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79. One-Step Purification of Bovine Adrenal Glucose-6-phosphate Dehydrogenase by Affinity Chromatography

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(5. II. 76)

Zusammenfassung. Das konventionelle Reinigungsverfahren von Glucose-6-phosphat-dehydrogenase (G6PDH) (E.C. 1.1.1.49) aus Rinder-Nebennierenrinde wird durch eine einzige affinitätschromatographische Trennungsstufe ersetzt. Die Anwendung einer mit Nicotinamid-Adenin-Dinukleotid-Phosphat (NADP) substituierten Sepharose ergibt aus dem Rohextrakt eine Protein-Fraktion, aus welcher nach Einengen die G6PDH unmittelbar kristallisiert. Die Reinheit des Enzyms wird durch analytische Gel-Elektrophorese sowie durch Vergleich mit schon bestehenden Literaturdaten geprüft. Vier weitere NADP-abhängige Dehydrogenasen werden im Eluat identifiziert und partiell gereinigt.

1. Introduction. – Bovine adrenal glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49) (G6PDH) has discriminatory properties towards adrenocorticotropic hormone (ACTH) [1], making it a good model for detailed binding studies with ACTH. For this reason we have begun to isolate the enzyme in our laboratory. G6PDH has already been purified from other sources such as yeast [2] [3] or erythrocytes [4] by conventional techniques including in some cases affinity chromatography. Bovine adrenal G6PDH was first isolated and crystallized by *Criss & McKerns* [5]. *Squire & Sykes* [6] have reported on an improved procedure, which appears to be the best available at present. Their six-step fractionation method still remains very laborious. This is partly due to the presence in the

[1] This work is a part of the doctoral thesis of *E. B.*
[2] Abbreviations: NADP (NADPH): oxidized (reduced) nicotinamide adenine dinucleotide phosphate; SDS: sodium dodecylsulfate; Tris: tris-(hydroxymethyl)-aminomethane; EDTA: ethylene diamine tetraacetate; bicine: N,N-Bis(2-hydroxyethyl)-glycine.

protein extract of inactivating factors like ascorbate [7] or proteases [8], which seriously reduce the yields.

The purpose of this work was to simplify the conventional method of *Squire & Sykes* and to improve its yield by making use of affinity chromatography. Experimental conditions were found under which NADP-Sepharose prepared according to *Wilchek et al.* [9] allows a one-step purification of bovine adrenal G6PDH starting from the crude extract. Elution was achieved either with free NADP or with increasing ionic activities in the buffer. The method of choice was the application of a gradient of increasing concentration of sodium chloride in the elution buffer. Four other NADP-dependent oxidoreductases (6-phosphogluconate dehydrogenase, E.C. 1.1.1.44; isocitrate dehydrogenase, E.C. 1.1.1.42; glutathione reductase, E.C. 1.6.4.2; malate dehydrogenase, E.C. 1.1.1.40) which could be identified in early fractions of the eluate are partially purified. A complete separation of G6PDH from the other dehydrogenases was achieved. Sufficiently high specific activity was obtained for the enzyme to crystallize. It appeared to be homogeneous by gel electrophoresis in the presence of SDS and as pure as reported by conventional purification [5] [6].

2. Materials and Methods. – *Chemicals.* Sepharose 4B was a product of *Pharmacia*, Uppsala, Sweden. NADP, glucose-6-phosphate, 6-phosphogluconate, DL-isocitrate, oxidized glutathione and L(–)-malate were purchased from *Sigma*. Periodic acid, glucose, adipic acid, ammonium sulfate and other inorganic salts were puriss. grade *Fluka* products.

Assay of dehydrogenases. – The dehydrogenase activity was estimated in aliquots of the protein solution by following the rate of NADPH production or consumption at 340 nm in a *Philips* Pye Unicam SP 1800 spectrophotometer. In the case of G6PDH, the optimum of pH and of Mg^{++} concentration was found to be respectively 8.8 and 30 mmol/l. These values don't fully agree with those previously described [5]. Nevertheless our assay solution was the same as in [6]. For the definition of an activity unit, see [6]. Enzymatic activity of the other oxidoreductases was evaluated using the classical assay solutions (see *e.g.* [10]). The estimation of the protein concentration according to *Lowry* [11] allowed the determination of specific activity.

