DISC ELECTROPHORESIS AS A METHOD OF FRACTIONATION AND QUANTITATIVE DETERMINATION OF HISTONES

by

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CONTENTS

ABSTRACT

Disc electrophoresis in polyacrylamide gels has been found to be a superior method for the analytical fractionation of histones. The details of the procedure developed for disc electrophoresis are discussed. The use of disc electrophoresis for the quantitative determination of histone subfractions has been found to be feasible. The binding of amidoschwartz stain is a linear function of protein concentration for the three major histone fractions and is independent of the fraction used; measurement of stain in the gels has been by densitometry using a Canalco Model E microdensitometer.

INTRODUCTION

Histones are basic proteins which occur complexed with the DNA (deoxyribonucleic acid) of a wide variety of plants and animals. Although they have been the subject of much discussion and investigation, there is yet no general agreement as to their function(s). Because histones are associated with the genetic material of higher organisms, the suggestion has been made that histones act as genetic regulators (2,3). Such a role for histones offers a means by which differentiation may be effected during the development of higher organisms.

Various studies of histones have shown that they are heterogeneous. If histones are fractionated by column chromatography on Amberlite CG-50 using an elution gradient of guanidinium chloride, they are separated into three major fractions (4,5). These fractions have been referred to as histones I, II, and III-IV, in order of their elution from the column. The assymmetry of the peaks of the chromatograph indicates that these fractions themselves are heterogeneous. Indeed it can be shown by other means, notably electrophoresis, that these fractions are composed of subfractions.

It should be mentioned that there is some variation in histone nomenclature, and no system of nomenclature which is entirely satisfactory has yet been adopted (6). The designations I, II, and III-IV, have been used by Rasmussen, Murray, and Luck (4). The histone subfractions are designated by small case letter subscripts (i.e. histone Ia, etc.). Such nomenclature has been used in our laboratories and will be used in this paper.

Because of this heterogeneity of histones, it was desired to seek an accurate and reproducible method of fractionation of histone subfractions. For this purpose, gel electrophoresis offered greatest promise. Gel electrophoresis differs from electrophoresis in free solution and on other supporting

media in that the molecular structure of the gel offers resistance to the movement of sample through it. Such resistance is referred to as molecular sieving. Because of the molecular sieving effect, the electrophoretic mobility of a protein through the gel is dependent not only upon its charge, but also upon its molecular size and structure.

The method of disc electrophoresis in polyacrylamide gel, which was introduced by Ornstein and Davis (7), was chosen for investigation rather than some other method of gel electrophoresis for several reasons. First, polyacrylamide is a synthetic polymer which is cross-linked by methylene bridges. The amount of polymer and the degree of cross-linking can be varied, thus a range of pore sizes for the gels is possible. This allows the variation of the degree of molecular sieving. Second, extremely fine resolution is possible by disc electrophoresis due to the initial concentrating of the protein sample into a thin band. The theory of this effect is extensively discussed by Ornstein (7). The essential feature is that the protein sample, which is applied over the gel, has relatively large mobility in the medium in which it is initially placed. When current is applied, the proteins move rapidly until they reach the gel. The resistance of the gel abruptly retards the proteins so that the sample becomes stacked into a very concentrated band prior to entering the gel. It can be seen that the protein stacking effect has the added advantage of making disc electrophoresis relatively independent of the initial concentration of the sample. Third, the method of disc electrophoresis is very sensitive to small amounts of protein; amounts on the order of micrograms are detectable by standard procedures. Fourth, polyacrylamide gels are transparent to visible light and very suitable for densitometry.

The present method for the disc electrophoresis of histones is a

modification of the procedure of Reisfeld, Lewis, and Williams (8) for the disc electrophoresis of basic proteins. The first section of this paper will describe and discuss the details of the method which has been developed.

In addition to the fractionation of histones, it was desired to use the method of disc electrophoresis for the quantitative determination of the histone subfractions. It is known that amidoschwartz dye is quantitatively bound by proteins, although different proteins do not necessarily have the same staining constant (amount of dye bound/unit weight of protein)(9). Thus, for the quantitative determination of the histone subfractions by disc electrophoresis, it becomes necessary to evaluate the characteristic staining constants for these subfractions. The quantification of histone subfractions will be discussed in the second part of this paper.

DISC ELECTROPHORESIS

MATERIALS AND METHODS

Reagents

Acrylamide (Eastman Organic Chemical #5521); NN' methylenebisacrylamide (bis)(Eastman #8383); NNN'N' tetramethylethylenediamine (TEMED)(Eastman #8178); ammonium persulfate (Mallincrodt Chemical Works #3460); β-alanine (Eastman #4638); amidoschwartz 10B (Schmid & Co. #10080).

Solutions

TEMED solution: 48 ml N KOH, 17.2 ml glacial acetic acid, 4 ml TEMED, deionized water to 100 ml.

