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DETERMINATION OF PROTEIN SPOTS SEPARATED BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

A simple method for quantitating proteins in the spots on two-dimensional polyacrylamide gel electropherograms is described. The system consists in three steps: (1) O'Farrell's two-dimensional gel electrophoresis of the proteins to be analysed; (2) staining of the gels with Coomassie brilliant blue; and (3) determination of the area and integrated density of the stained spots by the Joyce Loebl Magiscan-1 image analysis system.

The method can be used for the determination of proteins in the range 0.5–100 $\mu g/cm^2$; the amount of protein involved in most spots detected by the staining method actually falls within this range. As the minimum spot diameter that can easily be handled by the method is about 2 mm, as much as 30 ng of protein in such a spot can be determined. The method can also be applied to autoradiograms.

INTRODUCTION

Two-dimensional polyacrylamide gel (2-D gel) electrophoresis has been shown to be useful for the analysis of complex mixtures of proteins[1]. Cell extracts[2–5], chromosomal proteins[6–8], serum proteins[9,10] and gene products[11–15] have been analysed by the method of O'Farrell[2], using isoelectric focusing in the first dimension and discontinuous SDS gel electrophoresis in the second dimension. Computer analysis systems have been developed for the identification and quantitation of these spots in complex systems[16–19] and the methods have been applied to the analysis of protein properties such as appearance and disappearance and changes in p*I* or molecular size[3,10,13–16]. For the quantitation of spots in 2-D gel patterns, the integrated density of the spots stained with Coomassie brilliant blue or silver stain and of autoradiographs are analysed by a scanning or image analysis system, and various computerized systems for the quantitation of spots have been described[17–24].

We have tried to develop a simple method for determining the amount of proteins in spots using the Joyce Loebl Magiscan-1 image analysis system. In this paper, we describe a method that can be used for the quantitation of protein spots stained with Coomassie brilliant blue and its application to autoradiograms, and discuss the problem of the recovery of proteins during 2-D gel electrophoresis.

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EXPERIMENTAL

Enzymes and proteins

Bovine serum albumin (BSA), ovalbumin and soybean trypsin inhibitor were purchased from Sigma. Yeast alcohol dehydrogenase and bovine pancreatic deoxyribonuclease were products of Boehringer and Miles Labs., respectively. Rabbit actin was kindly provided by Dr. Sueo Matsumura of this Department. *Escherichia coli recA* protein was generously provided by Dr. N. Nakayama of the Institute of Medical Sciences, Tokyo University. T_4 DNA ligase was prepared according to the method of Tait *et al.*[25]. The amounts of BSA and ovalbumin were determined by both dry weight and the molar absorptivity ($A_{1\%}$) of 6.6[26] and 7.5[27] at 280 nm, respectively, and the amount of soybean trypsin inhibitor by dry weight. The amount of yeast alcohol dehydrogenase was determined by the molar absorptivity of 18.9 at 280 nm[28] and actin by the method of Lowry *et al.*[29] by using BSA as a standard. *Rec*A protein was determined according to the Bio-Rad Labs. protein assay using Coomassie brilliant blue[30]. The purity of each protein was determined from the electrophoretic pattern and the amount of protein applied to 2-D gel electrophoresis was then corrected accordingly.

2-D gel electrophoresis

2-D gel electrophoresis was performed by the method of O'Farrell[2] with some modifications. Isoelectric focusing gels (3 × 120 mm) contained 2% of Ampholines (1.6% of pH range 5–8 and 0.4% of pH range 3.5–10). The gels were extruded into 5 ml of SDS sample buffer[2] after isoelectrofocusing at 10°C and incubated at 37°C for 60 min with occasional shaking. They were then subjected to SDS slab gel electrophoresis in the second dimension or frozen rapidly with dry-ice–ethanol and stored at −20°C. When frozen, the gels could not be stored for more than 3 days before electrophoresis. The separation gel (135 mm wide × 105 mm long × 1 mm thick) for the second dimension was an exponential gradient gel of 10–16% acrylamide. Electrophoresis in the second dimension was performed at 20 mA until the tracking dye (Bromophenol blue) reached to the bottom of the gel.

