Journal of Chromatography, 287 (1984) 371–380
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,485

Note

Automated procedure for the recovery of nicotinamide-adenine dinucleotide phosphate: how to reduce costs of affinity elution

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(Received November 30th, 1983)

The elution of enzymatic proteins from chromatographic columns by means of ligands is used in many laboratories throughout the world. The reported yields are usually high and the purification factors obtained by means of affinity elution are consistently higher than for elution from group affinity columns or ion-exchange columns by means of varying pH or ionic strength, or by means of deforming buffers. However, the major drawback of affinity elution is the cost of the ligands used. Even if costs can be neglected in the research laboratory, they are important when the purification procedure is used in a repetitive process for large scale production. In the latter case, it is clear that recycling of the same batch of ligands would considerably reduce the costs, provided that the ancillary techniques required for recovery of the ligand in a form suitable for reuse are not, themselves, too complex.

In this paper we describe a method for the recovery of nicotamide-adenine dinucleotide phosphate (NADP) applied to the affinity elution of human 6-phosphogluconate dehydrogenase (6PGD) from a 2'5'ADP–Sepharose column. We believe that the procedure is of general applicability.

Basically, the procedure cvonsists of three successive chromatographic steps: an adsorption step on Amberlite XAD-4, followed by an ion-exchange step on DEAE Trisacryl M. The pure NADP eluted from the latter column is neutralized, desalted by chromatography and lyophilized. Because of the automation of the chromatography steps, the recovery of NADP is obtained without too great a demand on time and work.

MATERIALS AND METHODS

NADP was purchased from Boehringer (Mannheim, F.R.G.). The chemicals used as standards in the control of the recovered NADP (see text) were from Sigma (St Louis, MO, U.S.A.). Other reagent grade chemicals were from Merck (Darmstadt, F.R.G.), Carlo Erba (Milan, Italy), or Ega-Chemie (Steinheim, F.R.G.). Amberlite XAD-4 (50–60 mesh) was from Rohm & Haas (Philadelphia, PA, U.S.A.), DEAE Trisacryl from Pharmindustrie (Gennevilliers, France) and TSK HW 40 (F) gel was purchased from Toyosoda (Tokyo, Japan). The chromatographic columns were either from Wright (Croydon, U.K.) or made in this laboratory. The pumps

