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AUTOMATED PURIFICATION OF HUMAN ERYTHROCYTIC 6-PHOSPHOGLUCONATE DEHYDROGENASE

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SUMMARY

Human 6-phosphogluconate dehydrogenase (6PGD) was purified from hemolyzate by group affinity chromatography on 2′,5′-ADP-Sepharose, followed by buffer exchange chromatography on Sephadex G-25 and finally salting-out chromatography on Sepharose 6B. An apparatus was assembled from commercially available elements, in which the purification procedure can proceed and be completed unattended and under fully automatic control. The weekly production of the set-up is at present 10 mg of pure 6PGD. The choice of the purification procedure and the advantages of automation are discussed.

INTRODUCTION

6-Phosphogluconate dehydrogenase (D-6-phosphogluconate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.44) (6PGD) is an enzyme of the pentose shunt pathway. This dimeric enzyme (molecular weight of the monomer 53,000 daltons) catalyzes the transformation of 6-phosphogluconate into D-ribulose 5-phosphate. Numerous variants of human 6PGD have been described based on electrophoretic and functional studies of 6PGD from blood cells[1,2]. Human 6PGD has often been found to occur in post-translational modifications[3]. More information on the covalent structure of the native enzyme is required for the study of 6PGD variants or of post-translational modifications on a structural basis. If such a study of the enzyme is to be undertaken, relatively large amounts, that is several tens of milligrams, of protein must be purified, even though modern techniques of protein sequencing have reduced, by several orders of magnitude, the actual amount of material needed[4].

Since blood is easy to obtain, it is the preferred starting material for the purification of human 6PGD. Initially, two methods were considered for the purification of large quantities of 6PGD. The first was to start from a large amount of blood (20 or 30 l). The second was to purify the protein from a smaller volume of blood (that is less than 1 l), but repeat the procedure many times. The first choice was discarded because we did not possess the required equipment, namely centrifuges accommodating large volumes, or high-capacity chromatography columns. However, the second method is

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very time-consuming. The solution was to automate nearly all the purification procedure.

In this paper we describe our automated purification procedure. It is based on a technique described by Morelli[5], but with substantial modifications.

EXPERIMENTAL

Materials

Most chemicals (reagent grade) were purchased from E. Merck (Darmstadt, G.F.R.); NADP was obtained from Boehringer (Mannheim, G.F.R.). Chemicals for Dns derivatization were purchased from Pierce (Rockford, IL, U.S.A.) and polyamide plates from Schleicher & Schüll (Dassel, G.F.R.). The 2′,5′-ADP-Sepharose, Sephadex G-25, DEAE-Sephadex A-25 and Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden), DEAE-Trisacryl M from l'Industrie Biologique Française (Gennevilliers, France) and DE-22 from Whatman (Maidstone, Great Britain). Electromagnetic valves were obtained from ASCO (Scherpenzeel, The Netherlands), small-bore Kel F valves and their pneumatic actuators from Gilson (Villiers le Bel, France). Poly(tetrafluoroethylene) or polyethylene tubings were connected with standard 1 4-in. 28-thread fittings (Gilson), Swagelok fittings from Crawford Fitting (Solon, OH, U.S.A.) or with fittings made in the laboratory from parts of perfusion systems supplied by Vermed (Neuilly en Thelle, France). Polyethylene two- or three-way stopcocks were also obtained from Vermed. The columns were from Whatman or made in the laboratory. Most of the glassware used in the automatic chromatography system were custom-made, according to our design, with Sovirel SVL parts (Sovirel, Levallois Perret, France). The pumps used in the chromatographic system were purchased either from Gilson or from Ismatec (Zurich, Switzerland).

Top 2000 electric timers were obtained from Crouzet (Valence, France), relays, switches, electric plugs and sockets, as well as wiring elements, from local retailers. Human blood, from CNTS (Paris, France), consisted of freshly outdated hemoconcentrates.

Enzymatic assay

6PGD was assayed, at 30°C, in 50 m*M* triethanolammonium chloride buffer, pH 7.5, containing 0.1 *M* $MgCl_2$, 0.25 m*M* NADP and 10 m*M* 6-phosphogluconate.

Protein assay

The hemoglobin concentration was determined according to the method of Drabkin[6]. The protein concentration of pure 6PGD solutions was determined from the absorbance at 280 nm ($E^{\%}_{1\,cm}$ was found to be 10 with precisely weighed, pure, lyophilized 6PGD).

