REGULAR ARTICLE

A quantitative investigation into the losses of proteins at different stages of a two-dimensional gel electrophoresis procedure

Shaobo Zhou[1], Matthew J. Bailey[1], Michael J. Dunn[2], Victor R. Preedy[1] and Peter W. Emery[1]*

[1] Nutrition, Food and Health Research Centre, Kings College London, London, UK
[2] Department of Neuroscience, Institute of Psychiatry, Kings College London, London, UK

We report the results of a systematic investigation to quantify the losses of protein during a well-established two-dimensional polyacrylamide gel electrophoresis (2-DE) procedure. Radioactively labelled proteins ([^{14}C]bovine serum albumin and a homogenate prepared from the liver of a rat that had been injected with [^{35}S]methionine) were used, and recovery was quantified by digesting pieces of gel in H_2O_2 and subjecting the digests to liquid scintillation counting. When samples were loaded onto the first dimension immobilised pH gradient strips by in-gel rehydration, recovery of protein from the strips was 44–80% of the amount of protein loaded, depending on the amount of protein in the sample. Most of the unrecovered protein appeared to have adhered to the reswelling tray. Losses during isoelectric focusing (IEF) were much smaller (7–14%), although approximately 2% of the protein appeared to migrate from sample strips to adjacent blank strips in the focussing apparatus. A further 17–24% of the proteins were lost into the buffers during equilibration prior to running in the second dimension. Losses during the second dimension run and subsequent staining with SYPRO Ruby amounted to less than 10%. The overall loss during 2-DE was reduced by approximately 25% when proteins were loaded onto the IEF strips using sample cups instead of by in-gel rehydration. These extensive and variable losses during the 2-DE procedure mean that spot intensities on 2-DE gels cannot be used to derive reliable, quantitative information on the amounts of proteins present in the original sample.

Received: June 15, 2004
Revised: October 27, 2004
Accepted: November 30, 2004

Keywords:
Protein / Quantitative losses / Recovery / Two-dimensional gel electrophoresis

1 Introduction

The quantitative measurement of protein content in a tissue or cellular system is a complementary but important component of protein turnover studies [1]. In such studies emphasis is placed on determining absolute (*i.e.*, protein quantity *per* cell, region or organ) rather than relative protein quantities (*i.e.*, relative to wet or dry weight or a proportion of other proteins). The assessment of absolute amounts is necessary to establish whether pathophysiological or bioregulatory processes cause the net accretion or loss of specific or groups of proteins. Hitherto, isolation and quantification of proteins have employed a variety of techniques, such as homogenisation, subcellular isolation and acid-, solvent- or immuno-precipitation, coupled with colorimetric or fluorimetric assays. However, in recent years

Correspondence: Dr. Peter W. Emery, Nutrition, Food and Health Research Centre, Kings College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NN, UK
E-mail: peter.emery@kcl.ac.uk
Fax: +44-20-7848-4185

Abbreviation: PCA, perchloric acid

* Current address: Proteome Research Centre, Gonway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland

there has been an increasing usage of complex and multi-step proteomic methods to characterise changes in the abundance of individual proteins in *in vitro* or *in vivo* cellular systems. Inherent in many of these studies is the assumption that there are minimal or no loss of proteins during the proteomic procedures.

2-DE is now well established as the central proteomic procedure for separating and identifying proteins within complex mixtures. After the proteins have been separated, the gels can be stained and scanned and the proteins identified and quantified. It is generally assumed that the relative densities of the spots are proportional to the relative abundance of the different proteins in the original mixture. However, it has been shown that there are some losses of proteins at various stages of the 2-DE procedure, including loading the proteins onto the first dimension IPG strips, IEF and equilibration of the proteins prior to running on the second dimension gels [2]. It may thus not be possible to determine the absolute amounts of each protein in the original mixture with any certainty.

The magnitude of the losses during 2-DE has previously been investigated by Zuo and Speicher [2], who determined the losses indirectly by measuring the amounts of protein that could be recovered from the surfaces of the apparatus or from the various buffers and other solutions used. However, it would be more useful to measure the losses directly, by determining the amounts of protein that are actually present in the IPG strips and the 2-DE gels at each stage during the procedure.

