Glucose-6-phosphate dehydrogenase catalyzes the initial and rate-limiting step of the pathway that is the principal source of NADPH in many cells. Earlier studies of cells from several species indicated that the intracellular enzyme is under severe and unexplained restraint or inhibition. Moreover, the intracellular enzyme of human erythrocytes exhibits sigmoid kinetics, whereas the purified enzyme exhibits only classical kinetics. We here report that most of the NADP in the human erythrocyte is bound by soluble proteins. In addition, the fraction of unbound NADP that is in the oxidized form, [NADP⁺]/[NADP], varies in a sigmoid manner relative to the fraction of bound NADP that is in the oxidized form. These features of intracellular binding of NADP: 1) account for the previously unexplained inhibition and sigmoid kinetics of glucose-6-phosphate dehydrogenase within human erythrocytes and 2) represent a system in which activity of a rate-limiting enzyme is largely determined by the binding and release of substrate and product by intracellular proteins other than the enzyme itself.

**Experimental Procedures**

CF-25 ultrafiltration cones were the product of the Amicon Corp. Bio-Gel P-10, a molecular exclusion gel with an exclusion limit of 10,000 daltons, was obtained from Bio-Rad. Sephadex G-150 was a product of Pharmacia. d-[U-14C]Glucose 6-phosphate was obtained from the Amersham Corp. The composition of Krebs-Henseleit solution was given earlier (1). Test was purchased from Calbiochem-Behring. Normal erythrocytes were from healthy men whose glucose-6-phosphate dehydrogenase was known from earlier studies to be the usual or normal (G6PD-B) type. The obtaining of blood from a man with the Swiss type of acatalasemia was kindly arranged by the late Hugo E. Aebi of the Medizinisch-Chemisches Institut, University of Bern, Switzerland. Contamination of hemolysates with enzymes that degrade NAD(P) was minimized by two successive measures, as described earlier (1): leukocytes and platelets were removed by filtration of the blood through cellulose, and the stroma (membranes) of the erythrocytes was removed by centrifugation of the 1:10 hemolysate at 16,000 × g. Relative to NADP that was either intrinsic or added to a final concentration of 52 μM, the NADP concentration of hemolysates prepared in this manner remained constant over 18 h at 2 °C. The NADP of intact erythrocytes (1, 2) and freshly prepared hemolysate was almost entirely NADPH. Oxidation of much of the NADPH to NADP⁺ occurred over 12–24 h with molecular exclusion chromatography or dialysis, during which time enzymes of the hemolysate were separated from substrates, such as glucose 6-phosphate, that served to keep the NADP in the reduced form. The use of ultrafiltration cones (3), however, allowed the NADPH and NADP content of hemolysate proteins to be determined promptly after exposure to solutions of NADP with different NADP⁺/NADP ratios. Activities of erythrocytic enzymes and concentrations of protein were determined by methods previously described (3). Concentrations of hemoglobin were determined by the cyanmethemoglobin method (4). Determinations of NADP and NADPH were by the enzymic (cycling) method of Lowry and Passonneau (2, 5). As was confirmed by the use of internal standards (2, 5), artificial oxidation of NADPH of hemolysates at alkaline pH was prevented by the addition of cysteine and by the avoidance of certain maximum hemoglobin concentrations (5, 6). The latter precaution was made possible by the sensitivity of the cycling assay, which allowed extensive dilution of the sample (5).

**Results**

Evidence for Binding of NADP—Binding of erythrocytic NADP by macromolecules of the hemolysate was demonstrated by three different methods: molecular exclusion chromatography, dialysis, and ultrafiltration.

Molecular Exclusion Chromatography—In Fig. 1 may be

1The abbreviation used is: Tes, N-tri[hydroxymethyl)methyl]-2-amino-ethanesulfonic acid.

