Reaction of Folin–Ciocalteau Phenol Reagent with Purines, Pyrimidines, and Pteridines and Its Relationship to Structure

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Purines containing hydroxyl and/or amino groups at both the 2- and 6-positions react strongly with Folin–Ciocalteau phenol reagent. Methylation at either the 3- or 7-positions greatly reduces the reactivity. Analogously substituted pteridines containing hydroxyl and/or amino groups at both the 2- and 4-positions do not react strongly unless additional hydroxyl groups are present at the 6- and/or 7-positions. Analogously substituted pyrimidines containing hydroxyl and/or amino groups at both the 2- and 4-positions do not react. The evidence agrees with voltammetric interpretations that the 4- and 5-positions of the purine nucleus at the ring junctions are uniquely involved in the oxidation of the appropriately substituted purines. Folin–Ciocalteau reagent is a useful chromatographic spray reagent for the detection of small amounts (1 μg or less) or the strongly reacting purines.

It is generally recognized that Folin–Ciocalteau phenol reagent, which is used in the well-known Lowry et al. (1951) method for the estimation of protein, also reacts strongly with substances other than phenols (Peterson, 1979; Box, 1983). Lowry et al. (1951) also had recognized that uric acid, guanine, and xanthine reacted with the reagent, while adenine, hypoxanthine, cytosine, uracil, thymine, guanosine, adenosine, cytidine, and thymidine showed little or no reaction. In a study on the determination of protein contamination in DNA using the Lowry et al. (1951) procedure, Shepherd and Hopkins (1963) reported that guanine and xanthine gave an intense color with Folin–Ciocalteau reagent, that guanosine, guanylic acid, and hypoxanthine gave significant color, and that adenine, cytosine, uracil, and thymine and their nucleosides and nucleotides and orotic acid gave little or no color. The authors cautioned that the high color-yielding purines must be removed before ascertaining the protein contamination of DNA preparations. We have recently found that Folin–Ciocalteau reagent is useful for the detection of paralytic shellfish poisons (red tide poisons) (Mosley et al., 1985). The presence of a partially reduced purine ring system in these poisons prompted us to examine in more detail the effects of substituents on the reactivity of purines and related substances with Folin–Ciocalteau reagent and to establish a rationale for the reaction of the reagent with purines and related compounds.

EXPERIMENTAL SECTION

Materials. The adenine N^1-oxide, 2-hydroxypurine, 2-aminopurine, xanthine N^3-oxide, 2-(dimethylamino)-6-hydroxypurine, isoguanine hemisulfate, isoguanine N^1-oxide, 2,6-diaminopurine hemisulfate, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, theophylline, 1,7-dimethylxanthine, theobromine, caffeine, 1,3,9-trimethylxanthine, pterine, lumazine, xanthopterin, and leucopterin were purchased from Sigma Chemical Co., St. Louis, MO.

Stock Solutions. Stock solutions were made at concentrations of 1 or 0.5 mg/mL in water (2-aminopurine, 2-hydroxypurine, isoguanine), in 0.1 N HCl (hypoxanthine, adenine, cytosine, uracil, guanine, isocytosine, adenine N^1-oxide, isoguanine N^1-oxide, 2,6-diaminopurine, 2-(dimethylamino)-6-hydroxypurine), or in 0.1 N NaOH (the remainder of the compounds).

Procedure. The optimum conditions described by Box (1983) for color production by Folin–Ciocalteau reagent were used. To 2.5 mL of test solution were added 0.375 mL of a 20% solution of sodium carbonate (200 g of Na_2CO_3/L of solution in water) and 0.125 mL of 2 N Folin–Ciocalteau phenol reagent (Fisher Scientific Co., Medford, MA). (*Caution*! The phenol reagent is very corrosive and should not be pipetted by mouth.) The mixture was allowed to stand at room temperature for 1 h and the absorbance read on a Bausch and Lomb Spectronic 710 spectrophotometer at 745 nm in a 1-cm quartz cuvette against a blank containing 2.5 mL of water in place of the test solution. A series of dilutions were run until linear absorbance vs concentration plots were obtained, and results were calculated in terms of absorbance units/milligram of substance per of milliliter solution. Results were also calculated in terms of absorbance/micromole of substance per milliliter solution.

