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PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM RED BLOOD CELLS AND FROM HUMAN LEUKOCYTES

DESCRIPTION OF A NEW METHOD OF PURIFICATION BY ELECTRIC ELUTION OF THE ENZYME WITH $NADP^+$

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SUMMARY

The authors have highly purified glucose-6-phosphate dehydrogenase from erythrocytes, and leukocytes from a patient with chronic myeloid leukemia.

The stages of purification include a chromatography on DEAE-Sephadex and an elution of glucose-6-phosphate dehydrogenase from a CM-Sephadex column by its own coenzyme $NADP^+$.

This method permits an overall yield of 55%, and 80 to 90% for the stage of elective elution of a stable enzymatic extract, whose specific activity is 170 units/mg of proteins. This extract is homogeneous from an immunological point of view.

Sodium dodecylsulphate acrylamide gel electrophoresis shows the persistence of traces of impurities which could be completely eliminated by a chromatography on hydroxyapatite gel.

The mechanism of the fixation and elution of glucose-6-phosphate dehydrogenase on CM-Sephadex is discussed.

The utilization of leukemia leukocytes permits the purification of a quantity of enzymatic material sufficient for physicochemical and structural studies from a single donor.

Since such a tissue is always less affected by glucose-6-phosphate dehydrogenase deficiency than erythrocytes, it alone can permit the study of certain unstable variants.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from human erythrocytes was first completely purified in 1966 by Yoshida [1]. Starting with 15 liters of blood, this author obtained 15.6 mg of crystallized glucose-6-phosphate dehydrogenase in the course of 12 separate stages of purification. When abnormal and unstable enzyme variants are to be purified from small amounts of blood such a procedure is obviously not feasible.

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The same remarks can be applied to the methods established by Cohen and Rosemeyer [2] and by Bonsignore [3].

The method requiring an elective elution of the enzyme by one of its ligands attempts to simplify and shorten the purification procedure.

After the work of Matsuda and Yugari [4] on the hepatic glucose-6-phosphate dehydrogenase of rats, Rattazzi established the elution of erythrocytic glucose-6-phosphate dehydrogenase from a CM-Sephadex column with the natural substrate, glucose 6-phosphate [5].

In 1970 Yoshida described a method of purification using the elution of the enzyme also from a CM-Sephadex column, with a substrate analogue, the 6-phosphogluconate [6].

We describe the specific elution of erythrocytic and leukocytic glucose-6-phosphate dehydrogenase from the same ion exchanger with the coenzyme $NADP^+$.

This method allows us to obtain a highly purified, stable glucose-6-phosphate dehydrogenase with a high yield; it also allows the analysis of the mechanism of fixation-elution of the enzyme on this cationic ion exchanger.

Finally, we have also highly purified leukocytic glucose-6-phosphate dehydrogenase from the white blood cells of a patient with untreated chronic hyperleukocytic myeloid leukemia: since such a tissue is always much richer in glucose-6-phosphate dehydrogenase than red blood cells one can obtain enough enzyme from a single subject to allow a physico-chemical and structural study.

MATERIALS AND METHODS

Materiales

6 l of blood stored at +4 °C in acid–citrate–dextrose formula A and taken within 30 days were furnished by the blood bank of the Beaujon Hospital*. The blood of the leukemic patient used for the isolation of leukocytic glucose-6-phosphate dehydrogenase was collected in Fenwall bags for plasmapheresis.

The ion exchangers (DEAE-Sephadex A-50 and CM-Sephadex C-50) were supplied by Pharmacia and the hydroxyapatite gel by Clarckson-CC. The substrates for the enzymatic reactions, glucose 6-phosphate, 6-phosphogluconate, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), were supplied by Boehringer/Mannheim. The phenazine methosulphate and tetrazolium salt (MTT) came from Sigma Chem. Co., the amidoschwartz from Merck, and Coomassie blue R. 250 from E. Gurr. Acrylamide, bisacrylamide and sodium dodecylsulphate were furnished by Eastman-Kodak, and the agarose by "l'Industrie Biologique Française".

The enzymatic reactions were read with a Gilford 2400 spectrophotometer. The absorption at 280 nm of the eluate of the chromatographic columns was measured with a Beckman DB spectrophotometer coupled to a recorder. The conductivity of the buffers was measured with a Biolyon conductivity meter.

