High-Performance Liquid Chromatography of Preparations of Ribonucleic Acid Inactivator(s) from Cupric Ion and Hydroquinone Before and After Treatment with Histidine

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Abstract □ Preparations of viral RNA inactivator(s) produced during the cupric ion-catalyzed oxidation of hydroquinone were analyzed by high-performance liquid chromatography (HPLC) using UV and electrochemical (EC) detectors. In addition to hydroquinone and the main oxidation product (*p*-benzoquinone), which is known not to be the inactivator(s), the analysis showed three unidentified components (I–III). Partial UV absorption spectra of I–III were determined by HPLC with the UV detector set at various wavelengths. Components II and III, but not I, were highly unstable in the presence of L-histidine, which is an excellent chelator of cupric ion and can promptly stop ongoing viral RNA inactivation by the inactivator(s). The product *p*-benzoquinone was also highly unstable in the presence of L-histidine; the reaction between these two compounds (with or without copper) resulted in a cascade of products. The possibility that the inactivator(s) is II or III, or both, is discussed.

Keyphrases □ Hydroquinone—high-performance liquid chromatography of preparations of ribonucleic acid inactivator(s) from cupric ion after treatment with histidine □ Histidine—high-performance liquid chromatography of preparations of ribonucleic acid inactivator(s) from cupric ion and hydroquinone □ High-performance liquid chromatography—preparations of ribonucleic acid inactivator(s) from cupric ion and hydroquinone before and after treatment with histidine

Cupric ion and many lots of commercial reagent-grade phenol interact to produce a potent inactivator(s) of the naked RNA from poliovirions; even after redistillation, the purified phenol shows a strong capacity to interact with cupric ion to produce the RNA inactivator(s) (1, 2). This RNA inactivator(s) can be a serious problem when phenol is used for the isolation of RNAs.

However, phenol purified by steam distillation shows no activity, the activity being recoverable from the aqueous residue remaining after the steam distillation (1). The activity of this residue has been shown to be due, at least in part, to hydroquinone and catechol, which occur as impurities in the commercial reagent grade phenol (2, 3). In addition to hydroquinone and catechol, two other phenols have been shown to be active: pyrogallol and orcinol (2). Hydroquinone is the most active of the four phenols (2).

When cupric ion and hydroquinone are mixed together in buffer (pH 7.3) under air, the copper-catalyzed oxidation of the hydroquinone to *p*-benzoquinone starts promptly and proceeds rapidly, and inactivator activity rises in parallel with the rise in *p*-benzoquinone concentration (4). However, as shown by direct tests, *p*-benzoquinone is not the inactivator(s), nor is cuprous ion (4).

In the present report, preparations of inactivator(s) from cupric ion and hydroquinone were examined by HPLC using UV and EC detectors with the aim of detecting additional chemical species which could be candidates for the role of inactivator(s); three such species were found. Because of the effect of L-histidine, an excellent chelator of cupric ion (5), in promptly stopping ongoing inactivation of RNA by the inactivator(s) (1), the effect of L-histidine on the stability of these three species was determined.

EXPERIMENTAL

Chemicals—The following chemicals were used: *p*-benzoquinone, practical grade[1]; cupric chloride, reagent grade[2]; hydroquinone, purified[2]; 85% orthophosphoric acid, reagent grade[3]; potassium dihydrogen phosphate, reagent grade[3]; L-histidine monohydrochloride monohydrate[4]; tetramethylammonium hydroxide pentahydrate[5].

Distilled water was deionized, demineralized, and then double-distilled in glass before use.

The commercial hydroquinone was recrystallized twice from water before use. Solutions of the recrystallized hydroquinone in water were stored frozen under nitrogen.

Preparation of Viral RNA Inactivator(s)—Inactivator preparations were made by incubating cupric chloride at 1.11 m*M* and hydroquinone at 2.22 m*M* in buffer under air at 23° for 5 min (6). The buffer used was 137 m*M* NaCl, 2.69 m*M* KCl, 8.15 m*M* Na_2HPO_4, and 1.46 m*M* KH_2PO_4, pH 7.3. To obtain the UV absorption spectra, the incubation was for 20 instead of 5 min so that higher concentrations of the unknown products I–III would be obtained.

Treatment of Inactivator Preparation with L-Histidine—Solutions of buffer containing L-histidine hydrochloride at 167 m*M* and with the pH adjusted with sodium hydroxide back to 7.3 were prepared. One volume of the buffer with L-histidine, at 23°, was mixed with three volumes of fresh inactivator preparation. Samples were taken immediately and after various periods of incubation at 23°. Controls were done in the same way, except that plain buffer was used instead of buffer with L-histidine.