Monomer solution: 60 g acrylamide, 0.4 g bis, deionized water to 100 ml.

Persulfate solution: 0.2% (w/v) ammonium persulfate, 10 M urea in deionized water.

Tray buffer: 31.2 g β-alanine, 8 ml glacial acetic acid, deionized water to 1 liter (pH = 4.5).

Destaining solution: 40% (v/v) ethanol, 7% (v/v) acetic acid in distilled water.

Staining solution: 1% (w/v) amidoschwartz 10B in destaining solution.

Note: The TEMED solution and the monomer solution are stored in amber glass bottles and kept refrigerated a 0 - 5 degrees C. They are stable over a period of several months. The persulfate solution is prepared fresh before use. The other solutions may be kept at room temperature over a period of several months with no noticeable deterioration.

Procedure

Electrophoresis gels are prepared by adding 1 volume of TEMED solution and 2 volumes of monomer solution to 5 volumes of persulfate solution. The mixture is thoroughly agitated using a long-tip pipette equipped with a

rubber bulb. Nine-tenths ml of the mixture is then pipetted into each electrophoresis tube. This is then carefully overlayered with 0.1 ml of 3 M urea to allow the anaerobic polymerization of the acrylamide. Polymerization time is about 30 minutes.

Electrophoresis tubes are made of 6.5 cm lengths of 0.5 cm (I.D.) pyrex tubing. Prior to use they are thoroughly cleaned using any water soluble detergent or, if necessary, with acid-dichromate cleaning solution. They are then rinsed with copious amounts of water, followed by a single rinsing with acetone to facilitate their drying. The tubes are air dried.

Racks to stopper and support the electrophoresis tubes during polymerization of gel are made from one-eighth inch thick rubber sheeting cemented over a Plexiglas plate. The tubes are snugly held upright by holes in the rubber sheet.

After polymerization of the gels, the 3 M urea layer is removed using a long-tip pipette. Histone samples, usually dissolved in 10 M urea, are placed directly on top of the gels. Tray buffer is gently layered over the protein solution to the top of the electrophoresis tube. The tubes are then removed from the rack and placed in the holes of the upper tray of the electrophoresis apparatus. Tray buffer is placed in both the upper and lower compartments of the apparatus, and electrophoresis is carried out at 4 - 5 ma/tube (cathode down) for 1 - 2 hours using a regulated current power supply.

The electrophoresis apparatus is similar to that described by Ornstein and Davis (7) for disc electrophoresis. It consists of two cylindrical Plexiglas trays 10 cm in diameter and 8 cm deep. The upper tray is fitted with a lid and has eight holes in the bottom. The holes have rubber grommets which snugly hold the electrophoresis tubes. Platinum wire electrodes are

connected to both upper and lower compartments of the apparatus.

After electrophoresis, the gels are removed from the tubes by gentle rimming with water using a syringe with a blunted 22 gauge hypodermic needle. The gels are immmdiately placed in ca. 12 ml of staining solution, and the fixation and the staining of the protein bands are allowed to proceed for at least 4 hours.

After staining, the unbound dye is removed from the gels electrophoretically. Destaining tubes are made from 7 cm lengths of 0.6 cm (I.D.) glass tubing which are slightly tapered at one end. These tubes are plugged at the tapered end with 0.2 - 0.3 ml of polyacrylamide gel prior to use. The plugged tubes are filled with destaining solution, and the gels to be destained are inserted into them. Filling the tubes with solution prior to inserting the gels prevents the formation of air pockets which reduce the efficiency of destaining. The tubes are then placed in the electrophoresis apparatus, and the upper and lower trays of the apparatus are filled with destaining solution. From 2 to 5 ma/tube of current are applied (anode down) until the unbound dye migrates out of the gels. Destaining time is about 2 hours.

Any background color remaining in the gels after electrical destaining is leached out by storing the gels in destaining solution. The gels are then photographed and/or scanned using a Canalco Model E microdensitometer (Canal Industrial Corp., Rockville, Md.).

The procedure described above is for disc electrophoresis in gels containing 15% acrylamide and 6 M urea. The procedure for 7.5% acrylamide gels is identical except that the monomer solution is made of 30 g acrylamide,

0.8 g bis, and deionized water to 100 ml. Non-urea gels are made using persulfate solution without urea. In the case of non-urea gels, polymerization is carried out for 2 hours under a layer of water rather than 3 M urea.

RESULTS

The results of fractionation of whole pea bud histones by disc electrophoresis under three different gel conditions are shown by the photograph in figure 1. A typical microdensitometer scan of the fractionation of whole pea bud histone in 15% acrylamide gel containing 6 M urea is shown in figure 3,A. The electrophoretic pattern is typical of all preparations of whole pea histones and is completely reproducible in different electrophoresis runs.

