Synthesis of S-Lactoyl-Glutathione Using Glyoxalase I Bound to Sepharose 4B[1]

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Summary. Glyoxalase I bound to Sepharose 4B was used for synthesis of S-lactoyl-glutathione. The bound enzyme does not lose its activity during several months storing and can be used many times for synthesis of S-lactoyl-glutathione. This reaction product can be used as a substrate for glyoxalase II without any further purification.

RACKER[2] and WIELAND et al.[3] reported methods for obtaining S-lactoyl-glutathione as a substrate for glyoxalase II. The substrate is prepared from glutathione and methylglyoxal by enzymatic synthesis with glyoxalase I. The methods are troublesome to carry out, and in addition the protein of glyoxalase I requires separation and so cannot be used for further synthesis.

This paper describes the conditions for binding glyoxalase I to the organic bed of sepharose 4B and also some properties of the bound enzyme. The use of the attached enzyme for synthesis of S-lactoyl-glutathione is described. This method has proved of great practical value, since the enzyme can synthesize a large number of S-lactoyl-glutathione samples for a long time and without any loss of activity. When enzymatically synthesized, S-lactoyl-glutathione is free of those impurities which might influence the glyoxalase II reaction.

Materials and methods. Yeast glyoxalase I was purchased from Sigma (St. Louis, USA). Glutathione was from Merck (Darmstadt, West Germany). Sepharose 4B CNBr was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Liver glyoxalase I and liver glyoxalase II were obtained by molecular filtration of bovine liver homogenates (JERZYKOWSKI et al.[4]). Methylglyoxal was obtained by distillation of glyceraldehyde as described by PATTERSON and LAZAROW[5]. Methylglyoxal concentration in distillates was determined by the FRIEDEMANN method[6].

All other reagents were of analytical purity. Glyoxalase I, Glyoxalase II and S-lactoyl-glutathione were determined by the spectrophotometric method of RACKER[2], if not otherwise specified in the text. S-lactoyl-glutathione was determined spectrophotometrically, the extinction being measured at 240 nm. ($\varepsilon_M = 3370$). Units of enzyme activity were expressed as μmoles of S-lactoyl-glutathione produced (Gl. I) or decomposed (Gl. II) in 1 min (initial velocity) at room temperature under the experimental conditions used.

Preparation of the attached enzyme samples. 1 g Sepharose 4B CNBr was swollen and washed with 200 ml 0.01 *M* HCl and then filtered. After washing with water, it was suspended in 5 ml of 0.1 *M* sodium bicarbonate. Alternatively 0.06 *M* phosphate buffer pH 6.8 could be used instead of 0.1 *M* $NaHCO_3$ – without influencing the final enzyme activity yield. Glyoxalase I (Sigma) in a quantity of about 20 IU was then added to the suspension. The mixture was rotated end-over-end for 2 h at room temperature. During incubation, supernatant solution samples were taken and glyoxalase I activity was determined. In the same way bovine glyoxalase I (fractions with the maximum activity obtained by molecular filtration in the 0.066 *M* phosphate buffer[4]) was mixed directly with the washed gel in the proportion 1 g of gel + 12 ml of glyoxalase I solution – eluant from the column. Unbound material was washed away with coupling buffer, and any remaining active groups are reacted with 1 *M* ethanolamine at pH 8 for 1.5 h. 3 washing cycles were used to remove non-covalently adsorbed protein, each cycle consisting of a wash at pH 4 (0.1 *M* acetate buffer containing 1 *M* NaCl) followed by a wash at pH 8 (0.1 *M* borate buffer containing 1 *M* NaCl).

Determination of the activity of glyoxalase I bound to Sepharose 4B. Activity of the enzyme which had been bound to sepharose 4B was determined in the following way: the suspension of about 2 mg of bound enzyme in 0.1 ml H_2O was added to spectrophotometric cuvette which contained 1.7 μmol of glutathione, 2.4 μmol of methylglyoxal and 0.15 *M* phosphate buffer pH 6.8 to make a final volume of 3 ml (light path 1 cm). The gel rapidly fall to the bottom of the cuvette. Enzyme activity was calculated from the increase of extinction at 240 nm during the first 5 min. In the conditions of preparation described, the specific activity of the bound enzyme was about 10% of that of the free enzyme i.e. 2 IU per g of Sepharose.

Stability of the preparation. Stored at room temperature, the attached enzyme did not lose its activity for several months. Dried with acetone, it retained 75% of the initial activity for up to half a year.

Obtaining S-lactoyl-glutathione as a substrate for glyoxalase II. 12 mg of glutathione and 2.8 mg of methylglyoxal dissolved in 0.05 *M* phosphate buffer pH 6.8 were added to the suspension of 500 mg of insoluble enzyme (final volume 3 ml). The suspension was left until a sample diluted: 0.1 ml + 2.9 ml of phosphate buffer pH 6.8 or *Tris* buffer pH 7.4 showed extinction 1.3 at 240 nm. Reaction time depended upon the insoluble enzyme activity and took 2–5 h. 0.1 ml undiluted S-lactoyl-glutathione is enough for 1 determination of glyoxalase II by the spectrophotometric method of RACKER[2]. Results have shown that S-lactoyl-glutathione in solution obtained by this method remains stable for at least 2 months at temperature 4 °C.

