AN IMPROVED METHOD FOR THE DETECTION OF CAERULOPLASMIN*

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URIEL[1] showed that the copper-binding protein of blood, caeruloplasmin, could be detected and estimated by its ability to oxidise *p*-phenylenediamine, forming a dark quinonoid polymer. In the course of investigations into the heterogeneity of caeruloplasmin[2] in the populations of New Guinea, using the microtechniques of protein chromatography on DEAE cellulose paper[3] and acrylamide-gel electrophoresis[4] it became necessary to improve the sensitivity of the *p*-phenylenediamine oxidation method because of the low concentration of some of the minor components resolved in the acrylamide gel.

FISCHER[5] first described the formation of dyes by the oxidative condensation by silver halide in an exposed photographic plate of *p*-phenylenediamine with compounds containing active methylene or methine groups. This reaction is the basis of nearly all modern photographic colour processes and a very wide range of *p*-phenylenediamine derivatives and coupling agents have been described[6].

This paper describes a method of intensifying the colour produced by the action of caeruloplasmin on *p*-phenylenediamine by coupling with 2-cyano-acetylcumarone, a colour photographic coupling agent.

EXPERIMENTAL

Materials

DEAE cellulose paper (Whatman DE 20) was obtained from Messrs. Reeve Angel (London).

Acrylamide containing 5 % (w/w) N,N-methylenebisacrylamide was obtained as "AM-9 Chemical Grout" from Cyanamid (Australia).

p-Phenylenediamine was prepared by twice recrystallising B.D.H. reagent grade *p*-phenylenediamine from alcohol.

2-Cyano-acetylcumarone was a gift of Dr. N. LEWIS, Director of Research Laboratories, Kodak (Australasia) Ltd., Abbotsford, Victoria.

Caeruloplasmin was prepared by chromatography of Cohn fraction IV of human plasma on hydroxylapatite according to the method of BROMAN[7]. Both of the deep blue fractions obtained by this method were pooled.

Serum was prepared from blood which was obtained by venipuncture from nor-

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mal human donors and patients attending the Alfred Hospital, Melbourne, Victoria.

Buffers. The following buffers were prepared from analytical reagent grade salts according to CLARK[8].

pH 5.0, 0.05 *M* sodium acetate; pH 5.5, 0.05 *M* sodium acetate; pH 6.0, 0.05 *M* sodium phosphate; pH 6.5, 0.05 *M* sodium phosphate; pH 7.0, 0.05 *M* sodium phosphate; pH 7.5, 0.05 *M* sodium phosphate; pH 8.0, 0.05 *M* sodium phosphate.

Methods

Spot testing for intensity of colour development was carried out by pipetting 0.01 ml of serum or 0.01 ml of caeruloplasmin solution onto a piece of Whatman No. 1 filter paper. The paper was then incubated in the test solution at 37° for the desired period. The papers were dried in a current of air at 37° and the density of colour determined in an E.E.L. chromatography scanner.

Ion exchange paper chromatography was carried out on a Whatman DE20 paper, using the methods described previously[3].

Acrylamide gel electrophoresis was carried out in gels prepared by dissolving 6 g of AM-9 and 0.5 g of ammonium persulphate in 100 ml of pH 5, 0.05 *M* sodium acetate buffer, then adding 0.5 ml of dimethylaminopropionitrile. The solutions were poured into moulds identical to those used by SMITHIES[9] for two-dimensional filter paper–starch-gel electrophoresis. Electrophoresis in the gels was carried out under the same conditions as used for starch-gels by SMITHIES[9].