In this paper we report the results of a systematic study to quantify the losses of protein during a well established 2-DE procedure [3] that is in routine use in our laboratory. We used radioactively labelled proteins to facilitate the quantitation of protein losses at each stage, taking advantage of a recently developed procedure to measure the amount of radioactively labelled protein present in a polyacrylamide gel [4]. In the first experiment we used known amounts of a single, purified protein, [^{14}C]BSA. In order to extend the applicability of the results we then used a mixture of proteins from a homogenate of rat liver. The proteins had been labelled *in vivo* by injecting the rat with [^{35}S]methionine. It was therefore necessary to develop a procedure for reliably removing all the free [^{35}S]methionine (*i.e.*, that which had not been incorporated into protein) before quantifying the amount of labelled protein present in the samples. This was achieved by precipitating the proteins with dilute perchloric acid (PCA) or acetone, and our investigations centred on the efficacy with which subsequent washes with either PCA or acetone removed the remaining traces of free methionine. In subsequent experiments, the effects of loading different amounts of protein were investigated. Finally, the use of cup-loading as an alternative to in-gel rehydration as a method of sample loading was investigated.

Some of these results have been published previously in abstract form [5].

2 Methods

2.1 Experiment 1

2.1.1 Protein losses during reswelling, focussing and equilibration steps using a single protein

Methyl-[^{14}C]-methylated BSA (Perkin Elmer Life Sciences, Beaconsfield, Bucks, UK) was mixed with unlabelled BSA in water (2.7 mg/mL). One millilitre of ice-cold acetone was added to 300 μL of this solution and left to stand for 15 min at 4°C, then centrifuged at 1000 × *g* for 15 min at 4°C. The supernatant was discarded and the precipitate was washed three more times by resuspending in a similar volume of ice-cold acetone and recentrifuging. The final precipitate was dried with nitrogen gas and resuspended in reswelling buffer (8 M urea, 0.5% CHAPS, 0.2% DTT, 0.2% Pharmalyte pH 3–10 (Amersham Biosciences, Chalfont St. Giles, Bucks, UK)). The protein concentration was measured using a modified Bradford assay [6] and then adjusted to 0.0975 μg/μL with reswelling buffer. A solution containing a similar amount of [^{14}C]albumin and BSA that had not been washed with acetone was also made up to the same concentration with reswelling buffer. Aliquots of the two solutions of albumin in reswelling buffer (washed and unwashed) were mixed with 10 mL scintillation fluid (Ecoscint A; National Diagnostics, Hessle, Yorks, UK) in plastic vials, left until chemiluminescence had decayed and counted to an error value <1% in a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, High Wycombe, Bucks, UK). Quench correction was by the H number method.

Four hundred fifty microlitre aliquots of the two solutions of albumin in reswelling buffer, containing 44 μg protein, were loaded into the grooves of a reswelling tray (Immobiline DryStrip Reswelling Tray; Amersham Biosciences). Then, 18 cm IPG strips, pH 3–10 nonlinear (Immobiline™ DryStrip; Amersham Biosciences), were placed gel side down into the grooves containing the sample, covered with silicone oil and left overnight to absorb the protein. Additional blank IPG strips were loaded with reswelling buffer only. Two strips each containing the unwashed and washed solutions and one blank strip were then subjected to radioactivity analysis as follows: the strips were cut into small pieces and put into a plastic vial containing 2 mL H_2O_2, incubated at 70°C overnight, then 10 mL of scintillation liquid was added and the vials were counted as above [4].

The remaining rehydrated strips were placed gel side up in the grooves of a strip aligner, which was placed in a focussing tray (Immobiline Strip Tray; Amersham Biosciences). Strips of filter paper that had been soaked in deionised water were placed at each end to act as wicks and electrodes were placed on top of the wicks. Approximately 20 mL silicone oil was then poured into the tray, the electrodes were connected to a Multiphor II IEF unit (Amersham Biosciences) and the strips were focussed for 70 kVh.

The strips were then removed and placed in the channels of an equilibration tray (Immunoblot incubation and staining tray; Pierce Biotechnology, Rockford, IL, USA). The first equilibration buffer was added to channels of the tray, left for 15 min then removed, and the second equilibration buffer was added to the channels of the tray, left for 15 min then removed. Both buffers contained 6 M urea, 30% glycerol, 2% SDS, 0.05 M Tris HCl, pH 8.8; the first buffer also contained 1% DTT; the second buffer contained 4.8% iodoacetamide. After equilibration, the IPG strips were cut up, incubated with H_2O_2 and subjected to liquid scintillation counting as described above. This experiment was subsequently repeated using 7 cm IPG strips instead of 18 cm strips, but using the same apparatus. In this experiment, one batch of [^{14}C]albumin was washed three times with 1.5% PCA followed by one wash with acetone instead of three washes with acetone.