Staining and destaining of gels were carried out at 25°C by the method of Fairbanks *et al.*[31]. The gels were stained overnight in solution I (0.025% Coomassie brilliant blue R in 25% isopropanol and 10% acetic acid) with a volume of 150 ml per sheet of gel. The gels were then transferred to solution II (0.0025% Coomassie brilliant blue in 10% isopropanol and 10% acetic acid) with the same volume as above. After 6–9 h, the gels were transferred into solution III (0.001% Coomassie brilliant blue in 10% acetic acid) and incubated overnight. Finally, the gels were destained by several changes of 10% acetic acid. In these procedures, repeated use of solutions I and II was restricted to three times and solution III was freshly prepared each time. The destained gels were immersed in distilled water for 20 min to remove acetic acid and then dried on Whatman 3MM filter-paper *in vacuo*.

Preparation of ^{125}I-labelled proteins

Proteins were iodinated by the method of Hunter and Greenwood[32]. The iodinated protein mixtures were dialysed against several changes of 500 ml of phosphate-buffered saline (PBS) at 1–2 day intervals or was subjected to gel filtration on

a Sephadex G-25 column (7 × 0.5 cm I.D.) to remove free $Na^{125}I$. The ^{125}I-labelled proteins were purified by SDS polyacrylamide gel electrophoresis if necessary.

RESULTS

AREA and IDEN of protein spots

Various amounts of BSA, ovalbumin and *rec*A protein were subjected to 2-D gel electrophoresis and the pixels of the area (AREA) and the integrated density (IDEN) of the protein spots were determined with the Joyce Loebl Magiscan-1 image analysis system after the gels had been stained with Coomassie brilliant blue. It was found that the relationship between the logarithm of AREA or IDEN of spots and the logarithm of the amount of protein applied in electrophoresis is rectilinear within a limited range, as shown in Fig. 1. The slopes of the lines are the same for the three different proteins but the intercepts are not identical. The relationships can be represented by the following equation:

$$\log y = \alpha \log x + p \qquad (1)$$

where y and x are AREA or IDEN of the spots and amount of protein, respectively, and α and p are constants common to the three different proteins and that for each

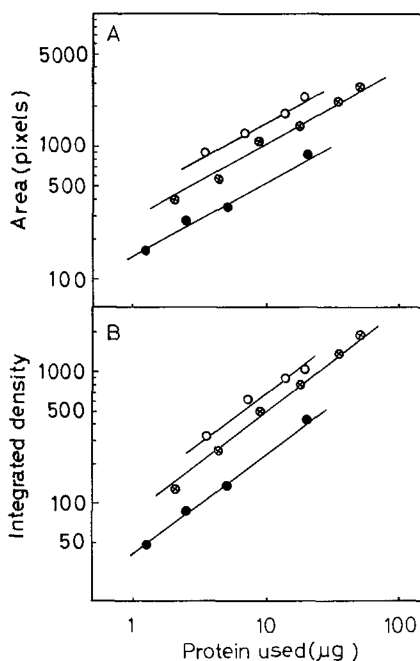


Fig. 1. AREA and IDEN of protein spots. Various amounts of proteins indicated on the abscissa were subjected to two-dimensional gel electrophoresis. AREA (A) and IDEN (B) of the protein spots were measured with the Magiscan-1 image analysis system using a program written in Fortran to measure both AREA and IDEN, simultaneously, after staining and destaining the gels. ○, BSA; ⊗, ovalbumin; ●, *E. coli rec*A protein.

protein, respectively. Although the number of proteins examined was limited, the equation is applicable to a variety of proteins as the relationship is valid for proteins having different molecular weights and p*I* values obtained from different sources, including a prokaryote. Hence the relative amount of a protein can be determined from the equation by the use of a value of α that is readily calculated from the slope obtained with a protein such as BSA. Fig. 1 shows that the linearity is maintained at least for amounts of protein between 2 and 20 μg, 2 and 50 μg and 1 and 20 μg for BSA, ovalbumin and *rec*A protein, respectively. Fig. 1 also shows that for these proteins the AREA and IDEN of spots per unit amount of protein are not identical with each other, indicating that these proteins have different p values in eqn. 1. The unequal p values for the AREA of these proteins may reflect either the difference in the degree of stacking of the proteins in the gel, which might be due to different characteristics of the individual proteins, or the different recoveries of proteins during 2-D gel electrophoresis, or both. As for IDEN, the different staining efficiencies and recoveries of proteins in the several steps involved may account for the different p values.

