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NOTE

Method and use of Recycled [^{35}S]Sulphate in metabolic labelling.

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Summary

Efficiency of [^{35}S]sulphate incorporation into macromolecules in metabolic labelling experiments has been uniformly poor, 0.0005-0.35% (1-6). A procedure is described for recovering approximately 90% of the unused [^{35}S]sulphate from such a labelling experiment in a form in which it can be used again, thus improving (asymptotic) incorporation efficiency up to tenfold.

Introduction

Although production of ^{35}S labelled glycoprotein, proteoglycan, and glycosaminoglycan by metabolic labelling of cells with [^{35}S]sulphate is a well-established procedure, it is very inefficient and only 0.005-0.35% of [^{35}S]sulphate is usually incorporated into macromolecules (1-6). More than 99% of the label ends up being thrown away.

Within certain limits, the quantity of radioactivity incorporated into total macromolecules will be proportional to the concentration of [^{35}S]sulphate in the labelling medium and

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inversely proportional to the concentration of [^{32}S]sulphate, so long as the enzymes responsible for sulphation is saturated with respect to sulphate.

The unused [^{35}S]sulphate must be recovered in a re-usable form. Therefore conditions which have to be met for the recovery method include: a) the ^{32}S/^{35}S ratio should not increase; b) toxic waste products should be removed; c) the concentration of ^{35}S should not decrease significantly. These conditions were fulfilled by the use of a suitable high-capacity ion-exchange column.

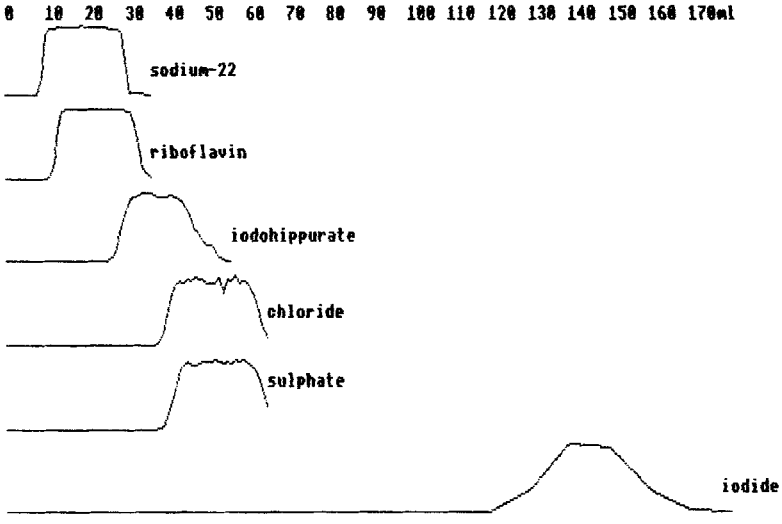
Experimental.

Pilot separation runs:

Two chromatography columns were connected in series with minimal intervening dead space; the first, 7mm x 5cm was packed with DEAE Sepharose CL6B, capacity approx 100μmol/ml, and the second, 5.6mm x 32cm, was packed with QAE Sephadex A25, capacity 500μmol/ml. Both gels were pre-equilibrated with 140mM NaCl, 10mM phosphate buffer, pH 7.4, with 2mM KI and 1mg/ml bovine serum albumin. Aliquots of 20ml buffer containing tracer quantities of [^{35}S]sulphate, [^{22}Na]sodium chloride, sodium [^{36}Cl]chloride, riboflavin, [^{131}I]iodohippuric acid, sodium [^{125}I]iodide, and sodium [^{99m}Tc]pertechnetate were passed through the columns at 0.6 ml/min. 1ml fractions were collected, and the elution profiles were noted. In each case a plateau of duration approximately 20 ml was noted (Fig 1).

Cell culture:

Bovine glomeruli were isolated and cultured as previously described (7). Glomeruli were plated out in 25 cm^2 plastic flasks in Dulbecco's Modification of Eagle's Medium (Flow Laboratories) containing 5% foetal calf serum (FCS), 2.5% Ewing's Sarcoma Growth Factor (Northumbria Biologicals) (ESG), 100μg/ml streptomycin sulphate, and 100 u/ml benzyl penicillin.



Elution profiles of 20ml aliquots containing various labelled substances. [^{99m}Tc]pertechnetate not shown as it elutes at 450ml. Vertical scales are optical density (riboflavin) or counts per minute (others, each on a different scale).