2.2 Experiment 2

2.2.1 Animal procedures

Female Sprague-Dawley rats weighing approximately 200 g were injected *via* a lateral tail vein with a flooding-dose of [^{35}S]methionine (0.150 mmol and 25 mCi in 0.5 mL *per* 100 g body weight). The rats were returned to their cages for 15 min, then killed by decapitation. The livers were rapidly dissected, frozen in liquid nitrogen and stored at −80°C prior to analysis. The animal work was carried out under the terms of United Kingdom Home Office Project Licence PPL70/5171.

2.2.2 Determination of the optimal method for removing free amino acids

Small pieces of liver (approximately 100 mg) were placed in tubes with 1 mL of ice-cold lysis buffer (9.5 M urea, 2% CHAPS, 0.8% Pharmalyte pH 3–10, 1% DTT) containing 50 μL protease inhibitors (Roche, Welwyn Garden City, Herts, UK)) and homogenised for 3–5 s using a Polytron homogeniser (Kinematica, Littlau, Switzerland). During homogenisation the tubes were kept on ice. An aliquot of the homogenate was taken for liquid scintillation counting. Further 100 μL aliquots of the homogenate were taken into a 1.5 mL Eppendorf tube with some small glass beads and 1.2 mL of ice-cold acetone or dilute PCA was added. The tubes were left on ice for 15 min then centrifuged at 1000 × *g* for 15 min at room temperature. The supernatant was removed and an aliquot was taken for liquid scintillation counting. The precipitate was resuspended in acetone or PCA and recentrifuged as before. Seven different variations of this treatment were tested, as follows: A, consisted of washing with acetone eight times; B, washing with 1% PCA five times; C, with 1.5% PCA eight times; D, with 2% PCA five times; E, with acetone four times followed by four more washes with acetone containing 300 mM methionine; F, washing with 1.5% PCA four times followed by four more washes with 1.5% PCA containing 300 mM methionine; G, washing with 1.5% PCA three times followed by two washes with acetone. In each case the supernatant from each wash was measured for radioactivity. At the end of each treatment the final precipitate was dried under nitrogen then further dried by vacuum centrifugation. The dry precipitate was then redissolved in 100 μL reswelling buffer and radioactivity measured by liquid scintillation counting.

2.3 Experiment 3

2.3.1 Protein losses during reswelling, focussing and equilibration steps using mixed tissue proteins

[^{35}S]methionine labelled liver tissues (0.44 g) (from rats treated as detailed in Section 2.2) were added to 4 mL lysis buffer and homogenised with a Polytron homogeniser as described in Section 2.2.2. Proteins were then precipitated with 1.5% PCA and the precipitate was washed three more times with 1.5% PCA and three times with acetone, as described in Section 2.2.2. The final supernatant was subjected to liquid scintillation counting to confirm that the radioactivity in the final wash was not significantly different from background. The precipitate was dried by vacuum centrifugation then redissolved in 2 mL of reswelling buffer. An aliquot of this solution was analysed for protein content using the modified Bradford method and the protein concentration was adjusted to 0.896 μg/μL with reswelling buffer. Two aliquots of 450 μL (containing 403 μg protein) were taken for liquid scintillation counting and six similar aliquots were loaded onto 18 cm IPG strips, pH 3–10 nonlinear. Five further strips were loaded with reswelling buffer only as blank controls. Reswelling, IEF and equilibration were then carried out as described in Section 2.1.1. Two sample strips and one blank strip were incubated with H_2O_2 and subjected to liquid scintillation counting (as described in Section 2.1) after the reswelling step; two sample strips and two blank strips were incubated with H_2O_2 and subjected to liquid scintillation counting after the focussing step; the remaining two sample strips and two blank strips were incubated with H_2O_2 and subjected to liquid scintillation counting after the equilibration step. This experiment was subsequently repeated with different protein loadings, 100 μg *per* strip and 1000 μg *per* strip.

2.4 Experiment 4

2.4.1 Protein losses during 2-DE

An homogenate of [^{35}S]methionine labelled liver tissue was prepared as described in Section 2.3. Proteins were precipitated using acetone and the precipitate was washed 12 times with acetone, the final precipitate was dried and redissolved in reswelling buffer, all as described above. Two aliquots of 160 μL, each containing 200 μg protein were

taken for liquid scintillation counting and eight similar aliquots were loaded onto 7 cm IPG strips, pH 3–10 nonlinear. Three further strips were loaded with reswelling buffer only, as blank controls. Reswelling, IEF and equilibration were then carried out as described above. Two sample strips and one blank strip were incubated with H_2O_2 and subjected to liquid scintillation counting (as described in Section 2.1) after the reswelling step; two sample strips were incubated with H_2O_2 and subjected to liquid scintillation counting after the focussing step; two sample strips were incubated with H_2O_2 and subjected to liquid scintillation counting after the equilibration step.

