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NEW STRATEGIES FOR THE SCREENING OF A LARGE NUMBER OF IMMOBILIZED DYES FOR THE PURIFICATION OF ENZYMES

APPLICATION TO THE PURIFICATION OF ENZYMES FROM HUMAN HAEMOLYSATE

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SUMMARY

A method is presented for screening immobilized dyes applicable to the purification of enzymes from haemolysate (haemolysate can be considered as a nearly pure solution of haemoglobin containing only marginal amounts of enzymes). Haemolysate is loaded on immobilized dye mini-columns until haemoglobin and the studied enzymes are found in the column eluate at the same concentrations as those present in the haemolysate. Such a frontal mode of screening allows those dyes to be selected which, displaying a higher affinity for the enzyme of interest than for haemoglobin, can be used to displace the unwanted protein (haemoglobin) from the column by the enzyme of interest (present at a much lower concentration).

INTRODUCTION

Pioneering work in the late 1960s[1] showed that enzymes could interact strongly with textile dyes. The potential for using this interaction for protein purification was rapidly appreciated[2,3] and has since shown great development[4]. A landmark came when Qadri and Dean[5] in the early 1980s introduced a concept that for purifying a given protein from a crude extract it could be extremely rewarding to search in a systematic way for a dye displaying selective affinity for the protein of interest. Subsequently Hey and Dean[6] stressed that it could be useful to use in a first purification step a negative column, that is a column that binds as many as possible of the unwanted proteins in the crude extract but not the protein of interest, and in a second and potentially final step an immobilized dye able to retain totally the protein of interest and the minimal number of unwanted proteins.

Scopes[7–9] later studied the influence of several factors that can play a role in protein retention by immobilized dye columns and gave precise guidelines intended to

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make the systematic screening of immobilized dyes a rapid, problem-solving procedure for the purification of proteins.

Protocols for screening indicate that one has to deposit on to the columns of immobilized dyes to be tested a finite volume of starting material and determine the amount of retained proteins as a whole, together with the retained amount of the protein to be purified. In this paper we present arguments which indicate that it can be fruitful to switch from such a zonal mode of screening to a frontal mode, particularly when the starting material in the purification procedure is haemolysate, which can be regarded as a nearly pure solution of a single protein (haemoglobin) containing only trace amounts of the proteins of interest. A study was undertaken with the initial aim of devising methods for the purification of two enzymes, namely 6-phosphogluconate dehydrogenase (6PGD) and glucose phosphate isomerase (GPI).

EXPERIMENTAL

Materials

Dyes were gifts from ICI France (Clamart, France) and from Hoechst France (Nanterre, France). Brand names of the different dyes used are given in Table I together with their abbreviations. Precise chemical structures of most of these dyes are unknown but some insight can be obtained from published information[4].

Agarose beads (Ultrogel A4) were obtained from IBF (Villeneuve la Garenne, France). Chemicals were purchased from Merck (Darmstadt, F.R.G.) and Carlo Erba (Milan, Italy). Bio-Rad Labs. (Richmond, CA, U.S.A.) polypropylene Econo columns were used for the screening procedure. Their funnels were cut 4 cm from the top. The columns were closed at the top with a simple piston-like Delrin part (machined by SAE, Amboise, France) allowing the dead space above the gel surface to be kept to a minimum.

Immobilization of dyes

The immobilization protocol is similar to those in common use[4]. To obviate the tedious manual procedure of rinsing the gels after dye immobilization, a simple machine was built from commercial components to permit rinsing of four different supports automatically. The precise protocol for dye coupling is as follows.

Wash 20 ml of Ultrogel A4 in a Büchner funnel with about 200 ml of deionized water followed by 100 ml of 0.2 *M* sodium hydroxide solution containing 2% (w/v) of sodium chloride.

Weigh in a screw-capped vessel 400 mg of the dye, and transfer the gel into the vessel together with 20 ml of 0.2 *M* sodium hydroxide solution containing 2% (w/v) of sodium chloride.