Gel electrophoresis. – Electrophoresis without SDS [12] was carried out in a 4% polyacrylamide gel (Ortec system) in a 0.025 M Tris-glycine buffer at pH 8.6, whereas with SDS [13] it was conducted on a 7.5% polyacrylamide gel in a 0.1% SDS and 0.1 M Tris-glycine buffer at pH 8.2. Temperature was in both cases 20° and current intensity 5mA per well. Protein was stained with Coomassie Brillant Blue or with the developing solution of *Rattazzi et al.* (G6PDH specific staining) [14]. The proteins for the electrophoresis on SDS-gels were dissociated in a buffer with the final concentrations: 0.04 M/1 Tris, 5% saccharose, 2% SDS and 2% mercaptoethanol by immersing the samples for 1.5 min in boiling water.

Affinity chromatography resin. – NADP was covalently bound to Sepharose 4B through the ribose ring on nicotinamide according to *Wilchek et al.* [9]. The spacer molecule between the Sepharose matrix and the nucleotide was adipic acid dihydrazide. The degree of substitution was 4 to 4.5 μmol nucleotide per ml packed gel. Repeated use of the columns resulted in a reduced binding capacity.

Crude protein extract. – The protein solution used for affinity chromatography was the product of a homogenization of bovine adrenal cortex and an extraction in a 0.1 M acetate buffer (pH 5.9). To this end the cortex was crushed in a mixer for 1 min, then agitated gently during 30 min in the buffer. The mixture was finally centrifuged at 3000 g over one hour and the pellet discarded. Starting with 500 g adrenal cortex, a typical run contained *ca.* 4500 G6PDH activity units in *ca.* 1300 ml solution. The specific activity of the crude extract varied between 0.08 and 0.22 units/mg.

Chromatography. – The protein fraction was applied to a 1.7 cm diameter column containing 25 ml of packed NADP-Sepharose. The flow rate was 3 to 4 ml/min. Fractions were collected and their protein content estimated by UV.-absorption at 278 nm. After no more protein was eluted

under these conditions, the resin was washed with 0.1 M NaCl in the same buffer. Then elution was started either with free NADP (200 mg NADP in 3 ml 0.1 M acetate buffer, pH 5.5) or with increasing ionic activities (0.1 M to 4 M NaCl) in the same buffer.

In the case of the purification of only G6PDH, the elution procedure was designed to obtain a maximal concentration of the enzyme. The loaded column was first washed with 1 M NaCl in the acetate buffer, which removed the other dehydrogenases simultaneously. Then G6PDH could be eluted in sharp fractions with a pulse of 4 M NaCl in the same buffer.

Crystallization. – Fractions containing G6PDH activity were pooled. Typical specific activity was 70 units/mg protein. In order to concentrate the solution, solid ammonium sulfate was added to final concentration of 60%. The precipitated protein was then dissolved in a small volume (*ca.* 5 ml) of phosphate buffer (0.08 M, pH 6.3) with stabilizing additives in the following concentrations: sodium azide, 0.02%; EDTA, 10^{-3} mol/l; mercapto-ethanol, 2.7×10^{-3} mol; NADP 2×10^{-5} mol/l. A small amount of insoluble material was removed by means of centrifugation.