Polyacrylamide gel electrophoresis

10% Acrylamide slab gel electrophoresis containing sodium dodecyl sulphate (SDS) was performed according to Laemmli's technique[7].

N-terminus study

The Dns derivatization of several samples of purified 6PGD (each 0.15 mg) was performed according to the method of Weiner *et al.*[8]. The acid hydrolysis of Dns-

protein was carried out with 5.7 *N* HCl, or with 3 *N* *p*-toluenesulphonic acid containing 0.2% 3-(2-aminoethyl)indole[9] in order to avoid the destruction of an eventual Dns-N-terminal tryptophan. The hydrolysis products of Dns-6PGD were submitted to thin-layer chromatography on polyamide plates[10].

Buffers used in the purification procedure

The buffers used in the purification procedure contained 10^{-3} *M* ethylenediaminetetraacetic acid, 10^{-2} *M* β-mercaptoethanol and 10^{-3} *M* ε-aminocaproic acid. They were prepared in large volumes and either stored at 4°C, or frozen in suitable aliquots if they contained labile substances like NADP or urea. Buffer A was 0.2 *M* potassium phosphate, pH 6.00; B was 0.2 *M* potassium phosphate, pH 7.85; C was 0.1 *M* potassium phosphate buffer, pH 7.85, containing 0.1 *M* KCl; D was 0.05 *M* potassium phosphate, pH 7.85.

The elution buffer was prepared by dissolving 400 mg of NADP in 100 ml of buffer D (final concentration of NADP, *ca.* 5 m*M*). Washing buffers containing 6 *M* urea and 1 *M* KCl were adjusted to either pH 6.5 (acid buffer) or pH 8.5 (basic buffer). Buffer E was 0.04 *M* potassium phosphate, pH 5.6, 45% saturated with ammonium sulphate; F was 0.04 *M* potassium phosphate, pH 5.6, 30% saturated with ammonium sulphate.

The amount of ammonium sulphate needed for the preparation of the buffers was calculated from tables[11].

Valves

Many valves were used in the chromatography system. They were: (a) four stainless-steel two-way valves (one normally open, three normally closed); (b) four three-way slide valves and two four-way slide valves made of acetal resin, their positioning being ensured by pneumatic activators which were connected either to compressed air or to the atmosphere, by electromagnetic three- or four-way brass valves.

Purification procedure

The procedure consisted of three chromatographic separations following the preparation of hemolyzate.

The first chromatogram was carried out in a 2′,5′-ADP-Sepharose column. The 6PGD was eluted with an NADP-containing buffer. The second chromatogram, on Sephadex G-25, was performed to exchange the elution buffer with buffer E (which contained ammonium sulphate at 45% saturation). During the third chromatogram on a Sepharose 6B column, the 6PGD was salted out at 45% saturation with ammonium sulphate, and eluted in a pure form with a linear gradient of decreasing ammonium sulphate concentration. The three chromatography columns were interconnected by tubings and valves. The operation of the valves and pumps was controlled by a programmer, and the whole chromatographic procedure was, thus, entirely automatic. A flow chart of the system is shown in Fig. 1, a photograph of the purification set-up placed in a cold room is shown in Fig. 2 and Fig. 3 shows the electric wiring diagram of the programmer.

The entire purification procedure shall now be described step by step.

Preparation of the hemolyzate. Two bags of packed red cells were pooled and two volumes of cold distilled water were added. The mixture was quickly frozen in a