Figure 3,B,C,D, show microdensitometer scans of the fractionation by disc electrophoresis of histones which had been previously fractionated by column chromatography on Amberlite CG-50. Chromatographically fractionated histone samples were provided by Douglas Fambrough; a chromatograph of such fractionation is shown in figure 2. Identification of the various bands observed in the disc electrophoresis of whole histones in terms of chromatographic fractions is straightforeward as can be seen in figure 3.

DISCUSSION

15% acrylamide gels containing 6 M urea are most useful for the disc electrophoresis of histones. The presence of urea in the gels enhances band resolution of histone subfractions compared to non-urea gels. In addition, urea gels have the advantage of shorter polymerization time. The pattern of fractionation of histones on urea and non-urea gels is quite

similar.

There is no difference in the banding pattern of histones run in 15% and 7.5% acrylamide gels. 7.5% acrylamide gels give greater band separation for runs of comparable times, however, they also show greater band diffusion. Furthermore, the lower concentration of acrylamide produces more fragile gels which require greater care in handling to avoid breakage. Because of their larger pore size, 7.5% gels undergo a noticeable amount of shrinkage and expansion during storage. This introduces variability in microdensitometer measurements of these gels, which is undesireable for quantitative determinations.

The use of sample and spacer gels as described by Ornstein (7) and as used by Reisfeld, Lewis, and Williams (8), in their procedure for disc electrophoresis has been found to be unnecessary. This has also been shown to be true by McAllister, Wan, and Irvin (10), who eliminated the use of sample and spacer gels for their disc electrophoresis procedure. The excellent resolution which has been obtained in the absence of a spacer gel supports the contention made by Hjerten, Gerstedt, and Tiselius (11) that protein concentration and stacking can occur in disc electrophoresis without the use of a multi-gel system.

There is reasonable latitude in the solvent for histone samples to be fractionated by disc electrophoresis. Solvents of high ionic strength, such as 8% guanidinium chloride, are unsuitable as they greatly reduce histone mobility and resolution. Ten M urea, 0.2 N HCl, and 0.2 N H_2SO_4 have been most useful. Ten M urea has generally been used because of its solvating strength for histones and because of its high density which facilitates the layering of tray buffer over it. The use of acid solvents has an advantage

in cases of histone samples with excessive non-histone protein contamination. A sample with such contamination is generally not completely soluble in 0.2 N acid, whereas it is in 10 M urea. The histone I and histone II fractions seem to be preferentially dissolved while histone III-IV and non-histone contaminants remain largely insoluble in acid solvents. The presence of a high degree of contamination by non-histone proteins could produce numerous bands upon electrophoresis, which could obscure the histone bands if 10 M urea is used as the solvent. Acid solvents alleviate much of this difficulty, albeit, at the expense of losing most of the histone III-IV fraction. When acid solvents are used, their densities are increased by making them 20% in sucrose to allow the layering of tray buffer over them.

A single band of histone containing as little as 0.1 microgram of protein may be detected by disc electrophoresis using amidoschwartz staining. The ideal amount of whole pea bud histone for electrophoresis by this method is 20 micrograms. Overloading occurs with over 50 micrograms of whole histone. As would be expected, the volume of the sample solution which is placed over the gel is not critical. Band resolution is not noticeably affected for volumes as small as 1 microliter or as large as 150 microliters.

Currents in excess of 6 ma/tube produce excessive ohmic heating within the gels during electrophoresis, which in turn leads to band curving and subsequent loss of resolution. Lower currents naturally require longer times for electrophoresis runs to give acceptible band separation. Band diffusion becomes significant for runs of longer than 3 hours. In this system a run of 1.5 hours at 4 ma/tube produces optimum results for pea histones.

Ethanol is used in the staining and destaining solutions as a dehydrating agent to prevent gel expansion due to absorption of water. Methanol

may be used in place of ethanol for the same purpose. The presence of the alcohol does not affect the staining and the fixing of the proteins. At least 4 hours of staining time are required, otherwise unfixed portions of protein bands may migrate during electrical destaining. Excessive current should be avoided in destaining gels. High current causes not only extensive ohmic heating, but also some removal of bound dye from the protein bands.

Gels may be stored in destaining solution in tightly capped glass vials for extended periods of time with no noticeable deterioration of either the gel or the stain. No change of gels has been noticed over a period of 6 months. It is likely that gels may be stored for much longer periods with negligible deterioration.

QUANTIFICATION

METHODS

Lyophilized histone fractions (prepared by Douglas Fambrough by column chromatography on Amberlite CG-50) were vacuum dried over phosphorus pentoxide at least 12 hours. They were then quickly weighed on a microbalance and dissolved in 10 M urea at a concentration of 1 mg/ml. The concentrations of these solutions were further checked by performing protein determinations by the method of Lowry, et al., (12). Carefully measured amounts of histone solutions were placed over 15% acrylamide gels containing 6 M urea, and electrophoresis was performed for 1.5 hours at 4 ma/tube.