Calibration of protein spots by the use of BSA standard

In order to examine the relationship between IDEN and amount of protein per unit area, 10% polyacrylamide slab gels uniformly containing various amounts of BSA were polymerized and BSA standard specimens were prepared (Fig. 2). The recovery of a standard BSA through the procedures was monitored by using ^{125}I-labelled BSA. In the experiment, about 90% of the labelled protein was recovered through the procedures and a correction was made for the calibration graph. As shown in Fig. 2, the ratio of IDEN to AREA of the BSA specimens plotted against the corresponding amount of protein (μg/cm^2) on logarithmic scales consists of three

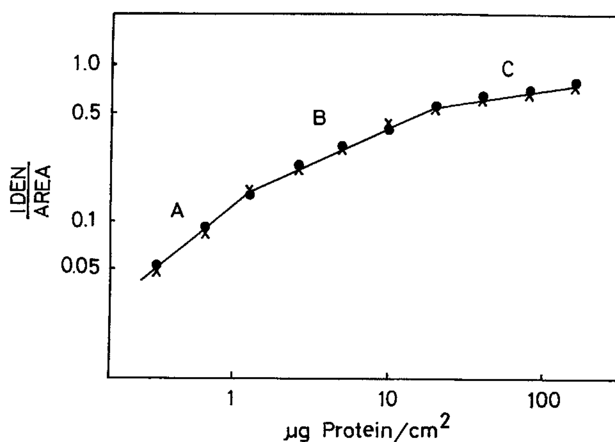


Fig. 2. Relationship between IDEN and amount of protein. SDS–10% polyacrylamide gel polymerized in the presence of various amounts of BSA at a thickness of 1 mm was cut into a disk of 20 mm diameter, stained and destained. The disks were then dried on Whatman NO 3MM filter-paper and the IDEN/AREA values of these disks were measured. The disk without added BSA was treated in the same way as those with BSA and used as a blank control. The value of the blank control was subtracted from the results. Two series of experiment (●, ×) were performed separately.

rectilinear ranges (lines A, B and C) and the relationship in these three different ranges can be represented by the equation

$$\log Y = \beta \log X + Q \quad (2)$$

where Y and X are IDEN/AREA of the BSA specimens and amount of protein per unit area, respectively, and β and Q are constants for the sample. Assuming that the staining efficiencies of all the proteins are identical, the average amount of protein per square centimetre, X, in a spot can be approximated from the IDEN/AREA value by use of eqn. 2 because the β and Q values for BSA can be used for other proteins. The amount of protein in the spots can therefore be obtained by multiplying X by the area of the spot obtained from the AREA by a calibration. The X values for the spots on the electropherogram used for the analysis shown in Fig. 1 were actually calculated in this way. The amounts of protein in the BSA and ovalbumin spots were then obtained by multiplying X by the area of the spot (cm^2) and are shown in Fig. 3. The IDEN/AREA values of the spots examined were confirmed to lie on line B in Fig. 2. Here, the IDEN/AREA value of the background was subtracted from the corresponding values for the spots.

