Synthesis of ^{35}S -labelled macromolecules by polymorphonuclear neutrophils

Evidence for the production of [^{35}S]sulphite which can modify both endogenous and exogenous proteins

Elizabeth E. GARDINER,* H. Clem ROBINSON,* Absorn SRIRATANA,* Su San MOK,*†
Dennis A. LOWTHER* and Christopher J. HANDLEY*‡

*Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia

The incorporation of [^{35}S]sulphate into macromolecules by rabbit peritoneal polymorphonuclear neutrophils (PMN) *in vitro* revealed that two major groups of ^{35}S -labelled macromolecules were synthesized by these cells. The first group did not bind to anion-exchange columns at pH 6.0 and contained 60–80% of the total incorporated radiolabel. The second group did bind to anion-exchange columns at pH 6.0 and eluted as a single peak of radioactivity at an ionic strength characteristic of sulphated proteoglycans; it accounted for the remaining incorporated radiolabel. Analysis of this material on Sepharose CL-6B demonstrated that ^{35}S -labelled macromolecules isolated from the cell extract migrated with K_{av} of 0.36, while corresponding material isolated from the medium migrated with K_{av} of 0.51. When subjected to electrophoresis on SDS/polyacrylamide gels the intact proteoglycan had a molecular mass of approx. 90 kDa and yielded two core proteins of molecular mass 31 kDa and 28 kDa after digestion with chondroitinase ABC. The peak of labelled macromolecules which did not bind to the anion-exchange column was found, by SDS/PAGE, to comprise ^{35}S -labelled proteins of various molecular masses. The ^{35}S label was displaced from this fraction by treatment with 0.1 M-sodium sulphite, suggesting that the radiolabel was in the form of an S-sulpho sulphite derivative. Using the sulphite-trapping agents *N*-2,4-dinitroanilinomaleimide and cyst(e)ine, [^{35}S]sulphite was detected in the incubation medium of PMN, indicating that these cells were able to synthesize [^{35}S]sulphite from [^{35}S]sulphate. The release of [^{35}S]sulphite from neutrophil cultures was calculated to be 78 pmol/h per 10^6 cells. When exogenous proteins were included in the incubation medium of cell cultures, the [^{35}S]sulphite reacted with these proteins to form a stable ^{35}S -labelled conjugate.

INTRODUCTION

Polymorphonuclear neutrophils (PMN) have a major role as mediators of the inflammatory response. PMN are involved in the phagocytosis and intracellular degradation of particles found at the site of tissue injury and infection (Barrett, 1978; Foster *et al.*, 1988) and use a variety of proteolytic enzymes, cytokines and free radicals to carry out these processes. PMN also synthesize a population of proteoglycans (Olsson *et al.*, 1968; Murata, 1974) and a number of proteins (Beaulieu *et al.*, 1987) which are associated with intracellular storage granules. The role of both the proteoglycans and the proteins in the biology of the PMN remains unclear.

The aim of this work was to characterize the ^{35}S -labelled macromolecules synthesized by PMN in order to ascertain their mechanism of synthesis.

MATERIALS AND METHODS

Materials

$\text{H}_2^{35}\text{SO}_4$ (carrier-free) was purchased from Du Pont (Wilmington, DE, U.S.A.), Sephadex G-25 (medium grade, pre-packed in PD-10 columns), Sephadex G-50 (fine grade), Sepharose CL-6B and Q-Sepharose were obtained from Pharmacia (Uppsala, Sweden), Dowex 1-X4 (200–400 mesh) from Bio-Rad (North Ryde, NSW, Australia), non-sterile plastic vials and centrifuge tubes from Disposable Products (Melbourne, Victoria,

Australia), urea, NH_4HCO_3 , sodium EDTA, NaCl, sodium acetate and NaN_3 from Ajax Chemicals (Auburn, N.S.W., Australia) and guanidinium chloride, glycogen (Type II, from oyster), mouse serum albumin, BSA, chondroitinase AC, soyabean trypsin inhibitor, phenylmethanesulphonyl fluoride, benzamidine hydrochloride, iodoacetic acid and aminohexanoic acid from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chondroitinase ABC (protease-free, from *Proteus vulgaris*) was obtained from ICN (Costa Mesa, CA, U.S.A.), *N*-ethylmaleimide and the anaesthetic Hypnorm from Janssen Pharmaceutica (West Germany), *NNN'*-tetramethylenediamine, bisacrylamide, SDS, ammonium persulphate, sodium sulphite and Triton X-100 from BDH Chemicals Ltd. (Poole, Dorset, U.K.), Amplify from Amersham International (Amersham, Bucks., U.K.) and Kodak X-Omat AR film from Kodak (Melbourne, Victoria, Australia). S-Sulphocysteine was prepared as described by Robinson & Pasternak (1964).