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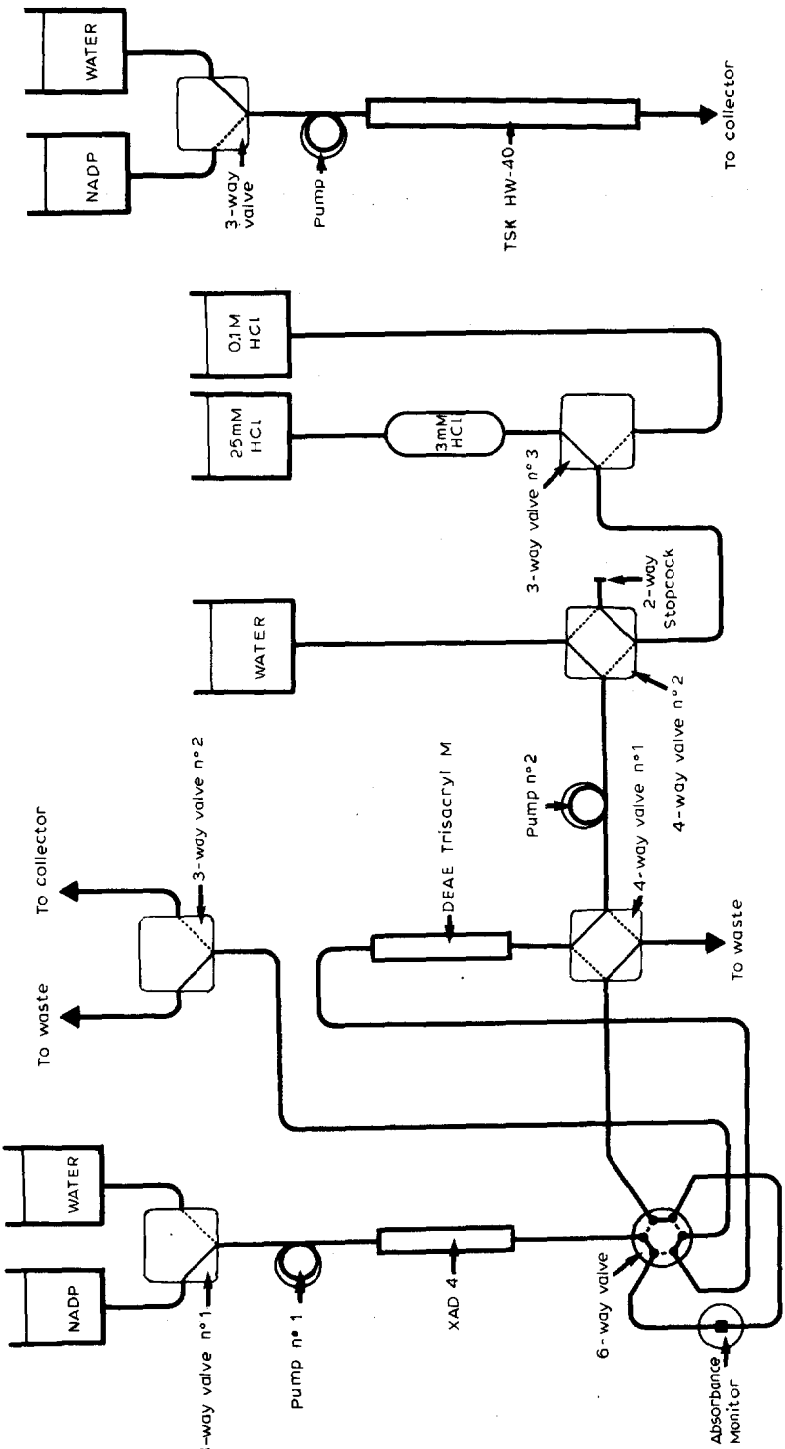

Fig. 1. Diagram of the two chromatographic systems: the flow chart of the purification set-up is on the left, that of the desalting set-up is on the right. The two positions of the valves are shown: the solid line through the valve shows the inactivated position, while the dotted line indicates the activated position. The three- and four-way valves, as well as the pumps, are numbered as in the text.

were from Gilson (Villiers le Bel, France) or from Ismatec (Zurich, Switzerland). The small bore Kel-F valves and the elements of the plumbing system were either from Rheodyne (Berkeley, CA, U.S.A.) or from Gilson. The pneumatic activators were from the same sources, or from FESTO (Rosny sous Bois, France). The electromagnetic pilot valves were from Jouvenel et Cordier (Rueil Malmaison, France). Top 2000 electric timers were from Crouzet (Valence, France) and the relays switches, electric plugs and sockets, as well as the wiring elements, from local retailers.

Recovery of NADP

In order to reduce the work required for the recovery of NADP, two set-ups were assembled from commercially available elements. In the first one, the first two chromatographic separations are carried out unattended, under fully automatic control: the columns are connected to each other and to appropriate reservoirs for NADP or developer solutions with tubings and valves. An electric programmer switches on and off the pumps and the fraction collector and activates the valves.

The flow diagram of the first set-up is shown in Fig. 1. Fig. 2 shows a photograph of the apparatus, of which several components (columns, NADP glass reservoir and fraction collector) were placed in a refrigerated cabinet. In the second set-up, the desalting of the purified NADP is carried out by gel filtration using a column of TSK HW 40 (F). Pumping and sample loading are carried out automatically. A flow chart of the set-up is also shown in Fig. 1.

The whole procedure will now be described step by step.

