Table 2. Incorporation of radioactivity into acid-precipitable material

| Reaction mixture* | Incorporation (cpm) | (%) |
|---|---|---|
| Nuclei (fresh) | 3030 | 100 |
| Nuclei (fresh) + 3 mM benzamide | 320 | 10 |
| Nuclei (fresh) 20 min incubation | 1180 | 40 |
| Nuclei (thawed) | 650 | 21 |

* Standard reaction mixture contained: 2 mM [3H] NAD (100,000 cpm); 80 mM Tris-HCl buffer, pH 7.5, containing 8 mM DTT, 3 mM NaF (20 µl); yeast nuclei (40 µg protein, 2 µl); water, up to 125 µl. Reaction was conducted at 25 °C for 5 min and it was terminated upon addition of 125 µl, ice-cold, 20% trichloroacetic acid. After 30 min at 0 °C the acid precipitated material was collected on a Millipore 0.45-µm filter and the radioactivity measured in a liquid scintillation counter.

When analyzed on native 5% disc gel electrophoresis all peaks migrated with the same Rm as the original, confirming the previous findings. However, when run on 5% slab gel, the active peak migrated slightly above the others, whose mobility remained unchanged, and which remained indistinguishable from each other. The native molecular weight was determined by gel filtration on a TSK 3000 FPLC column in the presence of 40 µg/ml of previously purified bovine serum albumin, for the preservation of the enzyme activity [5]. The result showed a single peak of activity eluted as a 200,000-dalton protein. The active peak (fig., A) was also rechromatographed on the same Mono-Q column, using a pH 8.0 buffer system; this allowed the identification of two active peaks as shown in (B) in the figure. The non-symmetrical appearance of the largest peak of activity suggests that it could also be composed of additional unresolved molecular forms.

When purified non-active peaks P 1, P 2 and P 3 were analyzed for adenine derivatives with the glyoxal procedure of Yuki et al. [7] using ADP-ribose as the standard, a value of 3, 3 and 4 moles of adenine derivatives per mole of protein was found for peaks P 1, P 2 and P 3, respectively. This finding allowed us to speculate that P 1, P 2 and P 3 could represent inactive, modified forms of the enzyme, possibly through poly(ADP-ribosylation).

The presence of poly(ADP-ribose) polymerase activity has not been clearly demonstrated in yeast, even though, for such a microorganism, incorporation of radioactivity from labeled NAD into acid-precipitable material has been reported [8]. Our preliminary experiments using yeast extracts failed to demonstrate appreciable incorporation of acid-precipitable radioactivity from NAD. Therefore nuclei were prepared and purified from cultured yeast cells. Yeast nuclei were isolated from exponentially growing yeast cells, with a procedure developed in our laboratory by Dr J. Schwencke, (Laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette, France) (unpubl. results). The purified nuclear preparation was tested for the presence of poly(ADPR)polymerase activity. [3H]NAD (adenine)a was incubated with crude nuclear preparations in the appropriate reaction mixture, and TCA precipitates were tested for incorporated radioactivity. The results showed that appreciable incorporation could only be detected with freshly prepared nuclei, and that prolonged incubation was detrimental to the extent of the incorporation (table 2). The effect exerted by benzamide, a known inhibitor of poly(ADP-ribose) polymerase [3], indicates that incorporation is due to the formation of the polymer. The accumulated evidence suggests that both poly(ADP-ribose) synthesizing enzyme and the synthesized polymer might be subjected to rapid degradation by specific enzyme activities present in yeast nuclei preparations, as already reported [9, 10]. In order to demonstrate unequivocally the modification of NMN adenylyltransferase through ADP-ribosylation, purification of yeast poly(ADP-ribose) polymerase appears then to be necessary. Work is in progress in our laboratory toward this goal.

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## Affinity chromatography of glucose dehydrogenase

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*Summary.* Porcine liver β-D-glucose dehydrogenase, a multi-functional protein, has been purified to apparent homogeneity. The enzyme has been separated from the endoplasmic reticulum using Triton X-114 and further purified using NAD to release glucose dehydrogenase from a NADP-linked sepharose column. The purified enzyme is capable of producing both NADH and NADPH in vivo as indicated by kinetic studies.
*Key words.* Glucose dehydrogenase; glucose; affinity chromatography.