RESULTS

Conditions for development of maximum colour intensity

The effect of pH. The spot test was carried out with the reagents dissolved in the pH 5.0, 6.0, 7.0, 8.0 buffers. The concentrations used were 0.05 g of *p*-phenylenediamine and 0.02 g of coupler per 100 ml, the incubation time was 30 min. The pH of maximum intensification in the presence of coupler appeared to be the same as for the develop-

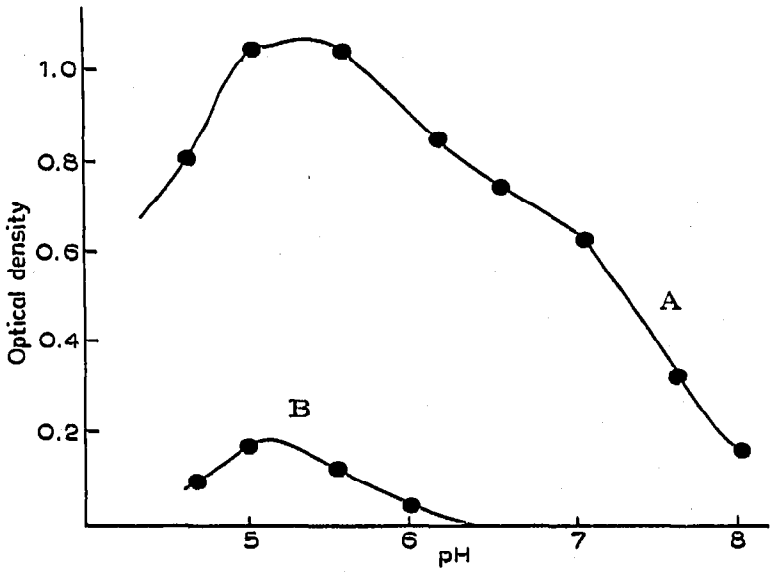

Fig. 1. The effect of varying pH on the colour produced by the oxidation of *p*-phenylenediamine by serum in the presence (A) and absence (B) of 2-cyano-acetylcumarone.

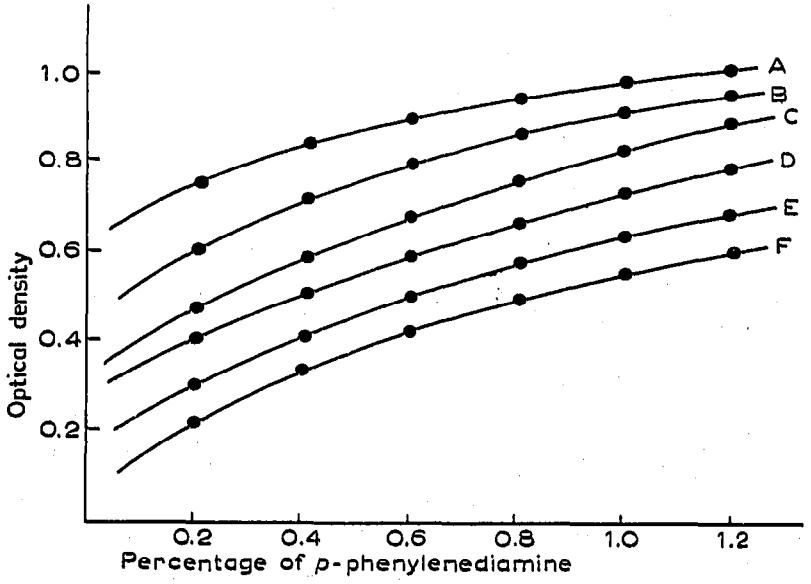

Fig. 2. The effect of varying *p*-phenylenediamine and coupler concentrations on the colour produced in the presence of serum at pH 5.5. (A) 0.2% coupler; (B) 0.16% coupler; (C) 0.12% coupler; (D) 0.08% coupler; (E) 0.04% coupler; (F) 0.02% coupler.

ment of the *p*-phenylenediamine colour alone as determined by the paper spot test (pH 5.5). The intensity in the presence of coupler at pH 5.5 appeared to be approximately 6 times that obtained with *p*-phenylenediamine alone (Fig. 1). After 4 h incubation this ratio fell to 3 to 1 in favour of the coupled reaction.

The effect of reagent concentration. The *p*-phenylenediamine and coupler concentrations could be varied over a wide range, at pH 5.5, without a great effect on the colour as estimated by the paper spot test (Fig. 2). As standards, a 0.1% (w/v) *p*-phenylenediamine and a 0.2% (w/v) coupler solution in pH 5.5, 0.05 *M* acetate buffer were adopted.