Cell cultures

PMN, collected from the peritoneal cavity of adult laboratory rabbits of mixed breeds as described by Cohn & Hirsch (1960), were washed in saline and freed of contaminating red blood cells by hypo-osmotic lysis. PMN were also isolated from the peripheral blood of rabbits according to the method of Pryce-Jones & Wood (1975), using acid citrate dextrose as an anticoagulant. Cells were counted and their viability found to be greater than 95% when assessed by Trypan Blue exclusion. Unless indicated

Abbreviations used: PMN, polymorphonuclear neutrophils; DNAM, *N*-2,4-dinitroanilinomaleimide.

† Present address: Department of Biochemistry, Rush-Presbyterian-St Lukes Medical Centre, 1653 West Congress Parkway, Chicago 60612-3864, IL, U.S.A.

‡ To whom correspondence should be addressed.

otherwise, all experiments reported in this paper utilized peritoneal PMN.

For experiments studying the kinetics of synthesis of sulphated macromolecules, 10^7 PMN were distributed into flasks and incubated, with shaking, at 37 °C in a water bath in 1.0 ml of a labelling medium of normal or sulphate-free Hanks balanced salt solution (Hanks & Wallace, 1949) containing 20 μ Ci of [35 S]-sulphate/ml. At various times samples of the incubation mixtures were taken and treated with a proteinase-inhibitor cocktail containing (final concentration): 50 mM-Tris/HCl, pH 7.4, 10 mM-sodium EDTA, 5.0 mM-benzamidine hydrochloride, 0.1 mM-phenylmethanesulphonyl fluoride, 1.0 mM-iodoacetic acid, 10 mM-aminohexanoic acid and 10 μ g of soya-bean trypsin inhibitor/ml. The cells and medium were then separated by centrifugation and cells were extracted with 4.0 M-guanidinium chloride containing 2% (v/v) Triton X-100 at 4 °C for 48 h.

In some experiments PMN were incubated in a similar incubation medium containing mouse serum albumin or BSA (1.0 mg/ml) and 0.1 mCi of [35 S]sulphate/ml. After 2 h proteinase inhibitors were added to the cell cultures which were then centrifuged and the resultant supernatants were analysed.

In experiments investigating the nature of granule proteoglycans, PMN (10^9 cells) were incubated for 1 h at 37 °C in 5.0 ml of sulphate-free Hanks balanced salt solution containing 1.0 mCi of [35 S]sulphate.

Analysis of 35 S-labelled macromolecules

Samples of medium or cell extracts were applied to columns of Sephadex G-25 eluted with 4.0 M-guanidinium chloride containing 0.05 M-sodium acetate (pH 6.0), 0.2% (v/v) Triton X-100 (pH 6.0) and 0.1 M-sodium sulphate. Fractions (1.0 ml) were collected and counted for radioactivity. Aliquots of incubation medium or cell extracts containing 35 S-labelled macromolecules were also subjected to size-exclusion chromatography on a column of Sephadex G-50 (16.0 cm $\times$ 1.5 cm) equilibrated in 6.0 M-urea containing 0.05 M-sodium acetate, pH 6.0, 0.15 M-sodium chloride and 0.5% (v/v) Triton X-100. The sulphated macromolecules appearing in the excluded-volume fractions were then applied to a column of Q-Sepharose (11.0 cm $\times$ 1.2 cm) equilibrated with the 6.0 M-urea solution at a flow rate of 30 ml/h. The column was washed with 60 ml of 6.0 M-urea solution and was then eluted with a linear gradient (0.15–1.5 M-sodium chloride; 100 ml total volume) in the 6.0 M-urea solution. Fractions (2.8 ml) were collected and 1.0 ml aliquots were counted for radioactivity.