Discussion. Immobilization of the enzyme to a solid phase matrix offers a number of advantages for practical applications. In many cases, it gives satisfactory results as far as stability of the enzyme is concerned. The bound glyoxalase remains active for a long time when stored at room temperature, as we have observed over a period of several months. A small quantity of immobilized glyoxalase I is easy to obtain in the laboratory by the method described. One sample of the enzyme can be used many times for S-lactoyl-glutathione synthesis, which provides convenient conditions for obtaining this compound as a substrate for glyoxalase II.

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It is noteworthy that in recent years considerable attention has been paid to glyoxalases since they are believed to participate in the regulation of cell division (SZENT-GYÖRGYI[7]) as well as in other metabolic processes (WEAVER and LARDY[8], URATA and GRANICK[9], GREEN and ELLIOTT[10], REEVES and AJL[11], JERZYKOWSKI et al.[12]).

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The Nature of DNA Synthesis by Isolated Nuclei from Cells of a Rat Tumour

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Summary. The DNA synthetic activity of nuclei isolated from a solid rat tumour was determined. The nuclei had DNA synthetic properties similar to nuclei from other sources but the time course of the reactions was different.

Recently[1-6] many workers have shown that nuclei isolated from mammalian cells contain a DNA synthesizing system which has features in common with that described for the membrane-DNA fragments obtained from bacteria[7-11]. These nuclei utilizing endogenous polymerases and chromatin, are capable of the continuation of DNA and RNA[2] synthesis in the presence of an adequate supply of precursors. Earlier workers (loc. cit) have investigated the DNA-synthetic properties of nuclei obtained from a wide range of mammalian cells and there is considerable evidence that it is possible to relate these properties to the status of the cells from which the nuclei were derived. The isolated nuclei provide a system by which the synthesis of macromolecules can be studied more directly than is possible in the whole cell system. For instance the effect of cytotoxic treatments on the nuclei can be determined without the involvement of the effect of the treatment on the cytoplasm.

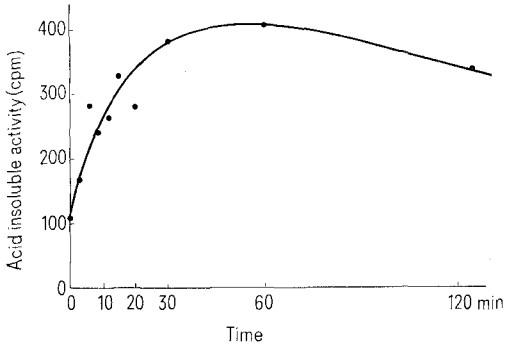

Fig. 1. The pattern of synthesis and degradation of new DNA as a function of time. Nuclei from rat fibrosarcoma RIB_{5c}.

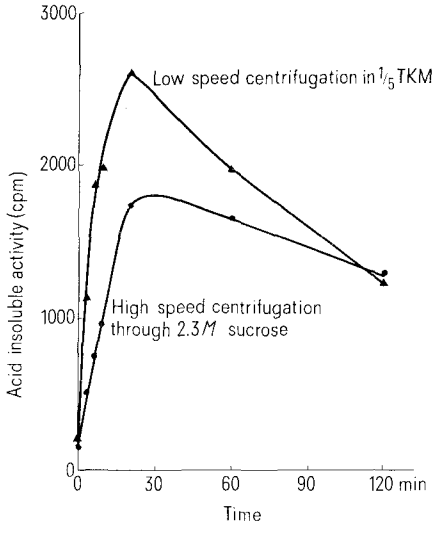

Fig. 2. Synthesis and degradation of new DNA by isolated nuclei. Final stages of isolation either through 1/5 TKM (▲); or through 2.3 *M* sucrose (●). The counts have been corrected for differences in initial concentrations of nuclei from RIB_{5c}.

To our knowledge cells from solid neoplasms have not been used as a source of nuclei. We have in our laboratory a series of transplantable rat tumours, of which, the gross responses to ionizing radiation have been studied in depth[12] and differences between these responses, not yet fully understood, may be due to differences in post-irradiation modifications of macromolecular synthesis. In this paper we present the results of experiments designed to establish the nature of the DNA-synthetic reaction in nuclei isolated from one of these rat tumours.

Isolation of nuclei from tumour tissues. The fibrosarcoma RIB_{5c} was excised from the flanks of an inbred strain of Wistar rat. They were immediately cooled in ice cold isotinic saline and all subsequent preparative procedures were at 0 °C. The tumour weighing 2–3 g was cut into small pieces and reduced to pulp by compression

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