Methods

All the steps of the purification were carried out at +4 °C. Unless otherwise indicated the buffers contained $2 \cdot 10^{-5}$ M $NADP^+$, 10^{-3} M EDTA, and 10^{-3} M

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ε-aminocaproic acid for the erythrocytic preparation and $2 \cdot 10^{-3}$ M for the leukocytic preparation, 10^{-3} M β-mercaptoethanol.

The activity of glucose-6-phosphate dehydrogenase was measured in a 0.1 M Tris–HCl buffer pH 8, 0.01 M $MgCl_2$, $2 \cdot 10^{-4}$ M $NADP^+$, and $6 \cdot 10^{-4}$ M glucose-6-phosphate at 30 °C.

The activity of 6-phosphogluconate dehydrogenase was subtracted from the activity of the two enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, combined [7].

One unit represents the reduction of 1 μmole of $NADP^+$ per minute at 30 °C.

The protein concentration was measured during purification by the method of Warburg and Christian [8], and at the end of purification by the method of Lowry [9] against a standard of crystallized beef albumin.

The purity of the enzymatic preparations was appraised by three methods:

(1) Immunological methods. An antiserum of rabbit anti glucose-6-phosphate dehydrogenase was obtained by injecting a total of 2.5 mg of the erythrocytic preparation mixed with an equal volume of complete Freund's adjuvant over 5 weeks. The strength of the antibodies thus obtained was 4 to 5 units of glucose-6-phosphate dehydrogenase neutralized per ml of antiserum and 12 to 20 units precipitated by electro-immunodiffusion (Kahn, A., unpublished).

This antiserum was tested by immunodiffusion according to the method of Ouchterlony [10] against the final extract and against extracts at different stages of purification.

(2) Polyacrylamide gel electrophoresis. The gel was 7.5% polyacrylamide in 0.01 M Tris–HCl, pH 8.5; electrode buffer was 0.05 M Tris–HCl, pH 8.5. On one half of the gels 50 μg of protein were deposited, and on the other half a dilution of the extract having a final activity of 0.002 unit.

The electrophoresis lasted 1.30 h at 3 mA/gel. The proteins were revealed by staining with amidoschwartz or Coomassie blue after fixing with trichloroacetic acid [11]. The glucose-6-phosphate dehydrogenase was specifically stained with a solution of 0.2 M Tris–HCl, pH 8, 0.01 M $MgCl_2$, $2 \cdot 10^{-4}$ M $NADP^+$, $6 \cdot 10^{-4}$ M glucose 6-phosphate, 2 mg/20 ml phenazine methosulphate and 4 mg/20 ml tetrazolium salt MTT.

(3) Sodium dodecylsulphate polyacrylamide gel electrophoresis. This was carried out after dissociation of the proteins by 1% sodium dodecylsulphate [12]. The deposits were from 20 to 50 μg of protein and the coloration was developed with Coomassie blue.

PURIFICATION PROCEDURE

The red blood cells were washed three times in 0.15 M NaCl with 10^{-3} M EDTA and haemolyzed with saponine (0.02% w/v). Four volumes of 0.005 M sodium phosphate buffer (pH 6.4) were added to the lysate and the stromas were eliminated by centrifugation at 16 000 $\times$ g for 45 min.

Batch of DEAE-Sephadex, pH 6.4 and precipitation by ammonium sulphate

20 to 30 g of DEAE-Sephadex A-50 previously equilibrated with a 0.005 M sodium phosphate buffer plus 0.02 M NaCl were added to the haemolysate. The ion

exchange resin was then gathered in a large Buchner funnel and washed with the same buffer plus 0.05 M NaCl until the eluate was completely colourless. The glucose-6-phosphate dehydrogenase was eluted with 1.5 to 2 liters of the same phosphate buffer in which the NaCl concentration was raised to 0.25 M. Ammonium sulphate was then added to a final concentration of 36% (w/v); the precipitate was collected by centrifugation and placed again in 50 to 100 ml of 0.005 M phosphate plus 0.05 M NaCl buffer (pH 6.4). It was dialyzed against 20 vol. of the same buffer that was replaced every 8 h until the conductivity of the dialysis bath remained practically equal to that of the original buffer.