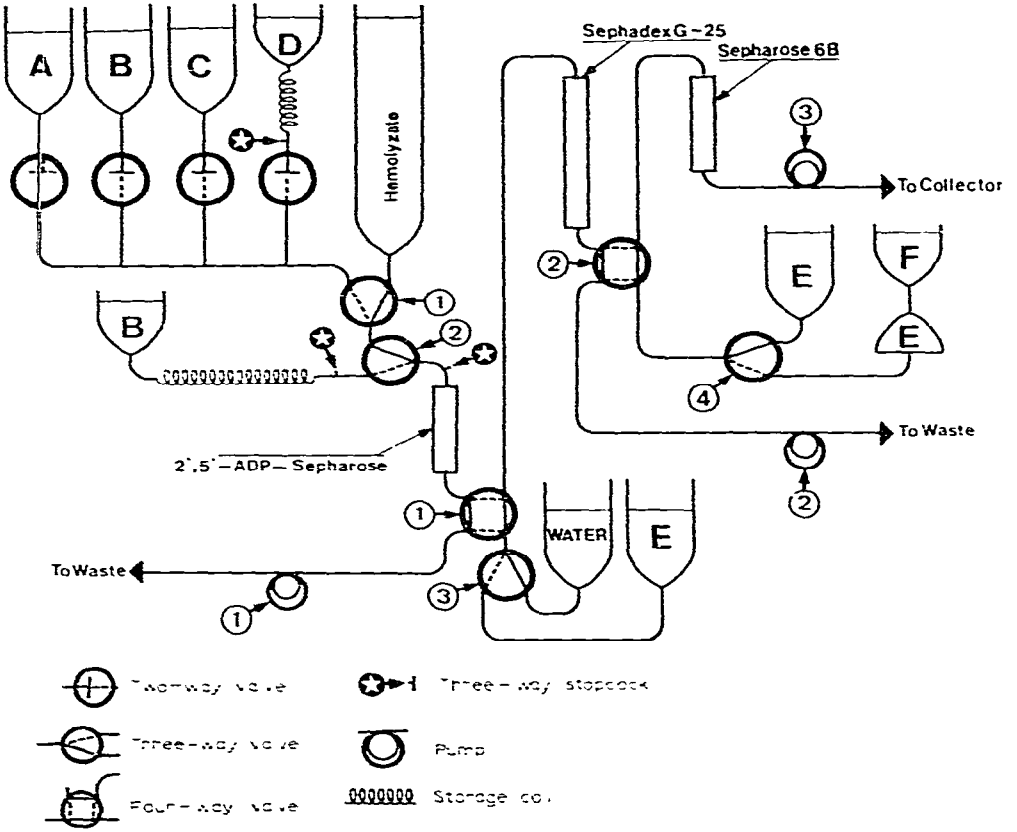

Fig. 1. Diagram of the chromatographic system. The two positions of the valves are shown: the solid line through the valve shows the inactivated position, while the dotted line indicates the activated position. The three- and four-way valves, as well as the pumps, are numbered as in the text. The absorbance monitor and the magnetic stirrer are not shown. Numerous two- and three-way stopcocks have also been deleted, as they are not absolutely necessary to operate the set-up, but simply convenient for cleaning or flushing the tubings and the buffer reservoirs.

large polyethylene basin floating on a dry ice–methanol bath. After 1 h, the polyethylene basin was removed from the bath and allowed to float in a sink filled with running tap-water. As the hemolyzate thawed, the liquid was removed with a peristaltic pump and pumped into a graduated cylinder surrounded with crushed ice. After all the hemolyzate had been transferred, it was diluted with an equal volume of buffer A–water (1:1). After homogenization, the hemolyzate was centrifuged for 45 min at 15,000 *g*. The supernatant, or part of it, was used in the first step of purification.

2',5'-ADP-Sepharose chromatography. When the automatic program was initiated by pressing the start button (Fig. 3), the hemolyzate was pumped on the column (2.5 cm wide, 2 cm long) through three-way valve 1 (Fig. 1) by peristaltic pump 1 (working at high flow-rate, 90 ml/h). Twelve hours later, the position of the three-way valves was changed, and the peristaltic pump was set at low flow-rate, 60 ml h. The column was then rinsed with buffers A, B and C for 1, 1 and 2.5 h respectively. The buffer selection was achieved through appropriate switching of the stain-