Labelling medium:

75% confluent cultures were transferred to medium composed as above except for the substitution of $MgCl_2$ for $MgSO_4$, and streptomycin chloride for streptomycin sulphate, and the addition of 500µCi/ml [^{35}S]sulphuric acid (carrier-free, New England Nuclear). 3.7 ml of medium was used for each 25cm^2 flask, and six flasks were labelled simultaneously.

Product and Label recovery:

Chromatography columns were set up as described for the pilot run, but this time both gels were thoroughly pre-equilibrated with vacuum-degassed labelling medium without ^{35}S (pH 8.4 approx). The 20 ml of pooled supernatant from the labelled cultures two days after labelling was degassed and then passed down the two columns in series, followed by degassed unlabelled labelling medium, at 160µl/min, and 1ml fractions were collected.

The 20 fractions containing most [^{35}S]sulphate were determined and pooled, and [^{35}S]sulphate was determined by scintillation counting of a small aliquot. The recovered label was then re-gassed in 5% CO_2 to a pH of 7.4, and the resultant medium was then topped up with fresh label to the original activity of 500μCi/ml, and used as labelling medium for the same cells.

The first column was disconnected from the second, and washed with phosphate-buffered saline (PBS; pH 7.0, ionic strength 150mM) until the effluent was free of unincorporated label. The column was then eluted with 1M NaCl 10mM phosphate pH 7.4. The total eluate was then dialysed against PBS and an aliquot counted.

The same procedure was then used again after a further two days, except that previously unlabelled cells were then labelled with the recovered material; altogether five such two day labelling experiments were performed successively with the same 10mCi of [^{35}S]sulphate (plus 4.2 mCi for topping up), leaving finally an aliquot of medium containing 9.5 mCi of sulphate which can be recycled again as required.

p-Nitrophenyl-beta-D-xyloside (NPX) as a chain initiator:
Similar experiments have been carried out using 2mM NPX in the labelling medium and column medium, as this has been reported to increase the yield of labelled product, and to alter its nature (8).

Results

Recovery of [^{35}S]sulphate was 85%, 87%, 95%, 90%, and 95% on successive runs. The fractions containing most [^{35}S]sulphate were from 43-62ml, and were thus delayed 11ml after the end of the fractions containing uncharged low-molecular weight constituents of the used medium.

Radiation dose to cells was calculated to be 700rads/24hours during labelling. We did not find evidence of morphological change in cultures after two days. After the second labelling on the same cells however, approximately 25% of the cells were showing morphological evidence of dysfunction. When the subsequently reprocessed medium was applied to a fresh set of cells, there was no morphological change after two days, but again after a further two days there was morphological change as 1before. We therefore ascribed these changes to the accumulated radiation dose of 2,800 rads rather than to being in reprocessed medium.

Yield of product recovered from the medium by ion-exchange on was 32.2 +/- 2.2 µCi. Percentage incorporation of sulphate (product/total sulphate fed to cells) was therefore 0.32 +/- 0.02 % respectively. With NPX yields were successively 182 +/- 23 µCi, and [^{35}S]sulphate were similar to the non-NPX values. Percentage incorporation of sulphate was therefore 1.82 +/- 0.23 % respectively.

Discussion.

Recovery of label from a metabolic labelling experiment is only worthwhile if the proportion otherwise incorporated into product is low and if the label would be prohibitively expensive in the quantities that would otherwise be required. In addition, it is only possible by the method described above if the label is ionic and suitably delayed on some ion-exchange column; the product must be poly-charged, or isolatable by affinity chromatography on the first column. The method as described was designed for the production of mCi quantities of metabolically labelled proteoglycan and glycosaminoglycan as secreted into the medium. When combined with NPX the asymptotic efficiency of label incorporation, expressed as

amount of label incorporated into product/amount of label not recovered for re-use was 18%, while without NPX it was 3.1%.

We were not able to measure total sulphate at the levels present in our labelling medium, but input-output balance considerations make it unlikely that sulphate concentration could have increased significantly as the columns were pre-equilibrated in medium containing the same concentration of sulphate as that in the medium being reprocessed, and continued to run in the same medium. In addition, had there been a steady increase in unlabelled sulphate concentration, a fall-off in yield of labelled product would have been expected.

Similarly we have not been able to demonstrate that the waste products of interest have definitely been separated from the label; our pilot run shows that wide separations of different anions may be expected, but we have only tested readily available radiolabelled anions. It is, however, likely that toxic substances are not accumulating as no deleterious effect on morphology or productivity of fresh cells incubated with the recovered label was observed.

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