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PAPER ELECTROPHORESIS OF BINARY MIXTURES OF COPPER(II) HISTIDINATE AND COPPER(II) COMPLEXES OF OTHER AMINO ACIDS

THE NATURE OF THE "THIRD SPOT"

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SUMMARY

The supernumerary (third) spots which separate when binary mixtures of copper(II) complexes of histidine and some other amino acids are subjected to paper electrophoresis were re-examined. It is concluded that, in the early stages of electrophoresis, the spots consist of mixed-ligand complexes composed of copper(II), histidine and other amino acid and that, as electrophoresis proceeds, the complexes undergo two different reactions, simultaneously. A portion of them re-equilibrates to form homogeneous copper(II) complexes of each of their constituent amino acids, while the remainder decomposes to the relatively stable 1:1 histidine–copper(II) complex and the other amino acid which is liberated as the free, uncomplexed ion. This interpretation is based on electrophoresis in mildly alkaline electrolytes of mixtures containing copper(II) histidinate and the copper(II) complex of either an acidic or a basic amino acid.

INTRODUCTION

WIELAND AND FISCHER[1] have reported the formation of a "third spot" during the paper electrophoretic separation of a mixture of the copper(II) complexes of histidine and lysine, and suggested that the spot consisted of the mixed-ligand complex, His–Cu(II)–Lys*. More recently, JURSÍK AND PETRŮ[2] found that a third spot also formed when mixtures of copper(II) complexes of L-histidine and other basic amino acids (L-ornithine and L-arginine) were subjected to electrophoresis, and they attributed its formation to a coulombic attraction between a free carboxylate group of the His_2–Cu(II) complex and the free ω-amino groups of the copper(II) complexes of the basic amino acids. Although much evidence now exists for the presence of free carboxylate groups in copper(II)–histidine complexes[3,4], it seems unlikely that an

* The complexes are conveniently formulated in this way using the following abbreviations: His = DL-histidine; Lys = L-lysine; Cyst = L-cysteic acid; Arg = L-arginine; Orn = DL-ornithine; Glu = L-glutamic acid; Asp = DL-aspartic acid.

ion-pair resulting from the proposed coulombic attraction would survive the process of electrophoresis as an isolated system in an indifferent electrolyte. It is true, however, that ion-pair formation has been demonstrated by paper electrophoresis[5–8], but only when one of the ions involved constituted part of a background electrolyte.

A third spot also formed during the electrophoresis of a mixture of the copper(II) complexes of histidine and glutamic acid[1] and, in the present work, His_2–Cu(II) was found to behave similarly in the presence of the copper(II) complexes of other acidic amino acids (DL-aspartic and L-cysteic acids)*. Clearly, ion-pair formation involving a free carboxylate group of the His_2–Cu(II) complex cannot be invoked to explain the supernumerary spots formed in the presence of the acidic amino acids. The results of the present work can be interpreted on the basis of the original suggestion of WIELAND AND FISCHER[1]. The third spot forming in each case appears to be due, primarily, to a mixed-ligand complex composed of copper(II), histidine and the other amino acid, but the complex re-equilibrates partially during electrophoresis to form homogeneous copper(II) complexes of each of its constituent amino acids. Simultaneously, a portion of it appears to decompose to the His–Cu(II) 1:1 complex and the other amino acid which is liberated as a free, uncomplexed ion. The latter reaction may occur predominantly during the separation of mixtures of the copper(II) complexes of histidine and the basic amino acids.

EXPERIMENTAL

Materials

Commercial samples of the amino acids, DL-histidine monohydrochloride, DL-ornithine monohydrobromide and DL-aspartic acid (B.D.H.), L-glutamic acid (Sigma Chemical Company), L-arginine monohydrochloride (L. Light & Co.), L-lysine monohydrochloride (E. Merck, Darmstadt) and L-cysteic acid (Calbiochem, Inc.) each gave a single spot on paper electrophoresis in borate buffer (pH 9.2). Cupric acetate (B.D.H.) was of analytical reagent grade.

Preparation of solutions of the copper(II) complexes. Solutions (0.05 *M*) of the 2:1 amino acid–copper(II) complexes were prepared by dissolving the calculated quantities of cupric acetate and an amino acid in a little water, adding 1 *N* NaOH to pH 9, and diluting appropriately with water.

Each of the required binary mixtures was prepared by dissolving calculated quantities of cupric acetate, histidine hydrochloride and another of the above selection of amino acids in a little water and bringing the solution to pH $\simeq$ 9 by the addition of 1 *N* NaOH. Sufficient water was then added to make the solutions 0.1 *M* with respect to each amino acid and to copper(II). Amino acid–copper(II) 2:1 complexes were thus formed in solution, each in 0.05 *M* concentration.