Treatment of *p*-Benzoquinone with L-Histidine—One volume of the above mentioned buffer with L-histidine, at 23°, was mixed with three volumes of a reference solution of 0.300 m*M* *p*-benzoquinone in buffer with cupric chloride at 1.11 m*M* or without cupric chloride. This reference solution had been preincubated under air at 23° for 5 min, in simulation of the incubation used in preparing the viral RNA inactivator(s). Samples were taken immediately and 15 min after adding the L-histidine. In the controls, plain buffer replaced the buffer with L-histidine.

HPLC—An isocratic HPLC system similar to that previously described was used (7). The fixed-volume injector[6] contained a 20-μl loop. A 250 × 4-mm reverse-phase column packed with Lichrosorb[7] RP-18, 10 μm, with a slurry packing technique was used. A guard column RP-18 MPLC[8] was incorporated into the system. An aqueous mobile phase containing 100 m*M* KH_2PO_4 and 12.5 m*M* tetramethylammonium hydroxide, pH adjusted to 3.9 using 14.7 *M* H_3PO_4, was prepared using the triple-distilled deionized demineralized water. This mobile phase was

[1] Eastman Kodak Co.
[2] Fisher Chemical Co.
[3] Mallinckrodt Chemical Works.
[4] Mann Research Laboratories.
[5] Sigma Chemical Co.
[6] Rheodyne.
[7] E. Merck Laboratories.
[8] Brownlee Laboratories.

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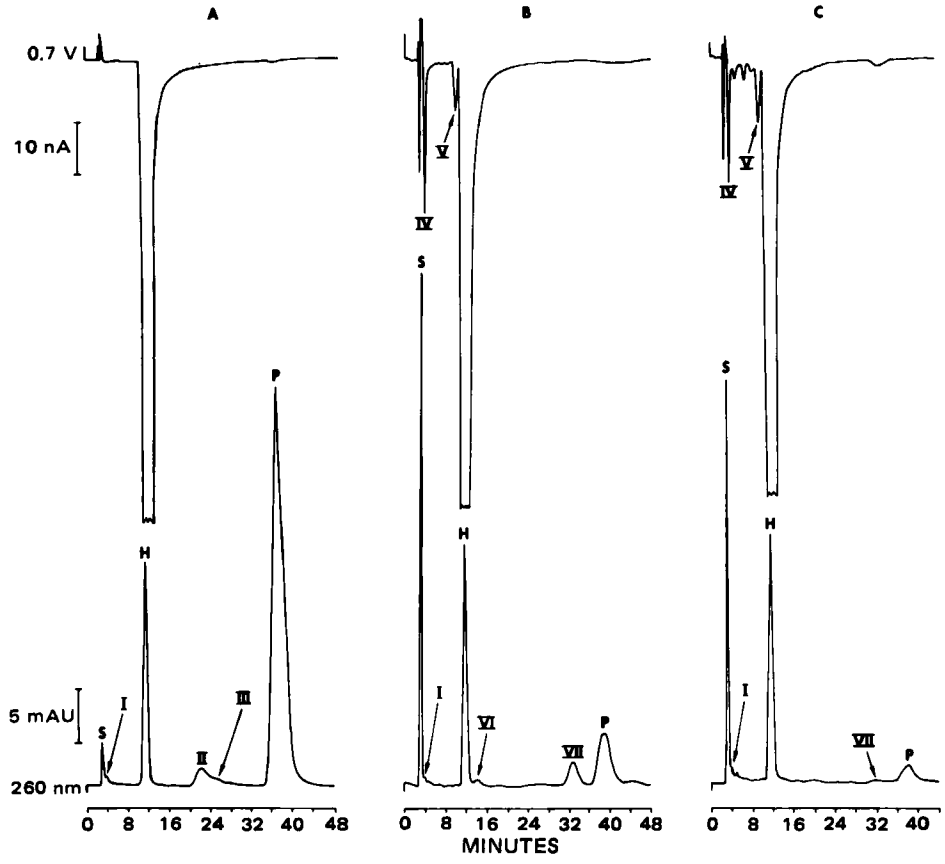

