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Double-label reductive methylation of tissue proteins for precision two-dimensional polyacrylamide-gel electrophoretic analysis

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Reductive methylation has been used to radioactively label crude-extract proteins with ^{3}H or ^{14}C. The procedure achieved good isotope incorporation and resolution of proteins on two-dimensional polyacrylamide gels. It allows high-precision comparison of tissue samples by double-labelling and should facilitate the study of tissue proteins by two-dimensional gel electrophoresis.

Precision two-dimensional gel electrophoresis separates up to 1000 polypeptides (O'Farrell, 1975). When proteins produced by cells in culture are being studied, double-isotope-labelling gives additional sensitivity and precision (McConkey, 1979; Choo *et al.*, 1980).

Tissue proteins have been studied on two-dimensional gels, generally with less sensitive stains like Coomassie Blue to detect the polypeptides. We report here the use of reductive methylation to achieve double-isotope-labelling of tissue proteins on gels. Reductive methylation (Means & Feeney, 1968) alkylates the amino groups of proteins including those present on lysine residues and at the *N*-terminus. Formaldehyde interacts with the amino group to form a Schiff's base; subsequent addition of a reducing agent such as $NaBH_4$ or $NaCNBH_3$ reduces the Schiff's base to a mono- or di-methyl-amine derivative. Radioactive labelling of proteins is achieved with the use of $NaB^{3}H_4$, [^{14}C]-formaldehyde or [^{3}H]formaldehyde (Rice & Means, 1971; Dottavio-Martin & Ravel, 1978).

Experimental

Preparation of tissue extract

Human livers, obtained at autopsy at the Royal Children's Hospital, Melbourne, and mouse livers, were homogenized in Kontes (Vineland, NJ, U.S.A.) ground-glass homogenizers; 1 g of tissue was used for approx. 1.5 ml of 0.2 M-sodium borate buffer, pH 9, at 4°C. The homogenate was centrifuged in 1 ml-capacity cellulose acetate tubes at 25000 *g* for 45 min at 4°C. A portion of the supernatant was taken for the measurement of protein concentration by the method of Lowry *et al.* (1951). The remaining portion was stored at −20°C until used for reductive methylation.

Reductive methylation

$NaB^{3}H_4$ (13 Ci/mmol) and [^{14}C]formaldehyde (20.2 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. $NaCNBH_3$ was a gift from Dr. N. Hoogenraad.

The method of ^{3}H-labelling was essentially that of Kumarasamy & Symons (1979), with some modifications. $NaB^{3}H_4$ was used instead of $KB^{3}H_4$. A portion (100 mCi) was dissolved in 100 μl of 100 mM-KOH. Portions (10 μl) were dispensed into vials, freeze-dried and stored at room temperature over NaOH under vacuum. Just before use the contents of each vial was dissolved in 100 μl of 100 mM-KOH. The reaction was performed on ice in a fume hood. A portion (30 μl; approx. 500 μg) of tissue extract was first treated with 3 μl of a 1 mg/ml solution of deoxyribonuclease and 3 μl of 5 mM-$MgCl_2$ for 5 min. To this, 5 μl of 100 mM-formaldehyde was added, followed 30 s later by 40 μl (4 mCi) of the prepared $NaB^{3}H_4$. After 20 min 25 μl of 100 mM-unlabelled $NaBH_4$ was added. After a further 10 min four times the volume of acetone was added. The precipitate was collected by centrifugation at 2720 *g* for 10 min and washed twice in 80% (v/v) acetone. The final pellet was freeze-dried and redissolved by boiling for 1.5 min in 40 μl of 0.01 M-Tris/HCl buffer, pH 8.0, with 4 μl of 20% (w/v) SDS and 2 μl of mercaptoethanol. This was stored at −20°C until used for two-dimensional electrophoresis. To determine the amount of radioactive incorporation into the proteins, 1 μl of the sample was added to a scintillation vial containing 10 ml of scintillation fluid {PPO (2,5-diphenyl-oxazole)/POPOP[1,4-bis-(5-phenyloxazol-2-yl)-

Abbreviation used: SDS, sodium dodecyl sulphate.

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benzene]/toluene/Triton X-100 (5.5 g:0.1 g:667 ml: 333 ml)}. Counting for radioactivity was done in a Packard liquid-scintillation counter.

The method of ^{14}C-labelling was a modification of that of Dottavio-Martin & Ravel (1978). Undiluted [^{14}C]formaldehyde was stored at −20°C. The reaction was again performed on ice. Tissue extract (30 μl) was treated with deoxyribonuclease and $MgCl_2$, as for the ^{3}H-labelling above. Next, 3 μl (45 μCi) of [^{14}C]formaldehyde was added, followed 30 s later by 10 μl of 1 M-$NaCNBH_3$. The reaction was allowed to proceed for 30 min before acetone was added and the precipitate collected and treated as described above.