A solution containing the His–Cu(II) 1:1 complex (0.1 *M* with respect to copper(II) and to histidine) was prepared by dissolving the calculated quantities of

* The third spot separating from these mixtures is not related to the supernumerary, copper-positive spot which separates from copper(II) salts of copper(II) complexes of the acidic amino acids[9] *e.g.* Cu(II)[Glu_2–Cu(II)]. In these compounds, the amino acid and copper(II) are present in 1:1 ratio, but only half the copper(II) is actually combined as the complex. The work described here was conducted with mixtures containing the acidic amino acids and copper(II) in 2:1 ratio and the supernumerary spots which separated from appropriate mixtures contained both copper(II) and amino acids.

cupric acetate and histidine hydrochloride in about 3/4 of the necessary volume of water. The addition of 1 *N* NaOH to pH $\simeq$ 7.5 caused a slight precipitate to appear which would not redissolve on shaking or by gentle warming. Water was then added to make the solution up to the required volume.

The continued addition of 1 *N* NaOH to such a solution (pH $\simeq$ 7.5) was accompanied, at first, by the appearance of a heavy precipitate. This redissolved completely on shaking, to give an intense blue-green solution when pH values a little in excess of 11 were reached (*cf.* ref. 9).

Both of the solutions (pH $\simeq$ 7.5 and pH $>$ 11) were used to demonstrate the electrophoretic properties of the His–Cu(II) 1:1 complex and gave comparable results when borate buffer was used as the electrolyte.

Electrolytes

The following electrolytes were used: (a) sodium borate buffer (pH 9.2) containing 0.2 gram-atom of boron per litre[10]; (b) acetate buffer (pH 4.6) containing 6.39 g $CH_3COONa \cdot 3H_2O$ and 3.2 g glacial acetic acid in 1 l of water. The solution was 0.1 *M* with respect to total acetate.

Spray reagents

The spray reagents used were: (A) ninhydrin (0.2 g) was dissolved in 96 ml *n*-butanol previously saturated with water, and 4 ml glacial acetic acid was added to the solution; (B) rubeanic acid (0.1 g) dissolved in 100 ml methanol.

Apparatus

Paper electrophoresis was conducted in the enclosed-strip apparatus described previously[11] using Whatman No. 4 paper in strips 13.5 $\times$ 61 cm, with 45 cm under pressure and cooled. Mains water at 18° was circulated through the coils of the cooling-plate and maintained the temperature of the paper at about 20°.

Procedures

General. Samples (0.5 μl) of the copper(II) complexes and other solutions were transferred by means of a platinum loop to papers impregnated with the borate electrolyte, and equilibrated for 15 min by enclosure within the apparatus. Caffeine was used as marker for zero migration without serious error[12], and rates of migration of test compounds were calculated relative to that of nitrobenzene-*p*-sulphonate applied to the same paper (M_N values[10]).

Electrophoresis was normally allowed to proceed for 1 h at about 21 V/cm. (Nitrobenzene-*p*-sulphonate moved 11.3 cm under these conditions.)

The papers were dried in the oven at 100°. Caffeine, nitrobenzene-*p*-sulphonate and copper(II) were located as dark blue spots under a Hanovia "Chromatolite" ultraviolet lamp.

The location of copper(II) was confirmed by spraying papers with rubeanic acid; the amino acids were detected with the ninhydrin reagent. Histidine in low concentrations on papers was difficult to detect in the presence of copper(II) unless the complex was first destroyed. The complex was effectively destroyed and free histidine liberated by brief exposure of partially dried papers to H_2S gas. Alternatively, papers were treated first with rubeanic acid, redried, sprayed with the ninhydrin reagent and then heated at 100° to develop the colour.

Procedure A. Identification of the contents of the spots and streaks which separate when a mixture of copper(II) complexes of DL-*histidine and* L-*cysteic acid is subjected to paper electrophoresis in borate buffer.* A solution containing a mixture of the copper-(II) complexes of histidine and cysteic acid was applied to the starting-line of a paper impregnated with the borate buffer, and electrophoresis was conducted for 30 min. The paper was partially dried in the oven, exposed to H_2S gas to precipitate copper(II) as CuS and liberate the amino acids. The pherogram was then dried completely, and one lane cut from it was lightly sprayed with the sodium acetate electrolyte (pH 4.6) and laid transversely across the middle of a fresh paper previously impregnated with the sodium acetate electrolyte and inserted in another apparatus. The assembly is represented in Fig. 2 where the lane removed from the borate pherogram is represented by the narrow strip, the original anode lying beyond the end marked "A". For reference, a solution containing histidine and cysteic acid (0.05 *M* with respect to each) was applied to the point marked "X", and electrophoresis was carried out for 25 min at 21 V/cm.

The second separation thus took place in a direction at right angles to the first. The paper, dried and treated with the ninhydrin reagent, showed that the reference mixture of amino acids had separated cleanly, cysteic acid migrating toward the new anode and histidine toward the cathode. The amino acids present in the three spots, as well as in the interconnecting streaks, on the lane of the original (borate) pherogram were thus readily identified. The spots and streaks still evident on the lane of the original pherogram are due to CuS precipitated on the cellulose fibres during the exposure of the pherogram to H_2S gas.

