APPLICATION OF THE ISOTOPE DERIVATIVE METHOD TO THE ANALYSIS OF PYRIMIDINES*

BY JACQUES R. FRESCO† AND ROBERT C. WARNER

(From the Department of Biochemistry, New York University College of Medicine, New York, New York)

(Received for publication, February 2, 1955)

In recent years several methods have been introduced for the analysis of the nitrogenous constituents of nucleic acids (cf. (2, 3)). However, a specific method is lacking for accurate analysis of microgram quantities of these substances in hydrolysates of biological specimens from which the nucleic acids are not readily isolated. A method which fulfills this need is presented. It applies the principles of the isotope derivative method (4, 5) to the determination of uracil and thymine, the pyrimidines characteristic of ribonucleic (RNA) and deoxyribonucleic (DNA) acids, respectively. The isotope derivative method, as employed here, involves the quantitative conversion of uracil or thymine to isotopically labeled p-iodophenylsulfonyl (pipsyl) derivatives, which are then quantitatively determined by carrier (4) or indicator (5) procedures without the necessity for quantitative isolation of the derivatives.

EXPERIMENTAL

Reaction of Pyrimidines and Purines with Pipsyl Chloride

Pipsyl chloride was explored for use as the analytical reagent because it was expected to react with compounds which possess amino or hydroxyl groups and it can be labeled with two distinguishable isotopes, I\(^{131}\) and S\(^{35}\). After examining a variety of aqueous and organic one- and two-phase reaction systems, it was found that uracil\(^{1}\), thymine, isocytosine, hypoxanthine, and 2,6-diaminopurine, but not 2-aminopyrimidine, cytosine, ade-

* A preliminary report of this work was presented at the Fortieth annual meeting of the American Society of Biological Chemists, April, 1949 (1). Taken, in part, from the thesis submitted by Jacques R. Fresco in May, 1952, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Science of New York University. This work was supported in part by grants to Dr. Milton Levy by the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. The iodine and sulfur isotopes used in this study were supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

† Present address, Department of Pharmacology, New York University College of Medicine, New York.

1 Substituted pyrimidines are numbered so that uracil is 2,4-dihydroxypyrimidine.

751
nine, and guanine formed monopipsyl derivatives in good yield. It would appear, therefore, that only those pyrimidines and purines which possess potentially enolic groups with $pK_a$ values in the region of 9 form those derivatives readily. A one-phase 75 per cent acetone system at room temperature, buffered at pH 8.5 to 9.0 with tetramethylammonium bicarbonate, was found to be the most effective medium for reaction of pipsyl chloride with uracil and thymine. A consistent 94 per cent conversion to monopipsyl derivatives has been obtained in recovery control analyses. This yield was not altered when the molar ratio of pipsyl chloride to pyrimidine was varied from 3 to 47 (Table I), indicating that dipipsyl derivatives are not formed.

The pipsylpyrimidines are stable at room temperature below pH 10; in stronger alkali they are readily hydrolyzed to $p$-iodophenylsulfonic acid (pipsyl acid) and the free pyrimidines. The derivatives are slightly soluble氯化物 with uracil and thymine. A consistent 94 per cent conversion to monopipsyl derivatives has been obtained in recovery control analyses. This yield was not altered when the molar ratio of pipsyl chloride to pyrimidine was varied from 3 to 47 (Table I), indicating that dipipsyl derivatives are not formed.

The pipsylpyrimidines are stable at room temperature below pH 10; in stronger alkali they are readily hydrolyzed to $p$-iodophenylsulfonic acid (pipsyl acid) and the free pyrimidines. The derivatives are slightly soluble.

\* Unless otherwise indicated, the amount of pipsyl chloride added was 5 to 10 times the amount of pyrimidine added.

† Uracil dissolved in 60 per cent HClO$_4$.

‡ Recovered as additional uracil when it was added to a nucleic acid hydrolysate previously analyzed.

