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Utilization of Folin−Ciocalteu Phenol Reagent for the Detection of Certain Nitrogen Compounds

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To determine in more detail the reaction of Folin−Ciocalteu phenol reagent with nitrogen compounds, a number of hydroxylamine-related compounds and a large number of guanidine-containing compounds were tested. In general, guanidine compounds did not react strongly unless they were hydroxyamino or hydrazino derivatives. The non-hydroxyamino paralytic shellfish poison saxitoxin, however, reacted to a significant extent. This may be due to the presence of a five-membered ring structure and its analogy to 2-aminopurines, which react strongly. A number of simpler amines were also tested. Tertiary aliphatic amines, but not primary, secondary, or quaternary amines, reacted strongly with the reagent. Primary, secondary, and tertiary aromatic amines all reacted strongly with the reagent. Reactivity was extended to pyrroles and indole derivatives but not to imidazole and benzimidazole derivatives. Defining the reactivity of Folin−Ciocalteau phenol reagent with nitrogen compounds extends the usefulness of the reagent for the detection and determination of certain nitrogen compounds in basic extracts by colorimetric means and by thin-layer chromatography.

KEYWORDS: Folin−Ciocalteu reagent; guanidines; paralytic shellfish poisons; hydroxylamine derivatives; amines

INTRODUCTION

Folin−Ciocalteu phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and phoshotungstic acids, in which the molybdenum and tungsten are in the 6^+ state. On reduction with certain reducing agents, the so-called molybdenum blue and tungsten blue are formed, in which the mean oxidation state of the metals is between 5 and 6 (*1*). Its reaction with the amino acids tyrosine and tryptophan is the basis for the widely used Lowry method for the colorimetric determination of protein (*2*). It is known that Folin−Ciocalteu reagent reacts not only with phenols but also with a variety of other types of compounds (*3*, *4*). The extensive review by Peterson (*3*) describes to what extent a large number of substances react with Folin−Ciocalteau reagent. The strongly reacting substances, other than phenols, include tertiary aliphatic amines, tertiary amine-containing biological buffers, tryptophan, hydroxylamine, hydrazine, certain purines, and other miscellaneous organic and inorganic reducing agents.

We have previously shown that Folin−Ciocalteu reagent can be used for the detection of paralytic shellfish poisons (PSPs) (*5*−*7*). The ability to utilize the reagent for this purpose appears to be somewhat remarkable when one considers the variety of compounds that may react with the reagent and the fact that PSPs occur at very low concentrations. Because of the similarity of the PSP ring system to the purine ring system, we have previously also studied the structural requirements for purines to react with Folin−Ciocalteu reagent (*8*) and shown the importance of an amino or hydroxyl group at the 2-position.

Our interest has been to better define the specificity of the reaction of Folin−Ciocalteu phenol reagent with nitrogen compounds in order to explore its possible usefulness for the detection and determination of certain nitrogen compounds in natural extracts by colorimetric means and by thin-layer chromatography (TLC). We tested a number of hydroxylamino and hydrazino compounds and also focused our attention on the guanidine-containing compounds, because these compounds occur so widely in nature and in very diverse structures (*9*−*13*). These include such substances as the biologically important phosphagens and the paralytic shellfish poisons. We have also further investigated the selectivity of the reaction of the reagent with aliphatic and aromatic amines in general.

MATERIALS AND METHODS

The method described by Ikawa et al. (*8*) using the optimum conditions described by Box (*4*) was used. To 2.5 mL of test solution were added 0.375 mL of a 20% (w/v) solution of anhydrous sodium carbonate and 0.125 mL of 2 N Folin−Ciocalteu phenol reagent (Fisher Scientific Co., Pittsburgh, PA) with a pipet. The mixture was allowed to stand at room temperature for 1 h, and the absorbance was read in a 1 cm quartz cuvette at 745 nm on a Bausch and Lomb Spectronic 710 against a blank containing 2.5 mL of water instead of the test