Procedure B. Formation of His_2–Cu(II) by in situ *reaction of the contents of the separated third spot with free* DL-*histidine applied directly to the pherogram.* A paper strip (which resulted in the pherogram reproduced in Fig. 3) was prepared for the experiment by drawing across it the line Y 2.4 cm to the anode side of line X which was to serve as the starting-line. The paper was impregnated with borate electrolyte and inserted in the apparatus; samples of the mixture of the copper(II) complexes of histidine and cysteic acid were applied to line X in lanes 1, 4, 5, 6, 7 and 9, and electrophoresis was conducted for 45 min. It had been ascertained, by means of a previous experiment, that the centres of the separated third spots would then coincide with the points at which line Y intersected the respective lanes. Electrophoresis was interrupted at this stage while the following solutions were applied to line Y in the lanes specified: histidine (0.05 *M*) in lanes 2, 4 and 6; histidine (0.01 *M*) in lanes 5 and 7; and the His_2–Cu(II) complex (0.05 *M*) in lanes 3 and 8.

Electrophoresis was resumed for 45 min after which the paper was partially dried and cut longitudinally into two strips containing, respectively, lanes 1–5 and lanes 6–9. The former was exposed to H_2S gas, completely dried, and treated with the ninhydrin reagent. The dried strip containing lanes 6–9 was sprayed with rubeanic acid.

RESULTS AND DISCUSSION

The phenomenon under review was found by WIELAND AND FISCHER[1] to occur during electrophoresis in sodium acetate electrolyte (0.1 *M*; pH 7.5), and JURSÍK AND PETRŮ[2] subsequently used the same electrolyte for their work, reporting that

the phenomenon was observed only at pH 7.5 and depended upon the composition of the buffer used. Sodium acetate solutions in this pH range have little or no buffering capacity, however. Hydroxyl ions present, for example, in 0.5 μl of 0.1 N NaOH solution applied to papers impregnated with the electrolyte (pH 7.5) survived as discrete spots or streaks after electrophoresis for more than 1 h, and it is believed that hydroxyl ions contained in the more alkaline test solutions used in the present work caused distortion of some pherograms, making interpretation of the results difficult. Furthermore, some amino acids such as histidine could be made to streak toward one or the other electrode according to the pH of the applied solutions. The poor buffering capacity of the sodium acetate electrolyte was also evident when a mixture of histidine and lysine containing copper(II) equivalent to only one half of the total amino acids present was subjected to electrophoresis. The appearance of the third spot was found to be dependent on the pH of the applied solutions. Only those solutions in excess of about pH 8.5 yielded the third spot. JURSÍK AND PETRŮ[2]