2Portions of this paper (including “Derivations,” part of “Results,” and Figs. 7–10) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2852, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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NADP-binding Proteins in Erythrocytes

FIG. 1. Distribution of NADP of hemolysate after molecular exclusion chromatography. After 1.5 ml of 0.75 M NaCl was mixed with 8 ml of 1:19 stroma-free hemolysate, 7.0 ml were applied to a 2 x 35-cm column of Bio-Gel P-10 that had been equilibrated with Krebs-Ringer solution/25 mM Tes, pH 7.4. Fractions of 7 ml were collected at a flow rate of 5 ml/hr during elution with the same buffer. NADP, NADPH, and protein were determined by methods given under "Experimental Procedures." N, NADPH; O, NADP; □, protein.

seen the distribution of NADPH and NADP + of human erythrocytes following chromatography of stroma-free hemolysate on a column of Bio-Gel P-10. The elution volume of the proteins corresponded to the exclusion (void) volume of the column. The NADP moved as two peaks: 27% had an elution volume equal to the bed volume and therefore corresponding to unbound NADP, but 73% of the NADP moved with the proteins. The total amount of NADP detected after chromatography was 98 nmol/g of hemoglobin, which was 90% of the NADP/hemoglobin ratio of the erythrocytes from which the hemolysate was prepared. Of the NADP present with the proteins after chromatography, 42% was NADP +. Only a very small fraction of the unbound NADP was NADPH (Fig. 1).

Dialysis—A 1.0-ml portion of hemolysate (23 mg of hemoglobin/ml) was dialyzed in freely spinning, 1-cm flat-width, dialysis tubing at 2-4°C against 125 ml of Krebs-Ringer buffer/25 mM Tes, pH 7.4. At the end of 18 h, the NADP content was 85 nmol/g of hemoglobin, which represented 78% of the content of the undialyzed hemolysate. Of the NADP in the hemolysate, 33% was NADPH. An accompanying bag of 1 ml of 3.35 mM NADP + (without hemolysate) contained 18% of its NADP + after the dialysis. The relatively low permeability of dialysis membranes of NADP, however, made dialysis-equilibrium studies impractical. Dialysis of either 40 µM NADP + or 40 µM NADPH (without hemolysate) resulted in removal of only 12-25% of the NADP in 1 h. The limited permeability of cellophane dialysis tubing to NADP was found with tubing of flat-width 1 cm as well as 2.5 cm and with tubing from two companies. The low permeability was found whether the tubing was boiled in solutions of EDTA and Na 2 CO 3 or not.

Ultrafiltration—NADP was retained with the macromolecular fraction when hemolysate was concentrated (3) by centrifugation in Amicon CF-23 ultrafiltration cones. The addition of 2 x Krebs-Ringer solution to an equal volume of freshly prepared, 1:10 stroma-free hemolysate caused the hemolysate to become Krebs-Ringer solution/25 mM Tes, pH 7.4, containing hemoglobin at a final concentration of 15.7 mg/ml. The NADP concentration of the preparation was 1.9 µM, 73% of the NADP being NADPH. A 6-ml portion of the hemolysate was concentrated by ultrafiltration to 0.5 ml. The concentrate contained 9.8 nmol of NADP, which corresponded to 86% of the original NADP content of the 6 ml of hemolysate. Another 14% of the NADP was found in the 5.5 ml of solution that passed through the ultrafiltration cone. Of the NADP in the concentrate, 76% was NADPH. Ultrafiltration of solutions of NADP in Krebs-Ringer solution/25 mM Tes, pH 7.4 (without hemolysate), revealed no tendency for the NADP to be concentrated on the ultrafiltration cone.

NADP Binding at Fixed Concentrations of NADP + and NADPH—Table I gives the amount of NADP bound by the macromolecules of a hemolysate following chromatography on a column of Bio-Gel P-10 (Fig. 1), equilibration against a defined concentration of NADP + or NADPH, and ultrafiltration. In contrast to the binding of NADP, no binding of 14 C labeled glucose 6-phosphate could be detected after equilibration with a wash solution containing [U- 14 C]glucose 6-phosphate at a final glucose 6-phosphate concentration of 50 µM.