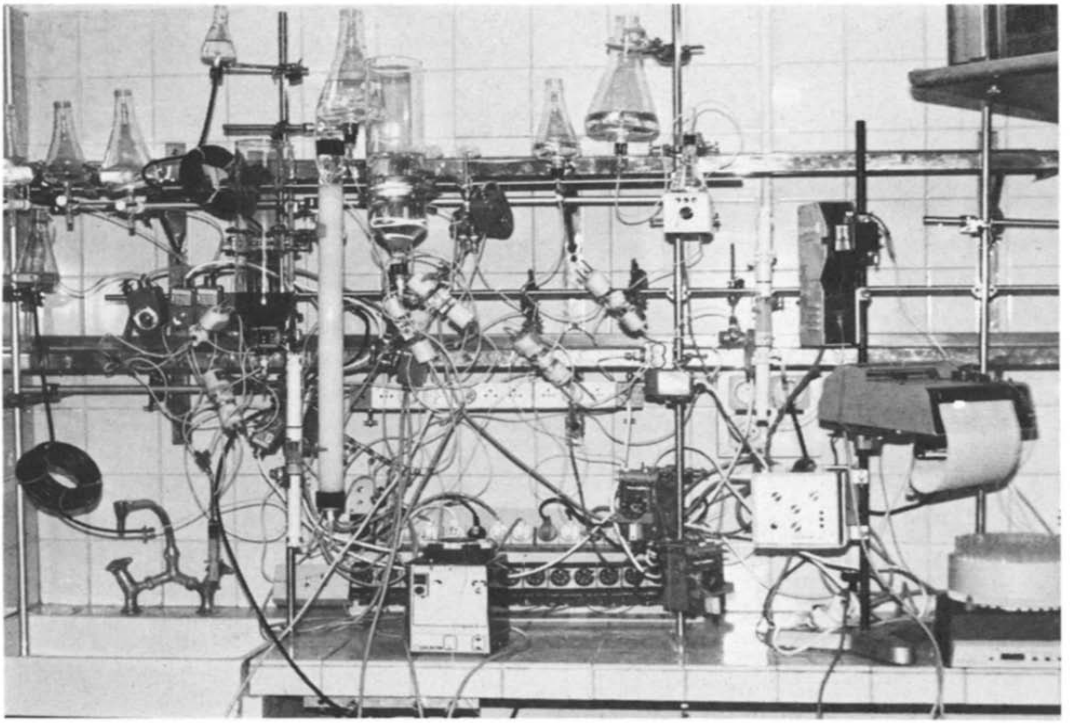

Fig. 2. Photograph of the purification set-up.

less-steel two-way valves situated ahead of the column. (The two-way valve for buffer A is normally open while the other two are normally closed.) The 6PGD was eluted by pumping the NADP-containing buffer on the column. Before the initiation of the program, using a syringe and a three-way stopcock, this buffer was placed in a storage coil (made of polyethylene tubing, 0.4 cm I.D.) connected with a glass reservoir filled with buffer D. In this way, all the NADP-containing buffer can be flushed on to the column with buffer D. When purified 6PGD was present in the effluent of the 2′,5′-ADP-Sepharose column, a four-way valve (1) in the effluent line was activated, and the outlet of the 2′,5′-ADP-Sepharose column thus connected to the inlet of the G-25 column and disconnected from the waste reservoir.

After sufficient time for the deposition of the 6PGD on top of the G-25 column, the four-way valve 1 was deactivated and the effluent line again connected to the waste reservoir. The 2′,5′-ADP-Sepharose column was then automatically rinsed sequentially with acid and alkaline urea-containing buffers, then with buffer B by actuation of three-way valve 2. The polyethylene coil situated ahead of this valve was filled successively with buffer B, alkaline and acid urea-containing buffers, using a syringe and a three-way stopcock as previously described for the NADP-containing elution buffer. This elegant method of programming buffer changes without any valve, by simply using storage tubing of small inside diameter, has been described and used for amino acid analysis by Hare[12]. The column was then re-equilibrated with buffer A by suitable positioning of the three-way valve 2 and of the two-way valves.

