six and eitht hours is due mainly to modification of existing 5-hydroxyuridine, it may be considered that the absorbance at 307 nm between six and eight hours is due entirely to an alkaline stable derivative of 5-hydroxyuridine. Figure 51 indicates the extent of modification of 5-hydroxyuridine by 2 1/2 hours in reaction aliquot $RI_{26}$. The faint absorbance of 5-hydroxyuridine in comparison to the strong absorbance bands of an unidentified material and Compound P, both derivatives of 5-hydroxyuridine, shows that by 2 1/2 hours most of the 5-hydroxyuridine present in the reaction had been modified.

The question of whether the 307 nm absorbing derivative, which appeared stable to alkali, was also stable to ammonia was answered in the following manner: Fifty microliters of about 23N ammonia were added to ten ml of a 1/10th dilution of the sixth hour reaction mixture; this solution was diluted to 1/100th with distilled water and returned to the water bath at $100^\circ$C for 45 minutes; then

its spectra was measured. The results are shown in Figure 57

for the 6 3/4th hour curves. There is complete loss of the

alkaline absorbance peak at 307 nm for the ammonia treated

sample, whereas the absorbance of Compound P, indicated by the

absorbance maxima at 250 and 265 nm is unchanged except for

a small drop due perhps to elimination of the absorbance of other

5-hydroxyuridine derivatives present in the mixture. From these

results it was concluded that one of the derivatives produced by

the action of alkali on 5-hydroxyuridine: a) possesses an absorb-

ance maximum in alkali at 307 nm, b) is somewhat resistant to

further modification by alkali, and c) readily reacts with ammonia,

in turn losing its alkaline absorbance maximum at 307 nm. This

derivative is not Compound R because acidic solutions of it do not

exhibit absorption maxima at 319-320 nm.

In the light of the above results, reaction mixtures $RI_{26}$ and

$RI_{28}$ from which Compound P and Compound $H_{28}$ were isolated

were tested for the presence of a derivative of 5-hydroxyuridine

that could react with or bind cytidine. It may be recalled that

cytidine, as well as a C-14 labeled nonabsorbing material termed

Compound B, and a spectrally odd substance, termed Compound

D, possibly a derivative of cytidine, could each be obtained from

Compound A, the principal labeled material isolated from a yeast

RNA hydrolysate which contained a spike of 5-hydroxyuridine-2-14C.

The migration of cytidine-5-3H, the absorbing components

of the non-labeled reaction mixtures $RI_{26}$ and $RI_{28}$, and a mixture

of cytidine-5-3H plus an aliquot of a reaction mixture, prepared

by spotting one material on top of the other on the paper chroma-
togram, were run in ascending chromatography in butanol-$H_2O$
(86:14). The migration of the label and absorbance of cytidine-
5-3H was compared with the migration of the cytidine label in
the reaction mixture. It may be seen from Figure 58 that both
reaction mixtures $RI_{26}$ and $RI_{28}$ contain a 5-hydroxyuridine der-
ivative which binds cytidine-5-3H. This is shown by the labeled
peak at the origin for the mixture of cytidine-5-3H plus $RI_{26}$ and
for cytidine-5-3H plus $H_{28}$. These peaks are not present in the
cytidine 5-3H sample radioprofile. The bound cytidine held up
at the origin in the butanol-$H_2O$ (86:14) solvent system chromato-
graphically behaves the same as Compound A. This may indeed
be the way Compound A arose, i. e. through the binding of cytidine
with a derivative of 5-hydroxyuridine which readily reacts with
either primary amines or ammonia.

The reactivity of commercial preparations of alloxan, allo-
xantin-$2H_2O$ and dialuric acid are each shown in Figure 58. These
compounds react with cytidine-5-3H forming ultraviolet absorbing
derivatives which do not migrate the same as cytidine or Compound
A. This is believed to be the first report of the reaction of these
components with cytidine, although the reactivity of alloxan with
amines has been known for some time and will be discussed in
detail below. Interest in these compounds generated from the fact
that $H_{28}$, although entirely different, migrates similarly to the
forward running absorbing areas of these compounds, and from the
observation that very different absorbing bands are produced when
these three compounds are each submitted to paper chromatography

on acetic acid washed versus unwashed paper. The unwashed
paper contains amino acids and peptides which apparently react
with these components to produce derivatives possessing differ-
ent chromatographic properties.

## Stability of Methylated Derivatives of 5-Hydroxyuridine in Alkali

Data above suggested that the stability of 5-hydroxyuridine
in solution depended on whether or not the 5-hydroxyl proton was
dissociated. In an attempt to examine this idea the stability of
methylated derivatives of 5-hydroxyuridine in alkali, at approx-
imately pH 13, was examined. The samples were heated in capped
polyethylene vials in a New Brunswick air convection incubator at
48-50°C. The spectra were recorded at zero time, 2 hours 40
minutes, 7 hours 40 minutes and 20 hours and are presented in
Figures 60-64. The course of reaction of 5-hydroxyuridine
control at pH 13.0 is shown in Figure 59. By 20 hours 5-hydroxy-
uridine has been completely destroyed. Figure 60 shows that
derivative a of 5-hydroxyuridine is completely destroyed by 7
hours 40 minutes at pH 13.0. Acidification of this solution,
curve AC, reveals the spectrum of a material with a maximum
at 270-273 nm. The original acid maximum of a was at 278 nm;
thus presumably a underwent conversion to the 270-273 nm max-
imum material as well as considerable loss in absorbancy. The
course of the reaction of 5-methoxyuridine at pH 12.7 is indicated
in Figure 61. There is no change in the absorbancy or maximum
of the samples spectrum after heating 20 hours in alkaline solution.

Acidification of the 20 hour sample produced a jump in extinction, which is also exhibited by untreated controls, see Figure 7. However, acidification did not produce the slight bathochromic shift, to a new maximum at 277.5 nm, which is characteristic of the untreated compound. Alkalization of the acidified sample resulted in a tremendous jump in extinction. The spectrum of this solution possessed a shoulder at 274 nm but was otherwise uninterpretable.

The course of the reaction of 3-methyl-5-hydroxyuridine in alkali at pH 13.1 is shown in Figure 62. In alkaline solution at zero time this sample characteristically possesses a hump or peak at 240 nm. Acidification of the 7 hour 40 minute solution yields a material possessing a spectral maximum at 277 which is the same as that of untreated controls, Figure 8, indicating that although there is a loss of ultraviolet absorption in alkali, those molecules which have not yet reacted still retain their identity. 3-Methyl-5-hydroxyuridine was destroyed by 20 hours.

The results presented in Figure 63 for the course of reaction of 3-methyl-5-methoxyuridine in alkali at pH 13.1 were totally unexpected. The zero time absorbance in alkali shifted from 0.53 at a maximum of 277 nm to 1.16 at 268.5 nm by 2 hours 40 minutes. Acidification of this solution, curve AC, resulted in a nonabsorbing material with an absorption maximum below 235 nm. Alkalization of this acidified solution, curve RA1, reestablished the 268.5 nm material, although on an absorbance basis half of it was lost. The alkaline absorbance of 1.16 at a maximum 268.5 for the 2 hour 40 minute solution, dropped to an absorbance of

0.43 by 20 hours, indicating the compound formed was only transiently stable in alkali. This unique compound somewhat resembled dihydrouridine in possessing a reversible spectra with absorbance in alkali but not in acid; and in its instability to prolonged exposure to alkali.

The course of the reaction of 3-methyl-5-methoxy-(2'-methoxy)-uridine at pH 12.9 is shown in Figure 64. The absorbance maximum changed from 276 to 267 nm by 2 hours 40 minutes and increased dramatically in extinction. This increase continued through 7 hours 40 minutes, a result not found with 3-methyl-5-methoxyuridine. Acidification of the 2 hour 40 minute sample caused a large drop in extinction but still retained the maximum at 267 nm. Alkalization of this solution produced a spectrum identical with that of the 2 hour 40 minute solution. Acidification of samples incubated 7 hours 40 minutes or longer resulted in non-absorbing solutions which are no longer able to yield characterizable spectra on alkalization.

A summary of the stability of the methylated derivatives of 5-hydroxyuridine in alkali at pH 13 at 48-50°C is presented in Figure 65. Isobarbituric acid, 5-hydroxyuridine, 5-hydroxydeoxyuridine, 5-hydroxyuridine a, and 3-methyl-5-hydroxyuridine each underwent a characterizable bathochromic shift on addition of alkali to acidic solutions, forming an isosbestic point at or near 294 nm. Their changes in absorbancy were measured at this wavelength in Figure 65. The other derivatives: 5-methoxyuridine, 3-methyl-5-methoxyuridine, and 3-methyl-5-methoxy-

(2'-methoxy)-uridine do not possess spectra resembling 5-hydroxy-
uridine; their stabilities toward alkali, shown in Figure 65, were
reflected by absorbancy changes at the wavelengths of their max-
ima. 5-Hydroxyuridine a, 5-hydroxyuridine, and 3-methyl-5-
hydroxyuridine lost 50% absorbance by 2 1/2 hours, 4 1/2 hours
and 6 1/2 hours respectively. Isobarbituric acid was the most
unstable and lost 50 per cent absorbance in less than an hour
whereas 5-hydroxydeoxyuridine was intermediate in stability
between 5-hydroxyuridine and 3-methyl-5-hydroxyuridine and
lost 50 per cent absorbance by 5 hours. The large increase in
absorbance of 3-methyl-5-methoxyuridine and 3-methyl-5-meth-
oxy-(2'-methoxy)-uridine may be observed. The latter appears
to be more stable in alkali and indicates the methylated ribose
may stabilize the loss of absorbance, but does not appear to be
involved in formation of the derivatives with increased extinction
and changed maxima. Differences in reactivity of the two imply
that the ribose is still present on each. 5-Methoxyuridine is
stable. This result is somewhat enigmatic in lieu of the ob-
servation that compounds additionally methylated at the N3
are modified and gain as well as lose absorbance.

Comparison of the Alkaline Hydrolysis Products of Uridine, 5-
Hydroxyuridine and Dihydrouridine

In order to determine whether 5-hydroxyuridine is formed,
perhaps in trace amounts, from uridine under alkaline hydrolytic
conditions the following experiment was carried out. Chromato-
graphically highly purified uridine-2-$^{14}$C of high specific activity

was heated in a water bath for ten minutes at 80°C in 0.3M potassium hydroxide. To compare the partial hydrolytic breakdown products of 5-hydroxyuridine and dihydrouridine with any which might arise from uridine, both compounds were subjected to the same hydrolytic conditions. Impure synthetically prepared 5-hydroxyuridine containing enough carrier to give a visible spot was employed. Hydrolytic conditions were chosen such that 5-hydroxyuridine would not completely break down. The uridine-2-$^{14}$C subjected to the alkaline hydrolytic conditions did not possess enough carrier to give an absorbing spot on the chromatogram. Unhydrolyzed dihydrouracil, dihydrouridine, 5-hydroxyuridine-2-$^{14}$C containing synthetic contaminants, and cold uridine were run as controls. Samples from each hydrolysate were applied directly to the paper (suffix A) or were neutralized (suffix AN) with hydrochloric acid and then applied to the paper. The chromatograms were run in four solvent systems and the results are graphed in Figures 66-69. The spray test employed for detecting the presence of dihydropyrimidine or β-ureido acids has been presented in Methods.

The migration of the hydrolysates and controls in 1butanol-H$_2$O-ethanol (5:2:3) is shown in Figure 66. The samples of hydrolysate designated A, which were applied directly to the paper, were chromatographed because it was felt that some of the products formed in alkali might be lost on neutralizing the solution and could be demonstrated in this manner. It was however observed that these hydrolysates migrate much differently from

the controls, even in cases where the sample was not altered during hydrolysis. Thus, consideration of the migration of these solutions will be omitted from the results. In Figure 66, uridine-2-$^{14}$C (C14UR) and the neutralized hydrolysate of uridine-2-$^{14}$C (C14UR AN) migrate in an identical manner with that of cold uridine (UR). No label arising from uridine was found in the area of (C14 5OHUR) migration. These results are shown in Figure 67, in 2-propanol-$H_2O$-conc. HCl, Figure 68 in 2-propanol-$H_2O$-conc. $NH_3$, and in Figure 69 in butanol-$H_2O$.

A comparison of the migration of C14 5OHUR and dihydrouridine (DHUR) reveals that in three of the four chromatograms the migration of DHUR is identical with that of C14 5OHUR. In the fourth solvent, 2-propanol-$H_2O$-conc. $NH_3$, Figure 68, in which both compounds are presumably unstable the migration of each differs somewhat.

There appears to be two major contaminants in C14 5OHUR as shown in Figure 69: a slow migratory peak, S, which runs parallel to a faint (minor) product of the hydrolysis of dihydrouridine; and a material, F, which migrates faster than 5-hydroxyuridine, as indicated by the shoulder on the 5-hydroxyuridine peak. This latter material also parallels a faint product of the hydrolysis of dihydrouridine. Material S appears to be one of the predominant alkaline hydrolytic products of C14 5OHUR and is definitely not the same as any of the major hydrolytic products of dihydrouridine. In addition, alkaline hydrolysis of 5-hydroxyuridine produces significant amounts of a non-absorbing product,

Z, which migrates similarly to dihydrouracil on all four chroma-
tograms. These derivatives of the alkaline hydrolysis of C1450HUR
are distinctly separated in Figure 66. Definitely one of these der-
ivatives, S, as seen in Figure 69, is present in the preparation
before hydrolysis, and is increased in amount subsequent to
hydrolysis. Compound F, in Figure 68, also appears to be present
in the sample, however it is not formed in the reaction. Neither
of these substances are identical to Compound Z which has ten-
tatively been identified as dihydrouracil, and is perhaps the prin-
cipal product of this hydrolysis of 5-hydroxyuridine. The Rf
values of these derivatives of 5-hydroxyuridine and those of
dihydrouridine, as well as the values for several controls are
presented in Table 17.

Chemical Alteration Products of 5-Hydroxyuridine

The migration of products of the action of ammonia, mer-
captoethanol, and potassium permanganate on 5-hydroxyuridine-
2-14C on ascending paper chromatography in butanol-$H_2O$ (86:14)
is shown in Figure 70. Migration of a 5-hydroxyuridine-2-14C
control and a labeled preparation, termed artifact, is also shown.
The latter preparation contains Compound A which was obtained
from the water wash of a celite partition column employed to
fractionate a digest of yeast RNA, spiked with 5-hydroxyuridine-
2-14C. Areas which gave a positive test for aldo-pentose are
also shown. The reactions in potassium permanganate and ammonia
were carried out in 0.66 M reagent at 20°C for 72 hours. The re-

action in mercaptoethanol was carried out with a one per cent
solution of the reagent under identical conditions. Neither
Compound A, the 5-hydroxyuridine-2-14C control, or the mer-
captoethanol reaction product gave positive tests for aldo-pentose,
whereas the potassium permanganate and ammonia reactions pre-
sumably produce a similar product, at least in terms of its mi-
gration, which does give a positive sugar test.

The chromatograms shown in Figure 71 were each divided
into three areas: Bands A, B, and C. The Band A materials
remained on the origin, migrated poorly if at all, and resembled
the migration of Compound A. In butanol-$H_2O$ (86:14) Band B
materials ran in the area of 5-hydroxyuridine migration and
Band C materials ran considerably faster. There was a signif-
icant quantity of label in the Band C area of the ammonium re-
action; but the other reactions produced very little material
which migrated into this area.

The migration of the Band A materials for the potassium per-
manganate and ammonia reaction are compared with the migration
of 5-hydroxyuridine-2-14C control and Compound A in 2-propanol-
conc. $HCl$-$H_2O$ (680:170:144) in Figure 71. It may be remembered
that Compound A, which has been termed artifact and is shown
in the bottom graph, is split in this solvent system into a cyti-
dine-like material (Compound C) and a non-absorbing labeled
material termed Compound B. The peak of the label migration
of Compound B is at 6 1/2 inches. It may be seen in Figure 71
that the Band A products of the potassium permanganate and ammonia

reactions, which remained on the origin, on chromatography in
butanol-$H_2O$ (86:14), shown in Figure 70, appear to contain major
non-ultraviolet-absorbing products which migrate similarly to
that of Compound B. The Band A material of the potassium per-
manganate reaction also contains an equal amount of an ultra-
violet absorbing product which is not 5-hydroxyuridine. Why the
non-absorbing product of both the potassium permanganate and
ammonia reactions remain at the origin, as does Compound A
on chromatography in butanol-$H_2O$ (86:14), but migrates as
Compound B in 2-propanol-$H_2O$-conc. HCl (680:144:170) may be
explained by considering the migration of the 5-hydroxyuridine-
2-14C control in each solvent system. In Figure 70 the migration
of 5-hydroxyuridine-2-14C control may be seen. An old solution
of this compound may be expected to contain some breakdown
products, however, it does not test positively for aldo-pentose.
In Figure 71 the same sample does test positively for aldo-pentose.
The highly acidic 2-propanol-$H_2O$-conc. HCl (680:144:170) solvent
can apparently degrade the control to some extent, releasing ri-
bose and dihydropyrimidine-type material. Apparently the same
hydrolytic (?) mechanism is responsible for splitting Compound
A and the products of the potassium permanganate and ammonia
reactions which remained on the origin in butanol-$H_2O$ (86:14)
chromatography.

The migration of Band B materials, Figure 70, from the
reactions of 5-hydroxyuridine in potassium permanganate, ammonia,
and mercaptoethanol, and of the 5-hydroxyuridine-2-^{14}C control,

in 2-propanol-H$_2$O-conc. HCl (680:144:170) is shown in Figure

72. Only the product of the ammonia reaction peaks at 6 1/2

inches and does not possess ultraviolet absorbance, characteris-

tic of Compound B. This material originally gave a positive test

for aldo-pentose, Figure 70. Band B materials of the other two

reactions appear to contain significant amounts of unaltered 5-

hydroxyuridine-2-$^{14}$C.

Migration of the Band C material is shown in Figure 73 along

with that of a 5-hydroxyuridine-2-$^{14}$C control and Compound A.

The latter is split into the cytidine-like component (Compound C)

and the labeled material, Compound B, at 6 1/2 inches. Very

little of the Compound B-like material was directly produced in

the potassium permanganate reaction. A large amount of the

Compound B-like material may be obtained by running Band A

material from the ammonia and potassium permanganate reaction,

Figure 57, in an acidic solvent as 2-propanol-H$_2$O-conc. HCl

(680:144:170). There is little intact 5-hydroxyuridine-2-14C

present in the ammonia reaction, and no other ultraviolet ab-

sorbing products. A significant amount of the Compound B mater-

ial is produced in the ammonia reaction and migrates into the

Band C area in the neutral solvent butanol-H$_2$O (86:14); however,

the label in the Band C area, even from the ammonia reaction,

is only a small proportion of that formed by subjecting Band A

and B materials from the ammonia and potassium permanganate

reactions, Figure 70, to ascending chromatography in 2-propanol-

H$_2$O-conc. HCl (680:144:170).

It is tempting to speculate from the general pattern of the above results that 5-hydroxyuridine is modified to a very reactive material, which possesses a high affinity to form complexes, both with other nucleosides, for example cytidine, or with 5-hydroxyuridine or with itself. Whereas, Compound B may be considered to be a fairly stable nonabsorbing species obtainable through very gentle hydrolysis of both the ultraviolet absorbing and nonabsorbing complexes found in Bands A and B. Thus it is postulated that Compound B itself does not form complexes and is only formed as a consequence of hydrolysis.

It is shown in Figure 60 that a material is produced in the reaction of 5-hydroxyuridine in both ammonia and potassium permanganate which is identical with Compound B. A comparison of the migration of Compound B and the common product of the two reactions with that of several controls, shown in Table 18 tentatively establishes the identity of the common unknown as dihydrouracil. This material tests positively for the presence of dihydrouridine or β-reido acid. In the case of Compound B, there is little label in the area of the positive aldo-pentose reaction. This pentose runs slightly ahead of the labeled dihydro derivative compound B in the 2-propanol-$H_2O$-conc. HCl (680:144:170) solvent.

In the ammonia and potassium permanganate reaction a sugar is released from 5-hydroxyuridine which migrates in the Band C area in the latter solvent but migrates in Band B area in butanol-$H_2O$ (86:14) with an Rf of 0.144. The Rf of D-ribose

in this solvent, as shown in Table 13, is 0.241, implying that the sugar released from 5-hydroxyuridine can not be considered to be ribose. This aldo-pentose represents a contaminant of both Compound B and the common dihydrouracil reaction product in chromatography in 2-propanol-$H_2O$-conc. HCl (680:144:170) and perhaps also in other solvents.

## 5-Hydroxyuridine as an Antimetabolite:  A Reevaluation

It was considered in the Introduction that because 5-hydroxy-
uridine immediately inhibits constitutive and induced synthesis of
β-galactosidase in bacteria and yeast, antimetabolic activity was not
dependent upon requisite incorporation into the genome.  The ability
to interrupt immediately and inhibit completely induced synthesis
of β-galactosidase at concentrations which had no effect on over-
all protein synthesis suggested its antimetabolic activity was at
the transcriptional level.  It may be recalled, however, that even
at extremely high 5-hydroxyuridine concentrations 30 per cent
residual RNA synthesis occurrs, i.e., only 70 per cent of normal
RNA synthesis is inhibited.  In addition, when a small amount of
5-hydroxyuridine is added to a log culture of either E. coli or
Saccharomyces cerevisiae growth is immediately inhibited.  An
important characteristic of the inhibition is that after a while the
cells recover and logarithmic growth, without a lag, continues at
the same generation rate as that preceeding the addition of the
inhibitor.  This unique and important characteristic of its in-
hibition may provide a powerful tool for understanding the cell-
ular mechanism and regulation of transcription.

All presently known RNA inhibitors, in sufficient concentra-
tions, inhibit RNA and protein synthesis completely after a short
incubation period (47).  Studies of this type have often included
many "open-ended" factors, e. g. it has only recently been

proven that, at least in <u>E. coli</u>, the RNA polymerase indeed repre-<superscript>127</superscript>

sents the genetic transcriptase (66). Nevertheless, various types

of inhibition of cellular transcription, involving the RNA polymerase

have been shown to occur: "Polymerization inhibitors" as actino-

mycin--which binds very strongly to DNA preventing RNA poly-

merase from moving along a region to which it is bound--and 3'-

deoxyadenosine-5'-triphosphate--which inhibits polymerization by

preventing further chain elongation--each act by blocking polymer-

ization of RNA chains (107). It is not impossible that 5-hydroxy-

uridine could also bind DNA and in so doing block chain elongation.

It was shown in this dissertation that cytidine will form a stable

covalent complex with either 5-hydroxyuridine or a derivative

which possesses an active C-5 carbonyl. Gentle hydrolysis of the

complex yielded a dihydro-derivative, Compound B, and cytidine.

Ninhydrin, which possesses an active carbonyl, also reacts with

cytosine (117). Determining the chemical state of labeled 5-hydroxy-

uridine in cells to which it had been added, which have recovered

from inhibition, might reveal the presence of similar complexes of

5-hydroxyuridine. Such a hypothetical complex might form at the

deoxycytidine-rich clusters shown to occur on the transcribing

strand of DNA which have been postulated (129) to function as

initiation points for the transcription process.

The finding that 5-hydroxyuridine is present in yeast RNA

permits the heretofore untenable assumption that such a dynamic

minor component may function in cellular mechanisms of macro-

molecular synthetic regulation. One could further speculate that

a minor component on one of the arms of transfer RNA molecules

could bind the cytosine containing clusters along the DNA strand and prevent initiation of transcription. In order to make repression of this type dynamic an enzyme which zips along the DNA, hydrolysing the transfer RNA from the DNA cluster, would also have to be postulated. Each transfer RNA which has presently been sequenced contains a dihydro-loop which apparently is not involved in charging or codon recognition. The presence of dihydrouridine in transfer RNA represents an enigma due to its chemical instability. Could it be replaced in cellular transfer RNA with 5-hydroxyuridine? On this basis it is suggested that 5-hydroxyuridine could interact under biological conditions with deoxycytidine clusters.

Several other types of transcription inhibition are known and each for the most part involves blocking of the initiation step. Proflavin, for example, binds very efficiently to DNA and alters its secondary structure preventing attachment of RNA polymerase (107). Other types of transcription inhibitors alter the RNA polymerase and thus prevent initiation. For example 0.5 molar salt concentration lowers the affinity of the enzyme for DNA in the initial attachment step, whereas many polynucleotides and polyanions including heparin, polyethylene sulfonate, and transfer RNA bind RNA polymerase either to the same site to which DNA binds or to an overlapping site (107).

Only one other type of transcription inhibitors are known, the rifamycin (141) and streptovaricin (90) derivatives. These antibiotics have proven to be extremely useful because they specifically inhibit the DNA-dependent RNA polymerase reaction of certain

cells. They do not bind DNA or affect the binding of chain poly-

merization properties of RNA polymerase but are believed to block

some step in initiation after binding (107). The most active der-

ivative, rifampicin (116)
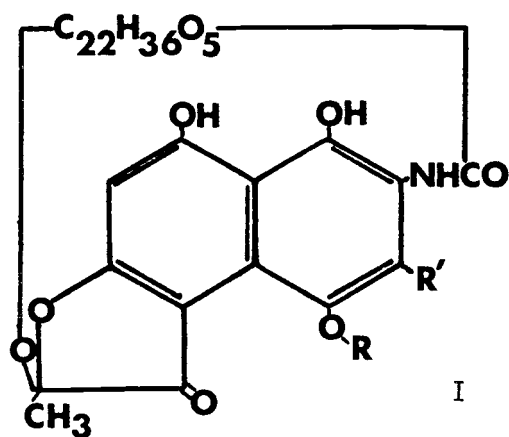
rifampicin, R=H, R'=-CH=N-
$N(CH_2)_2N(CH_3)CH_2CH_2$

Chromophore alone not an
inhibitor.

I

has been used to prove that transcription of the genome of certain

infecting bacteriophage, e. g. T7, SP01, and T4, involves at least

part of the host cells RNA polymerase (128).

How does 5-hydroxyuridine act as an antimetabolite? It has

been shown by Roy-Burman, et al. (1966) (113) that when 5-hydroxy-

uridine-5'-triphosphate-2-14C replaces uridine-5'-triphosphate-2-

14C in an in vitro RNA polymerase reaction it is incorporated

optimally at pH 7.0 at 1/27th the incorporation of uridine-5'-tri-

phosphate, but not at all when the analog exists predominantly in

the ionized form at pH 8.6 or 9.0. In competition with uridine-5'-

triphosphate the inhibitory effect of the 5-hydroxyuridine anion on

the incorporation of the latter into RNA is twice that for the un-

ionized molecule, reducing uridine-5'-triphosphate incorporation

by 60 per cent. As noted in the Introduction, the triphosphates of

other analogs as 6-azauridine, pseudouridine, ribothymine, 5,6-

dihydrouridine, 5-fluorouridine and 5-bromouridine do not inhibit RNA synthesis when they are added to an RNA polymerase reaction mixture containing all four natural substrates as does 5-hydroxy-uridine-5'-triphosphate. Roy-Burman et al. (1966) (113) suggested that the inhibitory effect of 5-hydroxyuridine is not directly related to the "inductive effect" but may be related to its affinity for RNA polymerase or a polymerase-DNA complex. These workers did not establish where the anionic charge was located on the molecule nor did they elaborate why they felt that inhibition was not due to an induction effect except that inhibition of the polymerase reaction still occurs with the unionized molecule.

Sterically substitution at position C-5 does not appear to be critically important to the functioning RNA polymerase. For example, if the incorporation of substituents with a bromine is sterically possible, then incorporation of a uridine analog with a C-5 hydroxyl should also be possible.

The increased inhibitory effect of the 5-hydroxyuridine anion can be more easily rationalized. On ionization 5-hydroxyuridine can transfer its anionic charge from the C-5 hydroxyl to C-6. The catalytic site of the RNA polymerase may require a certain charge homogeneity at C-6 of pyrimidines which would be upset by electron delocalization between C-5 and C-6 of ionized 5-hydroxyuridine. Orotidylic acid is known, for example, not to be incorporated into RNA.

Ionization of the C-5 hydroxyl proton may alter the electronic

character of 5-hydroxyuridine such that tautomerically a C-2 enol

rather than a C-2 keto would be formed. It has been shown (120)

that tautomerism in amine- and hydroxy-pyrimidines may be in-

fluenced by the electronic effects of different substituent groups,

and also by the quantitative degree of interaction through hydrogen

bonding. Uridine and cytidine have been shown by infrared spectro-

scopy to exist in the ketonic form (19). In fact it is essential that

the pyrimidine nucleoside triphosphates possess a C-2 keto group

to each properly hydrogen-bond with its homolog. Such an ability

is thought to be required for active incorporation of the base into

the forming polymer by RNA polymerase. Standard hydrogen

bonding occurs between adenosine and unionized 5-hydroxyuridine

with a C-2 keto group, however, only one hydrogen bond may be

formed by the ionized (or unionized) molecule possessing a C-2

enol and could be formed between either adenosine or guanosine.

It is difficult to see any similarities between the antimetabolic

activity of the 5-halo-analogs of uridine and the action of 5-hydroxy-

uridine. Bromouracil substitutes well for thymine because the

size of the bromine atom is close to that of the $CH_3$ group, and

the former can replace the bulk of thymine in bacteria or bacterio-

phage with little loss of viability (40, p. 308). 5-Fluorouracil is

incorporated into RNA but not detectably into DNA and is rapidly

bactericidal. It is converted to 5-fluorodeoxyuridylate which in-

hibits the conversion of deoxyuridylate, by thymidylate synthetase,

to thymidylate. This results in a block in DNA synthesis which

causes the cell to undergo thymineless death. In the presence of

thymine, 5-fluorouracil is no longer lethal and its incorporation

into messenger RNA results in errors in translation and in pheno-typic suppression of mutations (40, p. 309).

In this regard the ability of 5-hydroxyuridine to reduce uridylate incorporation might produce a kind of "uridine-less" slow down in transcription, however, as stated, even when it is present as an anion, uridine incorporation is only reduced by 60 per cent. It is difficult to see how 5-hydroxyuridine effects an immediate cessation of β-galactosidase synthesis without affecting overall protein synthesis. Apparently it blocks some step in the transcription mechanism. Exploitation of this fact may prove as valuable a tool for studying cellular genome regulation, and bacteriophage regulation of host genome transcription, as have the bulkier and presumably less specific antibiotics as rifampicin currently used in studies of such problems. It will be important to understand how cells recover from inhibition by 5-hydroxyuridine, whether it binds DNA or the catalytic site of the polymerase, and how the type of RNA in which it is normally incorporated may be involved in transcriptional regulation. Models of transcription, especially the newer ones (124), will have to account for inhibition, both of the in vitro and in vivo DNA dependent RNA polymerase reaction by 5-hydroxyuridine-5'-triphosphate.

The Binding of 5-Hydroxyuridine and Magnesium Cations

A comparison of the spectra of equimolar solutions of 5-hydroxyuridine at pH 8.70 in $5 \times 10^{-3}$ M $MgCl_2$ and in distilled water was shown in Figure 17. There was a pronounced difference in the

spectra, expecially in the area of the peak at 243 nm. This was

interpreted as strong ionic bonding between the 5-hydroxyuridine

C5-hydroxyl anion and $Mg^{++}$ which altered normal resonance

properties of the molecule. It is well known that $5x10^{-3}M$ to $10^{-2}M$

concentrations of magnesium cations are required to reassociate

or bind intact 30S and 50S ribosome particles, whereas in more

dilute solutions they come apart. No mechanism currently explains

the binding and dissociation of ribosome subunits due to changes in

magnesium concentration. It is not known whether the binding is

protein to protein, RNA to RNA, or between the two. There is a

possibility that 5-hydroxyuridine, present in ribosomal RNA, could

participate in binding ribosomal subunits. The magnesium cation

could bridge between two ionized hydroxyl groups in the following

manner:



II
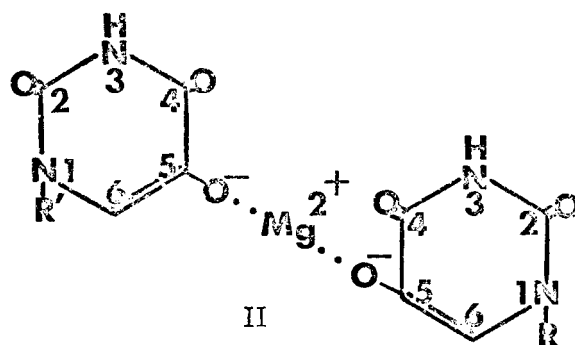
R= 50S ribosome subunit

R'= 30S ribosome subunit

Molecular models show that sterically this type of bonding is very

possible.

## Mutagenisity and the Ionic Character of 5-Hydroxyuridine

5-Hydroxyuridine has been shown to possess a dissociation

constant for the C5-hydroxyl proton of 7.83, and values of 10.57

for the dissociation of the N3-proton, and 11.70 for the C-2'-

hydroxyl of the ribose, Tables 1-3. The possession of an hydroxyl

at C-5 gives the molecule an extra dissociable proton absent in

both the C-5 halo derivatives and the major ribonucleosides. The

halo-analogs, as well as being incorporated into RNA are also in-

corporated into DNA and as such their potential for mispairing may

contribute to their mutagenic effect. In order to point out dissimi-

larities in the chemistry of 5-hydroxyuridine and 5-halo-analogs,

the former and 5-bromouridine may be compared. It has been

recognized that mutagenesis may be due to mispairing of 5-brom-

ouracil with guanine due to ionization of the N3 base proton being

more frequent for bromouracil than for thymine (134). This is

suggested by the increase in acidity of the N-3 proton from thymidine,

pKa 9.3, to 5-bromouridine, pKa 8.1. At neutral pH about eight

per cent of 5-bromodeoxyuridine exists as the anion, compared

with 0.16 per cent of thymidine. Anomalous base pairing of ionized

bromouracil with guanine is the result. (9, 137).

In comparing the pKa's of the N-3 proton for 5-hydroxyuridine,

thymidine, uridine, and bromodeoxyuridine, which are respectively

10.57, 9.8, 9.2[+], and 8.1 (134) it is obvious that the inductive effect

of the C-5 substituent influences the ionization. Electron donating

groups such as methyl, amino, mercapto, and dissociated carboxyl

and hydroxyl groups should show more basic pKa's for the N-3

proton than that of uridine. Whereas the halo-derivatives, as well

as other electrophilic substituents which lower the electron density

of the ring could be expected and do have more acidic pKa's than uridine. The ionized C-5 hydroxyl of 5-hydroxyuridine acts as an electron donating substituent in relation to the ring whereas the C-5 halo-substituents all exhibit an electrophilic behavior. Thus the anion concentration of the N-3 proton should be negligible at neutral pH, especially as compared to 5-bromouridine, and its mutagenic effect, due to its potential for mispairing, should be correspondingly lower.

It has also been recognized that the mispairing of deoxybromouridine with deoxyguanosine may be due to keto-enol tautomerization being more frequent for deoxybromouridine than thymidine (134). The tautomeric constant $K_t$, which is defined as the ratio of the acidity constants of the carboxyl form over the enol form gives an indication of the ease of ionization and stability of the anion formed. The tautomeric constant for 1-methyl uracil is $10^{3.3}$ whereas that for 1-methyl-5-bromouracil is $10^{1.7}$. The lower value for the latter compound indicates that the enol form predominates to a 40-fold greater extent for 1-methyl-5-bromouracil than for 1-methyl uracil. This effect may be considered to be due to the electrophilic character of the C-5-substituted bromine placing a larger positive electrostatic charge on the C-4-carboxyl carbon (134).

4-keto        4-enol

III         IV



V            VI

normal     vs     mutagenic
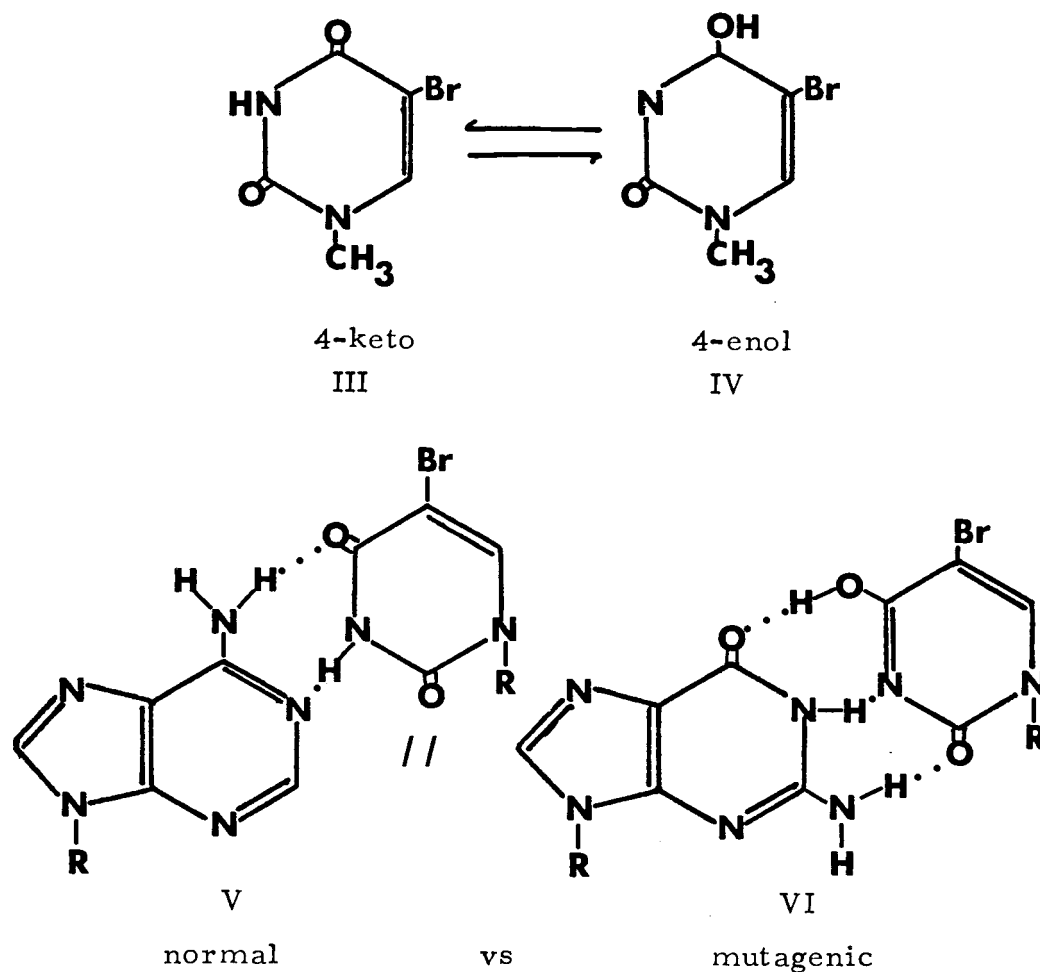
Whereas with 5-hydroxydeoxyuridine the adjacent electron donating hydroxyl would tend to strengthen the C-4-carbonyl, thus lowering the occurrence of the enol form and hence lowering the potential for mispairing. We may conclude that if 5-hydroxydeoxyuridine, incorporated into DNA, is mutagenic at physiological pH, then such an activity must be by another mechanism than that of mispairing, at least as that proposed for the 5-halo derivatives. The effect of the C-5-hydroxyl on the N-3-protons ionization thus is opposite to that of C-5-halo substituents. It retards the dissociation of the N-3 proton.

The pKa of 9.33 for the N-3-proton of 5-methoxyuridine

approximates that for uridine and is slightly less than that for thymidine. Thus by replacing the C-5-hydroxyl proton with a methyl group the inductive effect of the dissociated hydroxyl is eliminated. In turn, methylation of the N-3-proton appears to effect slightly the dissociation of the 5-hydroxyl proton as shown by the pKa of 8.33 for 3-methyl-5-hydroxyuridine. Almost an identical rise of about 0.5 in the pKa for the N-3-proton occurs by substituting a methyl at position C-5 of uracil; compare e. g. the pKa's of uridine and thymidine given above. In addition, the dissociation of 1-methyluracil with a pKa of 9.71 for the N-3 proton and 3-methyluracil with a pKa of 9.99 for the N-1-proton also show similar decreases in acidity on methylation as compared to the dissociation of the N-1-proton of uracil which possesses a pKa of 9.45 (73). Thus the substitution of the electron donating methyl group at N-3 of 5-hydroxyuridine must raise the electron density of the ring and strengthen the affinity of the C-5-substituted oxygen for its proton.

A Hypothesis on the Bathochromic Shift of Solutions of Ionized

5-Hydroxyuridine

Two resonance forms, excluding charge separation forms,

may be drawn for each of the three tautomers of the 5-hydroxy-

uridine monoanion and involve, in addition to the monoanion, VIIa,

a resonant C-6 carbanion, VIIb:
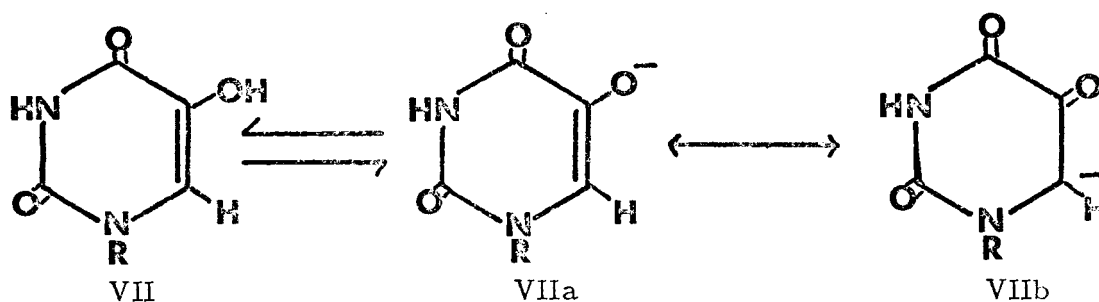


Dissociation of the 5-hydroxyl proton does not increase the con-

jugation of the molecule but does result in a bathochromic shift in the wavelength of its absorbance maximum. At least three other N-1 substituted nucleosides: 5-mercapto-2'-deoxyuridine (6), 5-aminouridine, and 5-methylamino-2'-deoxyuridine (119) also exhibit spectral bathochromic shifts when ionized. Resonance forms for the monoanion of each are:
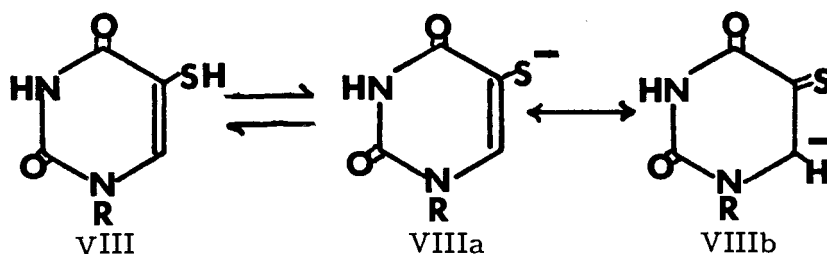


5-mercapto-2'-deoxyuridine, R= deoxyribose
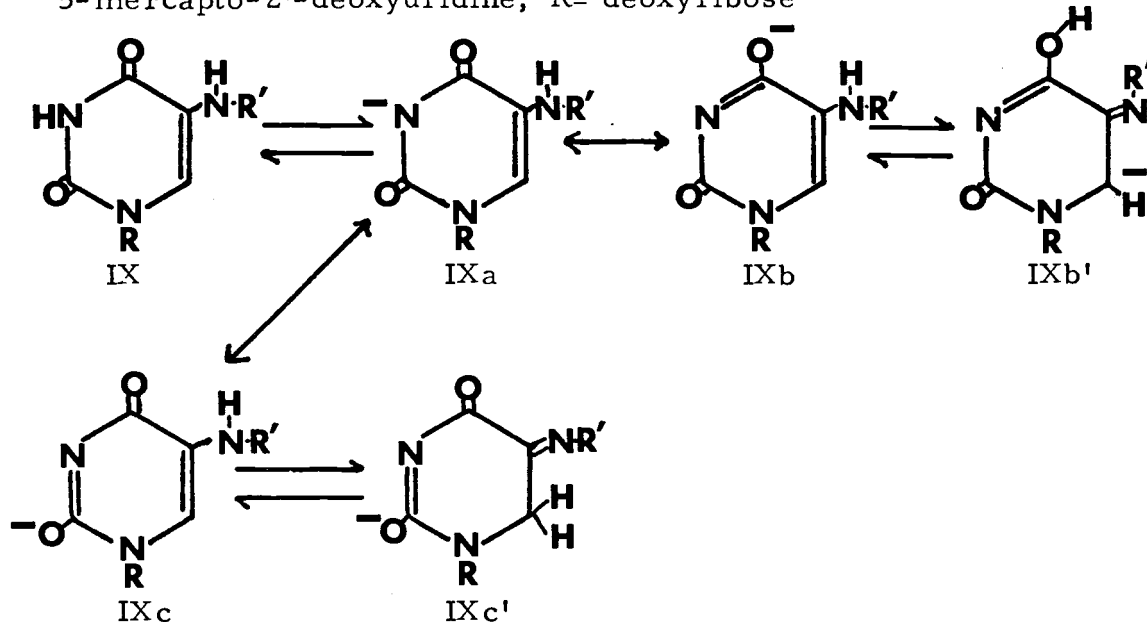


5-aminouridine, R= ribose, R'= H
5-methylamino-2'-deoxyuridine, R= deoxyribose, R'= CH₃

For 5-aminouridine, tautomerism between the 5-amino group and the resonant C-4 hydroxyl anion yields a C-5 imino, C-6 carbanion structure. This tautomerism is similar to that which occurs between the N-3 and the C-4 amino group in cytidine yielding a tautomer with a C-4 imino group.
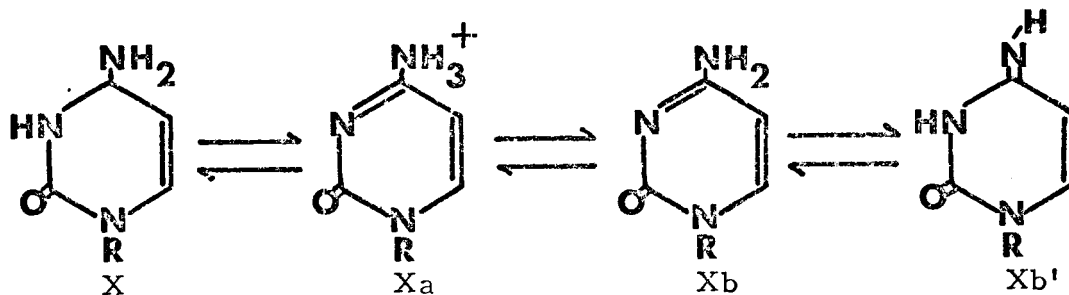
cytidine, R= ribose

Cytidine and 5-methyldeoxycytidine do not exhibit spectral batho-

chromic shifts when ionized. They are similar in this respect to

uridine and a number of other nucleosides including:

thymidine
5-fluoro-, 5-chloro-,
5-bromo-, and 5-iodouridine
5-methyluridine

5-hydroxymethldeoxyuridine
5-methyldeoxycytidine
1, 5-diribosyluracil

however the cytidine derivatives do not possess a dissociable N-3

proton, as do the latter substances.

One may ask what is the unique common property of the mono-

anion of 5-hydroxyuridine, 5-mercaptouridine, 5-aminouridine and

5-methylamino-2'-deoxyuridine causing the bathochromic shift, for as

stated, cytidine and uridine derivatives do not undergo spectral

bathochromic shifts. The resonance forms of the derivatives of

cytidine are similar to those for cytidine, presented above. Ion-

ization of uridine and the above related compounds to monoanions

results in a reduction in extinction at the absorbance maximum

rather than a spectral bathochromic shift. For each three resonance

forms can be drawn as those for uridine: R= ribose
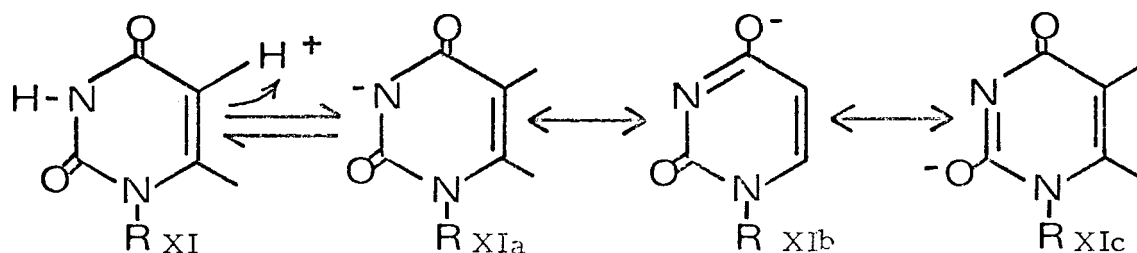
No tautomers are possible.

The basic difference between 5-aminouridine, 5-methylamino-2'-deoxyuridine, 5-hydroxyuridine, and 5-mercaptouridine, and the above pyrimidine nucleosides rests in an inability of the latter compounds to form a resonance hybrid similar to a phenoxide ion. Dissociation of the C-5 hydroxyl proton of 5-hydroxyuridine has been shown to give rise to its spectral bathochromic shift, thus the phenoxide type resonance occurring between C-5 and C-6, subsequent to dissociation of the 5-hydroxyl proton, _i. e._
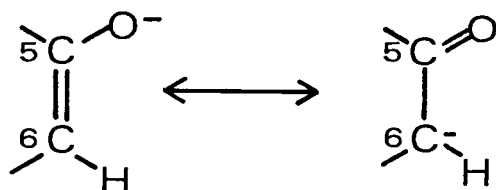
must be intimately associated with the spectral shift. Characteristic bathochromic shifts in the wavelength of maximum absorbance, similar to 5-hydroxyuridine, occur in spectra of solutions of pyrimidine bases of all the above nucleosides as well as other bases on ionization including:

| | |
|---|---|
| uracil | 5-methyluracil |
| bromouracil | 3-methyluracil |
| chlorouracil | cytosine |
| 5-ribosyluracil | 5-methylcytosine |
| 5-aminouracil | isocytosine |
| 5-mercaptouracil | 5-methylisocytosine |
| 5, 6-diaminouracil | isobarbituric acid |
| uracil-6-acetic acid | barbituric acid |
| 5-hydroxymethyluracil | 5, 6-diaminopyrimidine |

It has been proposed previously (14) that the bathochromic shift shown by these pyrimidine bases resulted from the dissociation of their N-1 proton. When the N-1 proton is substituted with a methyl group or a sugar the spectra of the subsequent derivatives on ionization of the

N-3 proton, only show a change in extinction at the wavelength of

the maximum and no longer exhibit bathochromic shifts. The mono-

anions of the above N-1 substituted uracil derivatives possess reso-

nance characteristics identical to those of uridine, whereas 3-methyl-

uracil resembles uracil in its spectral as well as resonance character-

istics, excluding charge separation forms:



XII                XIIa             XIIb             XIIc             XIId
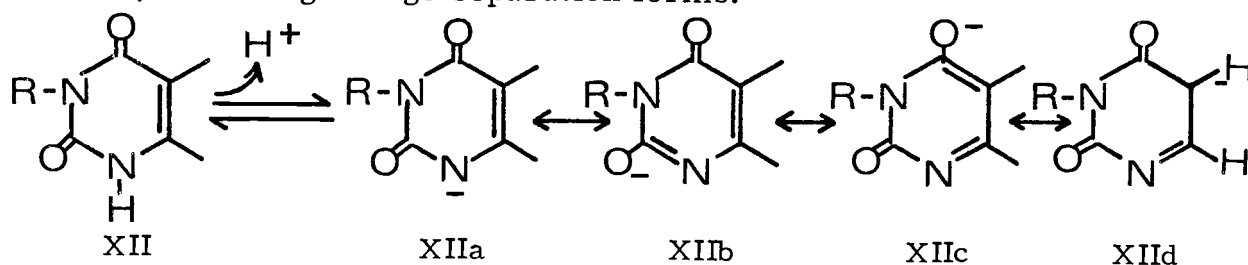
Uracil, R=H,   3-methyluracil, R= CH3

It has been mentioned in a previous section that the N-1 proton of

3-methyluracil is less acidic than the N-3 proton of 1-methyluracil

whose anion is stabilized by an additional resonance form. However,

the bathochromic shift of uracil characterizes a $pKa_1$ of 9.45 assumed

by analogy to be that for the N-1 proton. A comparison of the reso-

nance and tautomeric forms which may be written for the uracil

monoanion formed through the initial dissociation of either N-1 or

N-3 reveals: a) initial dissociation of the N-1 proton permits far

greater resonance stabilization of C-2 and C-4 enols, and b) twice

the number of C-2-enolic resonance forms can be drawn for uracil

when dissociation is written to occur first at N-3. Thus initial dis-

sociation of the N-1 proton of uracil and thymine is probably due to

the greater stability of the molecules enolic forms in alkaline solution.

From the above discussion it may be seen that if a resonance

structure can be formed for a pyrimidine anion in which the charge

is transferrable to C-5 or C-6 the compound can be predicted to

exhibit a spectral bathochromic shift on ionization. The following

resonance hybrid may be responsible for the bathochromic phenomenon:
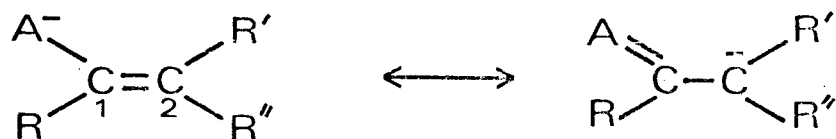


where A=N, O, or S; R=C, H, or N; R'=N, C; R''=C, H, or N; and

$C_1$ equals pyrimidine positions C-4 or C-5, and $C_2$ equals positions

C-5 or C-6.

This scheme works for all the above derivatives of uracil,

cytosine, and other pyrimidines for which it can be drawn. For

example the ability of 5-aminouridine to exhibit a bathochromic

shift on ionization is assumed to be due to its ability to form such

a resonance hybrid, where R=ribose:
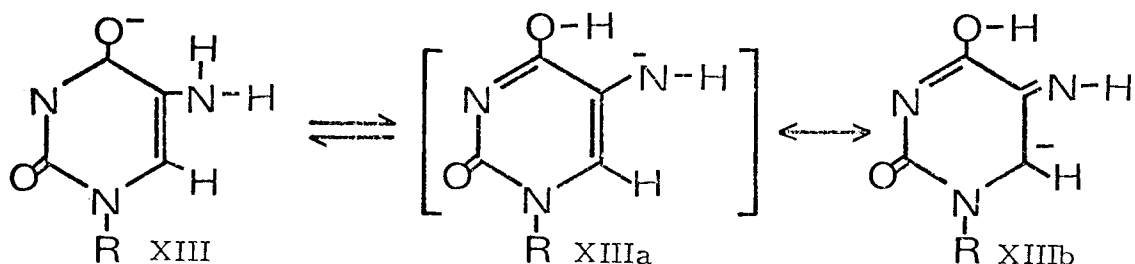


6Aminouracil exhibits a very small bathochromic shift in com-

parison with uracil due to the effect of the C-6 amino group  on the

C-5, C-6 double bond; thus the influence of substituent R' and per-

haps also substituents attached to R and R'' will affect the extent of

the bathochromic shift. The character of the chromophore formed

by A and its relation to adjacent groups in the ionized molecule can

effect an additional spectral absorbance maximum, generally around

240 nm. 5-Mercaptodeoxyuridine and 5-hydroxyuridine each exhibit

two spectral absorbance maxima on ionization of the C-5 substituent

proton. The additional absorbance maxima are presumably related to the resonant formation of either a C-5 thiol or C-5 carbonyl group.