Fractions containing 35 S-labelled macromolecules which did not bind to Q-Sepharose were pooled, dialysed against distilled water, freeze-dried and then redissolved in water. Some samples were treated with 0.1 M-sodium sulphite for 2 h at room temperature. Native and sulphite-treated samples were analysed by size-exclusion chromatography on a column of Sepharose CL-6B (115 cm $\times$ 1.5 cm) eluted at a flow rate of 6.0 ml/h with 4.0 M-guanidinium chloride containing 0.05 M-sodium acetate, pH 6.0, 0.1 M-sodium sulphate and 0.2% (v/v) Triton X-100. Fractions (2.0 ml) were collected and 1.0 ml aliquots counted for radioactivity. Samples of medium and cell extracts to be analysed by electrophoresis were applied to a column of Sephadex G-50 equilibrated in 0.05 M-ammonium bicarbonate and fractions containing radiolabelled macromolecules were pooled and freeze-dried. The dry material was resuspended in water and subjected to electrophoresis under non-reducing conditions on 5–20% gradient polyacrylamide/SDS gels (Laemmli, 1970). The gels were soaked in Amplify, dried and subjected to fluorography. Samples of media from PMN cultures containing either BSA or mouse serum albumin were also analysed by electrophoresis under non-reducing conditions.

Extraction of PMN granules and analysis of 35 S-labelled macromolecules

PMN granules were obtained by centrifugation using the method described by Sandy *et al.* (1981). The granules were extracted with 4.0 M-guanidinium chloride in the presence of proteinase inhibitors and the resultant extract applied to a column of Sephadex G-50 (20 cm $\times$ 1.5 cm) equilibrated in 0.05 M-acetate buffer, pH 6.0, containing 7.0 M-urea and 0.15 M-NaCl. The void volume was collected and applied to a column of DEAE-Sephacel (11 cm $\times$ 1.2 cm) equilibrated in the same buffer. The column was washed with 50 ml of buffer before a gradient of NaCl (0.15–1.5 M; 100 ml total volume) was applied. Fractions corresponding to the peak of radioactivity, which eluted at the position of proteoglycan (0.75 M-NaCl), were pooled, dialysed against distilled water and freeze-dried.

Portions of 35 S-labelled proteoglycan were redissolved in 0.2 ml of 0.1 M-Tris/HCl buffer containing 0.1 M-sodium acetate, pH 7.0. Some samples were treated with 0.06 unit of chondroitinase ABC in the presence of proteinase inhibitors for 24 h at room temperature (Oike *et al.*, 1980) and were then analysed by gel electrophoresis under reducing conditions. Samples of 35 S-labelled proteoglycan isolated from both the granules and incubation medium on DEAE-Sephacel were also analysed on Sepharose CL-6B. Some samples were treated with 1.0 M-sodium borohydride in 0.5 M-NaOH at 25 °C for 48 h, then neutralized with acetic acid before elution from Sepharose CL-6B.

Identification of inorganic [35 S]sulphite

Inorganic [35 S]sulphite was identified by reaction with *N*-2,4-dinitroanilinomaleimide (DNAM) according to the method of Robinson (1965). Samples of sonicated cell supernatant and medium (1.0 ml) were mixed with 0.5 ml of 0.01 M-DNAM in acetone. Aliquots of the reaction mix (30 μ l) were analysed by ascending paper chromatography on Whatman 3MM paper using one of the following solvents: A, butanol/pyridine/acetic acid/water (15:10:3:12, by vol.); or B, 2-methylpropan-2-ol/formic acid/water (14:3:3, by vol.). The chromatogram was then cut into 1.0 cm divisions and assayed for radioactivity. The sulphite addition compound formed with DNAM moved as a discreet spot with R_f values of 0.86 (solvent A) and 0.47 (solvent B).

Sulphite production by PMN was also characterized by reacting the culture medium from PMN, which had been incubated for 1 h with sulphate-free Hanks balanced salt solution containing 0.1 mCi [35 S]sulphate per 10^8 cells, with 10 mM-cyst(e)ine to form S-[35 S]sulphocysteine. Standard S-sulphocysteine was added to the reaction mixture and the solution was applied to a column (40 cm $\times$ 1.2 cm) of Dowex 1-X4 (formate form) equilibrated with water. The column was eluted with a linear gradient generated from 500 ml of water and 500 ml of 1.0 M-ammonium formate in 1.25 M-formic acid. Fractions of 20 ml were collected and aliquots (1 ml) counted for radioactivity. Aliquots of the fractions were also separated by paper chromatography in solvent A, stained with ninhydrin reagent and assayed for radioactivity.