The amounts of radioactivity left in the reswelling tray, focussing tray (including the paper wicks and silicone oil) and equilibration tray were measured by thoroughly washing them with Decon® 90 (Decon Laboratories, Hove, Sussex, UK) and subjecting the washings to liquid scintillation counting. Aliquots of the equilibration buffers were also subjected to liquid scintillation counting.

After equilibration, the two remaining sample strips and the one remaining blank strip were placed in contact with 7×7 cm^2 12% v/v polyacrylamide gels. The strips were sealed with a 1% w/v agarose solution and left to solidify before placing in a tank (Xcell SureLock™ Mini-Cell; Invitrogen, Paisley, UK) to separate in the second dimension. The running buffer contained 1.434% w/v glycine, 0.276% w/v Tris base and 0.096% w/v SDS. The gels were run at 20 mA *per* gel at 10°C until the dye front was at the bottom of the glass. The gels were then fixed for 1 h in 300 mL solution of 10% v/v methanol and 7% v/v acetic acid, followed by staining overnight in 300 mL SYPRO Ruby (Genomic Solutions, Huntingdon, UK) and finally washing in 300 mL of 10% v/v methanol and 7% v/v acetic acid for 1 h. The stained gels were scanned using a Storm scanner (Amersham Biosciences). They were then cut into pieces approximately 1×1 cm^2, and each piece was incubated with H_2O_2 and subjected to liquid scintillation counting. The IPG strips were removed from the gels, cut into several pieces, incubated with H_2O_2 and subjected to liquid scintillation counting. Aliquots of the running buffer and the fixing, staining and destaining solutions were also subjected to liquid scintillation counting.

2.5 Experiment 5

2.5.1 Comparison of in-gel reyhdration with cup-loading method

An homogenate of [^{35}S]methionine labelled liver tissue was prepared as described in Section 2.3. Proteins were precipitated with PCA and washed with PCA and acetone, dried and redissolved in reswelling buffer, again as described in Section 2.3. Aliquots containing 30 µg protein were then subjected to 2-DE on 7 cm IPG strips and 7×7 cm^2 gels using either in-gel rehydration or cup-loading procedures. For the in-gel rehydration samples, the procedure was the same as that outlined in Section 2.4, including measuring radioactivity left in the trays and equilibration buffers and measuring radioactivity in IPG strips after the focussing and equilibration. After staining with SYPRO Ruby, the gels were cut into pieces approximately 1×1 cm^2 and each piece was incubated with H_2O_2 and subjected to liquid scintillation counting. For the cup-loading procedure, IPG strips were rehydrated overnight with 160 µL reswelling buffer and immediately transferred to a strip aligner in a focussing tray and wicks were placed at each end. Sample cups were attached to a bar, which was fixed to the tray and positioned in contact with the strips approximately 5 mm from the cathodic end. The samples, containing 30 µg protein dissolved in 60 µL reswelling buffer, were added to the loading cups along with approximately 30 µL silicone oil. The rest of each strip was covered with silicone oil and the strips were focussed. The strips were then equilibrated and run out on second dimension gels as described in Section 2.4.1. After staining with SYPRO Ruby, the gels were cut into pieces approximately 1×1 cm^2 and each piece was incubated with H_2O_2 and subjected to liquid scintillation counting. The amount of radioactivity left in the sample cups was measured by thoroughly washing them with Decon 90 and subjecting the washings to liquid scintillation counting. The amounts of radioactivity left in the trays and equilibration buffers and in the IPG strips after the focussing and equilibration stages were all measured as described.

3 Results

3.1 Experiment 1

Results of this experiment are shown in Table 1. The amount of labelled albumin detected in the IPG strips after sample loading by reswelling was only 73–80% of the amount that had been placed in the reswelling trays. After focussing and equilibration the amount remaining in the strips had decreased to 54–71% of the amount loaded into the trays, or 74–89% of the amount present after reswelling. The amounts of radioactivity lost at each stage were slightly different for the samples that had been washed with acetone or PCA prior to loading compared with the losses from unwashed albumin, suggesting that acetone- or PCA-soluble fragments were taken up into the strips and retained during focussing and equilibration with different affinities from the bulk of the high molecular weight protein. For the unwashed albumin, the amount lost at each stage was somewhat greater when 7 cm strips were used than when the 18 cm strips were used, suggesting that the size of the IPG strip may have limited its capacity to absorb and retain protein.