**2,6-Diaminopurine is an exception to this generalization for which there is no apparent explanation.**
in acid and are soluble in alkali, alcohol, acetone, and ether. Some of their properties are presented in Table II.

Several considerations bear on the structure of these derivatives. (a) Nitrogen analyses are consistent with monopipsyl forms. (b) Coupling between pipsyl chloride and the pyrimidines does not take place below pH 8. (c) For uracil and thymine, dissociation occurs first at positions 1:2 (pK values 9.5 and 9.9, respectively) and then at positions 3:4 (pK values greater than 13) (6). (d) In the acetone medium referred to above, 1-methyluracil and 2-ethoxy-4-hydroxypyrimidine did not form pipsyl derivatives, while 4-ethoxypyrimidone did. Hydrolysis of the 4-ethoxy group from the pipsyl derivative of the latter in ethanolic HCl at room temperature yielded pipsyluracil. Thus, replacement of the dissociable proton of the 1:2 positions of uracil prevented reaction with pipsyl chloride, while corresponding replacement at the 3:4 positions did not. Therefore, attachment of the pipsyl moiety at the 3 or 4 position of these pyrimidines is eliminated. It is presumed that pipsyluracil and pipsylthymine are monosubstituted at the 1 or 2 position of the heterocyclic ring. The data do not permit a decision between these two positions. Benzenesulfonylpyrimidines were also prepared by treating uracil or thymine with benzenesulfonyl chloride in the acetone medium; they are presumed to be structural analogues of the pipsylpyrimidines.

**Table II**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Decomposition point, uncorrected</th>
<th>Eₘ(λₘₐₓ) (nm)</th>
<th>Nₙ, found</th>
<th>Nₙ, theoretical</th>
<th>Crystal form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipsyluracil</td>
<td>234-239</td>
<td>20,700 in 0.04 N HCl at 260 μm</td>
<td>7.41</td>
<td>7.40</td>
<td>Flat prisms</td>
</tr>
<tr>
<td>Pipsylthymine</td>
<td>229-231</td>
<td>20,230 in 0.04 N HCl at 264 μm</td>
<td>7.16</td>
<td>7.15</td>
<td>Fine long needles</td>
</tr>
</tbody>
</table>

Preparation of Compounds

**Non-Isotopic Pipsyluracil and Pipsylthymine Carriers**—Uracil or thymine was treated with a 2- to 3-fold excess of pipsyl chloride (4) in the buffered acetone medium (see below) for 1 to 2 hours. The reaction mixture was then acidified and the acetone removed *in vacuo*. The resulting pre-

---

3 The symbol 1:2 is used because the dissociating proton is involved in a lactam-lactim tautomerization and it has not been possible to determine the position from which it dissociates.

4 These compounds were kindly furnished by Dr. Jack Fox.
Precipitate was washed with water and recrystallized (several times from each solvent) with charcoal treatments, first from ammoniacal-alcohol buffer (0.36 M NH$_3$ and 0.12 M NH$_4$Cl in 80 per cent alcohol) by dilution with water and acidification, next from acetone, then from 95 per cent alcohol. It was finally washed with water and dried at 105°. The best evidence of the purity of these compounds is that the procedure described above yielded products of constant specific activity when a labeled reagent was employed (cf. "Carrier analysis"). It was further indicated by the reproducibility of nitrogen content and molar extinction coefficient observed in a number of preparations.

**Uracil and Thymine Standards**—Pure benzenesulfonyl or pipsyl derivatives of uracil or thymine were hydrolyzed in excess N alkali at 100°. The pyrimidines recovered on acidification of these hydrolysates were recrystallized several times from water with charcoal treatments and dried at 105°. The purity of these compounds was confirmed by determination of nitrogen content, molar extinction coefficient, and decomposition point.