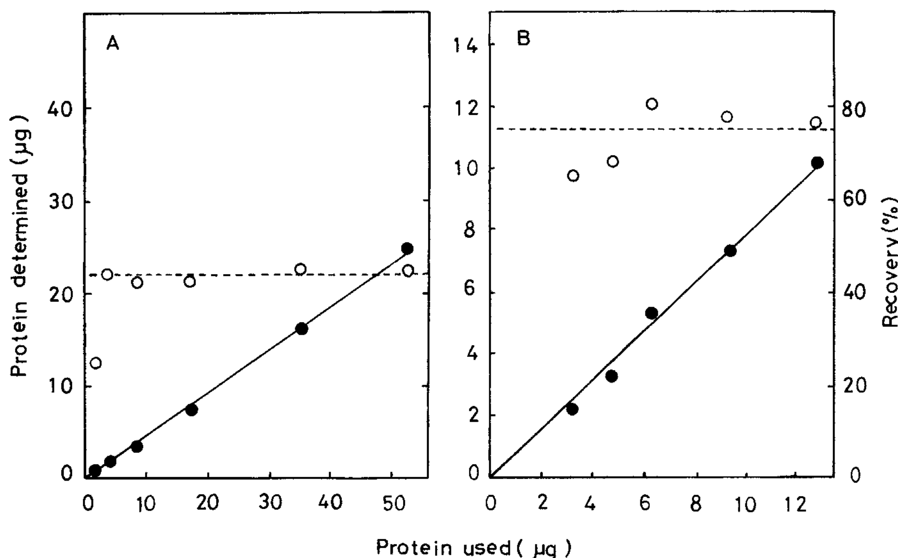


Fig. 3. Determination of authentic protein spots. The spots of ovalbumin (A) and BSA (B) of the electropherogram used for the determination of AREA and IDEN in Fig. 1 were calibrated by using eqn. 2. ●, Amount of protein in spots; ○, recoveries of proteins.

Recovery of proteins by 2-D gel electrophoresis

It was found from Fig. 3 that the amounts of ovalbumin and BSA recovered from the spots were proportional to the amounts applied in gel electrophoresis and the average recoveries of ovalbumin and BSA were 43% and 75% of the amounts applied, respectively.

The recoveries of various proteins, determined similarly are shown in Fig. 4 (experiment 1). For BSA, ovalbumin and soybean trypsin inhibitor, the recoveries were also determined by using ^{125}I-labelled preparations (Fig. 4, experiment 1). The recovery of BSA obtained with the ^{125}I-labelled preparation (73.5%) agrees well with the value obtained by the other method (75%), whereas the recoveries of ovalbumin and trypsin inhibitor determined by the IDEN/AREA are lower than those obtained from their radioactivities (Fig. 4). The data for the staining efficiency of ovalbumin and BSA, which were determined from both their IDEN and radioactivity, indicated that ovalbumin was stained about 20% less efficiently than BSA (unpublished data). This low staining efficiency may account for the difference in the recoveries of ovalbumin determined by the two different methods.

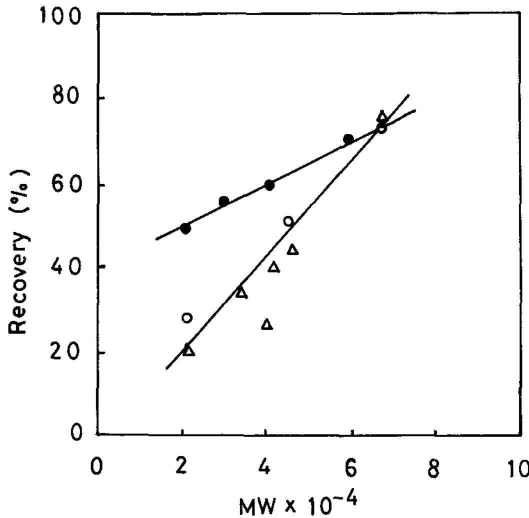
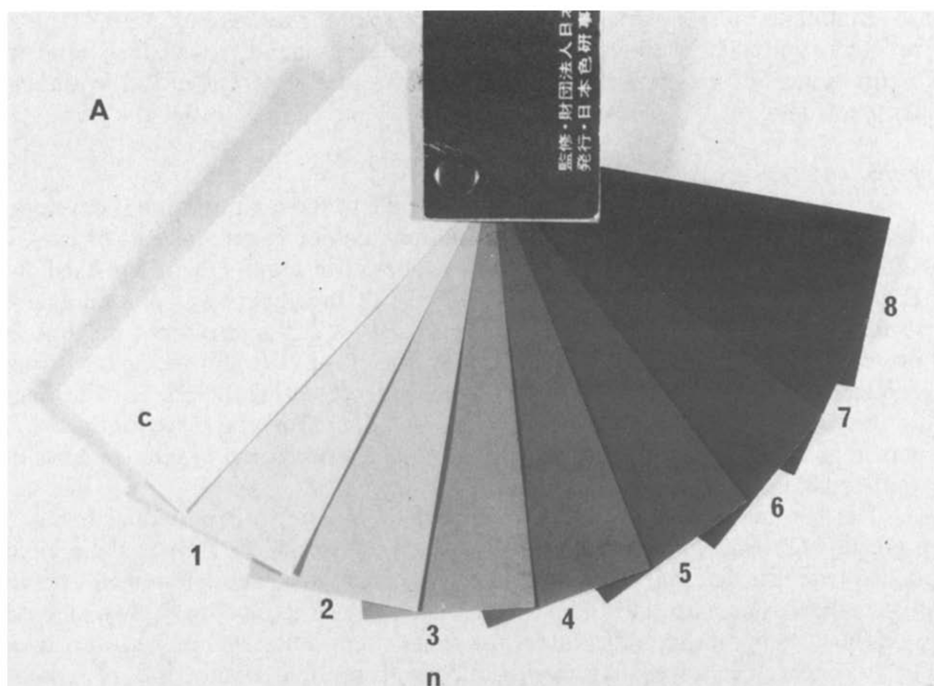