3.2 Experiment 2

Table 2 shows the amounts of radioactivity that were removed from the liver homogenate when the proteins were precipitated and washed with either PCA or acetone. The

Table 1. Radioactivity in IPG strips loaded with [^{14}C]albumin (Experiment 1)[a)]

	Size of IPG strip (cm)	Amount loaded into reswelling tray (dpm)	Amount present after reswelling		Amount present after equilibration	
			(dpm)	(% of amount loaded)	(dpm)	(% of amount loaded)
Untreated albumin	18	4470	3555	80	3177	71
Albumin washed with acetone	18	4080	3075	75	2619	64
Untreated albumin	7	1354	986	73	733	54
Albumin washed with PCA	7	971	765	79	615	63

a) Values are means of two samples after subtraction of appropriate blank values

Table 2. Radioactivity in supernatants after washing [^{35}S]methionine labelled liver homogenate with acetone or PCA (Experiment 2)[a)]

Treatment	A 8 × acetone	B 5 × 1% PCA	C 8 × 1.5% PCA	D 5 × 2% PCA	E 4 × acetone, 4 × acetone +met	F 4 × 1.5% PCA, 4 × 1.5% PCA +met	G 3 × 1.5% PCA, 2 × acetone
Wash number							
1	33	69	66	72	10	61	78
2	9	13	14	13	3	18	11
3	4	2	4	2	2	4	2
4	2	0.4	0.9	0.4	1	2	1
5	2	0.1	0.2	0.1	1	0.2	0.2
6	1		0.1		1	0.1	
7	1		0.1		1	0	
8	1		0.1		1	0	
Total	54	86	87	87	20	85	92
% remaining in precipitate	44	12	13	11	71	13	7

a) Values are expressed as % of radioactivity in the original homogenate, and are means of duplicates

majority of the radioactivity in the liver 15 min after an injection of labelled methionine was expected to be in the form of free amino acid which is soluble in PCA, and this was confirmed by the data shown in Table 2. Changing the concentration of PCA between 1 and 2% made little difference to the efficiency of removal of free methionine. Virtually all the free methionine appeared to have been removed by the first four washes, since subsequent washes contained less than 0.5% of the original radioactivity. Moreover, adding a high concentration of unlabelled methionine to the later washing solutions to 'chase out' labelled methionine resulted in only a marginal increase in the amount of radioactivity removed. In contrast, precipitation of the protein with acetone appeared to be a relatively ineffective way of removing the free amino acid, since barely half the radioactivity had been removed even after eight washes with acetone, whereas approximately 90% of the radioactivity could be removed with PCA. On the other hand, washing the final precipitate with acetone allowed it to be dried and redissolved in reswelling buffer much more effectively, since the PCA precipitate tended to remain in suspension as small particles when added to the reswelling buffer. Moreover, when the protein precipitate was washed only with PCA it tended to show a brown discoloration on storage, which may have indicated some oxidative damage. Hence, the protocol that was adopted for subsequent experiments involved precipitation with 1.5% PCA, three additional washes with 1.5% PCA, then three further washes with acetone.

3.3 Experiment 3

Table 3 shows the amounts of radioactivity remaining in IPG strips after the reswelling, focussing and equilibration steps when varying amounts of labelled proteins from a liver homogenate were loaded. The amounts recovered were considerably lower than was found for albumin. Losses during the reswelling procedure were in the range 38–56%, with the greatest losses being apparent when the largest amount of protein was used. Loss during the focussing step was much smaller, approximately 16%. Losses during the equilibration step amounted to 48–53% of the quantity present after reswelling. The loss at this stage appeared to be independent of the amount of protein initially loaded. Overall, when 100 μg protein was loaded, 69% of it had been lost by the

Table 3. Radioactivity in IPG strips loaded with [^{35}S]methionine labelled liver homogenate (Experiment 3)[a)]

Amount of protein loaded (μg)	Amount of radioactivity loaded	Amount present after reswelling		Amount present after focussing		Amount present after equilibration	
	(dpm)	(dpm)	(% of amount loaded)	(dpm)	(% of amount loaded)	(dpm)	(% of amount loaded)
100	4786	2943	62	nd	nd	1454	31
400	2462	1173	48	989	40	567	23
1000	56 071	24 877	44	nd	nd	11 688	21

a) Values are means of two samples after subtraction of appropriate blank values
nd, not determined