Figure 1—*Chromatograms of RNA inactivator(s) before and after treatment with L-histidine. To one volume of inactivator preparation made using the 5-min incubation was added one-third volume of either plain buffer (control) or buffer with L-histidine (experimental). Key: A, control immediately injected; B, experimental injected 15 min after adding L-histidine; C, experimental injected 3 hr after adding L-histidine. Upper and lower chromatograms: responses of EC and UV (260 nm), respectively. Key: (S) solvent front; (mAU) milliabsorbance unit; (H) hydroquinone; (P) p-benzoquinone; I–III, unknowns in inactivator preparation; IV–VII, unknowns appearing within 15 min after adding L-histidine. Offscale peaks: three H(EC) peaks and the S(UV) peak of panel B.*

filtered through a 0.2-μm porosity membrane filter[9], deaerated under vacuum, and kept at 40° during chromatography. The UV detector was set at 260 nm and 0.05 absorbance units full scale in most experiments. In some experiments, as described, the UV detector was set at wavelengths shorter or longer than 260 nm. The EC detector was set at 0.7 V and 100 nA full scale.

RESULTS

Components of Preparations of RNA Inactivator(s)—HPLC of RNA inactivator preparations made using the 5-min incubation of cupric chloride with hydroquinone showed six peaks (Fig. 1A): the solvent front peak, where cupric ion elutes; hydroquinone; *p*-benzoquinone; and three unidentified products (I–III). At the UV wavelength of 260 nm used for this chromatogram, product III appears as a trailing shoulder on the peak due to product II; but when 230-nm UV was used, II and III were seen as two peaks (Fig. 2). The peak due to component I was also more obvious when 230-nm UV was used (Fig. 2). The peak between component I and hydroquinone (Fig. 2) was due to one of the components, possibly unknown component XI (see below), appearing only after incubation of the cupric chloride and hydroquinone together for more than 5 min. Chromatograms of such inactivator preparations were found to be highly reproducible when the same conditions for production of inactivator(s), for separation, and for detection were used.

Partial UV Absorption Spectra of Components I–III—Partial UV absorption spectra of components I–III were determined from chromatograms of replicate inactivator preparations with the UV detector set at various UV wavelengths from 200 to 320 nm and are shown in Fig. 3.

Components Appearing upon Further Incubation of RNA Inactivator Preparations—In this study, attention was concentrated on components I–III, because of their presence after incubation of cupric ion with hydroquinone for only 5 min, and since previous experiments had shown that the production of RNA inactivator(s) from cupric ion and hydroquinone proceeds rapidly and without lag (4).

When the reaction between cupric chloride and hydroquinone was prolonged beyond 5 min, as in the controls where one-third volume of plain buffer was added at 5 min and the incubation continued at 23°, additional unknown products appeared. This result was shown by tests of samples that were taken after additional incubation periods of 5, 15, 30, 60, 180, and 420 min and analyzed by HPLC using both detectors with the UV detector set at 260 nm. These chromatograms showed seven additional unknown products (VIII–XIV) with retention times of <1 hr. Referred to by the earliest time of their appearance in min after starting the additional incubation, by the detector(s) *sensitive to them and by* their retention times in minutes and seconds, these seven products were: VIII, 5UV and EC5:45; IX, 5UV and EC17:15; X, 15UV4:45; XI, 15UV8:05; XII, 60EC4:15; XIII, 180UV14:15; XIV, 420UV41:35.

Effects of Histidine—The addition of L-histidine to inactivator preparations resulted in the rapid loss of components II and III but not of component I (Figs. 1 and 4). The synthesis of additional component I was, however, greatly diminished by L-histidine. The addition of L-histidine had other effects, which may be grouped in two sets.

The first set consists of effects that are connected or probably connected with the capacity of L-histidine to chelate cupric ion. One such effect was the very rapid formation of chelate complex(es) of cupric ion and L- histidine (8) (Fig. 1B); the offscale UV-absorbing peak at the solvent front (*S*) was due mainly to such complex(es), as shown by control chromatograms of L-histidine and cupric chloride chromatographed individually and after being mixed together. Another effect was the virtually immediate cessation of the oxidation of hydroquinone. A third effect was the blocking of the production of VIII, IX, XI, XIII, and XIV. Production of X was not blocked. Whether production of XII was blocked was not ascertained, due to interference from new compounds appearing after adding L-histidine (Fig. 1 and below).

The second set of effects of L-histidine consists of those which were

[9] Millipore.

