Stability of the Folin Phenol Reagent

By ROBERT C. PETERSON

A comprehensive study has been made of the stability of the Folin phenol reagent as the initial acidic concentrate. Even though refrigerated, an apparent continuing and linear decomposition of about 2 per cent per month occurs which should be considered in the determination of mcg. amounts of protein.

DURING work in which it was attempted to determine the specific activity of trypsin, the protein content of electrophoretically fractionated zones was determined with the Lowry (1) modification of the Folin-Ciocalteu (2) reagent.

A satisfactory standard curve could be prepared for protein concentration, but when a known sample was referred to it at a later time, the sample value was always low. It was found that the low values were produced by a slow but consistent deterioration of the Folin reagent whether it was from commercial sources[1] or prepared in our laboratory according to Folin-Ciocalteu (2). This deterioration occurred even though the reagent was refrigerated (6°) immediately after preparation and removed only when portions were desired for dilution in the protein tests.

The Folin phenol reagent is known to be rapidly destroyed by an alkaline solution (1), but this writer is not aware of any comments in the literature regarding its instability as the initially prepared acid.

Since this reagent is continually used, it seemed worthwhile to acquire more knowledge concerning its stability. It was felt that reagent prepared in the laboratory and used shortly thereafter would give the greatest response, and so a preparation was made as mentioned above and immediately refrigerated. The reagent titrated as 2.5 *N* acid with 0.5 *N* NaOH in the presence of phenol red; Fisher reagent gave the same value under these conditions. The laboratory reagent was tested approximately once a month for 6 to 7 months.

METHODS AND MATERIALS

The standard protein was N.F. Trypsin Crystallized Reference Standard, lot 6040 (3). A new solution, 5 mg. in 100 ml. of water, was made up for each test. A second standard was L-tyrosine.[2] A new solution, 3 mg. in 100 ml. of water, was made up for each test.

The phenol reagent was tested for optimal response according to Oyama and Eagle (4) and their Lowry reagent modifications were used. Reagent *A* consisted of 20 Gm. of Na_2CO_3, 4 Gm. of NaOH, and 0.2 Gm. of $K_2C_4H_4O_6 \cdot 1/2\ H_2O$ made to 1 L. with water. Reagent *B* was 0.5 Gm. of $CuSO_4 \cdot 5H_2O$ made to 100 ml. with water. Reagent *C* was made freshly before each test series from reagents *A* and *B*:50 to 1 by volume. For each test 0.5 ml. of each standard was diluted to 1 ml. with water and this solution was reacted with 2.5 ml. of reagent *C* for 10 min. Folin reagent, 0.25 ml., at various dilutions with water (4) was jetted in and mixed thoroughly. The absorbance was determined at 750 mμ every 30 min. for 2 hr. with a Beckman model DU spectrophotometer. For each test the optimum response of the Folin reagent was considered to be the absorbance value from the phenol reagent dilution which gave the greatest stability of color, *i.e.*, the absorbance values produced a plateau. These absorbance values, determined at approximately 1-month intervals, gave an indication of the stability of the phenol reagent.

With L-tyrosine, 15 mcg. per test, the curve in Fig. 1 was obtained when absorbance was plotted against time elapsed since preparation of the reagent. Figure 2 presents similar data for 25 mcg. of trypsin per test.

DISCUSSION

In all cases it was found that the 1 to 2 dilution of phenol reagent gave optimum response.

The continual deterioration of the phenol reagent is obvious. With trypsin this is a decrease in absorbance of 0.0044 every 30 days. With L-tyrosine, the decrease is greater.

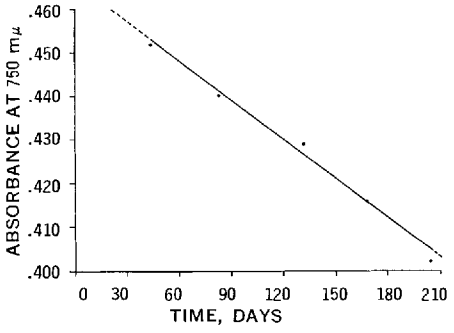

Fig. 1.—Lowry test with L-tyrosine as related to age of phenol reagent.

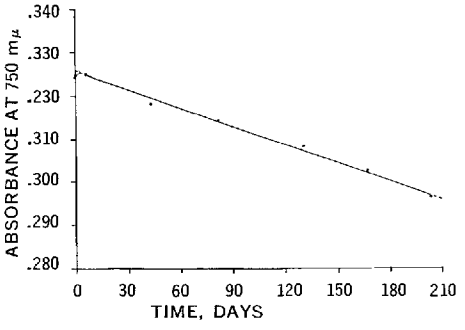

Fig. 2.—Lowry test with trypsin as related to age of phenol reagent.