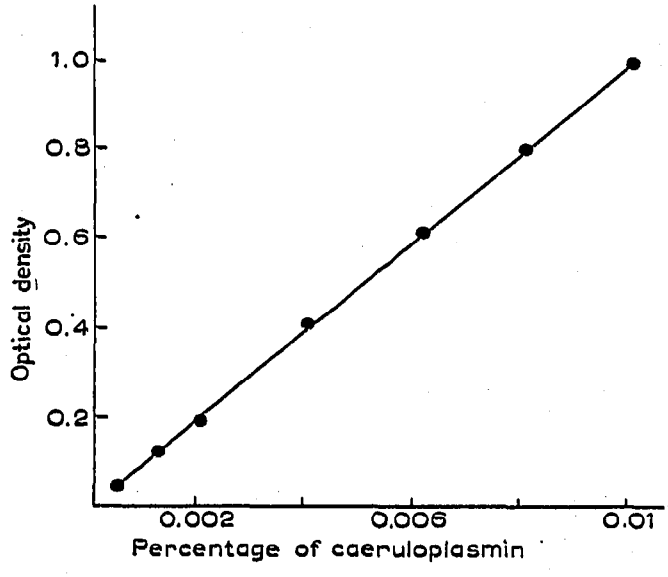

Fig. 3. The effect of the concentration of purified caeruloplasmin on the colour produced by the oxidation of *p*-phenylenediamine in the presence of coupler.

The effect of caeruloplasmin concentration. The spot test was applied to purified caeruloplasmin in a concentration ranging from 0.001 % (w/v) to 0.01% (w/v). The incubation time was 30 min. A linear relationship was found between the concentration of caeruloplasmin and the density of the colour produced at pH 6.0 (Fig. 3). The density of colour produced by each spot was of the same order as that produced by a spot of serum containing the same concentration of caeruloplasmin as determined by the method of URIEL[1].

Detection of caeruloplasmin variants by two-dimensional ion-exchange paper chromatography and acrylamide gel electrophoresis

Duplicate 0.1 ml samples of serum were submitted to ion-exchange paper chromatography under the conditions described previously[3]. One chromatographic strip was developed in the *p*-phenylenediamine coupler mixture. Using this as a guide the caeruloplasmin-containing area was cut from the other strip and placed in a slit cut in the acrylamide gel. Electrophoresis was carried out at 4° for 6 h at a potential gradient of 7 V cm^{-1}. At the end of the run the gel was removed from the tray, sliced into two halves as described by SMITHIES[9] for starch-gels, and one half incubated for 10 min in the standard *p*-phenylenediamine–coupler mixture and the other half in *p*-phenylenediamine solution alone. The two halves were then compared. In all cases enhanced staining of the minor caeruloplasmin components was observed. In some cases these components were so weak as to be unstained by *p*-phenylenediamine alone. A pair of such patterns is shown in Fig. 4.

DISCUSSION

Compared with conventional methods of staining proteins after zone electrophoresis or chromatography, stains based upon enzymatic activity possess one serious disadvantage. For conventional staining the proteins are immobilised in the support (by heat denaturation or protein precipitants) but with the enzymatic stains the proteins must be left in the native state and are free to diffuse. Hence the time required to effect reasonable staining is important in preserving the original resolution of the pattern. Even a modest increase in sensitivity is worthwhile, therefore, if it shortens the time of staining. The other important aspect of increased sensitivity is the ability to use reduced sample volumes and micromethods. This is most important in surveys of primitive populations where a great many different tests must be carried out on small single samples of blood, owing to the difficulty of collecting and transporting specimens.

The particular coupling agent used, 2-cyano-acetylcumarone, was selected by a number of criteria. Unlike the amine couplers it seemed unlikely to have any chelating properties which could affect the copper of the caeruloplasmin, nor did it have any strongly charged groups which could lead to protein binding, either inactivating the caeruloplasmin or making the coupler unavailable. Finally its structure indicated that condensation could take place at other than alkaline pH values. A full review of the chemistry of the coupling agents used in photographic colour development has been given by VITTUM AND WEISSBERGER[6].