Sephadex G-25 chromatography. The purpose of the chromatography on the

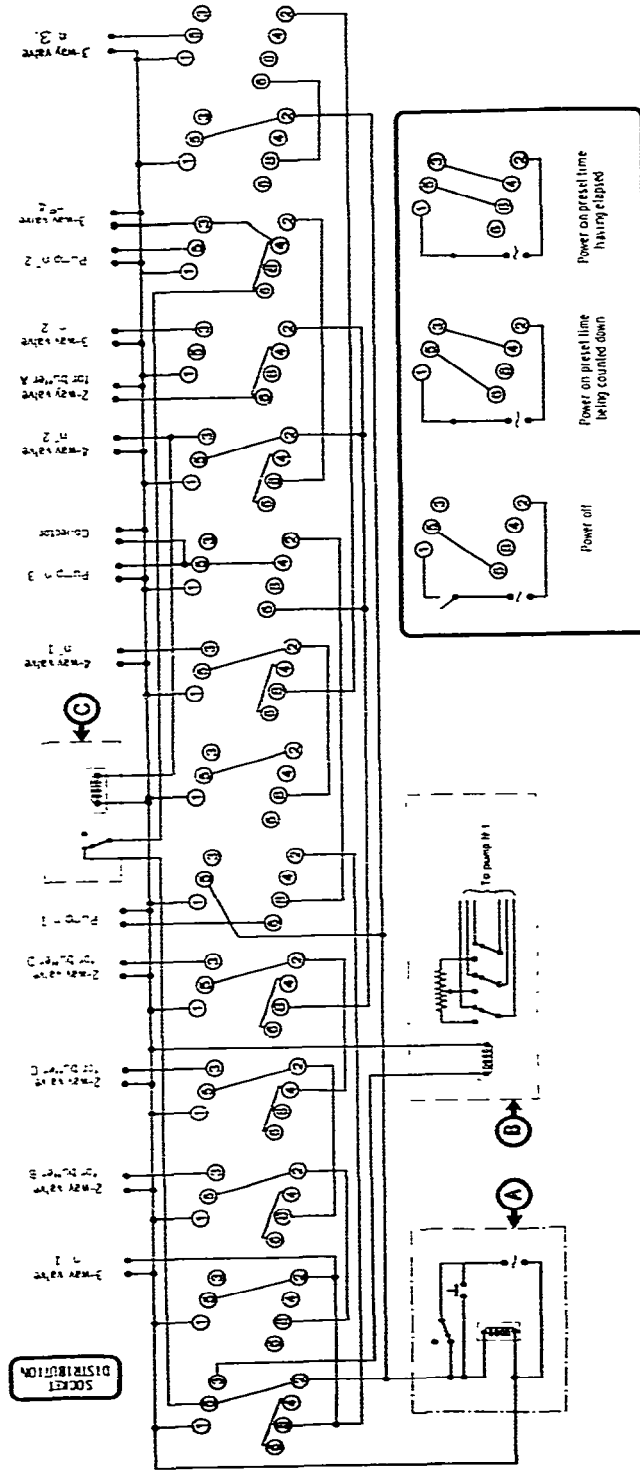

Fig. 3. Diagram of the wiring of the programmer. The main part shows the wiring: only the connections added to the timers are shown; the internal connections of terminal 5 to terminals 6 or 8, or of 3 to 4, are not indicated. The boxes show the three auxiliary assemblies whose functions are explained in the text. Assembly A is shown as running, that is with the relay excited. Some switches used for the manual activation of auxiliary assemblies, and some pilot lights, have been deleted to simplify the diagram. 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 preset time begins to be counted down while 3 and 4 are shorted together; when the preset time has elapsed, the contact between 5 and 6 is broken but 5 and 8 are shorted together. A reset of the timer is obtained by switching off the power to 1 or 2.

G-25 column equilibrated in buffer E is to exchange the 6PGD-containing buffer: the protein must be dissolved in buffer E before chromatography on Sepharose 6B. This chromatography was carried out at a constant flow-rate of 60 ml/h (peristaltic pump 2 or 3), on a column 3.6 cm wide and 65 cm long. The proteins, eluted from the 2′,5′-ADP-Sepharose, were deposited on top of the G-25 column after flowing through four-way valve 1. The four-way valve, in the effluent line of G-25 column, was activated in due course in order to let the proteins, eluted in the void volume of the G-25, be deposited on top of the Sepharose 6B column, while four-way valve 2 was activated, pump 2 turned off and the flow on both G-25 and Sepharose 6B columns ensured by pump 3 only.

It should be noted that the G-25 column must be rinsed with water after the buffer exchange. If this is not done, a retention of NADP on the column is observed, and large volumes of buffer E as well as a long time would be necessary to wash out completely the NADP. The washing of the G-25 column with water as well as its re-equilibration with buffer E for the next chromatographic step was effected through three-way valve 3. Of course, during washing and re-equilibration, the four-way valve 2 directs the G-25 effluent to the waste reservoir.

Sepharose 6B chromatography. The chromatography on Sepharose 6B was carried out at a constant flow-rate of 60 ml/h (peristaltic pump 3), in a column 1.6 cm wide and 15 cm long. The Sepharose 6B column was equilibrated in buffer E when the proteins eluted from the G-25 column were deposited on top of it due to the movement of four-way valve 2. After the automatic completion of sample application, the Sepharose 6B column was washed isocratically with buffer E for 15 min, then a linear gradient, made with 100 ml of buffer E and 100 ml of buffer F, was initiated through activation of three-way valve 4. The shape of the gradient, as well as the automatic rinsing of the column with limiting buffer, was simply obtained with an erlenmeyer flask containing 100 ml of initial buffer. The outlet of the flask was connected with the top of the column by three-way valve 4. The inlet was connected to a reservoir containing limiting buffer. Hence the flask is refilled with limiting buffer at the same rate as it is pumped on the column; magnetic stirring permits an efficient mixing of the initial and the final buffers.