Further description of NADP binding requires certain definitions. [NADP], [NADP + ] and [NADPH] will represent the concentrations of unbound dinucleotide. The subscript s will denote bound dinucleotide, and the subscript c will denote total dinucleotide (the sum of both bound and unbound dinucleotide). In addition, the fraction of NADP that is NADP + (the NADP + /NADP ratio) will be represented as f + . The following are examples of relationships that follow from these definitions: [NADP] s = [NADP] c + [NADP]; [NADP] c = [NADP] + [NADP]; f + = [NADP] c /[NADP] c and f c = [NADP] c /[NADP] c . Fig. 2 is a plot of f versus f c for an experiment in which the hemolysate was washed with Krebs-Ringer solution/25 mM Tes buffer, pH 7.4/12 µM NADP. The hemolysates used in the experiments of Table I and Fig. 2 consisted of the protein fractions obtained after chromatography in Bio-Gel P-10, as in Fig. 1. The proteins therefore had been separated from the

<table>
<thead>
<tr>
<th>NADP</th>
<th>[NADP]</th>
<th>[NADP]</th>
<th>NADP (bound)/hemoglobin</th>
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</thead>
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<tr>
<td>added</td>
<td>wash solution</td>
<td>ultrafiltrate</td>
<td>Ratio</td>
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<tr>
<td>µM</td>
<td>nmol/mg</td>
<td>µmol/340 g</td>
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<td>0.141</td>
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<td>10.1</td>
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metabolites that would have caused metabolic alteration of \( f \) or \( f_b \) during ultrafiltration. Several observations indicated that no major change in \( f \) or \( f_b \) occurred during the 30-60 min required for ultrafiltration: (a) The values for \( f \) shown in the ordinate of Fig. 2, which were those of the solution passing through the ultrafiltration cone, were similar to the \([\text{NADP}^+]/[\text{NADP}]\) ratios in the 12 \( \mu \)M NADP solutions with which the hemolysate was washed (legend of Fig. 2). (b) The addition of glucose 6-phosphate to the hemolysate before ultrafiltration resulted in an \( f \) not much less than that for hemolysate washed with NADP without glucose 6-phosphate (Fig. 2). In the presence of the glucose-6-phosphate dehydrogenase of hemolysate, this substrate should have served to keep most of the unbound NADP in the reduced form. (c) The study of Table I, with wash solutions of 2.0 \( \mu \)M NADP \( ^+ \) and 2.0 \( \mu \)M NADPH, was repeated but with determination of \( f \) and \( f_b \) both immediately after ultrafiltration and 1 h after ultrafiltration. The value of \( f \) from early assay was similar to the corresponding \( f \) from delayed assay, and all points fell close to the curve obtained with wash solutions of 12 \( \mu \)M NADP (Fig. 2).

No major effect on either \([\text{NADP}]_b\) or \([\text{NADPH}]_b\), as seen when hemolysate was washed with Krebs-Ringer solution/25 mM Tes, pH 7.4/2.5 \( \mu \)M NADP \( ^{+} \) containing any of the following at the final concentrations indicated: NAD\( ^{+} \), 40 \( \mu \)M; 2,3-diphosphoglycerate, 1 \( \mu \)M; or ATP, 1 \( \mu \)M. A comparison of Fig. 3 with Fig. 2 indicates, however, that the shape of the curve depends moderately on whether Tes or bicarbonate buffer is used. Although sigmoidicity of the \( f \) versus \( f_b \) curve can be seen for hemolysate washed with Krebs-Ringer solution/25 mM Tes, pH 7.4/12 \( \mu \)M NADP (Fig. 2), the sigmoidicity is even more noticeable with hemolysate washed with Krebs-Ringer solution/25 mM bicarbonate buffer, pH 7.4/15 \( \mu \)M NADP (Fig. 3).

Separation of NADP-binding Proteins by Molecular Size—

**Fig. 2.** Plot of \( f \) versus \( f_b \) for hemolysates exposed to NADP and Tes buffer. The hemolysate was washed with Krebs-Ringer solution/25 mM Tes, pH 7.4, containing NADP as described in Table I. Before the second ultrafiltration, however, five 6.0-ml solutions were obtained by the mixing of NADP\( ^{+} \)-washed hemolysate and NADPH-washed hemolysate so that the former represented 0, 25, 50, 75, and 100% of the 9.0 ml. The samples were ultrafiltered, and \( f_b \) was calculated, as in Table I. The symbol \( f \) represents the [NADP\( ^{+} \)/[NADP]] ratio of the solution passing through the ultrafiltration cone. \( O \) and \( O \), concentrates that had been washed with buffer containing NADP in a final concentration of 12 \( \mu \)M. \( O \), a concentrate of NADP\( ^{+} \)-washed hemolysate to which glucose 6-phosphate was added to a final concentration of 50 \( \mu \)M before ultrafiltration. \( I \) and \( II \) concentrates that had been washed with Krebs-Ringer solution/25 mM Tes, pH 7.4/2 \( \mu \)M NADP in which the NADP was either NADPH (lower pair) or NADP\( ^{+} \) (upper pair) with the determinations of NADP having being done either immediately after ultrafiltration (\( II \)) or 1 h after ultrafiltration (\( I \)).