Adsorption chromatography on Amberlite XAD-4. The NADP solution, which is obtained as a by-product during the automatic purification of human erythrocytic GPGD[1], contains NADP at a concentration varying from 0.27 to 0.34 mlg/ml, 2.5 m*M* 2-mercaptoethanol, various breakdown products of NADP, about 1 *M* ammonium sulphate and 0.05 *M* sodium phosphate (the pH is *ca.* 5.6). After the initiation of the program, the NADP-containing solution is pumped through the three-way valve 1, into the XAD-4 column (height 40 cm, inside diameter 3.8 cm). The flow-rate is 250 ml/h. The column effluent passes through the six-way valve and is directed to the waste through the four-way valve 1. After 0.6 l of NADP solution are loaded on the XAD-4 column, the three-way valve 1 is activated, and distilled water is pumped into the column. Ten minutes later the four-way valve is activated and the effluent from the XAD-4 column is switched to the DEAE Trisacryl M column (previously equilibrated with water). Shortly after, a large absorbance peak begins to elute from the XAD-4 column. The same configuration (that is with both the three-way valve 1 and the four-way valve 1 activated) is maintained for 4.5 h in order to allow all of the UV-absorbing peak to be loaded on the DEAE Trisacryl M column, and the latter to be rinsed with water. After the rinsing is completed, the four-way valve 1 is closed, the effluent of the XAD-4 column thereby disconnected from the DEAE Trisacryl column and again connected with the waste line. The six-way valve is activated and the effluent of the DEAE Trisacryl M column is directed towards the flow cell of the absorbance monitor. Pump 1 is switched off and pump 2 is switched on.

Elution of the DEAE Trisacryl M column. Water is passed via pump 2 into the DEAE Trisacryl M column for 20 min, at a flow-rate of 200 ml/h. The four-way valve 2 and the three-way valve 2, linked together, are activated, and the gradient