Glucose dehydrogenase (E.C. 1.1.1.47) is a multi-functional protein [1] capable of binding either NAD or NADP at its active site. This enzyme converts β-D-glucose (NAD) and α-D-glucose-6-phosphate (NADP) to the corresponding cyclic 1,5-lactones while producing either NADH or NADPH for the microsomal electron transport system [2, 3]. Glucose dehydrogenase is located on the luminal side of the endoplasmic reticulum [4] as is glucose-6-phosphatase. Removal of this lipo-protein from the endoplasmic reticulum is readily accomplished with Triton X-114, while its subsequent purification requires four separate chromatographic columns [1, 5].

In this work, we report the successful preparation and use of an affinity column, that takes advantage of glucose dehydro-

genase's unique ability to bind either NADP or NAD at its active site. An NADP-linked sepharose column is prepared and used to bind glucose dehydrogenase which is then later removed by the addition of NAD. The resulting protein yields the highest specific activity yet recorded and produces a single protein band when subjected to isoelectric focusing. This affinity column replaces a DEAE column and produces a protein that is completely stable for months at 4 °C. In addition, we have modified the use of a cation exchange column which has allowed the elimination of a Sephacryl S-200 column chromatographic step.

*Materials and methods.* Glucose dehydrogenase was purified as reported previously[1], with the following modifications. The protein solution obtained from the ammonium sulfate fractionation (30–45%) was applied in 3–4-ml portions to a Sephacryl S-300 column, omitting the S-200 column step used previously[1]. The S-300 column was equilibrated in 5.0 mM phosphate pH 7.0 buffer with 0.002% sodium azide. The resulting fractions were stored at 4 °C with 10 µM *p*-toluylsulfonyl chloride and later condensed with Lyphogel. The solution was filtered, the pH adjusted to 6.0, and a resulting precipitate removed by centrifugation at 10,000 × g for 10 min. The solution was then added to a Whatman SE-53 cation exchange column and eluted as described previously[1]. The active fractions were then added to an affinity column equilibrated with 50 mM pH 7.0 phosphate buffer. The affinity column was washed with additional 50 mM pH 7.0 phosphate buffer and the bound enzyme was eluted with 10 mM NAD in 50 mM pH 7.0 phosphate buffer. The active fractions were combined and added to an Amicon ultrafiltration cell equipped with a PM 30 DIAFLO filter. The solutions were condensed and washed using 5 mM pH 9.5 glycine buffer to remove residual NAD. The enzyme was stored at 4 °C and failed to lose any activity for several months.

Spectrophotometric assays were made at 37 °C in 0.05 M pH 7.5 tris/HCl or 0.05 M pH 10.0 glycine/NaOH buffer. All buffers were 1 mM in EDTA and 0.1 mM in dithioerythritol. Absorbance readings at 340 nm were recorded after initiation of the reaction by addition of the sugar solution. An extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$ was used to determine activity units and protein concentration was determined by the bichinchoninic acid method.[6]

*Affinity column.* The NADP-sepharose packing material was prepared using a modified version of the procedure described by Mosbach et al.[7]. Sepharose 4B (200 ml, representing 8 g dry wt) was activated by the cyanogen bromide method[8]. 6-Aminohexanoic acid (20 g), dissolved in 0.1 M $NaHCO_3$ (200 ml), was added, and the final pH adjusted to 8.5 with 4 M NaOH. After having been stirred for 15 h at room temperature, the gel was extensively washed with 0.1 M $NaHCO_3$, 0.01 M HCl, 0.5 M NaCl and water. The gel was then filtered off and 80 g of the wet gel, corresponding to 3.9 g dry wt, was washed on a filter with a large volume of aqueous 80% (v/v) pyridine. After filtration, there were successive additions of NADP (0.89 g), dissolved in water (24 ml), and dicyclohexylcarbodi-imide (40 g) dissolved in pyridine (96 ml) to the gel. The suspension was gently agitated on a rotary shaker for 10 days at room temperature to remove formed dicyclohexyl-urea. The gel was then filtered off and washed successively with water, ethanol, warm (40 °C) *n*-butanol, ethanol, water, and aqueous 80% (v/v) pyridine. The resulting gel was stirred for 3 days after adding an additional 10 g of carbodi-imide. The gel was then rinsed for 30 min with 1 mM HCl and for 5 min with ice-cold 0.1 M $NaHCO_3$, followed by extensive washings with 0.5 M NaCl and water. All washings were carried out at 20 °C. The preparation was stored as a moist gel at 4 °C.