Measurement of [35 S]sulphite production by PMN

Cultures of 1.6×10^7 PMN were incubated in Hanks balanced salt solution containing 0.1 mM-sodium sulphate and 50 μ Ci [35 S]sulphate/ml for 2 h. The cells and medium were then separated by centrifugation, and the cell pellet resuspended in 1.0 ml of Hanks balanced salt solution containing 0.1 mM-sodium sulphate. The cells were then sonicated and both the sonicated cell suspension and the medium were reacted separately with DNAM. Samples were analysed by paper chromatography using

solvent A. Radioactivity, which migrated to the same R_f value as the standard compound formed between DNAM and sodium sulphite, was considered to represent [^{35}S]sulphite remaining within the cell or released into the incubation medium after 2 h.

RESULTS

Kinetics of synthesis of ^{35}S -labelled macromolecules by PMN

Cultures containing 10^7 PMN were incubated with [^{35}S]sulphate in normal or sulphate-free Hanks balanced salt solution for up to 20 h. At the end of the incubation period, cells and medium were separated and the ^{35}S -labelled macromolecules were extracted and analysed by size-exclusion chromatography on Sephadex G-25. Fig. 1 shows the incorporation of [^{35}S]sulphate into macromolecules in either cell or medium fractions of PMN

Fig. 1. Incorporation of ^{35}S radiolabel from [^{35}S]sulphate into macromolecules in cultures of PMN

Duplicate cultures of PMN were incubated with [^{35}S]sulphate in normal ($\square$, $\blacksquare$) or sulphate-free ($\circ$, $\bullet$) medium for up to 20 h. Cell ($\blacksquare$, $\bullet$) and medium ($\square$, $\circ$) fractions were separated and the amounts of ^{35}S -labelled macromolecules in each fraction were determined by size-exclusion chromatography on Sephadex G-25 as described in the Materials and methods section.

Fig. 2. Separation on Q-Sepharose of ^{35}S -labelled macromolecules in the culture medium of PMN

^{35}S -labelled macromolecules were isolated from the medium of PMN incubated with [^{35}S]sulphate by size-exclusion chromatography on Sephadex G-50, applied to Q-Sepharose equilibrated with a buffer containing 6.0 M-urea, and eluted with a linear NaCl gradient as described in the Materials and methods section.

Fig. 3. Fractionation on Sepharose CL-6B of ^{35}S -labelled material from medium or granules of labelled PMN which bound to DEAE-Sephacel

^{35}S -labelled macromolecules from PMN granules (a) or medium (b) which bound to DEAE-Sephacel were pooled. Native samples ($\bullet$) or samples treated with 1.0 M-sodium borohydride ($\circ$) were fractionated on Sepharose CL-6B as outlined in the Materials and methods section.

cultures with time. The release of ^{35}S -labelled macromolecules from the cells occurred quickly and in the absence of any stimulatory agent. The distribution of the ^{35}S -labelled macromolecules in the cell pellet and the medium changed during the course of the incubation and also altered when 0.4 mM- MgSO_4 was added to the culture medium. The proportion of the total incorporated label recovered from the cell pellet was, however, within the range 20–40 % in four different experiments regardless of incubation time or addition of MgSO_4 . As expected, addition of 0.4 mM unlabelled MgSO_4 to the incubation mixture reduced the overall incorporation of ^{35}S , so in most of the following experiments sulphate-free medium was used.

Samples of cell extract and medium were subjected to ion-exchange chromatography on Q-Sepharose. Fig. 2 shows the elution profile of ^{35}S -labelled macromolecules in the medium fraction and indicates that approx. 25 % of total [^{35}S]sulphate incorporation eluted in the position characteristic of proteoglycan. The remaining 75 % of incorporated radiolabel did not bind to Q-Sepharose and eluted before the gradient commenced. When samples of guanidinium-chloride extracts of PMN were analysed on Q-Sepharose approx. 50 % of radiolabelled macromolecules bound to the column and eluted in the position of proteoglycan, while the remaining 50 % of incorporated label did not bind. Similar results were obtained when samples were analysed by ion-exchange chromatography on DEAE-Sephacel.