## Modifications of 5-Hydroxyuridine Under Standard RNA Chemical Hydrolytic and Fractionation Procedures

A general study of the stability of 5-hydroxyuridine under standard RNA chemical hydrolytic procedures was undertaken to determine whether the molecule may be modified under biological conditions, especially with regard to isolation. 5-Hydroxyuridine was found to be quite unstable under reducing conditions, as well as in alkali; however it was only slightly unstable in acid and toward weak oxidizing media. Preliminary studies suggested that the stability of the molecule, measured in terms of loss of ultraviolet absorbance, depended upon whether or not the 5-hydroxyl proton had dissociated but was also related to additional factors than merely pH, as for example the type of nucleophile present. This was shown by its increased instability in a pH 10.1 solution of $2 \times 10^{-2}$ M Tris, 3.5 molar in urea, as compared to the same tris solution without urea or to distilled water or magnesium chloride solutions at the same pH. 5-Hydroxyuridine has been found to be unstable under the following biological isolation conditions in which ribonucleic acid, or its major components are generally considered to be stable: (1.) 5-Hydroxyuridine is unstable in upper solvent G, ethylacetate-1-butanol-ligroin (bp 66-75°C)- water (1:2:1:1), the second eluting solvent employed in celite partition chromatography. This solvent, as mentioned above, is employed in a column technique for fraction-

ating mixtures of ribonucleosides into six substituent groups: $N^6$-methyladenosine, adenosine, uridine, methylated guanosines, guanosine, and cytidine. The technique was developed by Hall (1965) (52) who states that about 30 hours are required to complete the separation, "and, for convenience in laboratory scheduling" his "column was operated on three consecutive days, a procedure which did not diminish resolving power." Column HC #13, as described in the Results was continuously eluted for about 30 hours according to the schedule given by Hall (1965) (52). The ultraviolet absorbance and radioactivity of each fraction were measured manually as outlined in Methods. Subsequent to terminating the elution about 48 hours elapsed before it was found that none of the eluted fractions were labeled, indicating the 5-hydroxyuridine-2-14C spike had been held up on the column. As such, the 5-hydroxyuridine held on the column had been exposed to upper solvent G about 60 hours at room temperature. If a low concentration of a good nucleophile was present as a contaminant in one of the components of solvent G, then modification of 5-hydroxyuridine could result from its prolonged exposure to the solvent. It has been shown in Figures 52 and 53 that 5-hydroxyuridine in solution above pH 7.0 at 30°C was extensively modified by 5.86x $10^{-2}$M hydroxylamine. Presumably a similar reaction could occur on the column with an equally reactive nucleophile.

The elution profile of celite column HC #13, Figure 22, shows only a small trailing peak for cytidine when compared to the large cytidine peak obtained by Hall (1965) (52). This implied that a large portion of cytidine, as well as 5-hydroxyuridine, was held up on the

partition column. It was also found that significantly larger quantities of cytidine are routinely eluted from the celite column. Thus Compound A, the artifact of 5-hydroxyuridine-2-14C, was probably formed on the column.

(2.) 5-Hydroxyuridine is unstable in 50:50 (v/v) pH 8 phenol:water solutions heated at 60°C for 15 minutes. Technical difficulties encountered in extracting the strongly ultraviolet absorbing phenol from the aqueous phase of such a solution, however, preclude accurate quantitization of the extent of instability of 5-hydroxyuridine to the treatment. There is a shift in the absorbance maxima of the extracted solution to about 276 nm with considerably reduced absorbance at 307 nm. This has also been found to occur when about pH 8 aqueous solutions of 5-hydroxyuridine are exposed to low concentrations of phenylhydrazine hydrochloride or hydroxylamine hydrochloride. The ultraviolet absorbance spectra of these solutions were similar to the reaction spectra of 5-hydroxyuridine with hydroxylamine, shown in Figure 53. Fractionation of ribonucleic acid from a slightly alkaline biological milieu by a phenol-heat treatment can be expected to modify 5-hydroxyuridine residues contained therein.

(3.) Acidic as well as alkaline solutions of 5-hydroxyuridine lose considerable ultraviolet absorbance when autoclaved at 121°C for 15 minutes. The loss of absorbance of weakly acidic, pH 4.5, solutions was unexpected as 5-hydroxyuridine has been shown stable to N/10 HCl at 105°C for one hour. The loss of absorbance of weakly acidic solutions is probably due to saturation of the C-5, C-6 double bond rather than cleavage of the ring in lieu of the compounds stability in

in acid. It is likely that both saturation as well as ring cleavage and rearrangement occur on autoclaving alkaline solutions of 5-hydroxyuridine.

Different results were obtained, however, when similar solutions of 5-hydroxyuridine were autoclaved in $5 \times 10^{-3}$ M $MgCl_2$. Acidic as well as basic $MgCl_2$ solutions of 5-hydroxyuridine lost ultraviolet absorbance on autoclaving. In contrast to the above results, varying amounts of an ultraviolet absorbing derivative were formed in $MgCl_2$ solutions varying in pH from 4.5 to 9.5, indicating that the binding of a magnesium cation to the monoanion does not afford the same protection of the molecule against modification as the methyl group of 5-methoxyuridine. The derivative was identified due to its absorbance maximum at 304 nm in alkaline solution, and because its absorbance subsequent to autoclaving was atypical of control solutions at identical pH, e. g. as shown in Figures 2 and 16; as well as from the observation that the former solutions, on acidification, do not possess typical absorbance maxima at 280 nm. This derivative was not formed when solutions of 5-hydroxyuridine which did not contain $MgCl_2$ were autoclaved. In the presence of $MgCl_2$ acidic solutions became weakly alkaline, whereas alkaline solutions through pH 10.5 dropped in pH to about 8.5 on autoclaving. No alteration in pH of the 11.5 solution occurred, however, the ultraviolet absorbance of the solution was similar but not identical to the absorbance of the same solution prior to autoclaving, Figure 16. The spectra of the autoclaved $MgCl_2$ solutions above pH 9.5, i.e. of solutions at pH 10.5 and 11.5 are close enough to those of controls shown in Figure 16, as well as the 5-

hydroxyuridine derivative formed at and below pH 9.5 in Figure

42, that they may represent either the latter substance, or unaltered

5-hydroxyuridine. The atypical absorbance of these autoclaved

$MgCl_2$ solutions on acidification permits the tentative conclusion

that their spectra are of the 5-hydroxyuridine derivative whose form-

ation apparently is greatly accelerated above pH 9.5. Such spectral

interpretation is not unambiguous. Nevertheless, whether the ma-

terial present in large quantity subsequent to autoclaving $MgCl_2$

solutions at and above pH 10.5 is the same as the derivative material

formed below pH 9.5 or is in fact stabilized 5-OHUR, it is obvious that

a difference in reactivity of 5-OHUR below pH 9.5 and above pH 10.5

occurs. The sum of the reactivities of the monoanion versus the

dianion differ in the presence of magnesium cations.

Comparing the results from autoclaving with the stability of

5-hydroxyuridine in solution at 37°C in $5x10^{-3}$ M $MgCl_2$, Graph A,

Figure 41, it may be seen that there is a similar difference in the

character of the reaction of 5-hydroxyuridine below pH 9.5 and

above pH 10.5. These results also indicate a difference in reactiv-

ity of the monoanion versus the dianion. The heat treated solutions

between pH 4.5 and 9.5 steadily lose ultraviolet absorbance with time.

Solutions at pH 10.5 and above lose absorbance initially but subse-

quently exhibit a rapid gain in absorbance and then a second loss.

This fluctuation in solution absorbancy might be interpreted in terms

of an initial addition of $H_2O$ across the double bond, according to

Markownifoff's rule, resulting in loss in solution absorbance. The

hydrated dianion in the presence of a magnesium catalyst may auto-

catalytically eliminate the water reforming the 5-hydroxyuridine dianion whereas elimination of water from a hydrated monoanion may not be possible. Whether such a reaction could also occur on autoclaving is not clear. The products of the heat-treated and autoclaved $MgCl_2$ solutions of 5-hydroxyuridine were not isolated, thus a more detailed comparison of product formation via the two reaction conditions is not possible.

(4.) 5-Hydroxyuridine is modified by six routine (16, 29) RNA alkaline hydrolytic procedures: 0.3 N KOH at $37^\circ C$ for 16 hours (it is stable under the same conditions at $4^\circ C$, Figure 43), one normal KOH for one hour at $80^\circ C$, N/10 KOH for 20 minutes at $100^\circ C$, N/20 KOH for 40 minutes at $100^\circ C$, and one per cent (v/v) aqueous piperidine for one hour at $100^\circ C$, Figure 46. In the last five procedures 5-hydroxyuridine is modified to a new substance, termed Compound R, shown in Figures 45 and 47, which is formed with time.

Compound R possesses an alkaline ultraviolet absorbance maximum at 307-308 nm and an acid absorbance maximum at 319-320 nm. The acid and alkaline spectra of Compound R form isosbestic points between 273-275 nm and at 309 nm, and are reversible suggesting R possesses a new covalent character.

Two additional derivatives of 5-hydroxyuridine, Compounds P and $H_{28}$, Figures 48 and 49, have been obtained when concentrated, 90 $A_{280}$ units per ml, or dilute, 0.9 $A_{280}$ units per ml, solutions of 5-hydroxyuridine were refluxed in one or ten per cent,

or in five per cent (v/v) aqueous piperidine at $100^{\circ}C$, respectively.
The differences in reactivity depend primarily on the concentration
of 5-hydroxyuridine in the reaction and that in alkaline RNA hydro-
lytic procedures the sample is heated rather than refluxed. The
structure and mechanism of formation of Compound P will be con-
sidered in sections below in relation to the transformation of 5-
hydroxyuridine in alkaline media.

(5.) 5-Hydroxyuridine is modified in N and N/10 $NH_3$ at $37^{\circ}C$ and
at room temperature, Figure 43. At the higher temperature a
nonabsorbing product is formed, whereas at room temperature,
after initial loss of absorption an ultraviolet absorbing derivative
begins forming by 24 hours. The latter appears to be formed from
the nonabsorbing intermediary product rather than from a direct
modification of 5-hydroxyuridine. Biological desalting procedures
in which nucleotides are eluted from an activated charcoal column
with a solution of amoniated ethanol, as well as chromatographic
solvent systems containing $NH_3$ can be expected to modify 5-hydroxy-
uridine present in exposed samples. 5-Hydroxyuridine was also
shown to be quickly modified in the presence of two molar ammonium
carbonate at $37^{\circ}C$ at pH 9.0, Figure 43. This reagent is extensively
employed (131) for eluting oligonucleotides from DEAE-cellulose
and mononucleotides from Dowex-1 columns in a procedure for the
removal of urea and salt from these fractions. Native 5-hydroxy-
uridine present in mono- or oligonucleotide fractions exposed to
such procedures would be modified to non-ultraviolet-absorbing
substances.

(6.) Although hydroxylamine reacts specifically with some pyrimidines, particularly uracil and cytosine (68), its activity is considered in this section because its ease of reaction at low concentration with 5-hydroxyuridine differs from the character of its reaction with cytosine, uracil, and their 5-methyl derivatives. Cytosine reacts readily with hydroxylamine. 5-Hydroxymethylcytosine reacts to a lesser extent, while 5-methylcytosine and thymine are only slightly succeptible if at all (68). In neutral solution cytidine reacts with hydroxylamine forming N-4-hydroxycytidine and 6-hydroxyamino-5, 6-dihydro-N-4-hydroxycytidine, the latter compound presumably being formed via the intermediate 6-hydroxyamino-5, 6-dihydro-cytidine (75).

Uracil and its N-1-alkyl derivatives react slowly with hydroxylamine, pKa 5.96 (14), at neutral and slightly acid pH's and maximally at around pH 10.0, where cytosine is relatively inert (68). It has been shown that N-3 methyluridine, which cannot form an anion, maximally reacts with hydroxylamine at a progressively more alkaline pH (75). By analogy it was concluded (75) that dissociation of uridine was not a necessity for reaction and that the neutral form of uridine and the very reactive nucleophilic $NH_2O^-$ anion were the reacting species in the above reaction. However, at optimal reaction conditions, pH 10, uridine, pKa 9.2, predominantly exists as an anion, thus both reacting species for the most part may be considered to be anions.

Addition, as well as elimination of hydroxylamine to the C-5,

C-6 double bond of uridine readily occurs.  The short-lived adduct

is unstable with respect both to reversal to the parent uridine,  and

to irreversible ring closure with formation of a 3,4-dihydro-3-

ureidoisoxazol-5-one (100). This pyrazolone derivative formed by

ring closure yields isoxazolone and, ribosylurea which is subse-

quently transformed into ribosylhydroxylamine (75, 100). The

displacement of the equilibrium for the addition and elimination of

hydroxylamine anion from uridine by the irreversible isoxazalone

formation is evidenced by the slow decrease in ultraviolet absorbance

of the uridine reaction solution. The latter reaction only occurs

under basic condition. Basic catalysis is probably accomplished

through removal of the N-3 proton permitting the formation of

three resonance forms of the ionized adduct that are not possible

for the "neutral" adduct. It does not follow, however, that the same

reaction sequence prevails at neutral pH as the products of such a

reaction have never been isolated (68).

A comparison of the reaction products of uridine and cytidine

with hydroxylamine reveals that both the two functional groupings

of hydroxylamine are involved in the reaction with uridine whereas

only the amino group of hydroxylamine is involved in the reaction

with cytidine. This has been shown experimentally through the

observation that 0-alkylhydroxylamines do not attack the uracil

ring but readily modify the cytosine ring (100).

Cytosine (68) as well as uracil (100) and their N-1 alkyl der-

ivatives progressively lose ultraviolet absorption at their maxima on

exposure to hydroxylamine. Whereas 5-methylcytosine, its N-1

riboside, as well as 5-hydroxymethylcytosine do not exhibit any

disappearance of their characteristic absorption spectra, implying

that no addition of hydroxylamine to the C-5, C-6 double bond occurs.

5-Hydroxymethylcytosine and 5-methylcytosine undergo only exchange of the amino group to give the 4-hydroxylamino derivative. The spectra of each of these derivatives are quite similar and differ from the spectra of each unmodified base principally in their ultraviolet absorption below 260 nm (68).

Positions 2, 4, and 6 of pyrimidines are electron deficient and correspond to the $\alpha$ and $\gamma$ positions in pyridine whereas position 5 is less electron deficient and corresponds to the $\beta$ position. Electron-releasing groups at positions 2, 4, or 6 in pyrimidines activate position 5 to electrophilic attack and deactivate the other positions to nucleophilic attack. Strong electron withdrawing groups at C-5 increase electron-deficiency at other positions (135 p. 4-5).

There are two positions on the cytosine ring at which nucleophilic attack can occur, C-4 and C-6. In uracil nucleophilic attack occurs at C-6; whereas with both molecules electrophilic attack occurs only at C-5. Resonance structures can be drawn for the anion of either base, resulting from dissociation of the N-1 proton, such that the charge can be transferred to position C-5 but not to C-6. N-1 derivatives of these bases, e. g. where R equals methyl, 2'-deoxyribose, or ribose cannot transfer the anionic charge of their monoanions to either C-5 or C-6. Thus the unshared pair of electrons in the uracil or cytosine anion may act to orient rather than promote nucleophilic attack at C-6 since, for example cytidylic acid is nearly as susceptible to nucleophilic attack by hydroxylamine (100) as is

cytosine. The absence of activating substituent groups and the impossibility of drawing resonance forms in which an anionic charge can be transferred to either position C-5 or C-6 of the N-1 alkyl derivatives of uracil or cytosine indicates that hydroxylamine should add to the double bond of these derivatives at either C-5 or C-6. C-5 alkyl-substituted nucleosides, although chemically much less reactive, should add the hydroxylamine anion principally at C-5, according to Markownikoff's rule. The C-5 substituted pyrimidine bases, however, would still be able to transfer their anionic charge to C-5, and thus would add hydroxylamine at C-6 as is currently written.

In Figure 52 it was shown that at 37$^\circ$C in acid solution through pH 7.0, 8.4x10$^{-5}$M 5-hydroxyuridine was stable to 5.86x10$^{-2}$M hydroxylamine, whereas on increasing the pH to 8.0 or above a dramatic change in the reactivity of 5-hydroxyuridine with hydroxylamine occured. Reactions at pH 8-10.5 show: (a) a loss of absorbance at 293.5 nm, (b) a hypsochromic shift in the wavelength of maximum absorbance with time, (c) the derivatives are unstable on addition of acid to pH 1 and (d) increase in extinction and possess ultraviolet absorbance maxima at about 273 nm on addition of alkali to pH 13. The character of the absorbance at 293.5 nm of the reaction at pH 11.6 was not indicated in Figure 52 because the absorbance at this wavelength instead of dropping, as did the reaction solutions below pH 10.5 rapidly gained in absorbance, shown in Figure 55. There was no loss of absorbance but rather the rapid formation of a derivative which possessed a maximum at 277 nm which with time increased in absorbance and shifted to a maximum at 272 nm. The

derivative formed at pH 11.6 was as unstable in N/10 HCl as were the derivatives formed between pH 8 and 10.5.

Loss of ultraviolet absorbance at 293.5 nm at and above pH 8.0 indicates that hydroxylamine reacts at the C-5, C-6 double bond of 5-hydroxyuridine, but only after the C-5 hydroxyl proton has dissociated. The neutral or undissociated molecule resembles thymidine in its resistance to the addition of hydroxylamine.

From the resonance forms for the 5-hydroxyuridine monoanion it may be seen that nucleophilic addition is not likely at position C-6.



VIIa         VIIb

5-Hydroxyuridine, R= ribose
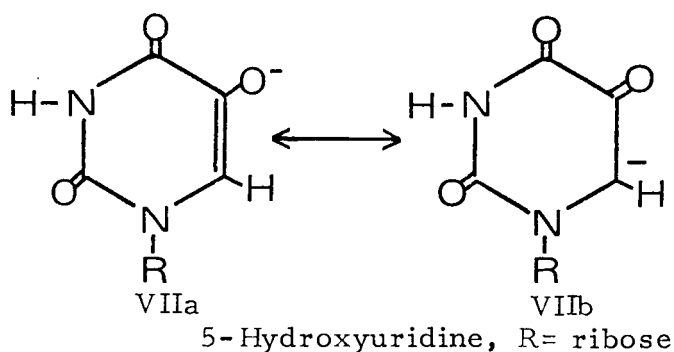
The loss of ultraviolet absorbance at 293.5 nm between pH 8 and 10.5 for the 5-hydroxyuridine anion indicates that addition of the hydroxylamine anion occurs at C-5 and probably attacks resonance Form VIIb. A similar addition of hydroxylamine to alloxan has been proposed (135. p.8):



XIV         XV         XVI

alloxan                     violuric acid

The reaction between the hydroxylamine anion and 5-hydroxyuridine

may be as follows:



VIIa          VIIb                    XVII              XVIII

R= ribose

Further addition of alkali may result in unsaturation at C-5, C-6

with the formation of a nitro-type compound, accounting for the

increase in extinction of the derivative in alkali.



R= ribose
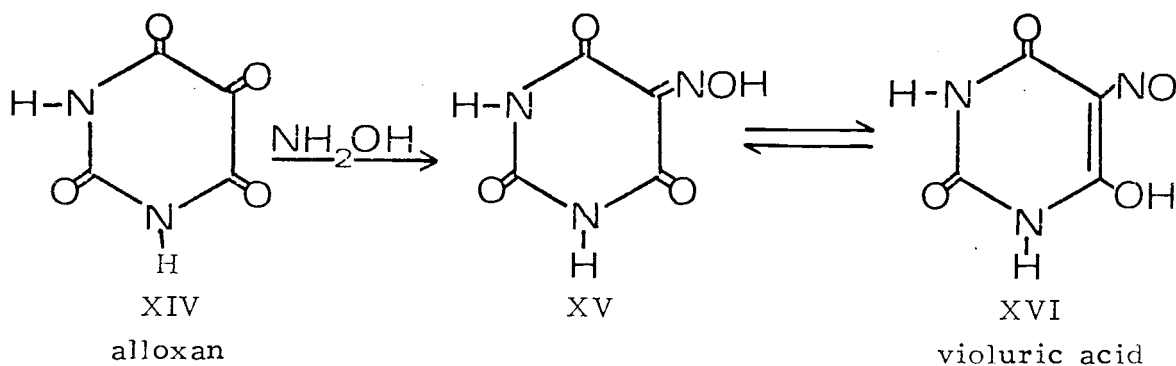
Acidification of the hydroxylamine adduct of 5-hydroxyuridine

results in loss of its characteristic ultraviolet absorbance between

270 and 280 nm. Acidification of the cytidine hydroxylamine adduct

6-hydroxylamino-5,6-dihydro-N-4-hydroxycytidine leads to the acid-

catalysed elimination of hydroxylamine with reformation of the C-5,

C-6 double bond to give N-4 hydroxycytidine (68). In the case of 5-

hydroxyuridine it is not known if hydroxylamine is eliminated, thus

the following reaction on acidification is postulated to occur:



XVIIIb          XVIII          XVIIIa'          XVIIIb'
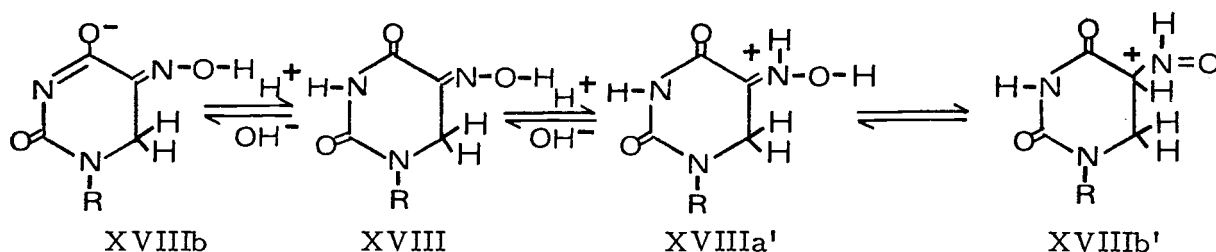
Studies on the Stability and Modification of 5-Hydroxyuridine

It has been suggested (85) that in RNA sequence studies en-

zymatic hydrolysis of all fragments and avoidance of acid and al-

kaline chromotographic systems is a necessity to be certain all

structurally unique nucleotides are obtained. These criteria were

established subsequent to a considerable amount of RNA sequence data. Alkaline hydrolysis has been employed by Holly (64), Zachau (144), and by other workers in this field to obtain the nucleotide composition of an oligonucleotide fragment. Other workers (52) have employed highly acidic and alkaline chromatographic systems for isolating minor nucleotides. Thus, these sequencing criteria reflect the ideal rather than procedures which have been routinely employed. Their justification stems from the observation that in the process of isolation a number of minor nucleosides are destroyed or modified by acid or alkali. These include dihydrouridine, 1-methyladenosine, $N6-\Delta^2$ isopentenyladenosine, and Y, and unknown, highly fluorescent nucleoside. Hall (52) has stated that the number of minor constituents of RNA known at present does not even closely approach the total number that actually exist, hence the importance of improving techniques for recovering progressively less stable constituents becomes a primary consideration.

A complicating factor in isolation is the problem that in conversion of intact macromolecules of RNA or DNA to their respective subunits by chemical and enzymatic procedures, conditionally stable constituents may be modified, perhaps even by an interaction of the components themselves. Since the original subunits each possess characteristic ultraviolet (UV) absorption, modification will either result in a change in their ultraviolet absorbance spectra or in loss of their absorbancy. The possibility for the latter modification, specifically with reference to pyrimidines, has perhaps not been fully appreciated. In the case of modified uridine derivatives this would involve saturation

of the C5-C6 double bond. Electron withdrawing substituents at C5 would lower the stability of this bond.

Naturally occurring saturated i. e. non-ultraviolet-absorbing minor components and other nonabsorbing derivatives of particularly labile minor nucleotides may be isolated together from biological sources. Determining their chemical identity requires techniques beyond characterization in chromatographic solvent systems and by nebulous low-UV, below 240 nm spectral data. Many of these compounds behave similarly under such conditions. Madison and Holley (1965) (86) in their initial isolation of 5, 6-dihydrouridine from "soluble" ribonucleic acid observed two other non-UV-absorbing materials which they did not identify. In subsequent experiments only about half of these materials were observed, thus they suggested they may have possibly been derived from dihydrouridylic acid during isolation. It is equally possible to state that they may have been other non-related dihydro derivatives because one gave a positive test for organic phosphate, and for a ureido group , but only after alkali treatment, indicating it was not a derivative of dihydrouridine in which the ring had been previously opened. The other compound did not test positively for ureido groups or organic phosphate indicating it could not likely have been derived from dihydrouridine.

Quantitative errors have been made in transfer RNA sequence studies involving the actual number of dihydrouridine residues present per given species (85). The number of moles of dihydrouridine is determined by pooling the suspected samples and dividing the

absorbance at 230 nm by either the extinction coefficient for di-

hydrouracil (8. 22x10$^3$) or that for dihydrouridine (approximately

10x10$^3$) (85), and then juggling the figures to account for losses

due to lability.  One severe criticism of this procedure is that

other non-UV-absorbing nucleic acid derivatives may also be present.

In addition, the major nucleic acid components, various salts and

reagents, and nucleic acid-related compounds, e. g. creatinine (84)

also absorb strongly at 230 nm and would obscure the significance

of the total absorbance at this wavelength.  Madison (1968) (85)

has stated that it is not impossible that a particularly labile nu-

cleotide could have been missed in the sequence studies which have

thus far been carried out.

Another type of nucleoside modification which may obscure

sequencing or isolation studies is exhibited by the thionucleosides,

since they can be oxidized or reduced.  The question of the behavior

of one of the sulfur containing bases 4-thiouridine and its disulfide

4, 4'-di(thiouridine) in conventional chemical hydrolytic procedures

for polyribonucleotide analysis was examined by Uziel (1966) (136).

He found that although 4-thiouridine was stable in 0.3 N NaOH for 13

hours at 37$^\circ$C the disulfide broke down into 10% uridine, 50% 4-

thiouridine and 40% compound X.  The absorbancy due to X disappear-

ed after 40 minutes at 30$^\circ$C in 0.3 N NaOH.  The loss of the absorbing

group could occur by ring-opening (33).  $Na_2S_2O_3$ has been found to

completely transform X into 4-thiouridine (26).  Uziel suggests that

the recovery of 4-thiouridine from alkaline hydrolysates does not un-

equivocally prove its presence in the parent molecule, especially since

other workers (80, 115) only obtained 50% recoveries of sulfur when

alkaline hydrolysates of tRNA were chromatographed on Dowex-1.

## Products Formed in the Reaction of Alkali with 5-Hydroxyuridine

Variations in heat, the extent of exposure, the concentration of hydroxyide ions, and the concentration of 5-hydroxyuridine lead to formation of a number of derivatives of the latter in alkali. These include:

(1.) Spectral concentrations, <u>i. e.</u> approximately $10^{-4}$ molar solutions of 5-hydroxyuridine, $5 \times 10^{-3}$ molar in $MgCl_2$, which are slightly alkaline yield a 5-hydroxyuridine derivative on autoclaving. The derivative possesses an absorbance maximum at 304 nm in neutral and alkaline solution but does not possess any absorbance at 280 nm on acidification.

(2.) In N KOH for one hour at 80°C, N/10 KOH for 20 minutes at 100°C, N/20 KOH for 40 minutes at 100°C and ten per cent piperidine for one hour at 100°C and one per cent piperidine for five hours at 100°C, at spectral concentrations, 5-hydroxyuridine is modified to a new substance termed Compound R. This material possesses an alkaline ultraviolet absorbance maximum at 307-308 nm and an acid absorbance maximum at 319-320 nm. Spectral reversibility suggests R is a unique stable derivative of 5-hydroxyuridine.

(3.) 100 X spectral concentrations of 5-hydroxyuridine in one or ten per cent piperidine at 100°C for 110 minutes yielded Compound P. This derivative possesses absorbance maxima at 263, 252, and 268 nm at pH 1, 7, and 14 respectively. Compound P possesses two pKa's at 3.1 and 11.9. Otter <u>et al.</u> (1969) (98) have recently found that 0.1 molar of either 2',3'-isopropylidine-5-hydroxyuridine or 5-hydroxyuridine

treated with either 0.1 N sodium hydroxide or unbuffered sodium bicarbonate at 100°C formed what has been termed here Compound P which they identified as 1-(β-D-ribofuranosyl)-2-oxo-4-imidazoline-4-carboxylic acid. The reaction conditions reported here to produce Compound P also result in the formation of a number of other 5-hydroxyuridine derivatives which have been tentatively identified, including: dihydrouracil, dihydrouridine, and an aldo-pentose positive, non-ribose sugar, by comparing their migration in ascending paper chromatography with control compounds.

(4.) Spectral concentrations of 5-hydroxyuridine in five per cent piperidine refluxed with bubbling at 100°C for 40 minutes facilitated the formation of Compound $H_{28}$. The latter exhibits a reversible absorbance spectra with an absorbance maximum in neutral and alkaline solution at 230 nm which disappears in acid, and characterizes two pKa's at 2.5 and 11.85. The material migrates very fast in ascending paper chromatography, an indication that perhaps it does not contain a bound sugar. Also present in the reaction, in addition to $H_{28}$ are presumably dihydrouracil, an unidentifiable dihydroderivative of 5-hydroxyuridine, and a sugar similar to the one obtained in the Compound P reaction mixture.

(5.) In the partial alkaline hydroxysis of 5-hydroxyuridine in 0.3 N KOH at 80°C for 10 minutes the principal product of 5-hydroxyuridine was Compound Z. This material migrated identically with that of a dihydrouracil control in four divergent ascending paper chromatographic solvent systems.

## Considerations of the effect of alkali on 5-Hydroxyuridine

Isopropylidine-5-hydroxyuridine, 5-hydroxyuridine, and 1-methyl-5-hydroxyuracil have been reported (98) to undergo base catalyzed rearrangements on refluxing in N/10 sodium hydroxide to 2-oxo-4-imidazoline-4-carboxylic acids. The reactions of 1,3-disubstituted 5-hydroxyuracils in sodium hydroxide were found by these workers (98) to differ from those of the 1-substituted compounds in that only small smounts of the corresponding imidazolines were formed directly. In the main, the 1,3-disubstituted 5-hydroxyuracils are slowly converted into non-ultraviolet absorbing intermediates which yield imidazolines after treatment with acid. Neither 5-hydroxyuracil nor 3-methyl-5-hydroxyuracil are converted under the same conditions into the corresponding imidazoline, either before or after acidification of the solutions. The reaction of those compounds which do form imidazolines have been reported (98) not to involve participation of a sugar hydroxyl group, or to proceed mechanistically via 5',6-anhydro a cyclic ureide intermediates as do the isopropylidene-5-halo-uridines(97). The following scheme, proposed by Otter et al. (1969) (98) shows the ring contraction of 5-hydroxyuracil derivatives as a benzylic acid type of rearrangement.
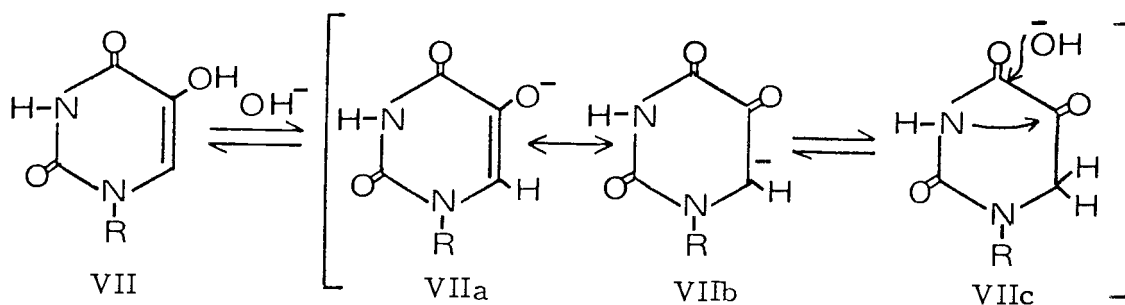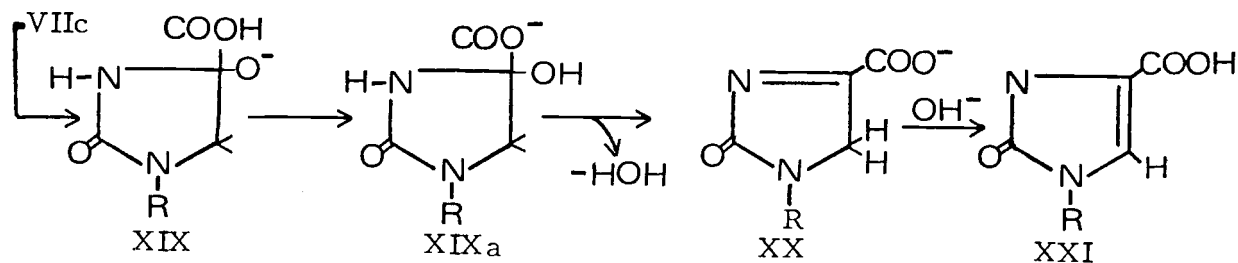
R= methyl, ribosyl, or 2',3'-0-isopropylidene-ribosyl groups

Either attack of hydroxide ion on C-5 of VIIc followed by migration of the C-5, C-6 bond, or attack on C-4 of VIIc and subsequent migration of the C-N bond, as written, would give intermediate XIX, which would undergo a proton shift to give the imidazoline XIXa. Base-catalyzed dehydration involving abstraction of the labile N-3 proton of XIXa would give the 2-oxoisoimidazolines, XX, which could rearrange to give the 1-substituted imidazolines XXIa (98).

The above rearrangement is similar to the benzylic acid rearrangement of isotopically labeled alloxan to alloxanic acid which has been shown (97) to involve exclusive C-N bond cleavage to the exclusion of a carbon shift. The rearrangement of alloxan and several of its derivatives to alloxanic acid and thence to parabanic acid occurs under widely differing conditions of pH.

XIV

alloxan

XXIII

alloxanic
acid

XXIV

parabanic
acid
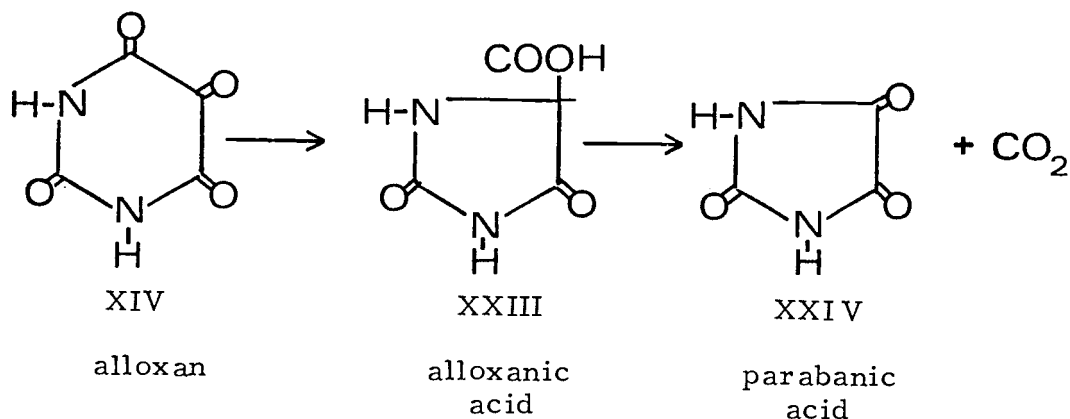
A prerequisite for the benzylic acid type of rearr-
angement of derivatives of 5-hydroxyuracil just considered
is that the        5-hydroxyuracils exist partly in the tautomeric
5-keto form VIIc. Otter et al. (1969) (98) have shown by deuterium
exchange studies that species VIIc exists under alkaline conditions
in which the 5-hydroxyl group is ionized. Deuterium exchange at
H-6 has been shown by a gradual decrease in the intensity of the
H-6 resonance of isopropylidene-5-hydroxyuridine relative to the
nuclear magnetic resonance signals of protons which did not under-
go exchange. They showed that exchange does not take place when
the enolate ion-keto equilibrium VIIa-VIIb--➤VIIc is precluded by
substitution of the 5-hydroxyl proton with a benzoyl group. It was
found that the rate of exchange of H-6 for deuterium was much
more rapid for isopropylidene-5-hydroxyuridine than for 1, 3-dimethyl-
5-hydroxyuracil. The former only undergoes 30 per cent exchange in
sodium deuteroxide during the complete deuteration of the latter.
These workers commented that the slower rate of exchange observed
for 1, 3-dimethyl-5-hydroxyuracil was consistent with the slower
rate at which it underwent ring contraction.

Nature of the Chemical Reactivity of 5-Hydroxyuridine in Alkali

It has been shown in this study that 5-hydroxyuridine does not gradually "rearrange" under basic conditions to a non-ultraviolet-absorbing product because of any inherent instability of the dianionic form of the molecule. 5-Hydroxyuridine is stable in five per cent triethylamine at $78^{\circ}$C in an aprotic solvent such as anhydrous diglyme, in which hydroxyl ions cannot be formed. The loss in absorbance of 5-hydroxyuridine in basic solution has been shown to be due to a fairly general mechanism since in aqueous piperidine, triethylamine, and in KOH or NAOH solutions absorbance is rapidly but not totally lost. Under each of these conditions an ultraviolet absorbing derivative, Compound R slowly begins forming. The loss of absorbance of 5-hydroxyuridine in basic solution requires the nucleophilic addition of an hydroxyl anion to the molecule, because when hydroxyl ions are excluded from a basic medium no loss of absorption occurs.

The actual loss of absorbance of the molecule may be due to attack of alkali at C-4 with consequent opening of the ring between N-3 and C-4 or, it may simply represent the nucleophilic addition of hydroxyide ion to the C-5, C-6 double bond. In general unsaturated pyrimidines are relatively resistant to ring opening at C-4, whereas dihydropyrimidines readily undergo such an amide hydrolysis.

Figure 65 compares the stability of isobarbituric acid and 5-hydroxy-2'-deoxyuridine in KOH, pH 13 at $50^{\circ}$C with 5-hydroxyuridine and with methylated derivatives of the latter. These two

compounds as well as 5-hydroxyuridine a[16], 3-methyl-5-hydroxy-uridine and 5-hydroxyuridine all rapidly lost absorbance. The order of decreasing reactivity was: isobarbituric acid>> (more rapidly loses absorbance than) 5-hydroxyuridine a > 5-hydroxyuridine > 5-hydroxydeoxyuridine > 3-methyl-5-hydroxyuridine. Under the same conditions 3-methyl-5-methoxyuridine and 3-methyl-5-meth-oxy-(2'-deoxy)-uridine rapidly increased in extinction by 120 and 160 per cent respectively, whereas 5-methoxyuridine neither gained nor lost absorbance. It may be concluded from these observations that the N-3, C-4 bond of 5-methoxy-uridine is resistant to alkaline amide hydrolysis and its C-5, C-6 double bond is resistant to the addition of water or attack by hydroxide ions. When the treated solution of 5-methoxyuridine was acidified after 20 hours of reaction the typical acid absorbance spectrum was obtained. Subsequent alkalinisation of the solution resulted in an immediate enormous 2X gain in the extinction at the characteristic absorbance maximum. The tremendous gain in extinction is very similar to the increase in extinction of e. g. 3-methyl-5-methoxyuridine in alkali and it may be concluded that the reaction mechanism for each substance is analogous. The mechanism does not represent a ring opening but does involve the participation of the hydroxide ion.
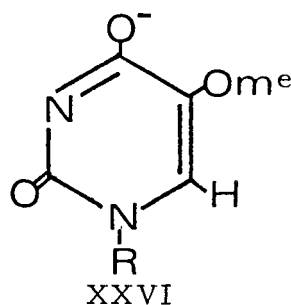
The basic difference between the latter two molecules in al-kaline solution is that 3-methyl-5-methoxyuridine retains a C-4 carbonyl whereas 5-methoxyuridine probably possesses an anionic

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[16] See Results section: Preparation, Absorbance and Ionization Characteristics of Methylated Derivatives of 5-Hydroxyuridine.

C-4 enol:

XXV          vs.          XXVI
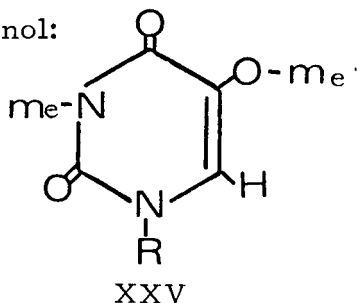
R= ribose;   me= CH$_3$

There is a greater increase in extinction of 3-methyl-5-hydroxy-(2'-methoxy)-uridine than for 3-methyl-5-methoxyuridine in alkali, perhaps reflecting greater reactivity of the former. 3-Methyl-5-hydroxy-(2'-methoxy)-uridine does not possess an anionic charge in alkali because all its dissociable hydroxyls have been methylated, whereas the ribose of 3-methyl-5-methoxyuridine can acquire an anionic charge in strong alkali. The proximity and strength of the electron donating hydroxyl anion at C-4 may obviate addition of alkali to the C-5, C-6 double bond in 5-methoxyuridine. Dissociation of the ribose hydroxyl of 3-methyl-5-methoxyuridine should increase the electron donating character of this substituent and restrict addition to the double bond in comparison to the reactivity of 3-methyl-5-methoxy-(2'-deoxy)-uridine.

It may be speculated, though without evidence, that addition of hydroxide ion to these molecules occurs at the C-5, C-6 double bond at position C-6. Addition at C-6 results in the formation of a resonant "common anion" in which one of the forms carries the charge on the ring, whereas addition at either C-4 or C-5 does not permit formation of a resonant ion.
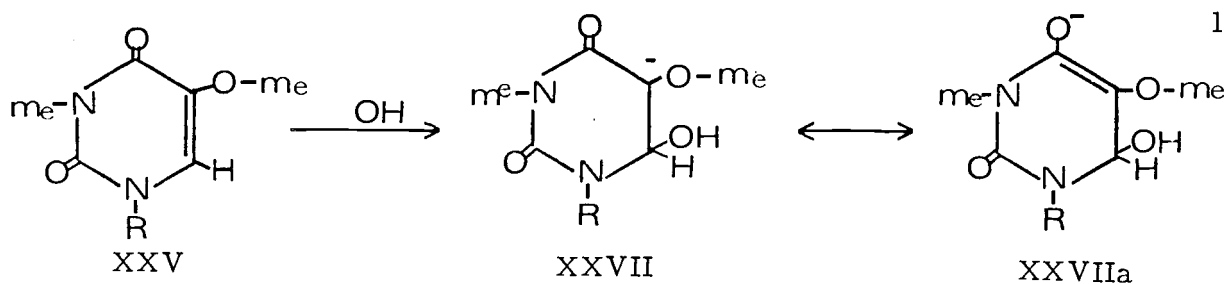
R= ribose; me= CH$_3$

A similar resonant "common-ion" structure in which the negative

charge can be placed on a ring carbon has been proposed by Fox

and Shugar (1952)[17] to account for the high extinction, i. e. approx-

imately 20,000, of the barbituric acid anion.
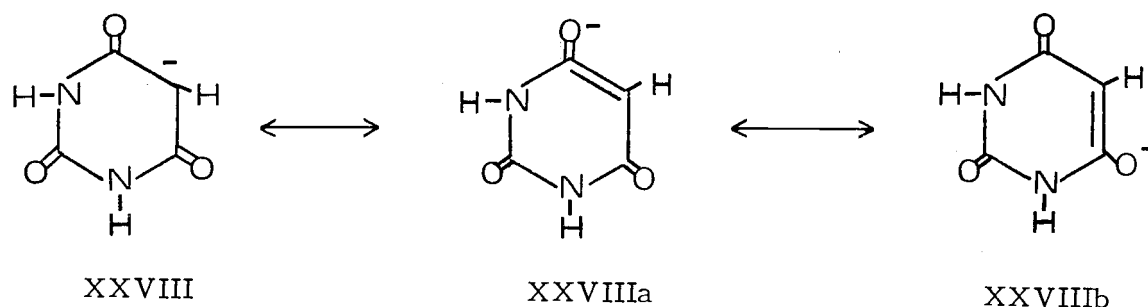


What makes such a resonance hybrid unique is that the anionic charge

can be transferred to a ring carbon whereas the resonant anionic

charge in molecules as for example uridine can only be transferred

between the hydroxyls and ring nitrogen. The extinction of the alkali

treated methylated 5-methoxyuridine products are about 16,000-

20,000 at their absorbance maxima, or around two to two and a half

times the extinction of the untreated derivatives.

17
    Fox, J. J. and D. Shugar. Absorption spectra and structure of bar-
bituric acid derivatives as a function of pH. Communique a la Societe
Chimique de Belgique 61: 44-63. 1952. As far as could be found this
is the only publication which postulates why certain nucleosides possess
extinctions double or triple those of the standard ribosides and deoxy-
ribosides.

The nature of the reactivity of the methylated derivatives of 5-hydroxyuridine in alkali strongly suggests that the loss of absorbance of 5-hydroxyuridine in alkali requires the dissociation of its C-5 hydroxyl proton and formation of the resonance hybrid:
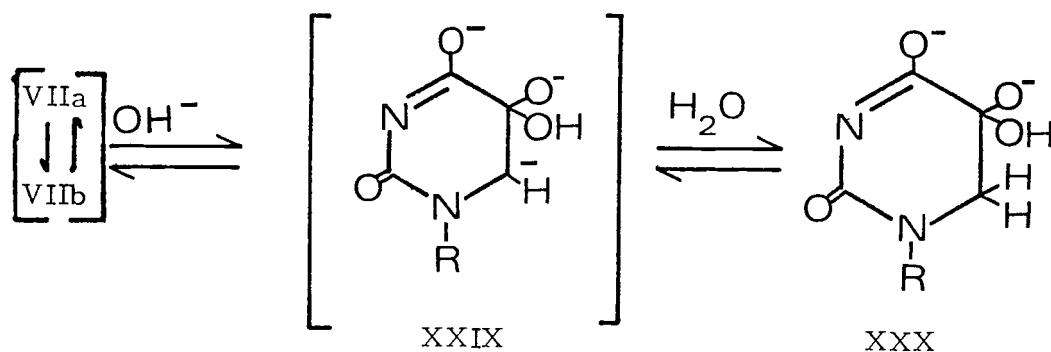


R= ribose
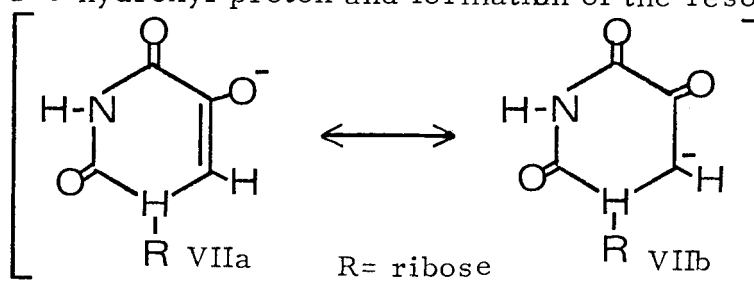
Otter et al. (1969) (98), as indicated above, proposed that 5-hydroxyuridine undergoes a benzylic acid type of rearrangement on treatment with alkali to give an 1-(β-D-ribofuranosyl)-2-oxo-4-imidazoline-4-carboxylic acid. Attack by hydroxide ion at either C-4 or C-5 gives the same imidazoline product.

There are indications from the reaction of 5-hydroxyuridine in alkali that a derivative is formed with an absorbance maximum in alkaline solution at 304 nm. Since this is approximately the absorbance maximum of 5-hydroxyuridine in alkali, measurement of the loss of absorbance at this wave length will not give an accurate picture of the extent of reaction. Perhaps this is why Otter et al. (98) reported that 5-hydroxyuridine rapidly lost absorbance at 55°C in N/10 NaOH but was stable to N NaOH. They found that when isopropylidene-5-bromouridine was heated in N NaOH at 55°C for 20 hours there was a gradual increase in the intensity of the ultraviolet absorbance at approximately 305 nm. They reasoned that since isopropylidine-5-hydroxyuridine is formed from isopropylidine-5-bromouridine that an increase in absorbance at 305 nm in-

dicated 5-hydroxyuridine was stable under these conditions.

The results presented here, however, have clearly shown 5-hydroxyuridine is unstable under similar reaction conditions. Treated solutions lose absorbance slightly at 303 nm and then gradually increase in absorbance at 307 nm. This increase is due to the formation of Compound R, and not to any stability of 5-hydroxyuridine. Acidification of the solutions absorbing at 304 nm, which dropped slightly in absorbance, reveals that such reaction mixtures do not exhibit absorbance at 280 nm characteristic of 5-hydroxyuridine, although they still show an absorbance maximum in alkali at about 304 nm. Acidification of the same solutions which are gradually gaining absorbance around 304-309 nm reveals an absorbance peak at 320 nm, characteristic of Compound R.

It is felt that the derivative of 5-hydroxyuridine which absorbs in alkali at about 304 nm but does not possess absorbance at 280 nm is the principal reaction intermediate which results from the treatment of 5-hydroxyuridine with alkali. Since the compound retains its absorbance maxima at 304 nm it is assumed the ring is still intact. Such a derivative may be seen in the spectra in Figures 45, 47, and 56. The ability of 5-hydroxyuridine to form the resonance hybrid indicated above precludes nucleophilic attack at C-6. Since attack at C-4 will result in rupturing the ring it is postulated that the initial loss of absorbance of 5-hydroxyuridine in alkali is due to the nucleophilic addition of hydroxide ion, according to Markownikoff's rule to C-5 of the C-5, C-6 double bond.

R= ribose

It is possible that XXX is the component noted in alkaline reaction mixtures which absorbs at 304 nm but does not absorb at 280 nm on acidification of the solution. Such an intermediate could undergo a number of different reactions and account for the diversity of products formed by the action of alkali on 5-hydroxy-uridine.

Compound P, the imidazoline which was also found by Otter et al. (1969) (98) can be formed through amide hydrolysis of the N-3, C-4 bond of XXX, opening the ring of the unsaturated compound.
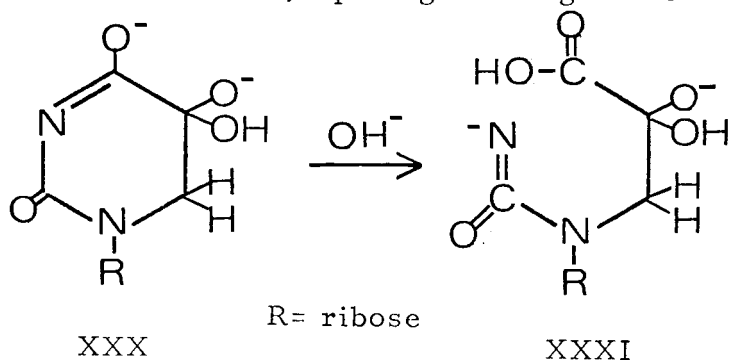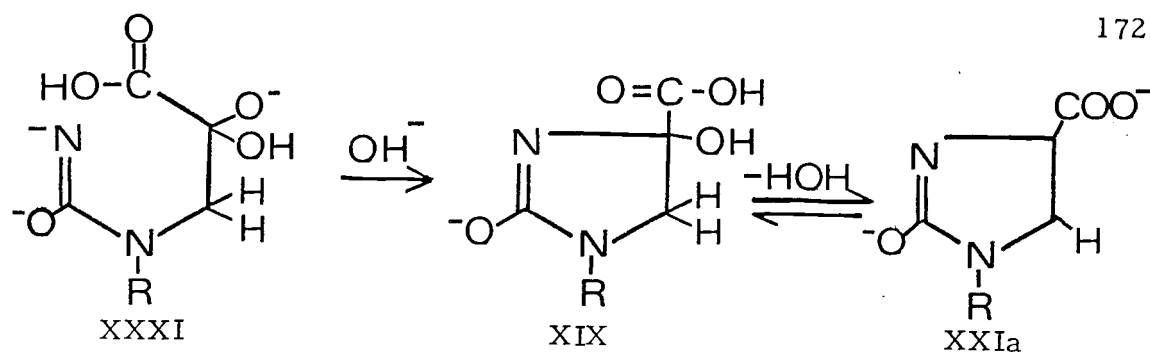


R= ribose

XXX                    XXXI

Ring closure of XXXI with elimination of one of the hydroxyls would give XIX an intermediate proposed by Otter et al. (98) which could rearrange with the elimination of water to give 1-(β-D-ribofuranosyl)-2-oxo-4-imidazoline-4-carboxylic acid, XXIa.

$$\text{XXXI} \xrightarrow{\text{OH}^-} \text{XIX} \xrightleftharpoons{-\text{HOH}} \text{XXIa}$$

XXXI    XIX    XXIa

R= ribose

There is not much difference between this mechanism and the benzylic acid rearrangement proposed by Otter et al. (98) because VIId and XXX

VIId    XXX
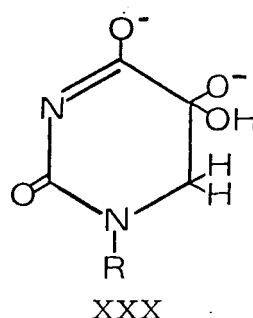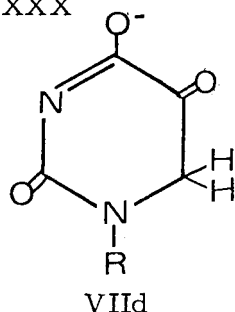
are chemically similar. The latter structure may not undergo as facile a benzylic acid rearrangement as when the hydroxide ion directly attacks C-4.

Figure 58 shows that a component of reaction mixtures $RI_{26}$ and $RI_{28}$, from which Compounds $H_{28}$ and P were respectively isolated, reacts with cytidine-5-3H. These reaction mixtures had not been neutralized with acid but were concentrated and washed with distilled water under vacuum as described in Methods, until they no longer contained piperidine. The small amount of unreacted 5-hydroxyuridine present in distilled water solutions of the concentrated reaction mixtures at pH 6.2 could not react with cytidine since dissociation of the C-5 hydroxyl proton does not occur at this

pH.

In Figure 57 it may be observed that the 304 nm absorbing substance reacts with ammonia and disappears whereas the absorbance of Compound P was unaffected. It is assumed that an unstable 304 nm absorbing derivative of 5-hydroxyuridine, formed in alkali, postulated to be X, was the species present in reaction mixtures $RI_{26}$ and $RI_{28}$ which reacted with cytidine-5-3H. Presumably such a derivative of 5-hydroxyuridine can also be formed under biological isolation conditions.