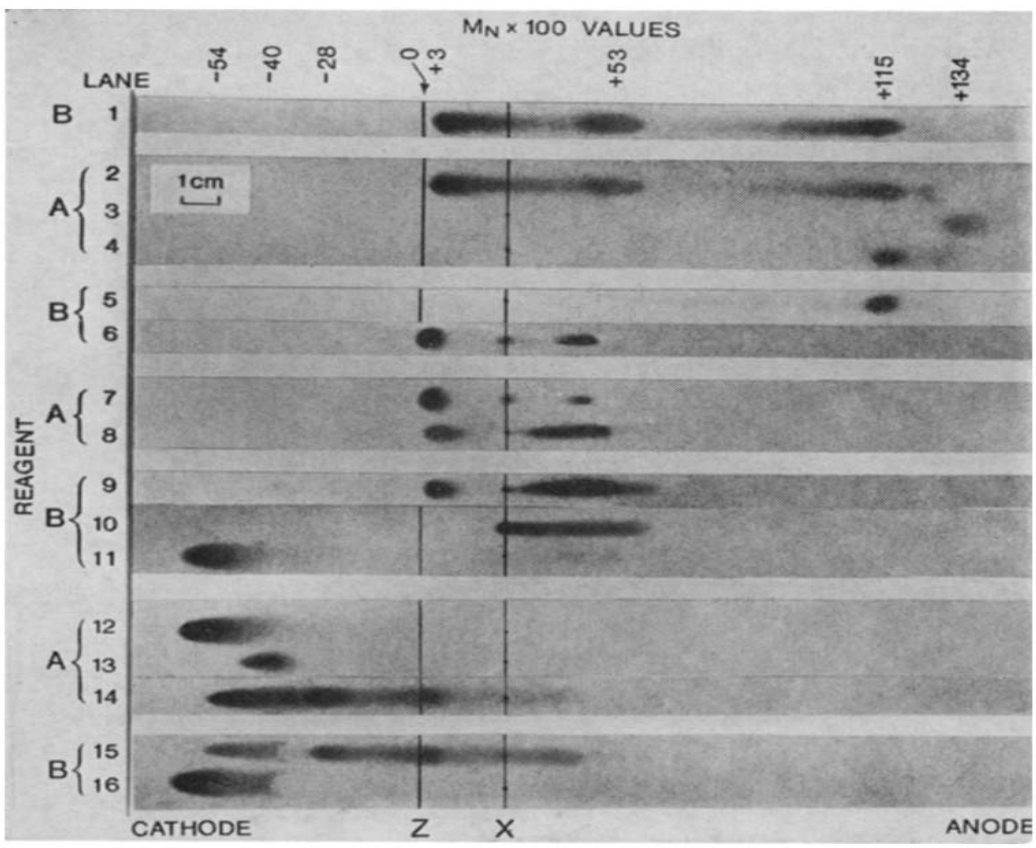


Fig. 1. Results of the paper electrophoresis of some amino acid–copper(II) complexes and mixtures thereof in borate buffer for 1 h at 20 V/cm and 20°. Solutions (0.05 M) of the following were applied to the starting line, X, as specified. Lanes 1,2: mixture of His_2–Cu(II) and $Cyst_2$–Cu(II); lane 3: L-cysteic acid; lanes 4,5: $Cyst_2$–Cu(II); lanes 6,7: His_2–Cu(II); lanes 8,9: the preparation, of pH > 11, of His–Cu(II) (1:1); lane 10: cupric acetate; lanes 11,12: Arg_2–Cu(II); lane 13: L-arginine; lanes 14,15: mixture of His_2–Cu(II) and Arg_2–Cu(II); lane 16: Arg_2–Cu(II) (0.05 M) containing free L-arginine (0.05 M). The dried papers were cut into strips and sprayed, as indicated, with either reagent A (ninhydrin) or reagent B (rubeanic acid) for the detection of amino acids and copper(II). The extent of the electroendosmotic flow, using caffeine as marker, is indicated by line Z. M_N values[10] express mobilities relative to the nitrobenzene-p-sulphonate ion (not shown on the pherograms).

reported that a third spot separated from a mixture of copper(II) histidinate and lysine but not from a mixture of copper(II) lysinate and histidine. The pH value of the latter was not specified, but it is possible that this puzzling result is explained by the above finding.

Other electrolytes with better buffering properties were therefore sought and, contrary to the experience of JURSÍK AND PETRŮ[2], a third spot was found to separate from appropriate mixtures of the amino acid–copper(II) complexes at pH values considerably in excess of 7.5 and in electrolytes other than sodium acetate. Carbamation of the amino acids caused complications however, when carbonate electrolytes were used[11,13], but carbamation does not occur in the presence of the borate buffer (pH 9.2) described in the EXPERIMENTAL section, and this was used for all the separations of copper(II) complexes reported here. Similar borate buffers were selected by SZWAJ AND KAŃSKI[14] for use in a related study.

The results of the electrophoresis of some copper(II)–amino acid complexes and mixtures thereof are shown in Fig. 1. The effect of the presence of $Cyst_2$–Cu(II) on the electrophoresis of His_2–Cu(II) (lanes 1 and 2) is typical of the complexes of other acidic amino acids (glutamic and aspartic acids), the third spot being anionic, in each case, and lying between the spots representing His_2–Cu(II) and the complex of the other amino acid*. The pherogram resulting from the separation of mixtures of the copper(II) complexes of arginine and histidine (lanes 14 and 15) exemplifies those obtained when the complexes of other basic amino acids replace that of arginine in the mixture, the third spot now being of intermediate cationic mobility. The

TABLE I

RELATIVE RATES OF MIGRATION OF THE AMINO ACIDS, THEIR COPPER(II) COMPLEXES, AND THE "THIRD SPOTS" WHICH SEPARATE FROM APPROPRIATE MIXTURES OF THE COMPLEXES

Compounds were detected after paper electrophoresis in sodium borate electrolyte (pH 9.2) at 21 V/cm and 20° for 1 h.

Pure compounds	$M_N \times 100$[a]	*Mixtures*	*"Third spot"* $M_N \times 100$
DL-Histidine	+ 26		
His_2–Cu(II)	+ 3		
His–Cu(II) (1:1)	+ 46		
L-Cysteic acid	+134		
$Cyst_2$–Cu(II)	+115	His_2–Cu(II) + $Cyst_2$–Cu(II)	+53
L-Glutamic acid	+ 92		
Glu_2–Cu(II)	+101	His_2–Cu(II) + Glu_2–Cu(II)	+50
DL-Aspartic acid	+ 99		
Asp_2–Cu(II)	+105	His_2–Cu(II) + Asp_2–Cu(II)	+51
L-Arginine	− 40		
Arg_2–Cu(II)	− 54	His_2–Cu(II) + Arg_2–Cu(II)	−28
DL-Ornithine	− 30		
Orn_2–Cu(II)	− 54	His_2–Cu(II) + Orn_2–Cu(II)	−33
L-Lysine	− 48		
Lys_2–Cu(II)	− 67	His_2–Cu(II) + Lys_2–Cu(II)	−37

[a] M_N values[10] express mobilities relative to the nitrobenzene-*p*-sulphonate ion, which moved 11.3 cm. Positive values represent anionic, negative values cationic mobilities.

* A third spot also separates from a mixture of copper(II) citrate and His_2–Cu(II) showing that the phenomenon is not limited to mixtures containing only amino acid ligands.

mobilities (as $M_N \times 100$ values) of the ionic species studied are given in Table I, positive values representing anionic mobilities, negative values cationic mobilities. The mobilities included in Fig. 1 serve to identify the respective spots.