Table 4. Amount of radioactivity recovered at each stage during 2-DE (Experiment 4)[a)]

	Amount left		Apparent loss[b)]	Amount recovered[c)]		Where recovered
	(dpm)	(% of amount loaded)	(dpm)	(dpm)	(%)	
Loaded onto IPG strip	3418					
After reswelling	2024	59	1394	1258 ± 35 (n = 7)[d]	90	Washings from reswelling tray
After focussing	1547	45	477	474 ± 47 (n = 7)	99	Washings from focussing tank
After equilibration	736	22	811	687 ± 31 (n = 5)	85	Equilibration buffers
After second dimension run	663	20	73	69	95	IPG strip (9 dpm); fixing and de-staining solution (60 dpm)

a) Values are means of duplicate samples after subtracting appropriate blanks
b) The amount lost was calculated by subtraction of the amount left from that present after the previous stage
c) The amount recovered represents what was found in the washings, buffers or other solutions, and is expressed as a percentage of the apparent loss
d) Where measurements were replicated more than twice values are given as mean ± SEM, with the number of observations in brackets

time the IPG strips had been reswollen, focussed and equilibrated ready to run on a second dimension gel. When the amount of protein loaded was increased to 1 mg, the amount lost increased to 79%.

When blank strips that had been subjected to IEF alongside strips that had been loaded with 400 μg labelled protein (containing 2462 dpm) were analysed for radioactivity, 27 dpm (mean of two strips) was found in each strip. This suggests that approximately 2% of the labelled protein may have migrated from one lane of the focussing tray to the next and been taken up by the IPG strip in the next lane.

3.4 Experiment 4

Table 4 shows the amounts of radioactivity present at each stage of the full 2-DE procedure together with the amounts recovered by carefully washing out the reswelling tray and focussing tray and counting samples of the equilibration buffers and the fixing and destaining solutions. The losses during the reswelling, focussing and equilibration stages (41, 24 and 48%, respectively) were comparable to those found in Experiment 3. Virtually all the radioactivity lost during the reswelling and focussing steps was recovered when the apparatus was carefully washed out with a strong detergent (Decon), suggesting that it represented labelled proteins that had been adsorbed to the walls of the trays. Similarly, 85% of the radioactivity lost during the equilibration step was recovered in the two equilibration buffers.

In marked contrast to the losses observed during the earlier stages of the procedure, approximately 90% of the radioactivity that was loaded onto the second dimension gel was still present in the gel after running, fixing, staining and destaining. A further 1% of the radioactivity was left in the IPG strip, and 8% was recovered from the fixing and destaining solution (the same solution was used for both processes). The amounts of radioactivity detected in the running buffers and the staining solution were indistinguishable from background levels.

Figure 1 shows the pattern of proteins in the gel as detected by staining with SYPRO Ruby. Proteins have been separated into clearly defined spots, as would be expected from this well established procedure. Scintillation counting

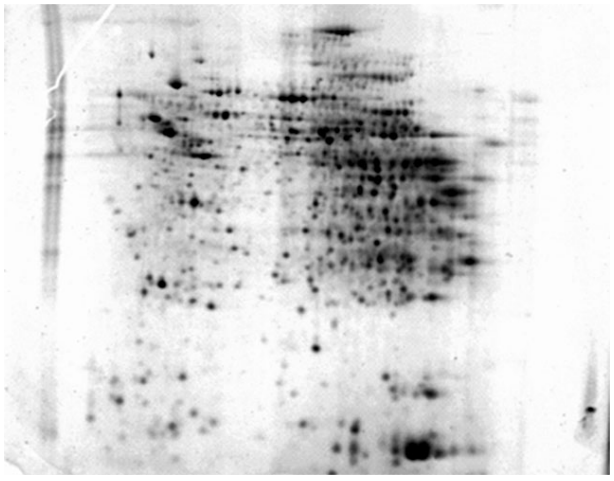

Figure 1. Scan of SYPRO Ruby stained 2-D gel of liver proteins (Experiment 4).

of the 1 cm^2 fragments of the gel confirmed that radioactively labelled proteins were present in all parts of the gel (data not shown).