The gels were stained in 1% amidoschwartz overnight and then destained electrophoretically at 2 - 3 ma/tube. The gels were left overnight in destaining solution to remove the last traces of background color. After complete removal of background dye, the gels were scanned several times in a Canalco Model E microdensitometer, each time rotating the gels in the microdensitometer sample holder. Densitometer tracings were recorded at constant chart speed and calibrated absorbance using the standard microdensitometer tungsten light source and a Wratten 38-A gelatin filter.

Areas under the peaks of the microdensitometer tracings were measured by either using a planimeter or by transferring the scans onto tracing paper of uniform weight, cutting out the areas, and weighing.

RESULTS

Microdensitometer scans of the electrophoresis of the three histone fractions which were quantified are shown in figure 3,B,C,D. Of the three fractions only one (histone IIa) was electrophoretically homogeneous. It was found that the staining constant for histone IIa remained constant for

amounts up to ca. 10 micrograms protein/gel. The linear relationship between staining and amount of protein was not valid for amounts of histone IIa greater than this value. Reproducibility of staining as measured by microdensitometer scan areas was quite good; the standard deviation for the scan areas of five different gels containing 4.5 micrograms of histone IIa was 4.3%.

Quantification of histones I and III-IV on disc electrophoresis was somewhat hindered by the inability to prepare workable quantities of these histones which were electrophoretically homogeneous. The preparation of histone I used for quantification contained two bands corresponding to histones Ia and Ib and a third band due to contamination by histone IIa. The amount of this contamination was determinable since histone IIa had already been quantified. The amount of histone IIa was determined to be 17.4%. It was found that the amount of amidoschwartz stain bound by the two bands of histone I was a linear function of the amount of protein for amounts up to ca. 5 micrograms protein/gel. The composite staining constant for histone I was virtually identical to that for histone IIa within the region where the linear staining relationship was observed.

The sample of histone III-IV used for quantification contained the two electrophoretic bands of these arginine rich histones and 5% contamination due to histone II. It was found that the composite staining constant for the two bands of histone III-IV was identical to that for histone IIa. The linear relationship between staining and amount of protein was observed for histone III-IV for concentrations of protein less than ca. 10 micrograms protein/gel.

The relationship between the intensity of staining with amidoschwartz dye and the amount of protein for these three histone fractions is represented

in figures 4 and 5.

DISCUSSION

It appears from these studies that the binding of amidoschwartz dye to histones is independent of the histone fraction. Furthermore, the amount of dye bound is linearly related to the amount of protein within certain concentration limits. The deviation from linearity of staining is not detrimental to the quantitative determination of the various histone subfractions under normal conditions used for disc electrophoresis. Unfractionated pea histones contain from 6% to 18% histone I and from 35% to 55% histone IIa. Since the ideal amount of whole histone for disc electrophoresis is 20 micrograms, the amount of each histone subfraction within a single band in the gel would be within the quantitative staining range.

Since the completion of this work, preparative fractionation of histones by disc electrophoresis has become possible with the advent of a preparative apparatus manufactured by the Canal Industrial Corporation. This method of histone fractionation has been used by Douglas Fambrough (personal communication) to confirm the identity of staining constants for the various histone subfractions. Direct measurements, by precipitation of protein with trichloroacetic acid (1,4), of the amounts of the various histone subfractions eluted from the preparative gel column for several different histone preparations have been in complete agreement with that determined from microdensitometer scan areas of gels of these preparations run on analytical disc electrophoresis and stained with amidoschwartz.

In addition, Hnilica, Edwards, and Hey (13) have recently shown that the staining constants for subfractions of calf thymus histones are identical when stained with amidoschwartz after electrophoresis on starch gels.

Thus, the available data are completely in agreement with the results of these present experiments.

The reason for the deviation from linearity of staining with respect to amount of histone I at lower amounts compared to the other histone fractions is not known. A possibility is that the affinity of binding between amidoschwartz dye and histone I is weaker than the binding affinities between the dye and the other histone fractions. It may also be possible, since histone I subfractions seem to migrate as finer bands in the gel during electrophoresis, that these subfractions become more concentrated within the gel so that concentration effects upon staining manifest themselves at lower amounts of protein compared to the other histone fractions. Nevertheless, this behavior of histone I in its staining does not affect the practicality of disc electrophoresis for use in quantitative analyses of histone subfractions.

CONCLUSIONS

Disc electrophoresis provides a simple method for the characterization of histones. Its absolute reproducibility of fractionation allows the unequivocal identification of the various histone subfractions. Its high sensitivity to small amounts of protein makes possible the analysis of microgram quantities of histone samples. This sensitivity is also valuable for the determination of the degree of purity of histone preparations with high accuracy; such determinations would be desireable prior to the performance of certain biological and chemical analyses of these preparations. Finally, the method is applicable for the quantitative determination of histone subfractions.