Tumble the vessel with the gel suspension for 1 h at 60°C.

Centrifuge the gel suspension for 2 min at low speed and discard the supernatant, which often contains a noticeable amount of undissolved dye.

Pour the gel into one of the four chromatographic columns of the rinsing machine, rinse automatically with 10 m*M* sodium hydroxide solution (200 ml), water (300 ml), 6 *M* urea (300 ml) and finally water containing 0.02% (w/v) of sodium azide. Store the gel in the latter solution.

When gels of higher dye substitution were required, after centrifugation the gels

TABLE I

BRAND NAMES OF THE DYES USED IN THE AUTOMATED SCREENING PROCEDURE AND THEIR ABBREVIATIONS

Procion is a Trade Mark of ICI and Remazol a Trade Mark of Hoechst.

Abbreviation	*Name*	*Abbreviation*	*Name*
A1	Procion Black P-2R	R1	Procion Red HE-3B
A2	Procion Black HE-XL	R2	Procion Red MX-8B
A3	Procion Black P-N	R3	Procion Red HE-7B
A4	Remazol Black B	R4	Procion Red H-EXL
A5	Diazol Black RN Quad	R5	Procion Red P-8B
		R6	Procion Red MX-7B
B1	Procion Navy P-2R	R7	Procion Red P-4BN
B2	Procion Navy MX-4RD	R8	Procion Red MX-G
B3	Procion Havy MX-RB	R9	Procion Red MX-5B
B4	Procion Havy HE-R150	R10	Procion Red H-3B
B5	Remazol Brilliant Blue R	R11	Procion Scarlet P-2R
B6	Procion Blue SP-3R	R12	Procion Rubine MX-B
B7	Procion Blue MX-G	R13	Remazol Brilliant Red 6B
B8	Procion Blue MX-R	R14	Procion Scarlet HE-3G
B9	Procion Blue MX-2GN	R15	Remazol Brilliant Red BB
B10	Procion Blue HE-RD	R16	Remazol Brilliant Red F3B
B11	Procion Blue MX-7 RX	R17	Remazol Brilliant Red RB
B12	Procion Blue MX-2R		
B13	Procion Blue P-4R	T1	Procion Turguoise H-A
B14	Procion Blue P-5R	T2	Procion Turquoise SP-2G
B15	Procion Blue MX-4GD	T3	Procion Turquoise MX-G
B16	Procion Blue HE-GN	T4	Procion Turquoise P-GX
B17	Procion Blue P-7RX		
B18	Procion Navy HE-RN	V1	Procion Violet P-3R
C1	Procion Brown MX-GRN	Y1	Procion Yellow P-3R
C2	Procion Brown P-4RD	Y2	Procion Yellow MX-4R
C3	Procion Brown H-3R	Y3	Procion Yellow P-4G
C4	Procion Brown P-GR	Y4	Procion Yellow MX-4G
C5	Procion Brown HE-XL	Y5	Procion Yellow Brown HE-XL
C6	Procion Brown P-2R	Y6	Procion Yellow MX-8G
C7	Procion Brown MX-5BR	Y7	Procion Yellow HE-6R
		Y8	Procion Yellow HE-4R
G1	Procion Green HE-4BD	Y9	Procion Yellow HE-6G
G2	Procion Olive P-7G	Y10	Procion Yellow MX-3R
G3	Remazol Brilliant Green 6B	Y11	Procion Yellow SP-8G
G4	Procion Green P-4BD	Y12	Procion Yellow HE-3G
		Y13	Procion Yellow MX-GR
H1	Diazol Light Grey 6-BLN	Y14	Remazol Yellow GR
		Y15	Remazol Yellow GL
O1	Procion Orange MX-2R	Y16	Remazol Yellow 4GL
O2	Procion Orange P-2R	Y17	Remazol Yellow RNL
O3	Procion Orange HE-R		
O4	Procion Orange MX-G		
O5	Remazol Brilliant Orange 3R		

were resuspended in a freshly prepared solution of dye. This procedure could be repeated several times (gels identified later in the text with the symbol III following the abbreviation were incubated three times with fresh dye solution).