An 2 × 6 cm column containing the immobilized NADP was prepared by mixing the affinity material with 50 mM pH 7.0 phosphate buffer in 1 M NaCl. The solution was allowed to settle at 4 °C, and any particles suspended at the top of the solution were discarded. The column was then poured by gravity, and thoroughly washed with 50 mM pH 7.0 phosphate buffer.

*Isoelectric focusing.* A Bio-Phoresis Horizontal Electrophoresis cell was used with a cold bath and a Bio-Rad power supply. Gel support film for agarose was purchased from Bio-Rad and applied to 100 × 125 mm glass plates. Agarose IEF gel was prepared by adding carrier ampholytes to a boiling agarose mixture, consisting of 1% agarose, 2% ampholytes, 5% sorbitol, and 10% glycerol. The hot gel was then poured on a plate, solidified, and aged in a humid chamber at 4 °C for 12 h. After aging, the plates were overlayed with blotting filter paper to remove excess water, and placed in the horizontal cell. Electrofocusing wicks were prepared for the anode and cathode. 0.1 M glutamic acid was used at the anode, and 0.5 M NaOH was present at the cathode. The pH range of the carrier ampholytes was 6–8. Samples, which were 2% in ampholytes, were applied in 15–25 µl streaks across the plate. The gels were run for 6 h at 10 °C at constant current. The voltage (~ 500 V) increased slightly during establishment of the pH gradient, an Ingold Isoelectric Focusing electrode was used to measure the pH gradient across the plate.

*Purification of enzyme.* Table 1 contains results for a typical purification of glucose dehydrogenase from 100 g porcine liver. The value for the final specific activity using affinity chromatography is almost double previous results from the laboratory[1]. The present scheme also establishes the integral role played by sulphoxyethyl cellulose cation matrix in filtering large portions of extraneous material from the S-300 eluant. This has reduced both the time and the number of columns needed for the overall purification. The enzyme, as

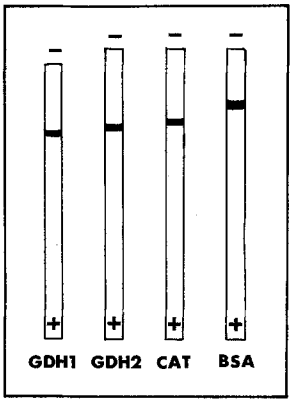
Table 1. Purification scheme and results

| | Step | Activity (units/ml) | Protein (mg/ml) | Volume (ml) | Specific activity (units/mg) | Overall purification |
|---|---|---|---|---|---|---|
| 1. | Homogenization, Triton X-114, centrifugation and ammonium sulfate fractionation | 0.504 | 56 | 28.6 | 0.0090 | – |
| 2. | Sephacryl S-300 | 0.116 | 1.49 | 50 | 0.078 | 8.7 |
| 3. | SE-53 cation column | 0.251 | 0.328 | 14.4 | 0.765 | 85 |
| 4. | Affinity column | 0.646 | 0.108 | 2.0 | 5.98 | 664 |

1 unit of activity is defined as the amount of enzyme required to produce 1 mole of NADH/min at pH 10, 37 °C.

Table 2. Kinetic constants at 37 °C

| Author | pH | $K_m$ (NAD) (μm) | $K_m$ (glc) (mM) | pH | $K_m$ (NADP) (μm) | $K_m$ (glc-6-P) (μm) | Hepatic source |
|---|---|---|---|---|---|---|---|
| This work | 7.5 | 2.8 1.1 | 3.2 0.6 | 7.5 | 2.2 0.3 | 3.6 0.6 | Porcine |
| | 9.0 | 2.9 0.6 | 4.6 0.3 | 10 | 7.2 0.4 | 8.3 0.5 | |
| Carper et al.[1] | 7.5 | 2.4 0.2 | 5.1 0.5 | 7.5 | 6.9 1.0 | 3.6 0.3 | Porcine |
| | 10 | 6.2 0.4 | 4.6 0.4 | 10 | 4.7 0.5 | 61.7 2.5 | |
| Campbell et al.[5] | 7.5 | 1.2 0.2 | 4.8 0.5 | 7.5 | 4.1 0.4 | 3.0 0.2 | Bovine |
| | 9.5 | 6.5 0.3 | 4.2 0.1 | 9.5 | 5.4 0.5 | 25.4 1.2 | |




Results of SDS electrophoresis for glucose dehydrogenase (GDH 1 – affinity chromatography, GDH 2 – DEAE column) catalase, and bovine serum albumin.

purified, is stable at room temperature for 24 h, and did not exhibit any appreciable deterioration when stored at 4 °C for 2–3 months. The Triton X-114 method coupled with the use of affinity chromatography is clearly a useful procedure for providing purified and stable glucose dehydrogenase.