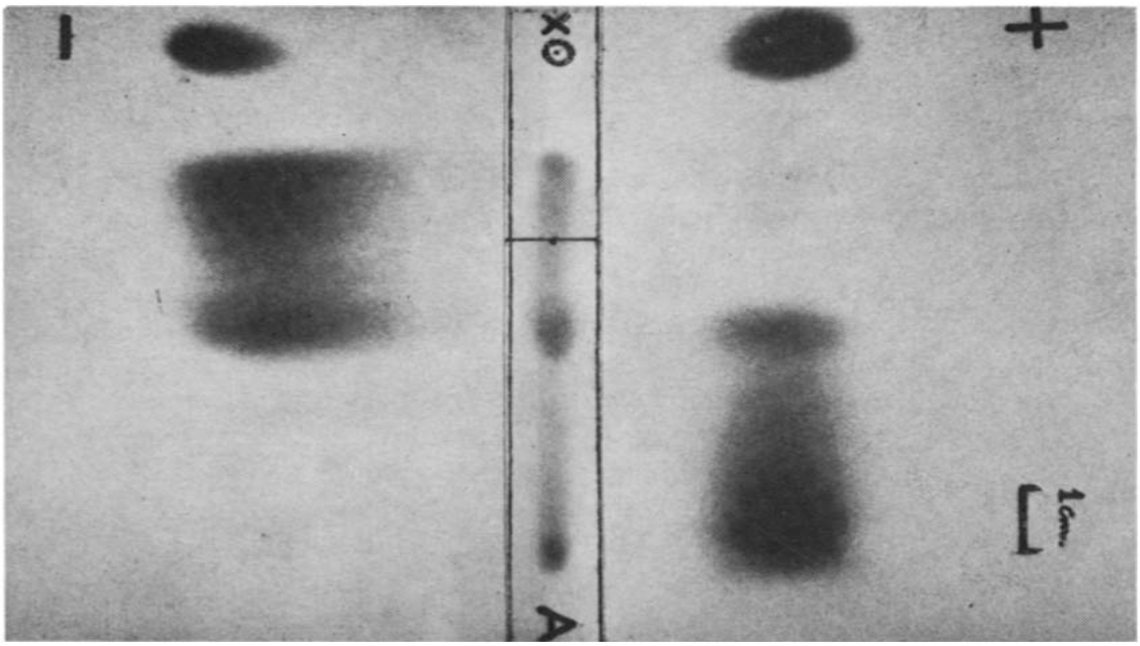


Fig. 2. Identification of the contents of the spots and streaks which separate when a mixture of the copper(II) complexes of DL-histidine and L-cysteic acid is subjected to paper electrophoresis in borate buffer for 30 min (procedure A, EXPERIMENTAL section). The lane removed from the borate pherogram is represented by the narrow strip, the original anode lying beyond the end marked "A". The spots and streaks still evident on the lane of the original pherogram are due to CuS precipitated there during the exposure of the paper to H_2S gas. The fastest (most anionic spot) of the borate pherogram is shown to contain copper(II) and cysteic acid, the slowest spot, copper(II) and histidine, and the intermediate (third) spot, a mixture of copper(II), histidine and cysteic acid. The streaks are shown to contain histidine (to the cathode side of the third spot) and cysteic acid (to the anode side) and both streaks also contain copper(II).

During the earlier stages of electrophoresis, the third spot separating from any mixture is connected on each side by streaks to the spots representing the copper(II) complex of histidine and of the other amino acid. When the other amino acid is cysteic acid, the streaks contain histidine (to the cathode side of the third spot) and cysteic acid (to the anode side) and both streaks also contain copper(II). The third spot contains a mixture of histidine, cysteic acid and copper(II). These facts were demonstrated by means of procedure A, outlined in the EXPERIMENTAL section, and the results of a typical experiment are reproduced in Fig. 2. In contrast to the streaks which form when a mixture of the complexes is subjected to electrophoresis, the complexes of cysteic acid and histidine, applied separately, migrate as compact spots as shown in Fig. 1, lanes 4, 5, 6 and 7. (A little free cysteic acid is seen to have separated, however, from the $Cyst_2$–Cu(II) complex.)

The above facts can be interpreted on the assumption that, in the early stages of electrophoresis, the third spot consists, at least partially, of a mixed-ligand complex of the type originally proposed by WIELAND AND FISCHER[1], and that this complex (for example, His–Cu(II)–Cyst) rearranges progressively, according to equilibrium (1).

$$2\ \text{His–Cu(II)–Cyst} \rightleftharpoons \text{His}_2\text{–Cu(II)} + \text{Cyst}_2\text{–Cu(II)} \quad (1)$$

The products of the rearrangement, the homogeneous amino acid–copper(II) complexes, then migrate away from the third spot with characteristic mobilities, giving rise to the observed streaks. As the electrophoresis proceeds, it is observed, again by means of procedure A, that the cysteic acid content of the third spot diminishes at a greater rate than that of histidine until, after 45 min, only histidine and copper(II) remain as detectable components of the third spot. (This now gives the slate-grey ninhydrin reaction typical of histidine as detected under our conditions.) This observation is not due to a greater sensitivity of histidine than cysteic acid to the ninhydrin reagent. In fact, the limits of detectability of these amino acids are almost identical, being equivalent to about 0.7 nmole of each per cm^2 of spot area.