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Table 1. Reaction of Various Substances with Folin–Ciocalteu Phenol Reagent

compound (source[a])	structure	*A*/mg/mL	*A*/μmol/mL
hydroxylamine and hydrazine derivatives, amides			
ammonium chloride	NH_3	<0.03	<0.01
hydroxylamine·HCl	H_2NOH	44	3.0
N,*N*-dimethylhydroxylamine·HCl (A)	$(H_3C)_2NOH$	48	4.7
O,*N*-dimethylhydroxylamine·HCl (A)	$H_3CNHOCH_3$	0.1	0.01
acetohydroxamic acid (A)	$H_3CC(O)NHOH$	85	6.4
N-hydroxysuccinimide (A)	$(CH_2CO)_2NOH$	0.06	0.01
formamidoxime (S)	$HC(NOH)NH_2$	116	7.0
hydroxyurea (S)	$H_2NC(O)NHOH$	68	5.1
semicarbazide.HCl	$H_2NC(O)NHNH_2$	2.4	0.27
formic hydrazide (A)	$HC(O)NHNH_2$	62	3.7
urea	$H_2NC(O)NH_2$	<0.03	<0.01
ethyl carbamate (EK)	$H_2NC(O)OC_2H_5$	<0.03	<0.01
formamide	$HC(O)NH_2$	<0.03	<0.01
formamidine acetate (S)	$HC(NH)NH_2$	<0.03	<0.01
allantoic acid (S)	$H_2NC(O)NHCH(COOH)NHC(O)NH_2$	<0.03	<0.01
allantoin (S)	see **Figure 1**	0.23	0.04
guanidines			
monosubstituted			
guanidine·HCl (EK)	$H_2NC(NH)NH_2$	<0.03	<0.01
methylguanidine·HCl (S)	$H_2NC(NH)NHCH_3$	0.15	0.02
L-arginine·HCl	$H_2NC(NH)NH(CH_2)_3CH(NH_2)COOH$	<0.03	<0.01
guanidinoacetic acid (S)	$H_2NC(NH)NHCH_2COOH$	<0.03	<0.01
streptomycin·1.5H_2O		0.35	0.26
hydroxyguanidine·0.5H_2SO_4·0.5H_2O (EK)	$H_2NC(NH)NHOH$	50	6.6
L-canavanine·H_2SO_4 (S)	$H_2NC(NH)NHO(CH_2)_2CH(NH_2)COOH$	11.5	3.2
aminoguanidine·0.5 H_2SO_4 (EK)	$H_2NC(NH)NHNH_2$	61	7.5
sulfaguanidine	$H_2NC(NH)NHS(O)_2[C_6H_4NH_2(p)]$	2.1	.45
disubstituted			
creatine·H_2O	$H_2NC(NH)N(CH_3)CH_2COOH$	0.05	<0.01
argininosuccinic acid,Ba·3.0H_2O (S)	$HOOCCH_2CH(COOH)NHC(NH)NH(CH_2)_3CH(NH_2)COOH$	0.08	0.04
phospho-L-arginine,Na	$(HO)_2P(O)NHC(NH)NH(CH_2)_3CH(NH_2)COOH$	1.12	0.31
N^G-hydroxyarginine acetate (S)	$C(NOH)NH(CH_2)_3CH(NH_2)COOH$	6.3	1.6
tri- and tetrasubstituted			
creatinine	see **Figure 1**	0.48	0.05
phosphocreatine,di Na·5.0 H_2O (S)	$(HO)_2P(O)NHC(NH)N(CH_3)CH_2COOH$	0.05	0.02
phosphocreatinine,Na·4.5H_2O (S)	see **Figure 1**	0.69	0.20
saxitoxin·2.0HCl	see **Figure 1**	~16	~6
neosaxitoxin·2.0HCl	see **Figure 1**	~68	~26
gonyautoxin-5·1.0HCl		~6	~2.5
aliphatic amines			
primary			
DL-alanine	$H_3CCH(NH_2)COOH$	<0.03	<0.01
L-lysine·HCl	$H_2N(CH_2)_4CH(NH_2)COOH$	<0.03	<0.01
D-glucosamine·HCl (CB)	$HOCH_2(CHOH)_3CH(NH_2)CHO$	0.05	0.01
putrescine·2.0HCl (S)	$H_2N(CH_2)_4NH_2$	0.04	<0.01
taurine (S)	$H_2N(CH_2)_2SO_3H$	<0.03	<0.01
secondary			
sarcosine·HCl (S)	H_3CNHCH_2COOH	0.05	<0.01
spermidine·3.0HCl (S)	$H_2N(CH_2)_4NH(CH_2)_3NH_2$	0.06	0.02
spermine·4.0HCl (S)	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	0.34	0.12
tertiary			
trimethylamine·HCl (S)	$(CH_3)_3N$	9.2	0.88
trimethylamine oxide·2.0H_2O(S)	$(CH_3)_3NO$	<0.03	<0.01
EDTA,di Na·2.0H_2O	$[-CH_2N(CH_2COOH)_2]_2$	0.81	0.30
PIPES (S)	$HO_3S(CH_2)_2N(CH_2CH_2)_2N(CH_2)_2SO_3H$	0.79	0.24
quaternary			
choline chloride (S)	$(CH_3)_3N^+(CH_2)_2OH$	<0.03	<0.01
tetramethylammonium OH·5.0H_2O (S)	$(CH_3)_4N^+OH^-$	<0.03	<0.01
betaine·HCl	$(CH_3)_3N^+CH_2COO^-$	<0.03	<0.01
aromatic amines			
aminobenzenes			
aniline	$(C_6H_5)NH_2$	72	6.7
N-methylaniline (A)	$(C_6H_5)NHCH_3$	215	23.0
N,*N*-dimethylaniline	$(C_6H_5)N(CH_3)_2$	137	16.6
pyrroles and indoles			
pyrrole (S)	see **Figure 1**	2.5	0.17
N-methylpyrrole (A)		0.61	0.05
DL-tryptophan	3-substituted indole	59	12.0
indole-3-acetic acid (S)	3-substituted indole	26	4.5