Paper Chromatographic Detection of Purines. After paper chromatography of dilute purine solutions, the chromatograms were dried, sprayed with a 1 to 10 dilution of 2 N Folin–Ciocalteau reagent, redried, then sprayed with 20% (w/v) Na_2CO_3 in water. Blue-gray spots against a light gray background were observed. Reversing the order of the sprays gave similar results.

RESULTS AND DISCUSSION

The results are shown in Table I. Looking at the unmethylated compounds, purines with only a hydroxyl (hypoxanthine) or amino (adenine) group at the 6-position gave very little color, purines with only a hydroxyl or amino group at the 2-position gave significant color, but all the derivatives containing hydroxyl and/or amino groups at both the 2- and 6-positions reacted strongly with Folin–Ciocalteau reagent. The reaction was of the same order of magnitude as the tyrosine reference standard. *N*-Oxides at the 1- or 3-positions did not appear to affect the color greatly. In agreement with Shepherd and Hopkins (1963), the pyrimidines gave virtually no reaction even though they were analogously substituted with hydroxyl and amino groups as the highly reactive purines. The pteridines pterine and lumazine, although similarly substituted in the 1,3-pyrimidine portion of the molecules as the highly reactive purines, also showed low reactivity to the reagent. Addition of hydroxyl groups at the 6- and 7-positions of the pteridine molecule (xanthopterin, leucopterin) made these compounds very reactive.

In order to rationalize these results, it may be appropriate to point out some similarities between the present results and the oxidation of these compounds by voltammetry. Under normal voltammetric conditions pyrimidines are not oxidized (Elving et al., 1973; Varadi et al., 1974), whereas most purines are, the order of ease of oxidation,

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Table I. Color Formation (Absorbance Increase) on Reaction of Folin–Ciocalteau Reagent with Purines, Pyrimidines, and Pteridines

pyrimidine	mol wt	position of substituent[a] 2	4	A/mg per mL[b]	A/μmol per mL
uracil	112.1	—OH	—OH	0.05	<0.01
cytosine	111.1	—OH	$—NH_2$	0.05	<0.01
isocytosine	111.1	$—NH_2$	—OH	<0.03	<0.01

purine	mol wt	position of substituent[a] 1	2	3	6	7–9	A/mg per mL[b]	A/μmol per mL
hypoxanthine	136.1				—OH		0.12	0.02
adenine	135.1				$—NH_2$		1.78	0.24
adenine N^1-oxide	151.1	→O			$—NH_2$		1.78	0.27
2-hydroxypurine	136.1		—OH				6.8	0.92
2-aminopurine	135.1		$—NH_2$				14.5	1.96
xanthine	152.1		—OH		—OH		80.0	12.17
xanthine N^3-oxide	168.1		—OH	→O	—OH		47.0	7.90
uric acid	168.1		—OH		—OH	(8)-OH	36.8	6.19
guanine	151.1		$—NH_2$		—OH		80.0	12.09
2-(dimethylamino)-6-hydroxypurine	179.1		$—N(CH_3)_2$		—OH		56.0	10.03
isoguanine hemisulfate	200.1		—OH		$—NH_2$		67.5	13.51
isoguanine N^1-oxide	167.1	→O	—OH		$—NH_2$		91.2	15.24
2,6-diaminopurine hemisulfate	199.1		$—NH_2$		$—NH_2$		58.2	11.59
1-methylxanthine	166.2	$—CH_3$	=O		=O		53.0	8.81
3-methylxanthine	166.2		=O	$—CH_3$	=O		0.72	0.12
7-methylxanthine	166.2		=O		=O	(7)-CH_3	0.09	0.02
theophylline (anhyd)	180.2	$—CH_3$	=O	$—CH_3$	=O		0.04	<0.01
1,7-dimethylxanthine	180.2	$—CH_3$	=O		=O	(7)-CH_3	0.14	0.03
theobromine	180.2		=O	$—CH_3$	=O	(7)-CH_3	<0.03	<0.01
caffeine (anhyd)	194.2	$—CH_3$	=O	$—CH_3$	=O	(7)-CH_3	<0.03	<0.01
1,3,9-trimethylxanthine	194.2	$—CH_3$	=O	$—CH_3$	=O	(9)-CH_3	0.20	0.04