3.5 Experiment 5

Table 5 shows the losses of protein at each stage of the 2-DE procedure using cup-loading as an alternative to in-gel rehydration for loading the samples. For the samples subjected to in-gel rehydration (the standard procedure), the amounts of protein remaining in the IPG strips after focussing and after equilibration were somewhat greater than in the previous experiments, presumably because the amount of protein loaded (30 μg *per* strip) was much lower than in any of the previous experiments. Nevertheless, when cup-loading was used, there was a considerable improvement in the amount of protein found in the strip after IEF, amounting to 85% of the amount that had been placed in the sample cup. On the other hand, a slightly greater proportion of the protein was lost during the equilibration stage, so that after equilibration only 51% of the protein that had been placed in the sample cup was present in the IPG strip.

Most of the radioactivity lost at each stage could be accounted for in the washings when the apparatus was washed with Decon and in the equilibration buffers. Less than 2% of the protein that had been placed in the sample cups was found when the cups were subsequently washed with Decon, but 10% was found in the focussing trays when they were washed with Decon. A further 3% was found in the wicks that were used during focussing. This may represent proteins with extreme p*I*s that have migrated off the end of the strip. For the in-gel rehydration samples, 29% of the amount placed in the reswelling trays was found when the trays were subsequently washed with Decon, 5% was recovered from the focussing trays and 5% was found in the wicks. The amounts lost during equilibration were approximately evenly divided between the two equilibration buffers.

Almost 90% of the radioactivity present in the strips after equilibration was recovered from the second dimension gels in the case of the samples that had been loaded by in-gel rehydration. This was very similar to the results of Experiment 4. In the case of the samples that had been loaded by cup-loading, 96% of the radioactivity present in the strips after equilibration was recovered from the second dimension gels.

3.6 Reproducibility

The data in the tables showing the amounts of radioactive protein present at each stage of the 2-DE procedure represent the means of duplicate values. In order to assess the reproducibility of the data we examined the differences between duplicates. The magnitude of the differences was found to be independent of the mean values. The mean difference between duplicate values for the amount of radioactive protein present after reswelling was 1.9 ± 0.9% of the mean value (mean ± SEM, $n = 3$); after focussing, it was 7.7 ± 1.7%

Table 5. Proportion of radioactivity recovered at each stage during 2-DE using either in-gel rehydration or cup-loading method (Experiment 5)[a)]

	Amount left				Apparent loss[b)]		Amount recovered[c)]			
	(dpm)		(% of amount loaded)		(dpm)		(dpm)		(%)	
	In-gel	Cup-loading	In-gel	Cup-loading	In-gel	Cup-loading	In-gel	Cup-loading	In-gel	Cup-loading
Loaded onto IPG strip	768	768								
After focussing	436	651	57	85	332	117	292	114	88	97
After equilibration	300	391	39	51	136	260	140	198	103	76
After second dimension run	263	375	34	49	37	16	nd	nd	nd	nd

a) Values are means of duplicate samples after subtracting appropriate blanks
b) The amount lost was calculated by subtraction of the amount left from that present after the previous stage
c) The amount recovered represents what was found in the washings and buffers, and is expressed as a percentage of the apparent loss

($n = 4$); and after equilibration 8.0 ± 3.6% ($n = 6$); after the second dimension run the mean difference between duplicates was 7.7 ± 2.0% ($n = 3$).

4 Discussion

The results of these investigations show that there are considerable losses of protein during each stage of a well-defined and generally accepted 2-DE procedure. This confirms several of the key conclusions of an earlier study [2], although the magnitude of the losses appears to be rather greater than has been reported previously. For example, Zuo and Speicher [2] reported that 5–38% of radioactively labelled protein remained in the reswelling tray after rehydration of the IPG strips whereas in the present experiment, losses between 20 and 55% of the radioactively labelled protein appeared not to have been taken up into the strips during the reswelling procedure. In the previous study [2] most of the radioactive protein that was not taken up into the strip was found in the small volume of liquid left in the tray. In the present study small amounts of liquid were also found in the tray, mainly as a thin film, but the volume was too small to quantify or count separately. It is possible that the method adopted in the previous study [2] may have underestimated the losses since some of the protein that had been adsorbed onto the surfaces of the apparatus may not have been removed by the authors' washing procedure, which utilised a 1% SDS solution. In the present experiment washing with Decon (an alkaline solution containing a mixture of anionic and nonionic surface active agents) was able to recover 90% of the radioactivity that had been lost (Experiment 4). A further difference between the present study and that of Zuo and Speicher [2] is that the latter used a dialysed extract of cultured *Escherichia coli* cells whereas the present study used homogenised rat liver, which may be regarded as representative of mammalian cell lysate.