Alloxan, alloxantin-$2H_2O$ and dialuric acid are shown in Figure 58 to react with cytidine-5-3H. Alloxan as well as ninhydrin form structures analogous to XXX which react readily with amines, e. g. reaction of alloxan with an amino acid has been postulated to occur as follows (50a).



$$RCHO + CO_2 + \quad XXXIII$$
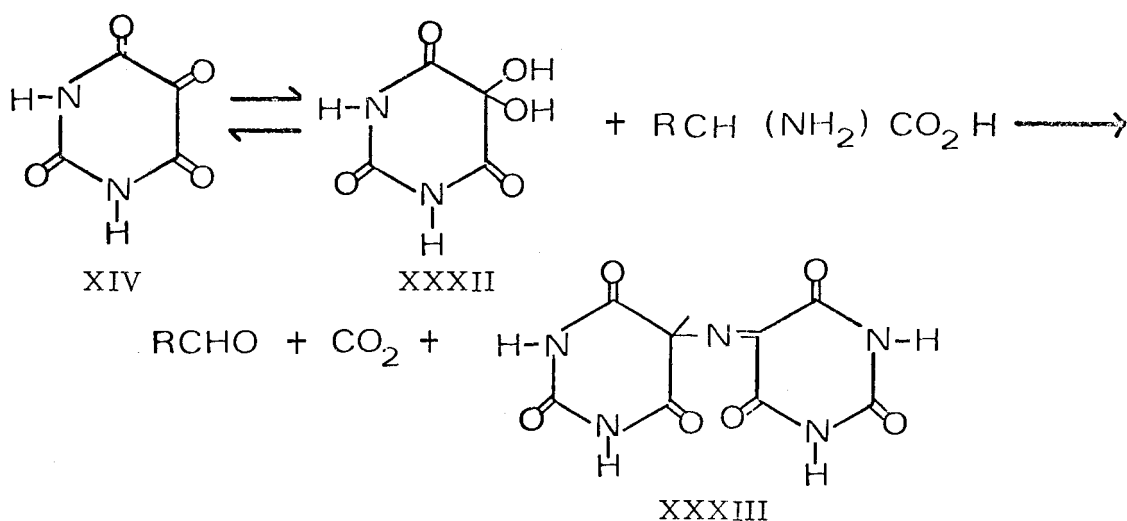
It is not realistic to postulate reaction mechanisms for the formation of several of the other alkaline derivatives of 5-hydroxyuridine as Compound R and $H_{28}$, since their structures have not been established.

## 5-Hydroxyuridine as a Component of RNA

The initial communication on the isolation of 5-hydroxy-uridine from yeast hydrolysate (83) did not establish the level of the component present, its potential for formation during isolation, or whether or not it was a cell product. These workers found that commercial uridine purchased from Schwarz Bio Research, Inc. Lot UN 5801 also contained 5-hydroxyuridine present at a level of 0.69 per cent. The significance of its presence as a contaminant of the commercial uridine preparation was not explained. The work attempted here has explored some of these questions.

The chemical synthesis of 5-hydroxyuridine-2-14C was obtained by heating highly labeled uridine-2-14C of high specific activity, mixed with chromatographically pure uridine carrier, in saturated $Br_2$-water solution over a boiling water bath as described in Methods. The crystallized material was purified by elution through Dowex-1-formate, recrystallization and paper chromatography. The purified preparation did not contain traces of labeled or absorbing contaminants.

Commercial preparation of uridine involves digesting yeast, usually Torula sp., RNA in 1 N NaOH at room temperature.[17] Bromine is not used commercially in the preparation of uridine,[17] nor have any of the RNA isolation techniques reported herein

17. Personal communication. A.W. Lis, Associate Professor Obstetrics and Gynecology, Univ. Oregon Medical School, Portland, Oregon; and Schwarz Bio Research, Inc. New York, New York.

been contaminated with it. The presence of 5-hydroxyuridine
in preparations of commercial uridine thus cannot be attribu-
table to bromine contamination. Aside from ionizing radiation,
bromine is the only oxidant known to participate in the synthesis
of 5-hydroxyuridine from uridine. The involvement of an un-
known oxidant participating in this reaction seems unlikely. The
possible formation of 5-hydroxyuridine from uridine under alkaline
hydrolytic conditions was investigated. A comparison of the par-
tial alkaline hydrolysis products of uridine, 5-hydroxyuridine
and dihydrouridine are shown in Figures 66-69 and in Table 17.
The data reveal a number things, but of importance here is the
observation that subjecting uridine to alkaline hydrolysis does
not produce even traces of 5-hydroxyuridine, although the latter
becomes extensively altered. It could be argued then that there
is a far greater potential for possible loss of biologically formed
5-hydroxyuridine during commercial isolation of uridine from
yeast RNA than for its formation. The possibility that 5-hydroxy-
uridine could be formed from a particularly labile minor nucleo-
tide present in RNA during alkaline hydrolysis cannot be ruled
out. A method of celite partition chromatography has been
found to separate uridine from 5-hydroxyuridine, Figure 20.

5-Hydroxyuridine has been considered a dynamic metabolite
of the yeast from which it was isolated (83). Clues to the function
and metabolism of 5-hydroxyuridine may be obtained by under-
standing how it is present in the cell. For example, if 5-hydroxy-
uridine exists as a component of RNA it should be obtainable by

nucleic acid hydrolysis. De novo synthesis, as occurs with

standard nucleotides, should be reflected by its presence in an

acid soluble fraction of the cell sap. Such clues would also con-

tribute toward understanding the intra-cellular functionality of

the RNA's, since the functionality of the RNA's must necessarily

equate with the metabolism of their minor components and the

regulation of active site interposition or codacilation into the RNA's.

5-Hydroxyuridine was not found in leachings from washed Candida

utilis cells, in the dialysates of ground cells, or in crude RNA

preparation dialysates. These results suggest that 5-hydroxy-

uridine is not formed de novo but is synthesized by enzymatic

modification of pyrimidines existing in 3',5'-phosphodiester

linkages in RNA; they are compatible with current theories

relating to biosynthesis of the minor components of transfer RNA.

Quantitization of the amount of 5-hydroxyuridine present

in yeast RNA was attempted by isotope dilution analysis on enzy-

matically prepared nucleosides from yeast RNA spiked with 5-

hydroxyuridine-2-14C. Fractionation was attempted by celite

partition chromatography. This was attempted because large

samples, i. e. greater than approximately 50 milligrams, of

nucleoside mixtures cannot practically be separated by column

chromatography on Dowex-1-formate, whereas as much as five

grams of nucleosides can be fractionated in one run on the celite

column employed. Practice column chromatography on celite

of 5-hydroxyuridine mixed with standard nucleosides had revealed,

Figure 19, that a small fraction of 5-hydroxyuridine was eluted

with the cytidine, guanosine fraction, whereas most of it was held up and could, i. e., at least the label could be removed from the column by washing it with water. It was felt this was not undesirable as the label could be separated from the standard nucleosides. When the spiked RNA digest was chromatographed it was found that the label complexed on the celite column and was not obtained throughout the full elution schedule but 89 per cent was recovered as a sharp peak in the first 425 ml of water wash. On submission of the labeled peak obtained, to paper chromatography in butanol-$H_2O$ (86:14), Figure 25, it was observed that the label migrated in two bands, one containing about twice the activity of the other, and neither paralleled the control. This was the first indication that the label was not behaving as 5-hydroxyuridine. In control experiments it was shown 5-hydroxyuridine-2-14C does not complex with standard mixtures of nucleosides and nucleotides, but it was altered however when added to a concentrated aliquot of the C14-labeled peak obtained from celite chromatography, and migrated in two labeled bands as above. It was concluded that 5-hydroxyuridine was either forming one or more molecular complexes or undergoing multiple chemical alteration or both.

Thus the labeled 5-hydroxyuridine-2-14C, which had been presumed would remain stable throughout the analysis could not be isolated intact. I was faced with abandoning a technique which had taken months to develop. It has been pointed out that with the partition column chromatographic method employed (52) one can

successfully recover 22 major and minor components as well as one of the three components from transfer RNA known to re-arrange. Since the isolation of 5-hydroxyuridine from RNA has not been confirmed, and since I was unable to isolate any quantity of 5-hydroxyuridine from a yeast nucleoside mixture to which it had been added, it was felt worthwhile to find out what had modified 5-hydroxyuridine and what were the products after modification.

The concentrated labeled peak from celite partition chromatography was accordingly examined in relation to 5-hydroxyuridine control by column chromatography on Dowex-1-formate, paper chromatography employing differential solvents, paper electrophoresis, ultraviolet spectroscopy, acid hydrolysis, and by various chemical spray tests. The label could be separated into three different labeled components: C73-74Aa was stable in acid, possessed an absorbance maximum at 272-274 nm in acid or distilled water, flocculated in alkali, and was probably a nucleophilic addition product of 5-hydroxyuridine. The latter conclusion is based on the similarity of its spectra to those of the reaction mixtures of 5-hydroxyuridine with hydroxylamine. C73-74Ab was a non-UV-absorbing derivative which possessed an Rf in butanol-$H_2O$-ethanol (5:2:3) similar to that of dihydro-uracil. C21b = Compound A was found to be a complex between cytidine, a dihydro-type material: Compound B, and a spectrally unique substance: Compound D. Compound A gave negative tests for dihydropyrimidine or β-ureido acid, β-amino acid, or for a

reducing sugar. Its inability to bind borate cannot be explained, since it appears to contain an intact ribose on cytidine. This result suggests the complex did not contain exposed vicinal hydroxyl groups. Presumably the 2',3'-hydroxyls of the ribose are tied up or complexed in some fashion. Compound B gives a positive test for dihydropyrimidine or β-ureido acid and negative tests for the presence of vicinal hydroxyls and a phenolic hydroxyl as the 5-hydroxyl of 5-hydroxyuridine. Compound B has been analyzed by high resolution mass spectroscopy and appears to be dihydrouracil or at least a very similar substance. It is impossible to make unqualified conclusions because of the small quantities of material purified for analysis. These materials were all formed under biological isolation conditions and were not available in quantities generally employed in routine organic analysis. Compound B was also formed in small amounts by treating 5-hydroxyuridine with ammonia or potassium permanganate and was the major product termed Compound Z of the partial alkaline hydrolysis of the latter.

Isotopic dilution, in terms of molar specific activity, of the C-14 label from the 5-hydroxyuridine-2-14C spike, into Compound B, was calculated by estimating the molecular weight of Compound B. These calculations were based upon the assumption that unlabeled 5-hydroxyuridine nucleosides present in ribosomal RNA were modified in the same manner and extent as the 5-hydroxyuridine-2-14C spike, and suggested that about four per cent of the ribosomal bases in ribosomal RNA are 5-hydroxyuridine. Such a value is hard to rationalize but nevertheless positively

suggests that 5-hydroxyuridine must be considered a minor constituent of RNA. Only one group (83) has isolated 5-hydroxy-uridine from RNA. Because, as reported in this work, even when 5-hydroxyuridine was added to an enzymatic digest of RNA it could still not be isolated unmodified; it is not hard to explain why the compound has not been found by other workers in the field, even if present in such an apparently large percentage of the ribosomal RNA bases.

The stage of alteration of 5-hydroxyuridine and formation of artifacts, Compounds A and B by the biological fractionation conditions employed were then investigated. Potential modification of 5-hydroxyuridine by the yeast RNA extraction procedure, by residual unappreciated enzymatic activity of the RNA hydrolysate, or through chemical reactivity with standard RNA components were for the most part experimentally ruled out. Examination of the partition column chromatographic solvents revealed, however, that exposure to upper solvent G, ethyl-acetate-1-butanol-ligroin (bp. 66-75°C)-water (1:2:1:1) significantly reduced the ultraviolet absorbance of 5-hydroxyuridine at 293.5 nm, thus the latter was modified on the partition column. The derivative formed, perhaps C73-74Aa, was apparently much more chemically reactive than 5-hydroxyuridine and favored reaction with cytidine. The complex formed, Compound A, appeared homogeneous toward a number of nucleoside separation techniques. These findings stimulated experiments to determine the stability of 5-hydroxyuridine under RNA chemical hydrolytic procedures.

## SUMMARY

This study involved an investigative characterization of physical and chemical properties of isobarbituridine ( iB, equals 5-hydroxyuridine ). iB has been considered a potential dynamic cellular product subsequent to its isolation (83) from yeast RNA hydrolysates. Earlier workers employed iB as an antimetabolite for controlling malignancies, considering it analogous to the 5-halo analogs of uracil and without potential as a natural cellular constituent. However, regardless of the molecules metabolic or antimetabolic activity the biological implications of its origin as well as the functional potential of a hydroxyl substituent at position five of a pyrimidine base necessitated a more fundamental study of the relationship of iB to yeast RNA, as well as of its potential for being formed or modified under standard procedures for isolation, hydrolysis, and component fractionation of RNA.

Various chemical characteristics of iB not reported in the literature were investigated. The ultraviolet absorption spectra were examined. Pka's of 7.83 for the 5-hydroxyl proton, 10.57 for the N-3 proton, and 11.7, were determined from spectral data. Assignments were made by studying the spectra as well as physical , chemical, and ionic properties of prepared methylated derivatives of iB including: 3-methyl-5-hydroxyuridine, 5-methoxyuridine, 3-methyl-5-methoxyuridine, 3-methyl-5-methoxy-(2'-methoxy)-uridine and the ionization characteristics of iso-barbituric acid and 5-hydroxy-(2'-deoxy)-uridine. In the course

of these studies another form of 5-hydroxyuridine, termed 5-hydroxyuridine a was demonstrated which possesses a pKa for the 5-hydroxyl proton of 8.18 but otherwise appeared unaltered. These two forms of iB can be identified by their spectra as well as the dissociation characteristics of their C-5 hydroxyl proton. Since β-nucleosides give positive cotton effects it was assumed that the form with a pKa of 8.18 might be an $\alpha$-nucleoside. The optical rotatory dispersion curve of 5-hydroxyuridine a was not obtained, thus such an hypothesis is unproven.

Studies of the ionic character of iB contributed to an understanding of its chemistry and provided insite to mechanisms of potential mutagenic or antimetabolic activity. The chemical effect of the C-5 hydroxyl is opposite to that of C-5 halo substituents, and it was concluded, if iB, incorporated into DNA, is mutagenic at physiological pH, then such an activity must be by another mechanism than that of misparing, at least as proposed for 5-halo derivatives as 5-bromouracil. Spectral studies in which iB has been considered a model compound have resulted in an hypothesis for predicting whether or not a pyrimidine will exhibit a spectral bathochromic shift on ionization. Spectral approaches have also been used to show that ionized iB binds $Mg^{2+}$ resulting in an alteration of its spectra. An hypothesis of the potential biological utility of this reaction was suggested.

Methods of column chromatographic analysis for the separation of iB from other nucleic acid components were explored in order to quantitatively assay cellular RNA fractions for its presence.

Confirmation of the presence of iB in commercial uridine preparations was obtained. Yeast nucleotide pools and ribonucleic acid analyses were conducted by paper chromatography, electrophoresis, and celite partition column chromatography. Isotopic dilution analysis at the nucleoside level, employing partition chromatography was carried out on yeast ribosomal RNA preparations spiked with iB-2-14C. The latter could not be isolated intact but the recovered label could be separated into three different species: C73-74Aa, an unstable substance with an absorbance maximum at 272-274 nm; C73-74Ab, a non-absorbing derivative with an Rf similar to dihydrouracil; and Compound A, an easily disrupted complex between cytidine, a dihydro-type material-Compound B, and a spectrally unique substance- Compound D. Compound B, which is probably the same as C73-74Ab, has been analyzed by high resolution mass spectroscopy and was tentatively identified as dihydrouracil. Compound B was also formed in small amounts by treating iB with ammonia or KMnO_4 and was the major product, termed Compound Z, formed when iB was exposed to mild alkaline hydrolytic conditions. Migration of Compound B and this product of the reactions, on paper chromatography, was identical to dihydrouracil in diverse solvent systems. It was concluded from these studies that iB was capable of forming one or more molecular complexes or undergoing chemical alteration or both under conditions in which neither heat nor pH extreme were encountered. On the assumption that unlabeled iB present in the above ribosomal RNA hydrolysate was modified in the same manner and extent as the spike iB-2-14C, calculation

of isotopic dilution suggested that about four per cent of ribosomal RNA bases are iB.

Alteration of iB by the RNA component fractionation procedure was examined. iB was found to be modified on exposure to one of the partition column chromatographic solvent systems employed in the procedure for fractionating component RNA nucleosides. These findings stimulated experiments to determine how the molecule is modified under present isolation conditions and to determine its stability to RNA chemical hydrolytic procedures. Preliminary studies suggested that the stability of the molecule, measured in terms of loss of ultraviolet absorbance depended upon whether or not the 5-hydroxyl proton had dissociated, but was also related to additional factors than merely pH as for example the type of nucleophile present. iB was shown to be unstable in weakly alkaline heated phenol solutions, to autoclaving, to six routine RNA alkaline hydrolytic procedures, ammonia, ammonium carbonate at pH 9.0, and hydroxylamine at and above pH 8.0, whereas, the molecule was stable to acid hydrolysis. RNA isolation or fractionation techniques employing any of these reagents would be expected to modify iB present. Under alkaline hydrolytic conditions iB was modified to an unidentified, previously undescribed substance, termed Compound R, with an acid absorbance maximum at 320 nm and an alkaline maximum at 307 nm. Reaction with hydroxylamine results in an unstable substance with an alkaline absorbance maximum at 272-274 nm with loss of absorbance on acidification. A mechanism for the reaction of iB with hydroxylamine was postulated.

Five products are formed in the reaction of iB with alkali: (a) a derivative which possesses an absorbance maximum at 304-307 nm in neutral and alkaline solution but does not possess any absorbance at 280 nm on acidification; (b) Compound R; (c) a dihydro-derivative, Compound Z which migrates identically with that of dihydro-uracil; (d) Compound $H_{2 8}$, an undescribed substance with a reversible absorbance spectra possessing a maximum in neutral and alkaline solution at 230 nm which characterizes pka's at 2.5 and 11.85; and (e) Compound P possessing absorbance maxima at 263, 252, and 268 nm at pH 1, 7, and 14 respectively. Its spectra describe pka's at 3.1 and 11.9. In a very recent study (98) on the stability of iB in alkali this was the only derivative of iB sited and was identified as 1-(β-D-ribofuranosyl)-2-oxo-4-imidazolone-4-carboxylic acid.

The nature of the chemical reactivity of iB in alkali was studied . It was shown that the loss of absorbance of iB in basic solution requires nucleophilic addition of an hydroxyl anion to the molecule and is not the result of any inherent rearrangement of the dianionic form of the molecule. Product (a) above of the reaction of iB with alkali reacts with ammonia and is perhaps the component present in alkaline reaction mixtures which complexes readily with cytidine at pH 6.2. This substance may be the common initial product of the reaction of iB with hydroxyl ions.

It was concluded that quantitative isolation of intact iB would not be possible at or above a neutral pH or in the presence of reactive nucleophiles from biological material. There is a far greater potential for loss of iB during isolation than for its formation.

Fig. 1. The absorbance spectra of 5-hydroxyuridine. The spectral curves for solutions at pH 5.4 ( --- ), 7.0 ( ...-... ), 8.0 (.......), 8.7 ( ..-..- ), 9.5 ( .-.-. ), 10.5 ( ——— ), and 11.5 ( 1-1-1 ) are shown along with isosbestic points a, b, and c. The structure of 5-hydroxyuridine numbered as indicated is shown.
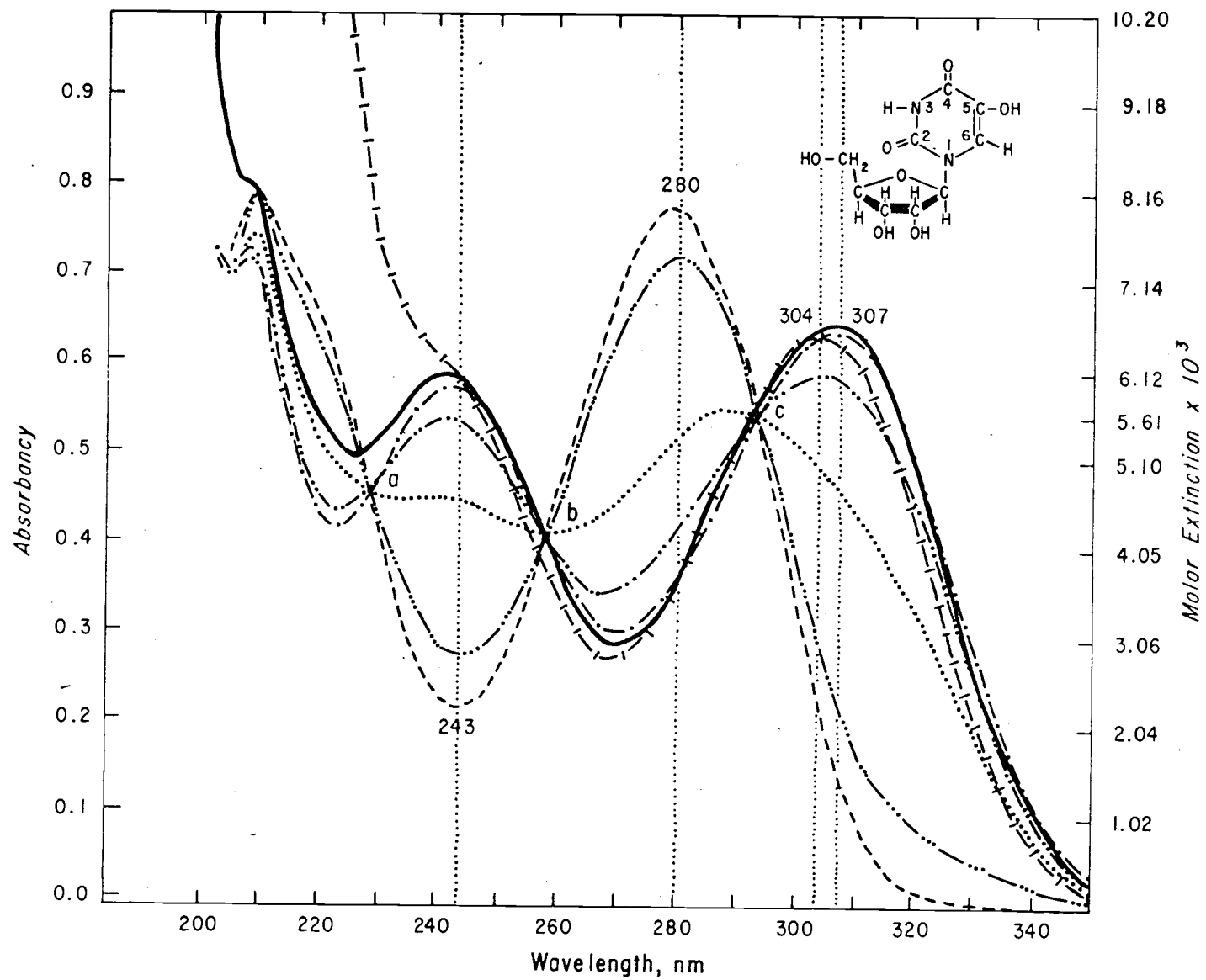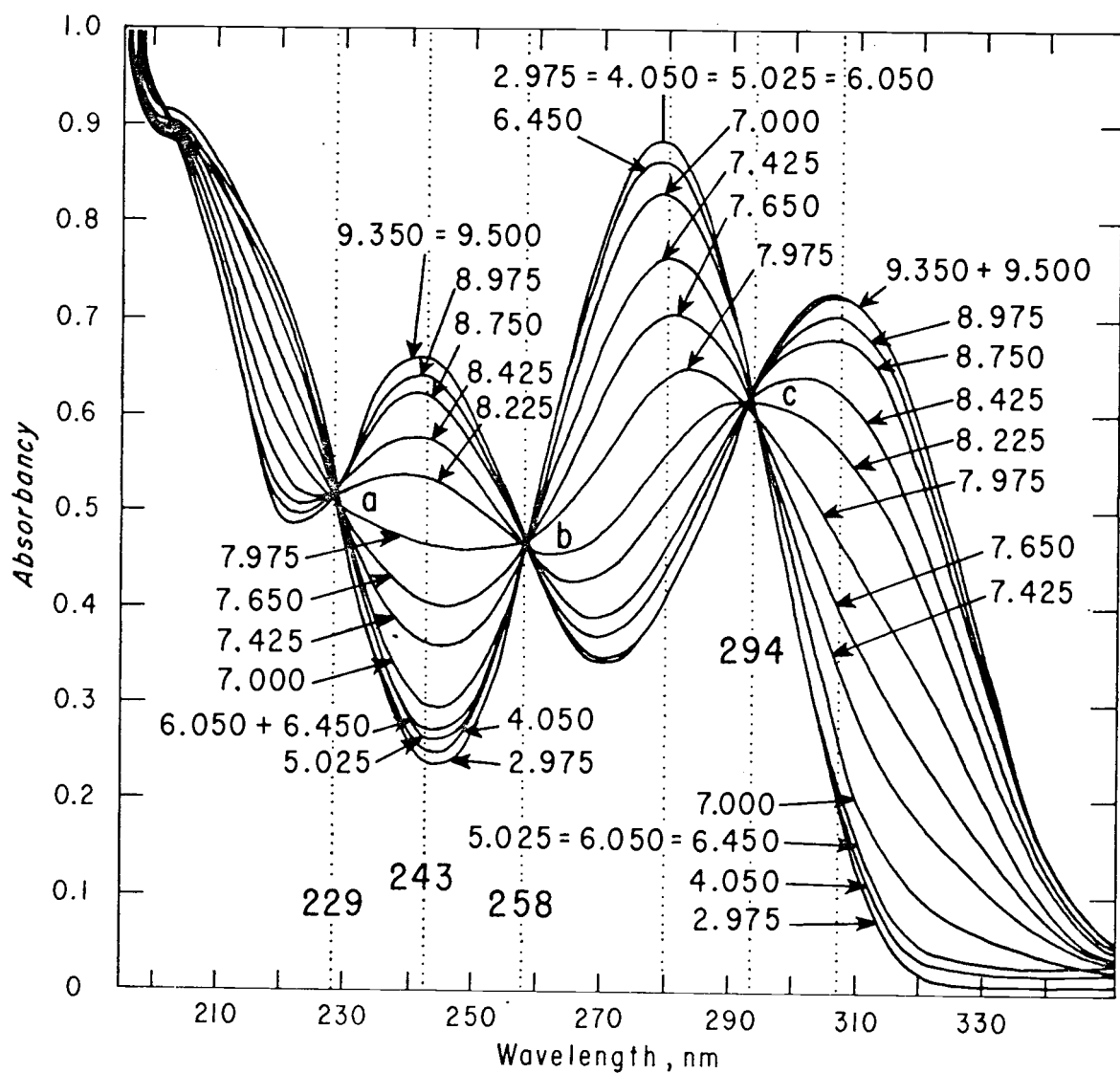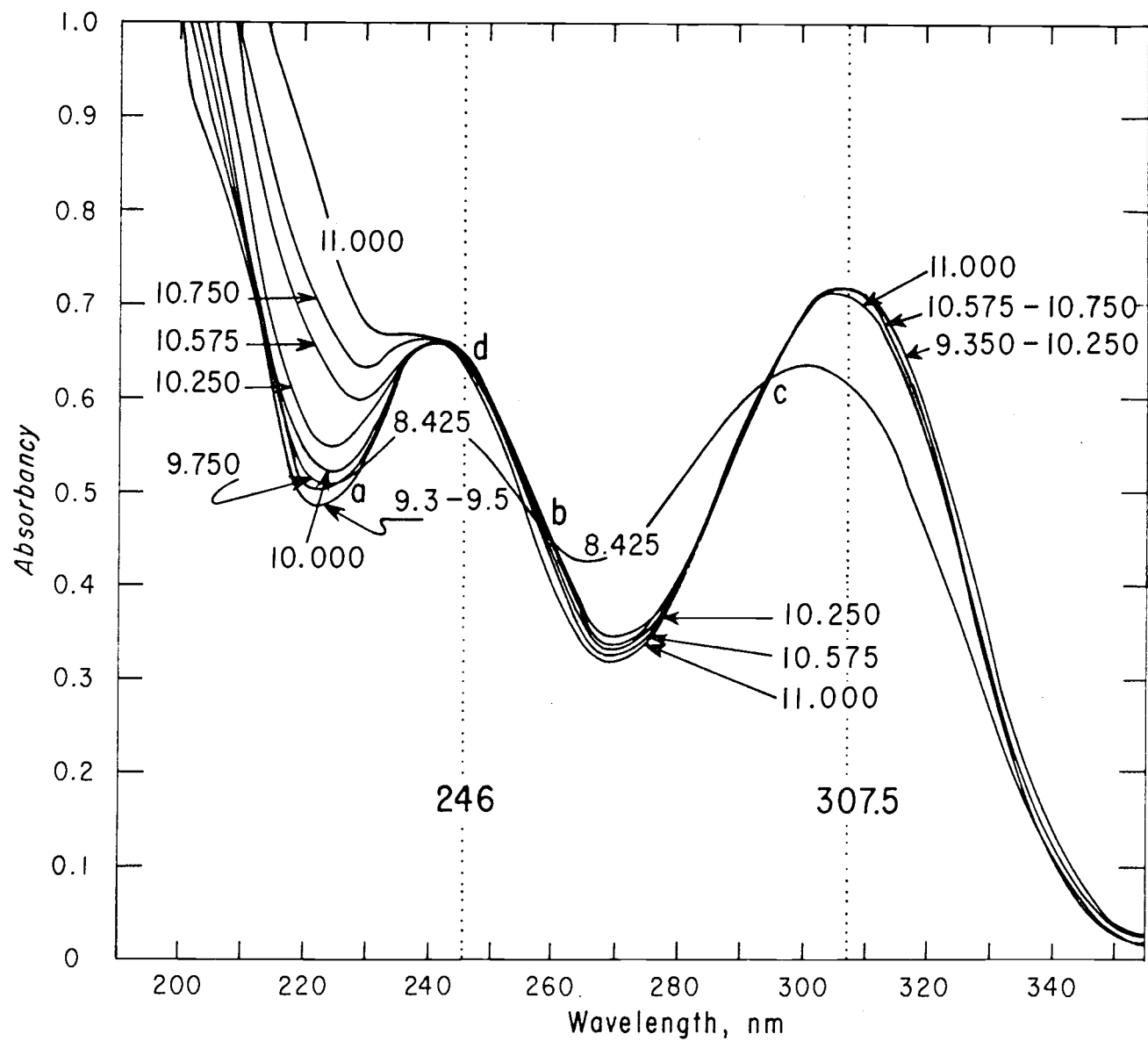
Fig. 2. Absorbance spectra of 5-hydroxyuridine, pH 2.975 to 9.500. The spectral curves for solutions between pH 2.975 and 9.500 define three isosbestic points a, b, and c at 229, 258, and 294 nm respectively which correspond to the 5-hydroxyl proton dissociation presented in the text.

Fig. 3.  Absorbance spectra of 5-hydroxyuridine, pH 9.500 to
11.000.  The spectral curves for solutions between pH 9.5 and
11.0 correspond to the N-3 proton dissociation presented in the
text.  The curve for pH 8.4 permits identification of isosbestic
points a, b, and c.  Spectra for solutions between pH 9.7 and
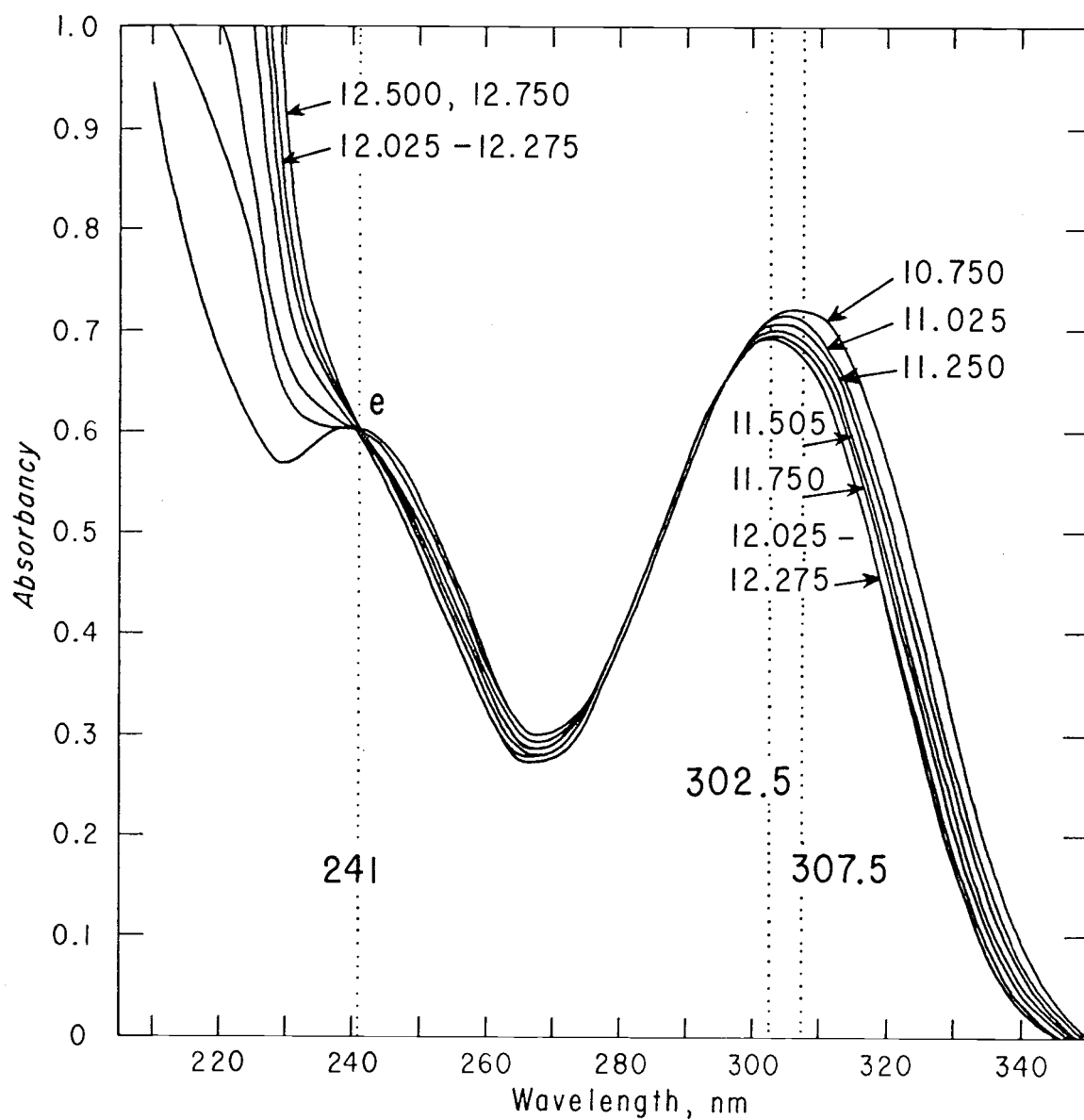10.7 pass through isosbestic point d.

Fig. 4. Absorbance spectra of 5-hydroxyuridine, pH 10.750 +
OPH 12.750. The spectral curves for solutions between
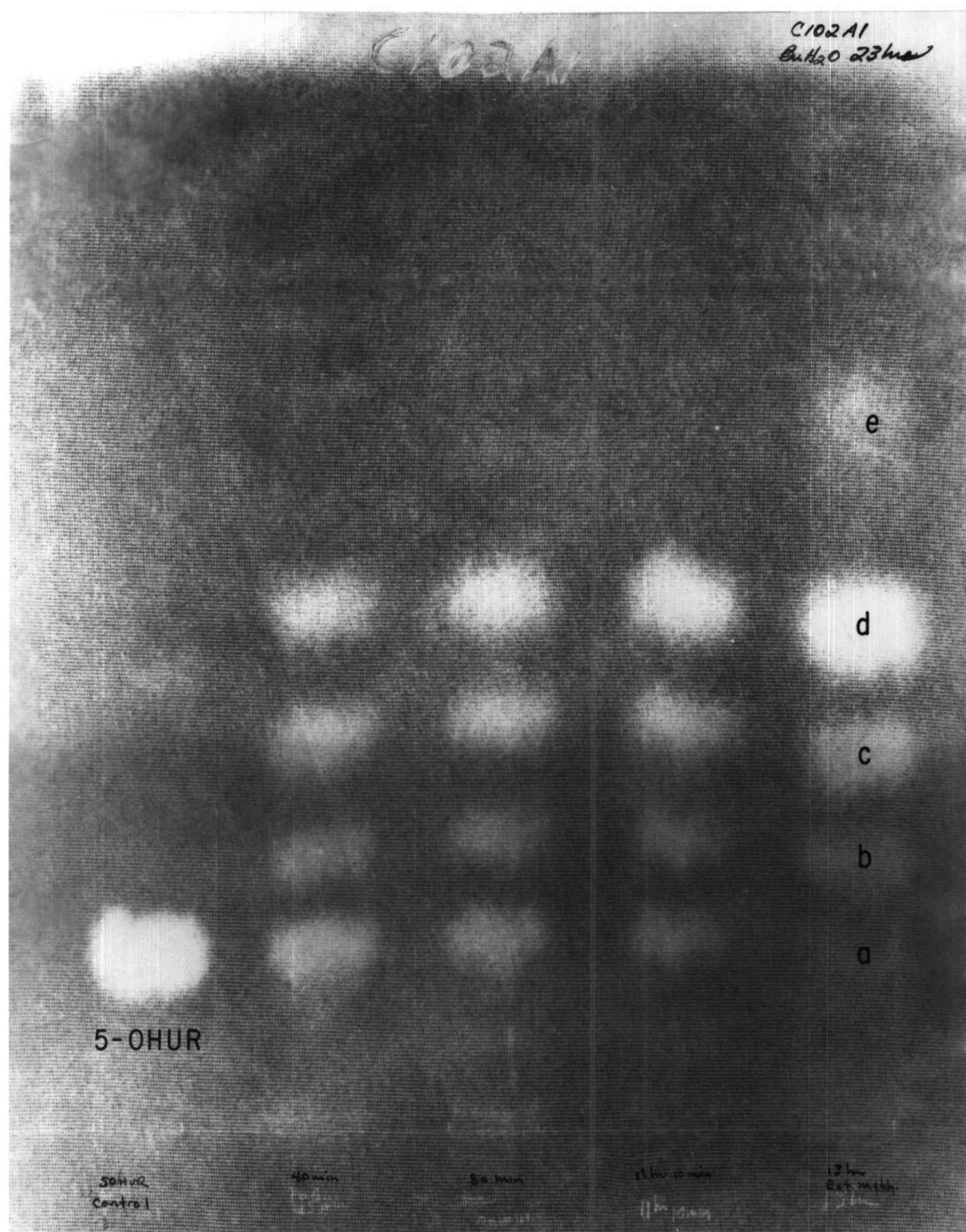pH 10.750 and 12.275 pass through isosbestic point _e_.

Fig. 5. Photograph of paper chromatogram showing production of methylated derivatives a through e with time. 5-OHUR equals 5-hydroxyuridine.
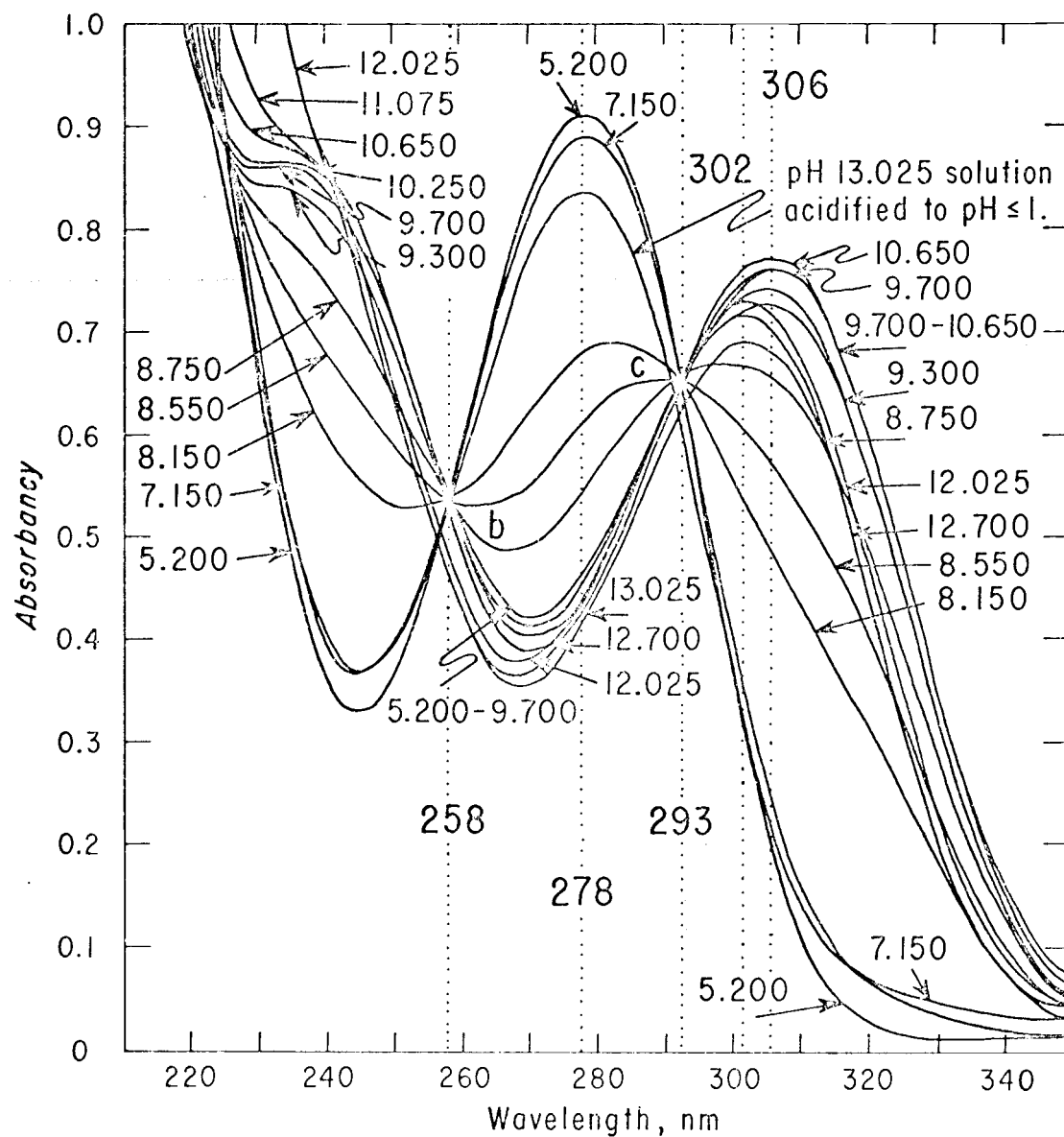
Fig. 6. Absorbance spectra of "methylated" derivative a, conformationally altered 5-hydroxyuridine.
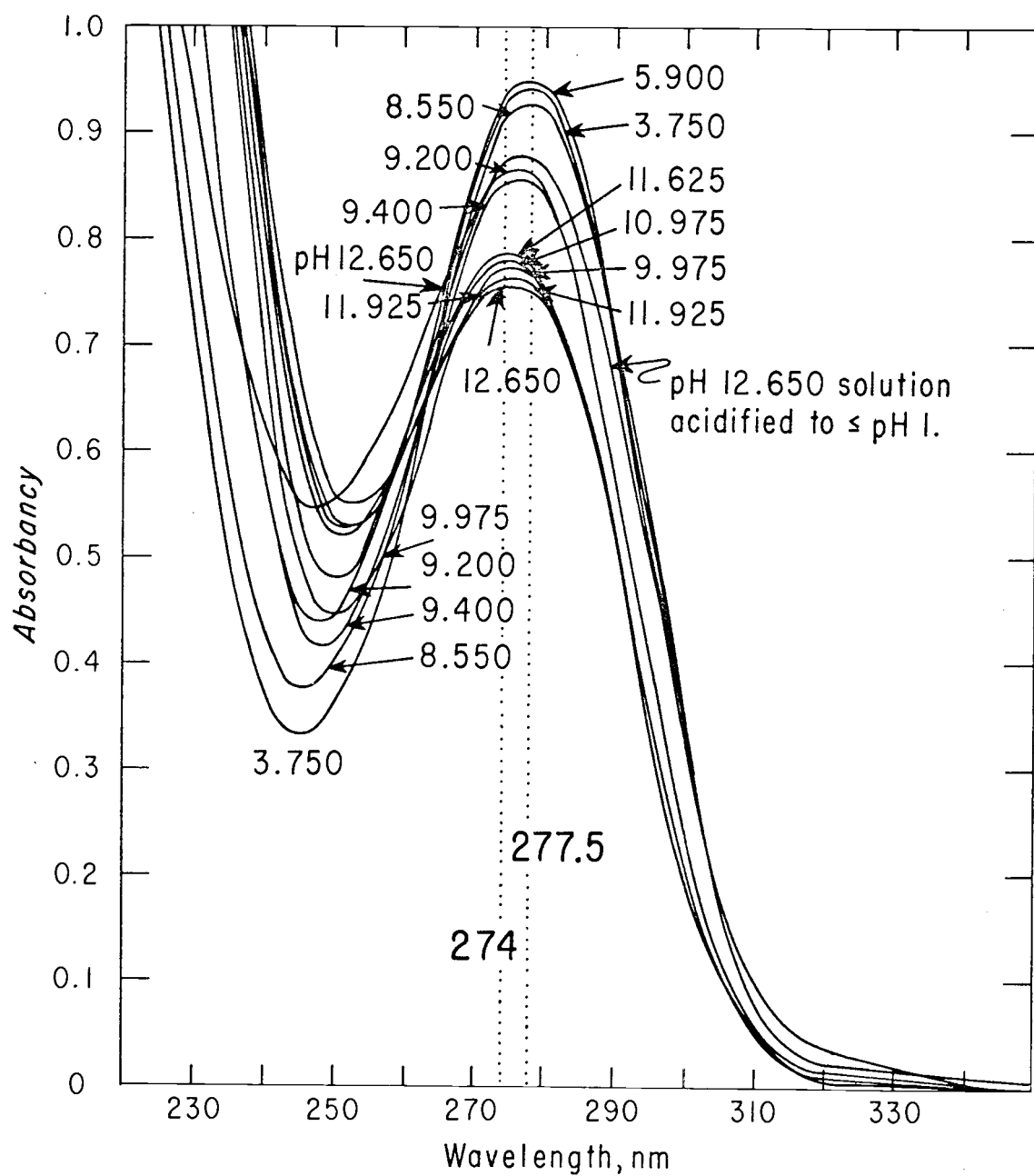
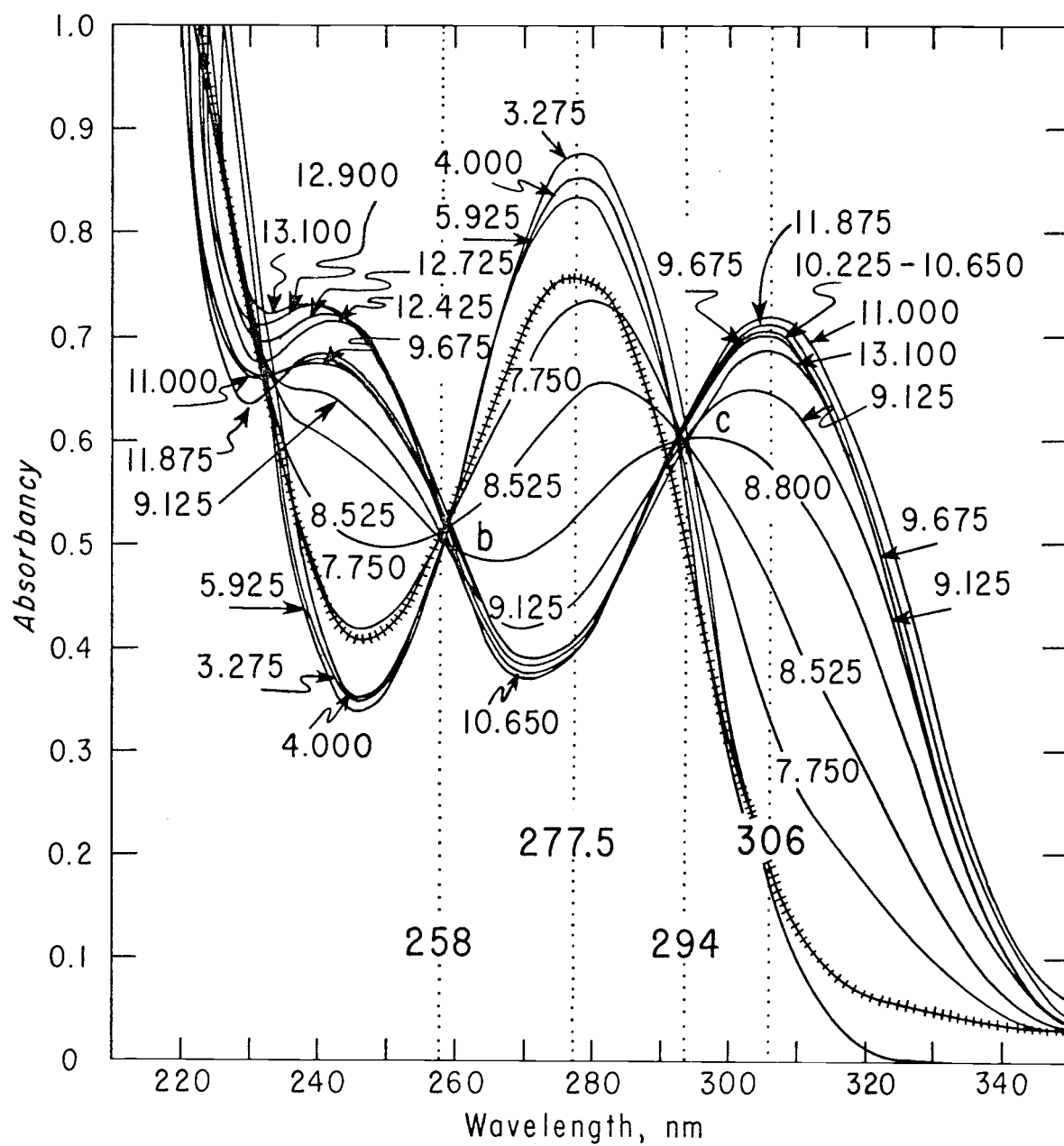Fig. 7. Absorbance spectra of methylated derivative b, 5-methoxyuridine.

Fig. 8. Absorbance spectra of methylated derivative c, 3-methyl-5-hydroxyuridine at solution pH values indicated.
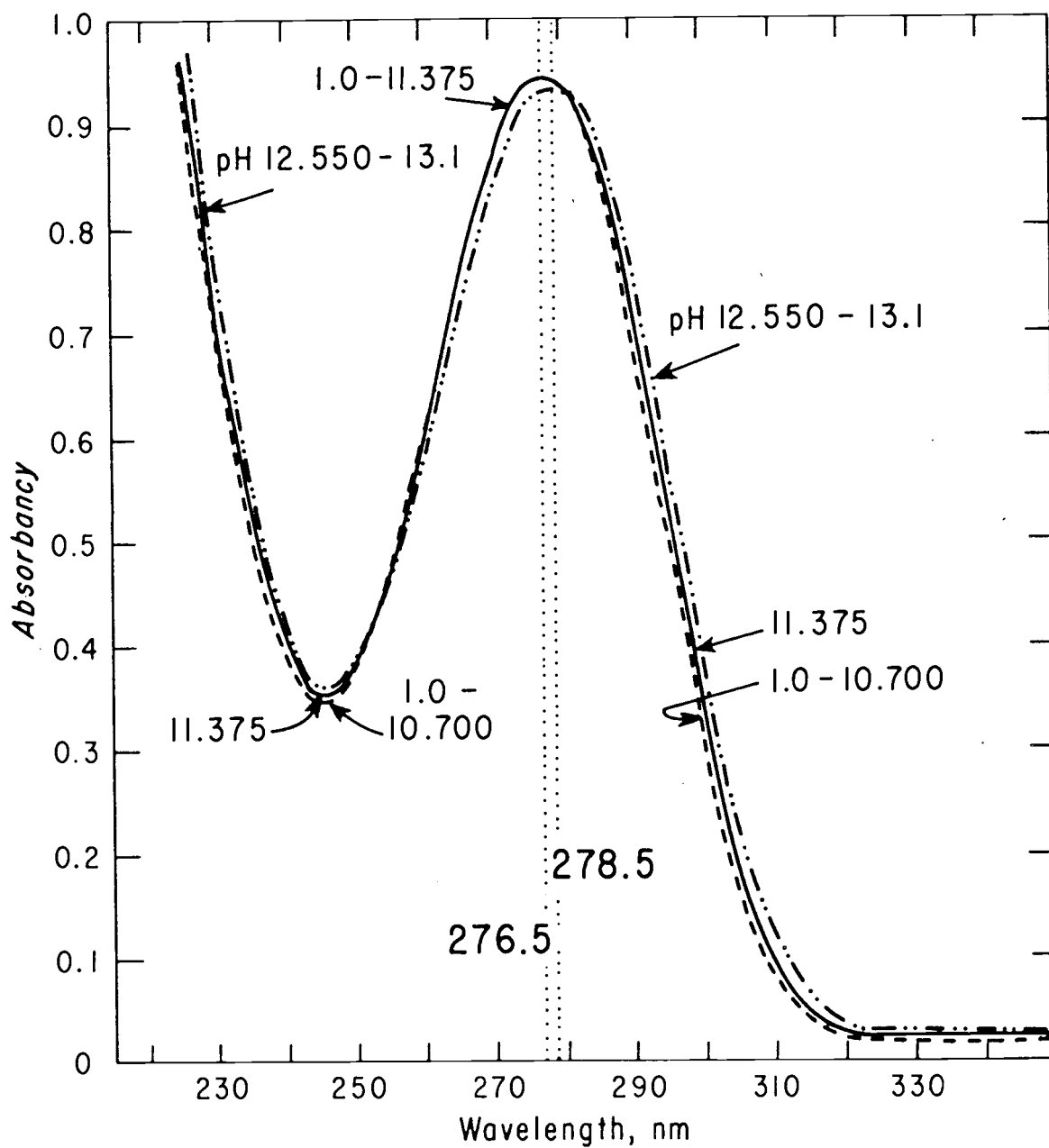
Fig. 9. Absorbance spectra of methylated derivative d,
3-methyl-5-methoxyuridine.