pteridine	mol wt	position of substituent[a] 2	4	5–8	A/mg per mL[b]	A/μmol per mL
pterine	163.1	$—NH_2$	—OH		0.08	0.01
lumazine	164.1	—OH	—OH		0.94	0.15
xanthopterin	179.1	$—NH_2$	—OH	(6)-OH	52.6	9.42
leucopterin	195.1	$—NH_2$	—OH	(6)-OH, (7)-OH	28.0	5.46

[a] See Figure 1 for pyrimidine, purine, and pteridine numbering systems. [b] Average of three or more separate runs on separate solutions, except for slightly reacting solutions, which were run twice. For comparison, A/mg per mL for tyrosine was 75 and A/μmol per mL was 13.6. Standard errors of the mean were generally 10% or less of the means.

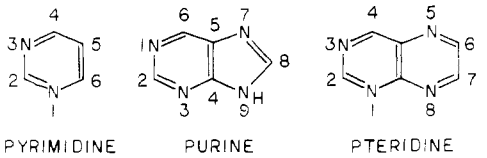

Figure 1. Numbering systems for the pyrimidine, purine, and pteridine ring systems.

from the most easily oxidized, being uric acid > xanthine > guanine > adenine > hypoxanthine. The oxidation by Folin–Ciocalteau reagent, although similar, appears to be more selective than voltammetric oxidation because of the need for hydroxyl and/or amino groups at both the 2- and 6-positions of the purine ring system for high reactivity. It is suggested [see Elving et al. (1973)] that in the case of uric acid the initial voltammetric oxidation step is the formation of a 4,5-dicarbonium ion or 4,5-diol, followed by cleavage of the 6-membered (or 5-membered) ring; in the case of guanine, oxidation at the 8-position, followed by oxidation at the 4,5-position, followed by cleavage of the 6-membered ring; and in the case of adenine, oxidation at the 2-position, followed by oxidation at the 8-position followed by oxidation at the 4,5-position, followed by cleavage of the 6-membered ring. The ease of oxidation at the 4,5-position appears to be crucial and may be expedited by certain strains imposed by the 5-membered ring in the purines. This would explain why the pyrimidines and the pteridines pterine and lumazine are not highly reactive to the reagent. The pteridines xanthopterin and leucopterin have additional hydroxyl groups at the 6- and 7-positions that seem to make them more susceptible to oxidation. The importance of the 4,5-positions is also indicated by our results with the N-methylated xanthines. Of these compounds only 1-methylxanthine reacted strongly with the reagent. The compounds containing methyl groups at the 3- or 7-positions reacted only slightly, and theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) did not react at all with the reagent. Here steric hindrance by the methyl groups at the 3- and 7-positions may be inhibiting the oxidation reaction at the nearby 4- and 5-positions.

When Folin–Ciocalteau reagent was used as a spray reagent (see the Experimental Section), the more highly reactive purines, such as uric acid, xanthine, and guanine, could readily be detected at the 1-μg level. Folin–Ciocalteau reagent is thus a useful reagent for the detection of specific highly reactive purines on chromatograms and in solution.