Fig. 4. Relationship between molecular sizes of proteins and their recoveries during 2-D gel electrophoresis. Experiment 1 (○, △): ○, recoveries of [^{125}I]BSA (68 kdalton), [^{125}I]ovalbumin (45 kdalton) and [^{125}I]trypsin inhibitor (21.5 kdalton). Radioactive spots were cut out after staining the gels and counted in a gamma counter. △, Those obtained with the Magiscan-1 using BSA, ovalbumin, actin (42 kdalton), *rec*A protein (40 kdalton), alcohol dehydrogenase (35 kdalton) and trypsin inhibitor. Standard deviations of determination of [^{125}I]BSA, [^{125}I]ovalbumin, [^{125}I]trypsin inhibitor, BSA, ovalbumin, actin, *rec*A protein and trypsin inhibitor are 2.4, 1.3, 2.4, 6.6, 9.2, 5.8, 11.8 and 2.4, respectively. Experiment 2 (●): the curve was obtained using [^{125}I]labelled BSA, T_4 DNA ligase (60 kdalton), actin, pancreatic deoxyribonuclease I (31 kdalton) and trypsin inhibitor. [^{125}I]BSA was always run as a standard at same time when the other [^{125}I]proteins were subjected to electrophoresis and the recoveries of the latter were calculated as a ratio to that of the former. The data are represented as the recovery of BSA is 73.5% as in experiment 1.

The recovery of protein during electrophoresis varies with the protein. However, the variation is not random, but is proportional to the molecular size (Fig. 4). This relationship was confirmed in an another experiment using ^{125}I-labelled BSA, T_4 DNA ligase, actin, pancreatic deoxyribonuclease I and soybean trypsin inhibitor (Fig. 4, experiment 2). The linearity between recoveries and molecular sizes of the proteins was maintained in a series of experiments, although the recoveries of proteins