Table 1 (Continued)

compound (source[a])	structure	*A*/mg/mL	*A*/μmol/mL
aromatic amines (continued)			
imidazoles and benzimidazoles			
imidazole (S)	see **Figure 1**	0.06	<0.01
L-histidine·HCl	4-substituted imidazole	0.18	0.04
benzimidazole (A)	see **Figure 1**	0.05	<0.01
other cyclic			
pyridine		<0.03	<0.01
other non-nitrogenous or inorganic reducing substances			
D-glucose		<0.03	<0.01
L-fructose		<0.03	<0.01
formic acid		<0.03	<0.01
sodium nitrite		0.11	<0.01
sodium sulfite		3.42 (max)[b]	0.43
stannous chloride·2.0 H_2O		23.4	5.3

[a] Source: A, Aldrich Chemical Co., Milwaukee, WI; CB, Calbiochem, La Jolla, CA; EK, Eastman Kodak Co., Rochester, NY; S, Sigma Chemical Co., St. Louis, MO. [b] Concentration dependent.

solution. From linear concentration versus absorption plots, results were calculated in terms of absorbance per milligram per milliliter (*A*/mg/mL) and absorbance per micromole per milliliter (*A*/μmol/mL).

For the use of the reagent as a TLC spray reagent, silica gel thin-layer chromatograms were allowed to dry and sprayed to visible dampness with 20% sodium carbonate solution and then with 2 N Folin−Ciocalteu phenol reagent. Reactive compounds gave blue spots against a white background.

RESULTS AND DISCUSSION

Hydroxylamine Derivatives, Hydrazine Derivatives, Amides. As shown in **Table 1**, all hydroxylamine derivatives containing the free −OH group reacted strongly with the Folin−Ciocalteu reagent. This included the *N*-acylated forms (hydroxamates) [RC(O)NHOH], which are the principal form in which hydroxylamines occur in nature. An exception was the diacylated compound *N*-hydroxysuccinimde. Numerous hydroxamates have been isolated from fungal sources (*14*, *15*), where they occur often as iron-binding siderochromes and are N^5-acylated derivatives of N^5-hydroxyornithine. Subramanian et al. (*16*) have proposed the use of Folin−Ciocalteu reagent for the estimation of siderochromes. Hydroxamates have also been isolated from bacterial sources, mainly *Pseudomonas*, *Streptomyces*, and *Mycobacterium*, as antibiotic substances (*15*).

Hydrazine derivatives, where the −OH group of hydroxylamines is replaced by the $-NH_2$ group, also reacted strongly with Folin−Ciocalteu reagent. Conventional amides appeared to react only weakly, if at all. Cyclization of amides into five-membered rings seems to favor a slight reaction, as in the case of saxitoxin.