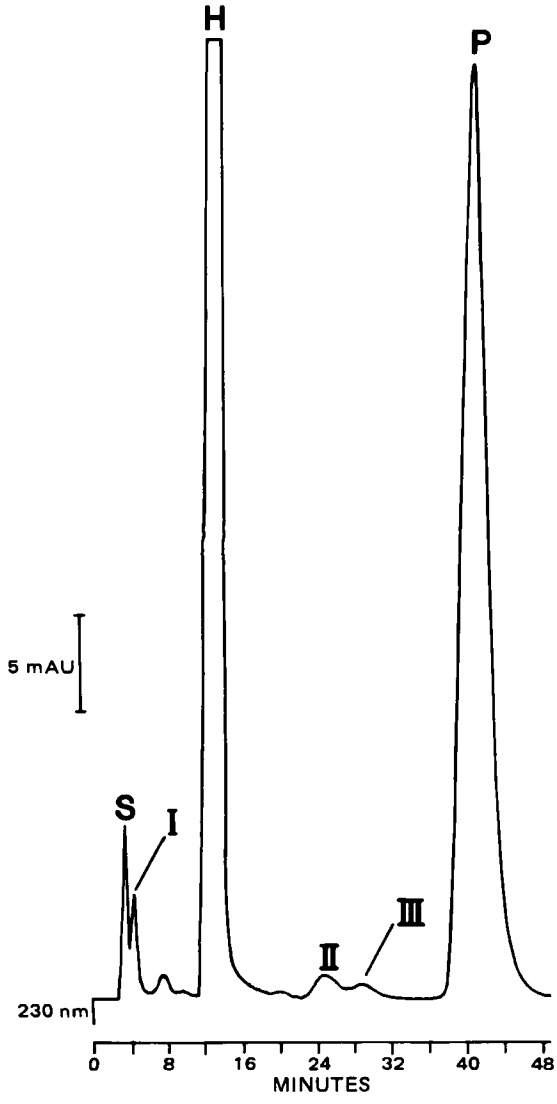

Figure 2—*Chromatogram of RNA inactivator prepared using 20-min incubation. Symbols as in Fig. 1.*

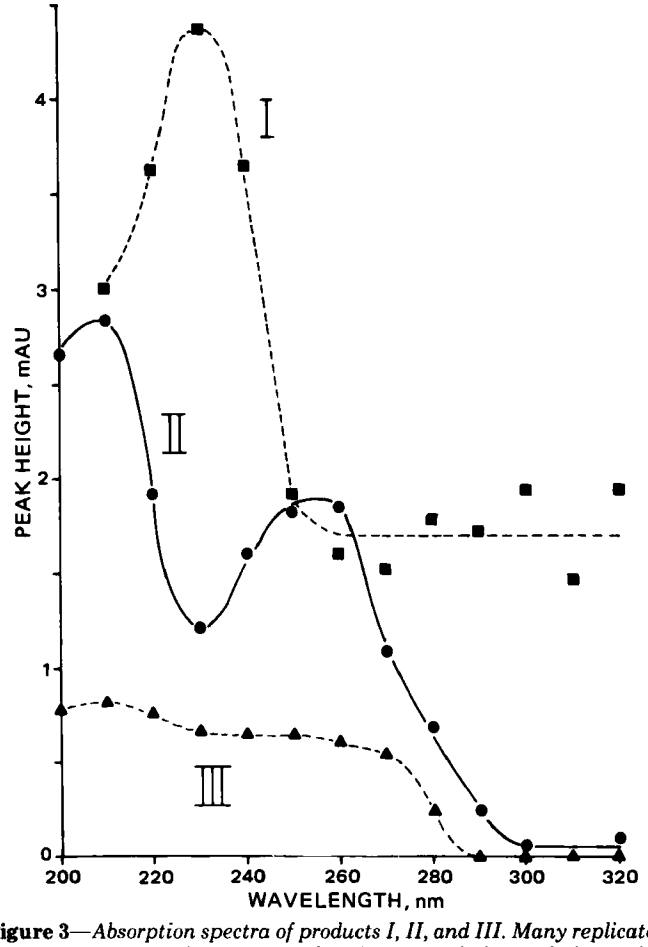

Figure 3—*Absorption spectra of products I, II, and III. Many replicate inactivator preparations were made using a 20-min instead of a 5-min incubation, the addition of plain buffer, and immediate sampling for analysis by HPLC using the UV detector, which was set at 200, 200, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, and 320 nm, respectively, for the replicate preparations. For each chromatogram, peak heights in milliabsorbance units (mAU) for unknown components I–III were estimated. The point for component I at 200 nm was missed due to interference from the offscale solvent front.*

found to be independent of the capacity of L-histidine to chelate cupric ion. These effects were (*d*) the rapid loss of *p*-benzoquinone (Figs. 1 and 4), (*e*) the increase in hydroquinone (Figs. 1 and 4), and (*f*) the appearance of a cascade of new compounds including IV–VII (Figs. 1 and 5).