DEAE-Sephadex column chromatography

The extract was then dialyzed and applied to a DEAE-Sephadex column (3 cm × 25 cm) which was previously equilibrated with the same buffer; the column was washed with 500 ml of this buffer (with a flow rate of 50 ml/h), then the glucose-6-phosphate dehydrogenase was eluted by initiating a gradient between 600 ml of the 0.005 M phosphate buffer, pH 6.4, plus 0.05 M NaCl and 600 ml of 0.05 M phosphate buffer, pH 6, plus 0.20 M NaCl. 10-ml fractions were collected. Fractions amounting to more than 90% of the enzymic activity were pooled and precipitated by 40 g % (w/v) ammonium sulphate. The precipitate was collected and dialyzed against a solution of 0.01 M phosphate, pH 6, containing $5 \cdot 10^{-5}$ M $NADP^+$.

CM-Sephadex chromatography with elective elution by $NADP^+$ (Fig. 1)

The preparation was applied to a CM-Sephadex C-50 column (2 cm × 2 cm) equilibrated with the same 0.01 M, pH 6, phosphate buffer. The column was washed with a 0.05 M, pH 6, phosphate buffer containing $5 \cdot 10^{-6}$ M $NADP^+$ until all proteins in the eluate disappeared (flow rate: 30 ml/h).

The elution of glucose-6-phosphate dehydrogenase was then caused by changing the $NADP^+$ concentration to $5 \cdot 10^{-4}$ M. To avoid all modifications, even slight, of the ionic strength $NADP^+$ was added in the form of a solution of the same conductivity as that of the washing buffer. The activity of the glucose-6-phosphate dehydrogenase is at its maximum as soon as an increase of the absorption at 280 nm appears in the eluate, which is due to $NADP^+$. It then decreases exponentially.

20-ml fractions were collected; those containing 90% of the enzymic activity were mixed (about 150 ml). The solution was vacuum dialyzed against a solution of 0.05 M Tris–HCl, pH 8, to form a volume of about 4 ml. The glucose-6-phosphate dehydrogenase was precipitated by 40 g % ammonium sulphate. The enzymatic preparation was conserved in this form at +4 °C. This purification procedure allows us to obtain 12 mg of protein of a specific activity of 170 units/mg, which thus gives a total yield of 55% and a purification of about 40 000 times.

Hydroxyapatite chromatography

5 mg of proteins from the preceding preparation were dialysed against a 0.01 M phosphate buffer, pH 6.8, and applied to an hydroxyapatite column (1 cm × 5 cm) equilibrated with the same buffer. The column was washed with 40 ml of this solution, then eluted with a discontinuous increase of ionic strength (flow rate: 20 ml per h, 5-ml fractions were collected): 20 ml of 0.03 M phosphate, 40 ml of 0.05 M phosphate, 40 ml of 0.07 M phosphate. A very small peak of inactive proteins was