$^{35}$S-labeled Pipsyluracil and Pipsylthymine Indicators—To conserve $^{35}$S-labeled pipsyl chloride (5), a 3-fold excess of the pyrimidine (10 mg.) was treated in the acetone medium with the reagent (10 mg.). The acidified reaction mixture (see below) was extracted several times with ether. The pooled ether extracts were washed with water and extracted several times with equal volumes of borate buffer (pH 10); the pipsylpyrimidines, precipitated from the borate extracts on acidification, was washed with water and recrystallized several times from ammoniacal-alcohol buffer. The final precipitate was washed with water and dissolved in 50 per cent alcohol or 50 per cent acetone for use in the indicator analyses. The purity of an indicator was checked by paper chromatographic analysis (cf. "Indicator analysis").

**Tetramethylammonium Bicarbonate Buffer**—Carbon dioxide was bubbled through tetramethylammonium hydroxide (1.1 M) until the solution indicated a pH of about 8.5 with Hydrion paper. Occasionally a gelatinous precipitate formed which was removed by centrifugation.

**Analytical Scheme for Uracil and Thymine**

Earlier presentations of the isotope derivative method (4, 5, 7) should be consulted for more explicit details and discussion of the methods and calculations described here.

**Reaction Mixture**—The unknown is dissolved in a minimal volume of tetramethylammonium bicarbonate buffer or is brought to about pH 8.5 by the addition of tetramethylammonium hydroxide. To this solution are added pipsyl chloride in at least a 10-fold excess over the reactive groups presumed to be in the unknown, enough tetramethylammonium bicarbon-
ate buffer to neutralize the acid which may be formed during the reaction, and acetone to a final concentration of 75 per cent, after which the reaction vessel is stoppered. This reaction mixture is shaken to insure solution, kept at room temperature for 1 hour, and then acidified with HCl in 75 per cent acetone.

**Carrier Analysis**—I\textsuperscript{131}-labeled pipsyl chloride (4) is employed for the reaction. The acidified reaction mixture is quantitatively transferred to a vessel containing an accurately weighed amount (50 to 100 mg.) of the carrier of the compound of interest. Homogeneous distribution of the carrier in the reaction mixture is effected by adding 75 per cent acetone to the mixture and heating until all the carrier is dissolved. Most of the acetone is then evaporated and the carrier precipitates on cooling.

When uracil and thymine are both presumed present in the unknown, a preliminary separation of their derivatives is effected after addition of both

| **Table III** |
| **Distribution Coefficients** |
| **Expressed as the ratio of concentration in the organic phase to that in the aqueous phase.** |

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Ether and 0.1 M borax (pH 9.2)</th>
<th>Ether and 0.2 M borate buffer (pH 10.0)</th>
<th>Ether and 0.01 N HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipsyluracil</td>
<td>1.1</td>
<td>0.14</td>
<td>38</td>
</tr>
<tr>
<td>Pipsylthymine</td>
<td>6.2</td>
<td>1.1</td>
<td>66</td>
</tr>
<tr>
<td>Pipsyl acid</td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

carriers by means of a modified seven plate counter-current distribution between 2 volumes of 0.1 M borax buffer (pH 9.2) and 1 volume of ether. This separation is based on the distribution coefficients given in Table III. The precipitated carriers are washed with water and dissolved in the ether phase of the first plate. Four aqueous phases are each completely transferred through the seven ether phases. The pooled ether phases of the first four plates, containing the bulk of the pipsylthymine, are extracted three times with 0.3 volume of 0.1 N NaOH. The alkaline extracts are pooled and immediately acidified to precipitate the pipsylthymine. The four aqueous phases are also pooled and the pipsyluracil therein precipitated by acidification.

The individual carriers are readily recrystallized in good yield from the ammoniacal-alcohol buffer by dilution with water and acidification with HCl to below pH 3. Generally, eight to ten recrystallizations of the carrier, with alternate charcoal treatments (Norit A), are sufficient for removal of coprecipitating pipsyl derivatives formed in reaction mixtures containing nucleic acid hydrolysis products (cf. Table V). A pure carrier
is indicated by constancy of specific activity through a series of crystallizations. Typical examples of such data are presented in Table IV.