Quantitative appreciation of dye fixation on agarose

To evaluate the incorporation of dye in agarose, we used an acid hydrolysis method[5]: one volume of gel was hydrolysed with ten volumes of 50% acetic acid at 110°C for 15 min. The amount of dye liberated was evaluated by absorbance measurements at a wavelength found appropriate on the basis of spectral recordings of free dyes.

Assays of proteins

The classical Drabkin method[10] was used to assay haemoglobin by automated flow injection analysis[11]. Enzymatic activities were measured with Zeiss PMQ II or Beckman DU 8 instruments essentially according to methods given by Beutler[12].

Chromatographic procedures

An automated system was devised to apply simultaneously to eight mini-columns (containing 1.6 ml of gel) the samples, buffers and washing solutions and to collect appropriately the eight eluates. Full details concerning the construction of the automatic set-up can be obtained from the authors on request.

Zonal procedure. Starting material was prepared as follows. Human erythrocytes were first washed in saline, then the cells were lysed by dilution (three times) in distilled water and by freezing and thawing. The resulting haemolysate was diluted 11-fold with initial buffer (see below) and centrifuged.

The buffers used for screening were those described by Scopes[9]. Buffer A contained 10 m*M* potassium hydroxide, 2 m*M* magnesium chloride and 30 m*M* sodium chloride and was adjusted to pH 6.5 with morpholinoethanesulphonate. Buffer B had the same composition as A except that it contained 2 *M* sodium chloride and no magnesium chloride.

The flow-rate was 3 ml/h. Before deposition of starting material, the columns were rinsed for 2 h with 6 *M* urea and then for 2 h with buffer B. They were then equilibrated with buffer A.

The zonal procedure itself was essentially that described by Scopes, *i.e.*, 3 ml of starting material were deposited on to the columns (1.6-ml bed volume), which were subsequently rinsed with 6 ml of buffer A, then they were automatically developed with 6 ml of, successively, buffer B, 6 *M* urea and water containing 0.02% (w/v) of sodium azide. Columns filled with immobilized dyes and fitted with their piston-like upper part could be stored as such and were ready for subsequent use.

The percentages of total protein and of enzymatic activity retained by the dyes were evaluated by assays of two fractions of 3 ml each, collected from the beginning of sample deposition.

Frontal procedure. The same instrument, buffers and flow-rates used in the zonal method were also employed for the frontal procedure. However, a large volume of haemolysate was deposited on to the columns, and the haemoglobin concentration and enzymatic activities were measured in 0.5-ml fractions.

Determination of bleed volumes. The bleed volume was considered to correspond to an eluent volume for which 50% of the concentration of haemoglobin or enzyme present in the haemolysate was found in the column effluent.

RESULTS AND DISCUSSION

Zonal procedure

The results are presented in Fig. 1. Such results can be appreciated in the light of Scopes' guidelines: "The object is to find two columns, one which binds as much as protein as possible without binding the enzyme, the other which just holds on to the enzyme, and as little protein as possible, so that it may be eluted with only a slight change to the buffer conditions"[9]. Dye Y14 can be seen as a likely candidate for a negative column. It has to be stressed that stripping the starting material of haemoglobin with this dye would need a column with a large volume.