Shepherd and Hopkins (1963) had cautioned that Folin–Ciocalteau-reactive purines must be removed before protein determinations using this reagent are carried out. The situation has been reversed in the determination of iron-binding siderochromes with Folin–Ciocalteau reagent by the removal of protein prior to the determination (Subramanian et al., 1965). This illustrates the general strategy that may be employed when the reagent is used for the analysis of reactive compounds other than proteins. This technique was employed by Mosley et al. (1985) in the analysis of paralytic shellfish poisons in shellfish with this reagent, where a simple chromatographic procedure was successful in removing interfering substances from a

very crude extract. These and present studies suggest the possible usefulness of Folin–Ciocalteau reagent as a postcolumn derivatization procedure in high-performance liquid chromatographic systems.

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Registry No. Uracil, 66-22-8; cytosine, 71-30-7; isocytosine, 108-53-2; hypoxanthine, 68-94-0; adenine, 73-24-5; adenine N^1-oxide, 700-02-7; 2-hydroxypurine, 2308-57-8; 2-aminopurine, 452-06-2; xanthine, 69-89-6; xanthine N^3-oxide, 13479-29-3; uric acid, 69-93-2; guanine, 73-40-5; 2-(dimethylamino)-6-hydroxypurine, 1445-15-4; isoguanine hemisulfate, 49722-90-9; isoguanine N^1-oxide, 7593-46-6; 2,6-diaminopurine, 69369-16-0; 1-methylxanthine, 6136-37-4; 3-methylxanthine, 1076-22-8; 7-methylxanthine, 552-62-5; theophylline, 58-55-9; 1,7-dimethylxanthine, 611-59-6; theobromine, 83-67-0; caffeine, 58-08-2; 1,3,9-trimethylxanthine, 519-32-4; pterin, 2236-60-4; lumazine, 487-21-8; xanthopterin, 119-44-8; leucopterin, 492-11-5.

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Comparison of Gravimetric and Chemical Analyses of Total Dietary Fiber in Human Foods

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Dietary fiber in five foods, which have significant compositional differences, was determined by the gravimetric procedure recently receiving AOAC first action and compared to values determined by chemical analysis of the fiber components in extractive free residue (EFR) of the same foods. The gravimetrically determined total dietary fiber value was within 10% of the chemically determined value for three of the five foods analyzed: peas, soy polysaccharide, wheat bran. The gravimetric method overestimated the fiber content of apple by 14% and of a food composite of a typical daily intake by 18%. Chemical analyses of the gravimetrically obtained residue for neutral and acidic sugars and Klason lignin content were generally similar to the amounts of these fiber components measured in the EFR. Samples of apple and food composite obtained by the AOAC procedure contained slightly more neutral sugars and the wheat bran more Klason lignin than did the EFR samples. Following analysis of the fiber residues for crude protein and starch, recoveries of 97–104% were obtained. Fiber fractions of peas obtained by both methods contained 8–9% starch.

A knowledge of the dietary fiber content of human foodstuffs in needed for several reasons. Identifying mechanisms of action of dietary fiber within the gastrointestinal tract requires detailed information about the composition of the fiber. Food industries are interested in a total dietary fiber value appropriate for quality control and nutrition labeling. Dietitians and nutritionists want a database of food fiber values that can be used to estimate daily fiber intakes of individuals and populations. Thus, there is a need for the characterization of the kind of fiber in a foodstuff as well as for total dietary fiber values. Two approaches to dietary fiber analysis are being pursued to meet these diverse needs: a detailed procedure similar to the methods of Theander, Englyst, or Southgate that will provide information about the kinds and composition of the fiber in foods and a more rapid gravimetric method that will yield a single value for total dietary fiber. To meet the latter objective, Asp, DeVries, Furda, and Schweizer developed a gravimetric procedure that was submitted for an interlaboratory study in early 1982; the interlaboratory study was coordinated by the Food and Drug Administration (FDA) (Prosky et al., 1984). The results of two interlaboratory studies have been reported (Prosky et al., 1984, 1985), and the method has been given first-action approval by AOAC ("Total Dietary Fiber in Foods", 1985).

In the first interlaboratory study the reproducibility of the method, assessed by calculating a coefficient of variation of data of 32 laboratories who participated, was greater than 15% for 8 of the 13 food samples (Prosky et

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