Two-dimensional polyacrylamide-gel electrophoresis

Samples for two-dimensional polyacrylamide-gel electrophoresis contained 6×10^6 d.p.m. in the case of ^{3}H-labelled proteins and 8.6×10^5 d.p.m. in the

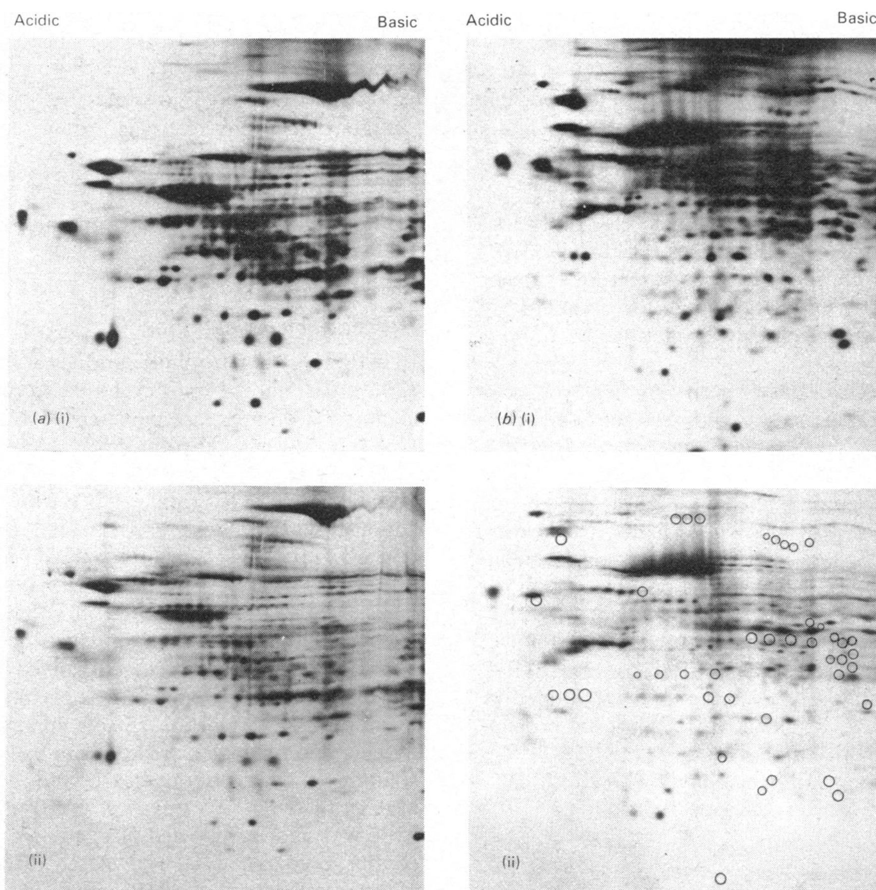

Fig. 1. *Two-dimensional polyacrylamide-gel analysis of radioactively labelled tissue proteins*
(*a*) Mouse liver crude extracts were labelled with ^{3}H by using NaB^3H_4 and formaldehyde and with ^{14}C by using $NaCNBH_3$ and [^{14}C]formaldehyde as described in the text. The ^{3}H-labelled extract and the ^{14}C-labelled extract were mixed at a d.p.m. ratio of 7:1 (6×10^6 d.p.m. of ^{3}H and 8.6×10^5 d.p.m. of ^{14}C) before being analysed on a two-dimensional gel; (i) fluorogram of the gel developed with Kodak X-Omat XR5 film for 24 h at −70°C, showing both ^{3}H- and ^{14}C-labelled-protein spots [in a separate experiment, it was found that at a 7:1 d.p.m. ratio of ^{3}H to ^{14}C, both ^{3}H- and ^{14}C-labelled proteins appeared with equal intensities on the fluorograms (results not shown)]; (ii) autoradiograph of the same gel developed with Ilford X-ray Ilfex 90 film for 20 days at ambient temperature showing ^{14}C-labelled proteins. Comparison of (i) and (ii) indicates that they are identical. (*b*) ^{3}H-labelled mouse liver crude extract was mixed with ^{14}C-labelled human liver extract (6×10^6 d.p.m. and 8.6×10^5 d.p.m. respectively) before being resolved on a two-dimensional gel; (i) fluorogram of the gel developed for 16 h, showing both the mouse (^{3}H-labelled) proteins and the human (^{14}C-labelled) proteins; (ii) autoradiograph of the same gel developed for 30 days showing ^{14}C-labelled human liver proteins. Spots present in (i) and absent in (ii) are therefore specific to the mouse liver extract. A number of major differences are indicated by circles.