The choice of a positive column is not as easy as it might seem. For instance, it could be possible to choose A1 or O2, which appeared to bind all of the 6PGD activity and nearly the same amount of haemoglobin. Nevertheless, before making a definite choice, it would be interesting to know which of the two immobilized dye columns has a greater capacity for the enzyme. Moreover, C6 and B9 seemed to behave in the same fashion with regard to 6PGD and haemoglobin (enzyme and haemoglobin are both totally retained). However, this experiment did not give any information on the relative affinities of the dyes for 6PGD and for haemoglobin. An immobililized dye with a greater affinity for 6PGD than for haemoglobin could be used in the displacement mode; we could plan to load the column with starting material until the

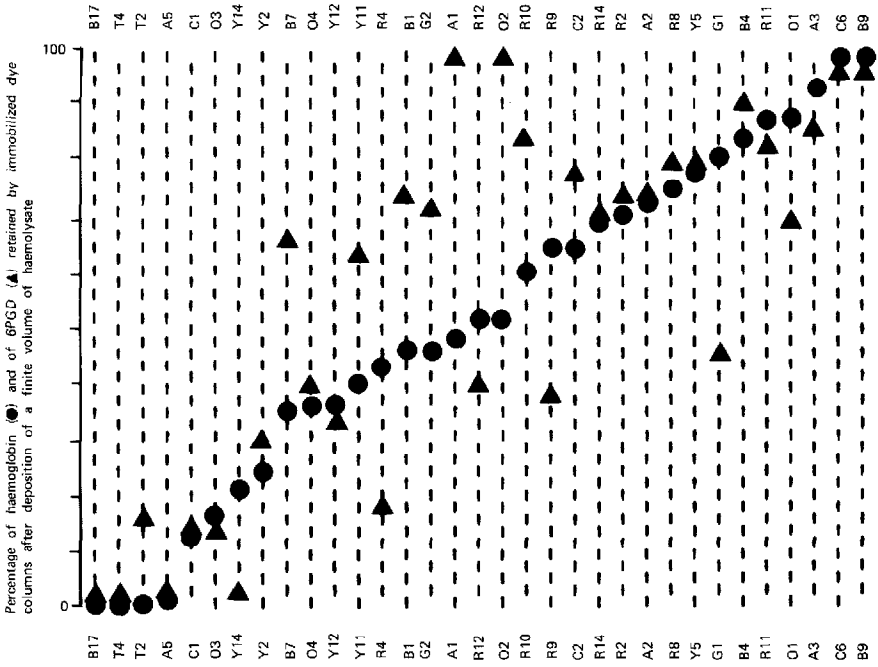

Fig. 1. Experimental results obtained with the automatic screening machine using the zonal method: small finite amounts of starting material were deposited on the columns (see Experimental). Abscissa: abbreviations as in Table I. Ordinate: percentages of whole protein (practically haemoglobin) retained from the starting material (●) and of enzymatic activity (6PGD; ▲).

enzyme begins to displace haemoglobin from the column. In this instance it would also be interesting to evaluate the absolute capacity of the dye columns for the enzyme because a greater capacity would mean a column of smaller volume. Displacement chromatography with a dye that has a lower affinity for 6PGD than for haemoglonin could also be used for 6PGD purification. However, a high concentration of haemoglobin in the starting material would obviously require a large column in order to treat a reasonable volume of haemolysate.

Hence, after zonal screening of haemolysate, for a few immobilized dyes little may be known about the relative affinities of the dyes for the enzyme and for haemoglobin or about the capacity of the columns for the enzyme, although such information would be useful to direct the sound choice of a dye for enzyme purification. This information can easily be obtained by frontal analysis[13].

Frontal procedure

Results for haemoglobin concentration and of enzymatic activity assays for two selected dyes are shown in Fig. 2. The data obtained with B18 show that haemoglobin is able to displace the studied enzymes from the column; the enzymatic activities in the