**Fig. 3.** Plot of \( f \) versus \( f_b \) for hemolysates exposed to NADP and bicarbonate buffer. The values of \( f \) and \( f_b \) are for concentrates that had been prepared as in Fig. 2, but with a wash solution of Krebs-Ringer solution/25 mM (sodium) bicarbonate, pH 7.4/12 \( \mu \)M NADP.

**Fig. 4.** Distribution of NADP binding factors after chromatography on Sephadex G-150 and equilibration with 5 \( \mu \)M NADP. A 15-ml sample of stroma-free hemoglobin/0.15 M NaCl, containing 380 mg of hemoglobin was passed by ascending chromatography up a 2.5 \( \times \) 96-cm column of Sephadex G-150 that had been equilibrated with Krebs-Ringer solution/25 mM Tes, pH 7.5/2.5 \( \mu \)M Tes, pH 7.5/25 \( \mu \)M NADP. Fractions of 7 ml were collected at a flow rate of 16 ml/h during elution with the same buffer. Following elution, 17.5 nmol of NADP were added to each fraction, and 6.5 ml of every other fraction was concentrated on ultrafiltration cones to a volume of 0.2 ml. [NADP\( ^{+} \)] and [NADPH], represent the difference in concentrations between ultrafiltrate and concentrate. O, [NADP\( ^{+} \)]; O, [NADPH]. Activities of catalase (a) and NADPH diaphorase (b), and concentrations of hemoglobin (Hb) are represented as fractions of the following peak values: catalase 3.4 s\(^{-1}\) ml\(^{-1}\), NADPH diaphorase 5.1 nmol min\(^{-1}\) ml\(^{-1}\), and hemoglobin 8.7 mg/ml. Arrows indicate the positions of (c) glucose-6-phosphate dehydrogenase, (d) glutathione reductase, and (e) 6-phosphogluconate dehydrogenase.

A slight difference can be noticed between the location of bound NADPH and bound NADP\( ^{+} \) in Fig. 1. That the two forms of the dinucleotide were associated with macromolecules of different molecular size was confirmed by chromatography of the hemolysate on a column of Sephadex G-150 (Fig. 4) in Krebs-Ringer solution/25 mM Tes buffer, pH 7.4/2.5 \( \mu \)M NADP\( ^{+} \). After chromatography and before ultrafiltration, each fraction received NADPH in the proportion of 2.5 nmol/ml. This addition served to expose the proteins of each fraction to equimolar concentrations of NADPH and NADP\( ^{+} \).
to allow discrimination between NADPH-binding proteins and NADP⁺-binding proteins. As was reported earlier with columns of Sephadex G-200 (3), the binding of NADPH coincided largely with activity of catalase, whereas the binding of NADP⁺ coincided with activity of NADPH diaphorase (NADPH: menadione oxidoreductase, EC 1.6.99.1). In contrast with the earlier studies with Sephadex G-200, however, small peaks of NADPH binding could be seen on each side of the hemoglobin peak (Fig. 4). As a consequence of dilution, the hemoglobin had an average molecular weight intermediate between that of the intact molecule (64,000) and dissociated hemoglobin (32,000). NADP binding of the smaller peaks was reduced when, 3 days after the chromatography, the concentrates were diluted, re-exposed to NADP, and re-concentrated. This observation served to confirm the suspicion that the smaller peaks had been missed in studies (3) with Sephadex G-200 as a result of the lability of these NADP-binding macromolecules and the slower flow rate in Sephadex G-200.

