Studies of the Differential Staining of Wheat Albumins, Globulins, and Gliadins in Polyacrylamide Gel by Aniline Blue-Black[1]

M. MINETTI, T. PETRUCCI, S. CATTANEO, F. POCCHIARI, and V. SILANO, Laboratori di Chimica Biologica, Istituto Superiore di Sanita, Rome, Italy

ABSTRACT

After gel electrophoresis on polyacrylamide gel and staining of the proteins with aniline blue-black, wheat albumins, globulins, and gliadins give blue-black, blue-green, and reddish-brown bands, respectively. To clarify the nature of this differential staining, we studied the influence of a number of physical and chemical parameters on absorption spectrums of protein bands, as well as the reversibility of the alterations induced by the protein moiety in the aniline blue-black absorption spectrum. We have shown that chromatic differences between albumin and gliadin bands derive from a differential binding to albumins and gliadins of differently colored components of the aniline blue-black. Gliadins bind preferentially some red impurities of the aniline blue-black, whereas in the albumin bands there is a higher percentage of the main blue component of the dye. We suggest that this different behavior of albumins and gliadins is not due to a higher affinity of the red impurities for gliadins as opposed to albumins, but to a lesser tendency of gliadins to coprecipitate the aniline blue-black during the staining process. The blue-green color, distinctive of globulin bands, is mainly due to reversible metachromatic phenomenons induced by globulins in the aniline blue-black spectrum. Since we have induced globulin-like alterations in the aniline blue-black spectrum by adding dehydrating agents (such as salts and strong acids) to a solution of the dye, we concluded that globulin-induced aniline blue-black metachromatism is due to interactions among dye molecules forced to suitable distances after binding to the protein chain.

A direct staining procedure for the differentiation of albumin, globulin, and gliadin-like fractions from wheat was recently described by our Laboratory (1-3). The albumin bands appeared blue-black, the globulin bands blue-green, and the gliadin-like bands reddish-brown after electrophoresis on polyacrylamide gel in a *tris*-glycine buffer system (pH 8.5) and staining of the proteins with a solution of aniline blue-black in 7.5% acetic acid. All fractions belonging to the same protein class gave the same color with aniline blue-black.

This paper deals with the influence of certain chemical and physical parameters on absorption spectrums of albumin, globulin, and gliadin-like electrophoretic bands. We assumed, as a working hypothesis, that the color differences might be due to some metachromatic phenomenons induced in the aniline blue-black spectrum by the protein moiety and/or to the presence in the dye of differently colored components with different affinities for the three protein classes.

As far as we know, the presence of differently colored bands has been described only in the disc-electrophoretic patterns of ribosomal proteins from *Escherichia coli* and higher plants (4-6). Many papers dealing with interactions between dyes and wheat proteins have been published (7-10).

[1]Aniline blue-black is a common name for the acid black I dye (C.I. n.20470) known by many other names, such as Buffalo black NBR, Naphtolblauschwarz, Naphthylamine black 10 BR, Naphthalene black, Pontacyl blue-black SX, Amido black and Amido Schwarz 10 B.

MATERIALS AND METHODS

Chemicals

All products used were of analytical grade by Carlo Erba (Italy). The reagents for polyacrylamide gel electrophoresis, including aniline blue-black, were obtained from Canal Industrial Corporation (U.S.A.). Naphtolblauschwarz was supplied by Fluka (Switzerland), Buffalo black NBR by Allied Chemical (U.S.A.), Amido Schwarz 10 B by Carlo Erba (Italy) or Bayer and Merck (Germany).

A purified α-gliadin sample was generously provided by D. D. Kasarda. This sample had been obtained according to the procedure described by Bernardin et al. (11).

Extraction and Purification of Albumin, Globulin, Gliadin-Like, and Gliadin Fractions

Dry whole seeds of *Triticum aestivum* (var. Turaro) wheat were finely crushed in a laboratory-type mill (Bühler-Switzerland) at stage 3.

Albumin, globulin, and gliadin-like fractions were extracted by shaking the whole meal with 0.15M NaCl (2:3 w./v.) for 3 hr. at room temperature. The extract was cleared by centrifuging at 45,000 × g. The gliadin-like fraction was precipitated by salting out the supernatant with 0.4M ammonium sulfate, and the albumin fraction was then obtained at 1.8M ammonium sulfate concentration. The 1.8M supernatant solution was dialyzed overnight against distilled water at 4°C. to precipitate the globulin fraction.