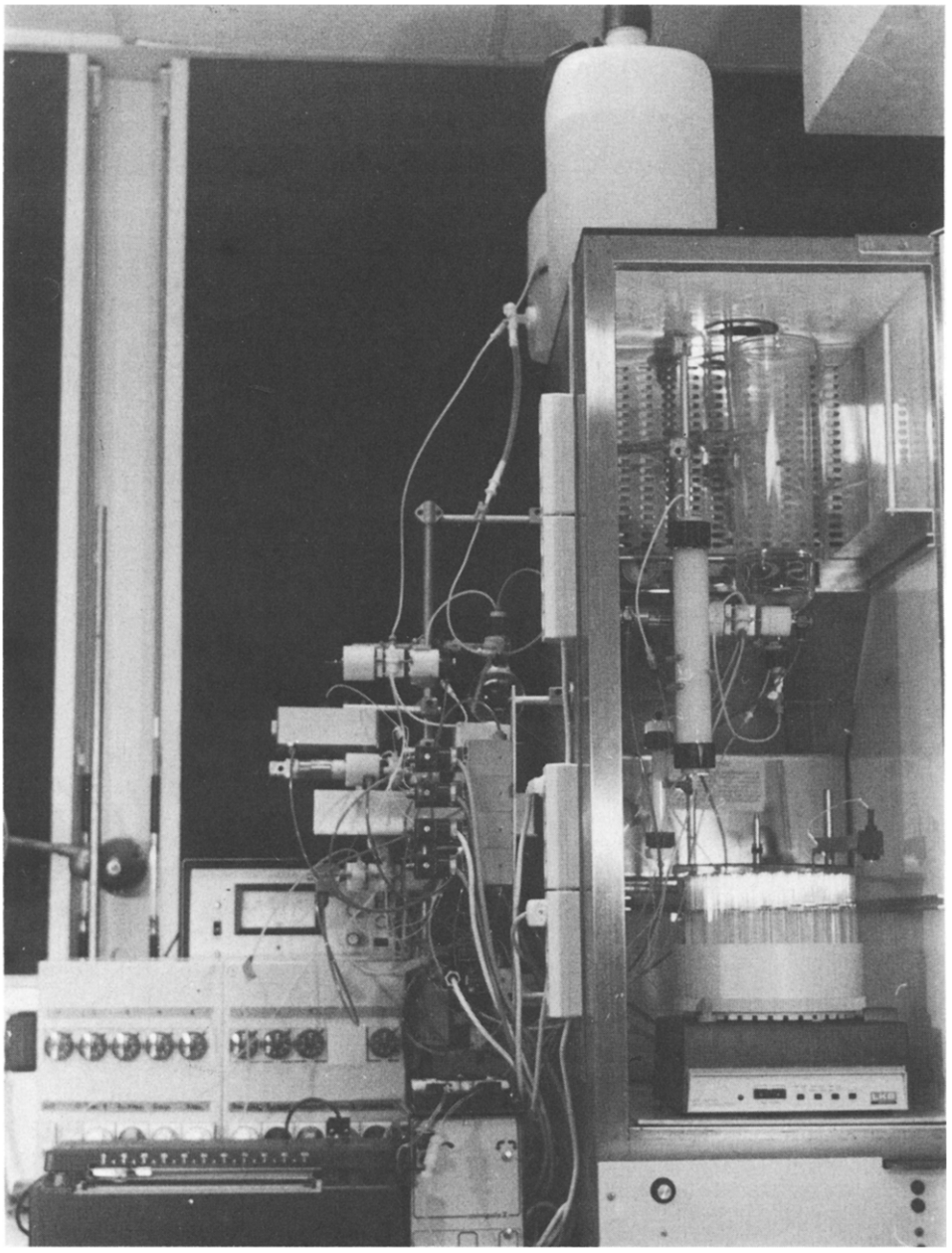

Fig. 2. Photograph of the purification set-up.

solution is pumped into the ion-exchange column, the effluent line being connected with the fraction collector which is switched on at the same time. The gradient between 250 ml of 3 m*M* HCl and 250 ml of 25 m*M* HCl is mixed in a vessel fitted with a two-hole stopper, filled with the initial eluent solution and connected through

one line with the column and through the other line with a reservoir containing the eluent of higher concentration. After elution for 5.5 h with the gradient followed by 25 m*M* HCl, the three-way valve 3 is activated. Hence 0.1 *M* HCl is pumped into the column for 2 h. The three-way valve 3 is closed, and the DEAE Trisacryl M column is re-equilibrated with water, thus making it ready for the next chromatographic cycle.

Chromatography on TSK HW 40 (F). The contents of NADP-containing tubes are pooled and adjusted to pH *ca.* 5.8 with dilute NaOH. NADP as its sodium salt is then obtained free from contaminating sodium chloride by chromatography on a column filled with TSK HW 40 (F) (diameter 4.4 cm, length 95 cm). Sample injection and subsequent column rinsing with distilled water are carried out automatically. NADP is loaded into the column when a three-way valve is in the activated position (see flow chart of the desalting set-up in Fig. 1); the sample volume is usually 25 ml; flow-rate 360 ml/h. The absorbance of the column outlet is monitored and the effluent collected in fractions. UV- absorbing fractions are pooled then frozen and lyophilized.

Programmers for automatic control of the chromatographics. The programmers for the two set-ups were assembled by connecting inexpensive electric timers, relays and electric sockets. Each programmed function is initiated by switching the current to the respective electric sockets in which are plugged the pumps, the electromagnetic pilot valves and the collector. A wiring diagram of the two programmers is shown in Fig. 3. Provisions were made to prevent an automatic restart of the programs if the electric current were to be shut down for a time. In the case of a temporary electric power failure, the pumps would, thus, remain off and the chromatography will proceed only if the main switch is manually operated. The wiring of both programmers provides opportunities for cyclic operation: if the switches designated by stars are left in an open position, the flow of the NADP solution on the XAD-4 column will start again (even before complete elution of the DEAE Trisacryl M) and similarly the flow of NADP on the TSK HW 40 column will automatically follow the rinsing of the column with water.

In the programmer for XAD-4 and DEAE Trisacryl separations, an electric clock makes possible an automatically delayed start of the apparatus. The program routinely used in the purification set-up is shown in Fig. 4.

Quality control of recovered NADP. A precisely weighed fraction of the recovered NADP is dissolved in water and injected in a liquid chromatography column (0.46 × 25 cm, Merck) filled with octadecyl silica (particle diameter 5 μm). The mobile phase[2] comprises 75 m*M* dipotassium hydrogen phosphate buffer adjusted to pH 4.12 with phosphoric acid containing 2 m*M* undecanoic acid and 12% (v/v) methanol.