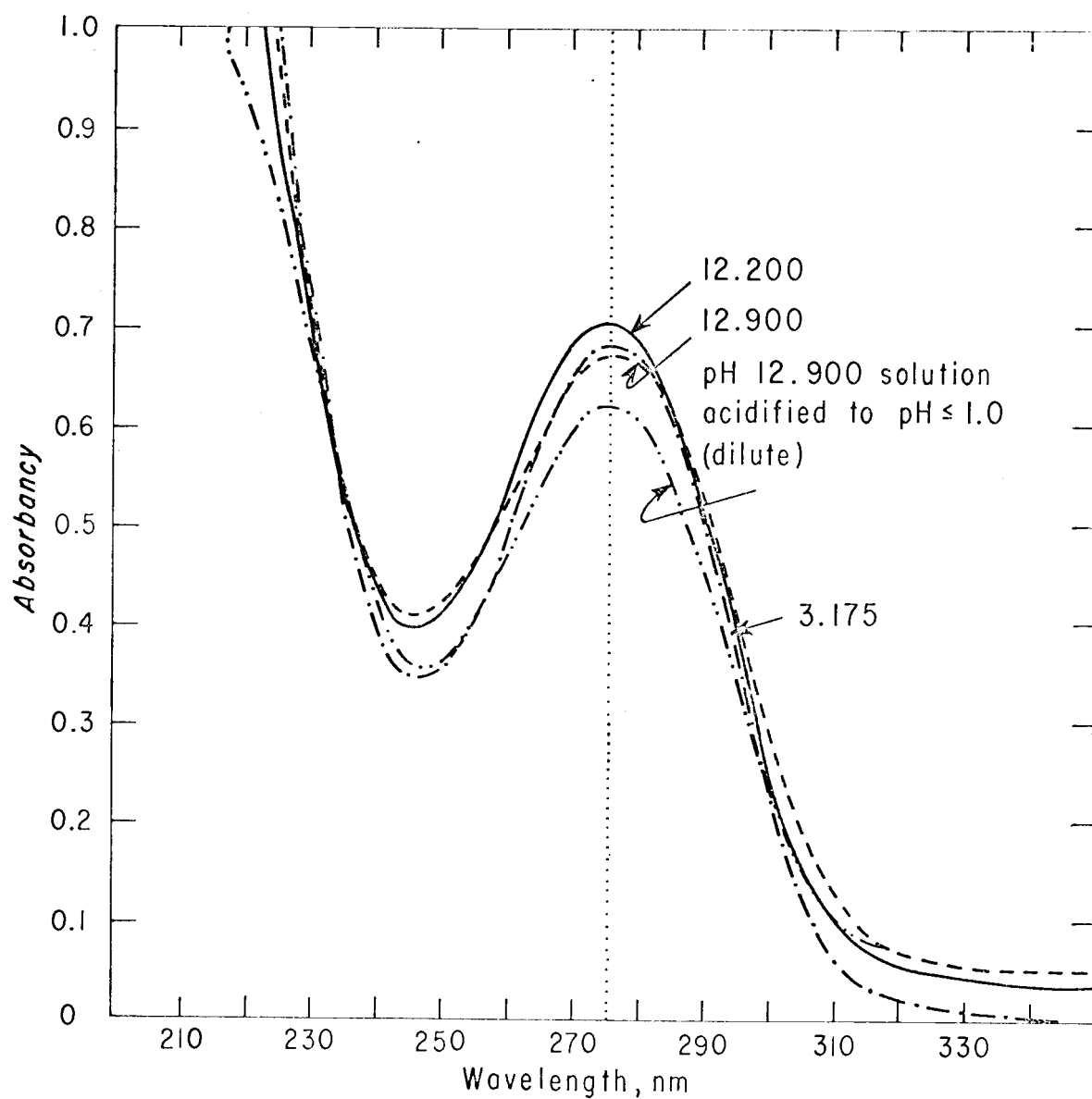Fig. 10. Absorbance spectra of methylated derivative e, "2'-o-methyl-3-methyl-5-methoxyuridine."
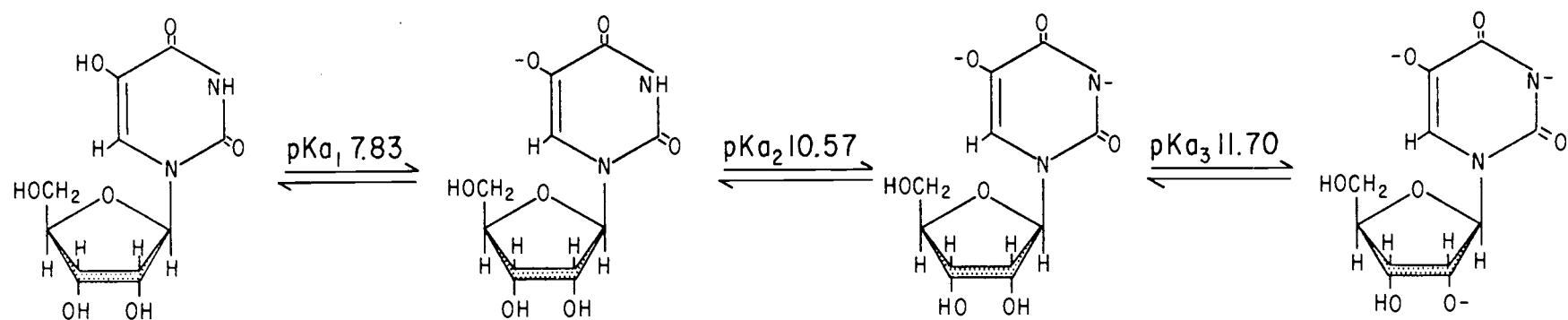
Fig. 11. Summary of the ionization characteristics of 5-hydroxyuridine.
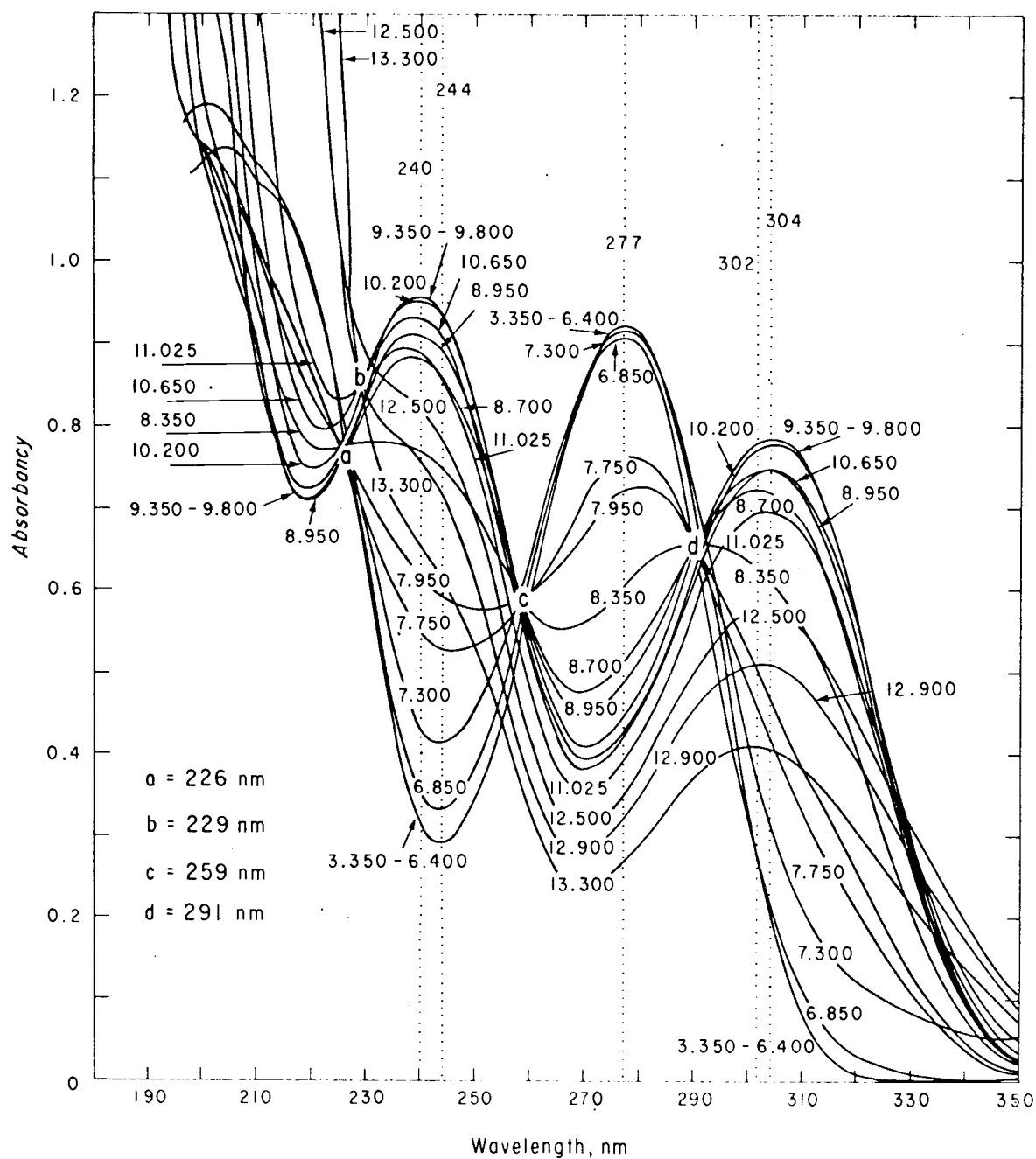
Fig. 12. Absorbance spectra of isobarbituric acid (5-hydroxy-uracil) at solution pH values indicated.
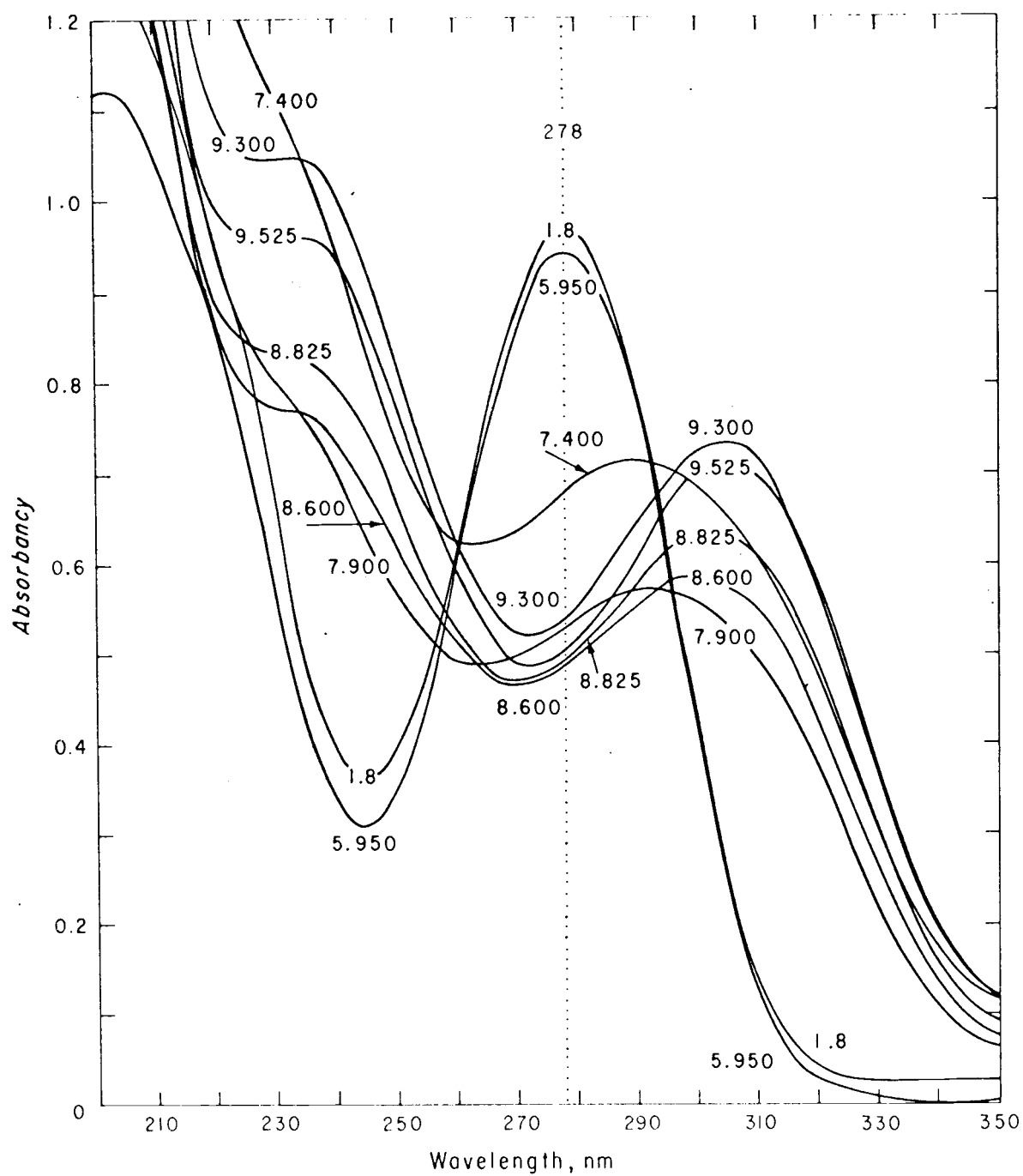
Fig. 13. Absorbance spectra 2'-deoxy-5-hydroxyuridine, pH 1.8 to 9.525.
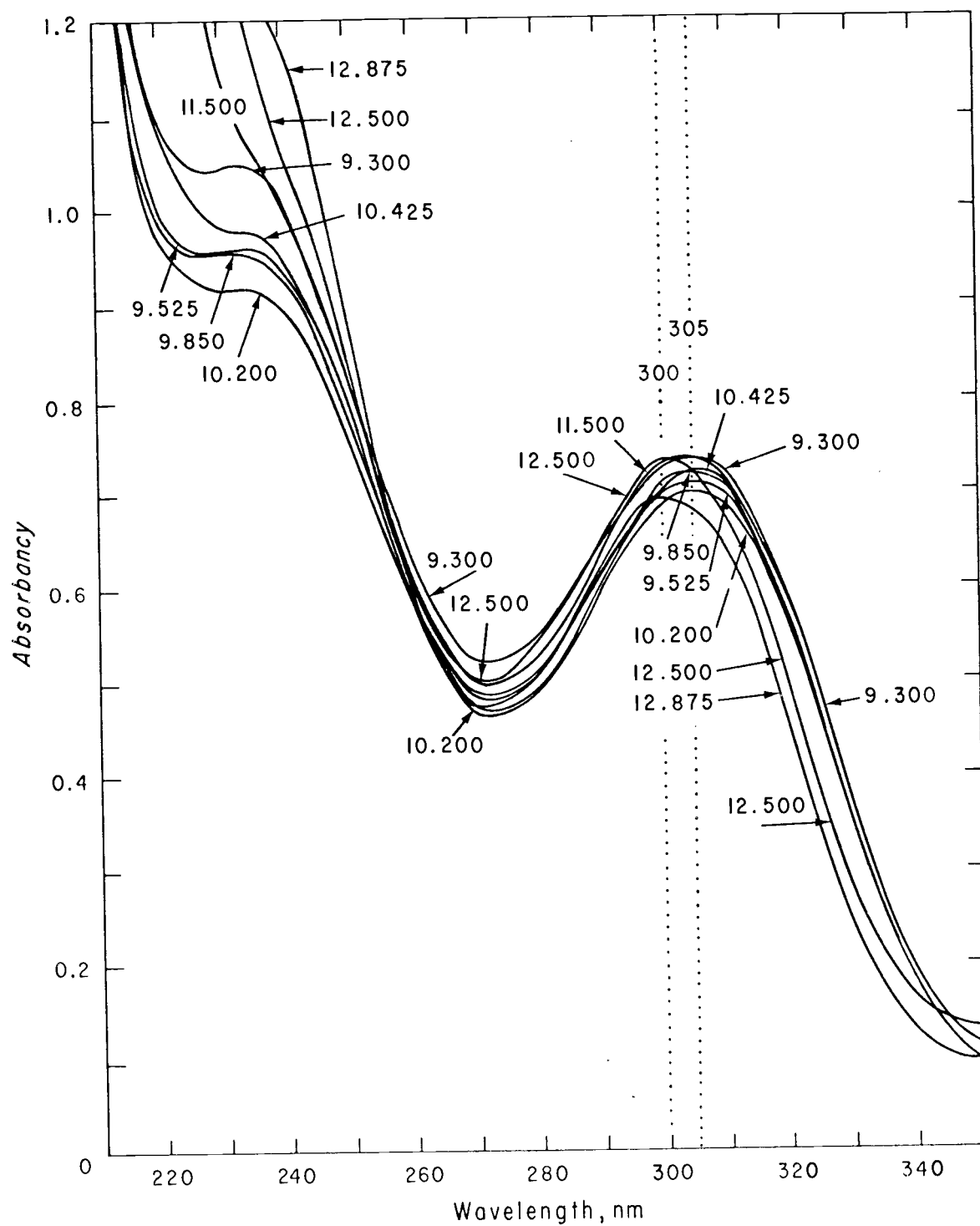
Fig. 14. Absorbance spectra 2'-deoxy-5-hydroxyuridine
pH 9.300 to 12.875.

Fig. 15. Optical rotatory dispersion curves of pH 7.0 distilled water ( ⸺ ) and $5\mathrm{x}10^{-3}$ molar magnesium chloride (..-..) solutions of 5-hydroxyuridine.
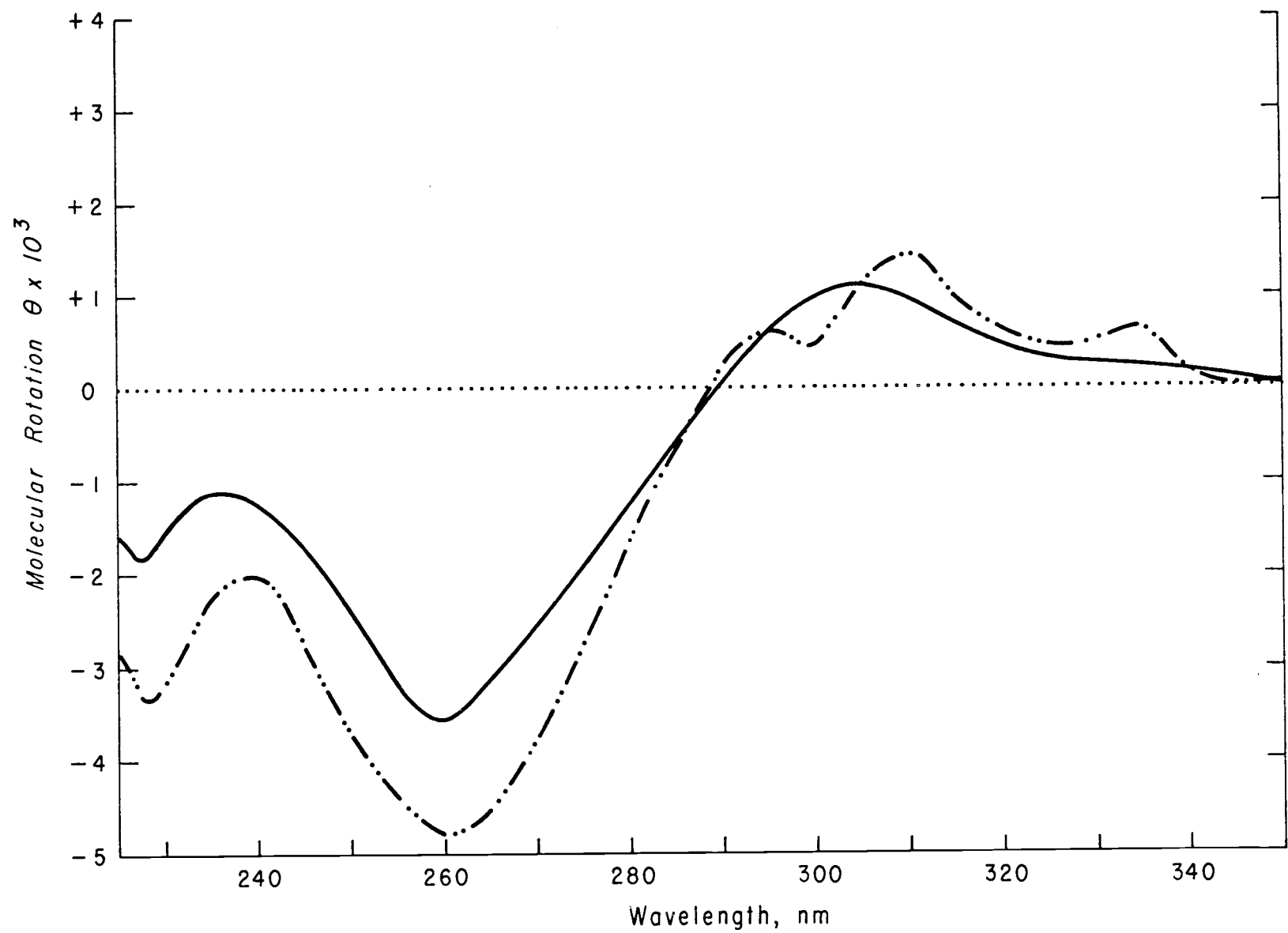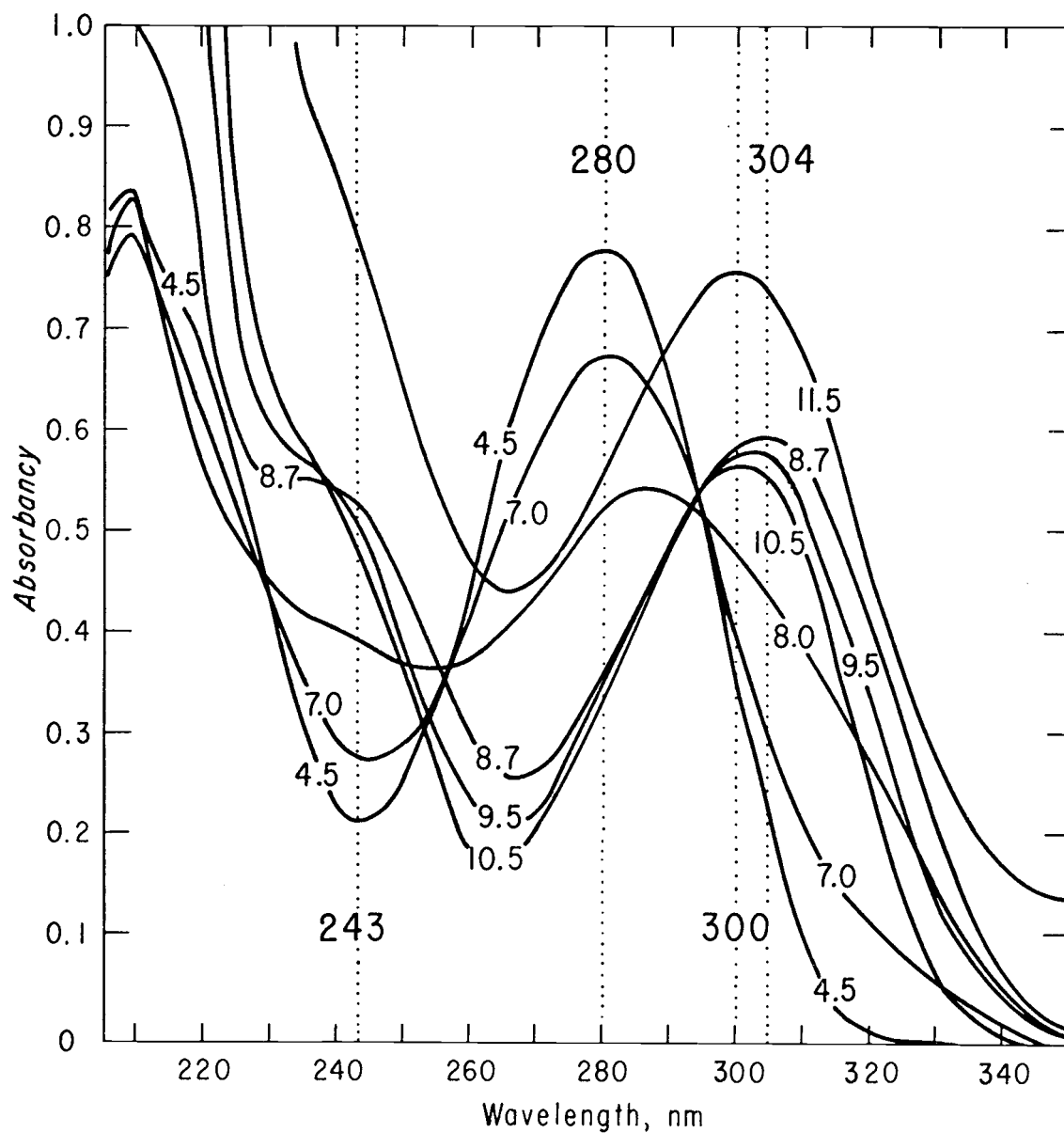
Fig. 16. Absorbance spectra of 5-hydroxyuridine in
5 x 10$^{-3}$ molar magnesium chloride at solution pH values
indicated.
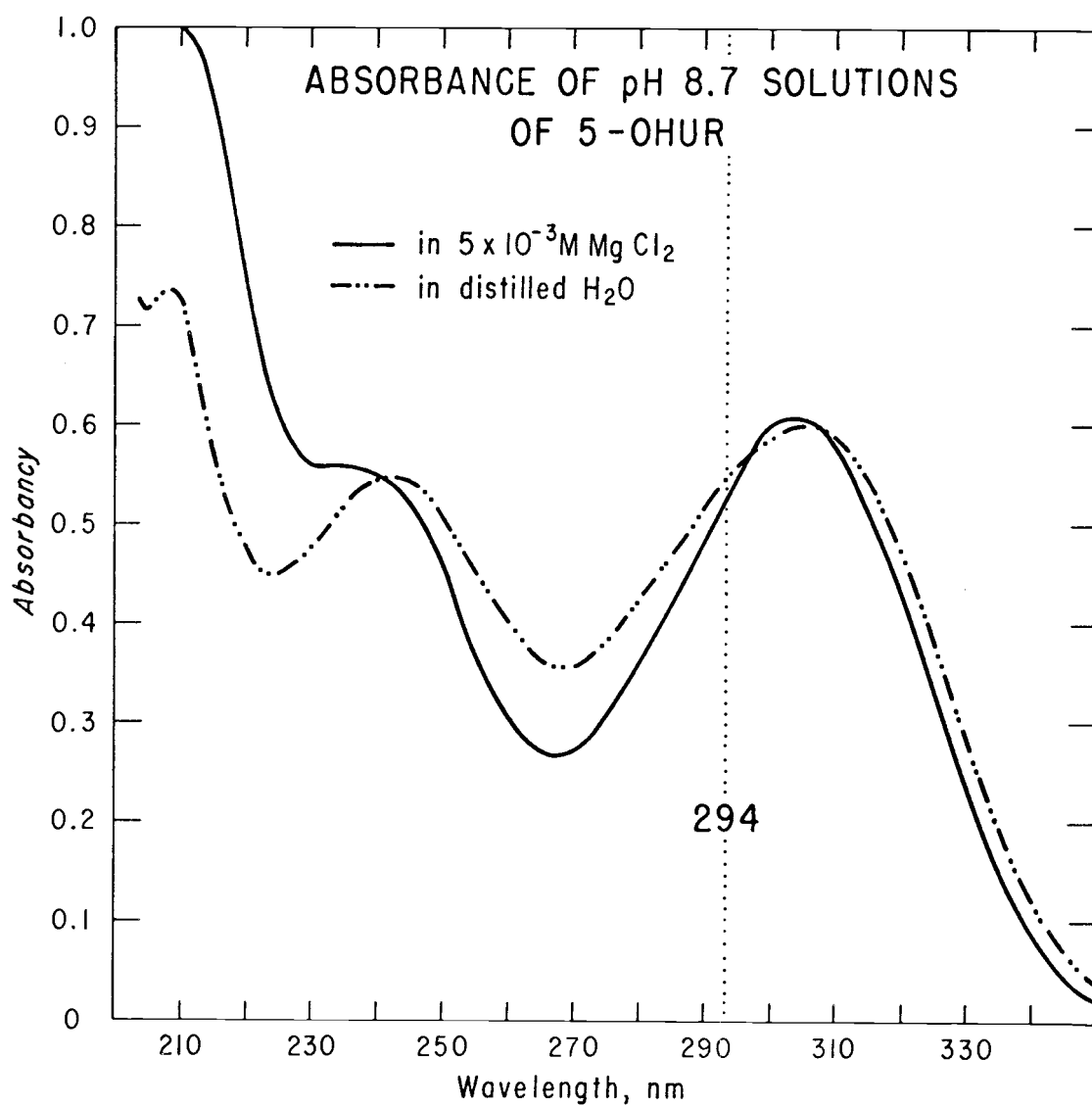
Fig. 17₃ Absorbance spectra of PH 8.7 distilled water and 5 x 10⁻³ molar magnesium chloride solutions of 5-hydroxyuridine.
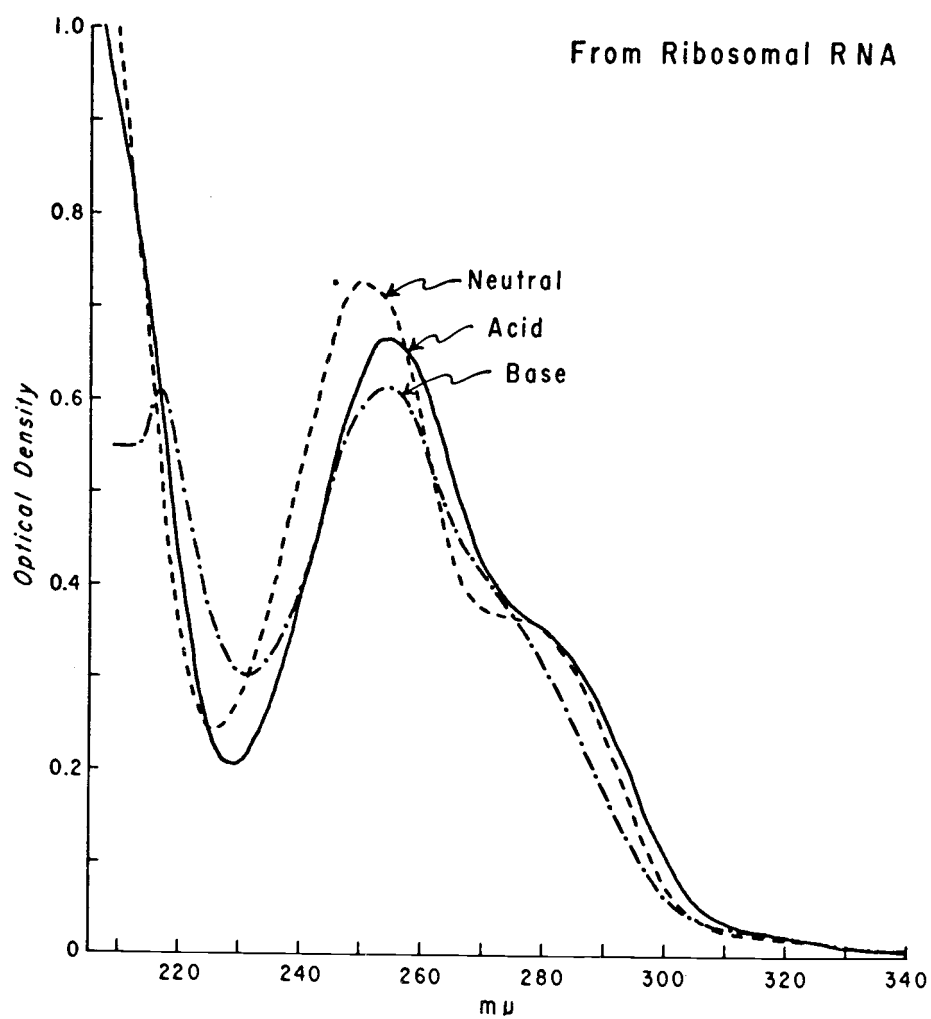
Fig. 18. Absorbance spectra of an unidentified component obtained from the dialysate of a commercial preparation of yeast RNA.
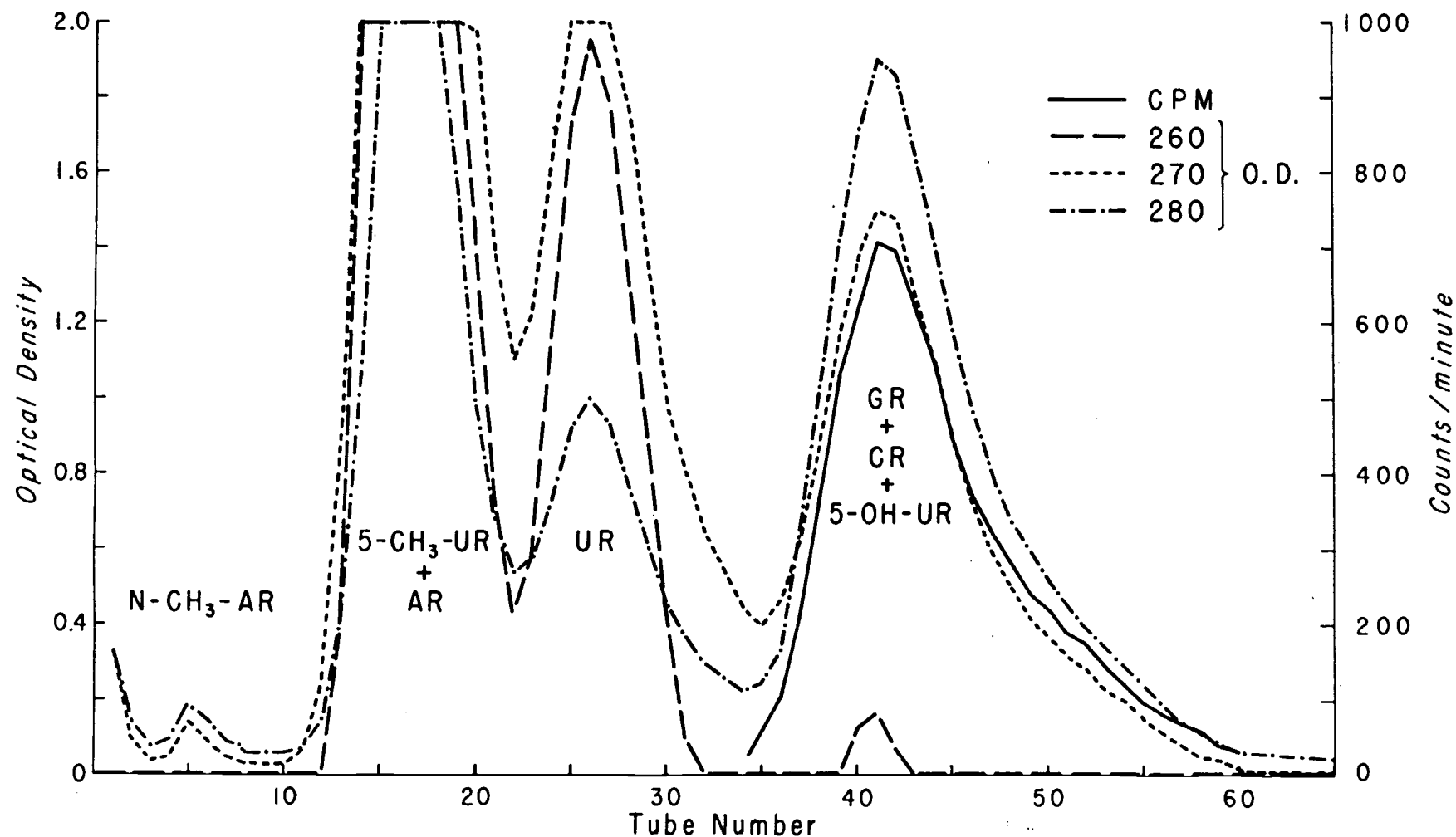
Fig. 19. Position of elution of 5-hydroxyuridine-2-14C (cpm for 5-OHUR), adenosine (AR) N-methyladenosines (N-CH3 -AR), cytidine (CR), guanosine (GR), uridine (UR), and 5-methyluridine (5-CH3 -UR) by celite partition chromatography, on small columns with the upper phase of solvent F. Absorbance of the fractions at 260, 270 and 280 nm is indicated.

Fig. 21. FLOW SHEET RNA FRACTIONATION

RNA Extraction From Yeast (Torula utilis)

Procedure of Crestfield, A.M. et al.
(J.B.C. 216, 185, 1955)
(Insoluble fraction)

Incubation Mixture (RNA mixed with 5-OHUR-2-14C)

Digest with snake venom and alkaline
phosphatase in $5 \times 10^{-3}$M $MgCl_2$, pH 8.6,
28 hr., 37°C., toluene added.

Hydrolysate

Centrifuged (18,800 x g for 3 hr. at 3°C.)
Lyophilized

Partition Column Chromatography (5 x 105 cm. column)

Collect Fractions (no label eluted)
1st Solvent (F) Upper phase
 ethylacetate-2 ethoxyethanol-$H_2O$ (4:2:1)
2nd Solvent (G) Upper phase ethylacetate-1-
 butanol-ligroin (bp 66-75°C.)-$H_2O$ (1:2:1:1)

Column Washed with $H_2O$

Label eluted first 425 ml single peak

Labeled Wash Fraction

Concentrate
Centrifuge
Concentrate to 20 ml

Wash Concentrate ( = HC #13 wash)

Fig. 20.  Celite partition chromatography of commercial uridine
spiked with 5-hydroxyuridine-2-14C and run on a 178 gram (2.54
x 86 cm) celite subfractionation column.  The column was eluted
with the upper phase of ethylacetate-glacial acetic acid-water
(5:1:2).
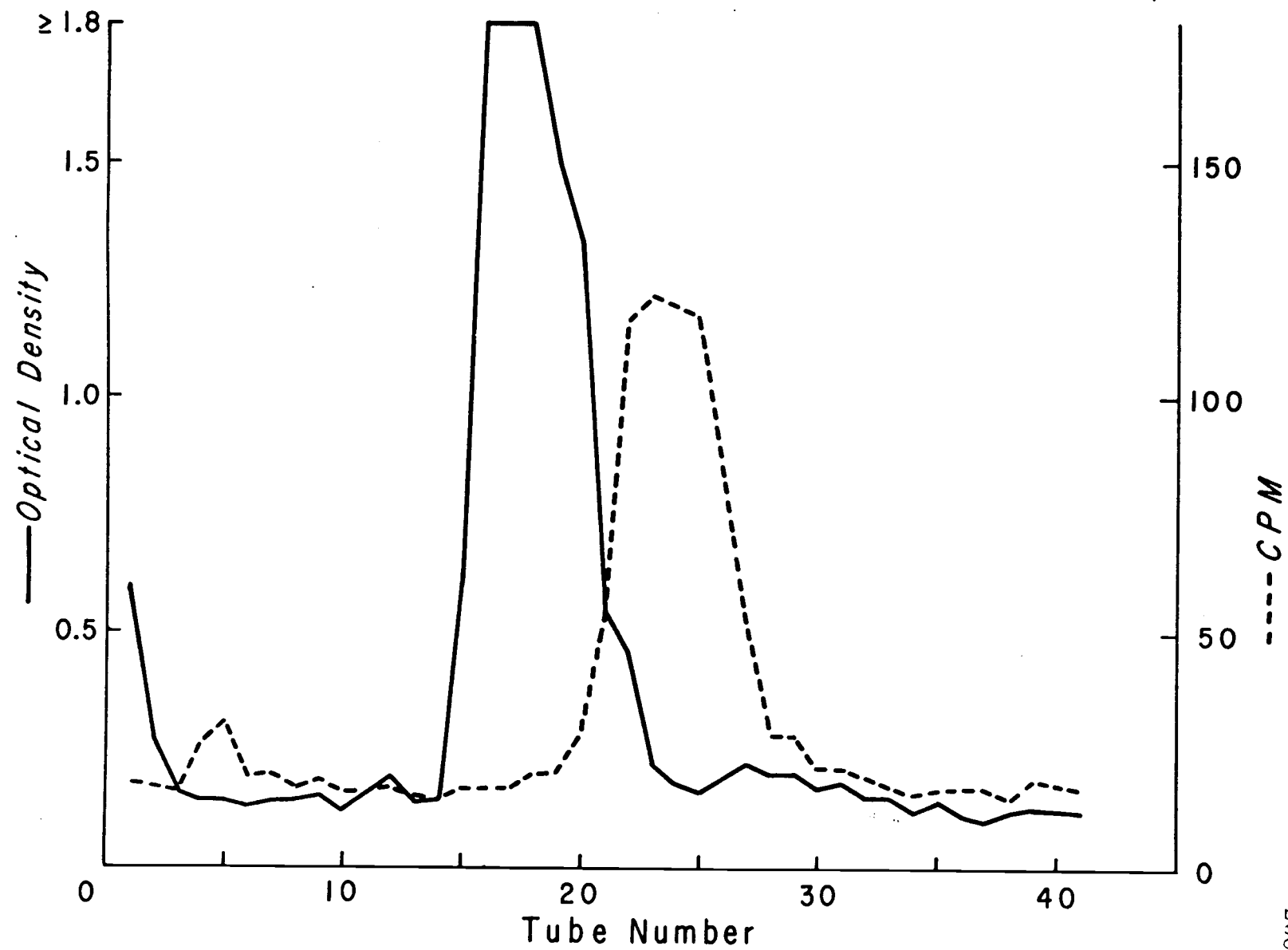
Fig. 22. Celite partition chromatography of a Crotalus ada-
manteus venom and alkaline phosphatase digest of yeast RNA
spiked with 5-hydroxyuridine-2-$^{14}$C. The 770 gram (5x105cm)
column was eluted with the upper phase of both solvents F and
G (see text).

Fig. 23. FLOW SHEET 1 SUBFRACTIONATION PROCEDURE

Wash Concentrate (HC #13)

Paper Chromatography
1-Butanol-H$_2$0 (86:14)
5-Submissions (22hr. each)

Cytidine-like component band    Guanosine-like component band    Label area    Nucleotides or oligonucleotides(?) and some label

Pool

Paper Chromatography
1-Butanol-g-acetic a.-H$_2$0
(5:2:3)
4 submissions(20hr. each)

Other bands    Labeled band    Small Labeled bands

Paper chromatography
Ethylacetate-1-pro-
panol —H$_2$0(4:1:2)
2 submissions

OD & Label remain at origin

Paper Chromatography
(Resubmit above)
2-propanol-1% aq
(NH$_4$)$_2$SO$_4$

OD Band    Major labeled band    Minor labeled band
Diffuse OD    Stronger OD

Paper Chromatography
1-butanol-H$_2$0-ETOH
(50:20:30)

Major labeled band    Minor labeled band

(Runs below 5-OHUR,    (Runs above 5-OHUR,
GR, & CR Controls)     GR, & CR controls)
(5-OHUR art. acid      C73-74Ab
max. 272 Mµ)

C73-74Aa

Paper Chromatography
1-butanol-g-acetic a.-H$_2$0
(5:2:3)
5 Submissions(20hr. each)

few fine bands (no label)    labeled band 100D units
C21b = Compound A

Paper chroma-    Paper electro-    acid
tography         phoresis          hydrolysis
9 solvent systems    3 buffers

Fig. 24 FLOW SHEET 2 SUBFRACTIONATION PROCEDURE

Wash Concentrate (HC #13)

Dowex-1-formate (2%) column
chromatography
Label elutes with $H_2O$
as single peak

Labeled Fraction
Concentrate

Paper Chromatography
1-Butanol-$H_2O$-ETOH

Other bands | Labeled band | Origin band

Paper Chromatography
2-Propanol-1% $(NH_4)_2SO_4$(2:1)

(One major band OD & L)

Paper Electrophoresis
Borate buffer, pH 9.2 4hr.

Positive migration (1/2 OD) | Negative migration (OD&L) (Labeled band)

Paper Chromatography
1-Butanol-$H_2O$(86:14)

Major labeled band remains on origin | Minor labeled band (= 1/3 OD&L) shows slight migration

Compound A

Negative tests for aldo-pentose (or aldo-hexose) and pyrimidine reduction products

Paper Chromatography
(Resubmit above)
2-Propanol—conc.HCl-$H_2O$(680:170:144)

Compound C | Non U.V.-absorbing band Positive test for aldo-pentose and for pyrimidine reduction products

Compound B

Paper Chromatography
2-Propanol-1% aq$(NH_4)_2SO_4$(2:1)

Other bands | Labeled band

Paper Electrophoresis
Borate buffer pH 9.2, 4 hr.

Positive migrating OD bands | Negative migration(OD&L) (Labeled band)

Paper Chromatography
1-Butanol-$H_2O$(86:14)

Faint migrating band (no L) | Labeled band and OD remain on origin

Compound A

Negative tests for aldo-pentose (or aldo-hexose) and for pyrimidine reduction products

Paper Chromatography
(Resubmit above)
2-Propanol-conc. HCl-$H_2O$ (680:170:144)

Compound C | Non U.V.-absorbing band Positive test for aldo-pentose (or aldo-hexose) and for pyrimidine reduction products
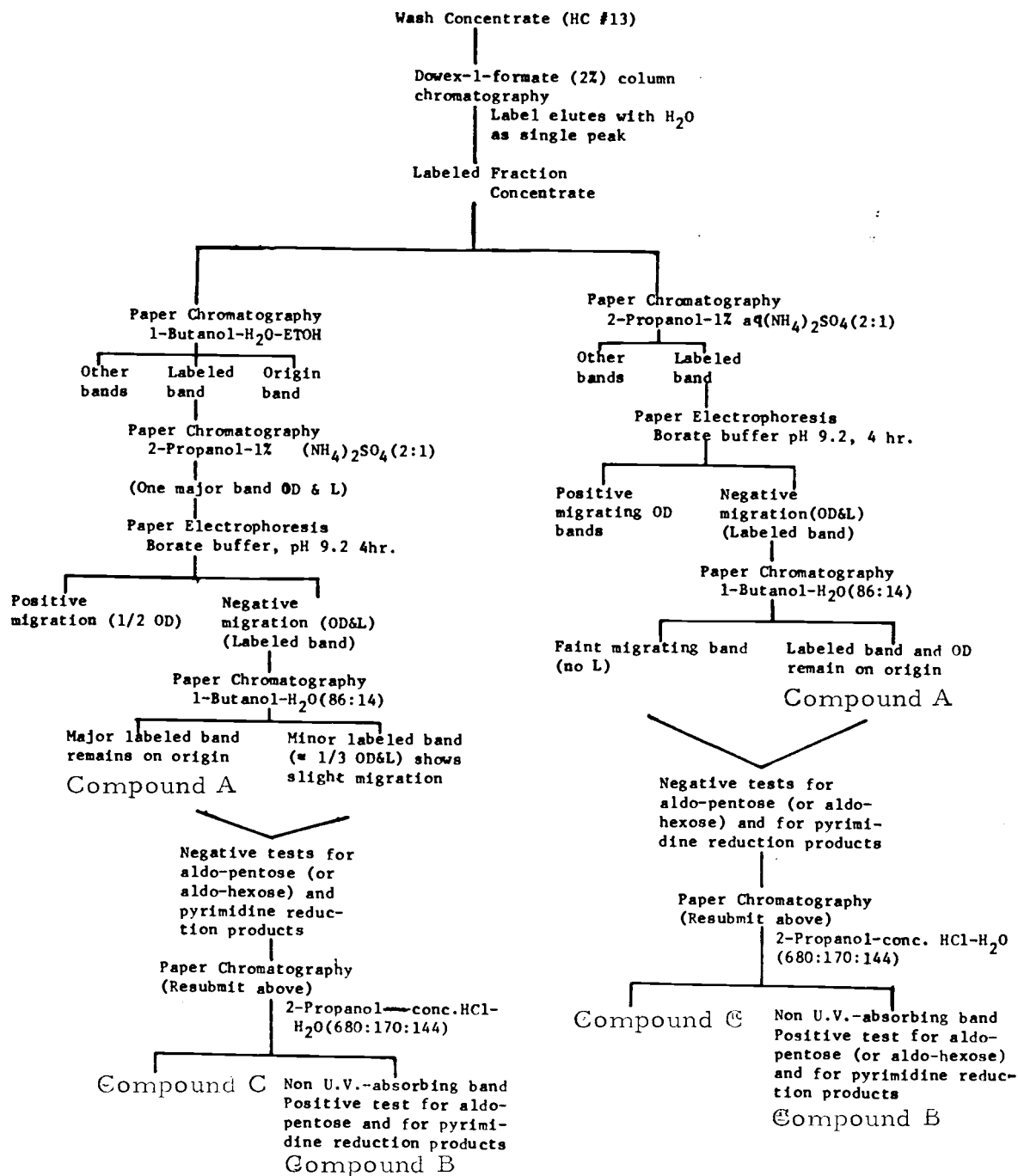
Compound B

Fig. 25. Migration of the labeled peak from celite partition
chromatography on ascending paper chromatography after
five submissions in 1-butanol-$H_2O$ (86:14).
This chromatogram gave the first indication that the lable was
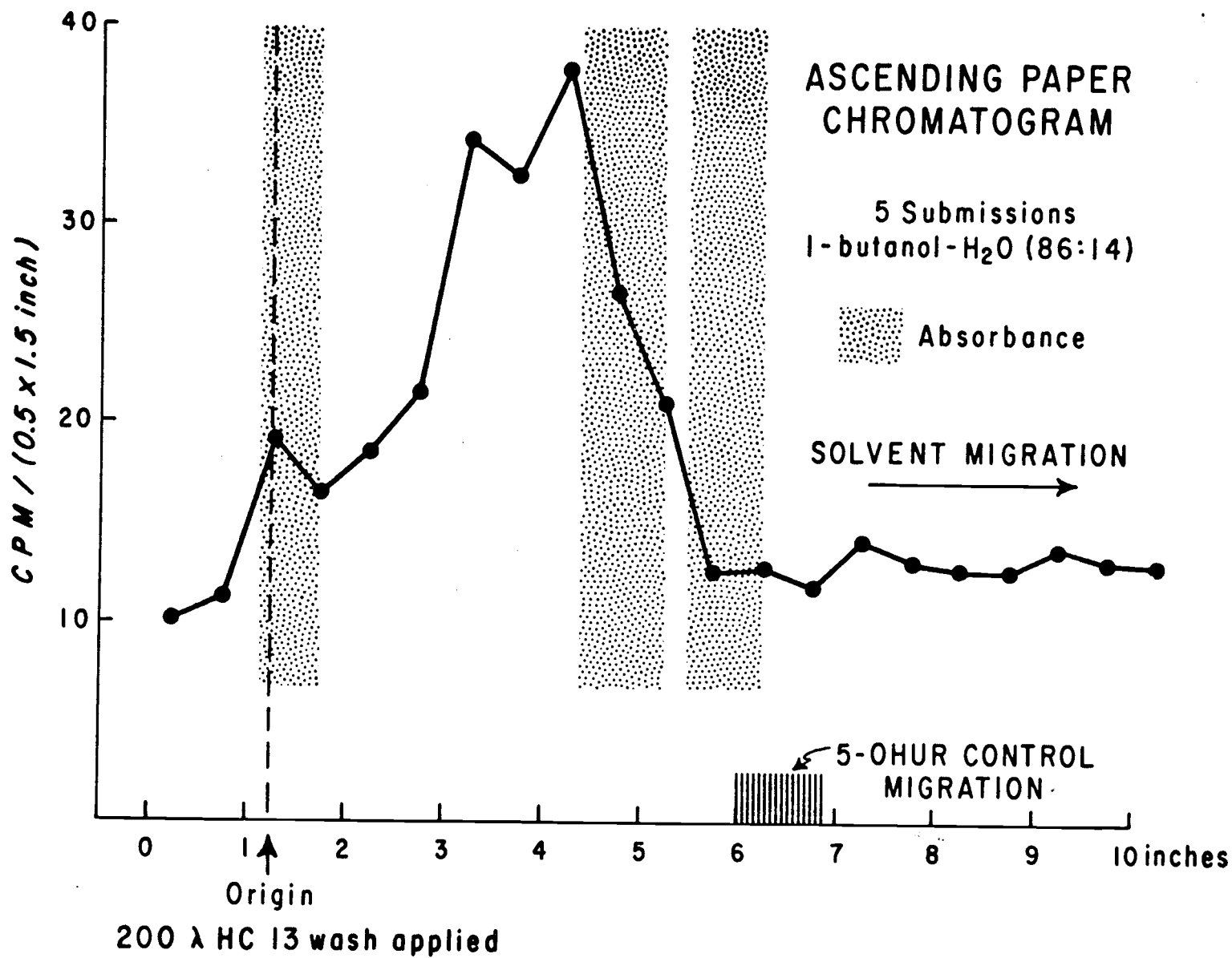not behaving as 5-hydroxyuridine.

ASCENDING PAPER
CHROMATOGRAM

5 Submissions
I-butanol-$H_2O$ (86:14)

Absorbance

SOLVENT MIGRATION

5-OHUR CONTROL
MIGRATION

Origin

200 λ HC 13 wash applied

Fig. 26. Migration of labeled 5-hydroxyuridine in an equimolar
mixture of standard mononucleotides and nucleosides on ascend-
ing paper chromatography in 1-butanol-$H_2O$ (86:14). This con-
trol experiment represents an attempt to ascertain whether 5-
hydroxyuridine complexes with standard nucleosides or nucleo-
tides.

ASCENDING PAPER CHROMATOGRAM

I Submission
I-butanol – H₂O (86:14)

Absorbance

SOLVENT MIGRATION →

MIGRATION OF CONTROLS

Origin
UMP, CMP, AMP, GMP,
CR, GR (comm. preps), 5-OHUR-2-14C applied
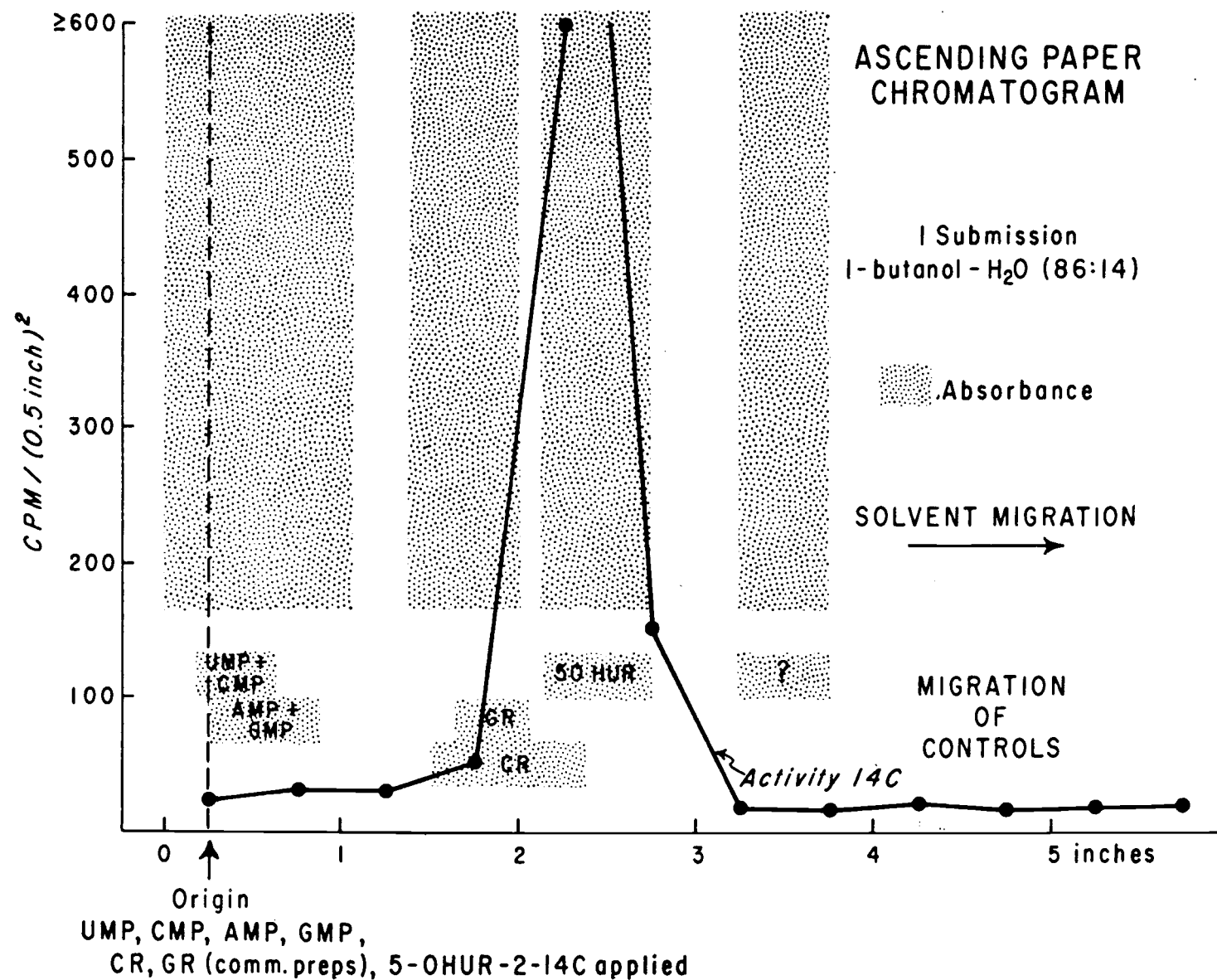
Fig. 27.  Migration of the labeled peak from celite partition chromatography spiked with 5-hydroxyuridine-2-$^{14}$C on ascending paper chromatography after 4 submissions in 1-butanol-H$_2$O (86:14).