The experimental procedure for the preparation of these protein fractions has been described in more detail in previous papers (1,2). Gliadins were extracted, under the same experimental conditions, by shaking the whole meal with 60% ethanol (2:3 w./v.) for 1 hr. at room temperature, after 20 extractions of the meal with distilled water containing 0.01% sodium azide to remove albumin and gliadin-like fractions. The ethanol extract was dialyzed at 4°C. against 0.1N acetic acid for 48 hr. and then against water for additional 48 hr. The dialyzed extract was lyophilized and stored at -20°C.

As bands of different color were not evident in the disc-electrophoretic patterns of the four protein preparations, we inferred (3) the homogeneity of the four protein classes.

Just before each experiment, fresh solutions were prepared from weighed portions of the lyophilized fractions. After centrifugation, the protein concentrations of the clear supernatants were determined spectrophotometrically according to Waddell (12).

Polyacrylamide Gel Electrophoresis

Anodic disc-electrophoresis was carried out on freshly prepared protein solutions in a vertical apparatus (Canal Industrial Corporation, Rockville, Md., U.S.A.) using glass columns (10 × 0.7 cm.).

The compositions of the gel and buffer solutions were as described in the gel formulation sheets (Shandon Scientific Co.) for a pH 9.5, glycine-*tris* buffer, 7.5% gel system. No ferricyanide was added to the gel. The protein samples (200 γ of albumins, 300 γ of globulins, gliadins, and gliadin-like fractions, unless otherwise indicated) were loaded on top of the gel in an 11% sucrose solution. A current of 5 ma. per channel was applied for about 95 min. for the albumins and globulins, and 135 min. for the gliadins and gliadin-like fractions. When albumin

and gliadin-like fractions were loaded together on the same gel, the run was carried out for 95 min.

At the end of the run, the gels were stained for 120 min. with 0.5% aniline blue-black in 7.5% aqueous acetic acid. When the staining was performed with a 0.004% aniline blue-black solution, the staining time was 5 days. The excess stain was usually removed by four 20-min. washings in 7.5% acetic acid, followed by an electrophoretic destaining in the same solution with 12.5 ma. applied per channel for 150 min. In other cases, excess stain was removed by continuous washing with 7.5% acetic acid for 3 days in a Decopol apparatus (Minivolt, Rome, Italy).

Absorption Spectrums

All spectrums reported were determined with a Beckman DB recording spectrophotometer. For direct measurement of absorption spectrums of protein bands, the stained gels were inserted upside down (after removal of the upper gel layer) in a 1-cm. path glass cell (3-ml. volume), and the empty space was filled with 7.5% acetic acid solution. In this way, the most intense albumin and gliadin-like bands would appear exactly in the optical path. With globulin, an additional 1.5 cm. of gel had to be cut from the top when the upper gel layer was removed to avoid interference from the low-mobility lipoglobulin fraction (13).

A solution of 7.5% acetic acid was used as blank without adding any gel, as the gel does not interfere with the absorption measurement between 400 and 750 nm.

The absorption spectrum of aniline blue-black was determined at acidic pH using a 0.0016% (w./v.) solution of the dye in 0.1M glycine-HCl buffer at pH 3 (glycine-HCl buffer as a blank) and at alkaline pH using a 0.0016% (w./v.) solution in 1N NaOH (1N NaOH as a blank).

Effect of Proteins on the Aniline Blue-Black Spectrum in the Absence of the Gel

Solutions (1%) of albumin, globulin, and gliadin-like fractions in 0.15M NaCl were added to a 0.0016% (w./v.) solution of aniline blue-black in 0.1M glycine-HCl buffer (pH 3). The final protein concentration was 100 γ per ml. The absorption spectrums of the suspensions were determined after a 20-min. incubation at 25°C. with a Beckman DB spectrophotometer using the glycine-HCl buffer as a blank.

Elution of the Dye from Polyacrylamide Gel Electrophoretic Bands

The dye was eluted from the bands by cutting the gels into small pieces, washing with water at 4°C. for 60 min., and soaking in a 1N NaOH solution at 4°C. for 48 hr. Five milliliters of the NaOH solution was used for each gel. The liquid was then decanted and the absorption spectrum measured using 1N NaOH as blank.

For the short alkaline treatment, the gels, stored in 7.5% acetic acid solution, were transferred to water at 4°C. for 25 min. to remove the bulk of acetic acid, and then placed in 1N NaOH for 90 min. To prevent overheating from neutralization, the temperature was kept at 4°C. for the initial 25 min. At the end of the incubation period in NaOH, the gels were transferred into cold water (4°C.) for 25 min. and finally returned into 7.5% acetic acid. The temperature was again kept at 4°C. for the first 25 min. The absorption spectrums of the residual bands in the gel were determined after 48 hr. as described above.