Under conditions in which the hemolysate was equilibrated to 10 µM NADP⁺/10 µM NADPH before ultrafiltration, even higher peaks of NADP binding were seen (Fig. 5). The weight of NADP bound in the catalase and NADPH diaphorase zones, however, remained constant when expressed per gram of hemoglobin (nmol/g). As with the experiment of Fig. 4, some variation and lability were observed in the ability of macromolecules between catalase and NADPH diaphorase to bind NADP. In three consecutive studies with erythrocytes from the same person, a shoulder was seen on the right-hand side of the hemoglobin peak (Fig. 4). As a consequence of dilution, the hemoglobin had an average molecular weight intermediate between that of the intact molecule (64,000) and dissociated hemoglobin (32,000). NADP binding of the smaller peaks was reduced when, 3 days after the chromatography, the concentrates were diluted, re-exposed to NADP, and re-concentrated. This observation served to confirm the suspicion that the smaller peaks had been missed in studies (3) with Sephadex G-200 as a result of the lability of these NADP-binding macromolecules and the slower flow rate in Sephadex G-200.

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![Fig. 5. Distribution of NADP binding factors after chromatography on Sephadex G-150 and equilibration with 20 µM NADP. A 15-ml sample of stroma-free hemolysate/0.15 M NaCl, containing 264 mg of hemoglobin was subjected to ascending chromatography and ultrafiltration of fraction as in the study of Fig. 4, except that the column was equilibrated with a buffer containing NADP⁺ at a final concentration of 10 µM, and 70 nmol of NADPH were added to each 1-ml fraction. Symbols are as in Fig. 4. The upper scale is derived from the regression line of elution volume versus log molecular mass, with dots on the upper scale representing the positions of the following protein standards: bovine catalase, 232,000; aldolase, 158,000; bovine serum albumin, 67,000; ovalbumin, 43,000; chymotrypsin, 25,000; and ribonuclease, 13,700.](image)

![Fig. 6. Effect of different values of f₁ on intracellular rate of glucose-6-phosphate dehydrogenase. Packed erythrocytes from an acatalasemic man were suspended in Krebs-Ringer solution/25 mM Tris, pH 7.4, containing either [1-¹⁴C]glucose or [2-¹⁴C]glucose at a final glucose concentration of 5 mM and methylene blue at a final concentration of 0, 0.5, 5, or 100 µM (1, 2). The total volume of each suspension was 2.3 ml. The suspensions were incubated in a metabolic shaker at 37 °C for 1 h at 37 °C. ¹⁴CO₂ was collected, rates of intracellular glucose-6-phosphate dehydrogenase were calculated, and concentrations of NADP and NADPH were determined as described earlier (1, 2). ○, results with erythrocytes of an acatalasemic man; •, previously reported (2) results with erythrocytes of a normal man.](image)
The results of the present study indicate that most of the NADP of the human erythrocyte is bound to soluble proteins within the cell. The hemoglobin concentration of packed cells used in the present study was 340 g/liter. Thus, the number of micromoles of NADP bound by hemolysate/340 g of hemoglobin, provides a micromolar estimate of [NADPb] within erythrocytes used in the study of Table I. From these calculations, described in the "Miniprint," one class having a greater affinity for NADP+ than for NADPH, the other having the opposite affinities. Mathematical considerations of conditions necessary for sigmoidicity are given in the "Miniprint."

The convolution portion of the curve in Fig. 2 is determined largely by the NADP-prefering protein ("Miniprint"). Since catalase is the major NADPH-prefering protein of human erythrocytes (3), the concavity of the curve in studies of acatalasemc cells (Fig. 6) is to be expected. The properties of purified human catalase (3) meet the requirements, described in the "Miniprint" for an NADP-prefering protein that could contribute to sigmoid binding characteristics. With the exception that the dissociation constants do not quite differ by 10-fold (Figs. 4 and 5), NADPH diaphorase has the required properties of an NADP+-prefering protein (9, 10). The exception regarding NADPH diaphorase would be readily offset by the presence of one or more minor proteins having the required preference for NADP+.

Whether the relative unavailability of NADP accounts for the reduced activity of intracellular glucose-6-phosphate dehydrogenase in other cells and species (1) remains to be determined. If the binding of NADP provides a biological advantage, the advantage is not seriously affected by genetic deficiencies of either of two proteins that separately account for 25-45% of the binding of NADP within human erythrocytes: deficiencies of neither catalase (13) nor NADPH diaphorase (14) seriously affect health. Nevertheless, certain long-term or evolutionary advantages of the binding can be envisioned. One possible advantage of such binding is a reduction in the rate of spontaneous hydration of NADP to form β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide phosphate (NADPH-X). As a result of this hydration, NADPH has a half-life of only 5-6 h when in aqueous solutions at 37°C and at pH 7.0-7.4 (15). The stability of catalase-bound NADPH during purification and storage of catalase indicates that this conversion does not occur while NADPH is bound to that enzyme (3). Another advantage is that the binding allows glucose-6-phosphate dehydrogenase to respond to a changing NADP+/NADPH ratio (specifically to f) as if the enzyme had sigmoid kinetics, decreasing its rate of production of NADPH greatly when more than half of the dinucleotide is NADPH and increasing its rate of production of NADPH greatly when less than half of the NADP of the cell is NADP. Alternatively, the sigmoid relationship can be regarded as one that maximizes the ability of the cell to have both NADP+ and NADPH in the cell.