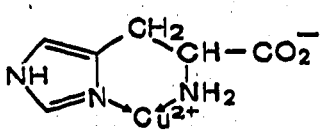
Although the composition of the third spot changes in this way during the course of electrophoresis, its mobility (M_N *ca.* 0.5) remains constant within the limits of experimental error. Evidently, the histidine and copper(II) which remain as the only detectable components of the third spot are not combined as the usual 2:1 complex, the mobility of which is quite different (*viz.*, M_N 0.03).

It is reasonable to assume that the elongated anionic spot which separates from mixtures containing histidine and copper(II) in equimolecular ratio (Fig. 1, lanes 8 and 9) is due to the His–Cu(II) 1:1 complex, and it has been designated as such in Table I. The complex is partially adsorbed by the cellulose support but the mobility, M_N 0.46, corresponding to the head of the elongated spot, probably approaches the true value for its mobility. This is comparable, therefore, with the mobility of the above third spot as well as with those separating from mixtures containing the copper(II) complexes of the other acidic amino acids (see Table I). The contents of the third spot separating from each of these mixtures tend to streak, indicating that they too, are partially adsorbed by the cellulose support. It is therefore suggested that, in the later stages of the electrophoretic separations, the third spot consists of the His–Cu(II) 1:1 complex and that the rearrangement described above (equilibrium (1)) occurs concurrently with the partial decomposition of the mixed-ligand complex represented by equilibrium (2).

$$\text{His–Cu(II)–Cyst} \rightleftharpoons \text{His–Cu(II)} + \text{Cyst} \quad (2)$$

MALEY AND MELLOR[15] have noted that copper(II) complexes much more strongly with histidine than with some other amino acids. It is not surprising, therefore, that the decomposition of the mixed-ligand complex, His–Cu(II)–Cyst involves the dissociation of the cysteic acid rather than the histidine moiety. The cysteic acid thus progressively liberated migrates rapidly toward the anode and ultimately leaves a rather stable residue of the His–Cu(II) 1:1 complex as the sole constituent of the third spot. This survives electrophoresis as a definite spot at the head of a faint streak for prolonged periods and is readily detectable after runs of 3 h or more.

The structure below is one of several which have been proposed for the 1:1 com-



plex and it has been shown to be a relatively stable species[16–19]. It has a formal net charge of +1, yet it behaves as an anion (Fig. 1, lanes 8 and 9). LEBERMAN AND RABIN[16,18] have postulated from their titration data that the 1:1 copper (II)complex takes up an hydroxyl ion from solution to form a neutral (zwitter-ionic) species. It appears, under the present conditions, to take up at least two hydroxyl ions from the alkaline electrolyte to acquire a net negative charge. It thus resembles the free cupric ion which is also anionic in the borate buffer, as indicated by the streak shown in Fig. 1, lane 10. This behaviour, typical of the cupric ion in other mildly alkaline electrolytes, is probably due to the ionisation of an aquo–metal complex of copper(II) (ref. 20) or to the presence of cuprate or bicuprate ions in the alkaline medium[21].

PERRIN[17] has concluded that the His–Cu(II) 1:1 complex is present in significant amounts in neutral and alkaline solutions containing the normal 2:1 complex and this is confirmed by the results shown in Fig. 1, lanes 6 and 7. The 1:1 complex present in the His_2–Cu(II) preparation has survived the process of electrophoresis to separate as a small, somewhat elongated anionic spot of characteristic mobility. YOSHINO AND MAKI[22] have made similar observations which led them also to suggest