Guanidines. As might be expected from the previous discussion, the guanidine derivatives reacting strongly with Folin−Ciocalteu reagent were the hydroxylamine and hydrazine derivatives (hydroxyguanidine, aminoguanidine, neosaxitoxin, N^G-hydroxyarginine) (**Table 1**). Exceptions were canavanine, in which the −OH group is not free but is alkylated and therefore not thought to be prone to reaction with the reagent, and saxitoxin, which does not possess a hydroxyamino group. *N*-Sulfones (sulfaguanidine) and *N*-phosphonates (phospho-L-arginine) appeared to show a slight reaction. Besides canavanine and the neosaxitoxin group of PSPs, other known hydroxyguanidines include hydroxyguanidine itself as a bacterial degradation product of canavanine (*17*), N^5-hydroxyarginine from *Bacillus* (*18*) and from a fungus *Nannizzia grypsea* (*19*), N^G-hydroxyarginine involved in the biosynthesis of nitric oxide from arginine (*20*, *21*), anatoxin-a(s) from the cyanobacterium

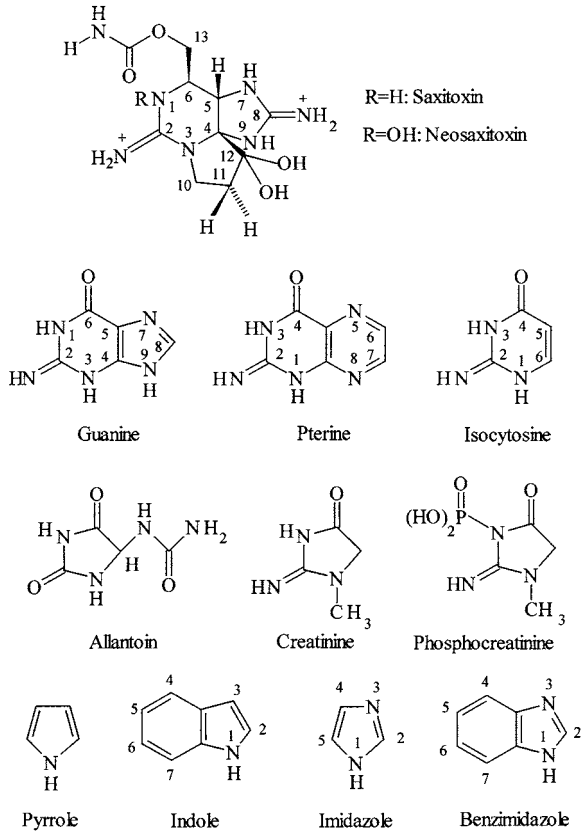

Figure 1. Structures of paralytic shellfish poisons and of compounds illustrating reactivity or nonreactivity to Folin–Ciocalteu phenol reagent.

Anabaena flos-aquae (*22*), and batzelladines G, H, and I from a Jamaican sponge (*23*). Guanidines are not likely to contribute to reaction to Folin−Ciocalteu reagent unless hydroxylamine derivatives, such as mentioned above, and other special structures (e.g., saxitoxin and aminopurines) are present.

Saxitoxin. Although saxitoxin lacks the N−OH group at position 1 (**Figure 1**), there was evidence that saxitoxin also does react significantly with Folin−Ciocalteu reagent. When extracts of the toxic cyanobacterium *Aphanizomenon flos-aquae* Kezar strain were chromatographed on an AG-50 resin column, small Folin−Ciocalteu reagent-reactive peaks corresponding to gonyautoxin-5 and to saxitoxin were observed (*6*; unpublished results). Neosaxitoxin, which slightly precedes saxitoxin in this chromatographic system, is absent in the Kezar strain. Gon-

yautoxin-5, like saxitoxin, is also not hydroxylated at position 1, yet gave a peak with Folin−Ciocalteu reagent. That a strain imposed by the five-membered fused ring system in saxitoxin may contribute to its reaction with Folin−Ciocalteu reagent is made likely by the fact that guanine is highly reactive to the reagent, whereas similarly substituted pterine and isocytosine (**Figure 1**) hardly react. 2-Aminopurine is also highly reactive (*8*). The 2-amino group of saxitoxin may be key to its reaction with Folin−Ciocalteu reagent. The effect of five-membered cyclization in promoting a reaction with the reagent can also be seen when a comparison is made between the cyclic/noncyclic pairs creatinine and creatine (*A*/μmol/mL of 0.05 versus <0.01), phosphocreatinine and phosphocreatine (*A*/μmol/mL of 0.20 versus 0.02), and allantoin and allantoic acid (*A*/μmole/mL of 0.23 versus 0.04). Thus, strains within a five-membered ring or caused by a fused five-membered ring may contribute to reaction with Folin−Ciocalteu reagent. It can thus be concluded that, although saxitoxin does not contain the N−OH group, the factors described above can be proposed to explain its reactivity to Folin−Ciocalteu reagent.