RESULTS AND DISCUSSION

A photograph of one recorded UV absorbance profile obtained during automatic chromatography on XAD-4 and DEAE Trisacryl columns is shown in Fig. 5. During loading of the NADP solution into the XAD-4 column the absorbance rose steadily at the outlet of the XAD-4 column (see the part of the tracing which is above solid bar 1). It was shown by means of high-performance liquid chromatography

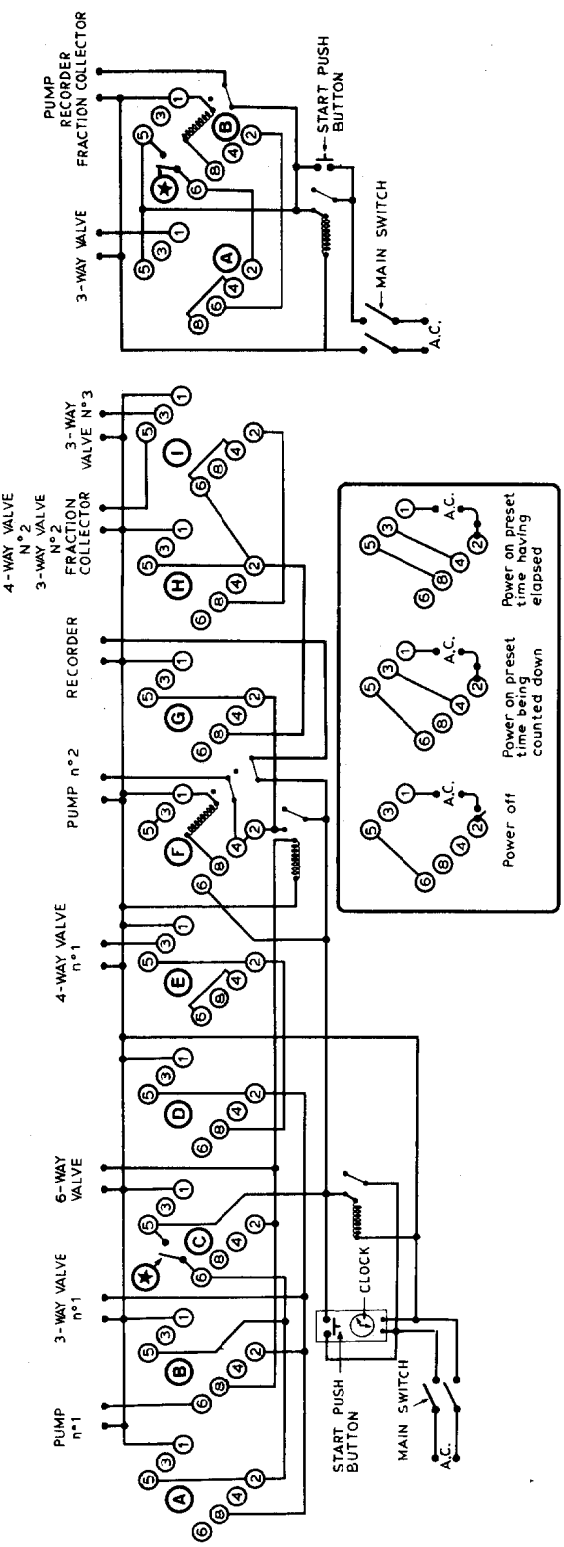

Fig. 3. Wiring diagram of the two programmers: the programmer for the purification set-up is on the left, that for the desalting set-up is on the right. The internal connections of the timers are not indicated (except in the insert): only the connections added to the timers are shown. The clock allows the program to be started at a preselected time, without the presence of an operator. Closing of the starred switches allows the NADP-containing solution to flow again into the XAD-4 column when the preset time of timer C has elapsed (purification set-up) or loading of the purified NADP to be resumed when the preset time of timer B has elapsed (desalting set-up). The insert shows the internal connections of the timers and how they work. When the electric power is connected to terminals 1 and 2, the count down of the preset time begins while the 3 and 4 terminals are shorted together. When the preset time has elapsed, the contact between 5 and 6 is broken, and 5 and 8 are shorted together. The timer is reset by switching off the power to 1 or 2.