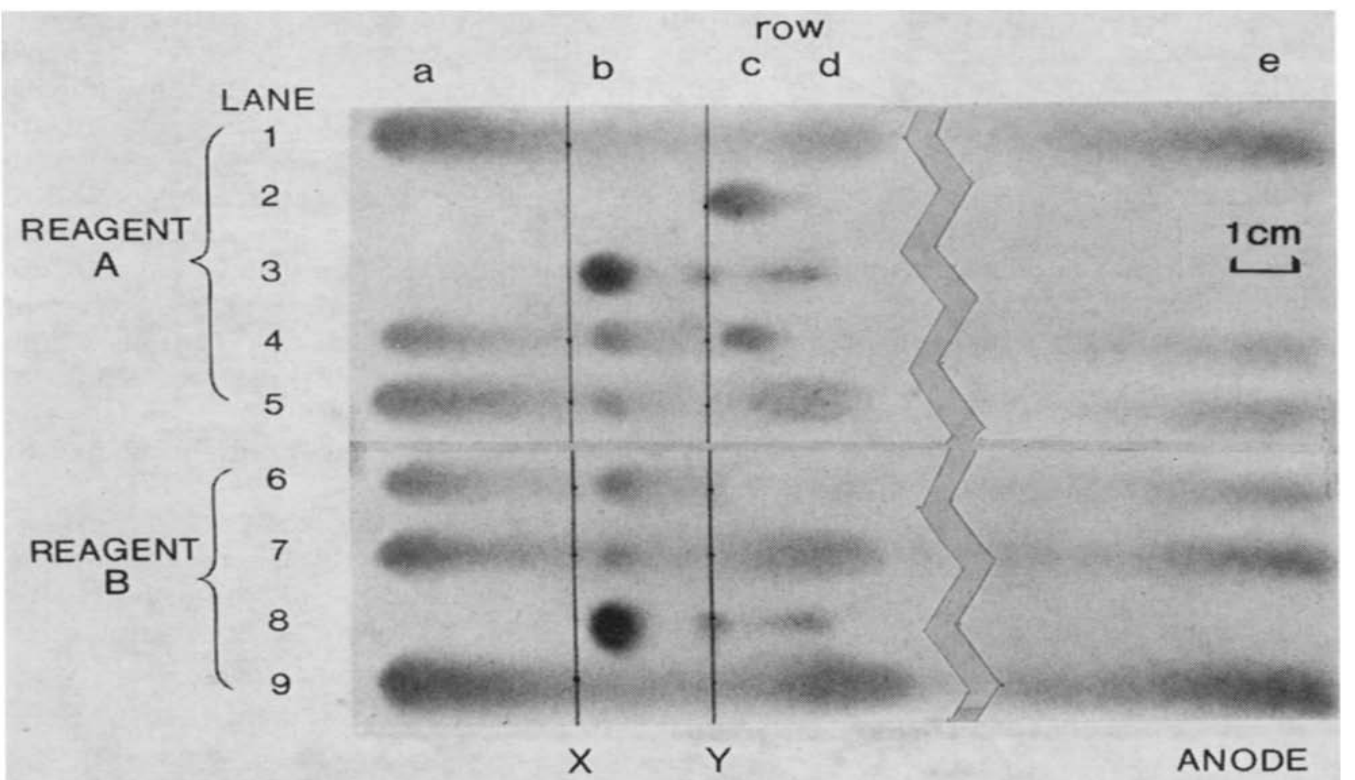


Fig. 3. Formation of His_2–Cu(II) by *in situ* reaction of the contents of the separated third spot with free DL-histidine applied directly to the pherogram (procedure B, EXPERIMENTAL section). A solution containing the mixture, His_2–Cu(II) and $Cyst_2$–Cu(II), was applied to the starting-line, X, in lanes 1, 4, 5, 6, 7 and 9, and subjected to electrophoresis for 45 min. The experiment was interrupted and the following solutions applied to line Y in the lanes specified: histidine (0.05 *M*) in lanes 2, 4 and 6, histidine (0.01 *M*) in lanes 5 and 7, and His_2–Cu(II) (0.05 *M*) in lanes 3 and 8. Electrophoresis was resumed for 45 min after which the paper was divided into two strips containing, respectively, lanes 1–5 and lanes 6–9. The amino acids were located in lanes 1–5 with reagent A (ninhydrin) and copper(II) in lanes 6–9 using reagent B (rubeanic acid).

that the 1:1 complex, co-ordinating with two hydroxyl ions, is present in alkaline solutions of the 2:1 complex.

Confirmation of the identity of the third spot was obtained from the results of procedure B, described in the EXPERIMENTAL section. The initial period of 45 min of electrophoresis brought about the separation of the third spot from the mixture of copper(II) complexes of histidine and cysteic acid which was applied to the starting-line, X, of the pherogram (Fig. 3) in the lanes specified. (It will be recalled that the results of procedure A, discussed above, showed that electrophoresis for a period of 45 min sufficed to yield a third spot which was entirely devoid of cysteic acid and contained only copper(II) and histidine.) The centres of the separated third spots, at the end of the initial period of electrophoresis, coincided with the points at which line Y intersects the respective lanes. The third spot contents were, therefore, effectively mixed with free histidine by interrupting the electrophoresis at this stage and applying the histidine solutions (0.05 *M* and 0.01 *M*) to line Y, as indicated. The resumption of electrophoresis for 45 min then resulted in the pherogram reproduced in Fig. 3 which, to facilitate discussion, is subdivided into the transverse rows a, b, c, d, and e, each containing spots of identical mobility. Rows a and e contain, respectively, His_2–Cu(II) and $Cyst_2$–Cu(II) and row d contains the third spot which separates from the mixture in the normal course of events, as shown in "control" lanes 1 and 9. Lanes 4, 5, 6 and 7 in which histidine was superimposed on the third spot at line Y contain a spot (row b) the mobility (M_N 0.03) of which is identical with that of His_2–Cu(II). Furthermore, its reactions to ninhydrin and rubeanic acid are identical with the reactions of the 2:1 complex; the comparison was made with samples of the latter applied to line Y (lanes 3 and 8) prior to the second period of electrophoresis. The spot in row b was therefore attributed to the 2:1 complex formed by *in situ* reaction on the pherogram between the contents of the third spot and the added histidine. Moreover, the spot in row b represents the only detectable product of the reaction, other spots (row d, lanes 5 and 7) being due to third spot contents which remained in excess after reaction with the more dilute (0.01 *M*) histidine solution. The spot (row c, lane 4) represents excess histidine applied to the third spot as the 0.05 *M* solution. (The movement of free histidine, applied to line Y and subjected to the second period of electrophoresis, is shown in lane 2.)