For determination of its specific activity, the carrier is dissolved in 7 ml. of the ammoniacal-alcohol buffer and then diluted to 10 ml. with water. An aliquot of 100 to 200 µl. is accurately obtained with a microconstriction pipette and diluted to a known volume with 0.04 M HCl. The optical density, measured against a suitable blank at the appropriate wave-length of maximal absorption, serves to determine the concentration of carrier.

### Table IV

*Specific Activities of Purified Carriers*

The carriers were isolated from pipsylation reaction mixtures of nucleic acid hydrolysates.

<table>
<thead>
<tr>
<th>Pipsyluracil</th>
<th>Pipsylthymine</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of crystallizations</td>
<td>Carrier remaining</td>
</tr>
<tr>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Average</td>
<td></td>
</tr>
</tbody>
</table>

* See foot-note 5.

Radioactivity can be measured in one of two ways. In one method, preferable when the specific activity of the isolated carrier is low, an accurately measured aliquot (3 to 10 ml.) of the ammoniacal-alcohol carrier solution is placed in an annular glass counting cell. After determining its radioactivity with a Geiger counter, the aliquot can be removed for further recrystallization. The measurable radiation of such liquid samples depends, in part, on the density (the salt and solvent concentration) of the sample. It is therefore necessary to dilute unknowns and reagent standards to the same concentration of ammoniacal-alcohol buffer and to take equal volumes of each for measurement of radioactivity. In the second method of radioactive analysis of the carrier solution an aliquot, the salt content of which is too low to incur an error because of self-absorption, less than 50 µl., is delivered onto a metal planchet already containing a 0.2 ml. drop of water. The sample is dried and counted under a thin mica window Geiger tube. The latter procedure is applicable only when the

---

* Isotope concentration or specific activity is defined as the ratio of counts per minute per micromole of sample to counts per minute per micromole of reagent standard.
specific activity of the carrier is so high that such a small sample can be taken for analysis. In either procedure an appropriate "reagent" standard consisting of a known amount of a pure pipsyl derivative, prepared with the same I\(^{131}\)-labeled pipsyl chloride that was used in the analysis, is counted at the beginning and end of each counting period. In addition to providing the specific activity of the reagent, this standard serves to correct for the decay of I\(^{131}\).

**Table V**

**Zero Control Analyses with Pipsyluracil Carrier**

5 \(\mu\)moles of the indicated substance were treated with I\(^{131}\)-pipsyl chloride in the acetone medium. 100 to 200 \(\mu\)moles of carrier pipsyluracil were then added. Purification of the carrier was conducted as indicated.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Apparent uracil found</th>
<th>No. of recrystallizations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)mole</td>
<td>per cent of added substance</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.0062</td>
<td>0.12</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.0061</td>
<td>0.12</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.0085</td>
<td>0.17</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.211</td>
<td>4.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
<td>Cytosine</td>
<td>0.0028</td>
<td>0.05</td>
</tr>
<tr>
<td>Thymine</td>
<td>0.04</td>
<td>0.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.022</td>
<td>0.4</td>
</tr>
<tr>
<td>Protein hydrolysate</td>
<td>0.046</td>
<td>0.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.0156</td>
<td>0.3</td>
</tr>
<tr>
<td>I(^{131})-Pipsyl acid†</td>
<td>0.0038</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* Partially purified by counter-current distribution prior to recrystallization.
† 10.5 \(\mu\)moles of pipsyl acid were used.

The amount of carrier added and the specific activities of the reagent and the pure isolated derivative are the data required to calculate the quantity of unknown (4).