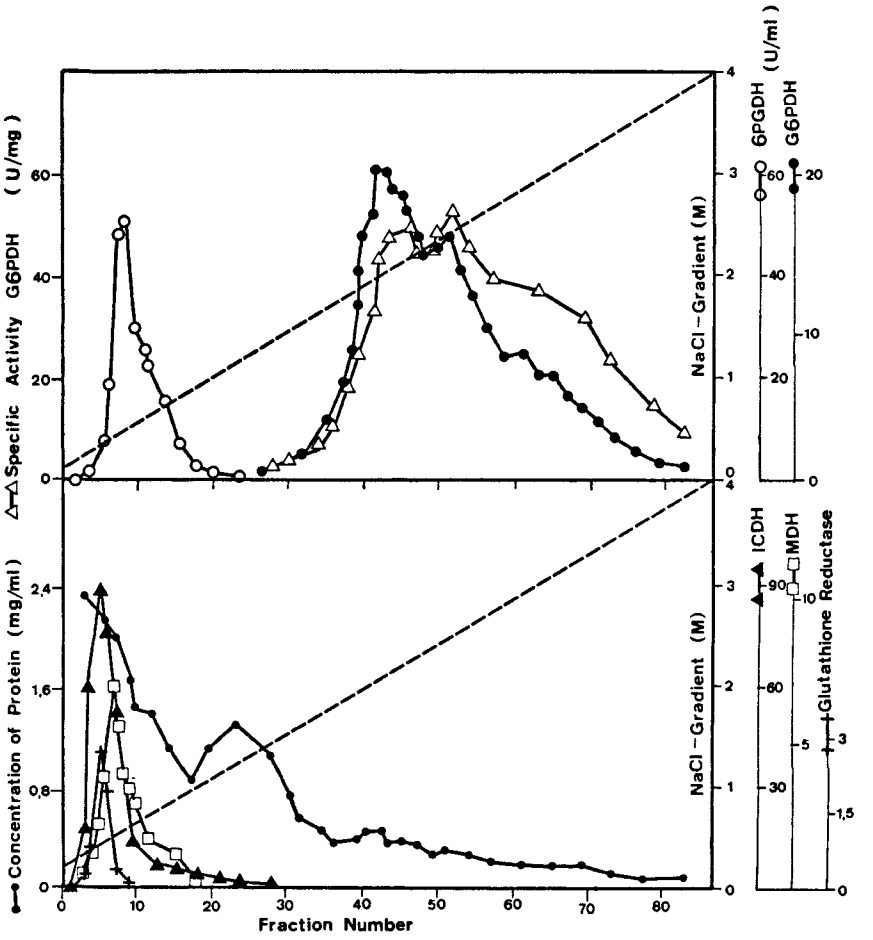

Fig. 1. *Elution profile of the five oxidoreductases by means of a gradient of NaCl.* Both parts of the figure are related to the same isolation and can be superposed. G6PDH: glucose-6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; MDH: malate dehydrogenase; ICDH: isocitrate dehydrogenase

Dialysis against a 60% ammonium sulfate solution (see [6]) started the crystallization which then procceded further over days. The pure crystalline part of the enzyme could be separated by centrifugation and the supernatant used again for further crystallization. Repeated crystallizations didn't improve the specific activity.

3. Results and Discussion. – When the elution was accomplished with NADP, about 65% of the total G6PDH activity was recovered with a high purification factor (about 500) and final specific activity of *ca.* 45 units/mg protein. However concentrations as high as 0.01 mol/l free NADP had to be applied, which made the procedure too costly. It was not used for routine isolation also because of the impossibility of removing the other dehydrogenases in one step. However it provided evidence that the dehydrogenases are retained in the column by true biospecific affinity forces.

The best results were obtained by increasing the concentration of sodium chloride in the elution buffer. The elution profile is shown in Fig. 1. Both the total enzymatic activity and the specific activity were followed as a function of the fraction number. A complete separation of G6PDH from the other oxidoreductases was achieved. The purification is summarized in Table 1. The G6PDH activity in the crude extract is corrected for 6-phosphogluconate-dehydrogenase activity only according to *Glock & McLean* [15].

Table 1. *Purification of bovine adrenal glucose-6-phosphate dehydrogenase by affinity chromatography*

Fraction	Volume ml	Concentration mg/ml	Activity units/ml	Specific activity units/mg	Yield % overall	Fold purification overall
Crude extract	1300	19.8	4.13	0.21	100	1
Affinity Chromatography	17	1.5	164	104	52	496
Crystallization	2	4.7	820	175	30.5	833

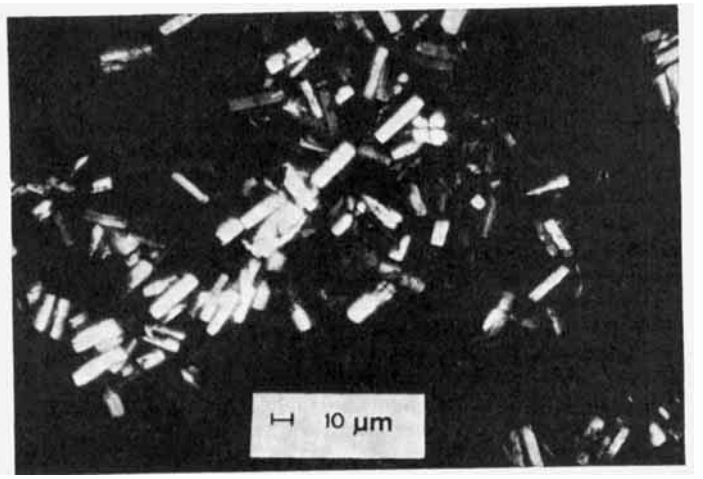

Fig. 2. *Crystals of glucose-6-phosphate dehydrogenase* (*Zeiss* Universal polarization microscope)

Only a partial purification of the four oxidoreductases which appear in the early fractions was achieved. Purification was 690fold for 6-phosphogluconate dehydrogenase (specific activity (SA) 31 units/mg), 610fold for isocitrate dehydrogenase (SA 39 units/mg), 2000fold for glutathione reductase (SA 1.2 units/mg) and 1370fold for malate dehydrogenase (SA 3.3 units/mg).