These findings are consistent with the conclusion that the His–Cu(II) 1:1 complex gradually accumulates as the main constituent of the third spot during the course of the electrophoretic separations. The spot contents would be expected to react with free histidine, according to equilibrium (3), to form the 2:1 complex, as observed.

$$His\text{–}Cu(II) + His \rightleftharpoons His_2\text{–}Cu(II) \qquad (3)$$

Electrophoresis of a mixture of the copper(II) complexes of arginine and histidine results in the separation of four spots (Fig. 1, lanes 14 and 15). The most anionic of these is perhaps more accurately described as the head of an undifferentiated streak. This extends back to the spot containing the His_2–Cu(II) complex, and the entire streak was shown, by a method similar to procedure B, to consist of the 1:1 complex. Some of this complex probably forms in the original mixture by a reaction analogous to that represented by equilibrium (2) and/or according to equilibrium (4).

$$Arg_2\text{–}Cu(II) + His_2\text{–}Cu(II) \rightleftharpoons 2His\text{–}Cu(II) + 2Arg \quad (4)$$

The formation of the 1:1 complex is thus accompanied by the liberation of free arginine, the presence of which was deduced from the following observations.

Unlike the copper(II) complexes of the acidic amino acids, the cationic copper complexes of arginine and other basic amino acids form comet-shaped spots and have a strong tendency to streak, as shown in lanes 11 and 12. Streaking of copper(II) does not occur, however, when the arginine complex is subjected to electrophoresis in the presence of free arginine. Copper(II) is then detected as a somewhat elongated spot with a concave trailing edge, as shown in lane 16, the characteristic shape of the spot being due to free arginine complexing with residual, streaked copper(II) and "sweeping" it along the lane in the wake of the main spot of the complex. (Lane 13 shows free arginine migrating as a compact spot rather less rapidly than its copper(II) complex.) The most cationic spot separating from the mixture of copper(II) complexes of arginine and histidine is due to the arginine complex and, when sprayed with rubeanic acid, it displays a concave trailing edge similar in appearance to the spot in lane 16. The spot adopts this characteristic shape even during the early stages of the separation, suggesting the presence of free arginine in the original mixture as applied. Although the space between this spot and the third spot is almost devoid of copper(II) at all times during the experiment, the amount of free arginine in the space gradually increases, indicating that only arginine, and none or little of its copper(II) complex, is liberated from the third spot. Inspection of lane 14 shows the liberated arginine as a node within the streak between the third spot and the Arg_2–Cu(II) spot. Assuming again that the third spot consists of a mixed-ligand complex, in this case His–Cu(II)–Arg, the reaction would be represented by equilibrium (5), analogous to equilibrium (2).

$$His\text{–}Cu(II)\text{–}Arg \rightleftharpoons His\text{–}Cu(II) + Arg \quad (5)$$

Being anionic, the His–Cu(II) 1:1 complex simultaneously formed during the experiment migrates back toward the origin, accounting for the heavy streak connecting the third spot with that due to the His_2–Cu(II) 2:1 complex. The contents of the third spot are thus gradually lost to this anionic streak as well as to the growing cationic spot of arginine, and when electrophoresis has proceeded for 90 min the third spot is no longer recognisable as such. It is then represented only by a tapering tail to the anionic streak.

Prolonged electrophoresis of mixtures containing the copper(II) complexes of basic amino acids thus results in the disappearance of the third spot*. It therefore differs from the rather stable third spot which separates from mixtures of the complexes of histidine and the acidic amino acids, the persistence of the spot in the latter case being aided by the fact that the His–Cu(II) 1:1 complex and the mixed-ligand complexes containing acidic amino acids are all anions of similar mobility.

* A mixture containing the copper(II) complex of ornithine, however, yields a much more stable third spot than those containing complexes of the other basic amino acids. The cationic mobility of the mixed-ligand complex, His–Cu(II)–Orn, which constitutes this third spot, is about the same as that of free ornithine; their M_N values are −0.33 and −0.3, respectively. Migrating in the presence of free amino acid, the mixed-ligand complex tends to be stabilized against decomposition of the kind represented in equilibrium (5).

Experimental evidence for the existence of mixed-ligand complexes of copper(II) containing two different amino acids has also been obtained by SARKAR and coworkers[23–25], and it is noteworthy that these complexes contain histidine as a common component. Other workers have obtained similar complexes in crystalline form[26]. Complexes of copper(II) containing mixed chelating ligands, only one of which is an amino acid, and yet others in which neither ligand is an amino acid, are well known and many of these also have been obtained in crystalline form[27,28].

In our experience, mixed-ligand copper(II) complexes containing histidine are unstable in aqueous medium and attempts to isolate them result in their decomposition according to the equilibria defined above. Their ultimate decomposition to the His–Cu(II) 1:1 complex is most easily demonstrated, under our conditions of electrophoresis, with those containing the acidic amino acids. Under other conditions of electrophoresis, however, it may be possible to obtain a stable spot consisting solely of the His–Cu(II) 1:1 complex from a mixed-ligand complex containing a basic amino acid, and this appears to have been achieved by JURSÍK AND PETRŮ[2]. Their results are consistent, therefore, with the present concept of the nature of the supernumerary spots and of the equilibria existing in the mixtures which give rise to them.

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