These properties of disc electrophoresis make it an attractive method for various studies of histones. Its simplicity makes its routine use for such purposes as histone identification or as checks of histone purity quite feasible. Furthermore, it should prove useful in such investigations as the comparative study of histones of intra- and interspecies origins and the quantitative evaluation of histone composition during various stages of organismic growth and development. Such investigations, in turn, may be useful in elucidating the biological function(s) of histones.

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Figure 1: Fractionation of whole pea bud histones by disc electrophoresis under three different gel conditions.

Left: 7.5% acrylamide gel containing 6 M urea.

Center: 15% acrylamide gel containing 6 M urea.

Right: 15% acrylamide gel without urea.

All gels run at 4 ma/tube for 1.5 hours. Microdensitometer scan of center gel is shown in figure 3,A.

FIGURE 1

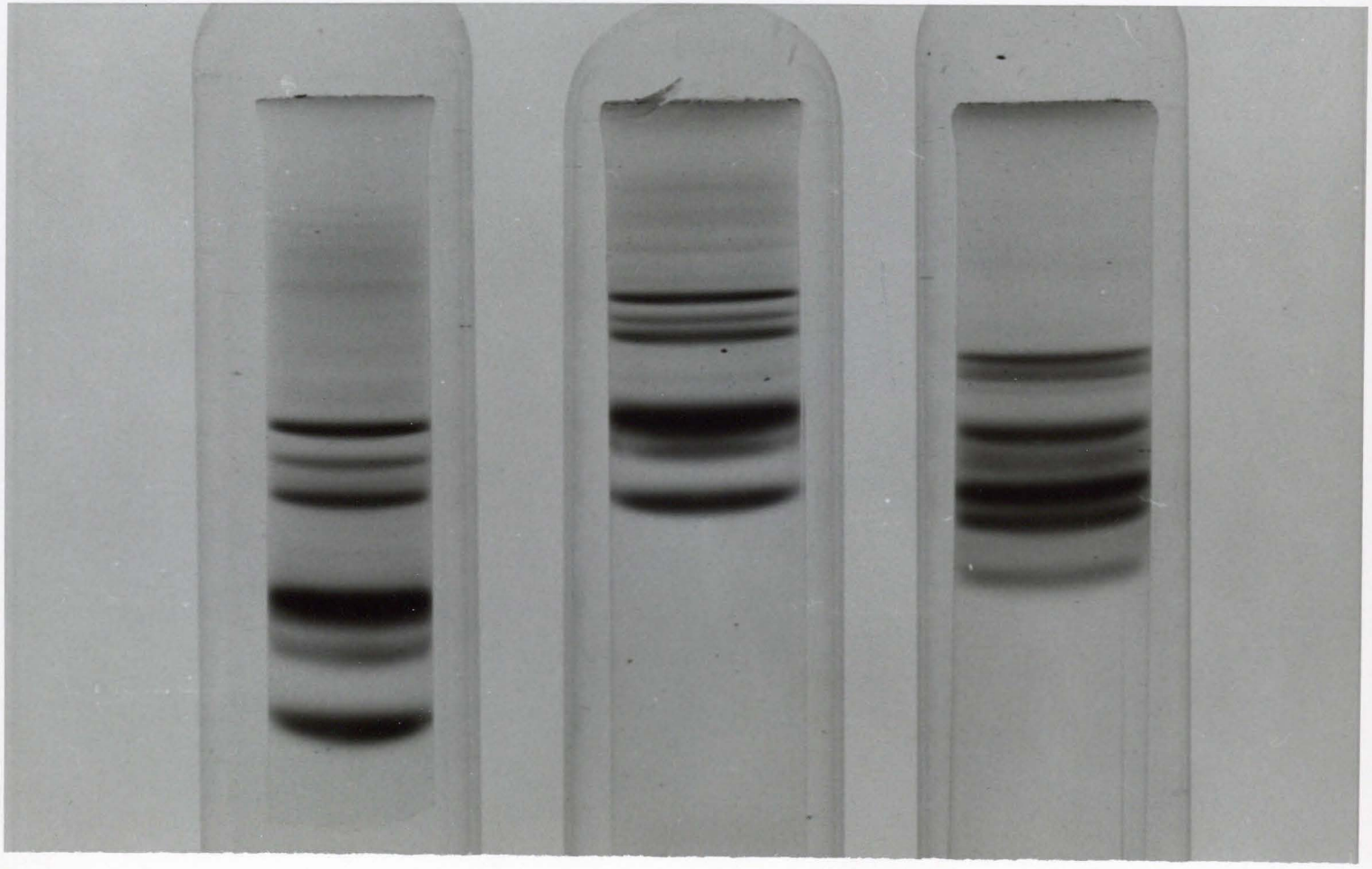


Figure 2

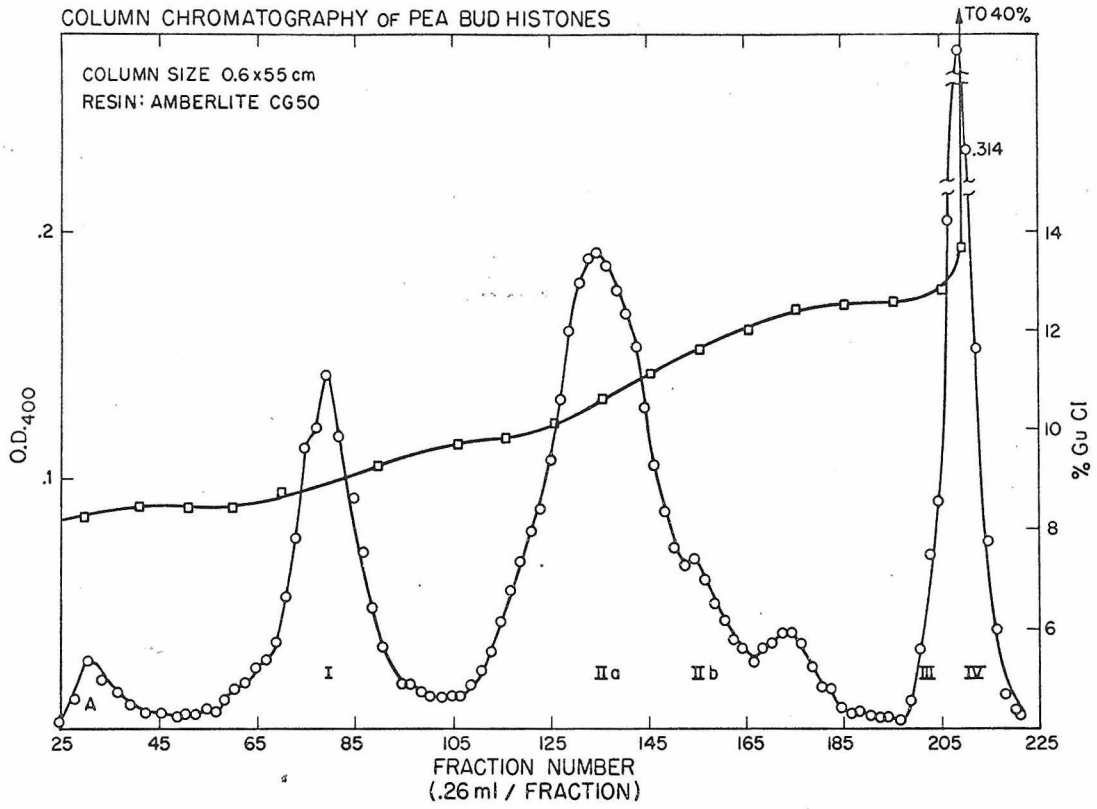


Figure 2: Fractionation of whole pea bud histones by column chromatography on Amberlite CG-50 using elution gradient of guanidinium chloride (GuCl).

o : Optical Density at 400 mu of fractions precipitated with trichloroacetic acid.

□ : Concentration of GuCl measured by refractive indices of fractions.

Data from Douglas Fambrough.

Figure 3

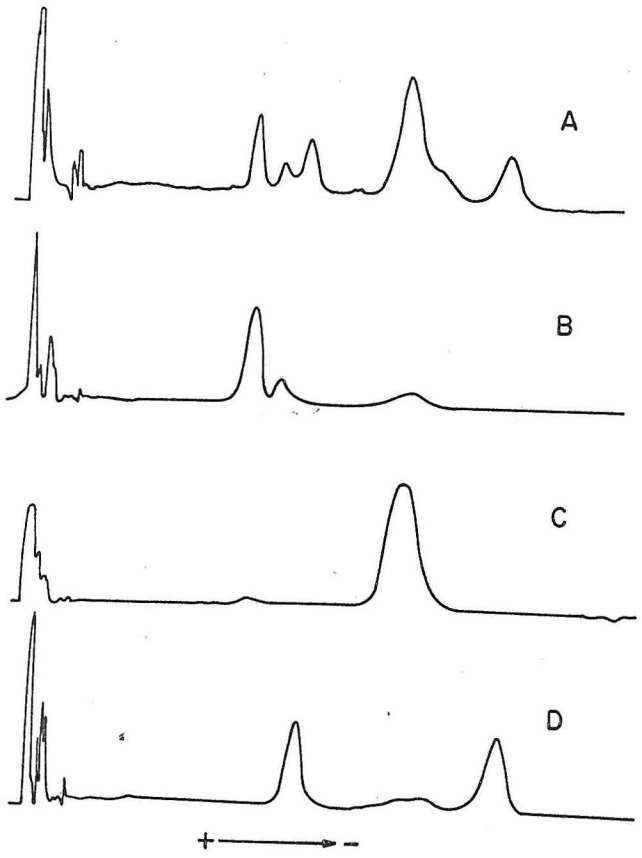


Figure 3: Microddensitometer scans of polyacrylamide gels after disc electrophoresis of whole pea bud histones and pea bud histone fractions. All gels contained 15% acrylamide and 6 M urea. Scan A shows fractionation of whole pea bud histone (see figure 1). Scans B - D show, respectively, fractionation by disc electrophoresis of histones I, IIa, and III-IV, all prepared by column chromatography. Peaks at far left indicate origin of gel, not stained material.