*Isoelectric focusing.* Isoelectric focusing was used to verify the purity of the final enzyme sample. Each run consisted of a known standard consisting of the enzymes, equine liver alcohol dehydrogenase and ribonuclease A. These standards, which have isoelectric points at 6.8 and 7.8, respectively, were run against the purified protein. All enzyme solutions contained 2% ampholytes to aid in formation of bands. A theoretical isoelectric point of 7.4 for glucose dehydrogenase was calculated by summation of $pK_a$'s for the amino acid residues[1]. The experimental isoelectric point of glucose dehydrogenase was 7.1.

*Electrophoresis.* Sodium dodecyl sulfate electrophoresis was carried out at pH 6.6 on 12% gels as described previously[1]. A single subunit band was observed for glucose dehydrogenase prepared by either affinity chromatography or our earlier method[1], as shown in the figure.

*Kinetics.* Preliminary studies on the kinetic properties of glucose dehydrogenase purified by means of affinity chromatography indicate reaction behavior quite similar to previous results. Table 2 demonstrates that at 37 °C, the apparent Michaelis constants of glucose dehydrogenase compare favorably to the previous results[1]. Although exhaustive studies concerning the kinetic properties of this enzyme have yet to be completed, it is apparent that the basic properties of glucose dehydrogenase have been preserved through our purification scheme.

Inhibition experiments were performed in relation to the effect of product (NADH, NADPH) inhibition of the glucose-NAD reaction. It was found that at the level, [NADH] = 3[NAD], there was 5% inhibition of the initial reaction velocity. Even at [NADH] = 6[NAD], the inhibition only increased to 20%, thus demonstrating that interference by the reduced NADH at normal substrate concentrations is only slight. NADPH exhibited an increase in inhibitory power, decreasing the initial velocity by half when its concentration was equal to the NAD concentration.

*Metabolic role.* Kinetic constants from steady state experiments[1, 5] have shown that the $K_m$'s for the substrates, glucose and glucose-6-phosphate, are less than their physiological concentrations when in reaction with the cofactors NAD and NADP, respectively. This relationship indicates that the enzyme catalyzes reactions between mutually phosphorylated or non-phosphorylated substrates and cofactors without significant cross-reaction in vivo. Consequently, glucose dehydrogenase appears to be a multi-functional enzyme capable of producing both NADH and NADPH from the oxidation of glucose and glucose-6-phosphate on catalytically distinct subunits.

The destination of these reduced cofactors is a matter of growing interest when various metabolic pathways are examined. Recent investigations have proposed that glucose dehydrogenase may be responsible as a supplier of NADPH and NADH to the microsomal electron transport system by the oxidation of glucose-6-phosphate[2, 3]. Kimura et al.[3] found that by the introduction of specific inhibitors of the microsomal electron system, latent activity of glucose dehydrogenase was detected in intact microsomes by the presence of NADPH. These observations indicate the immediate use of NADPH by the drug metabolizing enzyme, NADPH-cytochrome *c* reductase. As NADPH has been shown to be a major source of product inhibition, it appears quite possible that glucose dehydrogenase is responsible not only as a NADPH supplier, but also as its own regulator of coenzyme levels in the microsomal electron transport system.

Finally, studies by Ammon[9] have shown that reduced pyridine nucleotides may function as the intracellular trigger for the secretion of insulin. Since D-glucose is responsible for insulin secretion by pancreatic beta cells, the enzymatic apparatus, primarily those enzymes catalyzing rate-limiting reactions, assumes primary importance in the understanding of diabetes. Work by Malaisse and others[10–12] also support the role of NADH as a intra-cellular regulator, and have established that calcium efflux from the islet cell is directly related to glucose concentration. In particular, glucose-6-phosphate has been identified as a regulator of islet intracellular $Ca^{+2}$[13].

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## Interleukin 1 modulates collagen accumulation by rat granulation tissue cells both in vivo and in vitro

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*Summary.* In in vivo studies 0.5 U human interleukin 1 (IL-1) was inoculated daily into a subcutaneously implanted viscose cellulose sponge. IL-1 significantly decreased the dry weight (7.8%) and the hydroxyproline content (24.2%) of granulomas. When the cultured rat granulation tissue cells were exposed to IL-1 (0.5–2.0 U/ml) their collagen production decreased to 80% of that in controls. No effect on cell proliferation was detected.
*Key words.* Interleukin 1; granulation tissue.