The effluent from the Sepharose 6B column flows first through the cuvette of an absorbance monitor, then towards a fraction collector. The Sepharose 6B column is re-equilibrated with buffer E during the next chromatographic step.

Pure 6PGD was eluted nearly at the end of the gradient as a symmetrical optical density peak. The contents of the enzyme-containing tubes were pooled and the protein was concentrated to 4 ml with a Millipore immersible filter CX30. A 1-ml aliquot of glycerol was added, and the enzyme solution stored at 4°C.

Programmer for the automatic purification

The programmer comprised a number of inexpensive electric timers. Their mode of functioning is shown in the insert of Fig. 3.

The wiring of the fourteen timers is shown in Fig. 3. Three auxiliary assemblies were added to the timers. The function of assembly A is to prevent an automatic restart at the beginning of the program if the electric current were to be shut down for a time. Instead, in case of temporary electric power failure, the pumps will remain turned off and the purification will proceed only if the start push button is pressed

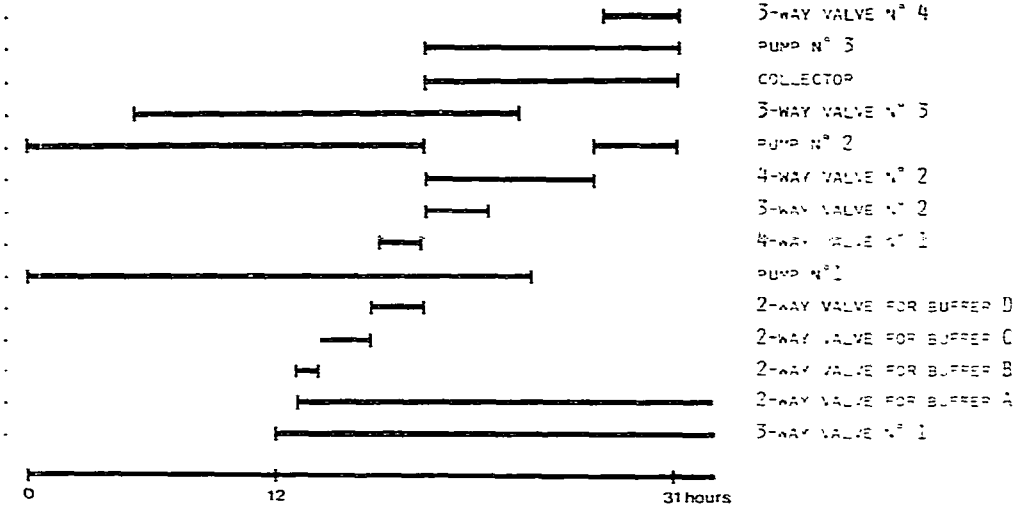

Fig. 4. Diagram of the program routinely used for the automatic purification of 6PGD. The solid lines parallel to the time axis show when the power is brought to the electric sockets feeding the valves, the pump or the collector in operation. For instance, buffer B flows on the 2',5'-ADP-Sepharose column if the power is brought to peristaltic pump 1 and if the two-way valve for buffer B (normally closed) is activated together with the two-way valve for buffer A (normally open) and the three-way valve 1 (four-way valve is inactivated).

manually after the necessary adjustments of the preset times of the fourteen timers. Auxiliary assembly B is used to control the high flow-rate of pump 1; C turns off pump 2 while four-way valve 2 is activated. The programmed functions, with the exception of the one depending on auxiliary assembly B, are obtained by switching on the current from the electrical sockets in which are plugged the power cords of the electromagnetic valves pumps or of the collector.

Fig. 4 shows a diagrammatic representation of the program used for the purification set-up.