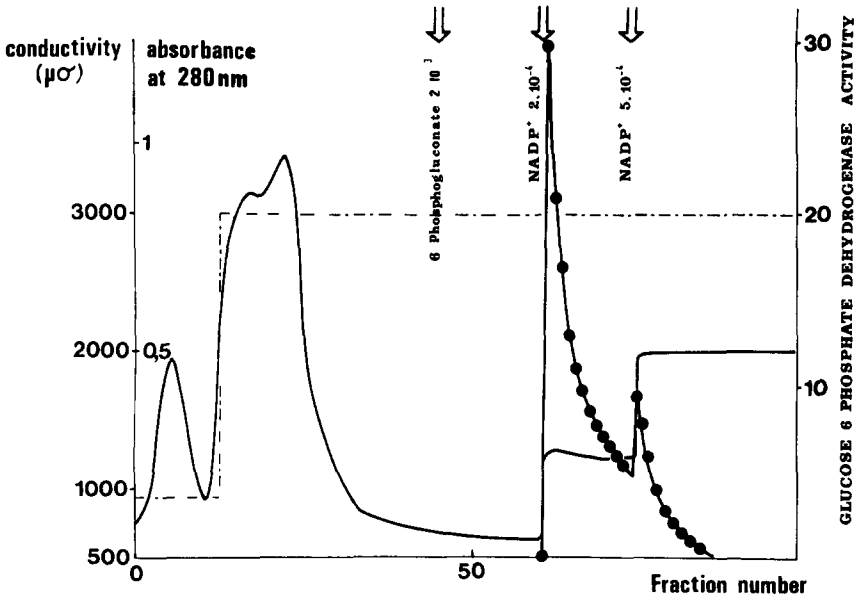


Fig. 1. Elution pattern of human glucose-6-phosphate dehydrogenase from a CM-Sephadex column. ———, Absorbance at 280 nm; ●—●, glucose-6-phosphate dehydrogenase activity; —·—, conductivity. A partially purified enzyme preparation was placed on a CM-Sephadex column (18 cm × 2 cm), equilibrated with 0.02 M phosphate buffer, pH 6.0, containing 10^{-3} M β-mercaptoethanol, 10^{-3} M ε-aminocaproic acid and $5 \cdot 10^{-5}$ M $NADP^+$. The column was washed with 0.05 M phosphate buffer, pH 6.0, containing also β-mercaptoethanol, EDTA, ε-aminocaproic acid and $5 \cdot 10^{-6}$ M $NADP^+$. The enzyme was not eluted by 10^{-3} M 6-phosphogluconate in the same buffer but was eluted by $2 \cdot 10^{-4}$ M $NADP^+$. The increase of $NADP^+$ concentration at $5 \cdot 10^{-4}$ M increased the elution rate. Flow rate 30 ml per h.

eluted at 0.03 M phosphate while the glucose-6-phosphate dehydrogenase was eluted at a narrow and perfectly homogeneous peak at 0.05 M. This procedure allowed the collection of 3.5 mg of protein with a specific activity of 180 units/mg.

Purification of leukocytic glucose-6-phosphate dehydrogenase

Isolation of the leukocytes and preparation of the crude extracts. 350 ml of whole blood from a patient with hyperleukocytic chronic myelocytic leukemia (250 000 white blood cells and 1 000 000 platelets/mm^3) were collected on acid–citrate–dextrose in Fenwall bags for plasmapheresis containing 125 ml of plasmagel (Roger Bellon). The white blood cells layer was gathered after 1 h of sedimentation at room temperature and the packed red blood cells were retransfused to the patient. Four bags were collected in 2 days. The packed leukocytes were isolated by centrifugation and resuspended in a solution of 0.15 M NH_4Cl which contained $2 \cdot 10^{-3}$ M EDTA and ε-aminocaproic acid.

Thus the haemolysis of the remaining red blood cells is complete in 15 min at room temperature. The leukocytes were washed 3 times in 0.15 M NaCl + EDTA and ε-aminocaproic acid, then lysed with a Potter homogenizer in $5 \cdot 10^{-3}$ M phosphate buffer, pH 6.4. The cellular remains were eliminated by centrifugation for

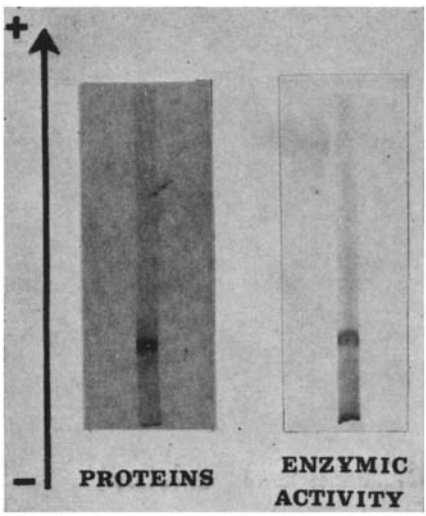
45 min at 37 000 × *g* and the supernatant was precipitated with 35.6 g % ammonium sulphate (w/v).

The rest of the purification was the same as that of erythrocytic glucose-6-phosphate dehydrogenase including a DEAE-Sephadex column and a CM-Sephadex column with specific elution by $NADP^+$. 12 mg of protein with a specific activity of 160 units/mg thus gave a yield of 50% and a purification of about 400 times.

Control of the purity of glucose-6-phosphate dehydrogenase preparations after the stage of elective elution (Figs 2 and 3). The specific activity of the erythrocytic and leukocytic preparations is very close to that of the crystallized enzyme of Yoshida [1]: 180 units/mg. This value is attained in the eluate of the CM-Sephadex column and after hydroxyapatite chromatography.

Immunodiffusion on agarose gel according to the method of Ouchterlony shows only one precipitation line with the immunizing extracts as well as with the leukocytic preparation and the less purified extracts. Polyacrylamide gel electrophoresis shows one major band and a minor slower band which both correspond to the active bands with the specific enzymic staining. Sodium dodecylsulphate polyacrylamide electrophoresis shows the existence of one clearly visible band accompanied by several more rapid micro-impurities, to the limit of visibility. These impurities completely disappeared from the erythrocytic extract after chromatography on hydroxyapatite.