Precipitation Behaviors in Polyacrylamide Gel of Albumin, Globulin, and Gliadin-Like Fractions by Aniline Blue-Black

Albumins (200 γ) and gliadin-like fractions (500 γ) were submitted to electrophoresis in the same gel for 95 min. as previously described. After electrophoresis, the gel was immersed in a 0.004% aniline blue-black solution in 7.5% acetic acid. At definite intervals, the gel was removed from the aniline blue-black solution and transferred into a 7.5% aqueous acetic acid solution, and densitometric scanning was performed by means of a Cromoscan densitometer (Joyce and Loeble & Co., Ltd., Gateshead, England). The band intensities were measured by corresponding peaks in the recording of the densitometric scanning, subtracting background absorption. There was no overlapping between gliadin-like and albumin bands because of lower mobility of gliadin bands in the buffer system chosen. Data reported are relative to the most intense albumin and gliadin-like bands.

Thin-Layer Chromatography

Twenty microliters of a 0.1% aqueous solution of the dye was developed on a silica gel plate (Merck). The solvent system consisted of n-butanol:ethanol:pyridine:ammonia, d. = 0.925 (40:10:30:20 by volume). After the run (ascending chromatography), the plate was dried at room temperature.

Aniline Blue-Black Purification

A 0.5% aqueous solution of aniline blue-black was made 2M with NaCl and stirred for 2 hr. at room temperature and for 5 hr. at 4°C. The precipitate, consisting of the purified blue fraction, was collected by centrifugation at 45,000 × g for 20 min. The red supernatant solution was discarded. The precipitate was redissolved in water and passed through a Sephadex G-10 column to remove the salt. The central cut was collected and lyophilized, and portions were dissolved in 7.5% acetic acid for protein staining.

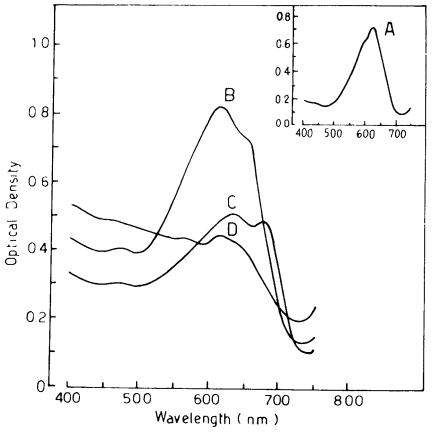
Metachromatism of Aniline Blue-Black Induced by Salts and Strong Acids

Metachromatic phenomenons were induced in a 0.0016% (w./v.) aniline blue-black solution in 0.1M glycine-HCl (pH 3) buffer by adding 5M NaCl or 5M HCl. Sodium chloride was added as solid and HCl as 37% solution. Any temperature increase was prevented by adding the acid very slowly and keeping the aniline blue-black solution in ice. The solutions were incubated for 20 min. at 25°C. and the absorption spectrums determined using 0.1M glycine-HCl buffer as a blank.

RESULTS

A) Influence of Some Physical Parameters on the Absorption Spectrums of Albumin, Globulin, and Gliadin Electrophoretic Bands

Absorption spectrums of albumin, globulin, and gliadin-like bands stained with 0.5% aniline blue-black solution in 7.5% acetic acid after disc electrophoresis on polyacrylamide gel in glycine-*tris* buffer (pH 8.5) are shown in Fig. 1. All albumin bands have an absorption maximum at 608 nm. (Fig. 1, curve B), all globulin bands have two maximums at 630 and 680 nm. (Fig. 1, curve C), while gliadin-like bands present an undifferentiated absorption between 400 and 550 nm. with the appearance of a slight peak at 610 nm. (Fig. 1, curve D). These



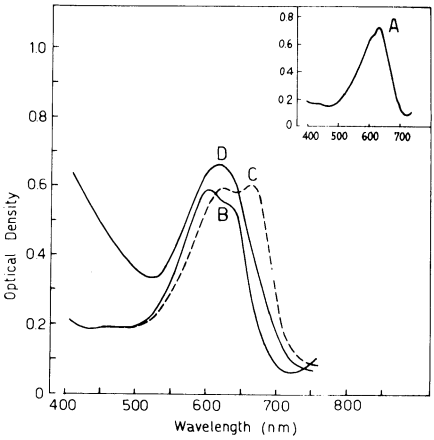


Fig. 1 (left). Absorption spectrums of aniline blue-black (A) and of albumin (B), globulin (C), and gliadin (D) bands in polyacrylamide gel stained with aniline blue-black.