In addition to the recovery control analyses (Table I) which checked the yield, the carrier procedure was tested for specificity in zero control analyses. The latter involved determinations of uracil on samples lacking uracil but containing a variety of possible contaminants. The samples were treated with I\(^{131}\)-pipsyl chloride. Pipsyluracil carrier was then added and purification was carried out by crystallization and counter-current procedures. In the examples presented in Table V, constant (zero) specific activity of the carrier had not yet been achieved at the time the analyses were terminated, but the amounts of carrier remaining were sufficient to have permitted many more recrystallizations.
Indicator Analysis—The unknown sample is treated with $^{131}$-labeled pipsyl chloride reagent. The acidified reaction mixture is diluted to a known volume with 50 per cent acetone. To an aliquot of this dilution are added aliquots of the appropriate $^{35}$S-labeled indicators. The solution is warmed to evaporate the acetone, diluted with 0.1 N HCl to a convenient volume, and extracted twice with equal volumes of ether. The pipsylpyrimidines pass into the ether phase, leaving most of the pipsyl acid, a major contaminant, in the aqueous phase. The ether extracts are pooled, evaporated to a small volume, and an aliquot containing an appropriate number of counts is applied to a Whatman No. 1 filter paper strip for chromatography. The chromatogram is developed with the organic phase of a freshly equilibrated mixture of tetrachloroethylene, c.p. (1 volume), butanol (0.02 to 0.03 volume), and HzO (1 volume), in which the retardation factors for pipsyl acid, pipsyluracil, and pipsylthymine are approximately 0.0, 0.5, and 0.7, respectively. The best resolution is achieved in this solvent when the amount of each derivative applied to a paper strip 3 cm. wide is less than 10 $\gamma$. It is sometimes desirable to remove a band and rechromatograph its eluate in the same solvent in order to remove tailing contaminants.

The developed paper chromatograms are air-dried and the components located by radioautography. The appropriate bands are cut out and divided into four or five transverse strips each. These are individually eluted with weak ammonia water or 50 per cent acetone, and the eluates are dried on metal planchets. The samples are almost weightless and are counted with a mica window Geiger tube without concern for self-absorption.

The radiation characteristics of $^{131}$ and $^{35}$S are distinguishable by differential penetration through thin aluminum filters. Each planchet is counted with ($\bar{a}$ counts) and without a filter ($\bar{s}$ counts), and the ratio of these respective counts, $\bar{a}/\bar{s}$, is compared for each of the several planchets made from the parallel strips in each band. If the ratios of consecutive bands show deviations from their average greater than predicted from the number of disintegrations measured, the band is considered impure. Examples of acceptable ratios are shown in Table VI. An $^{131}$ “reagent” standard (cf. “Carrier analysis”) and an $^{35}$S “indicator” standard containing a known aliquot of the particular $^{35}$S indicator are also counted with and without a filter along with each set of planchets.

The filter factors for pure $^{131}$ and $^{35}$S ($\bar{a}/\bar{s}$ for “reagent” and “indicator” standards), the specific activity of the reagent, the number of counts of indicator added, and the $\bar{a}/\bar{s}$ ratio of the particular band are the data required to calculate the quantity of unknown (5).

Accuracy of Analyses—The consistent recoveries of uracil and thymine
(94 ± 1 per cent) obtained in the control analyses (Table I) justify the use of the correction factor 1/0.94. This has been applied to the analytical data obtained by either carrier or indicator procedures (Tables VII and VIII).