Table I summarizes the results of the purification procedure.



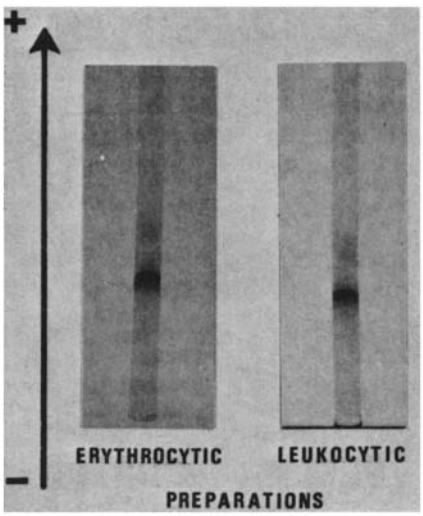


Fig. 2. Polyacrylamide gel electrophoresis of purified glucose-6-phosphate dehydrogenase of white blood cells (after selective elution with $NADP^+$). Proteins were stained with Coomassie blue [11]. Glucose-6-phosphate dehydrogenase was stained with a 0.2 M Tris–HCl buffer, pH 8, containing 0.01 M $MgCl_2$, $2 \cdot 10^{-4}$ M $NADP^+$, $6 \cdot 10^{-4}$ M glucose 6-phosphate, 2 mg/20 ml phenazine methosulphate and 4 mg/20 ml tetrazolium salt MTT.

Fig. 3. Sodium dodecylsulphate polyacrylamide gel electrophoresis of purified glucose-6-phosphate dehydrogenase of red blood cells and white blood cells (after specific elution with $NADP^+$). Proteins were stained with Coomassie blue [11].

TABLE I

	Proteins (mg)		Activity (units)		Specific activity (units/mg*)		Accumulative purification (fold**)		Yield (%)	
	Red blood cells	White blood cells	Red blood cells	White blood cells	Red blood cells	White blood cells	Red blood cells	White blood cells	Red blood cells	White blood cells
Haemolysate or crude extract of white blood cells	$875 \cdot 10^3$	9600	3750	3840	0.0043	0.4	1	1	100	100
DEAE-Sephadex Batchwise			3560						95	
Ammonium sulphate precipitation	2900	2800	3380	3340	0.86	1.2	200	3	90	87
DEAE-Sephadex column	216	173	2700	2600	8	15	1 850	37	72	68
CM-Sephadex column with selective elution	13	12.5	2460	2300	190	185	44 000	475	65	60
Vacuum dialysis and ammonium sulphate precipitation	12	12	2060	1920	170	160	40 000	400	55	50

* In the haemolysate only the protein was measured by assuming a protein concentration of 35 g/100 ml of cells.

** Ratio of specific activity of preparations, taking that of the haemolysate or of the leucolysate as 1.00.

DISCUSSION

The first part of the method proposed for the purification of glucose-6-phosphate dehydrogenase is derived directly from the techniques of Yoshida [1], Cohen and Rosemeyer [2] and Rattazzi [5]. It is necessary to note that the elution of glucose-6-phosphate dehydrogenase using a DEAE-Sephadex column at pH 6.4 by a decreased pH gradient and an increased ionic strength gradient, gives a glucose-6-phosphate dehydrogenase peak which is clearly narrower and more distinguishable than with a simple increase in ionic strength. As for the utilization of DEAE-Sephadex in "batch" for the first stage of purification we find according to Rattazzi that this permits a much greater yield than that obtained with cellulose-DEAE [1, 6], of the order of 95 to 100%.

The elution of glucose-6-phosphate dehydrogenase from a CM-Sephadex column by its coenzyme, $NADP^+$, is interesting for both practical and theoretical reasons. Practically, this technique is very simple in that it calls only for the application of two successive buffers without establishing a gradient; it is completely and easily reproducible, since between the conductivities of 3000 to 3500 $\mu\sigma$ of $5 \cdot 10^{-2}$ M phosphate, the glucose-6-phosphate dehydrogenase is not eluted at very small $NADP^+$ concentrations ($5 \cdot 10^{-6}$ M) and is eluted by strong concentrations of the same product.

The high $NADP^+$ concentration used for the elution of the enzyme probably explains the stability of glucose-6-phosphate dehydrogenase which even when diluted to pH 6 does not lose its activity after more than 48 h at +4 °C.