Fig. 2 (right). Modifications induced in the visible absorption spectrum of an aniline blue-black solution (A) by addition of albumins (B), globulins (C), and gliadins (D).

spectrums differ greatly from the absorption spectrum of a 0.0016% aniline blue-black solution in 0.1M glycine-HCl buffer (pH 3) which shows a maximum at 624 nm. and a shoulder at 590 nm. (Fig. 1, curve A). The absorption spectrums of gliadin bands obtained by electrophoretic analysis of 60% ethanol-gliadin extract and of a purified α-gliadin sample were identical to that of gliadin-like fractions. For this reason we will refer to gliadin-like and gliadin fractions as gliadins. It should be clear, however, that only gliadin-like preparations have been used for the experiments described in this paper.

Comparing sections of albumin, globulin, and gliadin bands obtained by cutting a horizontal section of the gel through the band, we observed the presence of a large unstained zone in the inner region of the gliadin bands. Staining time longer than 2 hr. did not affect the size of the unstained zone in the gliadin bands.

Upon adding albumins, globulins, and gliadins to an aniline blue-black solution, precipitation of protein-dye complexes was noted and the following changes were observed in the absorption spectrums of the protein-aniline blue-black suspensions. Albumins shifted the absorption maximum of the dye from 624 to 595 nm. and of the shoulder from 590 to 630 nm. (Fig. 2, curve B). In the presence of globulins, two maximums, at 624 and 670 nm., are evident (Fig. 2, curve C), while with gliadins the maximum is at 620 nm. and a strong, undifferentiated absorption has been observed below 520 nm. (Fig. 2, curve D). Comparing Figs. 1 and 2, it appears that chromatic differences among albumins, globulins, and gliadins stained with aniline blue-black are greater when the reaction between dye and proteins takes place in the polyacrylamide gel system than in free solution.

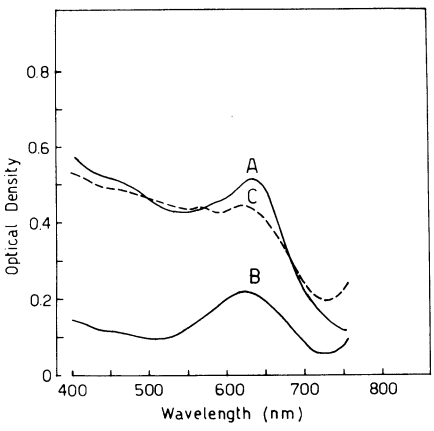
Visible spectrums of albumin-, globulin-, and gliadin-electrophoretic bands are

quite dependent both on the amount of protein loaded on the gel and on the concentration of the aniline blue-black staining solution. In fact, as shown for the gliadin bands (Fig. 3), we observed a significant absorption decrease at wavelengths below 520 nm. compared to that at longer wavelengths by doubling (Fig. 3, curve A) the protein load on the gel. The same effect was observed by decreasing (Fig. 3, curve B) the concentration of aniline blue-black from 0.5 to 0.004% (5 days staining time).

The absorption spectrum of protein bands was also affected by the destaining technique. Gliadin bands destained by washing with 7.5% acetic acid in a continuous-flux destaining apparatus instead of using the electrophoretic method gave a lower relative absorption at wavelengths below 520 nm. If the gel destained by washing was subsequently submitted to a 35-min. electrophoretic destaining, the spectrum returned to an appearance more typical of gliadin bands.

The absorption spectrum of the gliadin bands (Fig. 4, curve A) becomes very similar to that of albumin bands (Fig. 4, curve B) when 45% methanol is added to the aniline blue-black staining solution (Fig. 4, curve C).

Since many commercial preparations of acid black I (C.I. n.20470) other than aniline blue-black are available, we checked differences in the absorption spectrums of albumin, globulin, and gliadin bands using certain of those preparations that showed varying degrees of heterogeneity. Distinct differences among the absorption spectrums of albumin (B), globulin (C), and gliadin (D) bands were evident (Fig. 5) for all dyes tested. Significant differences in



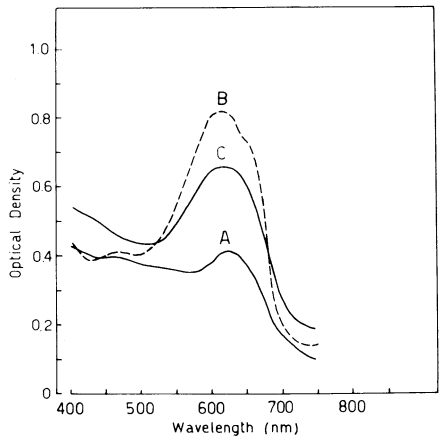


Fig. 3 (left). Absorption spectrums of gliadin bands in polyacrylamide gel: Dependence upon amount of protein loaded on the gel and aniline blue-black concentration. (A) gliadin amount = 570 γ (about twice the standard gliadin load). (B) aniline blue-black concentration = 0.004% (1/125 the standard aniline blue-black concentration). (C) standard conditions.