ASCENDING PAPER CHROMATOGRAM

4 Submissions
1-butanol–H$_2$O (86:14)

Absorbance

SOLVENT MIGRATION

Solvent Front

Origin

200 λ HC 13 wash
5-OHUR-2-14C } mixture applied

5-OHUR Control
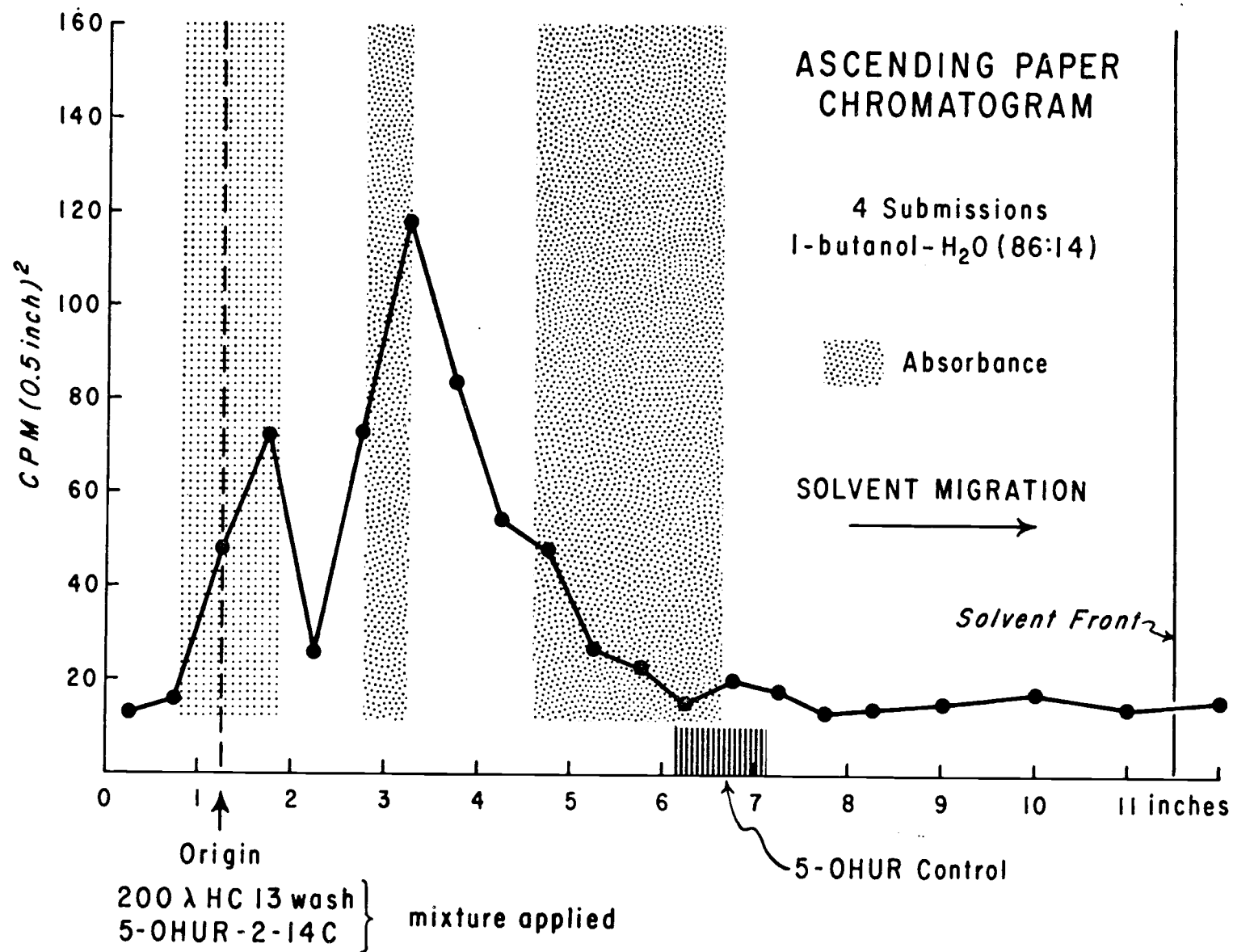
Fig. 28. The elution profile from a 103 cc Dowex-1-formate column of the labeled peak from celite partition chromatography. A, B, and C represent the position of elution of cytidine, guanosine, and cytidine 5'-phosphate respectively.
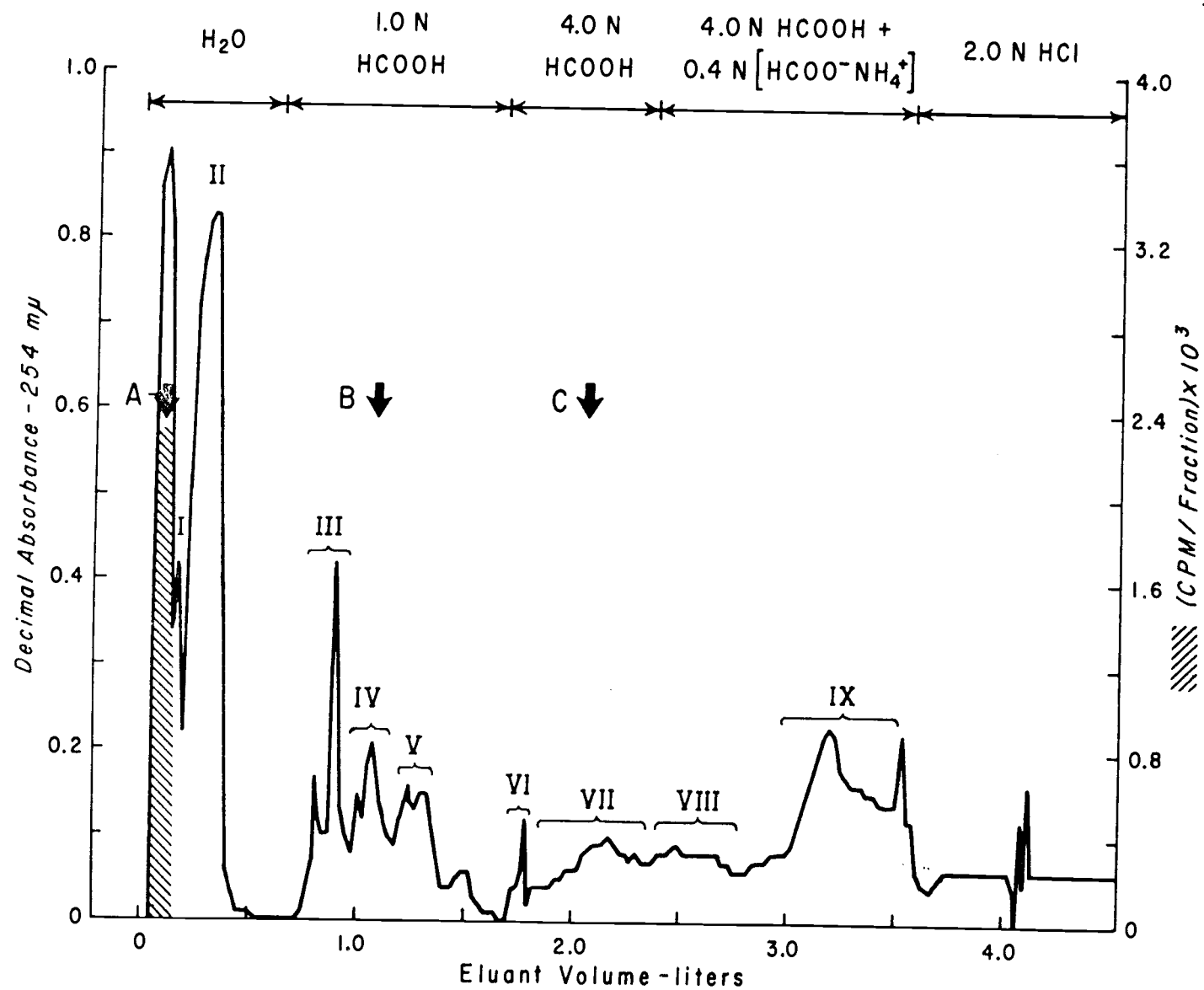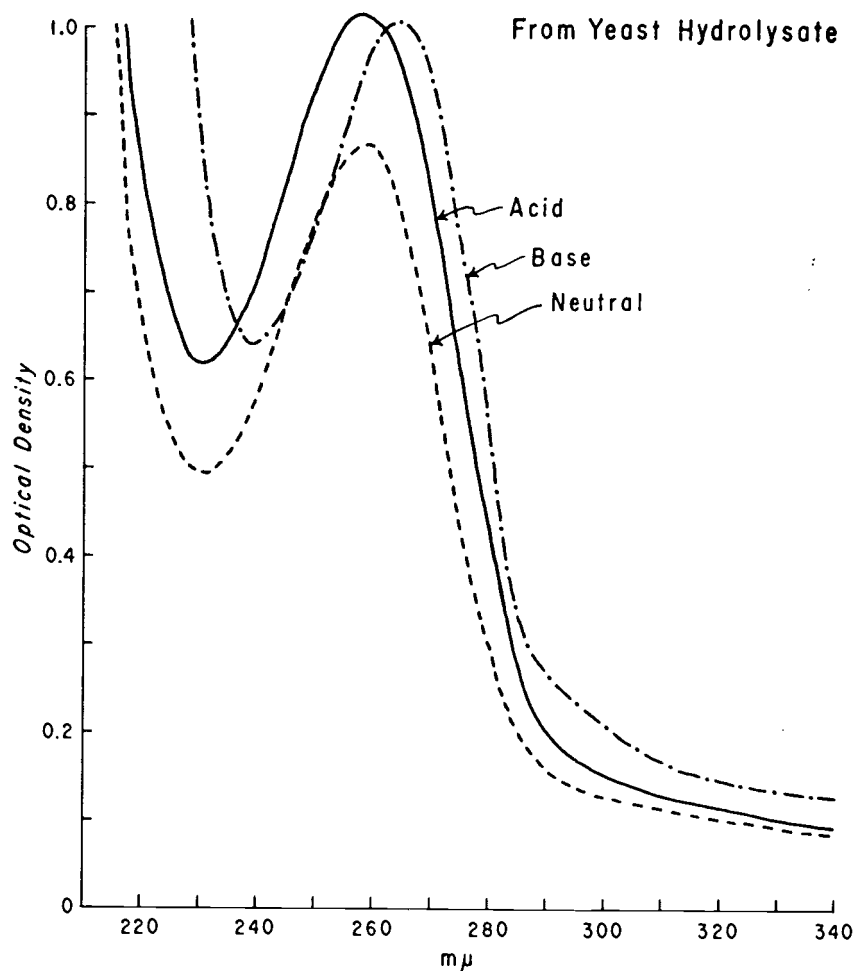
Fig. 29. Absorbance spectra of a purified preparation of Peak II
from the Dowex -1-formate column chromatographic profile shown
in Fig. 28. This unidentified nucleoside was obtained from an
enzymatic digest of yeast RNA. The material was not eluted during
the normal development of celite partition column HC #13, Fig. 22,
but was obtained, along with the C-14 labeled spike which had been
added to the RNA digest, after the column was washed with water.

Fig. 30. Elution profiles on 54 cc Dowex-1-formate columns of the labeled peak obtained from celite partition column HC #13, and of 5-hydroxyuridine-2-$^{14}$C and cytidine-5-$^3$H. The C14 label in the wash peak is designated as an artifact of 5-hydroxyuridine.
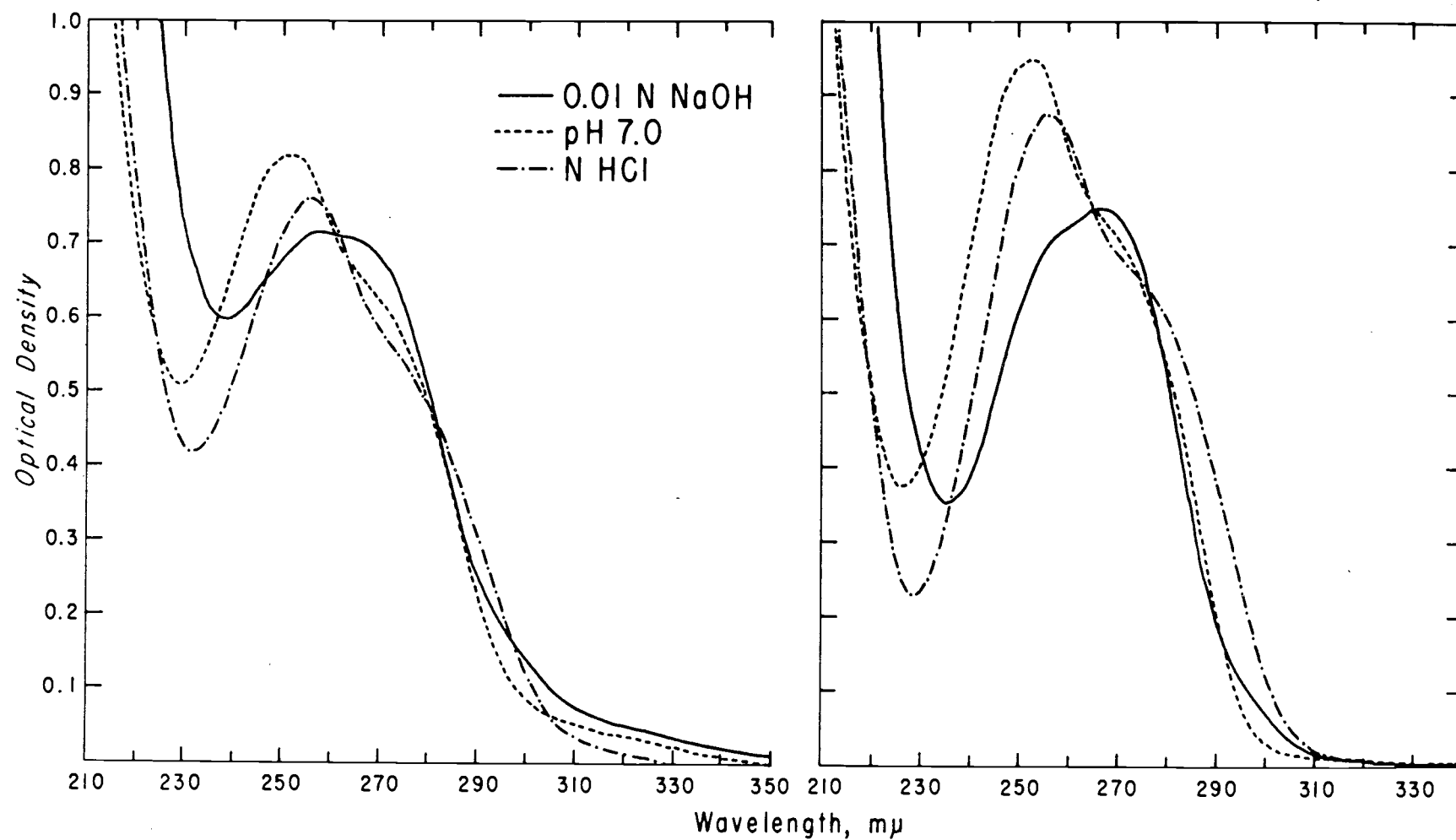
50 λ CR-5-3H
50 λ 5-OHUR-2-14C

CPM 3H (CR)
CPM 14C (5-OHUR)
CPM 14C (artifact)
OD 270 mµ

500 λ HC 13 wash

50 λ CR-5-3H
500 λ HC 13 wash

Optical Density 270 mµ

CPM/0.5 ml

Volume H₂O Eluant (ml)
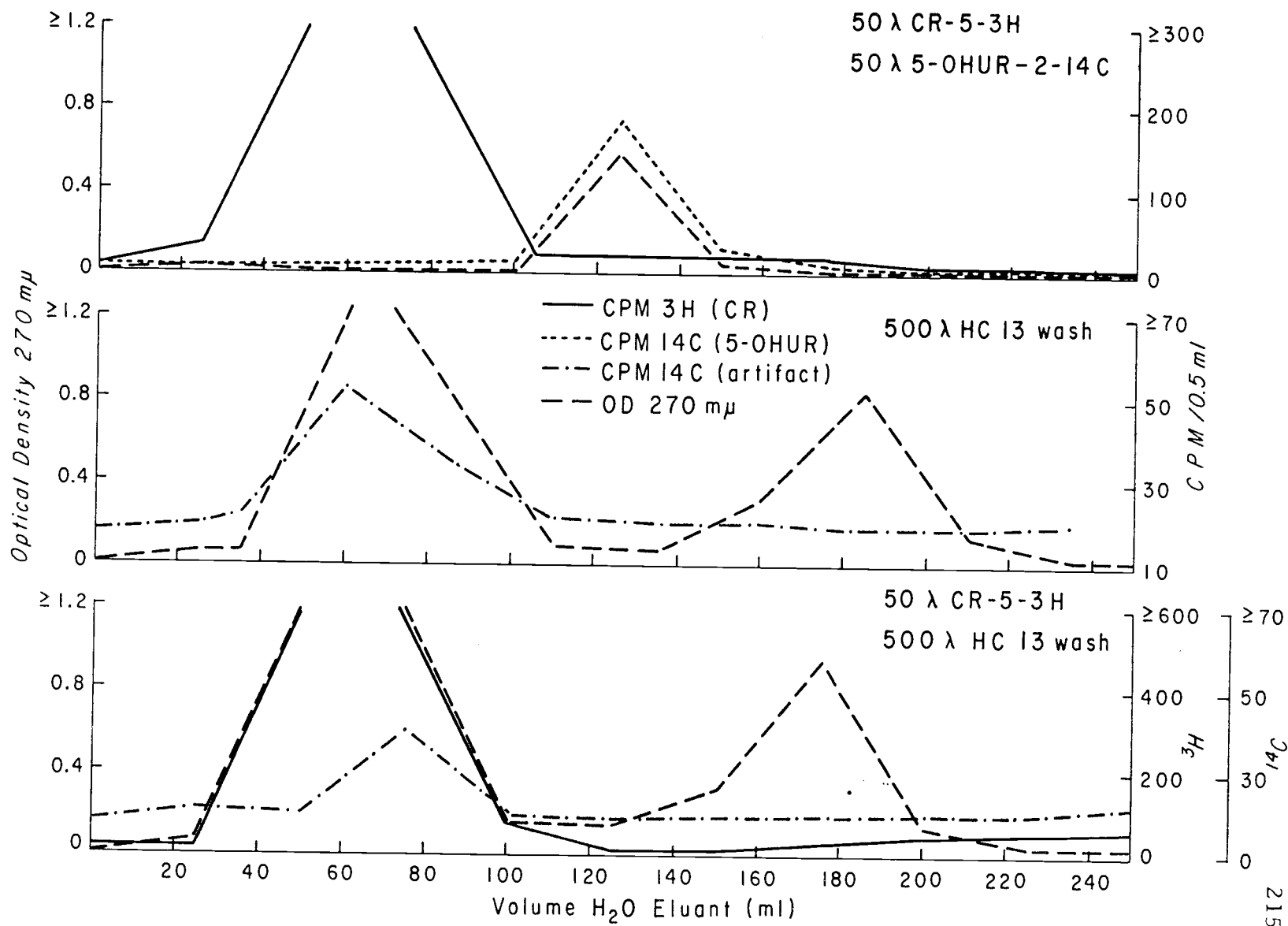
Fig. 31. Absorbance spectra of two highly purified $C^{14}$-labeled compounds obtained by different isolation procedures from a concentrate of the labeled peak that was eluted with water from celite partition column HC #13. The labeled material isolated by each method is the same and termed compound A.
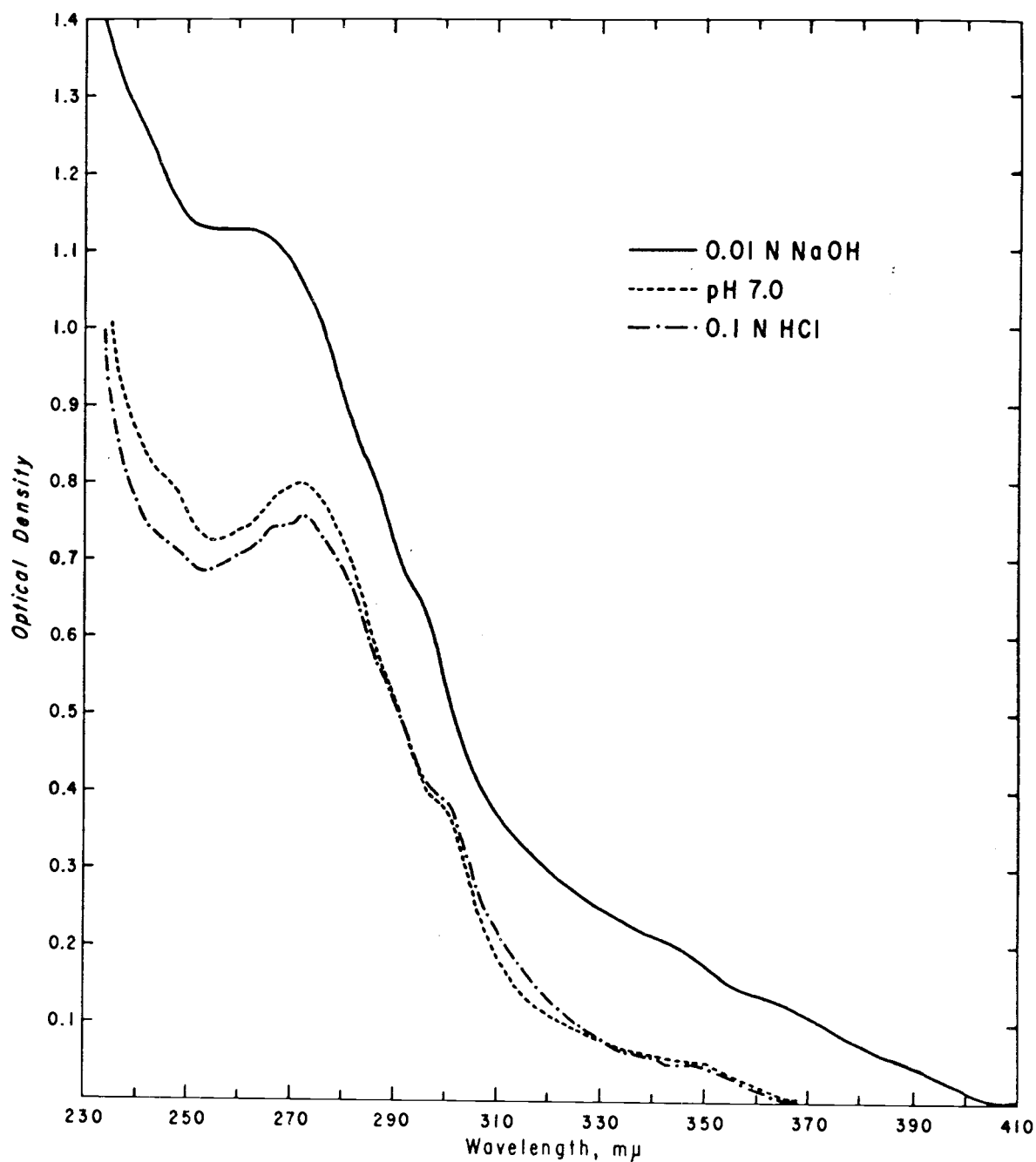
Fig. 32. Absorbance spectra of C-14 labeled compound C73-74Aa
obtained from a concentrate of the labeled peak eluted from
celite partition column HC #13 by washing the column with water
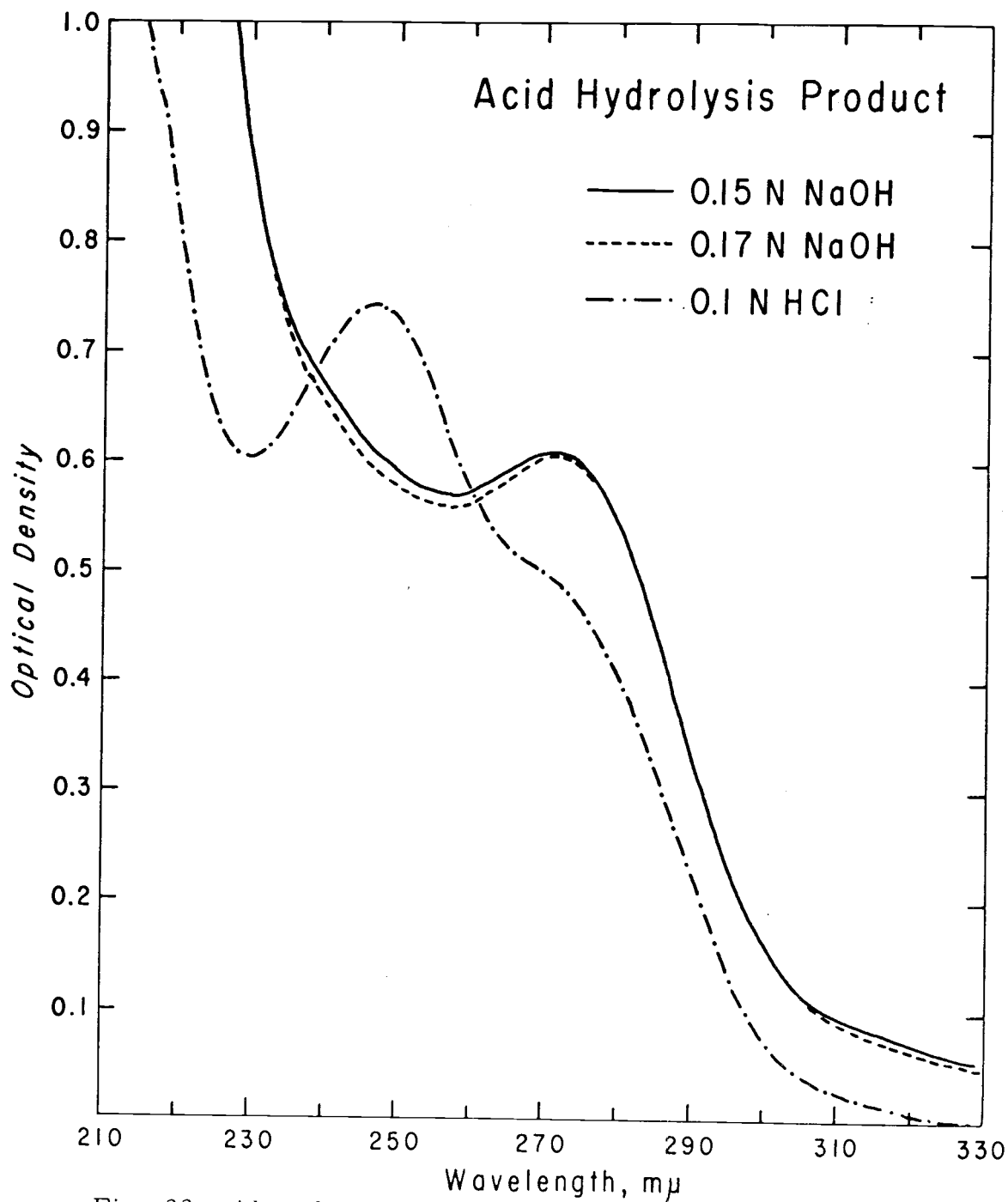(see text).

Fig. 33. Absorbance spectra of the acid hydrolysis product of Compound A. Hydrolysis was carried out at 105 C for one hour in N/10 HCL.

Fig. 34. Migration of Compound A and 5-hydroxyuridine-2-$^{14}$C control on ascending paper chromatography in 2-propanol-$H_2$O-conc HCL (680:144:170). Compound A migrates as two components: an ultraviolet absorbing spot (Compound C), and a non-absorbing labeled spot (Compound B). Areas, indicated along each chromatogram which give positive qualitative tests are shown; these include: a sugar test which detects aldo-pentose or aldo-hexose, a reduction test for the presence of dihydro-pyrimidines and B-ureido acids, and a ninhydrin test.
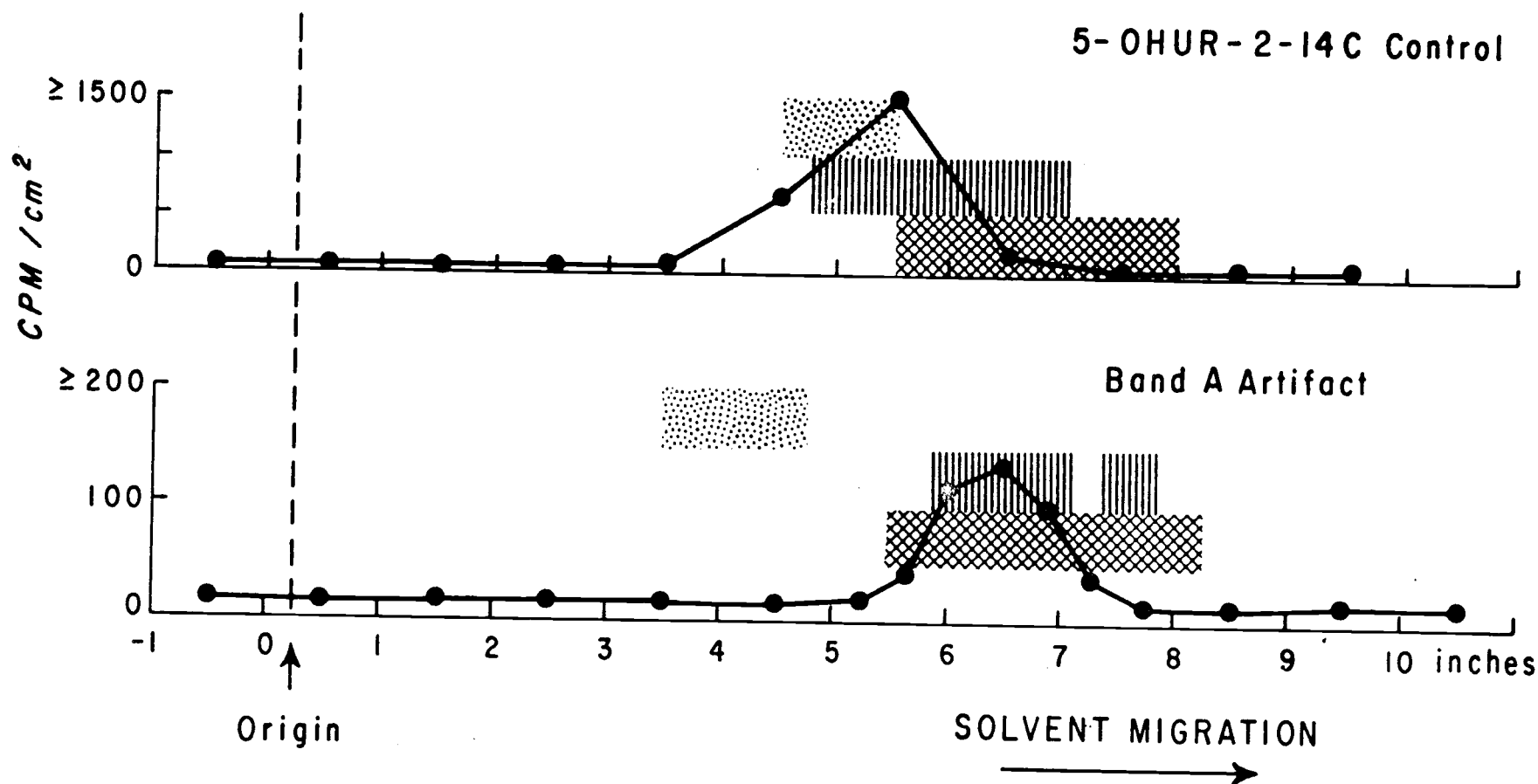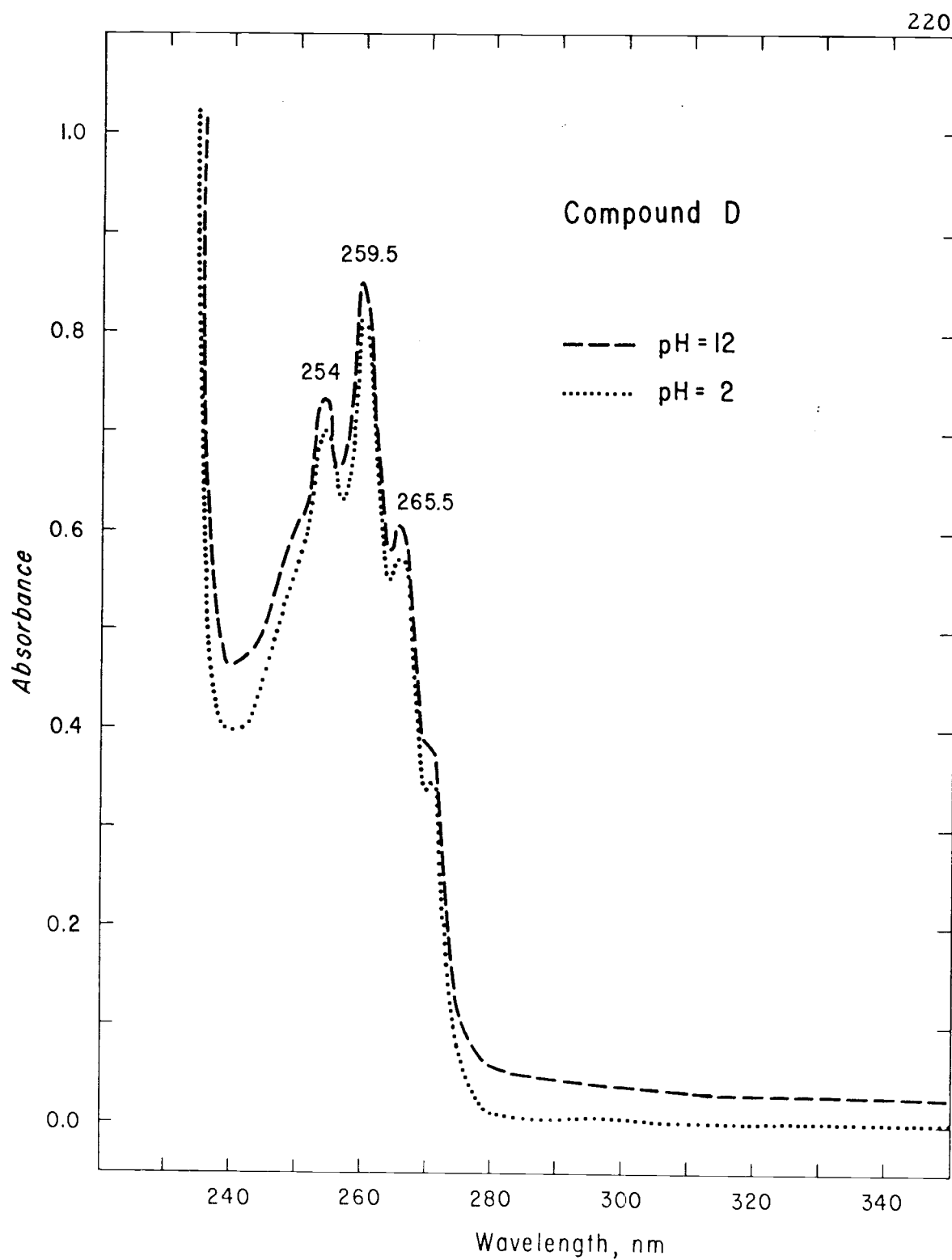
Fig. 35. Absorbance spectrum of Compound D, an unlabeled absorbing material obtained on chromatography of Compound C in butanol-$H_2$O-ethanol (5:2:3).
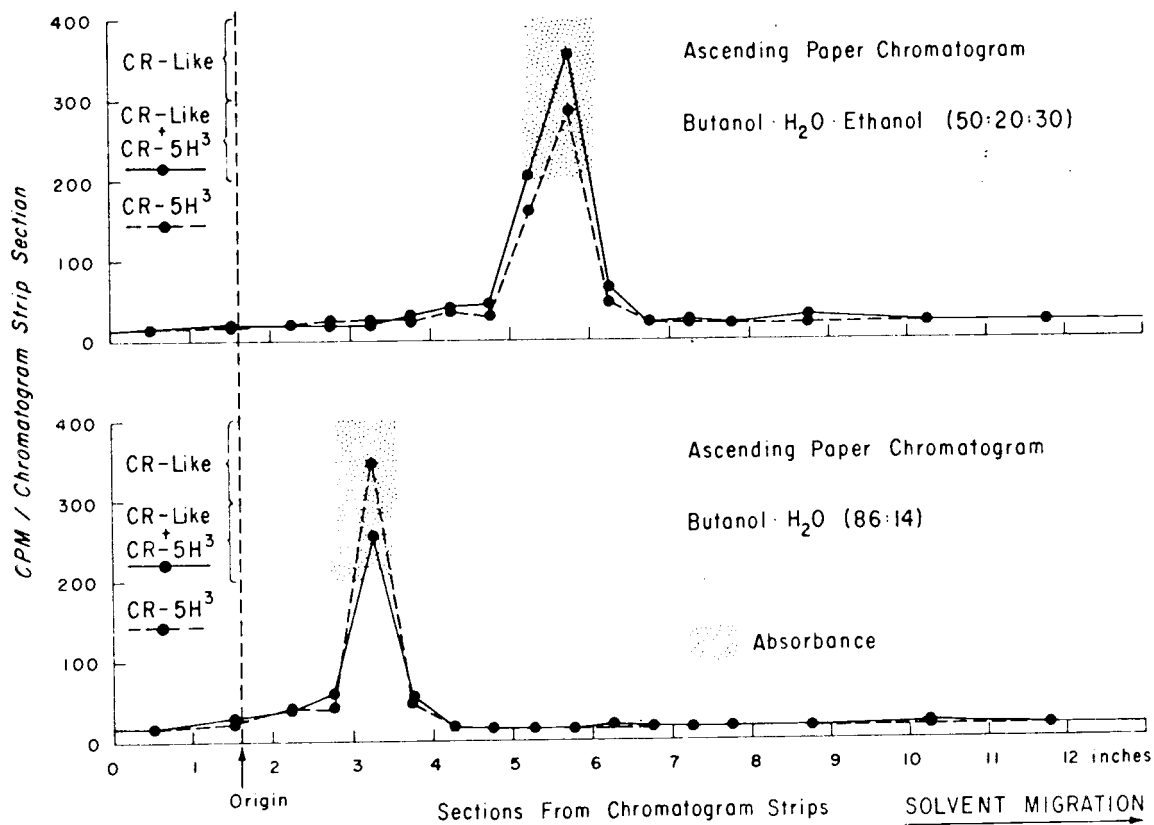
Fig. 36. Migration of a cytidine-like (CR-Like) substance, plus the CR-Like substance spiked with cytidine-5-3H, and a non-ultraviolet light absorbing quantity of cytidine-5-3H on ascending paper chromatography in both butanol-$H_2$O-ethanol (5:2:3) and butanol-$H_2$O (86:14).
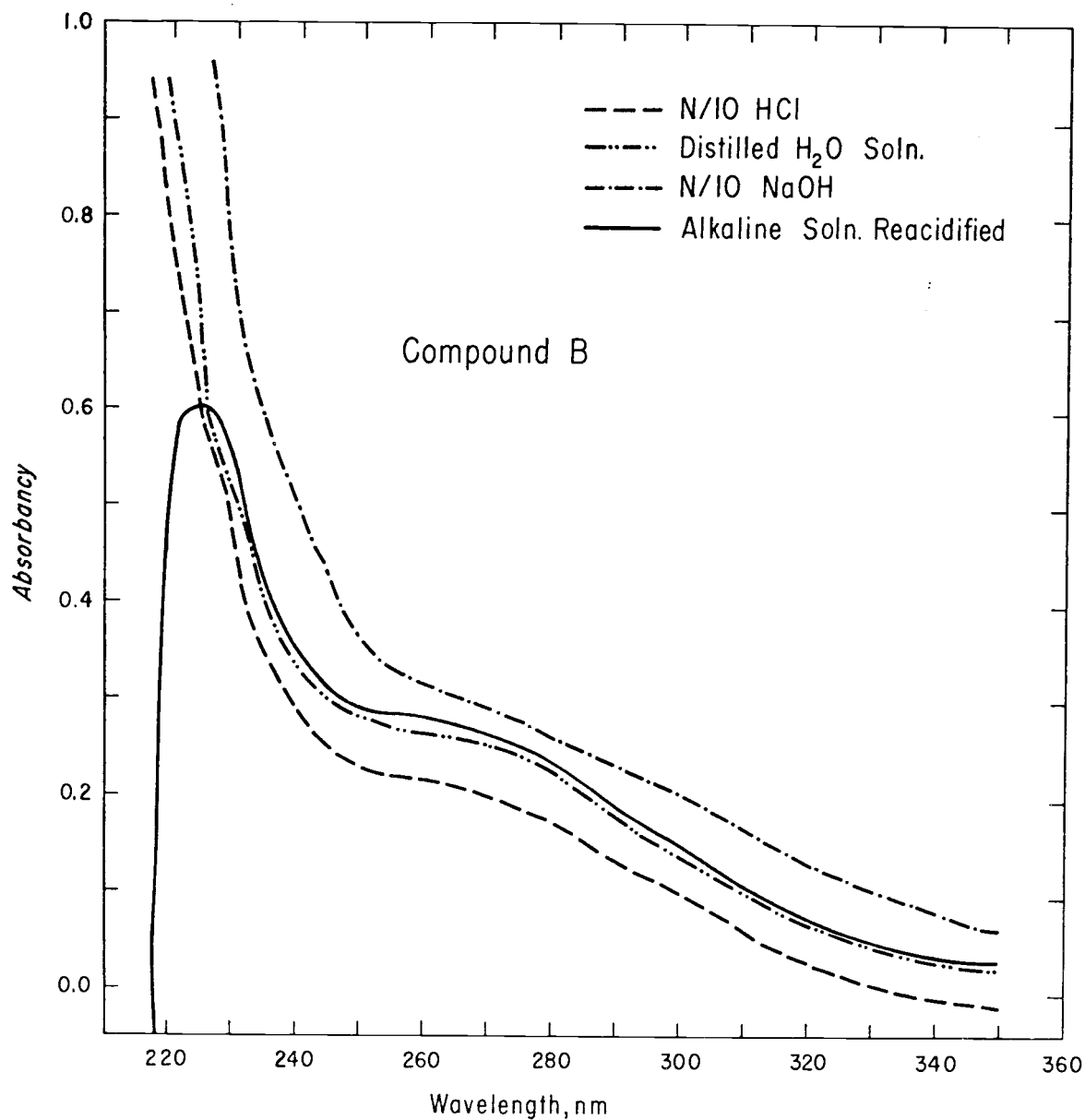
Fig. 37. Absorbance spectra of C-14 labeled Compound B. The chromatographically pure substance was a consistently obtainable derivative of the 5-hydroxyuridine-2-14C spike added to the yeast RNA enzymatic digest.

Fig. 38. Migration of Compound A, termed labeled artifact preparation, on ascending paper chromatography in 1-butanol-$H_2O$ (86:14). Graph A represents migration of 5-hydroxyuridine-2-14C; graphs B and C represent the migration of two different preparation of Compound A. The forward running material on Graph C appears to be an additional derivative of the label. The 5-hydroxyuridine-2-14C control gives a positive test for dihydropyrimidines or 3-ureido acids in the area of the major labeled peak. An explanation is presented in the text.

A,B,C Negative Sugar Test for Aldo-pentose and Aldo-hexose
B,C Negative Test for Dihydropyrimidines and β-Ureido Acids

5-OHUR-2-14C = Old H₂O Sample (Control)

Labeled Artifact Prep.

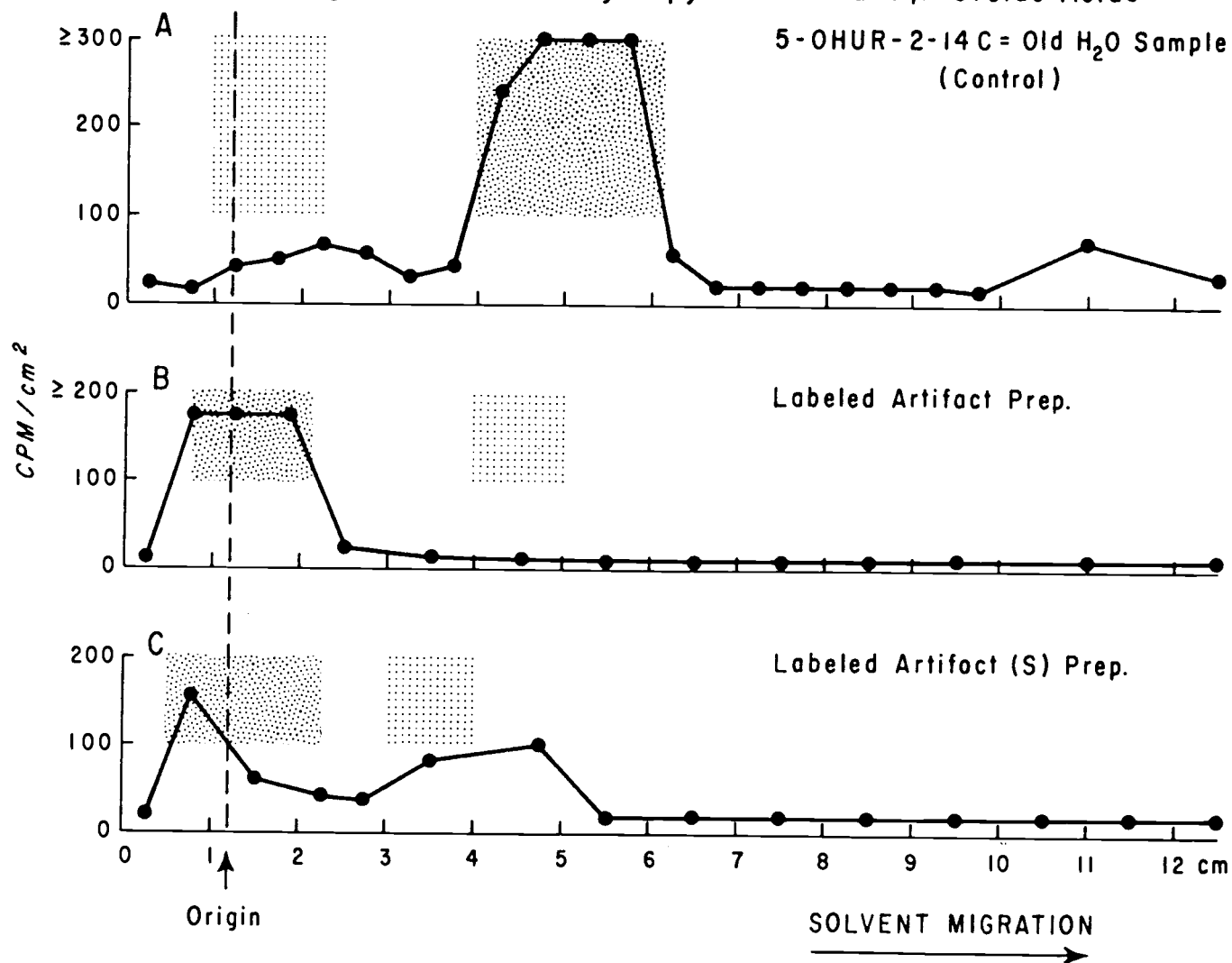Labeled Artifact (S) Prep.

Origin

SOLVENT MIGRATION

Fig. 39. Absorbance spectra of Compound M, a contaminant of a commercial preparation of guanylic acid.
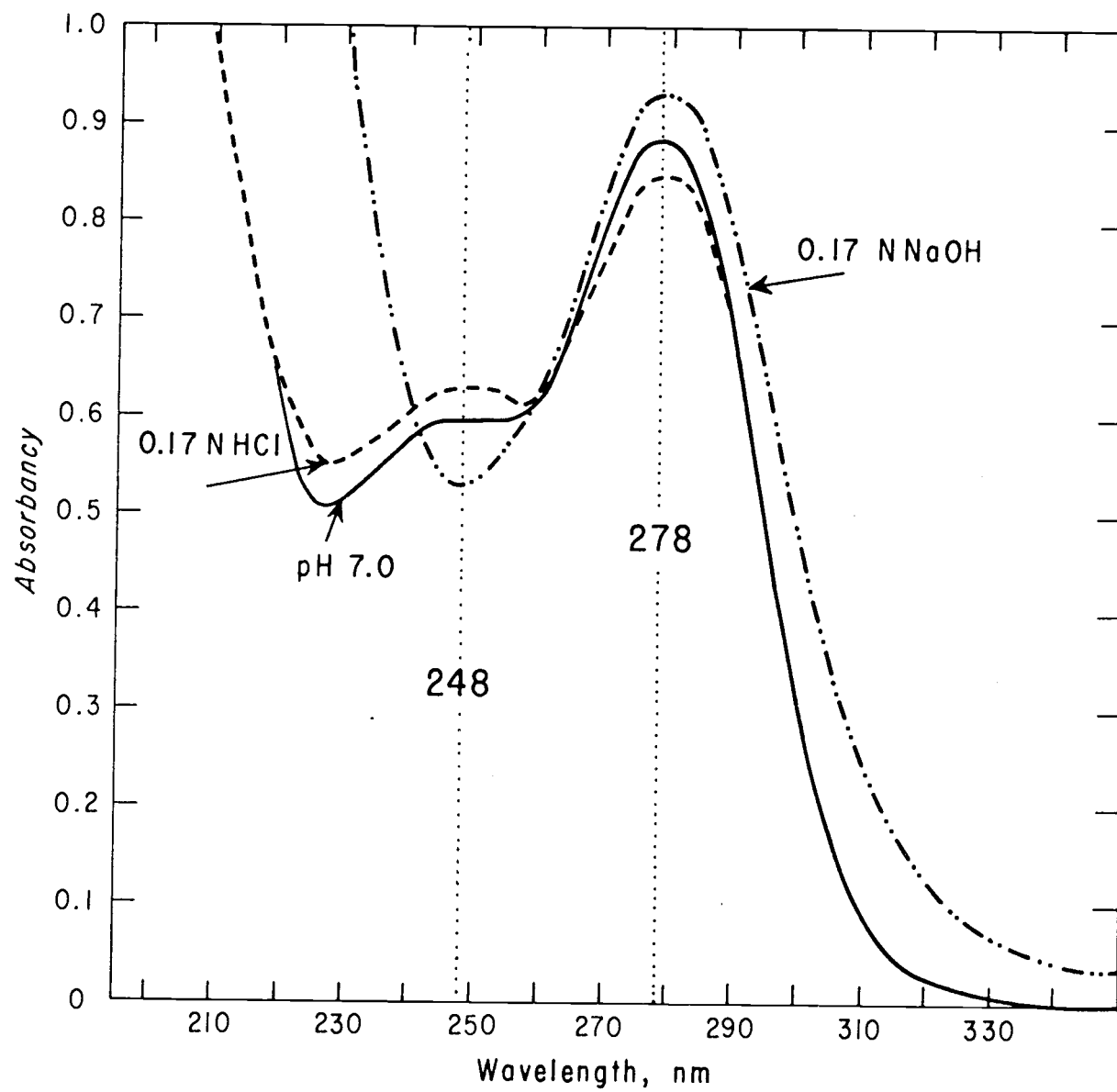
Fig. 40. Influence of partition column chromatographic solvents used to fractionate enzymatically digested RNA, on the stability of 5-hydroxyuridine at 23 $^{\circ}$C. The stability of 5-hydroxyuridine in N NH$_3$, NaOH, and 5x10$^{-3}$M MgCL$_2$ are included as controls.
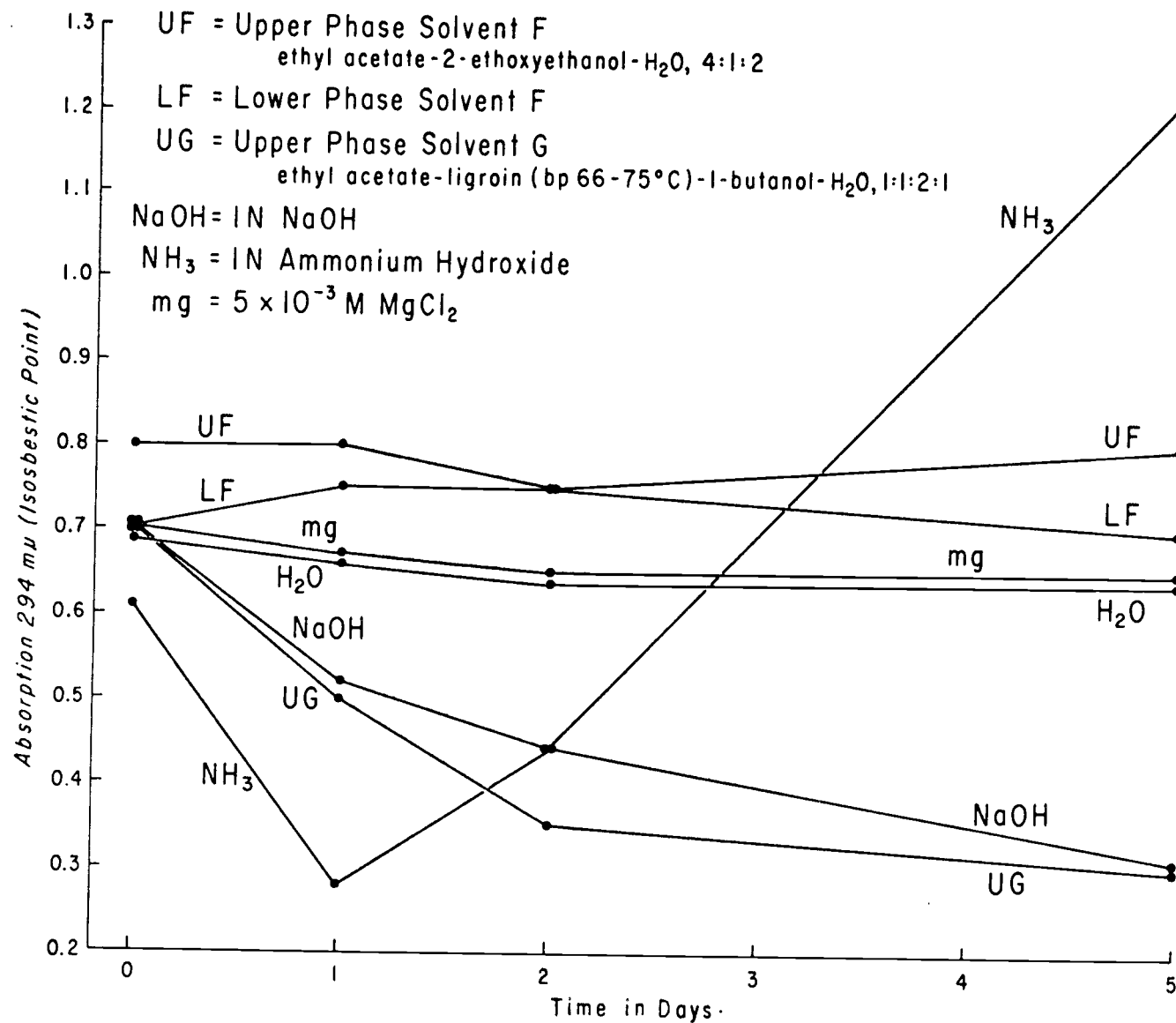
Fig. 41.   Decrease in absorbance at 2935 Å of 5-hydroxyuri-
dine at 37 °C in:  A, 5x10$^{-3}$ molar magnesium chloride; B,
distilled water; and C, 2.10 x 10$^{-2}$ molar Tris, 3.5 molar in
urea.   The inset shows the per cent change in optical density
with time at 60° C of 5-hydroxyuridine in distilled water at
indicated pH values.