case of ^{14}C-labelled proteins. Samples were freeze-dried and dissolved in 30μl of sample-dilution buffer [9.5 M-urea/2% ampholines (comprising 1.6% pH 5–8, 0.4% pH 3.5–10)/5% mercaptoethanol/8% (v/v) Nonidet P40 (Choo *et al.*, 1979)]. The two-dimensional electrophoresis was modified from that of Choo *et al.* (1979) by using first-dimension gels 120mm in length (acrylamide and *NN'*-methylenebisacrylamide were from Eastman, Rochester, NY, U.S.A.) and electrophoresis was at 10mA for 25min then at 25mA for 4–5h; in the second dimension, one running buffer containing 0.1% SDS (O'Farrell, 1975) was used throughout.

Gels were fixed overnight in 3.5% (v/v) $HClO_4$ at room temperature.

Double-label experiments and radioisotope detection

^3H- and ^{14}C-labelled proteins on gels were detected by fluorography. Gels were impregnated with PPO and dried as described by Bonner & Laskey (1974). Fluorographic exposure was made by placing Kodak X-Omat XR5 film over the dried gel at −70°C. In double-label experiments, ^3H and ^{14}C samples were mixed to a d.p.m. ratio of 7:1. This ratio gives equal intensity of ^3H and ^{14}C spots for a given fluorographic exposure time.

^{14}C-labelled proteins were detected on a double-label gel by the method of McConkey (1979) using no-screen film, which is insensitive to both the β-particles (autoradiography) and the photon flashes (fluorography) of ^3H decay, and detects only the ^{14}C isotope (McConkey, 1979). We have tested three different no-screen films (Ilford X-ray Ilfex 90 film, Kodak NS-2T and Kodak no-screen NS-5T films) and always detected a small amount of the ^3H in the major spots. It is not difficult to allow for this in interpreting the gels.

Results

By using the methods of reductive methylation described, a specific radioisotope incorporation of up to (2–4) $\times 10^5$ d.p.m./μg of crude liver extract protein was achieved with ^3H. With ^{14}C, the incorporation was approx. 2×10^4 d.p.m./μg of crude extract protein. This meant that as little as 10–20μg of protein was required for a gel run to enable detection of several hundred polypeptide spots over a 2–3-day exposure period (see below). Specific ^{14}C incorporation could be improved up to 2-fold by allowing the reaction to continue overnight, but for double-labelling experiments (see below), the reaction was limited to 30min in order to keep the ^{14}C- and ^3H-labelling conditions as similar as possible. [We have also tried ^{14}C-labelling with $NaBH_4$ and [^{14}C]formaldehyde (instead of $NaCNBH_3$ and [^{14}C]formaldehyde; see the Experimental section), but the specific radioisotope incorporation was relatively poor (2×10^3 d.p.m./μg of crude extract protein)].

Fluorograms of one sample radiolabelled on different occasions gave very consistent and reproducible labelling patterns (results not shown). The two-dimensional gel pattern of a sample labelled with ^3H was identical with that of the same sample labelled with ^{14}C (Fig. 1*a*). This indicates that labelling with ^3H and ^{14}C resulted in identical electrophoretic mobility of the polypeptides. The radiolabelled extracts were not affected by storage of up to 2 months at −20°C. Fig. 1(*b*) illustrates the use of the double-labelling method to compare polypeptide patterns between a human liver extract and a mouse liver extract. Many spot differences could be detected. High specific labelling and good resolution of proteins has also been achieved with extracts other than liver (results not shown).

Discussion

The present technique allows proteins present in tissue samples to be studied on two-dimensional gels with the full advantage of the sensitivity provided by radioactive labelling and the precision introduced by double-isotope-labelling, comparable with techniques already available for cells in culture.

The method of reductive methylation described has proved to provide: (i) high specific radioactivity of labelling, which decreases film exposure times, particularly those required for the detection of ^{14}C isotope in autoradiography, and decreases the quantity of protein applied to the gel, thereby improving the separation of proteins (O'Farrell, 1975); (ii) good reproducibility of protein-labelling patterns, allowing comparison of samples labelled at different times; and (ii) identical ^3H- and ^{14}C-labelling patterns, meaning that any polypeptide differences seen between two samples under comparison cannot be attributed to artefacts introduced during the radioactive-labelling procedure.

The procedure should facilitate the study of tissue proteins by two-dimensional polyacrylamide-gel electrophoresis.

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