Approximate values of *A*/mg/mL and *A*/μmol/mL for neosaxitoxin and gonyautoxin-5 (**Table 1**) were calculated from values given in terms of *A*/MU/2.5 mL in Ikawa et al. (*7*), assuming that neosaxitoxin has a potency of 3000 MU/mg and gonyautoxin-5 a potency of 300 MU/mg (*24*). The value for saxitoxin is based on a purified sample.

A common unusual feature of saxitoxin and neosaxitoxin is the carbamoyl group at position 13 (**Figure 1**). Because ethyl carbamate does not react with Folin−Ciocalteu reagent (**Table 1**), we can conclude that this group is not responsible for the reaction of PSPs with the reagent.

Aromatic Amines. The reactivity of tryptophan with Folin−Ciocalteu reagent prompted us to test a number of aromatic and aliphatic amines. Aniline, *N*-methylaniline, and *N*,*N*-dimethylaniline all reacted very strongly (**Table 1**), indicating that primary, secondary, and tertiary aromatic amines all react with the reagent. Box (*4*) reported that primary, secondary, and tertiary aromatic amines react with Folin−Ciocalteu reagent, but the extent of the reaction was not indicated. Indoles, which contain a pyrrole ring system fused to the benzene ring (**Figure 1**), also reacted strongly. Pyrrole itself reacted with the reagent, but its degree of reaction was considerably weaker than the indoles. *N*-Methylpyrrole reaction was even weaker. Benzimidazole, containing an imidazole ring fused to the benzene ring (**Figure 1**), showed almost no reaction to the reagent, and neither did imidazole itself. The imidazole-containing amino acid histidine showed only a slight reaction indicating a weak, if any, reaction of imidazoles with the reagent. Pyridine showed no reaction. Thus, of the aromatic nitrogen compounds tested, the primary, secondary, and tertiary benzenoid compounds, including indole derivatives, showed a high reactivity.

Aliphatic Amines. Primary, secondary, and quaternary aliphatic amines are not particularly reactive to Folin−Ciocalteu reagent (**Table 1**). However, reactivity was encountered with tertiary amines. This included some tertiary aliphatic amines that are commonly used in biological buffer systems. The reactivity of tertiary aliphatic amines with Folin−Ciocalteu reagent parallels the reactivity of tertiary aliphatic amines over secondary and primary amines to other oxidizing agents such as chlorine dioxide, ferricyanide, and permanganate (*25*). Trimethylamine, which is a very commonly occurring constituent in the marine environment, reacts particularly strongly. Its ready oxidation to trimethylamine oxide, which itself does not react with Folin−Ciocalteu reagent, may account for its ability to reduce the reagent.

Among the wide variety of nitrogen compounds that occur, high reactivity toward Folin−Ciocalteu phenol reagent has been found with tertiary aliphatic amines, (primary, secondary, and tertiary) aromatic amines, *N*-hydroxyl compounds, *N*-amino compounds, and compounds containing five-membered heterocyclic nitrogen. Folin−Ciocalteu reagent, therefore, has a potential of being a useful reagent in the detection of compounds of interest. It is fortuitous that such commonly occurring reducing substances as glucose and fructose do not react with the reagent (**Table 1**). Phenolic substances, on the other hand, would pose a serious problem in the application of the reagent to the study of nitrogen compounds. Not only do these substances themselves occur widely in nature, they also often occur in polyphenolic form as tannins and humic acids in many waters and extracts. If preliminary steps are taken to minimize the presence of phenolic substances, for example, through solvent extraction or the use of ion-exchange resins, reactivity to Folin−Ciocalteu reagent can be a valuable indication of the types of nitrogen compounds present. Steps in this direction have already been taken in studies on paralytic shellfish poisons, hydroxamic acid-containing siderochromes, and 2-amino- and 2-hydroxypurines (e.g., uric acid). Reactivity to Folin−Ciocalteu reagent is also a valuable indicator of the applicability of the reagent as a spray for the detection of compounds by TLC.

Although values for minimum detectable amounts are somewhat subjective, approximations may be made. The more reactive compounds have *A*/mg/mL of ≥50. At 1 μg/mL (1 ppm) these compounds would show higher than background absorbance. Although compounds with *A*/mg/mL of 10 are still considered to be reactive, they would have to be present at slightly higher levels to be readily detectable.

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