Fig. 4 (right). Absorption spectrums of gliadin bands in polyacrylamide gel: Dependence upon presence of methanol in the aniline blue-black solution. (Five hundred micrograms of gliadins were loaded per channel. Methanol, when present, was added to the 0.5% aniline blue-black solution in 7.5% acetic acid at a final concentration of 45% (v./v.). (A) gliadins, no methanol; (B) albumins, no methanol; (C) gliadins, with methanol.)

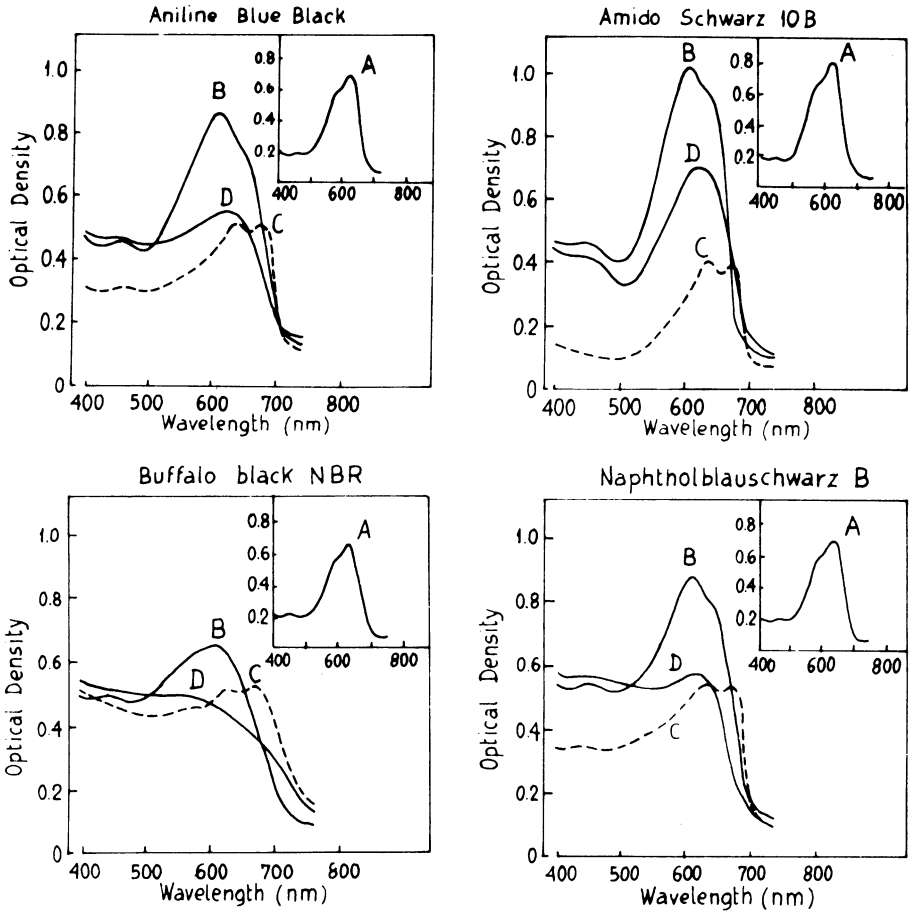


Fig. 5. Absorption spectrums of albumin (B), globulin (C), and gliadin (D) bands in polyacrylamide gel stained with different commercial acid black I products (A). (Two hundred micrograms of albumins and 500 γ each of globulins and gliadins were loaded per channel. Protein staining was performed in the conventional way using a 0.5% solution of each dye in 7.5% acetic acid.)

absorption spectrums of bands from the same protein class stained with different dyes were noted. The preponderance of the absorption at wavelengths below 520 nm. was lowest for the three protein classes with Amido Schwarz 10 B and highest with Buffalo black NBR.

Absorption spectrums of albumin, globulin, and gliadin bands stained with a solution of Amido Schwarz 10 B, aged in storage at room temperature for 6 months or by treatment at 72°C. with bubbling oxygen for 96 hr., were almost identical to that obtained with aniline blue-black.

B) Reversible and Irreversible Changes Induced in the Absorption Spectrum of Aniline Blue-Black by the Staining Process

When a stained gel is subjected to an alkaline treatment by immersion in a 1N

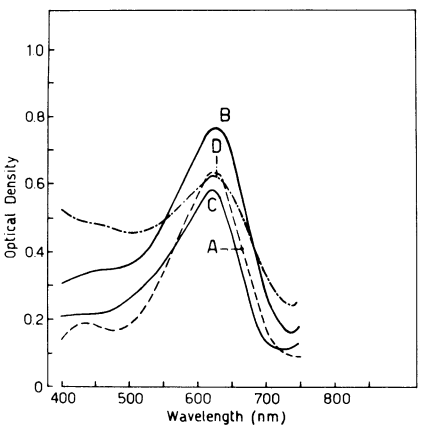
NaOH solution, the subsequent pH increase causes dissociation of dye from protein. With sufficient time (48 hr.), the dye diffuses out of the gel and complete elution of dye from protein can be obtained. With a shorter alkaline treatment (90 min.), only that part of the dye less tightly bound to proteins is removed.