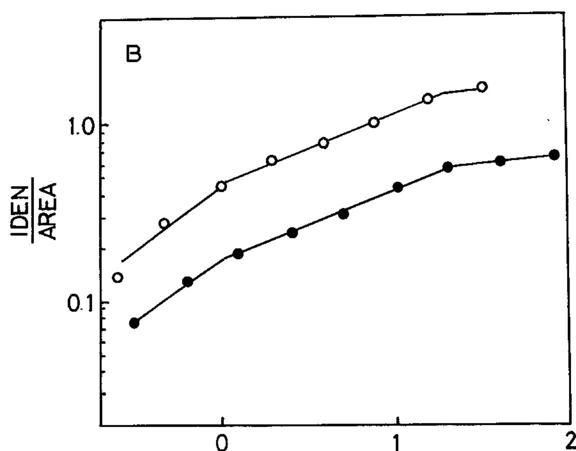


Fig. 5. IDEN/AREA of monotone colour papers having nine grades of darkness. (A) Monotone colour papers of Colour Cards 98a (Japan Color Research Institute, Tokyo, Japan). n, Number representing the degree of darkness tentatively assigned to the papers; c, background control. (B) Relationship between IDEN/AREA and degree of darkness of the colour papers. The IDEN/AREA value of the brightest paper (c) was subtracted from those of other papers as a background. The abscissa is $n' = 0.301(n - 3)$ for the colour papers and $\log(\mu g/cm^2)$ of the BSA standard shown in Fig. 2, where n is the degree of darkness shown in Fig. 4A. ○, IDEN/AREA of colour papers; ●, that of BSA standard.

varied in different series of experiments (for example, the average recoveries of BSA were 73.5% and 88% in the first and the second experiments, respectively) and the two curves are not identical even if the recoveries of BSA are identical in the two experiments (Fig. 4).

Application to autoradiograms

To examine whether this system is applicable to the quantitation of developed autoradiograms, we employed a set of monotone colour papers instead of BSA as the standard for the purpose of preparing a calibration graph (Fig. 5A). As shown in Fig. 5B, when the logarithm of IDEN/AREA of the papers was plotted against $n' = 0.301(n - 3)$ and the same abscissa was used for the logarithm of the amount of protein ($\mu g/cm^2$) in the BSA standard, the slope of the curve for the colour papers is identical with that for BSA, where n is a number representing the degree of darkness tentatively assigned to the colour papers (Fig. 5A). Therefore, the curve for the colour papers appears to be useful as a standard for the determination of relative amounts of spots or bands in an autoradiogram.

The method was then applied to the determination of individual bands of ^{32}P-labelled DNA fragments (Fig. 6). The relative amounts of DNA in these bands obtained from the determination of radioactivity and the values determined with the image analysis system are shown in Table I. The values obtained by the two methods agreed fairly well, although the values for spots 1 and 2 determined with the image analysis system were lower than those obtained from the determination of radioactivity. This does not seem to be due to the determination errors but to overexposure beyond the limit of the X-ray film used.

TABLE I

COMPARISON OF THE VALUES OF RADIOACTIVE SPOTS DETERMINED BY THEIR IDEN AND RADIOACTIVITIES

No. of spot in Fig. 5	*Radioactivity*		*IDEN*** *(relative values)*
	*Counts/min**	*Ratio*	
1	1480	1.89	1.53
2	1412	1.80	1.47
3	838	1.07	1.15
4	810	1.03	1.06
5	784	1.00	1.00
6	731	0.93	0.98
7	278	0.35	0.42
8	252	0.32	0.30
9	159	0.20	0.17
10	160	0.20	0.16
11	45	0.06	0.02

* Radioactive spots in Fig. 5 were cut out and counted in a scintillation counter. The background activity of the filter is subtracted from the values.

** The spots on the autoradiogram were measured as described in the text. The ratio is given relative to the amount in spot No. 5 = 1.00.

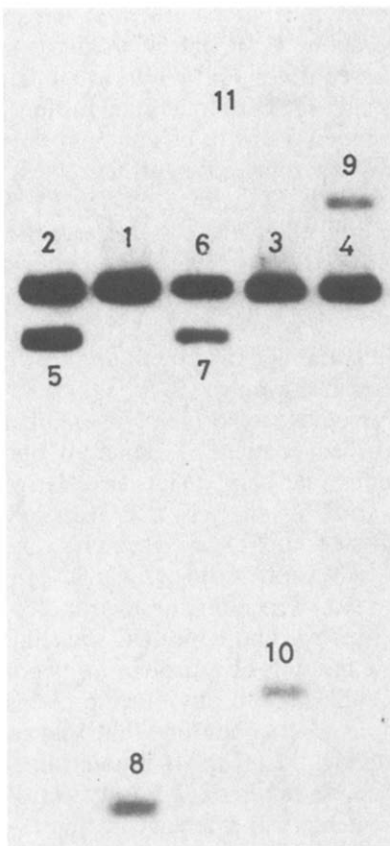


Fig. 6. Autoradiogram of DNA fragments hybridized with the rat aldolase cDNA probe. The genomic gene of rat aldolase was digested with various restriction enzymes and electropohoresed in an agarose gel. DNA from the agarose gel was transferred to a nitrocellulose filter and hybridized with a ^{32}P-labelled pRAB3031, a rat aldolase cDNA plasmid[38]. The filter was then washed and exposed for 1 day with an intensifying screen at −70°C.