Although addition of 0.4 mM unlabelled MgSO_4 to the incubation mixture was shown to reduce the overall incorporation of ^{35}S into macromolecules, it was found to have little effect on the proportion recovered in the proteoglycan component; thus the results presented in Fig. 1 were used to calculate that 35 % of the total incorporated label (cell pellet plus medium) was in

Fig. 4. Fluorogram of proteoglycan and proteoglycan core protein analysed by SDS/PAGE

^{35}S -labelled proteoglycan eluted from DEAE-Sephacel was analysed by electrophoresis on a 5–20% gradient polyacrylamide/SDS slab gel before and after digestion with chondroitinase ABC. Lane 1 shows native granule ^{35}S -labelled proteoglycan while lane 2 shows ^{35}S -labelled core proteins after digestion with chondroitinase ABC. The approximate molecular mass values of the proteoglycan and major core proteins are shown.

proteoglycan after incubation for 6 h in the absence of added sulphate, while 31% was in proteoglycan after the same period with added sulphate.

Characterization of ^{35}S -labelled macromolecules that bound to anion-exchange columns

Separate experiments established that most of the ^{35}S -labelled proteoglycan extracted from PMN could be recovered in a granule preparation isolated using the method of Sandy *et al.* (1981). The isolation and subsequent extraction of granules was therefore employed for the preparation of intracellular ^{35}S -labelled proteoglycan free from contaminating proteins.

^{35}S -Labelled macromolecules which bound to DEAE-Sephacel, from both the medium fraction and the granule extract, were pooled separately and subjected to size-exclusion chromatography on Sepharose CL-6B under dissociative conditions. Fig. 3(a) shows that ^{35}S -labelled macromolecules isolated from the PMN granules eluted from Sepharose CL-6B with a K_{av} of 0.37, while material isolated from the medium fraction migrated with a K_{av} of 0.51 (Fig. 3b). When treated with NaOH/sodium borohydride the ^{35}S -labelled macromolecules eluted from the column with K_{av} values of 0.63 and 0.71 respectively. This shift in the elution profile of the ^{35}S -labelled macromolecules on Sepharose CL-6B after alkali treatment and their polyanionic nature suggested that the labelled molecules were ^{35}S -labelled proteoglycans.

^{35}S -Labelled proteoglycans from the granule extract were analysed by electrophoresis on a 5–20% gradient polyacrylamide/SDS gel before and after treatment with chondroitinase ABC. Fig. 4 shows that native ^{35}S -labelled granule proteoglycan migrated as a broad band of radioactivity with a mean molecular mass of approx. 90 kDa, characteristic of a small proteoglycan. After digestion with chondroitinase ABC, two ^{35}S -labelled bands of molecular mass 31 kDa and 28 kDa

Fig. 5. Fractionation on Sepharose CL-6B of ^{35}S -labelled material from culture medium of ^{35}S -labelled PMN which did not bind to Q-Sepharose

^{35}S -labelled macromolecules from PMN medium which did not bind to Q-Sepharose were analysed by size-exclusion chromatography before and after treatment with 0.1 M-sodium sulphite. Control (a) and sulphite-treated (b) samples were eluted from Sepharose CL-6B using a dissociative solvent.

respectively were observed. In addition, exhaustive digestion of granule ^{35}S -labelled proteoglycan with chondroitinase AC in the presence of proteinase inhibitors, followed by analysis on Sephadex G-50, revealed that only 77% of the ^{35}S label eluted in the total volume, indicating the presence of dermatan sulphate glycosaminoglycan chains associated with the proteoglycan.

Characterization of ^{35}S -labelled macromolecules that did not bind to anion-exchange columns

^{35}S -Labelled macromolecules that did not bind to anion exchange columns, isolated from either medium or granules of labelled PMN, were treated with Pronase or trypsin and then analysed on a column of Sephadex G-50. More than 95% of the radiolabel in protease-treated samples eluted at the total volume of the column, indicating that the label had been associated with protein. Further analysis of this protein fraction, isolation from either medium or granules on Sepharose CL-6B, showed a broad peak of radiolabelled material with a K_{av} of 0.6 (Fig. 5a). When another sample was treated with 0.1 M-sodium sulphite and then analysed on Sepharose CL-6B (Fig. 5b) the radiolabel eluted at the total volume of the column, suggesting that incorporated ^{35}S label had been displaced by inorganic sulphite. When the control and sulphite-treated samples were separated by electrophoresis on polyacrylamide gels and then subjected to fluorography a similar loss of ^{35}S label was observed (Fig. 6). The track

Fig. 6. Fluorogram from SDS/PAGE of ^{35}S -labelled macromolecules from the medium of PMN which did not bind to Q-Sepharose

Aliquots of the samples analysed in Fig. 5 were subjected to electrophoresis on a 5–20% gradient polyacrylamide slab gel. Lane 1 shows controls and lane 2 shows sulphite-treated ^{35}S -labelled material.