The results of this study eliminate an apparent exception to the generalization (16) that the Michaelis constant for a substrate usually falls in the range of intracellular concentration of the substrate. The Michaelis constant for NADP+ is in the range of 3-7 μM for human glucose-6-phosphate dehydrogenase, yet the intracellular concentration of NADP was thought earlier to be 30-40 μM (7, 17). The present study, however, indicates that the concentration of unbound NADP is 2-5 μM. The results of this study also serve as a reminder that conditions within the cell can differ greatly from those in dilute lysates, even for the human erythrocyte, which has no nucleus or subcellular particles. A search for an explanation for this difference in human erythrocytes led to the present identification of a system in which the character and amount of activity of a rate-limiting enzyme depend largely on proteins that bind and release a substrate and product and thereby determine the availability of the substrate and product within the cell.

### TABLE II

<table>
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<td>[NADP]</td>
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</tr>
<tr>
<td>μM</td>
<td>μM</td>
<td>pM</td>
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<td>0.687</td>
<td>8.8</td>
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</table>

a Glucose 6-phosphate.

b Without correction for binding.

c With correction for binding.
REFERENCES

SUPPLEMENTARY MATERIAL

NADP-binding Proteins Causing Reduced Availability and Sigmoid Release of NADPH in Human Erythrocytes
Henry N. Kirkman, Glau F. Gaetani, and Evelyn H. Clemons

DERIVATIONS

Consider N types of proteins, each capable of binding either NADP+ or NADPH at a single binding site. An explicit solution for NADP+ and f, as well as for the concentrations of hemolysates (Table I, Figs. 2 and 3), can be obtained when NADP+ and f are fixed and known, as is very nearly the case with certain studies of hemolysates (Table I, Figs. 2 and 3). The following derivations will be carried out with symbols that are convenient for the writing of a computer program.

<table>
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<th>ITEM</th>
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<td>AT</td>
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<td>[NADPH]</td>
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<td>QB</td>
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<tr>
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<tr>
<td>f (= NADP*/NADPH ratio)</td>
<td>F</td>
<td>FB</td>
<td>FT</td>
</tr>
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</table>

Nh protein (PN) = \( N \times PT(N) \)

*AP(N)*: NADP-protein complex. 
*QPN*: NADPH-protein complex.

The total concentration of protein number N is:

Eq. 1  \( PN = PN + AP(N) + QPN \). The dissociation equilibria are:

\[ AP(N) + PN = KAP(N) \] and \[ QPN + PN = PKN \].

which, on rearrangement of terms, becomes:

Eq. 2  \( AP(N) = KAP(N) + K(N) \) and \[ QPN = PKN + Q(N) \].

Substitution of Eq. 2 and Eq. 3 into Eq. 1 and rearrangement yields:

\( PN = PT(N) + (A + Q(K) + Q(N)) \).

When this equation, in turn, is substituted into Eq. 2 and Eq. 3, solutions for AP(N) and QPN are obtained, as follows:

Eq. 4  \( AP(N) = (A(K) + Q(K) + Q(N)) + (A(K) + Q(N)) \).

Eq. 5  \( QPN = (Q(N) + K(N)) + (A(K) + Q(N)) \).

From these two equations, the values for A(Q) and FB can then be determined, given A, Q, and the constants for each protein: PT(N), KAP(N), and KQ(N). Since \( AB = ZAP(N) \) and \( QB = ZQ(N) \), then, Eq. 6:

\[ A(Q) = ZAP(N) + QPN \],

and Eq. 7:

\[ FB = \Sigma AP(N) + \Sigma QPN \].