DISCUSSION

In oder to perform quantitative analysis of spots, a calibration system is necessary. For this purpose, commercial optical density standards are used for the stained gel[17,33].

In this paper we have described a system for calibrating amounts of protein from both the area and the integrated density of spots stained with Coomassie brilliant blue. The method consists in two interrelated procedures using either eqn. 1 or 2. However, in the first method, which uses eqn. 1, only relative amounts of protein are obtained. On the other hand, the second method, which uses eqn. 2, would be more useful because the standards for the calibration can be readily prepared and the amounts of protein thus obtained are relatively close to the absolute values for the spots.

Although the second method appears to be more reliable than the first, it also has limitations for the quantitation of proteins in spots. First, the method has a calibration error owing to the ambiguity of the spot contours and the unequal distribution of protein in the spot. Second, the staining efficiencies of individual proteins are not identical. Although the protein being determined seems to be the most suitable as a standard, in general bovine serum albumin or some other protein is employed instead[34]. Therefore, errors due to the variability of staining efficiency are also introduced into the system[35]. In spite of these difficulties, the system described in this paper appears to be useful for the quantitative analysis of protein spots on the 2-D gel electropherograms as the reproducibility of the analysis appears to be satisfactory.

It is difficult to determine directly the absolute amount of protein applied in 2-D gel electrophoresis because the recovery of protein during the 2-D gel electrophoresis and the staining efficiency can vary. The recovery of proteins in 2-D gel electrophoresis has been monitored using ^{125}I-labelled proteins[36]. Hence, if one wants to determine either absolute amounts of individual components or relative amounts of different components in a preparation used for analysis, it is necessary to know the recovery of the protein(s) through all the steps of 2-D gel electrophoresis, including staining and destaining. The recoveries of several proteins, which were determined by two different methods, were found to vary depending on their molecular sizes and there is a direct relationship between recovery and molecular size (Fig. 4). Therefore, it would be possible to quantitate the amount of a protein in a spot by using a calibration graph if the relationship is applicable to any other proteins covered in the particular range. It is probably different gel conditions that lead to the recovery curves in the two experiments not being identical (Fig. 4). Standardization of reagents is important for obtaining reproducible results in 2-D gel electrophoresis[1]. The two curves in Fig. 4 should become identical if the reagents and experimental conditions are identical.

The method has been applied to the quantitation of embryonic chick serum proteins[37]. The amounts of individual serum proteins determined showed the range of the physiological level. For example, the amount of embryo-specific α-globulin (corresponding to mammalian α-fetoprotein) of a 13-day-old embryo was 4.75 mg/ml and the amount of albumin in an adult serum was 40 mg/ml (67% of total serum protein).

The method involving eqn. 2 can be used for the determination of proteins in the range 0.5–100 μg/cm^2. The amount of protein in most spots in 2-D gel electropherograms falls within this range. In O'Farrell's 2-D gel electrophoresis[2], the minimum spot diameter that can easily be handled by our method is about 2 mm, and as much as 30 ng of protein in such a spot can be determined by this method.

The method described here can be used efficiently and widely to follow the change of a single protein component under different physiological conditions such as cell cycle and differentiation or under normal and restricted conditions, and for comparison of the amounts of proteins among identical and/or non-identical proteins that have different positions in a 2-D gel electropherogram.

This method is also applicable to the quantitation of autoradiograms of proteins and nucleic acids. In this procedure, colour papers were found to be useful as a standard for the calibration of the degree of darkness.

The principle of the method should be also applicable to silver-stained gels.

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