5-OHUR in $5 \times 10^{-3}$M MgCl$_2$

5-OHUR in Distilled H$_2$O

5-OHUR in $2 \times 10^{-2}$M Tris, 3.5 M in Urea
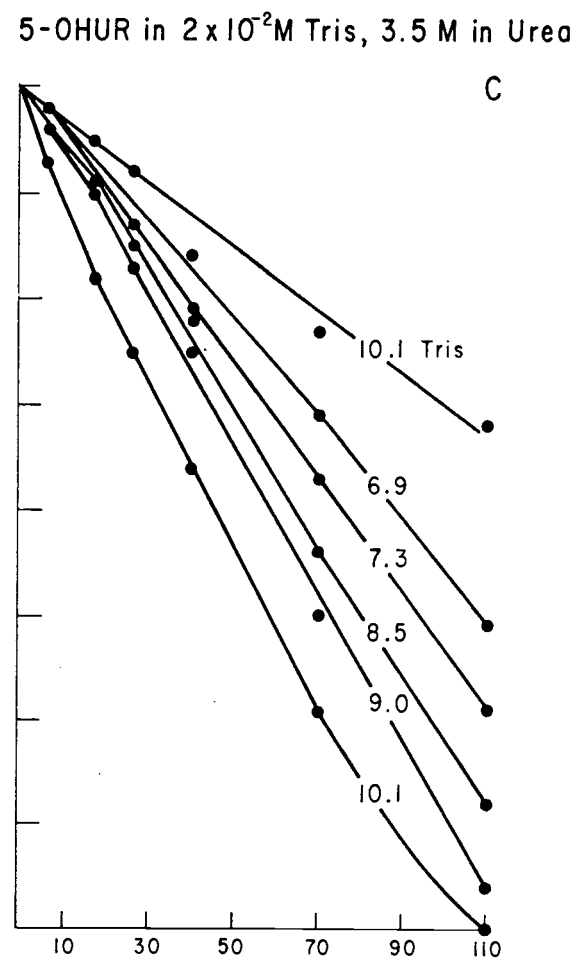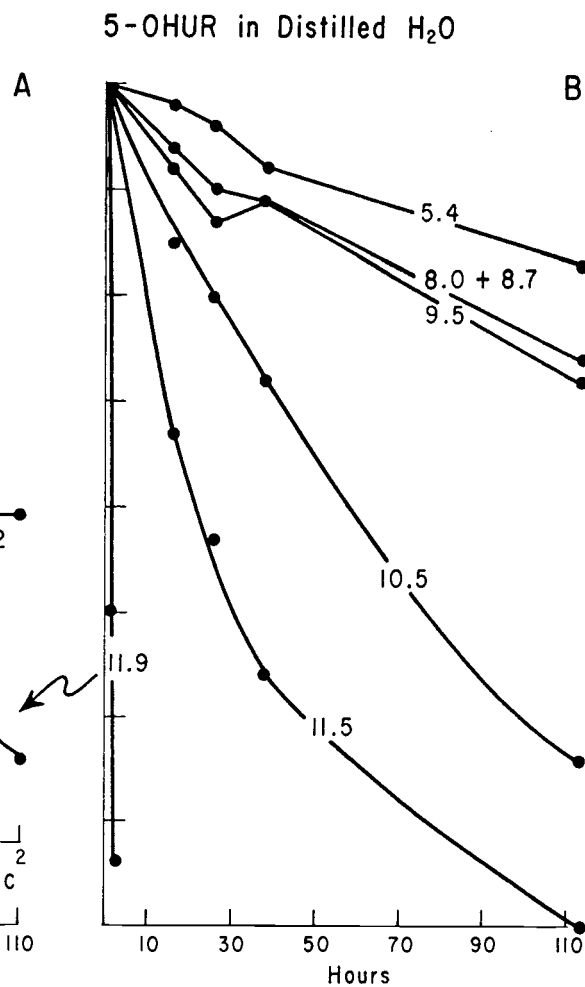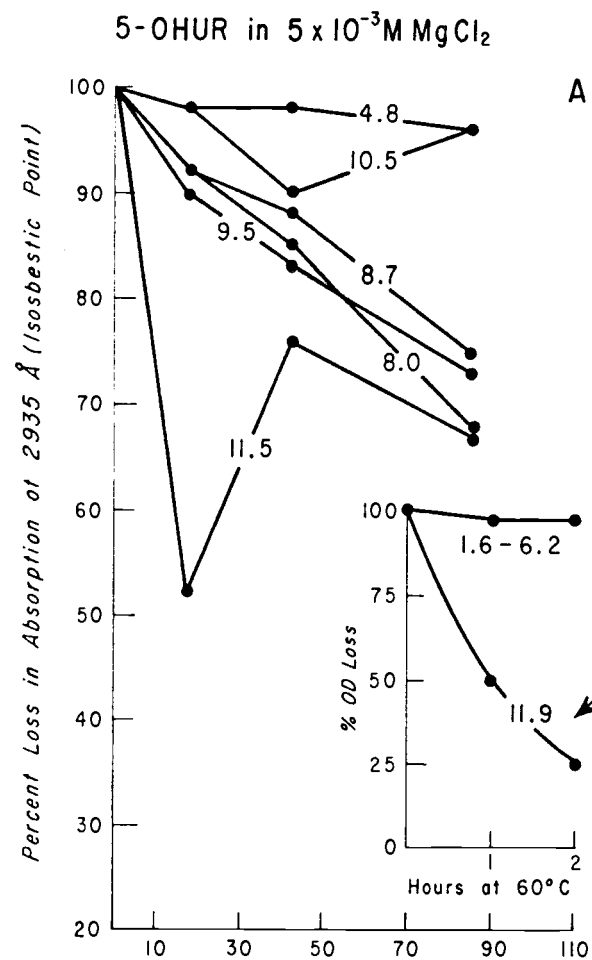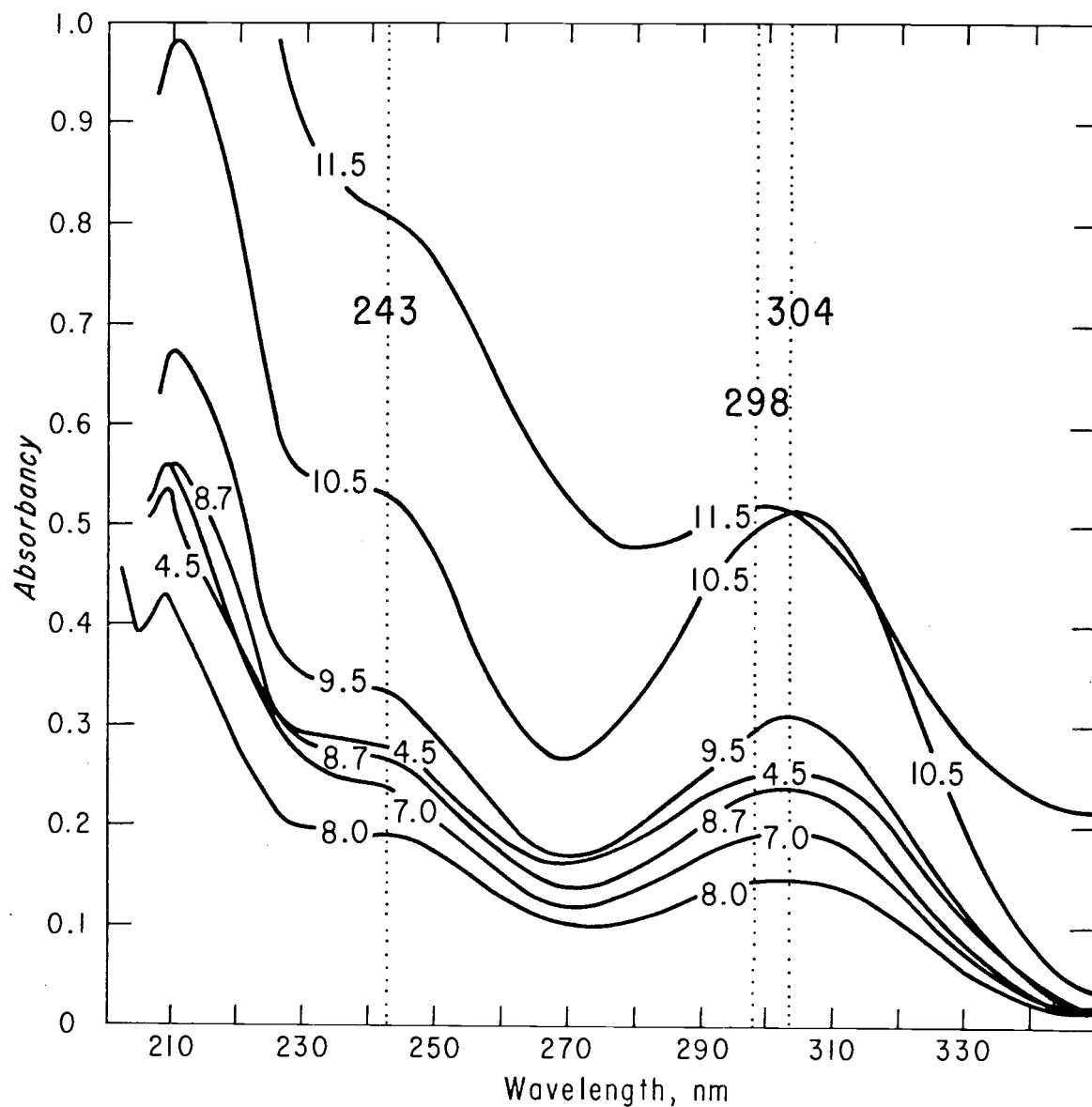
Fig. 42. Stability of 5-hydroxyuridine in 5x10-3 molar magne-
siumchloride under autoclaving at 121°C for 15 minutes at 15
pounds pressure. The origional pH of the solution is indicated.
On autoclaving these values changed from: 4.6 to 7.5, 7.0 to
8.0, 8.7 to 8.5, 9.5 to 8.4, and 10.5 to 8.7; the solution at pH
11.5 did not change, but crystals of magnesium hydroxide were
present (see text).

Fig. 43. Decrease in absorbance at 2935 Å of 5-hydroxyuridine in various solutions with time. A control in distilled water adjusted to pH 2.4 at 37° C; B control, 0.3N KOH at 4 °C; C 0.17M Tris, pH 8.8, 37° C; D N NH$_3$ at room temperature (23 °C); E 0.3N KOH at 37 °C; F N/10 and N NH$_3$ at 37° C; G 2M (NH$_4$)$_2$CO$_3$ pH 9.0 at 37°C.
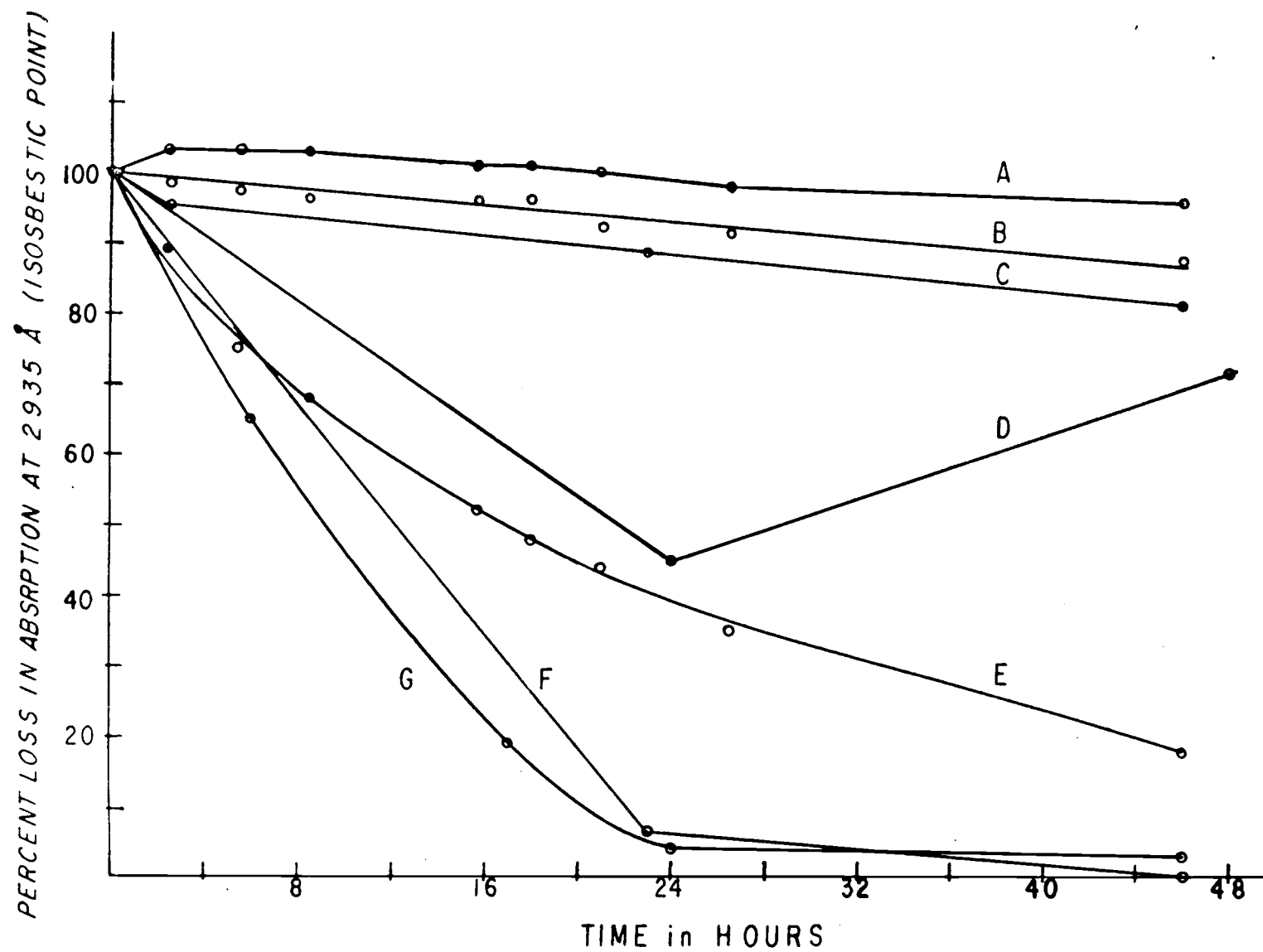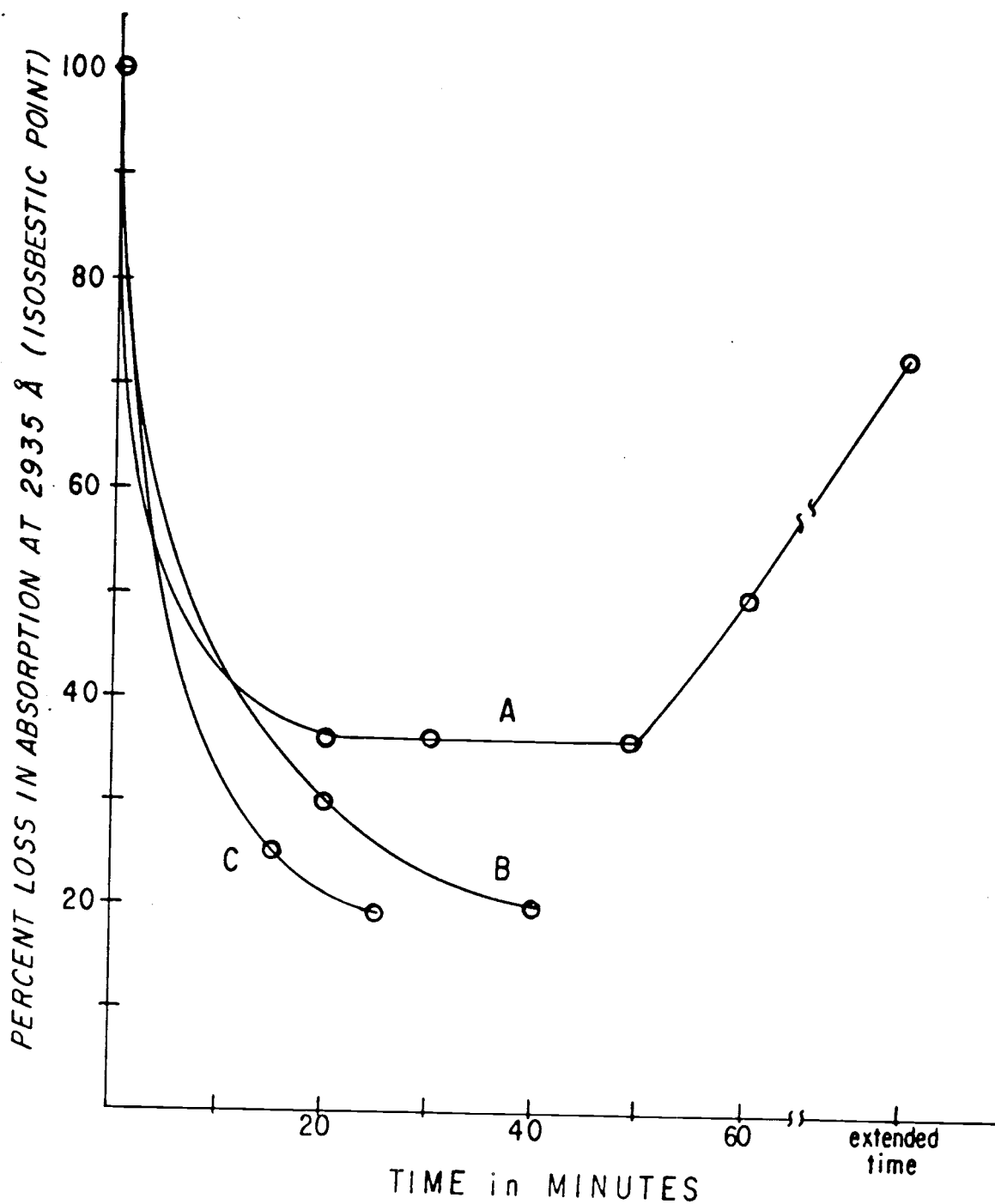
Fig. 44. Change in absorbance at 2935 Å of 5-hydroxyuridine with time under RNA hydrolytic conditions in KOH. A, in N KOH at 80° C, B, N/20 KOH at 100° C; C, N/10 KOH at 100° C.

Fig. 45. Course of reaction of 5-hydroxyuridine in N KOH at 80°C. Change in absorbance with time measured in N KOH (— curves B-D) and at pH 1 (....., curves B'-D'). Curve A is zero time (...-...); B and B', 20 minutes, C and C', 60 minutes; D and D', extended time; alkalization of acidified extended time sample ( ---- ) to approximately pH 11. The forming substance with absorbance maxima at 307 and 319 nm has been termed Compound R.
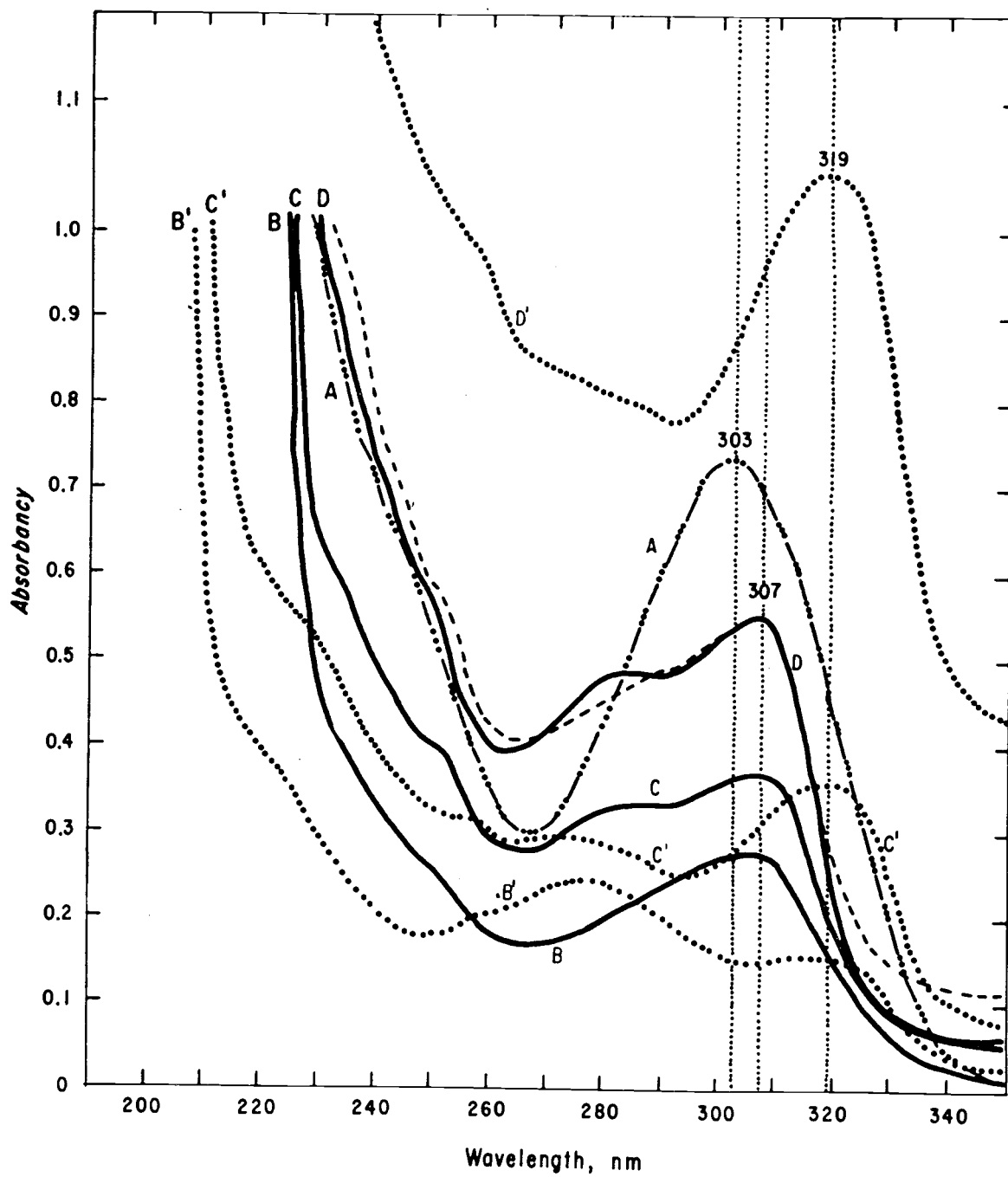
Fig. 46.  Change in absorbance at 2935 Å  of 5-hydroxyuridine
with time under RNA hydrolytic conditions in piperidine at
100° C. In 10 per cent (A) and 11 per cent (B) piperidine.
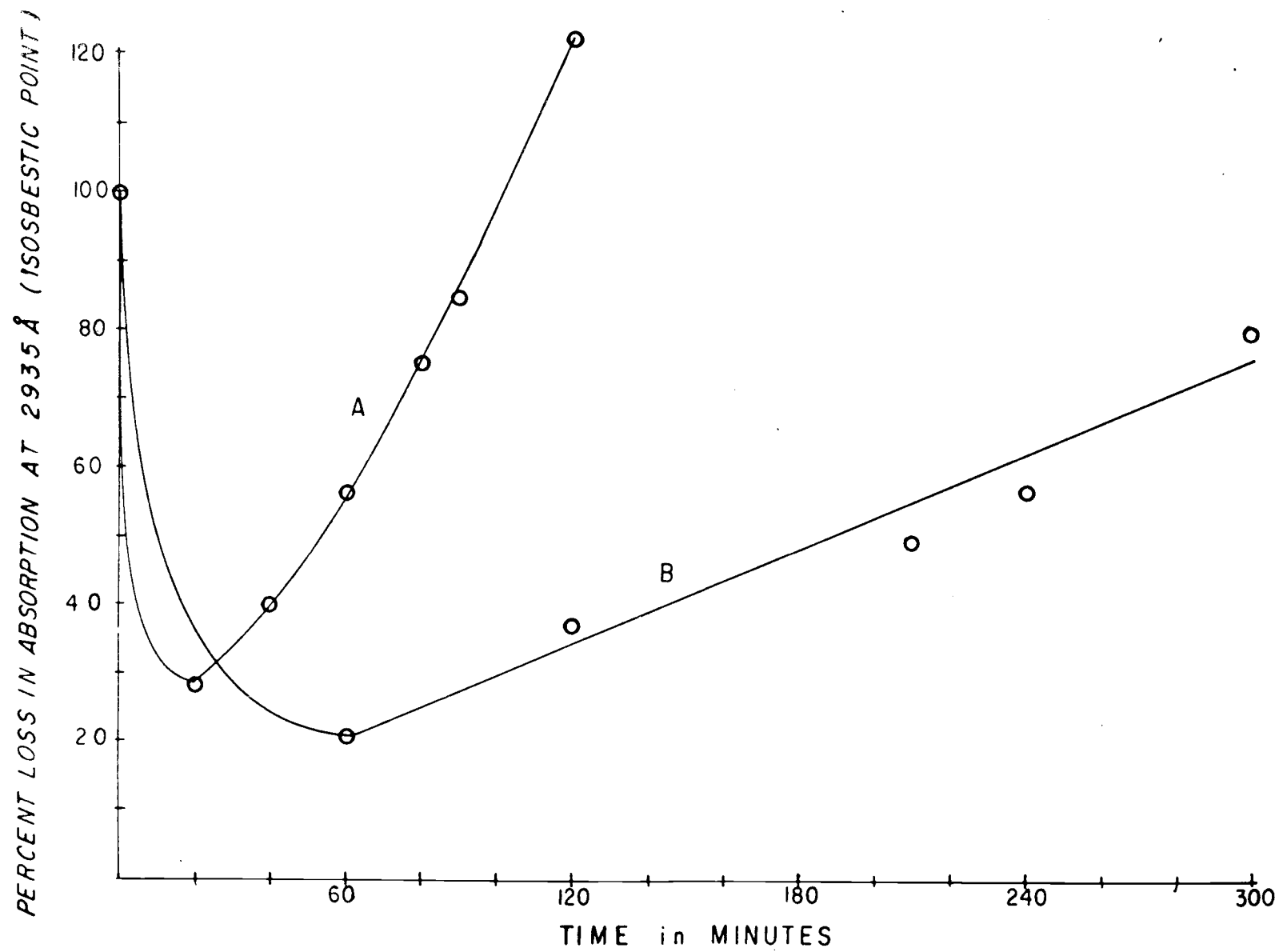
Fig. 47. Course of reaction of 5-hydroxyuridine in 1 per cent piperidine at 100° C. Changes in absorbance with time measured at pH 1 ( ...., curves B'-E'), and in 1 per cent piperidine (pH 12.0) ( ___, curves B-E). Curves A and A' (..._...), zero time, pH 1 and 12 respectively; B and B', one hour; C and C', 2 hours; D and D' 4 hours; E and E', 5 hours; difference spectrum of a 1 per cent solution of piperidine in blank and sample cell (.-.-.); alkalization of 5 hour acidified sample (---) to approximately pH 11. The forming substance with absorbance maxima at 308 and 320 nm has been termed Compound R.
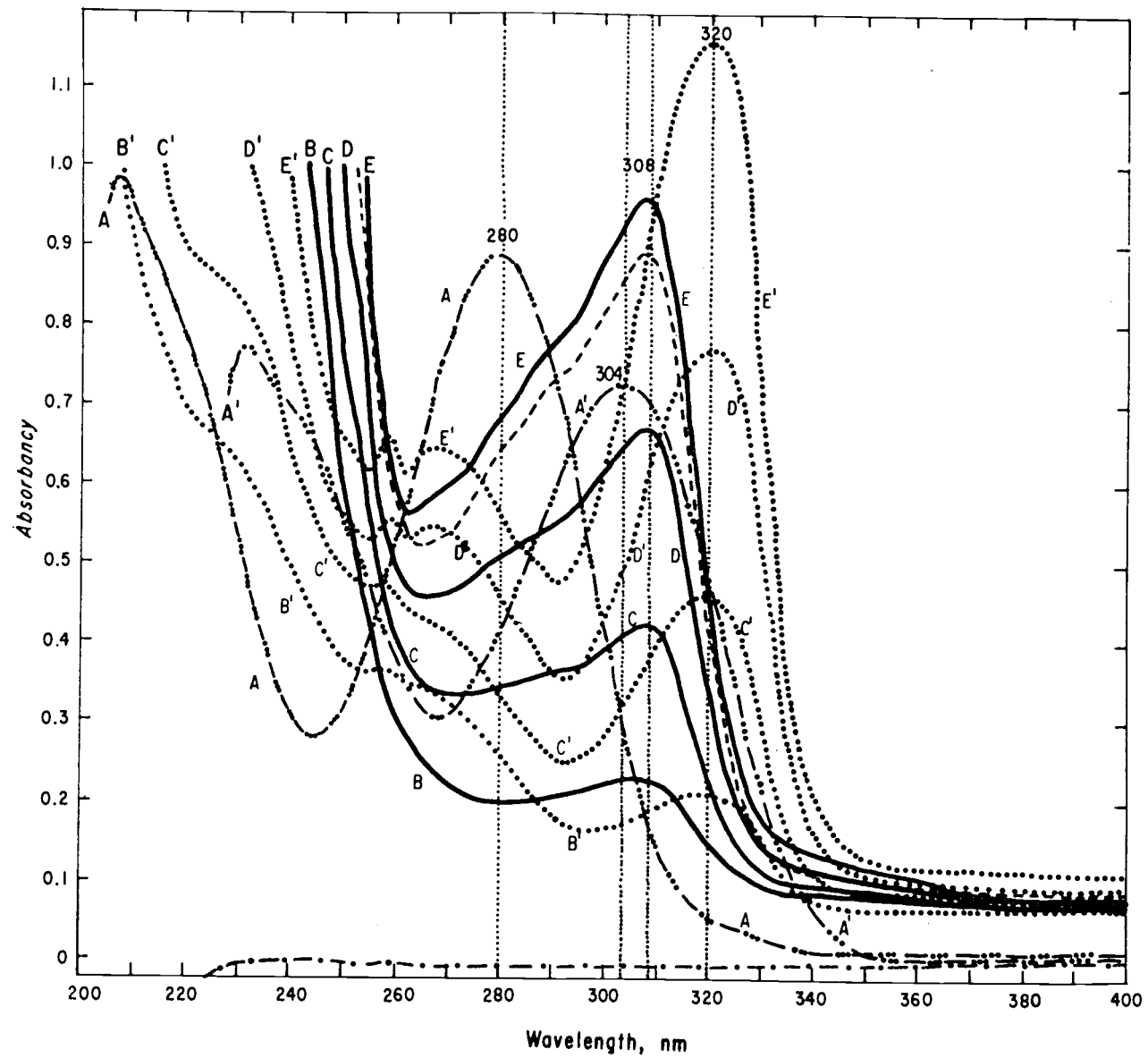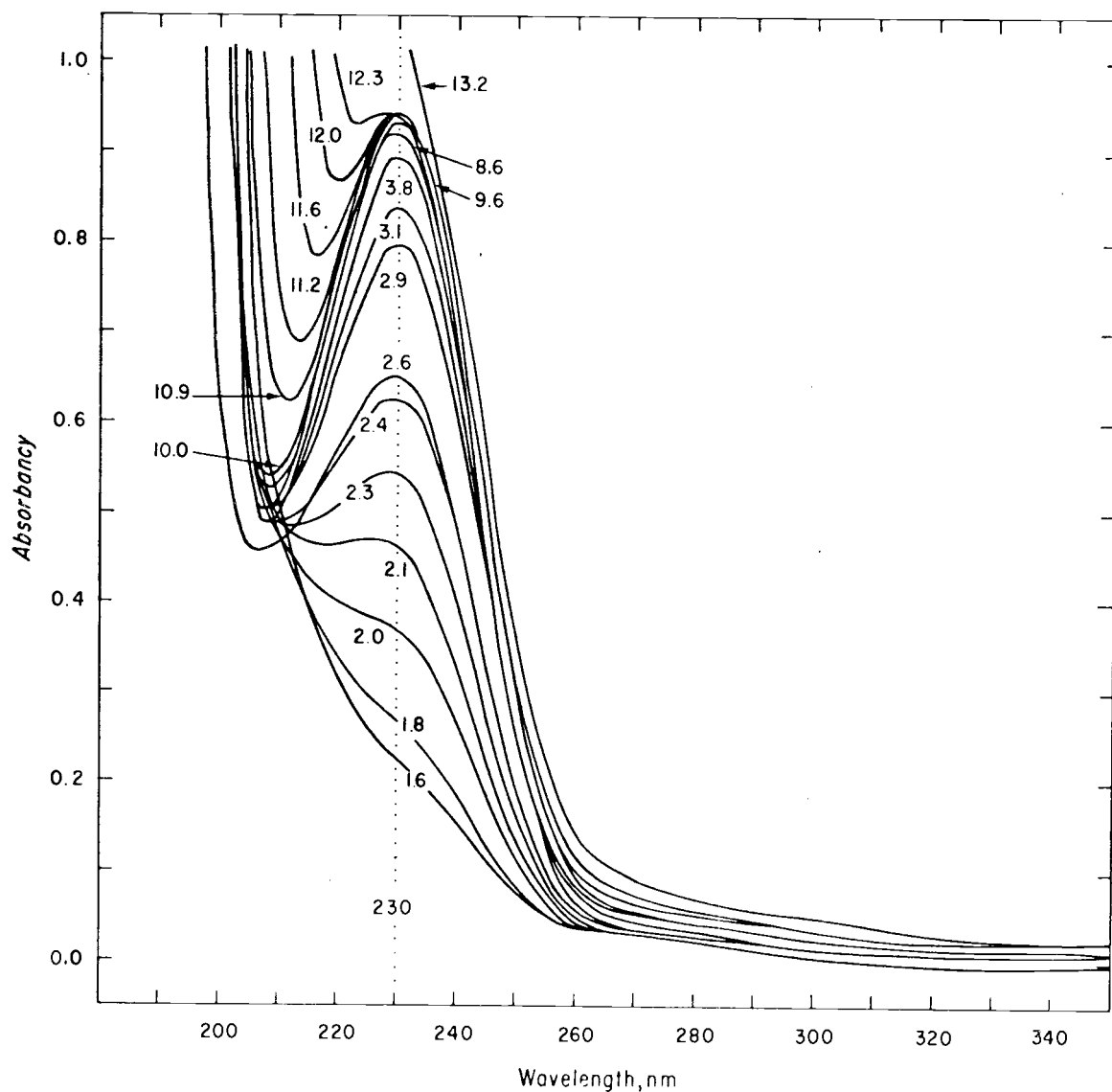
Fig. 48. Absorbance spectra of Compound H$_{28}$. This derivative of 5-hydroxyuridine was prepared by refluxing a 0.9A$_{280}$ units/ml/10mm solution of 5-hydroxyuridine in five per cent piperidine at 100°C for 40 minutes. The substance is stable in acid and alkali.
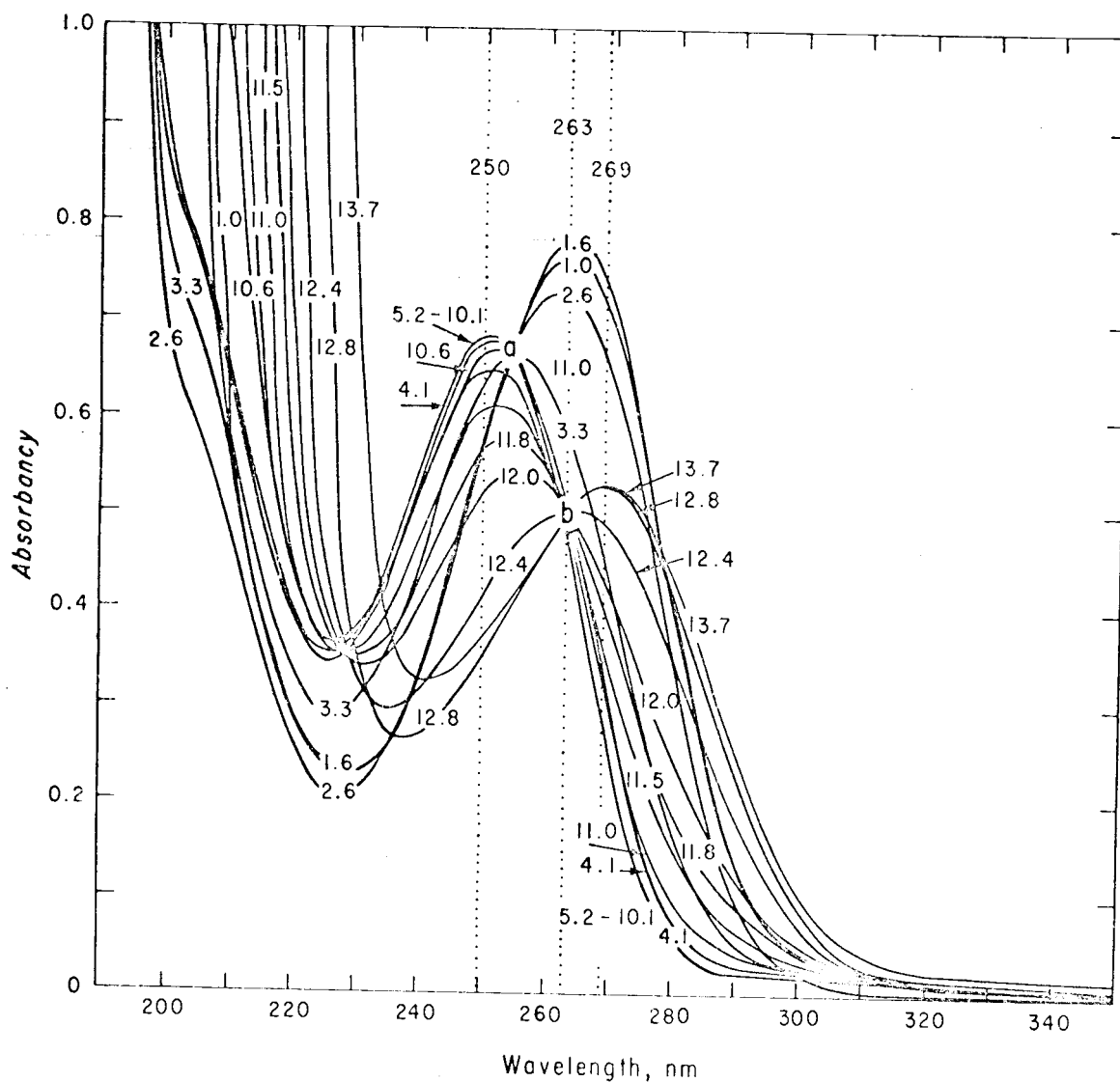
Fig. 49.  Absorbance spectra of Compound P (1-(β-D-ribofura-nosyl)-  -oxo-4-imidazoline-4-carboxylic acid).  This deriva-tive of 5-hydroxyuridine was prepared by reacting a $90A_{280}$ units/ml/10mm solution of 5-hydroxyuridine in one or ten per cent piperidine in a water bath at $98^{\circ}$ C for three hours.

Migration of 5·Hydroxyuridine & Synthetic Derivatives in Butanol · $H_2O$ (86-14)

|||||||| Positive Test for
Aldo-Pentose or Aldo-Hexose

Absorbance

Positive Test for
Dihydropyridines & β-Ureido Acids

5·OHUR

5·OHUR

Negative Sugar Test

$RI_{28}$

$RI_{28}$

$RI_{26}$

$RI_{26}$

Negative Test for
Dihydropyrimidine

0     1     2     3     4     5     6     7     8   inches

Origin

SOLVENT MIGRATION

Ascending Paper Chromatogram
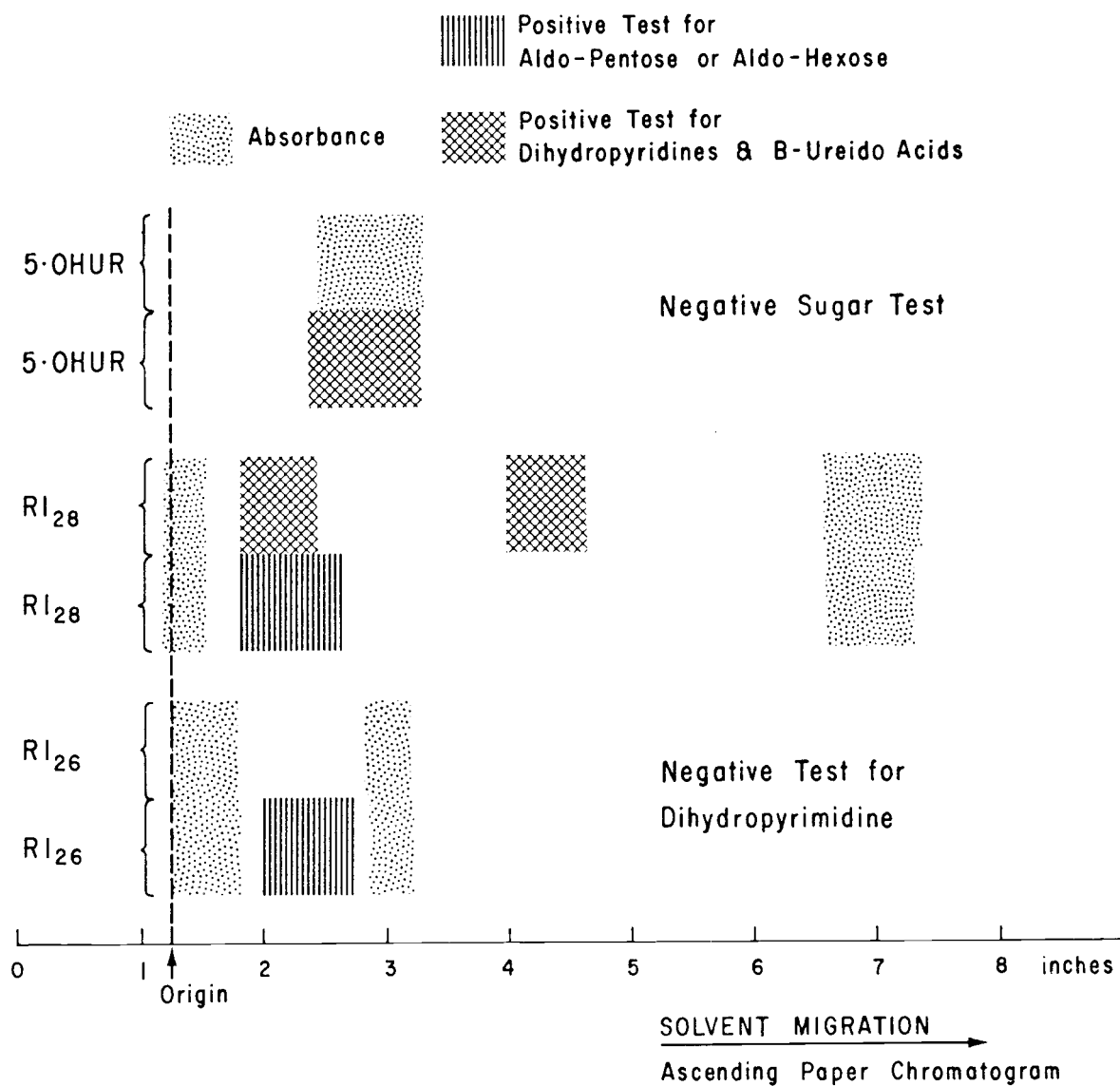
Fig. 50. Migration of 5-hydroxyuridine control and reaction mixture $RI_{28}$, containing compound $H_{28}$- the most forward running absorbing band - and reaction mixture $RI_{26}$, containing compound P - the absorbing band along the origin - on ascending paper chromatography in Butanol-$H_2O$ ( 86:14).
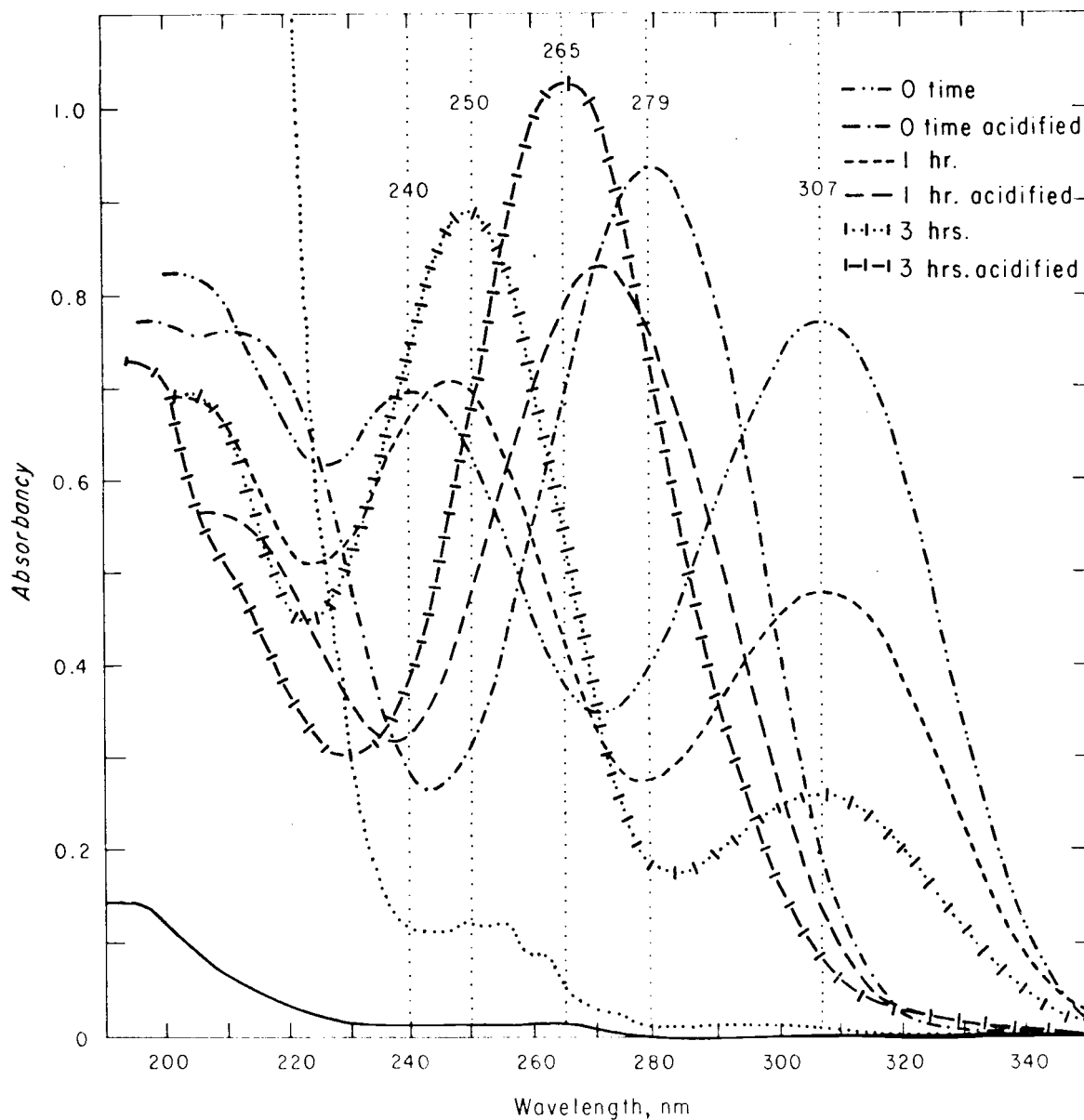
Fig. 51. Course of the formation of Compound P in one per cent piperidine with time at 98 C. All spectra represent a 1/100th dilution of the reaction mixture. The acidified spectra of zero time, one and three hours reaction aliquots were prepared by adding enough 12 N HCL to the diluted piperidine sample aliquot and blank to bring the aliquot to approximately pH one. The dotted line represents the absorbance of a 1/100th dilution of a one per cent piperidine solution against a water blank.
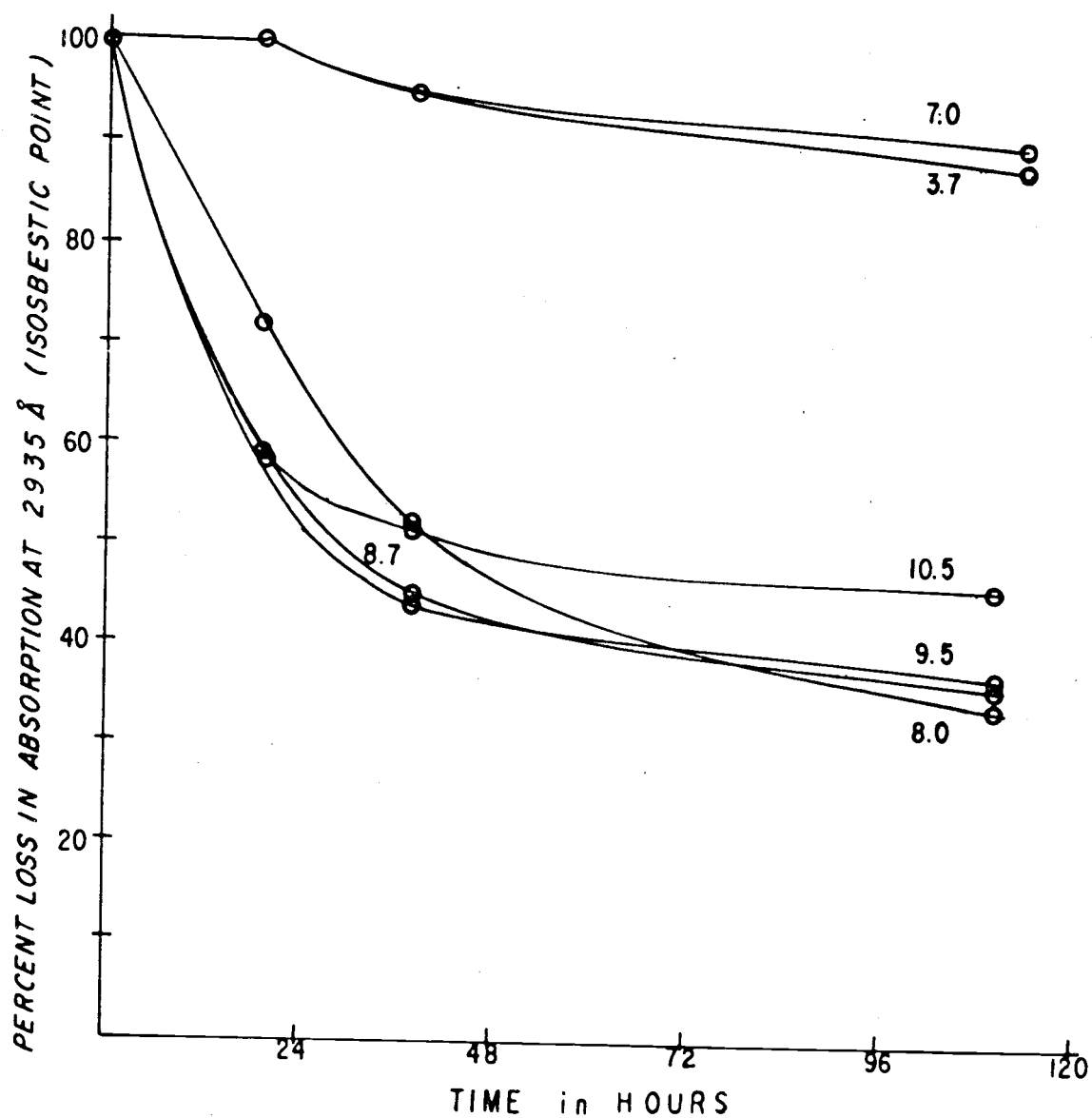
Fig. 52. Change in absorbance at 2935 Å of 5-hydroxyuridine with time at indicated pH's at 37 C in 5.86 x $10^{-2}$ molar hydroxylamine hydrochloride.
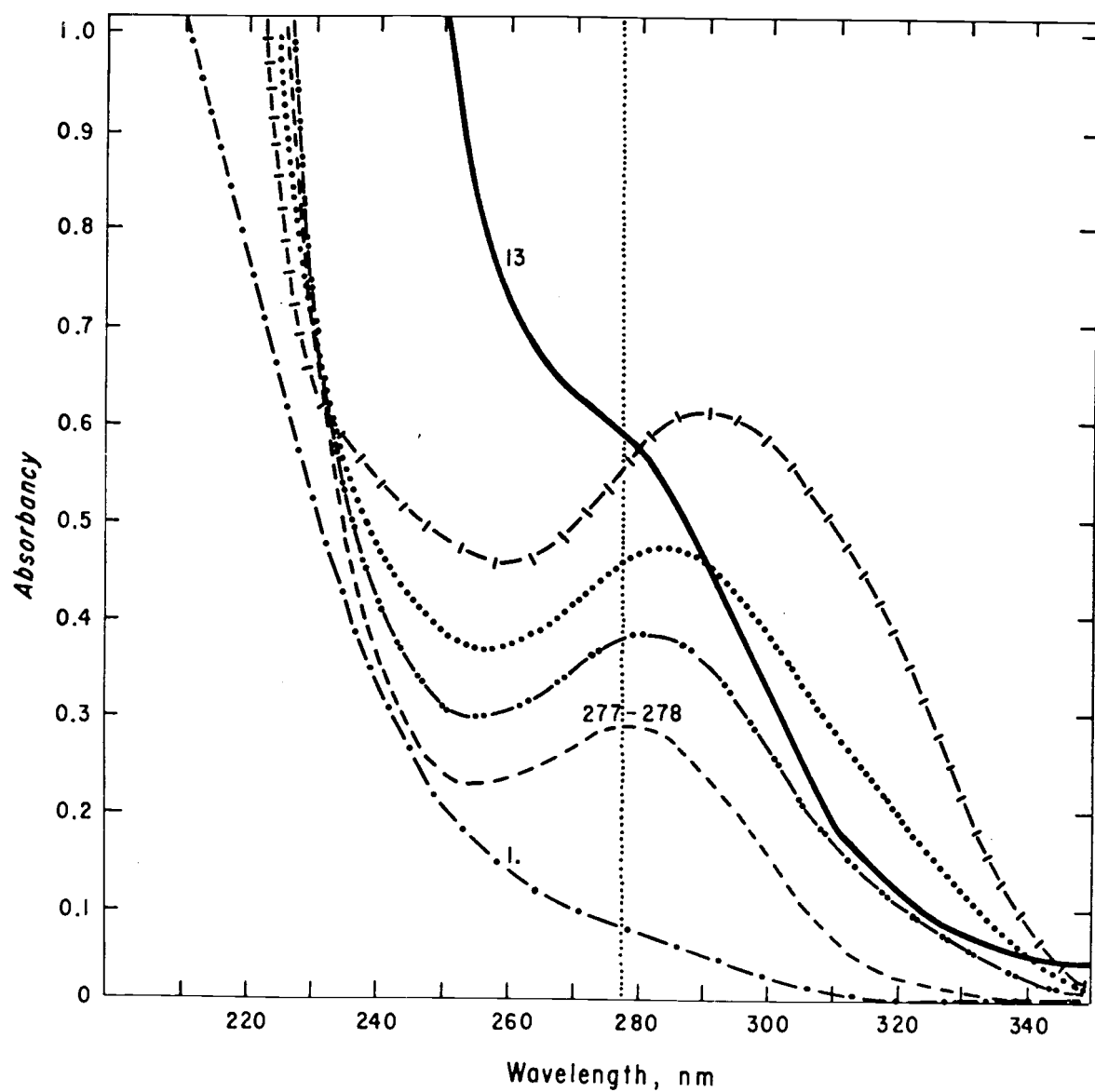
Fig. 53. Course of reaction of 5-hydroxyuridine in $5.86 \times 10^{-2}$ molar hydroxylamine at pH 8.0 at $37^{\circ}$ C. Absorbance, zero time ( l-l-l ), 19 hrs. ( .... ), 33 hrs. ( ..-.. ), 110 hrs. ( --- ).