In Fig. 6, the absorption spectrums of dyes eluted from albumin, globulin, and gliadin bands by treating gels with 1N NaOH for 48 hr. are compared with the absorption spectrum of a 0.0016% aniline blue-black solution in 1N NaOH. Dyes eluted from albumin (B) and globulin (C) bands have identical absorption spectrums differing from that of aniline blue-black (A) in higher relative absorption at wavelengths below 520 nm. The dye from the gliadin bands (D) exhibits similar, but more pronounced, differences from aniline blue-black.

These data show that dyes bound to protein bands differ greatly from aniline blue-black and suggest that the aniline blue-black composition has been changed by the staining process. This implies a heterogeneity of aniline blue-black and particularly the presence in this dye of at least one red or yellow impurity with higher affinity for the three protein classes.

We confirmed the presence in the dye of two components with different affinities for the proteins by performing spectrophotometric determinations directly on stained gels which had been subjected to a short alkaline treatment (90 min.). The absorption spectrums of the residual dye components were all very similar to one another regardless of the type of flour protein, and all had a gliadin-like shape.

Comparing the absorption spectrum of globulin bands (Fig. 1, curve C) with the spectrum of the corresponding dye eluted from the gel (Fig. 6, curve C), it is evident that staining of globulins induces some reversible alterations in the aniline



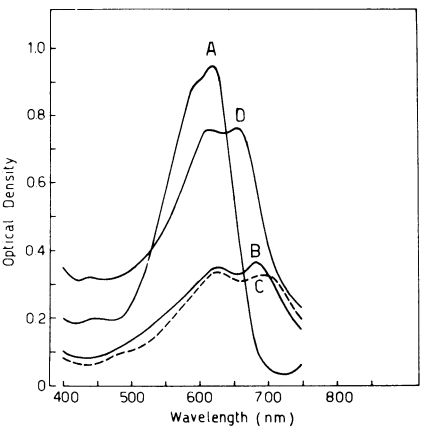


Fig. 6 (left). Absorption spectrums of aniline blue-black in 1N NaOH (A) and of dyes eluted by means of alkaline treatment from albumin (B), globulin (C), and gliadin (D) bands in polyacrylamide gel.

Fig. 7 (right). Aniline blue-black metachromatism induced by salts and strong acids. (A) aniline blue-black; (B) solution A made 5M in HCl; (C) solution A made 5M in NaCl; (D) polyacrylamide gel globulin bands.

blue-black spectrum. These reversible changes could be attributed either to electronic interactions among the molecules of the dye bound to the macromolecule (14) or to interactions between dye molecules and side-chain groups available on the protein molecule (15). As spectral changes due to the reciprocal influence of dye molecules can also be induced by adding small molecules able to compete with the dye for water molecules, we could discriminate between these two possibilities. As seen in Fig. 7, by adding 5M NaCl (B) or 5M HCl (C) to a 0.0016% aniline blue-black solution (A) in a glycine-HCl buffer (pH 3), the absorption spectrum of the dye became similar to that of globulin bands (D) stained with aniline blue-black. No significant differences in the character of the metachromatic effect were observed when we substituted (at about 5M concentration) KCl, $NaHSO_4$, $(NH_4)_2SO_4$, or $BaCl_2$ for NaCl and H_2SO_4 or P_2O_5 for HCl.

C) Heterogeneity of Aniline Blue-Black

When aniline blue-black was subjected to silica gel thin-layer chromatography, we found a main blue spot with R_f 0.22 and some faster spots (R_f 0.34, 0.57, 0.59) that were also blue. In addition, two red fractions were also present; one red spot with R_f 0.11 and another red substance traveling slightly slower than the blue main fraction (R_f 0.17).

A purification of aniline blue-black was attempted by salting a 0.5% solution out of the dye with 2M NaCl. The blue precipitate was separated from the red supernatant by centrifugation and then dissolved in water and passed through a Sephadex G-10 column. The purification treatment largely removed the red components from the aniline blue-black.

Absorption spectrums of albumin, globulin, and gliadin bands stained with partially purified aniline blue-black were similar to that obtained with Amido Schwarz 10 B.