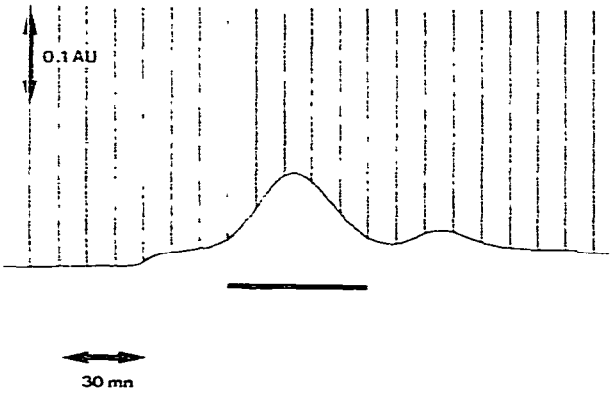

Fig. 5. Recorder tracing of the absorbance monitor. The chart paper speed was 4 cm/h. The vertical lines intersecting the recorder tracing are made by the event marker of the collector. The horizontal bar shows the 6PGD-containing tubes to be pooled.

RESULTS

Fig. 5 shows a part of a typical recorder tracing of the absorbance monitored at the Sepharose 6B outlet.

The contents of the tubes corresponding to the optical density peak, identified by the horizontal bar, were pooled. They contained the 6PGD activity; the smaller peak which follows the 6PGD peak corresponds to a protein as yet unknown. In the sodium dodecyl sulphate (SDS) gel system, it migrates slightly more slowly than 6PGD.

The overall yield of the purification is usually between 45 and 55%. The specific activity of the purified enzyme is of 25 IU/mg. This corresponds to a 5600-fold purification. SDS slab gel electrophoresis is used routinely to check the purity of each batch of automatically purified 6PGD.

Fig. 6 shows a photograph of one such slab gel which was loaded with 50 μg of 6PGD from eight different batches.

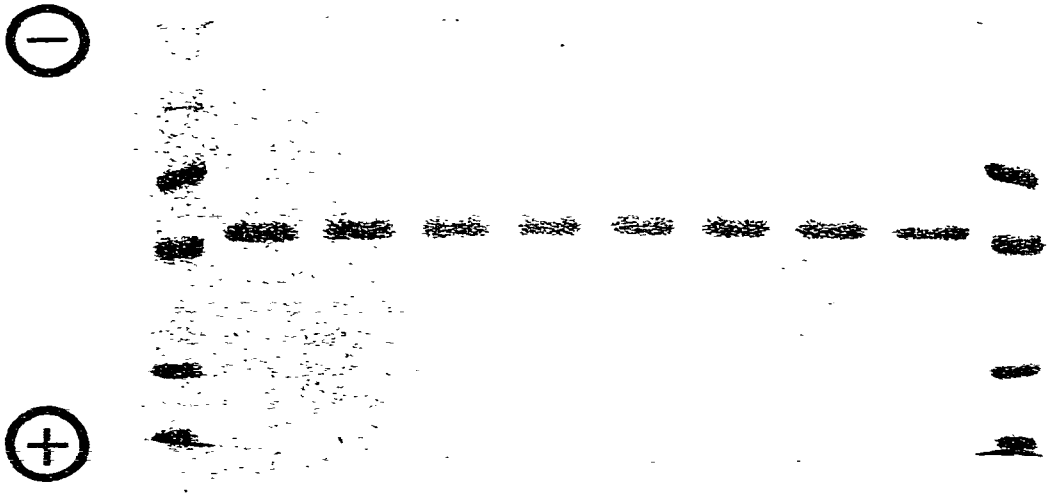

Fig. 6. SDS slab gel. Aliquots of 50 μg of purified 6PGD were loaded on each lane, with the exception of the left and right one where standards of known molecular weight were placed. The gel was coloured with Coomassie blue.

After Dns derivatization and acid hydrolysis of 6PGD, no Dns-amino acid, other than Dns-O-tyrosine and ε-Dns-lysine, was found.

DISCUSSION

The use of 2′,5′-ADP-Sepharose chromatography for purification of human 6PGD was first described by Morelli[5]. Actually, he described a complete procedure based on a 2′,5′-ADP-Sepharose chromatography followed by an ion-exchange step on DEAE-Sephadex A-25. The end product was said to be a pure protein, based on the results of SDS gel electrophoresis. In our hands this procedure did not work very

well: the resulting 6PGD was always contaminated with at least one slower migrating band. Other ion exchangers, DE-22 and DEAE-Trisacryl M, were tried in either manual or automated procedures.

Several modifications of the elution conditions were also tried. However, it was not possible to obtain reproducibly 6PGD with a single band on SDS gels. It should be noted, however, that Morelli prepared the 2′,5′-ADP-Sepharose employed and it is possible that we failed to reproduce his results because we used commercial 2′,5′-ADP-Sepharose with a different degree of substitution and, therefore, different chromatographic properties.