Fig. 54. Course of reaction of 5-hydroxyuridine in 5.80 x 10$^{-2}$ molar hydroxylamine at pH 10.5 at 37 C. Absorbance, zero time ( 1-1-1 ), 19 hrs. ( ..... ), 110 hrs. ( --- ). Changes in absorption at 19 hrs. measured at pH 1 ( .-.-. ) and 13 (——).

Fig. 55. Course of reaction of 5-hydroxyuridine in $5.86 \times 10^{-2}$ molar hydroxylamine at pH 11.6, at $37^\circ$ C. Absorbance, zero time (1-1-1-), 19 hrs. (...), 33 hrs ( - ), 110 hrs. ( ---). Absorbance 110 hrs., pH 1 (. -. -. -).
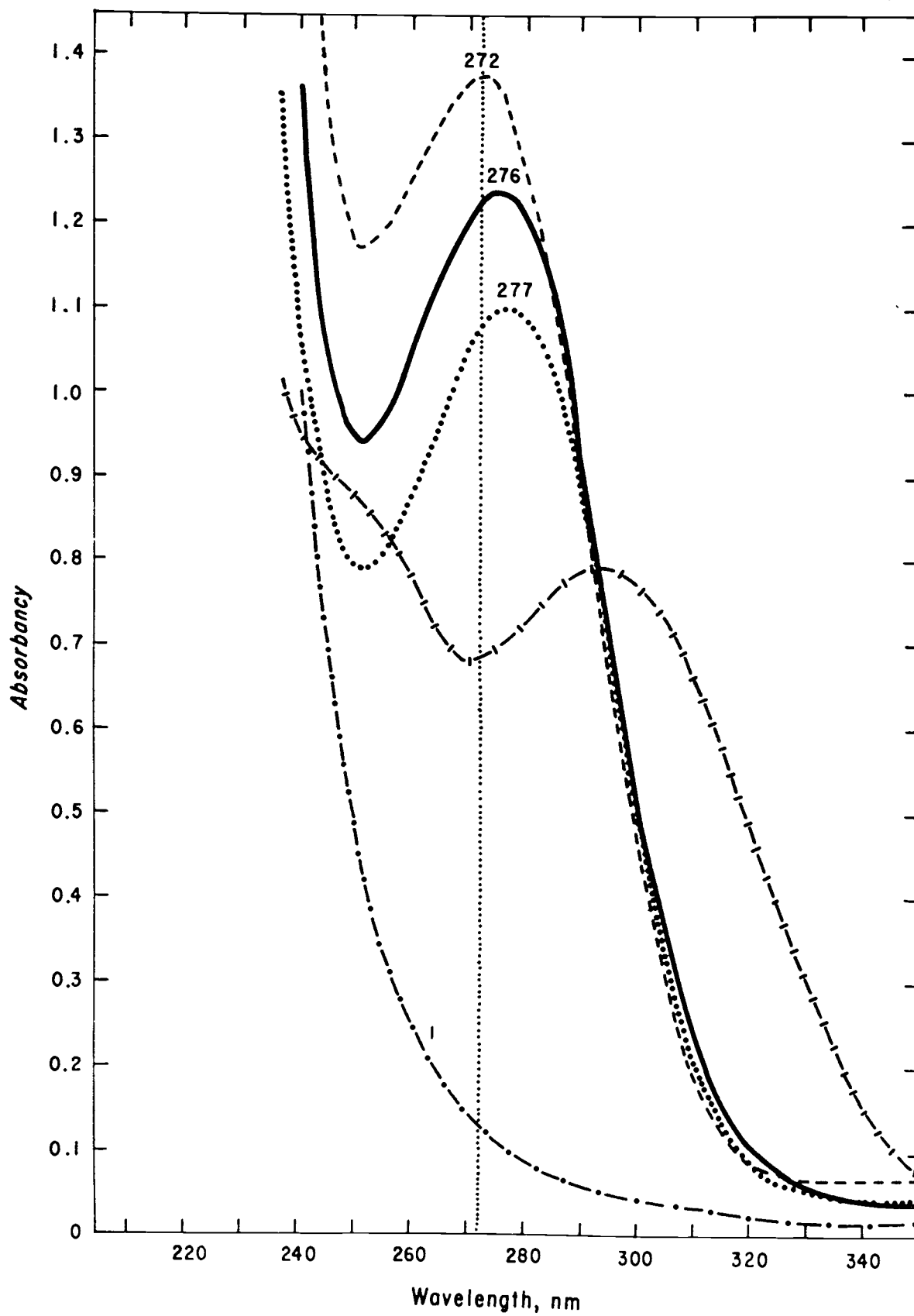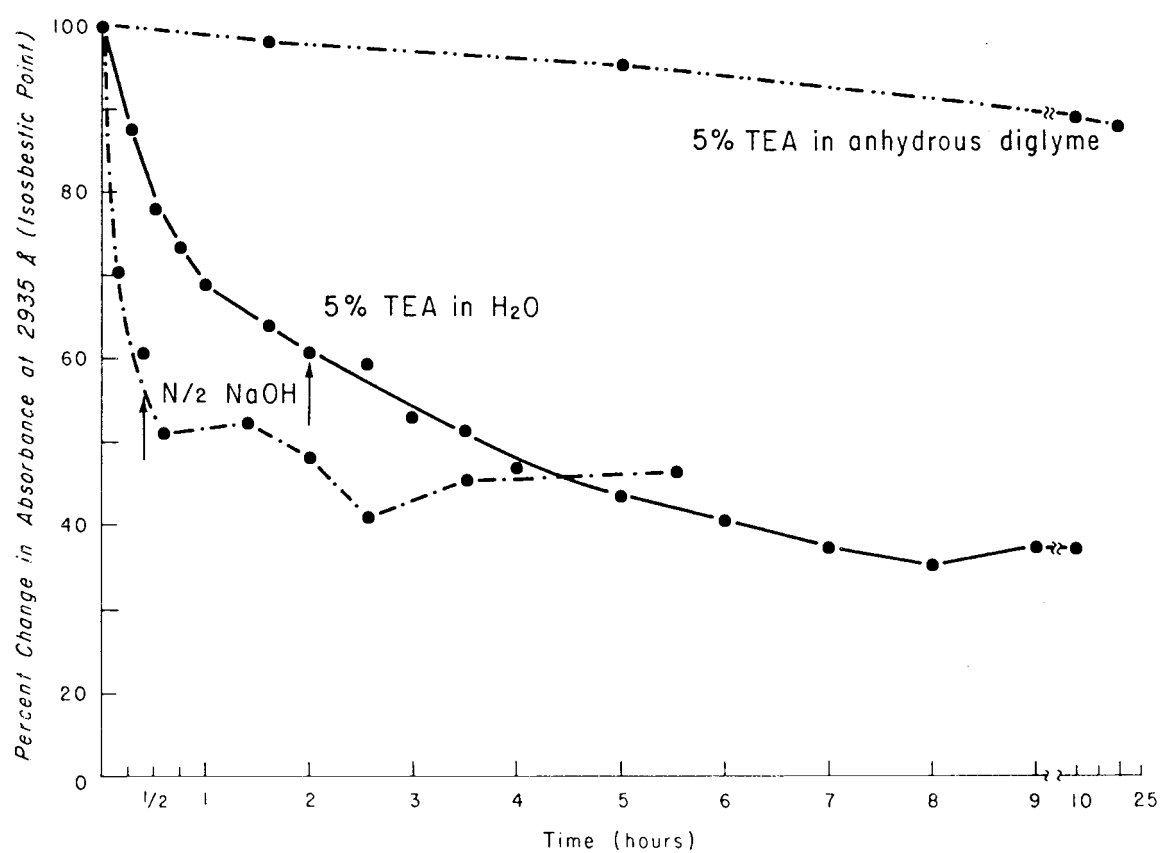
Fig. 56. Loss of absorbance of 5-hydroxyuridine in N/2 sodium hydroxide, aqueous five per cent triethylamine, and in anhydrous diglyme, 5 per cent in triethylamine at $78^{\circ}$ C.
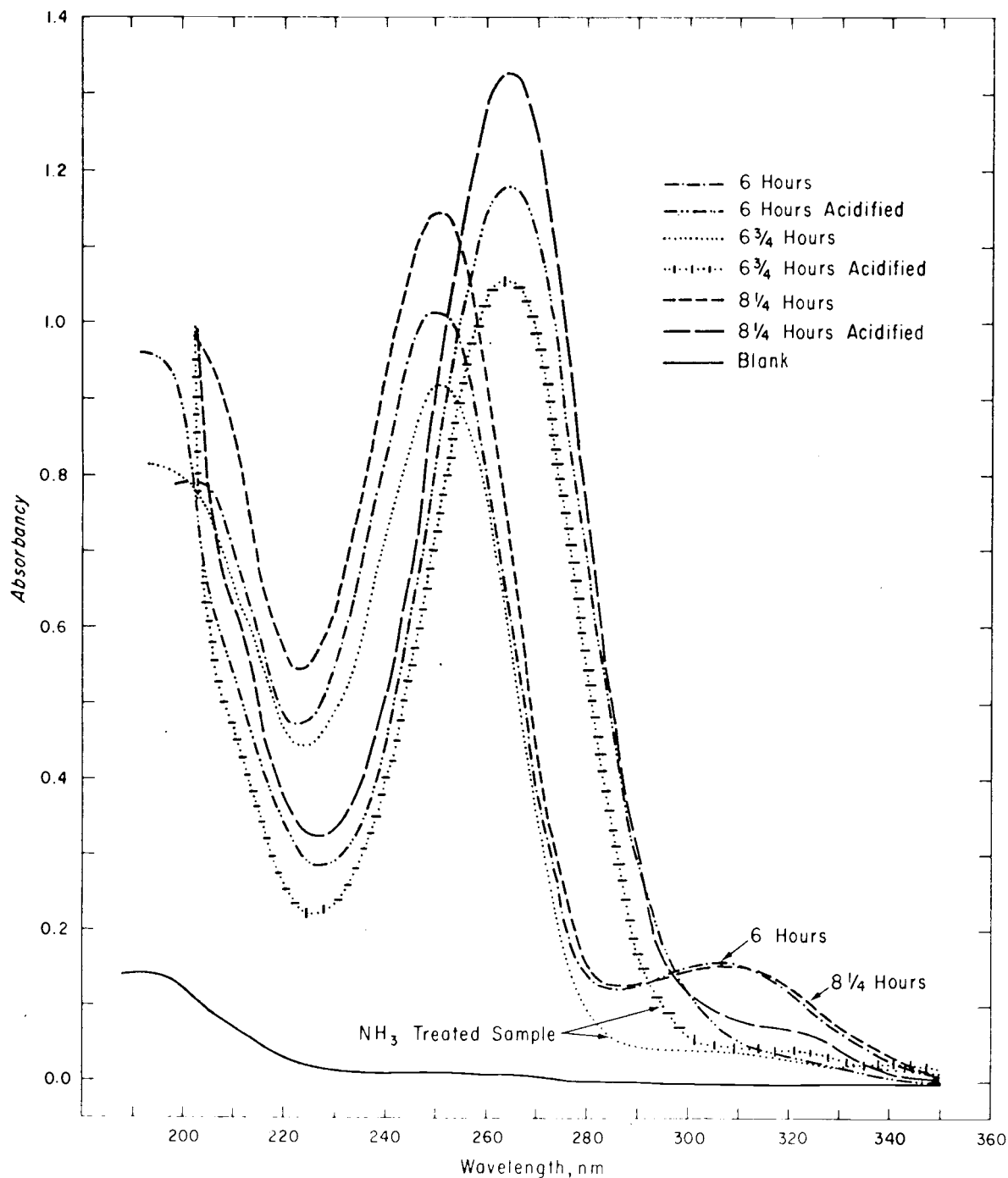
Fig. 57. Course of the formation of Compound P in one per cent piperidine between 6 and 8 1/4 hours. The spectra were obtained as in Fig. 51. The 6 3/4 hours spectra were obtained after heating an aliquot of the 6 hours reaction mixture, to which NH$_3$ had been added (see text), for 3/4th of an hour. The solid line represents the absorbance of a 1/100 dilution of a one per cent piperidine solution against a similar blank.

Fig. 58. Migration of non-labeled reaction mixtures $RI_{28}$, $RI_{26}$, and commercial preparations of alloxan, alloxantin $\cdot$ $2H_2O$, and dialuric acid both individually and mixed with cytidine-5-3H on ascending paper chromatography in Butanol-H O (86:14). The migration of the label and absorbance of cytidine-5-3H were compared with the migration of the cytidine label mixed with either a reaction mixture or commercial prep. to determine whether components or either "bind" cytidine.

Migration of RI₂₈, RI₂₆, & Commercial Preparations of Alloxan, Alloxontin·2H₂O, & Dioluric Acid Individually & with Cytidine-5H³ in Butonol-H₂O (86-14)
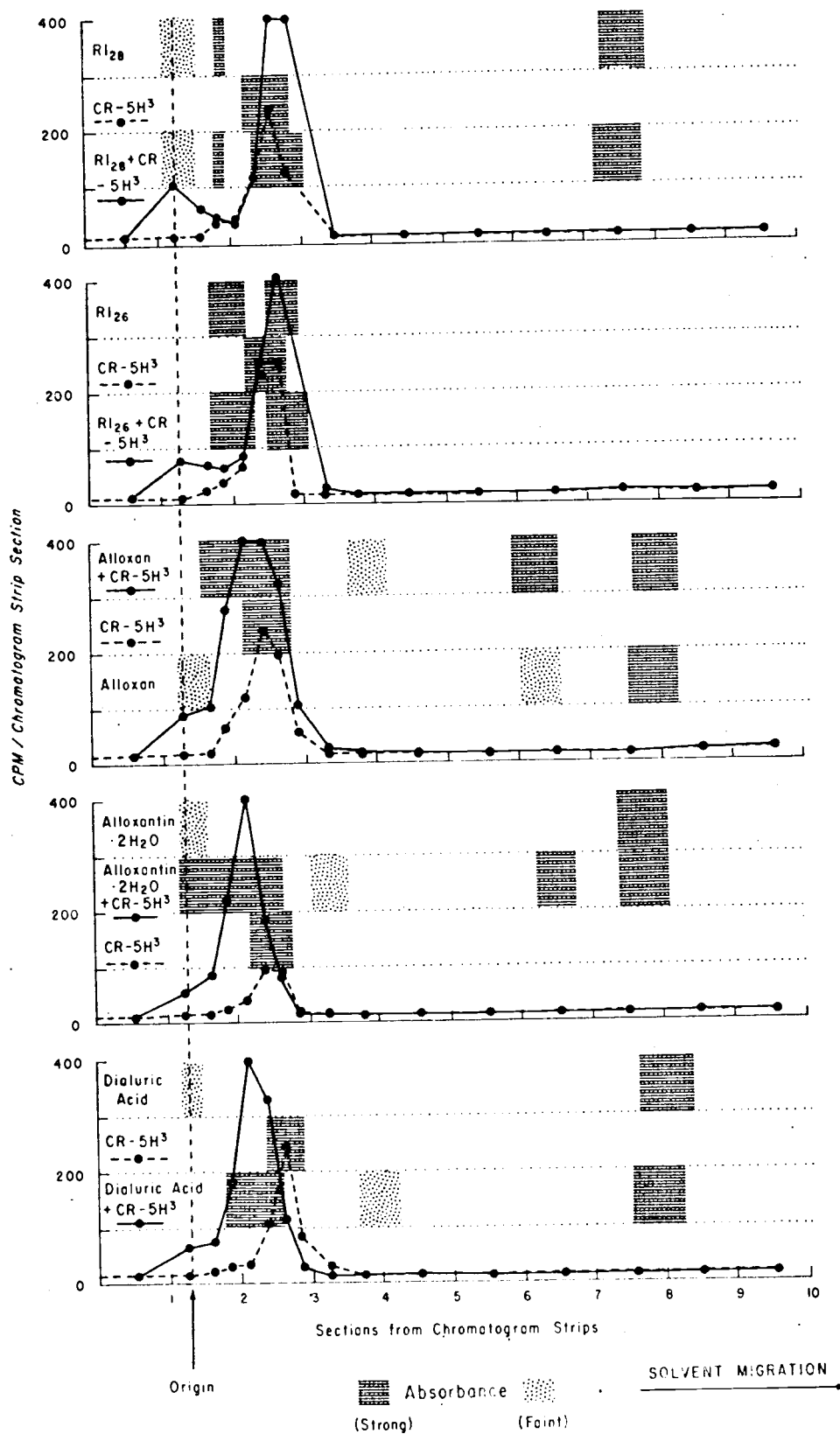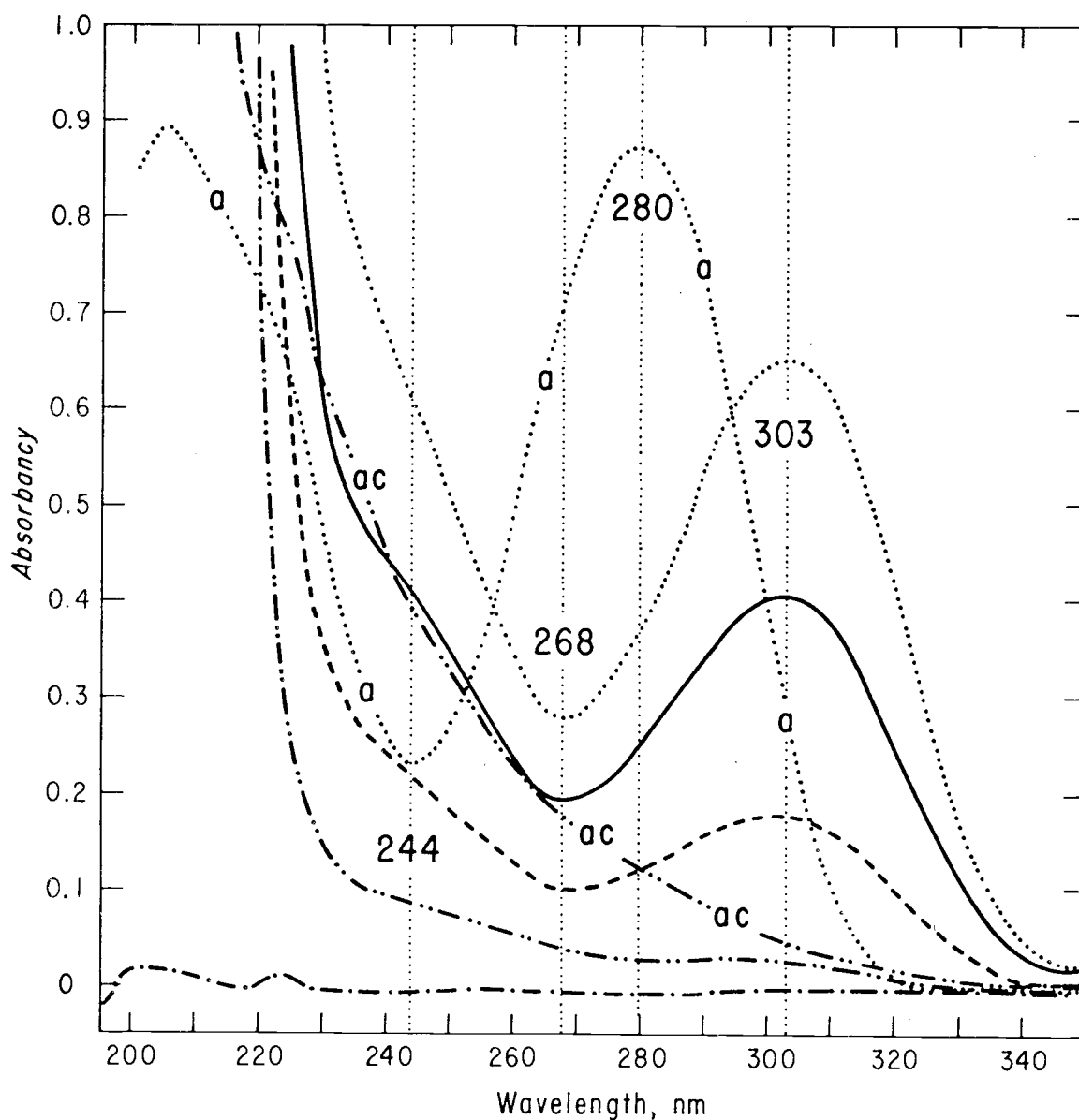
Fig. 59. Cource of reaction of 5-hydroxyuridine control in KOH
at pH 13.05 at 50° C. Absorbance, zero time (......), 2'40"
( —— ), 7'40" ( --- ), 20 hrs. ( -..- ). Ac indicates absorbance
of alkaline sample acidified to pH approximately one. The zero
time spectrum of 5-hydroxyuridine at pH 5.7 is shown by curve
a. Absorbance of KOH blank ( -.-. ).
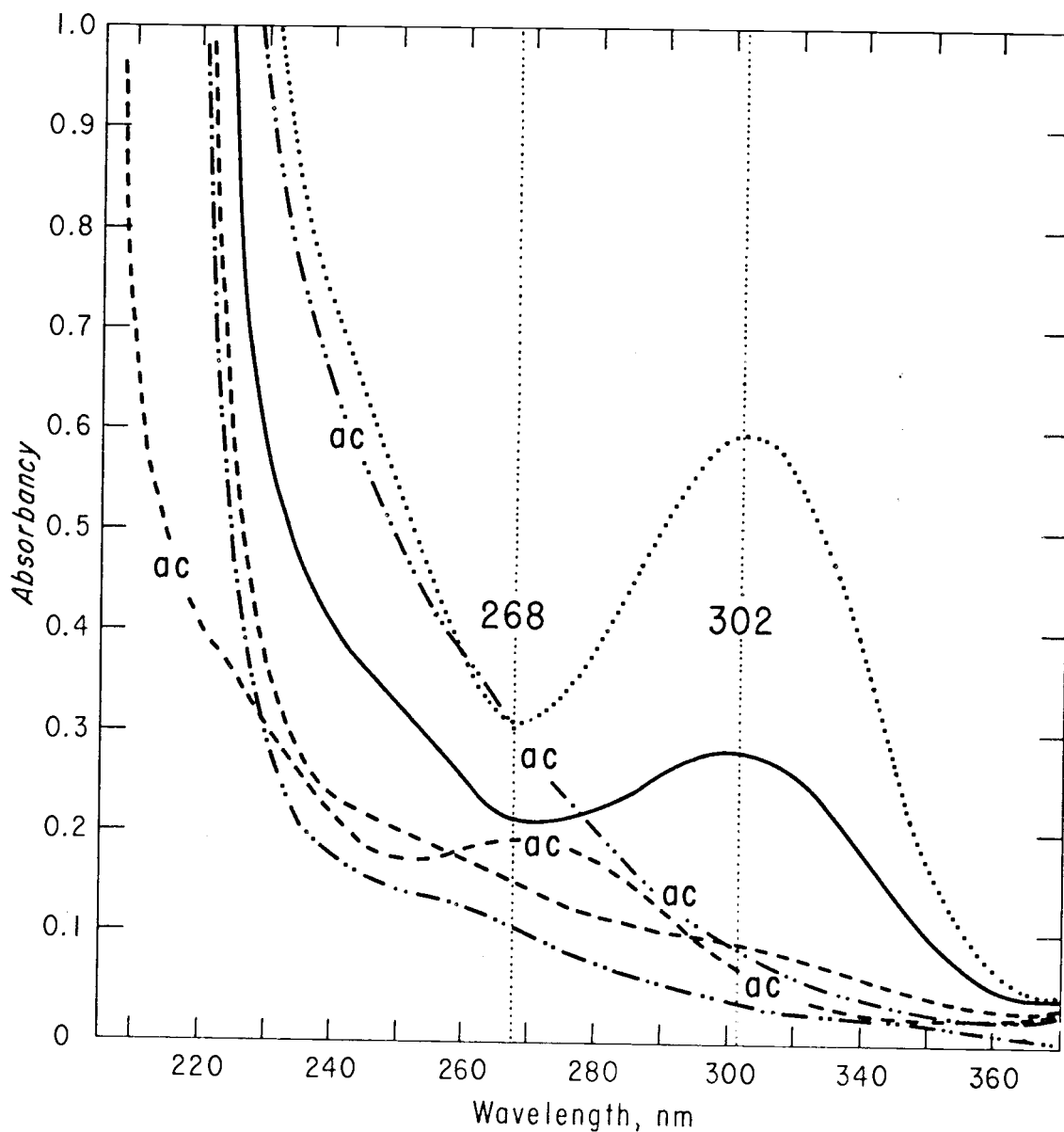
Fig. 60.  Course of reaction of 5-hydroxyuridine a_ (conforma-
tionally altered) in KOH at pH 13.03 at 50° C.  Absorbance,
zero time ( .... ), 2'40" ( ——), 7'40" ( --- ), 20 hrs.
( ..-.. ).  AC indicates absorbance of alkaline sample acidi-
fied to pH approximately one.

Fig. 61. Course of reaction of 5-methoxyuridine (5-hydroxyu-
ridine b) in KOH at pH 12.70 at 50° C. Absorbance zero time
(....), 2'40" (————), 7'40" (---), 20 hrs. (..-..). AC indi-
cates absorbance of alkaline sample acidified to pH approxi-
mately one; RAl represents alkalization of acidified sample
to approximately pH 11.

Fig. 62. Course of reaction of 3-methyl-5-hydroxyuridine (5-hydroxyuridine C), in KOH at pH 13.10 at 50° C. Absorbance zero time ( ..... ), 2'40" ( ——), 7'40" ( --- ), 20 hrs. (..-..). AC indicates absorbance of alkaline sample acidified to pH approximately one.
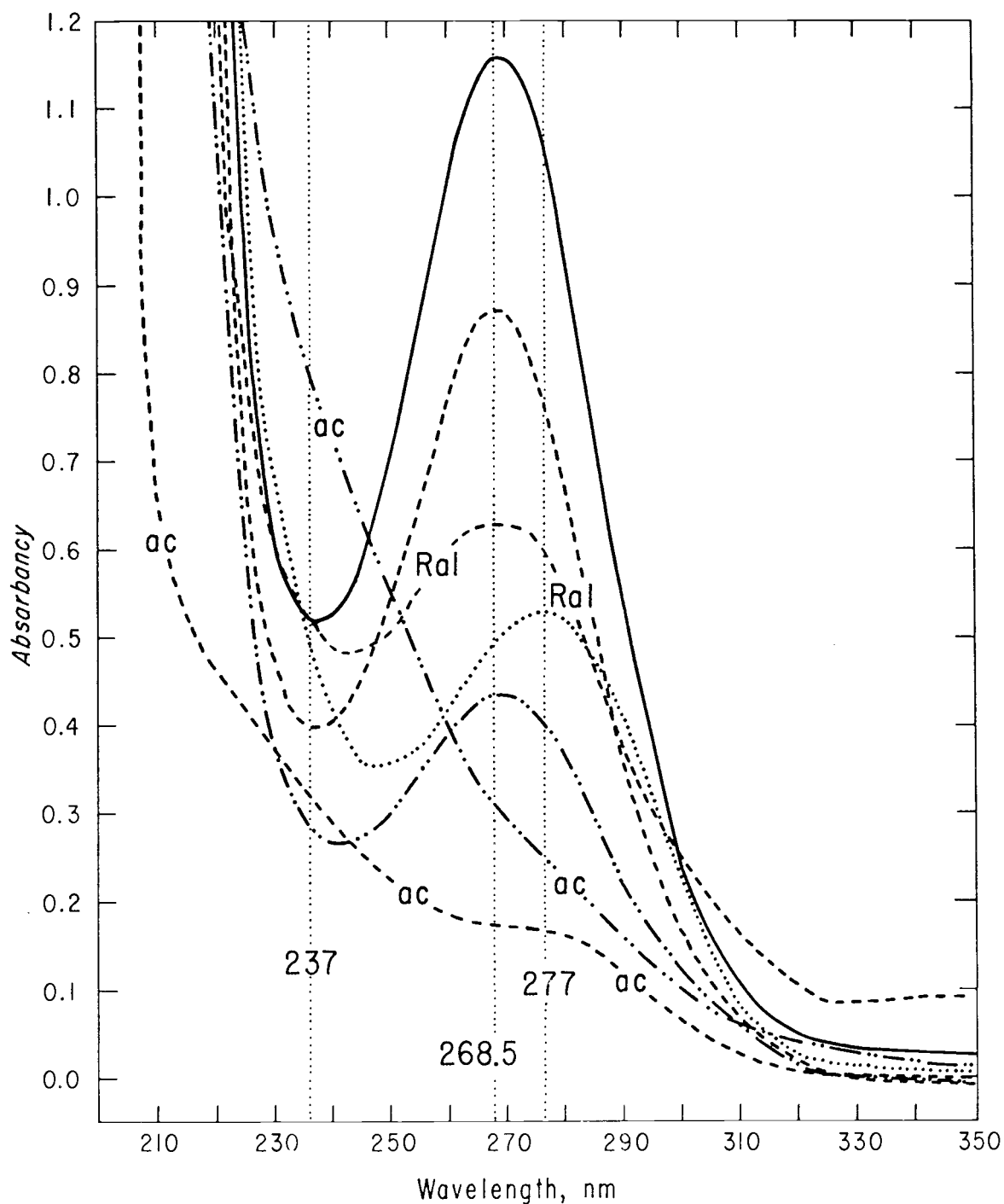
Fig. 63.  Course of reaction of 3-methyl-5-methoxyuridine
(5-hydroxyuridine d) in KOH at pH 13.05 at 50°C.  Absorbance,
zero time ( ..... ), 2'40" ( ——— ), 7'40" ( --- ); 20 hrs.
( ..-.. ).  AC indicates absorbance of alkaline sample acidi-
fied to pH approximately one; RAl represents alkalization of
acidified sample to approximately pH 11.

Fig. 64. Course of reaction of 2'-methoxy-3-methyl-5-meth-
oxyuridine (5-hydroxyuridine e) in KOH at pH 1290 at 50°C.
Absorbance, zero time ( ..... ), 2'40" ( —— ), 7'40" ( --- ),
20 hours ( ..-.. ). AC indicates absorbance of alkaline sample
acidified to pH approximately one; RA1 represents alkalization
of acidified sample to approximately pH 11.
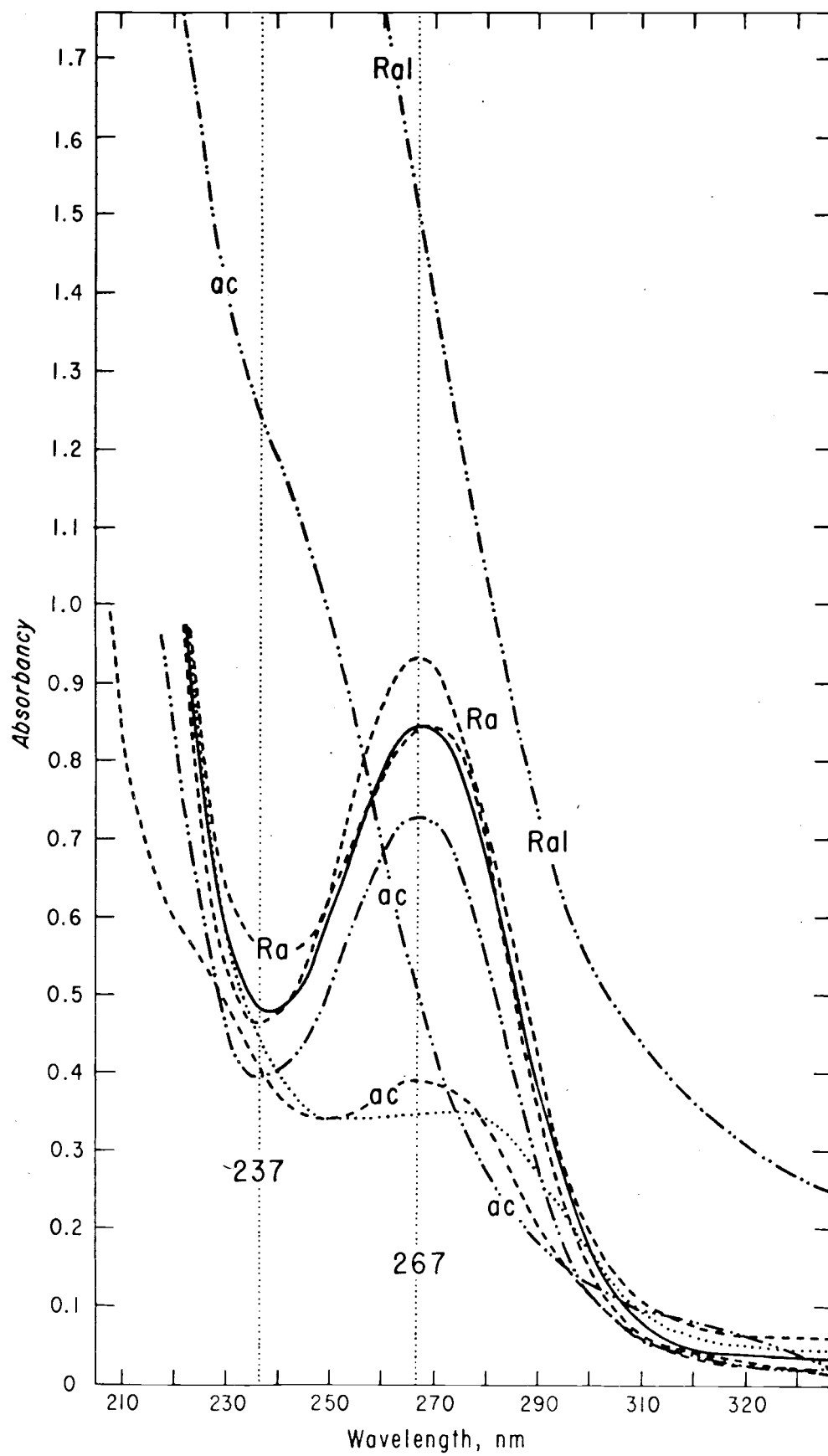
Fig. 65. Stability of isobarbituric acid (Iba), 2'-deoxy-
5-hydroxyuridine, 5-hydroxyuridine; a, 5-hydroxy-
uridine a ; b, 5-methoxyuridine; c, 3-methyl-5-hydroxy
uridine; d, 3-methyl-5-methoxyuridine; and e, 2'-deoxy-
3-methyl-5-methoxyuridine in KOH, pH 13, at 48-50°C.
Change in ultraviolet absorbance measured at wavelength
of maximum absorption for b, d, and e, and at 2935Å for
the other substances (see text).
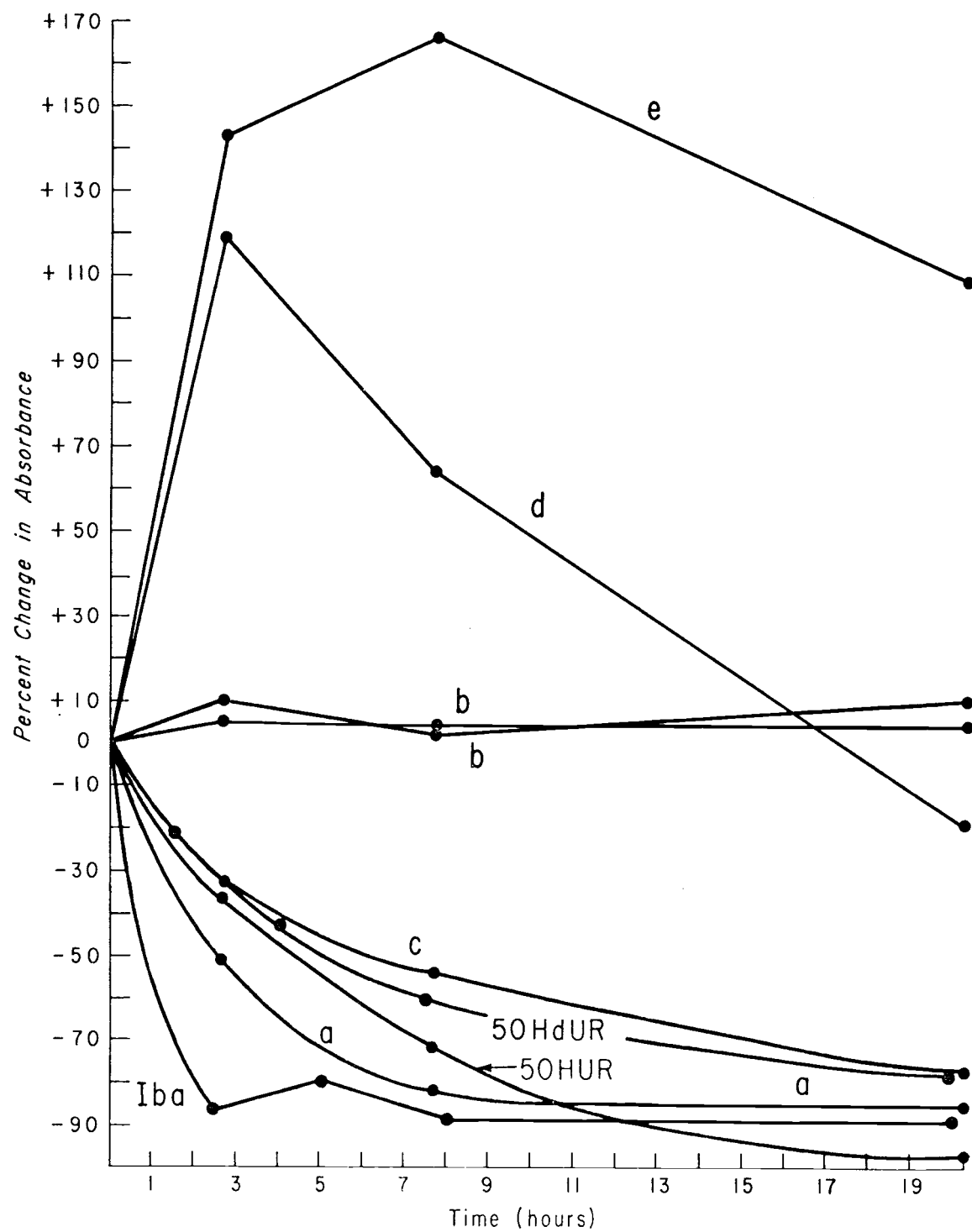
Fig. 66. Migration of uridine, 5-hydroxyuridine, dihydrouridine, and their partial alkaline hydrolysis products on ascending paper chromatography in 1-butanol-$H_2$O-ethanol (50:20:30) for 19 hours. Twenty microliter spots were applied to the paper; the suffixes A and AN indicate that subsequent to partial hydrolysis in 0.3N KOH at 80° C for 10 min. the hydrolysates were directly applied or neutralized with HCl and applied respectively.

ASCENDING PAPER CHROMATOGRAM

I butanol – H₂O – ethanol – (19 hrs.)
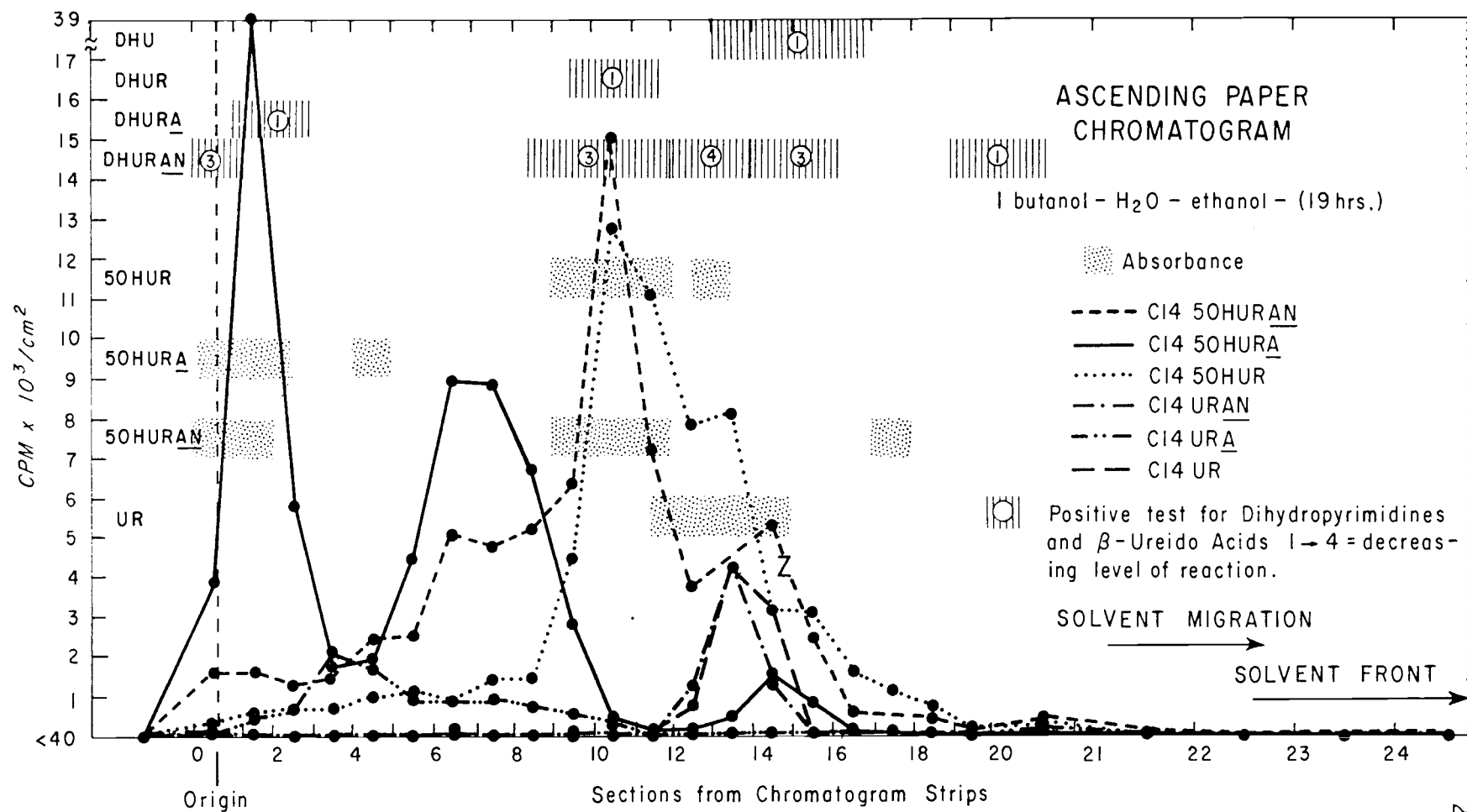
Fig. 67. Migration of uridine, 5-hydroxyuridine, dihydrouridine and their partical alkaline hydrolysis products on ascending paper chromatography in 2-propanol-$H_2O$-conc. HCl (680: 144:170) for 19 hours. Twenty microliter spots were applied to the paper; the suffixes A and AN indicate that subsequent to partial hydrolysis is 0.3N KOH at 80° C for 10 min. the hydrolysates were directly applied or, neutralized with HCl and applied respectively.
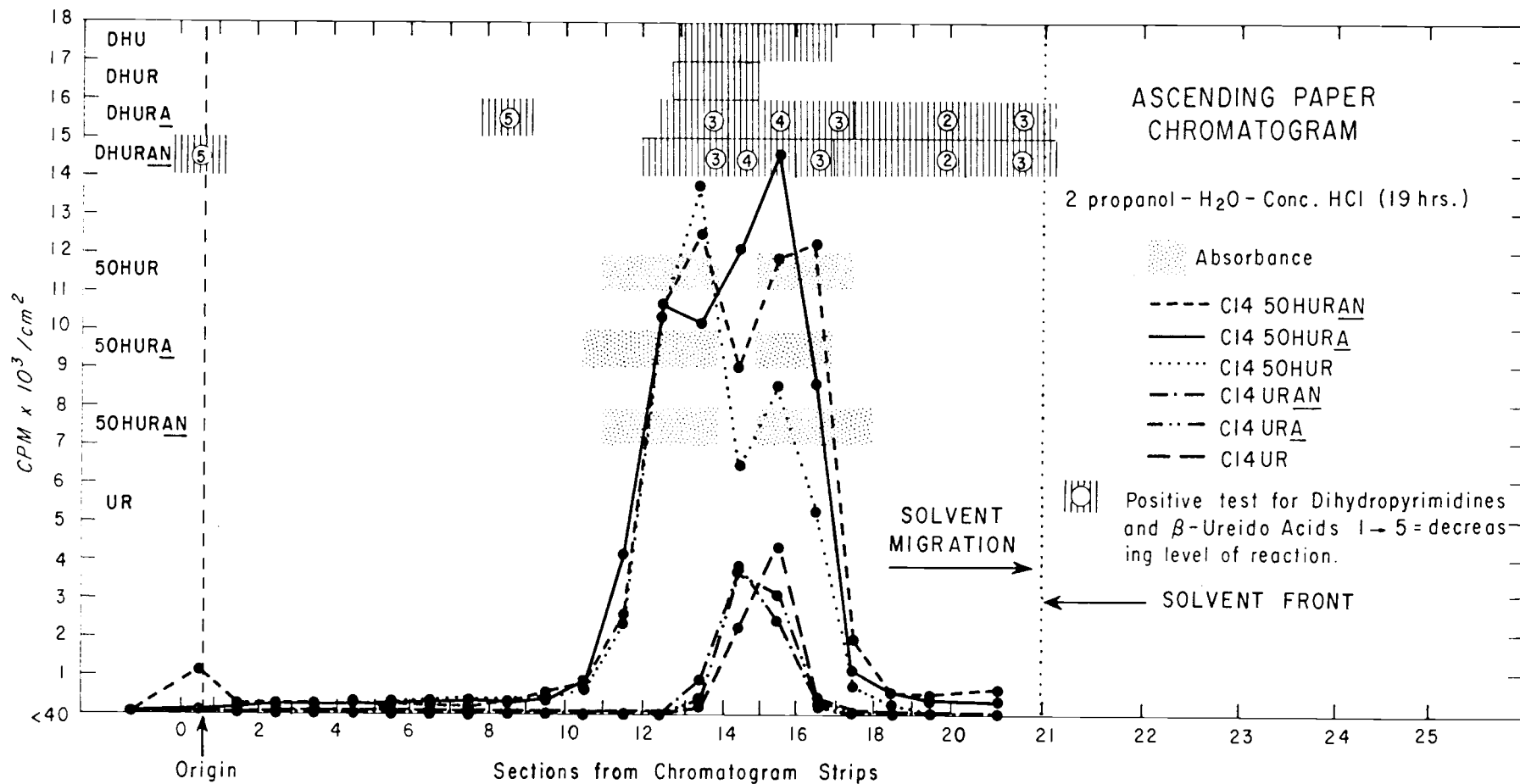
Fig. 68. Migration of uridine, 5-hydroxyuridine, dihydrouridine and their partial alkaline hydrolysis products on ascending paper chromatography in 2-propanol-$H_2$O-conc.$NH_3$ (7:2:1) for 19 hours. Twenty microliter spots were applied to the paper; the suffixes A and AN indicate that subsequent to partial hydrolysis in 0.3N KOH at 80°C for 10 min. the hydrolysates were directly applied or, neutralized with HCl and applied respectively.

ASCENDING PAPER CHROMATOGRAM

2 propanol – $H_2O$ – Conc. $NH_4OH$ – (19 hrs.)

Absorbance

- - - - C14 5OHURAN
——— C14 5OHURA
········ C14 5OHUR
–·–·– C14 URAN
–··–··– C14 URA
– – – C14 UR

Positive test for Dihydropyrimidines and β-Ureido Acids 1→5 = decreasing level of reaction.

SOLVENT MIGRATION

SOLVENT FRONT

$CPM \times 10^3/cm^2$

Sections from Chromatogram Strips

Origin
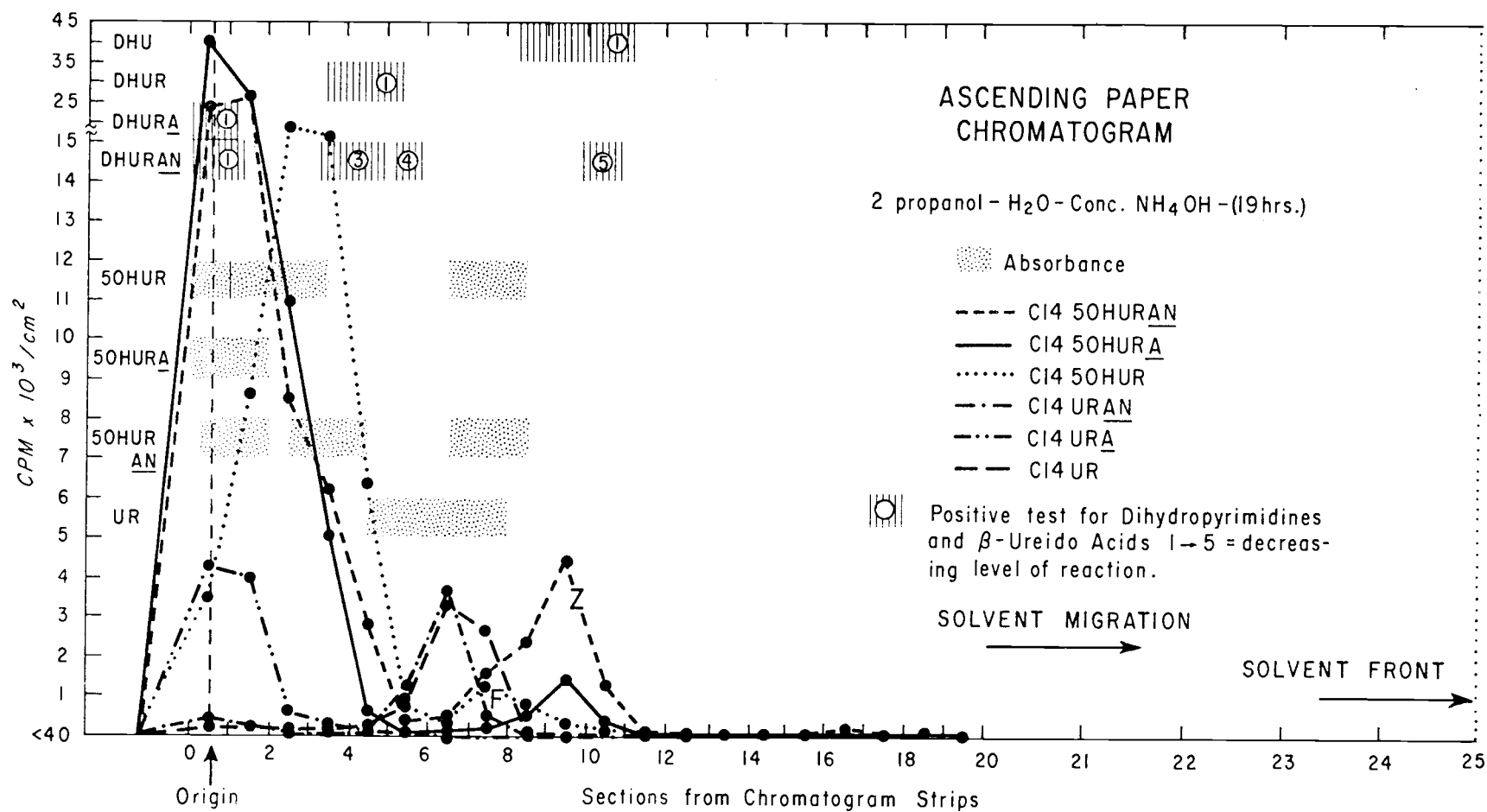
Fig. 69. Migration of uridine, 5-hydroxyuridine, dihydrouridine and their partial alkaline hydrolysis products on ascending paper chromatography in 1-butanol-H$_2$O (86:14) for 19 hours. Twenty microliter spots were applied to the paper; the suffixes A and AN indicate that subsequent to partial hydrolysis in 0.3N at 80°C for 10 min. the hydrolysates were directly applied or, neutralized with HCl and applied respectively.

ASCENDING PAPER
CHROMATOGRAM

1-butanol – H₂O (19 hrs)

Absorbance

- - - - C14 50HURAN

——— C14 50HURA     —··— C14 URA

········ C14 50HUR     ——— C14 UR

—·—· C14 URAN

Positive test for Dihydropyrimidines
and β-Ureido Acids 1→5 = decreas-
ing level of reaction.