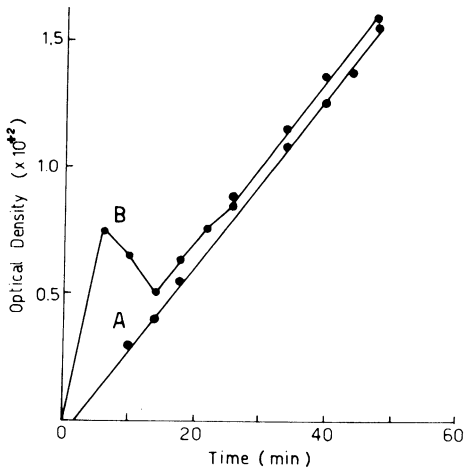


Fig. 8. Precipitation behavior in polyacrylamide gel of albumins (A) and gliadins (B) by aniline blue-black.

D) Precipitation Behaviors in Polyacrylamide Gel of Albumins, Globulins, and Gliadins during the Staining Process

Differences in precipitation behaviors of albumins, globulins, and gliadins in polyacrylamide gel were studied by immersing the gels, after electrophoresis, in a 0.004% solution of aniline blue-black and measuring the precipitation of protein bands by densitometry. Typical precipitation behaviors representative of the rate at which the staining solution penetrated the gel and interacted with albumins (A) and gliadins (B) to form a protein-dye coprecipitate are shown in Fig. 8.

Albumins exhibited a linear slope, after a short latency period, while precipitation of gliadins started immediately after immersion of the gel in the staining solution and reached its maximum after about 5 min. Successively, the gliadin precipitate partly redissolved and a subsequent precipitation was observed similar to that of the albumins. Since gliadins precipitated as white bands at first and bound the dye only later, it was evident that aniline blue-black was not responsible for the immediate precipitation of gliadin bands. This precipitation took place also in the absence of aniline blue-black by immersion of the gel in a 7.5% aqueous acetic acid solution, while the linear albumin-like precipitation was not observed. Under these staining conditions, at the times reported, globulin bands did not precipitate at all, showing a higher solubility in the presence of such mild precipitating agents.

These data show that gliadin precipitates most rapidly during the staining process. A similar conclusion could be inferred from the previously reported finding of a large unstained zone in the inner region of the gliadin bands.

DISCUSSION

The data show that the protein-staining process causes alterations in the aniline blue-black absorption spectrum. Certain of these alterations can be reversed by dissociation of the protein-dye complexes (reversible), and others are not altered by removal of the dye from protein bands (irreversible).

Chromatic differences between albumin and gliadin bands are related to irreversible alterations, whereas differences between albumin and globulin bands are mainly due to reversible modifications of the aniline blue-black absorption spectrum.

The absorption spectrum of the globulin bands has two maximums at 630 and 680 nm. that are not affected by the amount of protein loaded on the gel or by purity and concentration of aniline blue-black. The spectrum is not affected by the polyacrylamide gel matrix (Figs. 1, 2), methanol in the aniline blue-black solution, or the destaining technique. Globulin-like alterations induced in the aniline blue-black spectrum by means of dehydrating agents such as salts and strong acids (Fig. 7) suggest that the globulin-induced aniline blue-black metachromatism is mainly due to interactions among dye molecules forced to suitable distances after binding to the protein chain.

Irreversible alterations of the aniline blue-black spectrum caused by the staining of albumins, globulins, and gliadins consist mainly of an absorption increase in the yellow and red regions below 520 nm. of the spectrum compared to that at higher wavelengths. We suggest that these alterations are due to the presence in the dye of differently colored impurities with different affinities for proteins.

We have shown (Fig. 6) that there are at least two dyes bound to albumin,

globulin, and gliadin bands and that the dye responsible for the gliadin-like absorption spectrum is more tightly bound to proteins. Also, the influence of the destaining technique on the absorption spectrums of protein bands suggests the presence of more than one dye.

The heterogeneity of aniline blue-black has been shown by thin-layer silica gel chromatography, and the removal of red impurities affected the absorption spectrums of the protein bands.

Different amounts of impurities are likely responsible for the differences observed among the absorption spectrums of albumin, globulin, and gliadin bands stained with different commercial products of acid black I. As shown by results obtained with a solution of Amido Schwarz 10 B aged by storage or oxidation, the impurities responsible for the differences observed, using different commercial products of acid black I, are probably produced by degradation of the main blue component.

By staining protein bands with a very dilute aniline blue-black solution (Fig. 3, curve B), we found that chromatic differences between albumin and gliadin bands are reduced. We suggest that this is due to insufficient amounts of characteristic red impurities available for binding to proteins under these experimental conditions.