The total yield of this stage is of the order of 80 to 90%. This higher yield and above all, this stability seem to represent the major advantage of our method compared to that described by Rattazzi [5]. This author eluted glucose-6-phosphate dehydrogenase from the same CM-Sephadex column with 2 mM of glucose-6-phosphate in a phosphate buffer 0.02 M, pH 6, 0.016 M NaCl, and $2 \cdot 10^{-5}$ M $NADP^+$. This enzyme was unstable and lost its activity rather rapidly, probably because all of the $NADP^+$ in the solution had been rapidly reduced to NADPH [5, 13]; the enzyme–NADPH complex is much less stable than the enzyme–$NADP^+$ complex [14, 15]. In the case of the purification of a variant which is itself unstable, such an inconvenience could become determinant.

The specific activity obtained by Yoshida [6] by eluting glucose-6-phosphate dehydrogenase with a substrate analogue, the 6-phosphogluconate, is half as much as that which we obtained and electrophoretic analysis shows the presence of two major protein constituents in the eluate. Moreover this elution by 6-phosphogluconate is closely dependent on experimental conditions, since in the phosphate buffer $5 \cdot 10^{-2}$ M, pH 6, the 6-phosphogluconate cannot elute the enzyme in the absence of $NADP^+$ nor increase the elution in its presence (Fig. 1).

Theoretically, the fixation and elution mechanism of glucose-6-phosphate dehydrogenase is clarified by various experiments. In the absence of $NADP^+$ under our experimental conditions, the fixation of the enzyme on the CM-Sephadex is highly insufficient or absent, which seems to indicate that only the molecules bound to $NADP^+$ are fixed. However, free $NADP^+$ in the form of a sodium salt is not at all retained by the column. Elution caused by $NADP^+$ is maximum as soon as it appears in the eluate; it then decreases exponentially, indicating that at every instant the elution of glucose-6-phosphate dehydrogenase is proportional to the quantity of free $NADP^+$ in the buffer. Thus there seems to be a competition between the ionic groups of the resin and the free $NADP^+$ for the linking to glucose-6-phosphate dehydrogenase. At the ionic strength chosen, the first type of interaction (enzyme-exchanger) is sufficiently repressed so that the second predominates.

In the light of this hypothesis, the problem of the actual mechanism of the elution of glucose-6-phosphate dehydrogenase by glucose 6-phosphate in the experiments of Rattazzi must be examined: at the very high glucose 6-phosphate concentration used, it is probable that almost all the glucose-6-phosphate dehydrogenase is rapidly found in a form "linked to NADPH" (thus accounting for its instability) which no longer has any affinity for glucose 6-phosphate [16]. Thus it seems that it is in fact the reduction of $NADP^+$ to NADPH which is responsible for the elution of the enzyme. The loss of the cationic radical "quaternary ammonium" of the nicotinamide residue which accompanies such a reduction could intervene in the weakening of the interactions with the negatively charged groups of the ion exchanger.

The enzymatic product obtained from leukocytes as from red blood cells is confirmed as being highly purified according to immunological and electrophoretic criteria. However sodium dodecylsulphate acrylamide gel electrophoresis shows the persistance of very small quantities of material which migrates more quickly than the protomer of glucose-6-phosphate dehydrogenase and which could easily be eliminated

by hydroxyapatite chromatography or gel filtration [6]. This material is not antigenic and probably represents less than 5% of the enzymatic proteins. It results from the difficulty of obtaining an eluate completely free of impurities absorbing at 280 nm at the time of the washing of the CM-Sephadex with the $5 \cdot 10^{-2}$ M phosphate buffer.

The granulocytes of patients with "chronic myeloid leukemia" represent an important source of glucose-6-phosphate dehydrogenase which could in this way be isolated in notable quantities (more than 10 mg) from a single patient. If one considers that the activity of glucose-6-phosphate dehydrogenase is always much less weakened in the case of a deficiency in white blood cells than in red blood cells, one can understand the interest in such a material in order to isolate certain variants which are completely inactive in the red blood cells. Some common labile variants could be detected in such patients and thus purified. The richness of granulocytes in enzymes of anaerobic glycolysis could make it possible to use such a tissue in order to obtain from a single donor a sufficient quantity of other enzymatic proteins than glucose-6-phosphate dehydrogenase for physicochemical and structural studies.

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