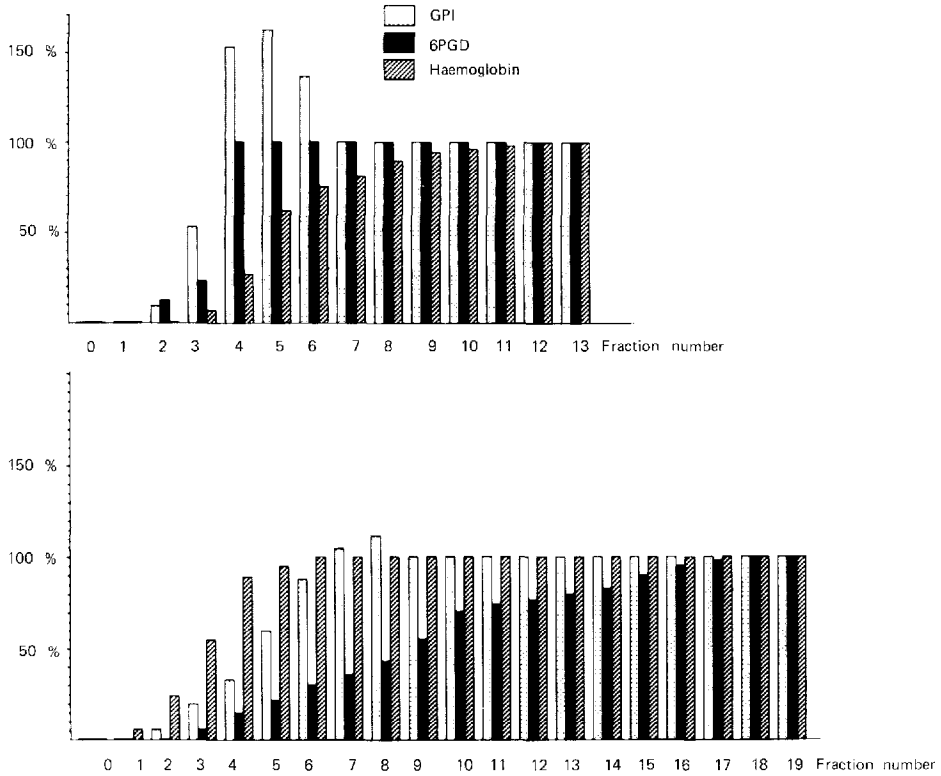

Fig. 2. Haemoglobin concentrations and enzymatic activities measured in 0.5-ml collected fractions with two different immobilized dyes. The value of 100% on the ordinate corresponds to the value measured in the original starting material. Top, results obtained with B18; bottom results obtained with A3.

collected fractions preceding haemoglobin breakthrough rise to higher levels than in haemolysate. With dye A3, 6PGD leaves the column later than haemoglobin, hence the enzyme probably displaces haemoglobin from the column. The low concentration of 6PGD compared with that of haemoglobin explains why the haemoglobin level does not rise to an appreciably higher level before breakthrough of the enzyme.

The results of the measurements of the bleed volumes of haemoglobin and of the two studied enzymes with 79 different dyes are shown in Fig. 3.

Comparison of the results obtained with dyes O2 and A1 demonstrate the interest in using frontal analysis for studies of dye–protein interactions in haemolysate; they could have been considered as equivalent on the basis of the results of zonal analysis because both dyes retained all 6PGD activity present in the 3-ml sample of haemolysate and nearly the same amount of haemoglobin (Fig. 1). The results of frontal analysis (Fig. 3) show, however, that the O2 column has a much greater capacity than the A1 column.

We should point out that the criteria for the choice of a positive column and the strategy for its use that we propose are not identical with those defined by Scopes. Thus, we no longer search for a dye that "just holds on to the enzyme" and use it for elution chromatography[9]. Instead, we look for an immobilized dye with a higher affinity for the protein of interest than for the unwanted protein (haemoglobin) and deposit the starting material in such a way that permits the displacement of the unwanted protein as much as possible by the protein of interest. Obviously bleed volume measurements are not rigorous measurements of the affinity of the proteins studied, but nevertheless it can be inferred from theoretical studies on frontal affinity chromatography[13] that among a mixture of proteins it is the one with the greatest affinity for the immobilized ligand that will have the greatest bleed volume.

The precise ranking of different dyes on the abscissa of Fig. 3 is probably strongly dependent on the actual amount of each dye immobilized on agarose. Immobilization of B16, O1, B4 and C3 at different concentrations showed that an increasing dye loading did not change the order of elution of the proteins, although the protein capacities increased.