Although chromatic differences between albumin and gliadin bands evidently are mainly due to the presence in gliadin bands of more aniline blue-black impurities that absorb at wavelengths below 520 nm., this is not conclusive evidence for a higher affinity of these impurities for gliadins as opposed to albumins. These chromatic differences may be due rather to a lesser tendency of the gliadins to coprecipitate with the major component of the aniline blue-black during the staining process. Experimental conditions able to increase the coprecipitation of the dye during protein precipitation by aniline blue-black can also minimize differences between the absorption spectrums of albumin and gliadin bands. This has been shown by increasing protein load on the gel (Fig. 3, curve A) or by staining the proteins in a gel-free system (compare Figs. 1 and 2) where occlusion phenomenons are not dependent on the diffusion of dye into protein bands.

Although no direct determinations of the ratio of the amount of absorbed dye to amount of proteins in albumin and gliadin bands have been performed, it seems reasonable to assume that the amount of dye absorbed is directly related to the diffusion of the dye into the inner region of protein bands. As shown by the large unstained zone observed in the central region of the gliadin bands, the diffusion of the dye into gliadin bands is less efficient than diffusion into albumin bands. This is a consequence of the lower solubility of gliadins under our experimental conditions (Fig. 8) and probably also because reaction with the dye takes place after gliadins have been precipitated. When 45% methanol is added to the staining solution, gliadin solubility increases as well as diffusion of dye into gel, and the spectrums of gliadin bands become similar to that of the albumin bands.

In conclusion, we suggest that the chromatic differences between albumin and globulin bands are mainly due to metachromatic phenomenons induced in the aniline blue-black spectrum by globulin moiety. Aniline blue-black heterogeneity

and differences in protein solubilities must be taken into account to explain the chromatic differences between albumin and gliadin bands.

Acknowledgments

This work was partly supported by the Consiglio Nazionale delle Richerche, Rome.

We would like to thank D. D. Kasarda for his stimulating advice and F. Malatesta for his skilled technical assistance.

Literature Cited

1. SILANO, V., DE CILLIS, U., and POCCHIARI, F. Varietal differences in albumin and globulin fractions of *Triticum aestivum* and of *Triticum durum*. J. Sci. Food Agr. 20: 260 (1969).
2. MINETTI, M., PETRUCCI, T., POCCHIARI, F., SILANO, V., and AVELLA, R. Varietal differences in water-soluble gliadin fractions of *Triticum aestivum* and of *Triticum durum* seeds. J. Sci. Food Agr. 22: 72 (1971).
3. SILANO, V., and POCCHIARI, F. A specific color reaction of albumin, globulin, and gliadin preparations from wheat. Cereal Chem. 48: 445 (1971).
4. TRAUT, R. R. Acrylamide gel electrophoresis of radioactive ribosomal proteins. J. Mol. Biol. 21: 571 (1966).
5. GESTELAND, R. F., and STAEHELIN, T. Electrophoresis analysis of proteins from normal and cesium chloride-treated *Escherichia coli* ribosomes. J. Mol. Biol. 25: 149 (1967).
6. GUALERZI, C., and CAMMARANO, D. Species specificity of ribosomal proteins from chloroplast and cytoplasmic ribosomes of higher plants; electrophoretic studies. Biochim. Biophys. Acta 199: 203 (1970).
7. UDY, D. C. Dye-binding capacities of wheat flour protein fractions. Cereal Chem. 31: 389 (1954).
8. UDY, D. C. Estimation of protein in wheat and flour by ion-binding. Cereal Chem. 33: 190 (1956).
9. MOSSBERG, R. Some problems concerning the determination of crude-protein content in cereals by dye-binding. Agr. Hort. Gen. 23: 206 (1965).
10. LAWRENCE, J. M., LIU, S. C., and GRANT, D. R. Dye-binding capacity and amino acid content of wheat-protein gel electrophoresis bands. Cereal Chem. 47: 110 (1970).
11. BERNARDIN, J. E., KASARDA, D. D., and MECHAM, D. K. Preparation and characterization of α-gliadin. J. Biol. Chem. 242: 445 (1967).
12. WADDELL, W. J. A simple ultraviolet spectrophotometric method for the determination of proteins. J. Lab. Chim. Med. 48: 311 (1956).
13. SILANO, V., DE CILLIS, U., and POCCHIARI, F. Composizioni elettroforetiche di estratti proteici di *Triticum aestivum* e *Triticum durum*. Ann. Ist. Super. Sanita 3: 559 (1967).
14. KLOTZ, I. M. The effects of salts and proteins on the spectra of some dyes and indicators. Chem. Rev. 41: 363 (1947).
15. SCHUBERT, M., and HAMERMAN, D. Metachromasia; chemical theory and histochemical use. J. Histochem. Cytochem. 4: 159 (1956).

[Received April 24, 1972. Accepted October 5, 1972]