The accuracy of the analyses is limited primarily by the error in counting. In the carrier method, this error can be held to 1 per cent by determining 10,000 counts on samples in which the radioactivity is at least 10 times that of background. In the indicator analyses, the errors in several counts can combine to give a larger than additive over-all counting error. To prevent a total error due to counting of greater than 1 per cent it is therefore necessary to determine 20,000 to 40,000 counts on most samples. For a filter which holds back about 99 per cent of the $^{35}$S activity and 35

<table>
<thead>
<tr>
<th>Table VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratios of Counts Measured with and without Filter (ɛ/δ) of Eluates of Consecutive Strips of Pure Bands Obtained from Paper Chromatograms</td>
</tr>
</tbody>
</table>

The pipsyluracil and pipsylthymine were isolated from pipsylation reaction mixtures of nucleic acid hydrolysates.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Strip 1</th>
<th>Strip 2</th>
<th>Strip 3</th>
<th>Strip 4</th>
<th>Strip 5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipsyluracil</td>
<td>0.384</td>
<td>0.392</td>
<td>0.395</td>
<td>0.388</td>
<td>0.396</td>
<td>0.391</td>
</tr>
<tr>
<td>Pipsylthymine</td>
<td>0.381</td>
<td>0.371</td>
<td>0.376</td>
<td>0.381</td>
<td></td>
<td>0.377</td>
</tr>
</tbody>
</table>

per cent of the $^{31}$I activity, high accuracy is favored when the $\varepsilon/\delta$ ratio for the sample is about 0.3.

Analysis of Nucleic Acid Hydrolysates

Hydrolysis with Perchloric Acid—The quantitative aspects of the perchloric acid method of hydrolysis of nucleic acids (9) were reexamined by carrier and indicator procedures to follow the liberation of uracil and thymine from RNA and DNA, respectively. These analyses also demonstrated that the pipsylpyrimidines formed in reaction mixtures containing partial hydrolysis products of nucleic acids can be purified to constant specific activity. A slightly modified procedure for derivative formation was required for the $\mathrm{HClO}_4$ hydrolysates. This procedure, described below, yielded satisfactory recovery analyses of uracil dissolved in $\mathrm{HClO}_4$ (Table I).

An aliquot of unknown is transferred to a Folin sugar tube and the $\mathrm{HClO}_4$ therein precipitated by adding an equivalent amount of tetramethylammonium hydroxide; the other reagents required for pipsylation are then added. The tube is stoppered and shaken for 1 hour on a high
frequency vibrator in order to disrupt the large tetramethylammonium perchlorate crystals in which small amounts of the bases are occluded. Upon acidification of the reaction mixture, the salt crystals dissolve. The analysis is then pursued by carrier or indicator techniques.

For the hydrolysis study, nucleic acid samples of approximately 100 mg. were accurately weighed into 10 ml. volumetric flasks, and 2 ml. of the desired concentration of HClO₄ were added. The flasks were stoppered and placed in a boiling water bath; at various intervals the flasks were removed, cooled in an ice bath, and diluted with water to 10 ml. The hydrolysates were then centrifuged to remove the caramel-like residue formed during the hydrolysis. Aliquots of 50 to 100 μl. (0.5 to 1 mg. of nucleic acid) were then taken for analysis. The results obtained (Table VII) are comparable to those reported by Marshak and Vogel (9). It is evident that DNA is more rapidly hydrolyzed than RNA and that treatment of these nucleic acids for 1 hour in 70 per cent HClO₄ effects complete release of uracil and thymine. The plateau region from 1 to 3 hours indicates that uracil and thymine are stable under the conditions of hydrolysis and that cytosine is not converted to uracil. The latter possibility was further checked by heating cytosine in 70 per cent HClO₄ at 100° for periods of 1 and 2½ hours and analyzing these solutions for uracil by the indicator technique. No significant conversion was detected (<0.01 per cent).

A comparison of some analyses obtained by both isotope derivative and conventional non-isotopic paper chromatographic methods (9, 10) on the same nucleic acid specimens hydrolyzed in 70 per cent HClO₄ (Table VIII) indicates good agreement between these two types of analysis.

Isotope derivative analyses have also been carried out on hydrolysates
of cell fractions in which compounds derived from constituents other than nucleic acids predominated. The pyrimidine content in some cases was less than 1 γ. It was nevertheless possible to purify the pipsylpyrimidine to constant specific activity, demonstrating that quantitative analyses for pyrimidines can be achieved without a preliminary isolation of the nucleic acid.