It is worth noting that some immobilized dyes columns exhibited a very high capacity for proteins; for instance, 105 mg of haemoglobin were retained by a 1.6-ml column of dye B4 III before that any haemoglobin had left the column, and O1 III retained 57 mg of haemoglobin (the two dyes have the same bleed volume but the breakthrough curve of O1 III is less steep than that of B4 III).

Inclusion of ligands in the haemolysate buffer was used to check rapidly if affinity elution would be effective in desorbing the enzymes from the dye columns (as shown in Table II, the bleed volumes were in fact lower in every instance although the effect was more dramatic with dyes with which a large bleed volume had been found).

NADP was also seen in some instances to be able to lower significantly the bleed volume for haemoglobin, even though haemoglobin it is not known as an NADP-binding protein.

The results of frontal screening did not allow us to devise a purification procedure for GPI using a dye column in the first step (the bleed volumes for GPI and haemoglobin, at least in the buffer system used, were far too close). However, a simple purification procedure for 6PGD derived from the results described in this paper is presented in the following paper[14].

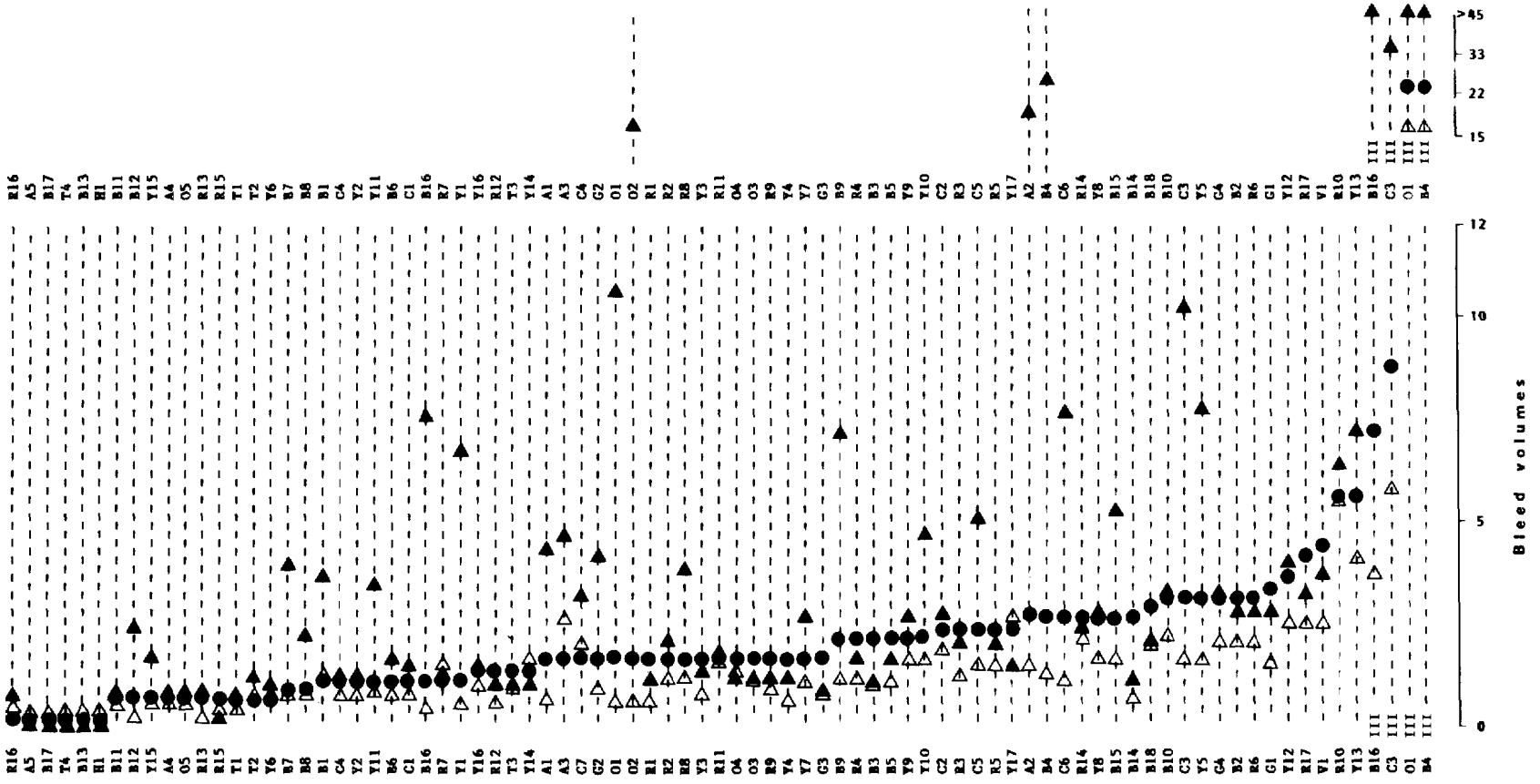

Fig. 3. Bleed volumes obtained with 79 immobilized dyes for haemoglobin (●), 6PGD (▲) and GPI (△). Bleed volumes were measured with 1.6-ml columns. Volumes (expressed in ml) are on the ordinate: for graphical representation, experimental values were reduced by the bleed volume of an unsubstituted agarose column. Abscissa: abbreviations of dyes as in Table I. The different dyes are arbitrarily ranked in order of their increasing affinity for haemoglobin. The dyes B16, C3, O1 and B4 were immobilized on agarose at two different levels of substitution (dye incorporations were respectively 1.5, 1.3, 1.0 and 0.8 mg per ml of gel for the lower level of substitution and 6, 2.6, 7.5 and 3.6 mg per ml of gel for the higher level of substitution; these dyes are followed by the symbol III).

TABLE II