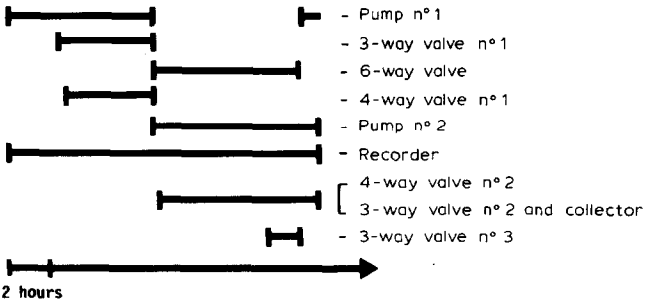

Fig. 4. Program routinely used for the automatic purification of NADP. The solid lines parallel to the time axis indicate the time at which power is brought to the electric sockets which feed the valves, the pumps and the collector. As is seen, the loading of the NADP-containing solution on the XAD-4 column is restarted automatically, even before re-equilibration of the DEAE column is completed.

(HPLC) that this was due to elution from the column of an as yet unknown UV-absorbing product, no NADP being present. The solid bar 2 shows the profile obtained when the effluent from XAD-4 was injected onto the DEAE Trisacryl. Solid bar 3 shows the profile of the pooled NADP. It has to be pointed out that the position and size of the NADP peak are similar in all chromatographic runs and the NADP-containing tubes can be directly identified by referring only to the absorbance profile, without using any other analytical procedure.

Fig. 6 shows the chromatograms obtained after injection into the high-performance chromatograph of a standard test mixture of the various products which can possibly contaminate the purified NADP (upper trace) of an aliquot of the start-

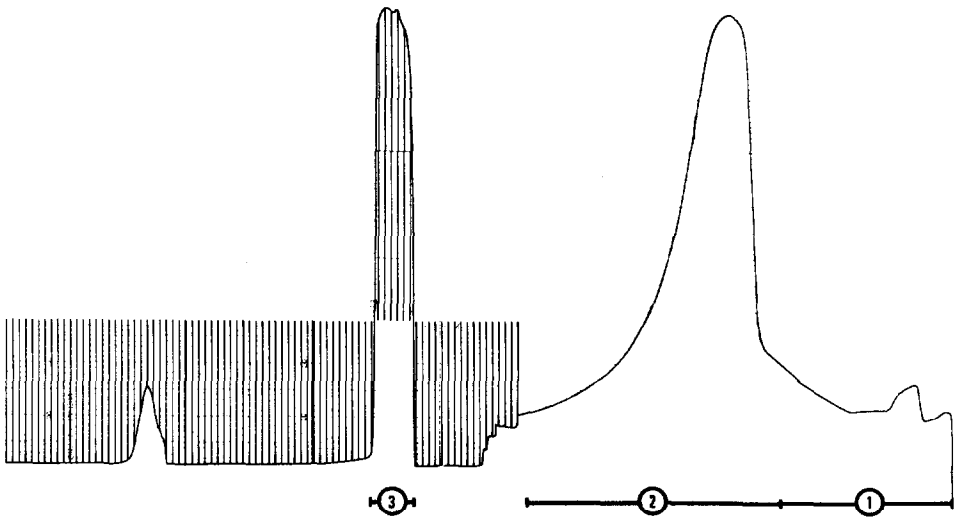

Fig. 5. Absorbance profile recorded in the purification set-up. The part of the record above lines 1 and 2 was recorded with the six-way inactivated and hence shows absorbance variations at the outlet of the XAD-4 column. The other part of the record (taken with the six-way valve activated) shows absorbance variations at the outlet of the DEAE Trisacryl M column (the vertical lines intersecting the recorder trace were made by the event marker of the collector).