Fig. 7. Isolation of S - ^{35}S sulphocysteine on Dowex 1-X4 from the medium of ^{35}S -labelled PMN which had been treated with cyst(e)ine

A preparation of PMN was incubated for 1 h with ^{35}S sulphate, the medium was then separated and cyst(e)ine added. The sample was applied to a column of Dowex 1-X4 (formate form) and eluted with a linear ammonium formate gradient. Peak 2 (indicated by bar) was shown to be S - ^{35}S sulphocysteine after further analysis by paper chromatography.

containing the untreated sample revealed a number of ^{35}S -labelled proteins, whereas the lane containing sample treated with inorganic sulphite yielded no detectable radioactive bands. This suggested that PMN have the ability to generate ^{35}S sulphite from ^{35}S sulphate which may then react directly with endogenous proteins.

The production of inorganic sulphite by PMN

Experiments were performed to demonstrate the production of inorganic sulphite by PMN. Cultures of PMN were incubated with sulphate-free Hanks balanced salt solution containing

Fig. 8. Rate of production of ^{35}S sulphite by PMN

Duplicate cultures of PMN were incubated with ^{35}S sulphate for up to 2 h, then the cells and medium of each incubation were separated and the cells lysed by sonication. The cell lysate (○) and medium (●) fractions were treated with DNAM. Samples were subjected to paper chromatography using solvent A and the amount of ^{35}S label associated with the addition compound formed with DNAM was determined.

^{35}S sulphate and then were reacted with cysteine or DNAM. In each experiment a ^{35}S -labelled sulphite addition compound was detected by paper chromatography. The S - ^{35}S sulphocysteine product was characterized by ion-exchange chromatography on Dowex 1-X4 with an ammonium formate gradient, and by paper chromatography in either solvent A or B. Fig. 7 illustrates the elution profile of PMN labelling medium, which was treated with cyst(e)ine solution and then applied to a column of Dowex 1-X4. The sample eluted as three main peaks of ^{35}S radioactivity. The first peak eluted at a low ammonium formate concentration and was also present in the control labelling medium. This unknown peak has not been characterized further; however, its elution position suggests that it is not S - ^{35}S sulphogluthathione. Paper chromatography performed in solvent A confirmed that the second radioactive peak (fractions 75–79) migrated to the same position as standard S -sulphocysteine. The third peak of ^{35}S -labelled material was unincorporated ^{35}S sulphate.

The radiolabelled adduct formed with DNAM was yellow in colour and moved as a discreet spot with the same R_f value as the addition compound formed between DNAM and unlabelled inorganic sulphite. DNAM was used routinely for measurement of sulphite production by cell cultures.

Rate of production of sulphite by PMN

Fig. 8 illustrates that there was linear production of ^{35}S -sulphite with time. The rate of sulphite synthesis was calculated to be 78 pmol/h per 10^6 cells. Under experimental conditions, this was equivalent to a concentration of $2.5 \mu\text{M}$ -sulphite present in the incubation medium after 2 h. In a similar experiment using peripheral PMN isolated from rabbit blood treated with anti-coagulant, the rate of sulphite release into the culture medium was estimated to be 61 pmol/h per 10^6 cells.

Incorporation of ^{35}S label into exogenous proteins in the labelling medium of PMN

PMN were incubated in sulphate-free Hanks balanced salt solution containing ^{35}S sulphate in the presence of either mouse

Fig. 9. Fluorogram from SDS/PAGE of ^{35}S -labelled proteins derived from PMN incubated with ^{35}S -sulphate and mouse serum albumin or BSA

Separate preparations of PMN were incubated with ^{35}S -sulphate and either mouse serum albumin or BSA for 2 h. The medium was separated from the cells and analysed by electrophoresis on a 12% polyacrylamide/SDS slab gel. Lane 1, medium alone; lane 2, medium containing mouse serum albumin; lane 3, medium containing BSA. Arrow heads indicate the major bands corresponding to mouse serum albumin (67 kDa) and BSA (65.5 kDa).

serum albumin or BSA for 2 h. Aliquots of medium containing ^{35}S -labelled macromolecules were analysed by gel electrophoresis on a 12% polyacrylamide/SDS gel, and then subjected to fluorography. Fig. 9 shows that the two exogenous proteins present in the medium of PMN became labelled. Further treatment of the labelled medium containing BSA with 0.1 M-sodium sulphite for 2 h at 20 °C resulted in the loss of the ^{35}S label from the BSA band.