SOLVENT MIGRATION

SOLVENT FRONT

CPM x 10³/cm²

DHU
DHUR
DHURA
DHURAN
50HUR
50HURA
50HUR AN
UR

Sections from Chromatogram Strips

Origin
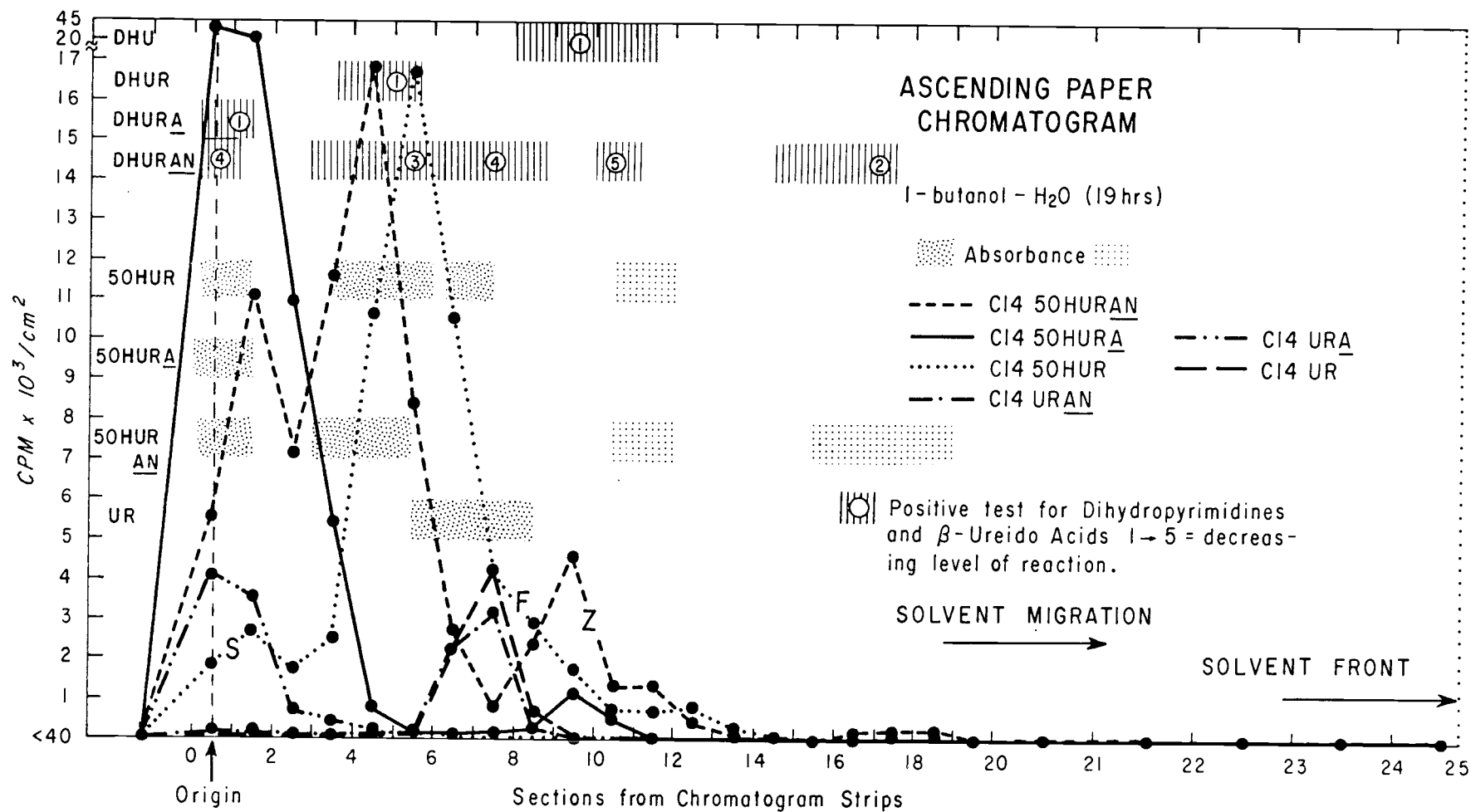
Fig. 70. Migration of products of the action of ammonia (graph 2), mercaptoethanol (graph 3), and potassium permangamate (graph 1) on 5-hydroxyuridine as well as 5-hydroxyuridine control (graph 4), and an artifact preparation from celite column HC #13 (graph 5) on ascending paper chromatography in butanol-$H_2O$ (86:14). Areas which gave a positive test for aldo-pentose are indicated.

3,4,5 Negative Sugar Test for Aldo-pentose and Aldo-hexose
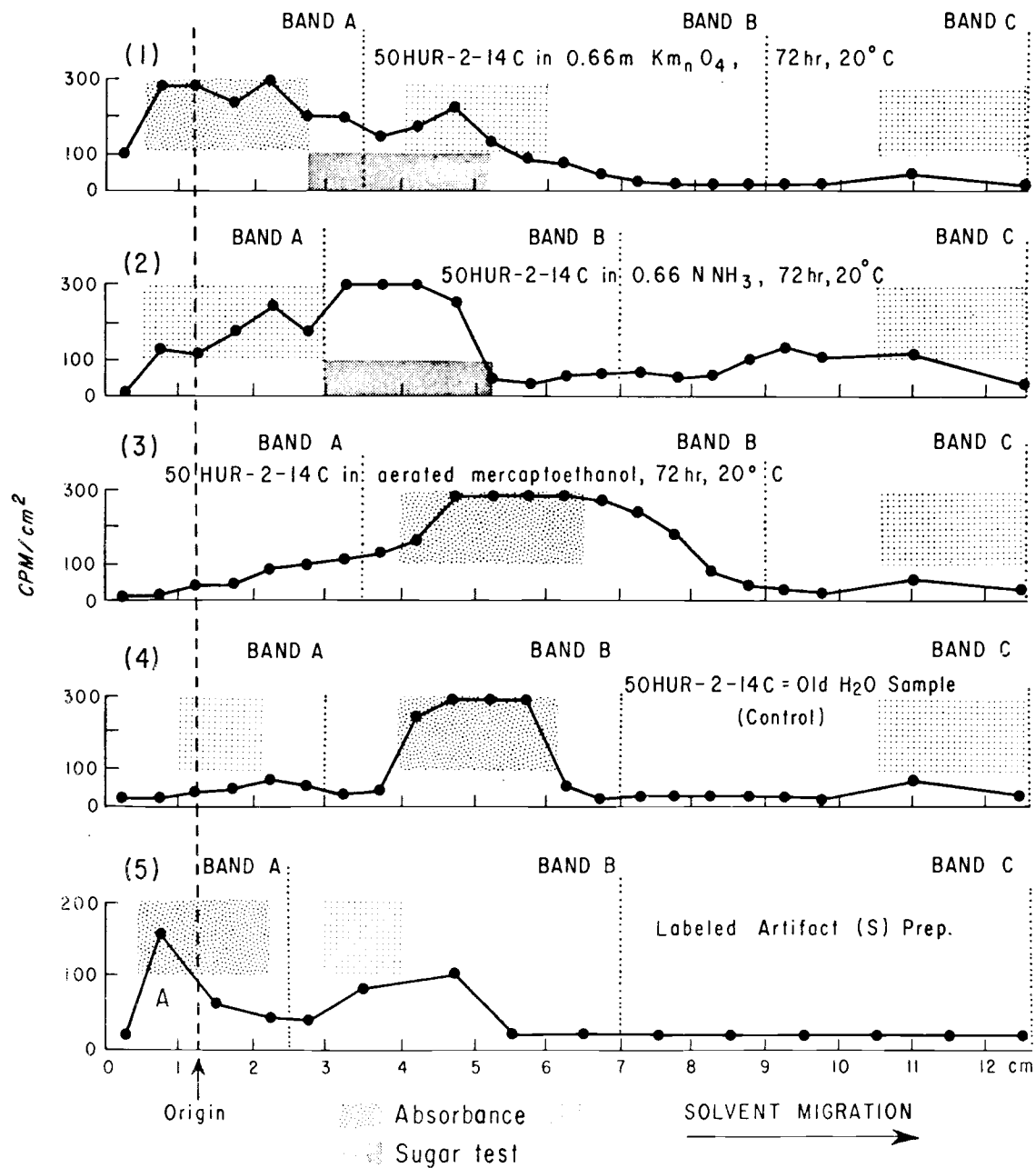
*Migration of Chemically Altered 5-hydroxyuridine + Butanol – H₂O (86:14)*

Fig. 71. Migration of Band A material from Fig. 70 representing the reaction of 5-hydroxyuridine with potassium permanga-mate (graph 2) or ammonia (graph 3) on ascending paper chro-matography in 2-propanol-$H_2$O-conc. HCl (680:144:170). The migration of a 5-hydroxyuridine control, & the band A artifact material from Fig. 70, are shown. The areas along each chro-matogram which gave positive qualitive tests for aldo-pentose, dihydropyrimidines or 3-ureido acids, as well as a ninhydrin test are indicated.

I-4 Ninhydrin Tests
(negative)

5-OHUR-2-I4C CONTROL

(1)

Optical Density

Reduction Test

Sugar Test

BAND A
KM$_n$O$_4$ REACTION

(2)

Optical Density

Reduction Test

Sugar Test

$CPM / (1/2 x 1 Inch)$

BAND A
NH$_3$ REACTION

(3)

Reduction Test

Sugar Test

BAND A
ARTIFACT

(4)

Optical Density

Reduction Test

Sugar Test

SOLVENT FRONT

Origin

Inches

SOLVENT MIGRATION

256
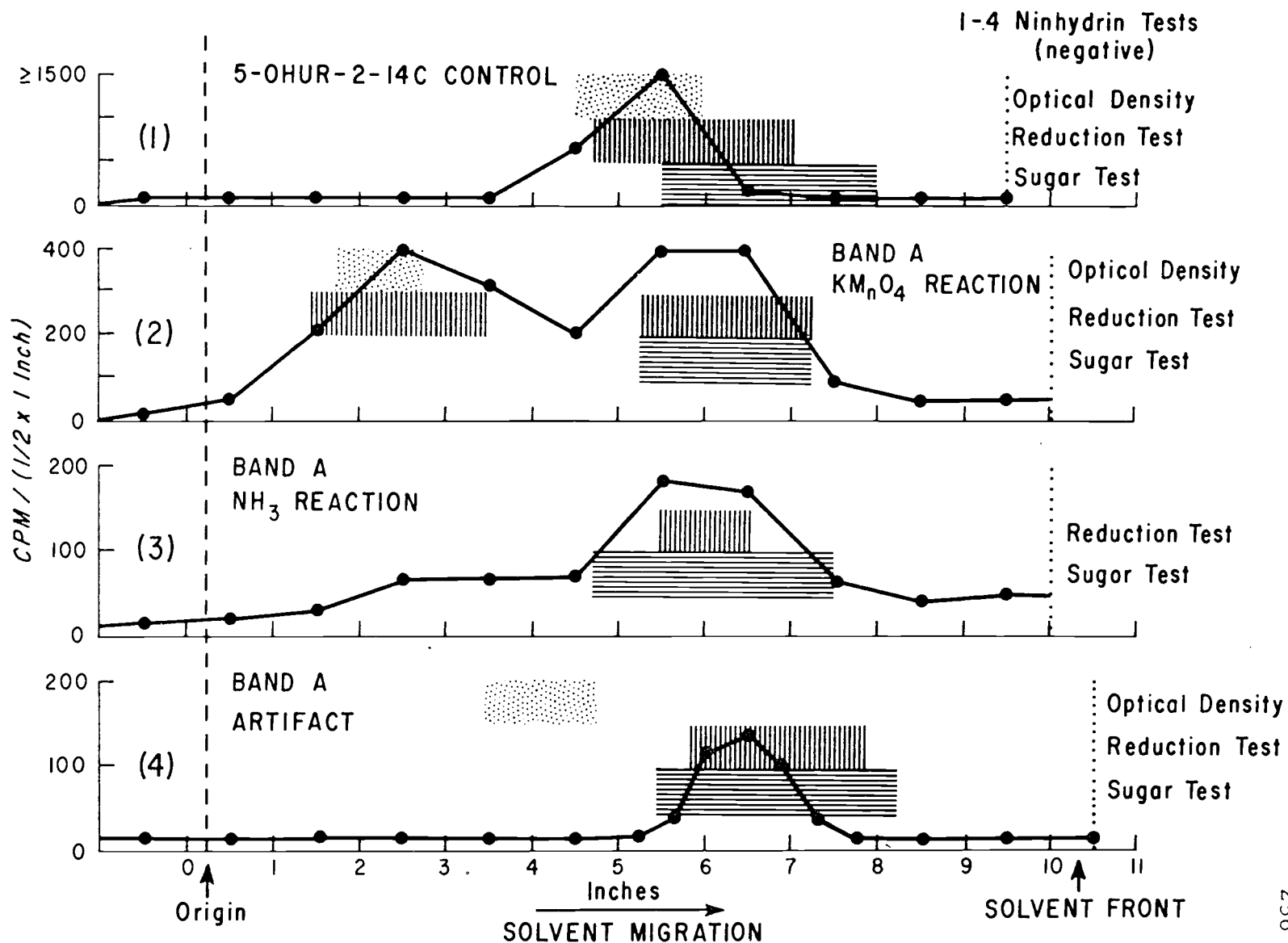
Fig. 72. Migration of Band B material from Fig. 70 representing the reaction of 5-hydroxyuridine with potassium permangamate (graph 2), ammonia (graph 3), or mercaptoethanol (graph 4) on ascending paper chromatography in 2-propanol-$H_2O$-conc. HCl (680:144:170). The migration of a 5-hydroxyuridine control is shown, in graph 1. The areas along each chromatogram which gave positive qualitative tests for aldopentose, dihydropyrimidines or 3-ureido acids, as well as a ninhydrin test are indicated.
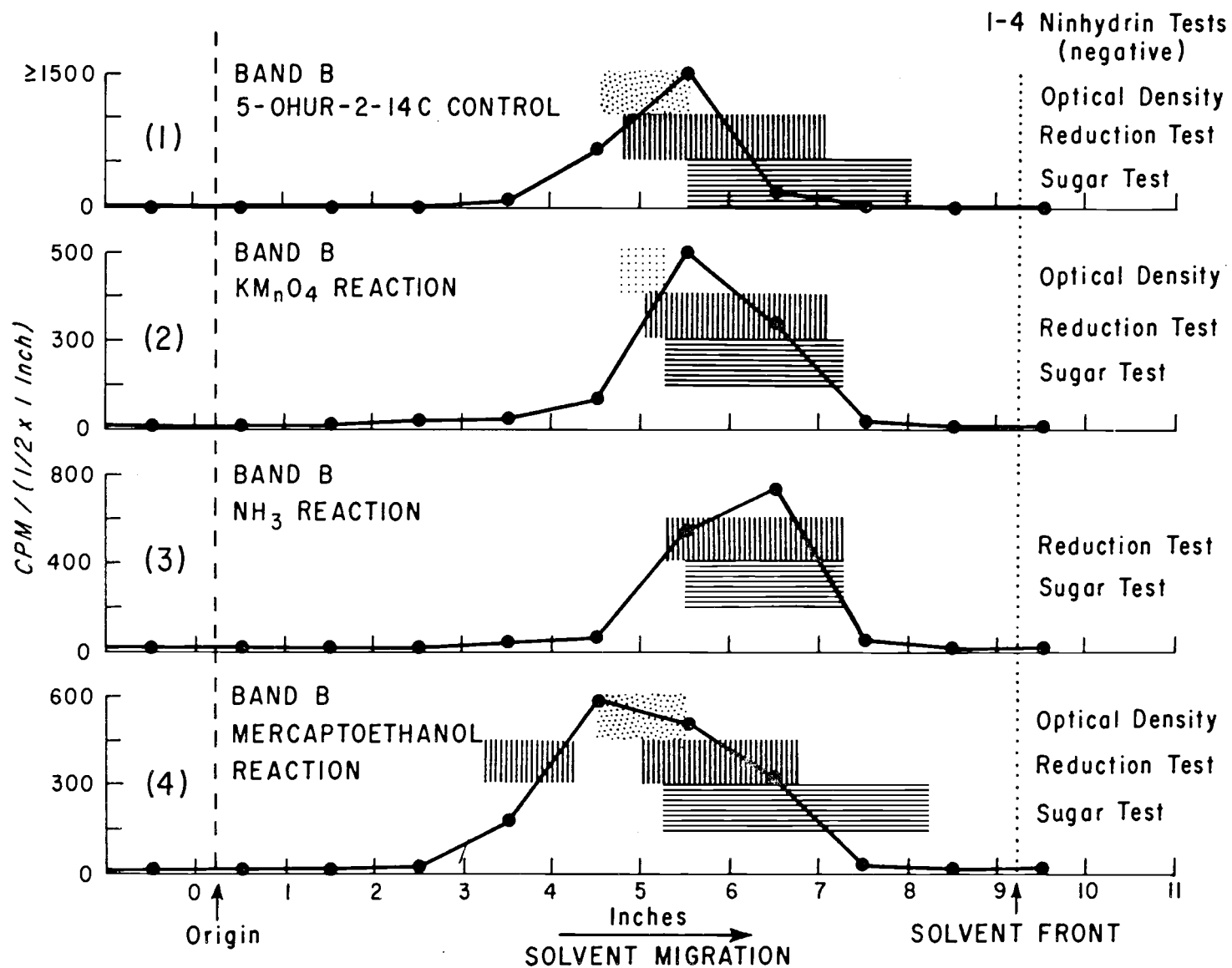
Fig. 73. Migration of Band C material from Fig. 70 representing the reaction 5-hydroxyuridine with potassium permangamate (graph 3) or ammonia (graph 4) on ascending paper chromatography in 2-propanol-$H_2$O-conc. HCl (680: 144:170). The migration of a 5-hydroxyuridine control and Compound A are also shown. The areas along each chromatogram which gave positive qualitative tests for aldo-pentose, dihydropyrimidines or 3-ureido acids, as well as a ninhydrin test are indicated.
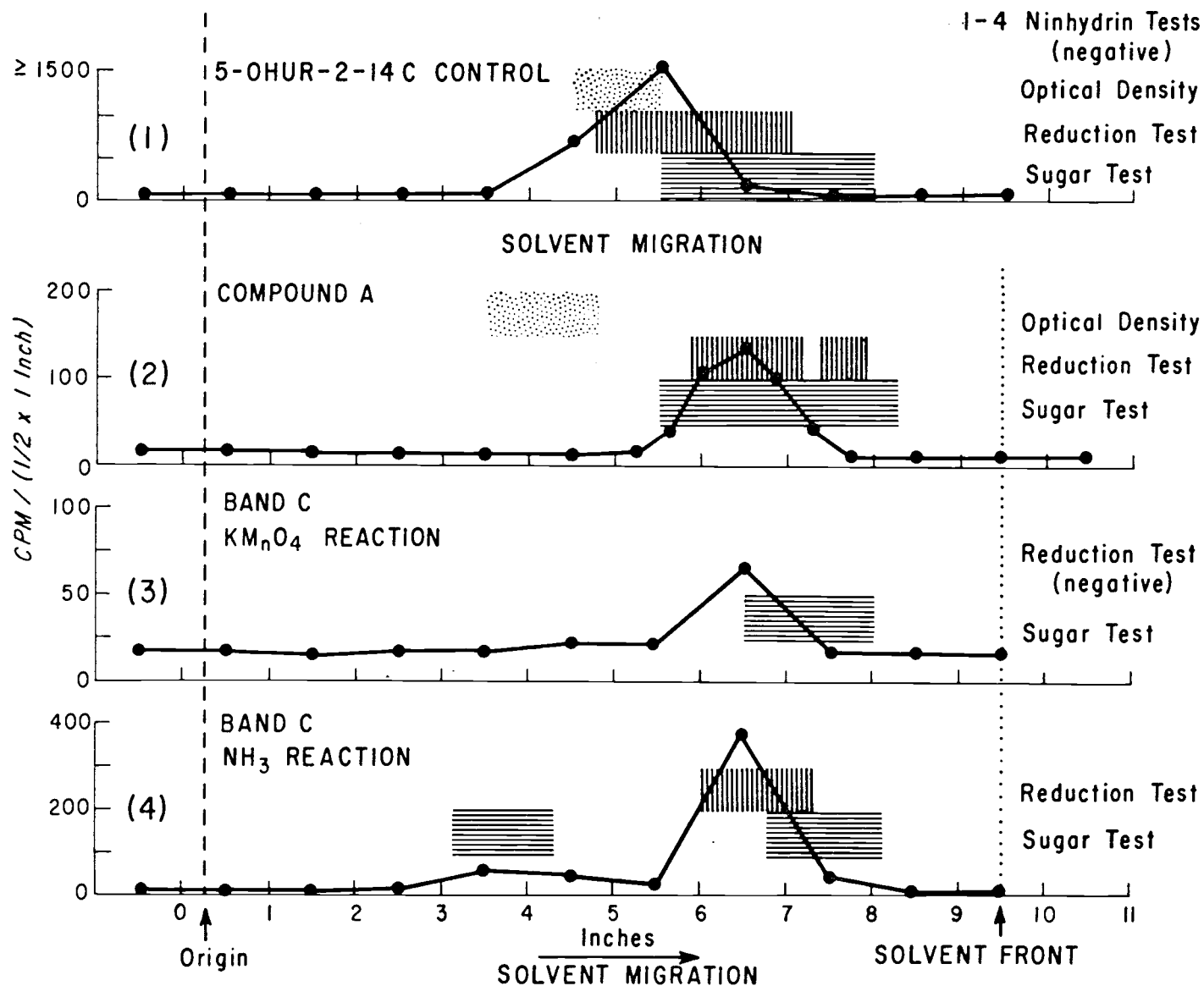
## TABLE 1

### Ionization Constant of 5-Hydroxyuridine*

calculated pKa$_1$ values

| wavelength nm | pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7.000 | 7.425 | 7.650 | 7.975 | 8.225 | 8.425 | 8.750 |
| 240 | 7.760 | 7.837 | 7.857 | 7.921 | 7.849 | 7.848 | 7.793 |
| 245 | 7.657 | 7.809 | 7.822 | 7.911 | 7.842 | 7.843 | 7.783 |
| 250 | 7.778 | 7.823 | 7.802 | 7.850 | 7.827 | 7.796 | 7.771 |
| 270 | 7.915 | 7.895 | 7.920 | 7.960 | 7.884 | 7.837 | 7.836 |
| 280 | 7.919 | 7.860 | 7.870 | 7.947 | 7.881 | 7.863 | 7.829 |
| 305 | 7.840 | 7.803 | 7.846 | 7.929 | 7.846 | 7.763 | 7.713 |
| 315 | 7.778 | 7.823 | 7.832 | 7.906 | 7.844 | 7.819 | 7.684 |
| 325 | 7.566 | 7.798 | 7.817 | 7.919 | 7.852 | 7.856 | 7.775 |

Average = 7.83

\* Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbancies at each wavelength and pH were obtained from the spectral curves of Fig. 2. UI = absorbance at pH 2.975, I = absorbance at pH 9.350 (9.500).

## TABLE 2

### Ionization Constant of 5-Hydroxyuridine *

calculated pKa$_2$ values

| wavelength nm | pH | | | |
|---|---|---|---|---|
| | 9.750 | 10.000 | 10.250 | 10.575 |
| 215 | 10.811 | 10.551 | 10.550 | 10.025 |
| 220 | 10.954 | 10.731 | 10.551 | 10.195 |
| 225 | 10.954 | 10.763 | 10.570 | 10.131 |
| 228 | 10.771 | 10.677 | 10.524 | 10.123 |
| 230 | 10.980 | 10.903 | 10.551 | 10.149 |

Average = 10.57

## TABLE 3

### Ionization Constant of 5-Hydroxyuridine *

calculated pKa$_3$ values

| wavelength nm | pH | | | | |
|---|---|---|---|---|---|
| | 11.250 | 11.525 | 11.750 | 12.025 | 12.275 |
| 230 | 12.125 | 11.715 | 11.486 | 11.602 | 11.852 |
| 235 | 11.977 | 11.665 | 11.415 | 11.451 | 11.701 |

Average = 11.70

* Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbancies at each wavelength and pH were obtained from the spectral curves of Figures 3 and 4. Table 2, UI = absorbance at pH 9.350 (9.500), I = absorbance at pH 10.750. Table 3, UI = absorbance at pH 11.025, I = absorbance at pH 12.500 (12.750).

TABLE 4

Ionization Constants of 5-methoxyuridine (=5-hydroxyuridine b)*

| wavelength nm | calculated pKa$_1$ values pH | | | wavelength nm | calculated pKa$_2$ values pH | | |
|---|---|---|---|---|---|---|---|
| | 8.550 | 9.200 | 9.400 | | 10.975 | 11.625 | 11.925 |
| 270 | 9.435 | 9.552 | 9.468 | 240 | 11.348 | 11.507 | 11.493 |
| 274 | 9.187 | 9.253 | 9.345 | 245 | 11.321 | 11.346 | 11.506 |
| 277.5 | 9.264 | 9.176 | 9.329 | 250 | 11.402 | 11.383 | 11.499 |

Average pKa$_1$ = 9.33          Average pKa$_2$ = 11.42

\* Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbancies at each wavelength and pH were obtained from spectral curves of Fig. 7. For pKa$_1$, UI = absorbance at pH 3.750 (= 5.900), I = absorbance at pH 9.975 (= 10.975). For pKa$_2$, UI = absorbance at pH 9.975, I = absorbance at pH 12.650.

TABLE 5

Ionization Constants of 3-methyl-5-hydroxyuridine (5-hydroxyuridine $\underline{C}$)[*]

calculated pKa₁ values

| wavelength nm | pH | | | |
|---|---|---|---|---|
| | 7.750 | 8.525 | 8.800 | 9.125 |
| 240 | 8.784 | 8.574 | 8.277 | 8.232 |
| 245 | 8.255 | 8.511 | 8.295 | 8.146 |
| 250 | 8.227 | 8.471 | 8.366 | 8.150 |
| 255 | 8.427 | 8.487 | 8.348 | 8.104 |
| 270 | 8.068 | 8.525 | 8.322 | --- |
| 275 | 8.072 | 8.497 | 8.317 | --- |
| 280 | 8.080 | 8.525 | 8.322 | --- |
| 285 | 8.031 | 8.470 | 8.308 | --- |
| 300 | 8.177 | 8.472 | 8.305 | --- |
| 305 | 8.139 | 8.499 | 8.317 | --- |
| 310 | 8.136 | 8.496 | 8.283 | --- |
| 320 | 8.134 | 8.476 | 8.267 | --- |

Average pKa₁ = 8.32

calculated pKa₂ values

| wavelength nm | pH | |
|---|---|---|
| | 12.425 | 12.725 |
| 235 | 12.688 | 12.658 |

Average pKa₂ = 12.67

[*] Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbancies at each wavelength and pH were obtained from spectral curves of Fig. 8. For pKa₁, UI = absorbance at pH 3.275 ( = 5.925), I = absorbance at pH 9.675. For pKa₂ UI = absorbance at pH 11.875, I = absorbance at pH 13.100.

## TABLE 6

### Ionization Constants of Isobarbituric Acid

| Wavelenght | Calculated $pKa_1$ values | | | | |
| | pH | | | | |
| nm | 7.300 | 7.750 | 7.950 | 8.350 | 8.700 |
| --- | --- | --- | --- | --- | --- |
| 240 | 7.979 | 8.010 | 8.038 | 7.996 | 7.823 |
| 244 | 7.919 | 8.055 | 8.094 | 8.059 | 7.942 |
| 305 | 7.911 | 8.004 | 8.042 | 7.970 | 7.846 |
| 315 | 7.897 | 7.977 | 8.023 | 7.970 | 7.782 |

Average $pKa_1$ = 7.97

| | Calculated $pKa_2$ values | | | | | | | | | |
| pH | wavelenght, nm | | | | | | | | | |
| | 235 | 240 | 244 | 250 | 265 | 270 | 277 | 300 | 304 | 315 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 10.650 | 10.871 | 10.904 | 10.825 | 10.950 | 10.525 | 10.650 | 10.746 | 10.746 | 10.746 | 10.870 |

Average $pKa_2$ = 10.78

| | Calculated $pKa_3$ values | | | | | | | | | |
| pH | wavelenght, nm | | | | | | | | | |
| | 240 | 244 | 250 | 265 | 270 | 277 | 300 | 304 | 315 | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 12.500 | 12.404 | 12.410 | 12.441 | 12.200 | 12.200 | 12.240 | 12.303 | 12.216 | 12.345 | |

Average $pKa_3$ = 12.3

\* Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbance at each wavelength and pH were obtained from spectral curves of curves of Figure 12. For $pKa_1$ UI = absorbance at pH 3.350, I = absorbance at pH 9.600. For $Pka_2$ UI = absorbance at pH 9.800, I = absorbance at pH 11.025. For $pKa_2$ UI = absorbance at pH 11.025, I = absorbance at pH 12.900.

## TABLE 7

Stability of 5-Hydroxyuridine in Solution at $23^{\circ}C$

<table>
<tr><td colspan="2"><u>Stable in</u></td><td colspan="3"><u>Unstable in</u></td></tr>
<tr><td colspan="2">Solutions exhibit less than<br>5 per cent loss in ultravio-<br>let absorbance at 2935Å by<br>5 days</td><td colspan="3">Per cent change in ultravio-<br>let absorbance at 2935Å by<br>5 days</td></tr>
<tr><td>1.</td><td>$H_2O$ pH 6.2</td><td>1.</td><td>1 per cent(V/V) 2-mer-<br>captoethanol</td><td>+200</td></tr>
<tr><td>2.</td><td>$10^{-6}$-$10^{-8}$M $KMnO_4$</td><td>2.</td><td>1M HCl</td><td>- 16</td></tr>
<tr><td>3.</td><td>$4 \times 10^{-6}$ M ascorbic acid</td><td>3.</td><td>1M $H_2SO_4$</td><td>- 23</td></tr>
<tr><td>4.</td><td>0.4M ammonium formate<br>4M in formic acid</td><td>4.</td><td>$10^{-4}$M $K_3Fe(CN)_6$</td><td>- 17</td></tr>
<tr><td>5.</td><td>$10^{-2}$-$5 \times 10^{-3}$M $MgCl_2$, pH 6.2</td><td>5.</td><td>$10^{-6}$M $K_3Fe(CN)_6$</td><td>- 12</td></tr>
<tr><td></td><td></td><td>6.</td><td>1M NaOH</td><td>- 66</td></tr>
<tr><td></td><td></td><td>7.</td><td>1M $NH_4OH$</td><td>+200</td></tr>
</table>

Table 8. Results of Acid Hydrolysis (0.10NHCl, 105° C., 1 Hr.)

| Spectra Prior to Hydrolysis | | | | | Spectra Subsequent to Hydrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|
| pH Curve | λ max. (mμ) | λ min. (mμ) | Isosbestic Points pH Curve | λ (mμ) | pH Curve | λ max. (mμ) | λ min. (mμ) | Isosbestic Points pH Curve | λ (mμ) |
| 5-Hydroxyuridine-2-14C (Same as cold prep.) | | | | | | | | | |
| 1.7-7.0 | 280 | 245 | 1.7-7.0 | 257,294 | 1.0 | 279 | 246 | 1.0-12.8 | 257,294 |
| 7.5 | 286 | 254 | 1.7-7.5 | 258,294 | 12.8 | 303 | 269 | | |
| 12.3 | 303 | 268 | 1.7-12.3 | 257,294 | | | | | |
| | | | 7.5-12.3 | 254,294 | | | | | |
| Isobarbituric Acid | | | | | | | | | |
| 1.7 | 277 | 244 | | | 1.0 | 218 | 214 | 1.0-12.8 | 256,293 |
| 7.0 | 206 277 | 244 | 1.7-12.3 | 258,294 | 12.8 | 276 238 | 243 269 | | |
| 12.3 | 239 304 | 270 | | | | 302 | | | |
| Uridine | | | | | | | | | |
| 1.7 | 206 261 | 230 | 1.7-12.3 | 244,291 | 1.0 | 260 | 229 | 1.0-12.8 | 245,287 |
| 7.0 | 260 | 228 | | | 12.8 | 260 | 243 | | |
| 12.3 | 261 | 241 | | | | | | | |

Table 9.   Results of Acid Hydrolysis (0.10NHCl, 105° C., 1 Hr.)

| Spectra Prior to Hydrolysis | | | | | Spectra Subsequent to Hydrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|
| pH Curve | λ max. (mμ) | λ min. (mμ) | Isosbestic Points pH Curve | λ (mμ) | pH Curve | λ max. (mμ) | λ min. (mμ) | Isosbestic Points pH Curve | λ (mμ) |
| **C21b** | | | | | | | | | |
| 7.0 | 253 273(s) | 229 | 1.7-7.0 | 220,259 269,280 305 | 1.0 | 247 274(s) | 230 | 1.0-14 | 238,261 |
| 1.7 | 256 281(s) | 232 | 1.7-12.3 | 247,262 281,297 | 12.8-14 | 272 | 257 | | |
| 12.3 | 257(b) | 238 | 7.0-12.3 | 237,261 | | | | | |
| **C21b** | (Sample heated at pH 7.0, 105°C., 1 hr.: no acid added.) | | | | 7.0 | 253 273(s) | 231 | 1.0-12.3 | 236,259 281,291 |
| | | | | | 1.0 | 249 274(s) | 231 | 1.7-12.3 | 245,267 275 |
| | | | | | 1.7 | 255 281(s) | 233 | | |
| | | | | | 12.3 | 254 267(s) | 238 | | |

Note:   s=shoulder, b=broad peak

Table 10.   ELECTROPHORETIC MIGRATION (mm)*

| COMPOUND | 0.05M Potassium Borate Buffer pH 9.2, 19v/cm | | | 0.01M Ammonium Formate Buffers 19v/cm | | | |
| | | | | pH 9.3 | | pH 3.5 | |
| | 2-1/2 hr | 4 hr | Charge of compound in field | 2-1/2 hr | Charge of Compound in field | 2-1/2 hr | Charge of compound in field |
|---|---|---|---|---|---|---|---|
| Adenosine | | +54 | - | | | | |
| Cytidine | | +90 | - | | | | |
| Guanosine | | +115 | - | | | | |
| Isobarbituric acid | +80 | | - | +105 | - | | |
| Uracil (PK. 9.5) | -30 | | + | -39 | + | | |
| Uridine | +45 | | - | -29 | + | | |
| 5-Hydroxyuridine-2-14C | +115 | +214 | - | +51 | - | | |
| Artifact (Labeled) | -28 | -73 | + | -58 | + | -35 | + |

*Values represent average migration determined from several chromatograms.

Table 11.  Rf Values of Artifact (C21b) Isolated from HC#13 Wash

| SOLVENT SYSTEM | Rf (Solvents) | | | |
|---|---|---|---|---|
| | C21b (OD&L)* | 5-OHUR | UR | U |
| 1-Butanol-g.Acetic acid-$H_2O$ (50:25:25) | 0.33 OD&L | 0.33 | 0.43 | 0.53 |
| 1-Butanol-$H_2O$-conc.$NH_4OH$ (86:14:5) | 0.03 OD&L | 0.01 | 0.07 | 0.19 |
| 1-Butanol-$H_2O$ (86:14) in $NH_3$ gas | 0.014 OD&L | 0.005 | 0.05 | 0.15 |
| 2-Propanol-1% aqueous$(NH_4)_2SO_4$ (2:1) | 0.59 OD&L | 0.67 | 0.74 | 0.74 |
| 1-Butanol-$H_2O$ (86:14) | 0.04 OD&L | 0.18 | 0.30 | -- |
| Upper phase Ethyl acetate-1-propanol-$H_2O$ (4:1:2) | 0.08 OD&L | 0.23 | 0.32 | 0.62 |
| 1-Butanol-$H_2O$-abs. Ethanol (50:20:30) | 0.20 OD&L major 0.45 L | 0.33 | 0.42 | 0.53 |
| 2-Propanol-$H_2O$-conc. HCl (680:144:170) | 0.26 OD 0.59 L | 0.49 | 0.60 | 0.62 |
| 2-Propanol-$H_2O$-conc. $NH_4OH$ (7:2:1) | 0.34 OD 0.52 L | 0.30 | 0.49 | 0.54 |

* OD = absorbing spot at 254 m$\mu$: L=Label (14C) migration.

TABLE 12

Survey of the Potential of 5-Hydroxyuridine to Form a Complex
With Standard Nucleic Acid Enzymatic Digestion Products

| Reaction Mixture* | Formation of Compound A (Artifact) |
|---|---|
| 1. 5OHUR + s.v. + a.p. in MgCL$_2$, pH8.6, 12 hr.,30 hr. | no |
| 2. 5OHUR +a.p. in MgCL$_2$, pH 8.6, 12 hr., 30 hr. | no |
| 3. 5OHUR + s.v. + a.p. in H$_2$O, pH 8.6, 12 hr., 30 hr. | no |
| 4. (1) + GMP, 30 hr., (2) + GMP, 30 hr. | no |
| 5. (1) + UMP, 30 hr., (2) + UMP, 30 hr. | no |
| 6. (1) + AMP, 30 hr., (2) + AMP, 30 hr. | no |
| 7. (1) + AMP, CMP, GMP, UMP, 30 hr. | no |
| (2) +AMP, CMP, GMP, UMP, 30 hr. | no |
| 8. GMP in MgCL$_2$; + a.p., 4 hr., + 5OHUR, 4 hr. would show if A. from GR + 5OHUR | no |
| 9. GMP in MgCL$_2$, 4 hr.; + 5OHUR + a.p., 4 hr. would show if A. from GMP + 5OHUR | no |
| 10. GMP in MgCL$_2$, 4 hr.; + 5OHUR, 4 hr., would show if A. formed nonenzymatically with GMP | no |
| 11. 5OHUR in MgCl$_2$, 4 hr., + a.p., 4 hr., would show if A. formed by modification of 5OHUR by a.p. | no |
| 12. GMP in MgCl$_2$, 4 hr.; + a.p., 4 hr., would show if A. formed from only GMP by a.p. | no |
| 13. GR in MgCl$_2$, 4 hr.; + a.p., 4 hr., would show if A. formed from only GR by a.p. | no |
| 14. GR in MgCl$_2$, 4 hr., + a.p., 4 hr.; + 5OHUR, 4 hr., would show if A. formed from modified GR + 5OHUR by a.p. | no |
| 15. 5OHUR in MgCl$_2$, 4 hr., + a.p., 4 hr.; + 5OHUR, 4 hr, would show reverse of reaction (9) predominates | no |

* All reaction mixtures incubated at 37°C in 5X10$^{-3}$M MgCl$_2$ and submitted to ascending paper chromatography in 2-propanol-H$_2$O- conc. Hcl (680:144:170); concentration of each substrate equals 1mg/ml; abbreviations: A, artifact or complex; a.p., alkaline phosphatase; s.v., snake venom; 5OHUR, 5-hydroxuridine; AMP, adenylic acid; CMP, cytidylic acid; GMP, guanylic acid; UMP, uridylic acid; GR, guanosine .

TABLE 13

Rf Values of 5-Hydroxyuridine  Artifacts &  Controls

| Compound | Rf Values in Chromatographic Solvent Systems * | | | |
|---|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol- $H_2O$ conc. HCl(680:144.170) | Identity of Component |
| 1.  Compound A,  UAM | -.041-.030 UAM + L | .20 UAM + L + .45 L | .26 UAM + .67 L | Comp. B + CR-Like + Comp. D |
| 2.  Compound B | - | .471 | .657-.670 | dihydrouracil? |
| 3.  Compound C,  UAM | - | .20 | .26 | CR-Like+Comp. D |
| 4.  CR-Like,  UAM | .234 | .383 | - | cytidine |
| 5.  Compound D,  UAM | - | .733 | nd | ? |
| 6.  5-Hydroxyuridine | .136-.156 | .300-.311 | .587 | - |
| 6a.  DH-spot | .137-.145 | .300-.311 | | fp-DH-al of 6 |
| 7.  5-Hydroxy 2-deoxyridine | .253 | .459 | | - |
| 8.  Dihydrouridine | .089 | .297 | .610 | - |
| 9.  Cytidine | .229 | .376 | | - |

*  Ascending paper chromatography on washed paper, see methods.
   UAM, ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance giving
   positive test for dihydropyrimidine or 3-ureido acid; DHSC, definitely a contaminant which gives a
   positive test for dihydropyrimidine or 3-ureido acid; nd, not detectable; fp-DH-al, definitely false
   positive dihydropyrimidine test through breakdown of substance indicated by alkali used in test.

TABLE 13 (continued)

Rf Values of 5-Hydroxyuridine Artifacts & Controls

| Compound | Rf Values in Chromatographic Solvent Systems * | | | |
|---|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol-$H_2O$ Conc. HCl(680:144:170) | Identity of Component |
| 10. Uridine | .224 | .412 | .683 | - |
| 11. D-ribose | .241 | .455 | | - |
| 12. B-alanine -= DHS | .033 | .165 | | - |
| 12a. DHSC | .165 | nd | | same as 12a, 22f, 23c |
| 13. B-aminoiso butyric acid = DHS | .065 | .245 | | - |
| 14. B-carbamyl alanine=DHS | .444 | .582-.598 | | - |
| 14a. DHSC | .289 | .480 | | dihydrouracil |
| 15. Dihydrouracil | .300 | .474 | .665 | - |
| 16. Isobarbituric acid | .285 | .421 | | - |
| 16a. DHSC | .250-.306 | nd | | dihydrouracil? |

* Ascending paper chromatography on washed paper, see methods.
UAM, ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance giving positive test for dihydropyrimidine or 3-ureido acid; DHSC, definitely, contaminant which gives a positive test for dihydropyrimidine or 3-ureido acid; nd, not detectable; fp-DH-al, definitely false positive dihydropyrimidine test through breakdown of substance indicated by alkali used in test.

TABLE 13 (continued)
Rf Values of 5-Hydroxyuridine Artifacts & Controls

| Compound | Rf Values in Chromatographic Solvent Systems * | | | |
| --- | --- | --- | --- | --- |
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol-$H_2O$ Conc. HCl (680:144:170) | Identity of Component |
| 17. 5-Hydroxymethyluracil | .274 | .428 | | – |
| 17a. UAC | .117 | .292 | | ? |
| 18. Alloxan | .400-.423 | .542-.579 | | – |
| 18a. DHSC 1 | .427 | .524 | | fp-DH-al of 18 |
| 19b. DHSC 2 | .265 | nd | | dihydrouracil |
| 20. Alloxantin· $2H_2O$ | | | | |
| 20a. DHSC 1 | .296-.303 | .473 | | dihydrouracil |
| 20b. UAM 1 | .404 | .585 | | alloxan |
| 20c. DHSC 2 | .075 | nd | | same as 13 |
| 20d. UAM 2 | .513 | .672 | | parabanic acid? |
| 20e. DHS over 20 d | .513 | .672 | | fp-DH-al of 23? |

* Ascending paper chromatography on washed paper, see methods.
  UAM, ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance giving positive test for dihydropyrimidine or 3-ureido acid; DHSC, definitely a contaminant which gives a positive test for dihydropyrimidine or 3-ureido acid; nd, not detectable; fp-DH-al, definitely false positive dihydropyrimidine test through breakdown of substance indicated by alkali used in test.

TABLE 13 (continued)

Rf Values of 5-Hydroxyuridine Artifacts & Controls

| Compound | Rf Values in Chromatographic Solvent Systems * | | | |
|---|---|---|---|---|
| | Butanol-$H_2$O (86:14) | Butanol-$H_2$O Ethanol (5:2:3) | 2-propanol-H O Conc. HCl(680:144:170) | Identity of Component |
| 21. Barbituric acid | .274-.286 | .424 | | - |
| 21a. DHSC | .256-.266 | .457 | | fp-DH-al of 21 |
| 21b. UAC | .076 | .206-.221 | | dialuric acid ?as 22a |
| 22. Dialuric acid (a-i?) | | | | |
| 22a. UAM 1 | .092 | .237 | | dialuric acid ?as 21b |
| 22b. UAM 2 | .400 | .576 | | alloxan |
| 22c. DHSC 1 | .292 | .468 | | dihydrouracil |
| 22d. UAS 3 | .513 | .668 | | parabanic acid |
| 22e. DHS over 22d | .513 | .668 | | fp-DH-al of 23 |
| 22f. DHSC 2 | .158-.166 | nd | | same as 12a, 23c |
| 22g. DHSC 3 | .087-.100 | nd | | dihydrouridine |

* Ascending paper chromatography on washed paper, see methods.
UAM, ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance giving positive test for dihydropyrimidine or 3-ureido acid; DHSC, definitely, a contaminant which gives a positive test for dihydropyrimidine or 3-ureido acid; nd, not detectable; fp-DH-al, definitely false positive dihydropyrimidine test through breakdown of substance indicated by alkali used in test.

## TABLE 13 (continued)

### Rf Values of 5-Hydroxyuridine Artifacts & Controls

| Compound | Rf Values in Chromatographic Solvent Systems* | | | |
|---|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol-$H_2O$ conc. HCl(680:144:170) | Identity of Component |
| 22h. UAM 4 | 0 | nd | | ? |
| 22i. DHS over 22h | 0 | nd | | ? |
| 23. Parabanic acid | .519 | .663 | | - |
| 23a. DHS over 23 | .512 | .668 | | fp-DH-al of 23 |
| 23b. DHSC 1 | .295 | .473 | | dihydrouracil |
| 23c. DHSC 2 | .162 | nd | | same as 12a, 22f |
| 23d. DHSC 3 | .091-.100 | .303 | | dihydrouridine |

* Ascending paper chromatography on washed paper, see methods.
UAM, ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance giving positive test for dihydropyrimidine or 3-ureido acid; DHSC, definitely a contaminant which gives a positive test for dihydropyrimidine or 3-ureido acid; nd, not detectable; fp-DH-al, definitely false positive dihydropyrimidine test through breakdown of substance indicated by alkaline used in test.

TABLE 14

Ionization Constants of Compound $H_{28}$[*]

Calculated $pKa_1$ values

| Wavelenght nm | pH | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1.8 | 2.0 | 2.1 | 2.3 | 2.4 | 2.6 | 2.9 | 3.1 | 3.8 |
| 235 | 2.822 | 2.592 | 2.384 | 2.391 | 2.388 | 2.420 | 2.305 | 2.303 | 2.578 |
| 240 | 2.960 | 2.740 | 2.378 | 2.380 | 2.268 | 2.402 | 2.240 | 2.242 | 2.453 |

Average $pKa_1$ = 2.5

Calculated $pKa_2$ values

| Wavelenght nm | pH | | | |
|---|---|---|---|---|
| | 10.9 | 11.2 | 11.6 | 12.0 |
| 220 | 11.757 | 11.815 | 11.932 | 11.892 |

Average $pKa_2$ = 11.85

[*] Ionization constants were calculated as pKa values according to the procedure described in Methods. Absorbancies at each wavelenght & pH were obtained from spectral curves of Figure 48. For $pKa_1$ UI = absorbance at pH 1.6, I = absorbance at pH 8.6. For $pKa_2$ UI = absorbance at pH 8.6 = 10.0, I = absorbance at pH 12.3.

TABLE 15

Ionization Constants of Compound P *

| | Calculated pKa$_1$ values | | | | | |
| | Wavelenght nm | | | | | |
| pH | 245 | 250 | 265 | 269 | 275 | 280 |
|---|---|---|---|---|---|---|
| 2.6 | 3.712 | 3.660 | 3.217 | 2.919 | 3.259 | 3.185 |
| 3.3 | 3.174 | 3.123 | 3.000 | 2.925 | 3.027 | 2.979 |
| 4.1 | 3.120 | 3.034 | 2.969 | 2.987 | 2.957 | 2.892 |

Average pKa$_1$ = 3.1

| | Calculated pKa$_2$ values | | |
| | Wavelenght, nm | | |
| pH | 250 | 275 | 280 |
|---|---|---|---|
| 11.5 | 12.082 | 11.952 | 11.992 |
| 11.8 | 12.079 | 12.030 | 11.632 |
| 12.0 | 11.652 | 11.963 | 12.000 |
| 12.4 | 11.661 | 11.810 | 11.906 |

Average pKa$_2$ = 11.9

* Ionization constants were calculated as pKa
values according to the procedure described
in Methods. Absorbance at each wavelenght
and pH were obtained from spectral curves
of Figure 49. For pKa$_1$   UI = absorbance
at pH 1.6   UI = absorbance at pH 5.2.
For pKa$_2$ UI = absorbance at pH 5.2 = 10.6,
I = absorbance at pH 13.7.

## TABLE 16

Rf Values of Products from Piperidine Hydrolysis of 5-Hydroxyuridine

| Compound | Rf Values in Chromatographic Solvent Systems * | | |
|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ (86:14) | Tentative Identity ** of Unknown Component |
| 1. Reaction Mixture 22 (10 per cent piperidine, 100° C, 105 minutes, 3.85 x $10^{-3}$ molar substrate) | | | |
| 1a. UAM 1 | .066 | .259 | Compound P |
| 1b. UAM 2 | .140 | .300 | 5-hydroxyuridine |
| 1c. DHS | .096-.120 | nd | dihydrouridine |
| 2. Reaction Mixture 26 (one per cent piperidine, 100° C, 40 minutes, 3.85 x $10^{-3}$ molar substrate) | | | |
| 2a. UAM 1 *** | .026, .064-.073 | .263 | Compound P |

* Ascending paper chromatography on washed paper, see methods.
** Compared with migration of controls, Table 11; UAM ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance, giving positive test for dihydropyrimidine or β-ureido acid; nd, not detectable; dna, data not available.
*** Rf values for Compound P and 5-hydroxyuridine, respectively: 0.36, 0.068, in butanol-$H_2O$ (86:14) in $NH_3$ gas; 0.219, 0.181 in butanol-$H_2O$-glacial acetic acid (5 0:25:25); 0.648, 0565 in 2-propanol-$H_2O$-$NH_3$ (7:2:1)

## TABLE 16 (continued)

### Rf Values of Products from Piperidine Hydrolysis of 5-Hydroxyuridine

| Compound | Rf Values in Chromatographic Solvent Systems * | | |
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | Tentative Identity ** of Unknown Component |
| --- | --- | --- | --- |
| 2b.  UAM 2 | .136-.161 | .301-.324 | 5-hydroxyuridine |
| 2c.  Sugar Spot | .099 | nd | not ribose, same as 3c |
| 2d.  DHS 1 | .107 | .310 | dihydrouridine |
| 2e.  DHS 2 | nd | .463 | dihydrouracil |
| 3.  Reaction mixture 28 (one per cent piperidine, $100^{\circ}C$, 40 minutes, $1.08 \times 10^{-4}$ molar substrate | | | |
| 3a.  UAM 1 | .013 | nd | ? |
| 3b.  UAM 2 | .025 | nd | ? |
| 3c.  DHS 1 | .062-.076 | .256 | dihydroderivative of Compound P ? |

\* Ascending paper chromatography on washed paper, see methods.
\* \* Compared with migration of controls, Table 11; UAM ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance, giving positive test for dihydropyrimidine or β-ureido acid; nd, not detectable; dna, data not available.

TABLE 16 (continued)

Rf Values of Products from Piperidine Hydrolysis of 5-Hydroxyuridine

| Compound | Rf Values in Chromatographic Solvent Systems * | | |
|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | Tentative Identity ** of Unknown Component |
| 3d.  DHS 2 | .286 | .460 | dihydrouracil |
| 3e.  Sugar Spot | .091 | dna | not ribose, same as 2c. |
| 3f.  UAS 3 | .127 | nd | 5-hydroxyuridine |
| 3g.  UAS 4 | .503-.532 | .686 | Compound $H_{28}$ |

\* Ascending paper chromatography on washed paper, see methods.
\*\* Compared with migration of controls, Table 11; UAM ultraviolet absorbing material; UAC, ultraviolet absorbing contaminant; DHS, substance, giving positive test for dihydropyrimidine or β-ureido acid; nd, not detectable; dna, data not available.

TABLE 17

Rf Values for Partial Alkaline Hydrolysis of 5-Hydroxyuridine & Controls[3]

| | Rf Values in Chromatographic Solvent Systems | | | | |
|---|---|---|---|---|---|
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol-$H_2O$ Conc. HCl(680:144:170) | 2-propanol-$H_2O$ ConcNH (7-2-1) | Tentatively Identifiable Components |
| 1. Dihydrouracil | .299 | .458 | .665 | .290 | — |
| 2. Dihydrouridine | .128 | .320 | .610 | .123 | — |
| 3. 5-Hydroxyuridine | .157 | .317 | .587 | .075 | — |
| 4. Uridine | .221 | .413 | .683 | .186 | — |
| 5. Partial alkaline hydrolysis products of dihydrouridine, (5a-5g)[1] | | | | | |
| 5a. | .495 | .628 | .834 | .009 | |
| 5b. | .128 | 0 | .573 | .107 | |
| 5c. | nd[2] | .310 | .729 | nd | |
| 5d. | nd | .471 | .963 | nd | |
| 5e. | 0 | .400 | .669 | .154 | |

1. In order of decreasing intensity of dihydro-pyrimidine spray reaction and increasing Rf.
2. nd, not detectable
3. See also Figures 70-73; hydrolytic conditions: ten minutes at 80°C in 0.3 M KOH

TABLE 17 (continued)

Rf Values for Partial Alkaline Hydrolysis of 5-Hydroxyuridine & Controls[3]

| | Rf Values in Chromatographic Solvent Systems | | | | |
| --- | --- | --- | --- | --- | --- |
| | Butanol-$H_2O$ (86:14) | Butanol-$H_2O$ Ethanol (5:2:3) | 2-propanol-$H_2O$ ConcHCl(680:144:170) | 2-propanol-$H_2O$ ConcNH$_3$ (7-2-1) | Tentatively Identifiable Components |
| 5f. | .318 | nd[2] | 0 | .309 | |
| 5g. | .225 | nd | nd | nd | |
| 6. Partial alkaline hydrolysis products of 5-hydroxyuridine, (6a-6e) | | | | | |
| 6a. 5-hydroxyuridine remaining | .125 | .317 | .587 | .015 | |
| 6b. Alkali product Z | .286 | .445 | .678 | .280 | dihydrouracil |
| 6c. Forward running shoulder of Z | .350 | nd | .729 | nd | |
| 6d. Alkali trailing product S | .061 | .189 | 0 | nd | |
| 6e. Additional alkali trailing product | nd | .016 | nd | nd | |

2. nd, not detectable
3. See also Figures 70-73; hydrolytic conditions: ten minutes at 80°C in 0.3 M KOH.

TABLE 18

Rf Values of Chemical Alteration Products of 5-Hydroxyu-
ridine & Controls

| | Compound | Rf Values 2-propanol-$H_2O$ conc. HCL(680: 144:170) | Tentative Identity of unknown Component |
|---|---|---|---|
| 1. | Dihydrouracil | .665 | - |
| 2. | Dihydrouridine | .610 | - |
| 3. | 5-Hydroxyuridine | .587 | - |
| 4. | Uridine | .683 | - |
| 5. | Compound B | .669 | Dihydrouracil |
| 6. | Band C material, Fig. 70 reaction 5-hydroxyuridine in ammonia, migration Fig. 73. | .669 | Dihydrouracil |
| 7. | Band C material, Fig. 70 reaction of 5-hydroxyuridine in polassium permangamate, migration Fig. 73. | .669 | Dihydrouracil |
| | | Butanol-$H_2O$ (86:14) | |
| 8. | D-ribose | .241 | - |
| 9. | Aldo-pentose positive material released in reaction of 5-hydroxyuridine in ammonia or potassium permangamate in Fig. 70. | .144 | not ribose |

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