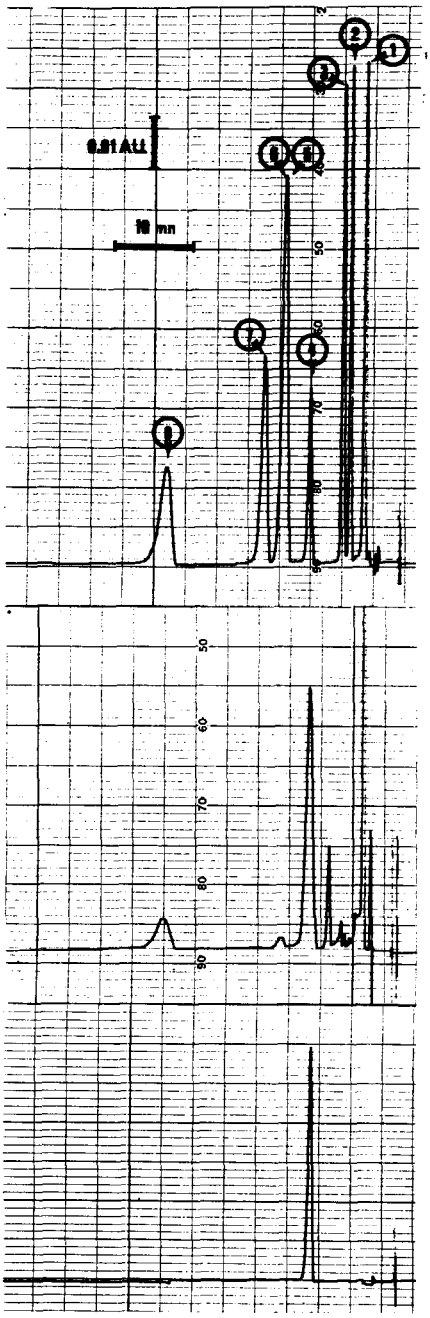

Fig. 6. Photographs of HPLC recorder tracings. The upper tracing shows the chromatogram of a standard mixture containing 2 μg of each numbered component, except for components 1 (0.45 μg) and 2 (1 μg). The middle tracing shows the chromatogram of 20 μ of the starting material loaded on the XAD-4 column. The lower tracing shows the chromatogram of 3 μg of the end product of the described purification procedure. Components: 1 = adenine; 2 = adenosine; 3 = adenosine; 5′-monophosphate; 4 = NADP; 5 = adenosine 2′-monophosphate; 6 = adenosine 2′,5′-diphosphate; 7 = adenosine 5′-diphosphate; 8 = adenosine 2′-monophospho-5′-diphosphoribose.

ing NADP solution used for purification (middle trace) and of an aliquot of the final product (lower trace).

The automatic chromatographic set-ups are used 6 days a week, thus permitting treatment of 3.6 l of an NADP-containing solution to produce 0.75 g of NADP. The time and work devoted to NADP recovery are reduced to a minimum by automation. The maintenance of the automatic set-up consists in putting clean tubes into the fraction collectors, and filling the respective gradient vessels and reservoirs with NADP solution, water and eluents. This method enabled us to reduce the production cost of pure erythrocytic human 6PGD considerably.

Adsorption on activated charcoal is a widely used method for the purification of pyrimidine coenzymes and related substances[3]. In an early version of the described procedure, the first chromatographic separation was on activated charcoal, but XAD-4 was later found to be more convenient mostly because of its much better flow properties. The XAD-4 resin has previously been used for the purification of nucleotides, nucleosides and their bases, from mixtures[4]; it was also used for desalting nucleosides and bases[5].

Anion-exchange chromatography has been widely used for the purification of nucleotides and coenzymes. Probably, all kinds of anion exchangers have been used for the same purpose for which we chose DEAE Trisacryl M: the latter ion exchanger is mechanically stable, can withstand a relatively high flow-rate and is not degraded by the eluent solutions even on a long-term basis. The polystyrene resins, which also possess these properties, were tried and discarded because the concentrations of HCl needed for elution are much higher (data not shown). Numerous mobile phases (including dilute HCl[6]) have been proposed to elute NADP or related substances from anion exchangers. It might have been throught that, when eluted with dilute HCl, NADP could be recovered free from solvent by simple evaporation (for example, by lyophilization). It should be noticed, however, that all our attempts to do so were unsuccessful because of a massive degradation of NADP (data not shown); a similar NADP degradation was also observed when trifluoroacetic, formic or acetic acid was used instead of HCl for the elution of NADP from DEAE columns. The only way to prevent this degradation was to raise the pH of the pooled NADP fractions to about 5.80 prior to lyophilization.