DISCUSSION

PMN were shown to incorporate ^{35}S -sulphate into macromolecules in a linear manner over a 6 h time period *in vitro* and ^{35}S -labelled macromolecules were detected in the incubation medium with no appreciable lag time. Analysis by ion-exchange chromatography on either Q-Sepharose or DEAE-Sephacel demonstrated that there were two groups of ^{35}S -labelled macromolecules present in both medium and granule fractions with differing ionic properties. ^{35}S -Labelled macromolecules from granules of PMN which bound to anion-exchange columns were estimated, by PAGE, to have a molecular mass of approx. 90 kDa. Digestion with chondroitinase ABC yielded two radiolabelled polypeptides with molecular masses estimated to be 31 kDa and 28 kDa respectively. Several phagocyte populations have been reported to synthesize chondroitin sulphate proteoglycan, including macrophages (Takasu *et al.*, 1982; Uhlin-Hansen *et al.*, 1989), mast cells (Stevens *et al.*, 1988) and

basophils (Orenstein *et al.*, 1978). Lohmander *et al.* (1990) studied the intracellular proteoglycan produced by HL-60 human promyelocytic leukemia cells, a cell line which exhibits many synthetic characteristics of several haematopoietic cell types and showed core proteins of similar sizes to those reported in this paper. The occurrence of two core proteins suggests that there may be differences in the *N*-glycosylation of the two core proteins (Hey *et al.*, 1990). ^{35}S -Labelled proteoglycans isolated from the granule extract or the medium fraction of PMN were also analysed by size-exclusion chromatography on Sepharose CL-6B. Comparison of K_{av} values suggests that the granule proteoglycan is larger than proteoglycan isolated from the medium after incubation of PMN.

The majority of the radiolabel, however, was observed to be incorporated into proteins which did not bind to anion-exchange columns. The ^{35}S label was not affected by mild acid treatment which is known to cleave tyrosine *O*-sulphate ester links (Huttner, 1984), but was displaced from the macromolecules by treatment with sodium sulphite or cysteine at neutral pH. The latter reagents were used to investigate the possibility that the ^{35}S label was associated with an *S*-sulpho derivative (Roy & Trudinger, 1970). This displacement with sulphite was demonstrated using both size-exclusion chromatography and PAGE, and is consistent with the radiolabel being present in the protein as an *S*-sulpho derivative. The formation of such derivatives is equivalent to the production of inorganic sulphite and may even be the consequence of it, because the reaction

is readily reversible under physiological conditions and needs no enzyme catalyst (Cecil, 1963).

This paper demonstrates that PMN secrete inorganic sulphite into the incubation medium, since ^{35}S -sulphite was detected in both the medium and sonicated cell supernatant of PMN after incubation with ^{35}S -sulphate using DNAM as a sulphite-trapping agent. Similar results were also achieved using cyst(e)ine or phenacyl bromide to trap sulphite. The production of ^{35}S -sulphite by PMN was linear with time for up to 2 h and its release into the incubation medium was rapid. Similar experiments using PMN isolated from peripheral blood indicated that activation of PMN was not essential for sulphite production. Results presented in this paper demonstrate that ^{35}S -sulphite was then capable of reacting with both exogenous and endogenous proteins present in the medium.

Studies on sulphite production by cells have focused mainly on sulphate-reducing systems in micro-organisms (Schwenn *et al.*, 1988; Kletzin, 1989). Sulphate reduction has been reported in mammalian tissues (Waley, 1959; Wortman, 1963; Robinson & Pasternak, 1964); however, little is known about the mechanism. It is likely that it involves a 3'-phosphoadenylylsulphate sulphotransferase and at least one carrier protein, speculated to be thioredoxin (Schwenn *et al.*, 1988).