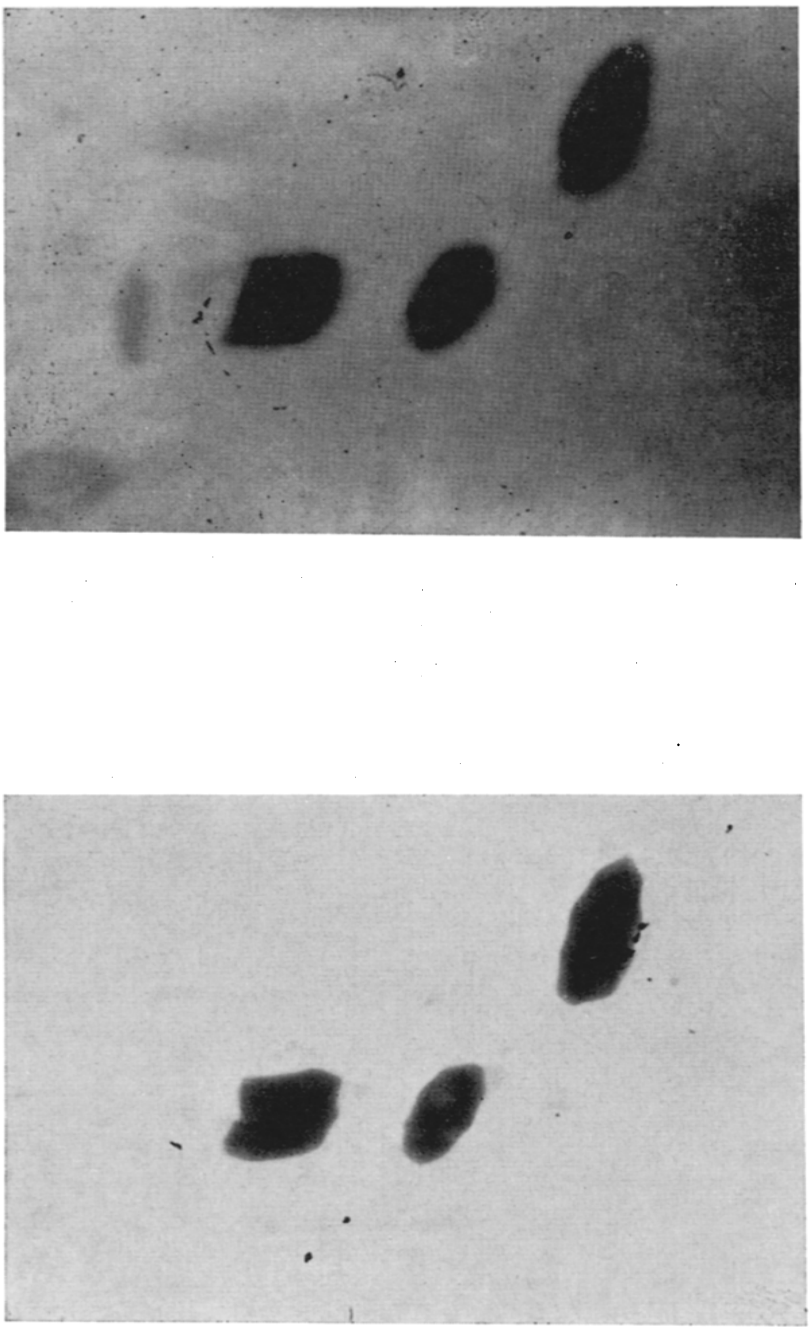

Fig. 4. Patterns produced by two-dimensional ion-exchange paper chromatography and acrylamide gel electrophoresis of serum after staining with (a) the standard *p*-phenylenediamine–coupler solution and (b) in the presence of 0.1 % (w/v) *p*-phenylenediamine in pH 5.5, 0.05 *M* acetate buffer.

SUMMARY

An improved method for the detection of caeruloplasmin is based upon the intensification in the presence of a coupling reagent, 2-cyano-acetylcumarone, of the colour produced by its oxidation of *p*-phenylenediamine. The colour produced was approximately 6 times denser than that produced in the presence of *p*-phenylenediamine alone. The method has the advantage that shorter incubation times can be used, thus preserving resolution by minimising diffusion after zone electrophoresis.

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