**Hydrolysis with Formic Acid; Conversion of Cytosine to Uracil**—It was found that cytosine is converted to uracil when nucleic acids are hydrolyzed in 92 per cent formic acid at 130° for long periods (20 to 400 hours). This observation suggested the possibility of determining cytosine by the isotope derivative method as additional uracil, since uracil alone can be determined from HClO₄ hydrolysates. The rate of conversion of cytosine to uracil was then investigated. In 92 per cent formic acid, 0.3 ml. of formic acid per micromole of cytosine, at 140°, cytosine could be quantitatively converted to uracil in 120 hours. While cytosine has not been regularly determined in nucleic acids as additional uracil, this method has been used in a few instances for both RNA and DNA. Under the conditions for conversion of cytosine to uracil it is to be expected that 5-methylcytosine will be converted to thymine, and therefore it may be determined as additional thymine.

**DISCUSSION**

The theoretical basis for isotope derivative analysis (4) provides for a high degree of specificity and sensitivity which is required in many microanalytical problems in biology. Its theoretical and practical advantages
over isotope dilution and other types of analytical procedures have been demonstrated in its application to amino acid and peptide analysis (4, 5, 7, 11, 12).

In the isotope derivative method the accuracy of analysis is dependent on three fundamental factors: reproducible yield of isotopic derivative, isolation of pure isotopic derivative, and accurate isotope analysis (4). Stoichiometric yield is perhaps desirable, but the uncertainties surrounding the quantitative aspects of the reaction between pipsyl chloride and the pyrimidines need not be a cause for concern in view of the consistency of the recoveries. While the reason for the incompleteness of the yields is not known, certain factors may be eliminated; i.e., formation of disubstituted derivatives, impure carriers or indicators, and impure pyrimidine standards. Constancy of specific activity has been taken as a sufficient criterion of the purity of the isolated isotopic derivative. This test, when carefully applied, is far more sensitive than the usual analytical or physical criteria of purity, and its adequacy has been demonstrated in previous work with the isotope derivative method (4, 5, 11, 12). Its application to the analysis of pyrimidines is supported by the data on the purification of isotopic derivatives, by the agreement between carrier and indicator analyses, and by the zero control analyses. It is evident from these considerations that the procedures described for the determination of pyrimidines fulfil the requirements stated above.

Previous attempts at accurate microanalysis of nucleic acid components in the presence of a wide variety of other compounds in hydrolysates of biological specimens have met with little success. Conventional paper chromatographic and microbiological methods have proved to be too insensitive or non-specific (9, 13), and, when isotope dilution was used (14), the determination was subject to gross error, owing to the very small dilution of the isotopic carrier (cf. (15)). On the other hand, the applicability of the isotope derivative method to these mixtures has been demonstrated by the attainment of constant specific activity of the pipsylpyrimidines isolated when such hydrolysates were analyzed.

SUMMARY

A scheme is presented for the analysis of microgram quantities of uracil and thymine by the isotope derivative method, with isotopic p-iodophenylsulfonyl (pipsyl) chloride as the analytical reagent. The method has been applied to the determination of uracil and thymine in nucleic acid hydrolysates. Data are also presented on the liberation of these pyrimidines from nucleic acids hydrolyzed in perchloric acid and on the conversion of cytosine to uracil in formic acid.
Complementary studies on the reaction between pipsyl chloride and pyrimidines and purines are reported.

BIBLIOGRAPHY

APPLICATION OF THE ISOTOPE DERIVATIVE METHOD TO THE ANALYSIS OF PYRIMIDINES
Jacques R. Fresco and Robert C. Warner


Access the most updated version of this article at http://www.jbc.org/content/215/2/751.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/215/2/751.citation.full.html#ref-list-1