Sulphite production by PMN may be an additional weapon against bacterial invasion. Sulphite has been shown to be toxic to cultures of bacteria at a concentration of 1.0 mM (Shapiro, 1977). However, the biological role of sulphite may be somewhat more complex than simply toxicity. Sulphite is known to react with disulphide bonds of proteins in plasma to form thiols and *S*-sulpho compounds (Cecil, 1963). Work by other groups has shown that sulphite in the presence of peroxidase/ H_2O_2 or Co(II) ions can cause site-specific fragmentation of BSA (Ito & Kawanishi, 1991) and DNA (Kawanishi *et al.*, 1989). PMN may release sulphite to chemically modify the structure of a protein, and consequently enhance the antigenicity of that protein. It is indeed possible that the effects of sulphite released by an influx of PMN within the confines of an articulated joint could lead to

modification of proteins within the joint tissues sufficient to cause a chronic autoimmune response of the type characteristic of rheumatoid arthritis.

This project was supported by the National Health and Medical Research Council of Australia.

REFERENCES

- Barrett, A. J. (1978) *Agents Actions* **8**, 11–17
- Beaulieu, A. D., Lang, F., Belles-Isles, M. & Poubelle, P. (1987) *J. Rheumatol.* **14**, 656–661
- Cecil, R. (1963) in *The Proteins* (Neurath, H., ed.), pp. 379–476, 2nd edn., Academic Press, New York
- Cohn, Z. A. & Hirsch, J. G. (1960) *J. Exp. Med.* **112**, 983–1004
- Foster, S. J., Cunliffe, C. J. & McCormick, M. E. (1988) *Biochem. Pharmacol.* **37**, 1181–1183
- Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200
- Hey, N. J., Handley, C. J., Ng, C. K. & Oakes, B. W. (1990) *Biochim. Biophys. Acta* **1034**, 73–80
- Huttner, W. B. (1984) *Methods Enzymol.* **107**, 200–223
- Ito, K. & Kawanishi, S. (1991) *Biochem. Biophys. Res. Commun.* **176**, 1306–1312
- Kawanishi, S., Yamamoto, K. & Inoue, S. (1989) *Biochem. Pharmacol.* **38**, 3491–3496
- Kletzin, A. (1989) *J. Bacteriol.* **171**, 1638–1643
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–681
- Lohmander, L. S., Arnljots, K. & Yanagishita, M. (1990) *J. Biol. Chem.* **265**, 5802–5808
- Murata, K. (1974) *Clin. Chim. Acta* **57**, 115–124
- Oike, Y., Kimata, K., Shinomura, T. & Suzuki, S. (1980) *Biochem. J.* **191**, 203–207
- Olsson, I., Gardell, S. & Thunell, S. (1968) *Biochim. Biophys. Acta* **165**, 309–323
- Orenstein, N. S., Galli, S. J., Dvorak, A. M., Silbert, J. E. & Dvorak, H. F. (1978) *J. Immunol.* **121**, 586–592
- Pryce-Jones, R. H. & Wood, G. C. (1975) *Biochim. Biophys. Acta* **397**, 449–458
- Robinson, H. C. (1965) *Biochem. J.* **94**, 687–691
- Robinson, H. C. & Pasternak, C. A. (1964) *Biochem. J.* **93**, 487–492
- Roy, A. B. & Trudinger, P. A. (1970) in *The Biochemistry of Inorganic Compounds of Sulphur*, pp. 16–19. Cambridge University Press, Cambridge
- Sandy, J. D., Sriratana, A., Brown, H. L. G. & Lowther, D. A. (1981) *Biochem. J.* **193**, 193–202
- Schwenn, J. D., Krone, F. A. & Husmann, K. (1988) *Arch. Microbiol.* **150**, 313–319
- Shapiro, R. (1977) *Mutat. Res.* **39**, 149–176
- Stevens, R. L., Fox, C. C., Lichtenstein, L. M. & Austen, K. F. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2284–2287
- Takasu, Y., Hasumi, F. & Mori, Y. (1982) *Biochim. Biophys. Acta* **716**, 316–323
- Uhlén-Hansen, L., Eskeland, T. & Kolset, S. O. (1989) *J. Biol. Chem.* **264**, 14916–14922
- Waley, S. G. (1959) *Biochem. J.* **71**, 132–137
- Wortman, B. (1963) *Biochim. Biophys. Acta* **77**, 65–72

Received 14 April 1992/28 May 1992; accepted 25 June 1992