G6PDH was obtained in a pure crystalline form. Crystals which are shown in Fig. 2 compare well with those described earlier [4] [5]. They gave two active protein bands when examined by gel electrophoresis without SDS and when stained specifically according to *Rattazzi et al.* [14]. However they gave a single band in the presence of SDS. This seems to exclude the possibility of the existence of two isozymes in the crystalline material and is consistent with an enzyme composed of four identical subunits as claimed by *Squire et al.* [16].

It is not clear why partial retention of the enzyme occurs on the resin. A sample of NADP-Sepharose, when mixed to the assay solution immediately after the chromatography shows a well detectable dehydrogenase activity. This residual activity cannot be eluted either with free NADP or with a high ionic strength buffer. Furthermore the binding capacity of the column material is reduced after repeated use of the column. These facts might be due to a covalent attachment of part of the enzyme to the spacer or the ligand molecules. Our attempts to verify this hypothesis by using alcaline conditions known to release both the spacer and the ligand from the resin [17] [18] were unsuccessful as they destroyed the enzymatic activity, too.

Introduction of affinity chromatography under the conditions described here allows a purification of bovine adrenal G6PDH which is both rapid (3 days instead of 3 weeks [6]) and efficient (overall yield: 30.5% instead of 13% [6]). It should accelerate the current characterization of the enzyme from this species [16] [19] as well as the elucidation at the molecular level of its interaction with ACTH [1].

We thank Dr. *R. Schwyzer* for his advice and counsel during this investigation and Dr. *P. G. Squire* for having introduced us to the use of his G6PDH fractionation method. Thanks are also due to Mr. *G. M. Pelican* and to Dr. *W. Schlegel* for preliminary investigations and to Miss *Th. Zeier* for technical assistance. This work is supported by the Schweizerischer Nationalfonds für die Förderung der wissenschaftlichen Forschung (Grant 3.3330.74 to Prof. *R. Schwyzer*).

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80. Synthesis of 3,7-Diacetoxy-benzo-bullvalene[1])

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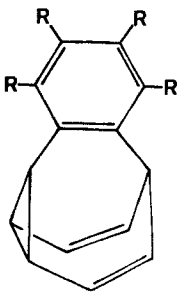
(29. XII. 75)

Zusammenfassung. Durch partielle reduktive Fragmentierung von 9,9a-Benzo-9a-homotriaster-9-en-3,7-dion (**6**) mit Zinkstaub in Eisessig wurde 61% 9,10-Benzo-tricyclo[3.3.2.$0^{2,8}$]-dec-9-en-3,7-dion (**3**) und daraus mit Isopropenyl-acetat 47% 3,7-Diacetoxy-benzo-bullvalen (**4**) erhalten. Eine relativ schnelle degenerierte *Cope*-Umlagerung in **4** liess sich durch die Temperaturabhängigkeit von dessen ^{1}H-NMR.-Spektrum aufzeigen. Mildere Acetylierung von **3** lieferte das Mono-enolacetat **9**.

Unter etwas energischeren Bedingungen der Zink/Eisessig-Reduktion wurden beide Cyclopropanringe in **6** aufgespalten, wobei 90% eines Gemisches, hauptsächlich bestehend aus 9,10-benzo-bicyclo[3.3.2]dec-9-en-3,7-diol (**7**) entstand. Behandlung von **6** mit Zinkstaub in Acetanhydrid lieferte – offenbar wegen noch unübersichtlichen Unterschieden in den Reaktionsbedingungen – einmal 50% des oben erwähnten tricyclischen Diketons **3**, ein andermal 45% 3,8-Diacetoxy-9,10-benzo-bicyclo[3.3.2]deca-2,9-dien-7-on (**10**) und sonst Gemische von **3** und **10**, sowie von einem Produkt, für das die Struktur eines 3,7-Diacetoxy-9,10-benzo-tricyclo[3.3.2.$0^{2,8}$]dec-9-en – Diastereomerengemisches **11** in Betracht gezogen wird.

Benzo-bullvalene **1** has been prepared from benzobarbaralone over a ring expansion [1] and from 7,8-benzo-bicyclo[4.2.2]deca-2,4,7,9-tetraene by a photochemical method [2]. It was shown, from the ^{1}H-NMR. spectrum, that the benzene ring of benzo-bullvalene **1** does not participate in the degenerate *Cope* rearrangement, as



1 R = H
2 R = C_6H_5

[1]) The systematic name is: 3,7-diacetoxy-9,10-benzo-tricyclo[3.3.2.$0^{2,8}$]deca-3,6,9-triene, which is used in the Experimental Part.

[2]) Post-doctoral fellow, University of Zürich, 1972–1975.