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[1] Fisher Scientific Co.; Hartman-Leddon Co.
[2] M. A., lot MJ999, Mann Research Laboratories.

For accurate quantitative use of the Folin phenol reagent, either its response as related to a standard decomposition curve must be considered or its activity must be correlated with a known amount of standard protein or amino acid.

It follows that to get sensitivity with the reagent with microgram quantities of protein, the state of decay of the reagent must be known. If deterioration is sufficiently great, either fresh reagent must be used or the quantity must be increased.

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Nuclear Magnetic Resonance Spectra of Amines II. Identification of *N*-Phenyl Amines

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The effects of a strongly acidic solvent on the chemical shift and spin-spin splitting of the phenyl protons in *N*-phenyl amines are characteristic for this functional group. These effects provide the basis for the identification of the *N*-phenyl group in primary, secondary, and tertiary amines.

THE EFFECT of protonation of amines containing *N*-methyl groups has been recently reported by these laboratories (1). The authors wish to report on the *N*-phenyl group as characteristically identified by examination of the NMR spectrum of free base in deuterated chloroform and the spectrum in trifluoroacetic acid.

EXPERIMENTAL

All NMR spectra were recorded on a Varian A-60 spectrometer using Varian sample tubes. Deuterated chloroform and trifluoroacetic acid were used as solvents. Spectra were obtained on samples at room temperature at a concentration of 50 mg./ml.

The *N*-phenyl amines used were Eastman organic chemicals as purchased from Distillation Products Industries, Rochester, N. Y., or K and K chemicals as purchased from the K and K Laboratories, Plainview, N. Y.

RESULTS AND DISCUSSION

The pronounced change in the aromatic proton pattern of an *N*-phenyl amine free base on conversion to the amine cation is illustrated in Fig. 1, curve A, diphenylamine in deuterated chloroform, and curve B, diphenylamine in trifluoroacetic acid. This phenomenon, the collapse of a complex A_2B_2C aromatic pattern into a simple peak (or narrow band of peaks), is general for any protonatable *N*-phenyl group with no other substituents on the *N*-phenyl ring. The collapse of this pattern can be attributed to equalization of the chemical shifts of the phenyl protons. The principal cause of inequality of chemical shifts for the phenyl protons in an amine free base is conjugation of the amine group with the phenyl ring. Protonation of the amine blocks this conjugation and results in nearly uniform chemical shifts for the protons on the benzene ring. The reduction of conjugation of an *N*-phenyl amine on formation of the amine cation is well established in the theory of ultraviolet spectra for anilines (2).

The authors have found the collapse of the A_2B_2C spectral pattern of the *N*-phenyl group useful for determining whether one or more *N*-phenyl groups in an unknown compound have other substituents on the *N*-phenyl ring. For example 3-chloro-*N*-phenyl aniline shows 2 species of protons in trifluoroacetic acid due to the *meta* substituted benzene ring.

The NMR data for 5 representative *N*-phenyl reference compounds are listed in Table I. It should be noted that acidic solvents, such as aqueous hydrochloric and sulfuric acids, all influence the NMR absorption pattern of the *N*-phenyl group in the same fashion, regardless of whether the amine is primary, secondary, or tertiary.

If the *N*-phenyl group is close to another aromatic ring, the asymmetric magnetic field generated by the second aromatic ring may prevent observation of collapse of the A_2B_2C pattern on cation formation. For example, the NMR spectra for *N*-methyl-*N*-phenyl-benzylamine (Fig. 2) show a more complex pattern for the ion (Fig. 2, B) than for the free base (Fig. 2, A). The authors have observed a similar

TABLE I.—NMR CHEMICAL SHIFTS FOR *N*-PHENYL GROUPS IN ANILINE AND *N*-SUBSTITUTED ANILINES

Compd.	Appropriate Range of Complex A_2B_2C Aromatic Pattern in $CDCl_3$, p.p.m.	Chemical Shift Downfield from Tetramethylsilane for Single Aromatic *N*-Phenyl Peak in CF_3COOH, p.p.m.
Aniline	6.7–7.4	7.52
Diphenylamine	6.5–7.5	7.60
N,N-Dimethyl-aniline	6.3–7.4	7.62
N-Methylaniline	6.6–7.4	7.55
N-Methyldi-phenylamine	6.7–7.4	7.65

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