Interleukin 1 (IL-1) is a 17.5 kDa polypeptide factor. It has been suggested that it regulates connective tissue breakdown in chronic inflammation. In cell culture conditions, IL-1 is known to stimulate prostaglandin $E_2$ ($PGE_2$) [1], collagenase [2] and plasminogen activator synthesis [3] by fibroblasts and it also enhances the synthesis of enzymes degrading proteoglycan complexes [4] and extracellular fibronectin [5]. Recent studies have suggested that IL-1 may induce matrix reformation by increasing the synthesis of fibronectin [6] and hyaluronic acid [7]. The studies on collagen synthesis suggest that experimental procedure, and the origin of the cell line, affect the results. IL-1 seems to stimulate collagen production in skin fibroblasts by elevating collagen mRNA levels [8, 9]. Especially in synovial cells and chondrocytes IL-1 may decrease collagen synthesis [8, 10, 11]. This may by due to indirect effects mediated via $PGE_2$. IL-1 has also been reported to induce cellular proliferation in skin fibroblast and synovial cell cultures [12, 13]. In the present study, the effects of IL-1 were tested both in cell cultures and in vivo in the viscose cellulose sponge model of experimental granulation tissue. We have previously shown that the same model is suitable to test the in vivo effects of human epidermal growth factor (EGF) [14–16].

*Material and methods.* A standardized experimental wound model described by Niinikoski, Heughan and Hunt was used [17]. Cylindrical viscose cellulose sponges (Säteri Oy, Valkeakoski, Finland) were implanted under the skin in the dorsal midline of Male Sprague-Dawley rats. Each rat received one sponge cylinder. Altogether 12 rats were studied in two groups of 6 animals. In the control group the implants were treated immediately after implantation by injecting 0.05 ml of 0.9% saline solution into the central tunnel of the implant. The implant of the test group was injected correspondingly with 0.05 ml saline solution containing 0.5 U of IL-1. Ultrapure human IL-1 purified from glass-adhered human monocytes was obtained from Genzyme Corporation (Boston, MA, USA). Injections of the two groups were repeated daily under strictly aseptic conditions. Seven days postoperatively rats were sacrificed, and the implants were dissected free from the surrounding tissue. Bacteriological examinations of wound fluid were performed in both groups at the end of the experiments and no infections were observed. Nucleic acids were extracted from the implants according to the method of Schmidt and Thannhauser [18]. DNA was determined by the diphenylamine reaction [19] and RNA was assayed as RNA-ribose by the method of Ceriotti [20]. Aliquots were taken for the determination of nitrogen [21], hydroxyproline [22], hexosamines [23], and uronic acids [24].

Granulation tissue cells were isolated from experimental granulomas induced in adult rats. The cells were detached from the cellulose sponge matrix slices by digestion with collagenase and trypsin [25] and then cultured in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% fetal calf serum [26].

The rate of collagen synthesis was measured using [$^3$H]proline (Amersham) as the precursor. Confluent cultures were first preincubated for 24 h in the presence of 1.0 U/ml IL-1 and ascorbic acid (50 µg/ml). After preincubation fresh ascorbic acid and IL-1 were added with the isotope and the hydroxyproline bound radioactivity was measured after 24 h [27].

In the cell proliferation assays the number of cells in culture dishes was counted with a Bürker's hemocytometer, after the cells had been detached by trypsin treatment.

*Results.* Data for the effects of IL-1 on various wound healing parameters in experimental granulation tissue are shown in the table. After daily application of 0.5 U of IL-1, statistically significant reductions were observed in the try weight of the sponge (– 7.8%) and hydroxyproline (– 24.2%). Considering the mean amounts of DNA, RNA and protein, measured as total nitrogen, no significant reductions were observed between the groups. The mean amounts of hexosamines and uronic acids, reflecting the amounts of glycosaminoglycans, were almost similar in both groups.

The collagen production in cultured granulation tissue cells exposed to IL-1 was about 80% of that in controls (fig. 1). In the presence of 14 µM indomethacin, IL-1 had no effect on the rate of collagen synthesis (fig. 1).

IL-1 had no effect either on the early or on the late proliferation of granulation tissue cells when this was measured as a change in the number of cells (fig. 2).