It had been hoped that, by using formic or acetic acid for elution of the DEAE column and ammonia for titration, it would be possible to get rid of the volatile salts ammonium formate or acetate and to recover pure NADP by lyophilization. In fact, it was found difficult to obtain absolutely salt-free NADP using this approach. Hence, we preferred to switch back to HCl elution, to use sodium hydroxide for raising the pH of the NADP pool, to desalt then NADP by gel filtration and to recover the sodium salt form of NADP by lyophilization. pH adjustment of the NADP pool is done manually and the purification and desalting set-ups are separate units. We did not link the two set-ups in a single fully automatized unit because the resulting apparatus would be too complex and it would also probably be necessary to use a huge gel filtration column: actually the size of the sample deposited on top of the gel filtration column is limited to 25 ml, that is approximately one fourth of the volume of the NADP pool. Nevertheless, the desalting set-up can keep pace with the output of the purification set-up because of a much shorter cycle time.

A small, precisely weighed aliquot is routinely taken to monitor the quality of

the various NADP batches thus produced. On several occasions the NADP was also subjected to other tests: assay of Cl^- in order to check the efficiency of salt removal[7]; enzymatic assay of NADP[8]; spectroscopy; analytical chromatography on AGMP 1[9]; gradient HPLC on Partisil 10 SAX[10]; thin-layer chromatography on PEI cellulose[11]. However, for routine use, isocratic HPLC[2] was found to be the most informative and convenient way to ascertain the purity of the product (see Fig. 6). The quality of the purified NADP was also confirmed by the fact that the same results were regularly obtained with either recovered NADP or with an authentic sample.

Although the described method of NADP recovery has been tailored to fit our needs, we think that it can be used as described whenever NADP is present, along with its breakdown products, in a salt solution of high molarity. If NADP is present in a salt solution of low molarity, the XAD-4 chromatography step can be omitted and the NADP-containing solution directly applied to the DEAE Trisacryl column, after dilution and/or pH adjustment. In repetitive procedures designed for medium or large scale purification of NADP-requiring enzymes, the affinity elution procedure could be made more economical by recovering NADP after use.

ACKNOWLEDGEMENTS

We wish to thank Mr. R. Kernemp (INSERM U 129, Paris) for skilfully making some of the glass parts used in this study. We gratefully acknowledge the financial support of the Paris 7 University (UER Xavier Bichat).

REFERENCES

1 Y. Kroviarski, S. Cochet, P. Boivin and O. Bertrand, *J. Chromatogr.*, 243 (1982) 111–121.
2 J. H. Knox and J. Jurand, *J. Chromatogr.*, 218 (1981) 341–354.
3 R. B. Hurlbert, *Methods Enzymol.*, 3 (1957) 785–805.
4 T. Uetmatsu and R. J. Suhadolnik, *J. Chromatogr.*, 123 (1976) 347–354.
5 G. C. Mills, *J. Chromatogr.*, 242 (1982) 103–110.
6 D. S. Hsu and S. S. Chen, *J. Chromatogr.*, 192 (1980) 193–198.
7 J. Ruzicka and E. H. Hansen, *Flow Injection Analysis*, Vol. 62, Wiley, New York, 1981, pp. 132–133.
8 E. Beutler, *Red Cell Metabolism: a Manual of Biochemical Methods*, Grune & Stratton, New York, 1971, pp. 99–102.
9 J. T. Axelson, J. W. Bodley and T. F. Walseth, *Anal. Biochem.*, 116 (1981) 357–360.
10 E. Harmsen, P. Ph. De Tombe and J. W. De Jong, *J. Chromatogr.*, 230 (1982) 131–136.
11 E. Bömme and G. Schultz, *Methods Enzymol.*, 38 (1974) 27–38.