Evidence of the cascade of new compounds was seen soon after the addition of L-histidine. Within 15 min after this addition, four new components, IV–VII, appeared. Components IV and V were detected by the EC detector and VI and VII by the UV detector (Fig. 1B). The kinetics of production of IV–VII and the kinetics of the later decrease or disappearance of IV, VI, and VII are shown in Fig. 5. These four components did not appear in the controls not containing L-histidine. At times > 15 min after adding L-histidine, additional new compounds appeared which did not appear in the corresponding controls that had received only plain buffer. At least four of these additional compounds are shown in Fig. 1C, where the inactivator preparation had been incubated for 3 hr after adding the L-histidine. Such chromatograms are highly reproducible. To avoid cluttering the figure, these additional unknown compounds have not been numbered.

That the above effects (*d*), (*e*), and (*f*) are independent of copper was shown by tests of the effect of L-histidine on *p*-benzoquinone with and without cupric ion. Buffer with L-histidine, or plain buffer for the controls, was added to solutions of commercial *p*-benzoquinone in buffer with and without cupric chloride; and the mixtures were analyzed either immediately or after 15 min at 23°. The chromatograms showed that L-histidine, with or without copper, caused a rapid decrease in the concentration of *p*-benzoquinone. In two experiments, after 15 min only 14 and 9.3% of the starting *p*-benzoquinone remained when cupric chloride was not added; when cupric chloride was added the corresponding figures were 17 and 21%. In the controls where plain buffer was added, there was no significant change in the concentration of *p*-benzoquinone in 15 min with or without cupric chloride. In the commercial *p*-benzoquinone used there was a very small amount of hydroquinone, detectable by the EC detector. In 15 min after adding L-histidine, the amount of hydroquinone increased five- to eightfold with or without cupric chloride. In the controls where plain buffer was added, there was no significant change in the concentration of hydroquinone in 15 min without cupric chloride; but with cupric chloride there was, as expected, a marked reduction in the concentration of hydroquinone after 15 min, due to cupric ion-catalyzed oxidation. The chromatograms of samples taken 15 min after the addition of L-histidine to the *p*-benzoquinone with and without cupric chloride showed peaks virtually identical to unknown components IV–VII both in retention time and in detector response ratios of the UV and EC detectors.

These experiments show that L-histidine reacts with *p*-benzoquinone, that direct or indirect products of the reaction are hydroquinone and unknown components IV–VII, and that copper is not necessary for this reaction or for the production of these five products. These effects of the reaction between L-histidine and *p*-benzoquinone can account for the above-described effects (*d*), (*e*), and (*f*).

DISCUSSION

The most significant aspects of this work are that components other than hydroquinone and *p*-benzoquinone were sufficiently stable to be detected by HPLC in preparations of RNA inactivator(s) and that two of these components, II and III, were unstable in the presence of L-histidine. This instability casts II and III as candidates for the role of inac-