Figure 4: Quantification of histone fractions on disc electrophoresis. Intensity of staining with amidoschwartz is plotted with respect to the amount of protein from 0 - 6 micrograms/gel. Intensity of staining measured in terms of the areas under the peaks of the microdensitometer scans. Scan areas are in arbitrary units.

⊹ : Histone I.

o : Histone IIa.

△ : Histone III-IV.

The solutions of histones I and IIa, which were placed on the gels for electrophoresis, were at the initial concentration of 0.1 mg/ml. Solution of histone III-IV was at initial concentration of 1 mg/ml.

FIGURE 4

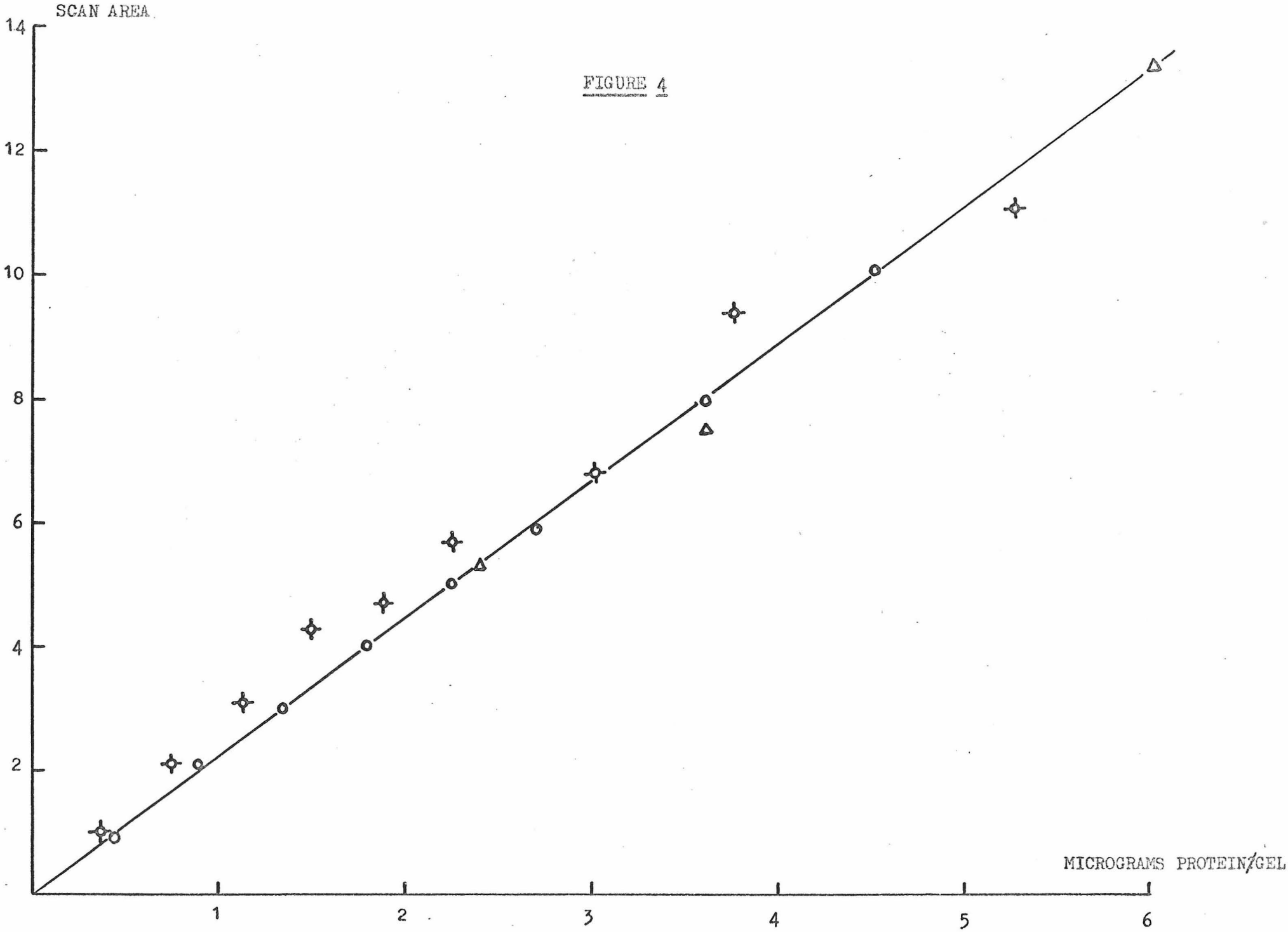


Figure 5: Quantification of histone fractions by disc electrophoresis. Intensity of staining with amidoschwartz is plotted with respect to the amount of protein from 0 - 12 micrograms/gel. Intensity of staining measured in terms of microdensitometer scan areas. Scan areas are in arbitrary units.