The results of Experiments 1 and 3 show that protein losses during reswelling increase as the amount of protein loaded increases, in line with the data of Zuo and Speicher [2]. This suggests that the capacity of the IPG strips to absorb protein is limited, although the manufacturers recommend that 600 μg protein can be taken up by each 18 cm strip and previous authors have claimed that 5 mg protein can be loaded by this method [7, 8]. Moreover, it is likely that the different proteins will be taken up into the strips to different extents, depending for example on their molecular mass, as shown by Zuo and Speicher [2]. Hence the proportions of different proteins detected at the end of the 2-DE procedure may not be truly representative of the mixture that was loaded.

The volume of rehydration solution used, 450 μL, was in line with the manufacturer's recommendations, although some authors recommend a smaller volume such as 350 μL [11]. Zuo and Speicher [2] found that slightly less protein was lost during the reswelling stage when using 350 μL than when using 450 μL.

The in-gel rehydration procedure has been widely adopted as a means of loading relatively large amounts of protein onto IPG strips while avoiding the problems of protein precipitation around the site of a loading cup and the consequent tendency to form a series of horizontal streaks in the second dimension [7, 8]. However, the data from Experiment 5 showed that in-gel rehydration was associated with less complete uptake of protein into the strips than cup-loading, in line with previous observations [2]. On the other hand, the cup-loading procedure was associated with a greater loss of protein during the subsequent equilibration stage. This may suggest that more of the protein was nearer the surface of the strips after cup-loading than after in-gel rehydration, and was thus more readily removed during the equilibration process than if it had penetrated more deeply into the gel. Hence, overall just over 50% of the protein had been lost by the end of the second dimension run using the cup-loading method compared with 66% loss when a comparable amount of protein was loaded by in-gel rehydration.

Protein losses during the IEF step were less extensive than those during sample loading, but still significant (7% of the amount loaded in Experiment 3, 14% in Experiment 4). This is in line with the 5–10% losses reported by Zuo and Speicher [2]. In the present experiment we also found evidence that a small amount of protein had migrated from sample strips to adjacent blank strips after focussing. This may have been because all strips are in contact with a layer of silicone oil, or it may have been a result of contamination when the oil was poured off at the end. Clearly this could result in inaccurate amounts of different proteins being identified in 2-DE gels if different samples are focussed in the same tray, though the extent of this problem appeared to be very slight (2% of protein taken up in the blank strips).

The work of Zuo and Speicher [2] showed that the amount of protein lost during the sample loading and IEF could be reduced by using one piece of apparatus for both procedures, such as the IPGphor. This also allows 'active' rehydration by applying a low voltage (30–50 V), which may improve uptake of some proteins [11], although Zuo and Speicher found that this did not improve overall protein recovery [2]. However, under some circumstances the protein separation achieved using the IPGphor is not entirely satisfactory, so that some authors transfer the loaded IPG strips to a Multiphor prior to IEF [12].

The loss of protein during the equilibration step was not surprising, since the purpose of the equilibration buffers is to facilitate the elution of proteins from the IEF strips into the second dimension gels. However, the magnitude of the loss was greater than had been anticipated, since Zuo and Speicher [2] reported that only 7–10% of the protein that had originally been loaded was lost at this stage. In the present experiments we found that 17% of the protein that had originally been loaded was lost at this stage in Experiment 3 and 24% was lost at this stage in Experiment 4. Equilibration was carried out using 10 mL portions of each buffer with gentle shaking, as recommended elsewhere [11].

The loss of protein during the second dimension electrophoresis was minimal, as reported by Zuo and Speicher [2]. However, we did find a small but significant loss of protein during the procedure for fixing, staining with SYPRO Ruby and destaining. This has not been reported before as far as we are aware, but may reflect incomplete protein precipitation by the methanol/acetic acid fixing reagent.

In conclusion, this study has shown that up to 80% of the protein being analysed can be lost during a conventional 2-DE procedure. The particular procedure we examined has been used in numerous previous studies and has yielded useful qualitative information on protein expression in a variety of tissues, including heart [9] and skeletal muscle [10]. The present study does not invalidate conclusions drawn from comparisons between samples that have been subjected to identical procedures, although it may help to explain some of the variability that is commonly seen between replicate analyses. However, it would clearly not be possible to relate the amounts of proteins detected on a 2-DE gel to the amounts originally present in a tissue sample with any degree of certainty.

Financial support form the Wellcome Trust and the Biotechnology and Biological Sciences Research Council is gratefully acknowledged.

5 References

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