Since we did not succeed in obtaining a pure protein by ion exchange after group affinity chromatography, we decided to adopt, for the next purification step, a completely different technique. We tried salting-out chromatography. In order to define the precise conditions of this purification step we followed the guidelines of Von der Haar[13,14]: at pH 5.6, 6PGD precipitated in a 55% saturated ammonium sulphate solution. When 6PGD, dissolved in 45% ammonium sulphate, at the same pH, was applied to a Sepharose 6B column, the enzyme was adsorbed by the Sepharose. The protein could then be desorbed from the column by a decreasing ammonium sulphate gradient. The enzyme obtained after this salting-out chromatography was pure, as shown by SDS gel electrophoresis and N-terminal end group determination (no free N terminus could be detected).

Salting-out chromatography on unsubstituted Sepharose has been used for preparative purposes by several authors[13–15]. In the particular case of the purification of halophilic enzymes extracted from Halobacterium, this technique was thought to be particularly satisfactory since the preservation of these enzymatic activities requires the use of high salt concentrations throughout the whole purification procedure[15]. The work of Von der Haar[13,14], as well as our own, shows that salting-out chromatography on unsubstituted Sepharose can be a useful alternative to other, more common, chromatographic procedures, even for proteins which do not share the special properties of the halophilic enzymes. It should be noted that our yield from salting-out chromatography on Sepharose 6B is somewhat low (ranging from 50 to 60%), compared to the yields published by other users of this method; the salting-out chromatography on Sepharose of halophilic enzymes (ref. 15 and relevant references cited therein) resulted in yields of 74% or higher. Von der Haar[13] obtained 88 and 94% for two different proteins, but only 59% for a third one. The missing enzymatic activity in the pooled peak of 6PGD cannot be found in any other fraction, and cannot be desorbed from the Sepharose 6B column by rinsing it with low ionic strength buffer. It should be pointed out that when 6PGD activity in effluent of G-25 column is searched manually, it appears that the activity peak is eluted later than the excluded volume of the G-25 column measured when the latter is equilibrated with water. The activity peak is also somewhat tailing. It can be postulated that such an abnormal behaviour of 6PGD on G-25 at high ammonium sulphate concentration results from the same mechanism which governs salting out on Sepharose 6B. The yield from the G-25 chromatography is nevertheless quantitative.

We feel that the automation of the major part of the purification procedure constitutes a very important improvement: the purification set-up is started three times a week, thus producing nearly 10 mg of pure enzyme per week. Two and a half hours are sufficient to: pool the contents of 6PGD-containing tubes from the preced-

ing purification, prepare a fresh hemolyzate and set the purification machine in starting conditions. The preparation of the hemolyzate is made as simple as possible; the centrifugation before placing the hemolyzate on the 2′,5′-ADP-Sepharose is necessary to avoid clogging the column with cellular debris. Our attempts to substitute centrifugation by filtration were unsuccessful; of course, loading the centrifuge and collecting the supernatants remains a manual procedure. Before starting the purification procedure, one must check the level of the buffer reservoirs and refill them if necessary. (As mentioned in Experimental all buffers were prepared in bulk, thus reducing the work of operating the set-up.) It is necessary to fill the two storage coils and the gradient generator, to put new clean tubes in the fraction collector, to fill the cleaned hemolyzate reservoir with the new hemolyzate and to flush the tubing between the hemolyzate reservoir and the 2′,5′-ADP-Sepharose column by use of a syringe and a three-way stopcock (see Fig. 1). The start button is then pushed, and the purification can proceed unattended for 31 h, as shown in Fig. 4.

The success of the automation was made possible by the small number of chromatographic steps. If a precipitation in the liquid phase had been one of the necessary steps of purification, automation would have been considerably more difficult. In addition, the fact that all columns could be operated at the same flow-rate of 60 ml/h was advantageous. Of course, the possibility of using the columns several times without repacking makes automation more attractive by decreasing the maintenance work. To date, our 2′,5′-ADP-Sepharose column has not needed repacking although it has been used three times a week for 3 months; G-25 and Sepharose 6B columns have been used for 8 months without repacking.

To summarize, we have described a purification procedure for 6PGD from human erythrocytes, based on group affinity chromatography on 2′,5′-ADP-Sepharose followed by salting-out chromatography on Sepharose 6B. The automation of the chromatographic procedure permits the production of 10 mg of pure native enzyme per week, with a minimum amount of time and work.

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