BLEED VOLUMES MEASURED WITH A SMALL NUMBER OF DYES IN THE ABSENCE AND PRESENCE OF NADP

Dye	*6PGD*		*Haemoglobin*	
	Without NADP	*With NADP*	*Without NADP*	*With NADP*
A2	18	0.5	2.5	2.5
B4	27	0.5	2.5	1.5
Y8	2.5	1	2.5	2.5
B14	1	0.5	2.5	2.5
C3	10	1	3	2.5
R10	6.5	2	5.5	3.5
Y13	7	1.5	5.5	3.5
B16 III	>45	1	7	2.5

In conclusion, we should stress that the modifications that we have described to the screening procedures proposed previously by Scopes[9] and Hey and Dean[6] are of the greatest use in instances where haemolysate (*i.e.*, a quasi-pure solution of haemoglobin) is the starting material of the purification procedure. Choosing an immobilized dye with a greater capacity for the enzyme means that column volume needed to treat a given volume of haemolysate will be minimized. However, there are also some drawbacks to such a choice, as has been stressed by Scopes[15], *i.e.*, one could experience some difficulties in trying to elute a protein from a too tight binding immobilized dye.

Obviously these modifications could also be applied in more common situations such as the purification of proteins from bacterial extracts. However, in such instances, zonal screening has proved to be efficient for the selection of both a "negative" and a "positive" column to be used in tandem. Zonal screening is obviously much more easy to perform as a large number of enzymatic assays are needed in order to complete a frontal screening procedure, whereas zonal screening needs only one assay to evaluate the amount of retained enzyme. The choice of frontal analysis would therefore seem justified only if one would like to select a dye for a positive column with a high affinity for the protein of interest with the inherent drawback that possibly this stronger binding column would no longer allow the protein of interest to be "eluted with only a slight change to the buffer conditions"[9]. It is worth noting that in one procedure described for the purification of proteins of bacterial origin, displacement of one protein from a dye column by another of greater affinity was used advantageously[16].

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