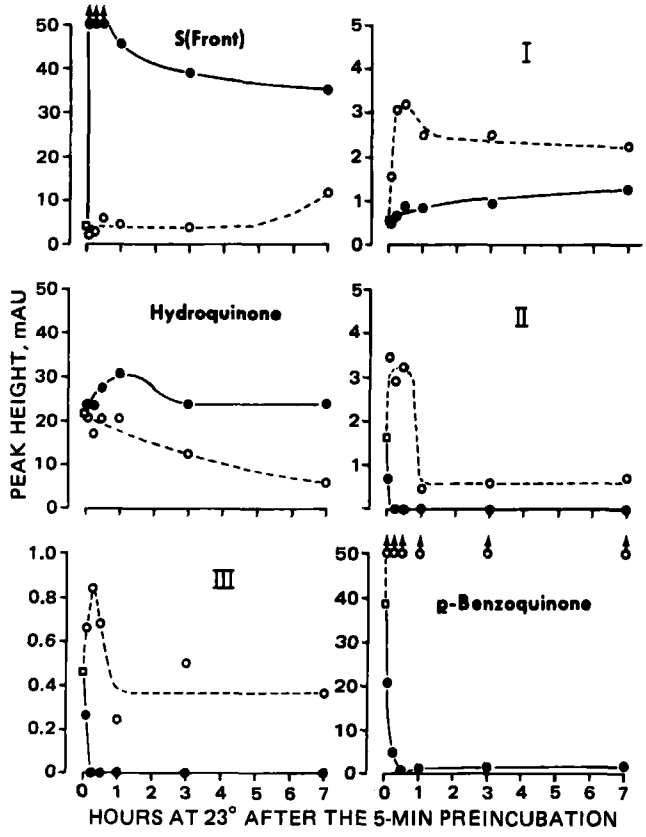

Figure 4—*Effect of L-histidine on six UV-absorbing peaks from inactivator preparation. The inactivator preparation was made and the control and experimental additions were made as in Fig. 1. A sample of the control was immediately injected for analysis by HPLC, using the same chromatographic conditions as in Fig. 1. Incubation of control and experimental continued at 23°, under air, and samples taken at the various times shown were analyzed by HPLC. Key: mAU, milliabsorbance unit; S, peak at solvent front; I–III, three unknown components shown in Fig. 1A. (□) immediate sampling of buffer control; (○) incubated buffer control; (●) incubated histidine experimental. The arrows indicate offscale peaks.*

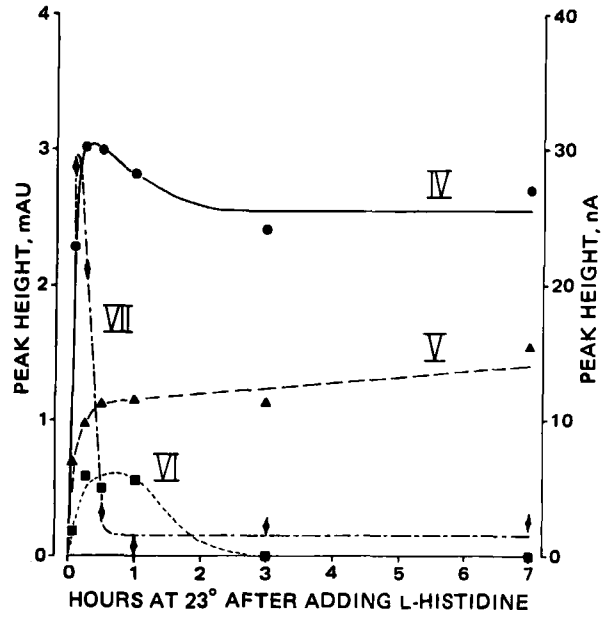

Figure 5—*Kinetics of appearance and disappearance of unknown components IV–VII after addition of L-histidine. Inactivator preparations were made and L-histidine then added, as in experimentals of Fig. 1B and C. Samples were analyzed by HPLC 5, 15, 30, 60, 180, and 420 min later. Components IV and V were analyzed by EC detector, VI and VII by UV detector, as in Fig. 1. mAU, milliabsorbance unit.*

tivator(s). What connection, if any, components II and III may have with the free radical species at the one-electron oxidation step, namely, *p*-benzosemiquinone and its anion as well as the complex of this anion with cupric ion (9), is presently unknown.

Another important aspect of the work is that it shows the multiplicity of the effects of L-histidine on RNA inactivator preparations. It appears that most or all of these effects can be attributed to just two properties of L-histidine: its capacities to chelate cupric ion and to react with *p*-benzoquinone.

For more than a century, various amines have been known to react with *p*-benzoquinone (10). The reaction leads successively to several products. For example, reaction with an aromatic amine may yield 2-arylaminohydroquinone, 2,5-diarylaminohydroquinone, and from these products the corresponding quinones through oxidation by remaining *p*-benzoquinone; moreover, this oxidation would in turn also yield hydroquinone. If the reacting amine were L-histidine, the substituent on the hydroquinone and on the quinone would be relatively polar, and the substituted compounds would be expected to show shorter retention times in the HPLC system used here than the corresponding unsubstituted compounds. Also, it would be expected that the substituted quinones would be detectable by the UV detector at 260 nm but probably not detectable by the EC detector as used here, whereas the substituted hydroquinones would probably be more readily detected by the EC detector. Thus, such a series of reactions could explain several of the observations regarding the reaction between *p*-benzoquinone and L-histidine, namely: (*a*) the rapid production of four unknown components (IV–VII), (*b*) that two of these were detected by EC, whereas the other two were detected by UV, (*c*) the retention times of IV–VII relative to the retention times of hydroquinone and *p*-benzoquinone, and (*d*) the increase in hydroquinone.

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