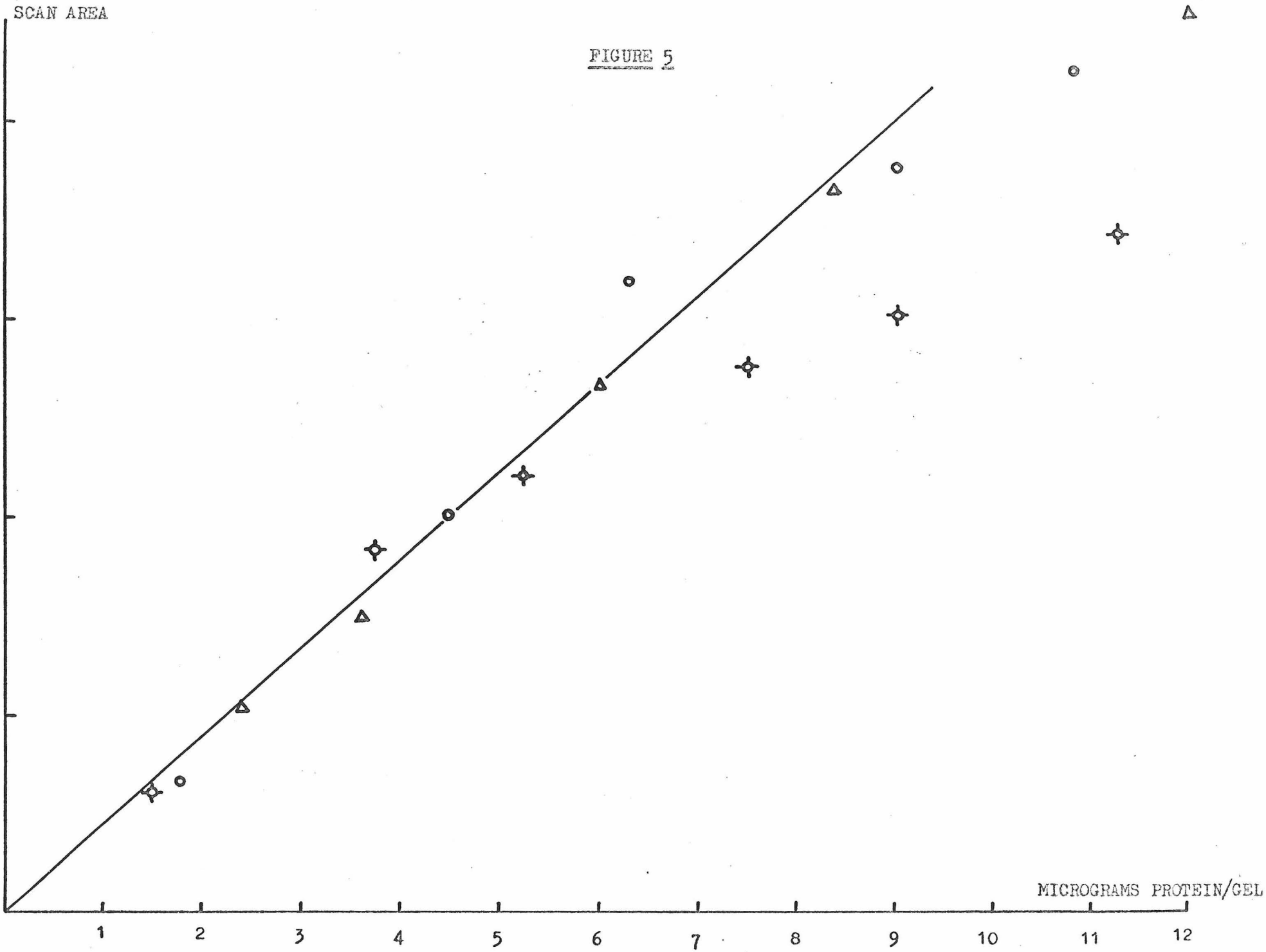
⊹: Histone I.

o : Histone IIa.

△ : Histone III-IV.

All three histone solutions, which were placed on the gels for electrophoresis, were at the initial concentration of 1 mg/ml.

FIGURE 5


SCAN AREA
20
15
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MICROGRAMS PROTEIN/GEL
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