The situation with erythrocytes is the opposite of that with hemolysates. In erythrocytes, AQ is relatively constant, and FT is measurable, but A and F are unknown. Since

Eq. 8  \( FT = A*P(N)/(A + Q + \Sigma AP(N) + \Sigma QPN) \)

and

Eq. 9  \( FT = (A + \Sigma AP(N)/(A + Q + \Sigma AP(N) + \Sigma QPN)) \)

then (through Eq. 4 and Eq. 5) AGT and FT are functions of A, Q, and the constants of the proteins. The expression on the right in Eq. 8 will be symbolized by E8, whereas AGT will denote the known value. Similarly, the expression on the right in Eq. 9 will be symbolized by E9, whereas FT will represent that known value. Iteration provides a mean of estimating the values of A and Q that lead to agreement between E8 and AGT and between E9 and FT. Methods of fitting nonlinear equations by iteration are presented by Cleland (16). The equations provide the improved estimate of A and Q, for each step in the iteration, are:

\( E8 = (\Delta A) \frac{\Delta E8}{\Delta A} \) and \( E9 = (\Delta Q) \frac{\Delta E9}{\Delta Q} \).

Let the prefix DA represent the partial derivative with respect to A, the prefix Q represent the partial derivative with respect to Q. Also, let the prefix DL represent the partial derivative with respect to \( \Sigma QPN \). Thus, the two previous equations become a pair of simultaneous equations in the two unknowns: DLDA and DLQ:

\( D\Sigma AEB = D\Sigma AEB + D\Sigma AEB \) and \( D\Sigma AEB + D\Sigma AEB - D\Sigma AEB + D\Sigma AEB \).

A and Q are altered by their corresponding deltas at each cycle of iteration. After the deltas become sufficiently small, the values of A and F are calculated from the definitions AGT = A + Q and FB = A + Q.

A computer program in BASIC has been written for both situations: the determinations of AGT and FB when A and Q are specified and the determinations of AGT and FB when AGT and FT are specified. For the latter condition, the expected rate of glucose-6-phosphate dehydrogenase (relative to Vmax is calculated from the kinetic equation and from constants for the human enzyme (2). Estimates of the enzymatic rate require statements as to the concentration of glucose-6-phosphate. The programs present the results as both tables and graphs. Printouts of the programs are available from the senior author upon written request.

RESULTS

The following are the results of two simulations in which AQ was fixed at 2 pM. For both simulations, the resultant AGT was found to vary from 32 to 35 pM.
Experience with numerous simulations indicated that sigmoid curves are generated when all of the following conditions are satisfied:

(a) Most of the bound NADP at any given time is bound by two or more proteins in which the dissociation constants differ by 10-fold. That is, $K_A(N)$ exceeds $10K_D(N)$ for at least one protein, and $K_C(N)$ exceeds $10K_A(N)$ for at least one other protein.

(b) The majority of the binding sites of the proteins mentioned above are occupied by NADP over the range of values of $F$ or $FT$.

(c) Sigmoidicity of the $F$ versus $FT$ plot requires that most of the NADP is bound. When this is true the $F$ versus $F_6$ plot resembles the $F$ versus $F_6$ plot.

Conditions (b) and (c) imply that $A_Q$ should not greatly exceed the total binding capacity of the proteins and that the dissociation constants should not exceed $A_O$. Two of the four simulations failed to give sigmoid curves as a result of violation of these conditions: condition (b) with Fig. 8 and condition (a) with Fig. 9.

The variations in $A_Q$ with $F$ were not presented here. When conditions (a) through (c) were met, the $A_Q$ versus $F$ plots tended to be either straight lines or slightly convex. Consequently, the $A_Q$ versus $F$ plots tended to be either straight lines or concave when $A_Q$ was constant. Certain other models also gave sigmoid plots but were more complicated than the model presented here and were difficult to reconcile with the data from studies on hemolysates and erythrocytes. One such model, which tended to give severe convexity of the $A_Q$ versus $F$ plot at constant $A_Q$, allowed the protein to have two binding sites, the affinity for NADPH at a second site being increased when NADP$^+$ was present at the first site and vice versa. Severe convexity of the $A_Q$ versus $F$ plot was also seen if each protein had only one binding site and was capable of binding only one of the two forms of NADP (either NADP$^+$ or NADPH).