Genetic Variants of Human Erythrocyte Glucose 6-Phosphate Dehydrogenase. I. Regulation of Activity by Oxidized and Reduced Nicotinamide–Adenine Dinucleotide Phosphate*

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ABSTRACT: The dependence of reaction velocity on oxidized nicotinamide–adenine dinucleotide phosphate ($NADP^+$) concentration was studied in five genetic variants of glucose 6-phosphate dehydrogenase: the three common variants A, B, and A^- and the two rare variants Ijebu-Ode and Ita-Bale. All these variants exhibit nonhyperbolic kinetics, with transition taking place from a state I of low affinity to a state II of high affinity for $NADP^+$ as the concentration of the latter is increased.

The enzyme–substrate dissociation constants for the two states (K_{s_1}, K_{s_2}) were determined. For the five variants, K_{s_1} ranged from 21 to 170 μM, and K_{s_2} from 1.3 to 13 μM. The inhibitory effect of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) on the glucose 6-phosphate dehydrogenase reaction was studied in the three common variants and in Ijebu-Ode. The values of the inhibition constant, K_i, ranged from 16 to 210 μM. The type of inhibition is not purely competitive, in that NADPH also affects the transition of the enzyme from state I to state II. An inverse relationship between K_{s_2} and K_i was found on comparing a set of four variants. This suggests that the pyridine nucleotide binding structure is different in each variant in such a way, that the closer the fit for $NADP^+$, the looser the fit for NADPH. The findings on the "deficient" variant A^- indicate that, contrary to previous views, it differs in kinetic behavior from the "normal" variants A and B, in two important respects: (a) A^- has the highest affinity for $NADP^+$; (b) A^- is the least sensitive to inhibition by NADPH. Because of these properties, it is inferred that this variant will normally operate, within the intact erythrocyte, at a nearly maximal velocity, thus compensating in part for the reduced enzyme concentration.

Glucose 6-phosphate dehydrogenase plays a key role in the metabolism of the erythrocyte as the first enzyme of the pentose phosphate pathway and the rate-limiting step for the production of NADPH.[1] One of the variants of this enzyme found in human erythrocytes, designated A (for nomenclature, see World Health Organization, 1967), has a "sigmoid" saturation curve with respect to $NADP^+$, which is markedly affected by the presence of NADPH (Luzzatto, 1967). This led us to suggest that one possible regulatory mechanism for the activity of glucose 6-phosphate dehydrogenase in human red cells is based on its interaction with $NADP^+$ and NADPH. Here we report studies consistent with this view, based on kinetic measurements of the interaction with $NADP^+$ and NADPH of four other variants of glucose 6-phosphate dehydrogenase. It will be shown that genetic changes affect markedly the interaction of the enzyme with the substrate and with the product of the reaction. In particular, the saturation kinetics of one of the variants, A^-, is such as to enable it to utilize the substrate much more effectively at physiological substrate concentrations. This behavior may be advantageous to the red cell and thus compensate in part for the "enzyme deficiency" associated with this variant.

Materials and Methods

Reagents. Glucose 6-phosphate (Boehringer und Soehne), $NADP^+$, and NADPH (Sigma Chem. Co.) were all standardized spectrophotometrically at 340 nm.

Enzyme Variants. The nomenclature followed is as recommended by World Health Organization (1967). The variants common in Nigeria A, B, and A^- (Luzzatto *et al.*, 1965) have been obtained from unselected blood donors, and identified by starch gel electrophoresis (Luzzatto and Afolayan, 1968). The two rare variants Ijebu-Ode and Ita-Bale (electrophoretically slower than B) have been previously described (Luzzatto and Afolayan, 1968).

Purification. Variants A, B, and A^- were partially purified from pooled blood units according to Chung and Langdon (1963a), Yoshida (1966), and Cohen and Rosemeyer (1969). The two rare variants were purified from the two original blood samples, as described in World Health Organization (1967). The specific activities (units/mg of protein) of the preparations on which the kinetic experiments were carried out were as follows: A, 2.8; B, 1.8; A^-, 0.8; Ijebu-Ode, 0.2; Ita-Bale, 0.2. All enzyme preparations were stored at $-20°$ in 70% saturated $(NH_4)_2SO_4$.

Enzyme Kinetics. Before kinetic determinations, enzyme preparations were dialyzed at 4° for 6–12 hr against three changes of 300 volumes of 0.05 M Tris-borate buffer (pH 8.0) containing 0.1 mM EDTA and 2 μM $NADP^+$. Assay conditions and precautions for the determination of reaction

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[1] Abbreviations used are: NADPH, reduced nicotinamide–adenine dinucleotide phosphate. $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate.

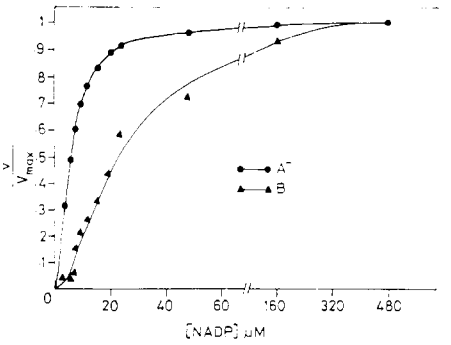

FIGURE 1: Relative reaction velocity of two common variants of glucose 6-phosphate dehydrogenase as a function of the concentration of the substrate, $NADP^+$. The concentration of the other substrate, glucose 6-phosphate, was saturating. V_{max} (expressed in ΔA_{340}/min) was between 0.05 and 0.06. The triangles (variant B) and the full circles (variant A^-) represent experimental points. The full lines are plots of eq 1, calculated for the values of parameters listed in Table I.

TABLE I: Kinetic Parameters of Glucose 6-Phosphate Dehydrogenase Variants.

Variant	$NADP^+$ Binding		NADPH Binding
	K_{s_1} (μM)[a]	K_{s_2} (μM)[a]	K_i (μM)[b]
A[c]	45	13	16
B	21	12	30
A^-	23	1.3	210
Ijebu-Ode	22	3	38
Ita-Bale	170	2.7	

[a] Values of the binding constant in state I (low affinity) and state II (high affinity), obtained from the data of Figures 1 and 2, according to eq 1 (see Luzzatto, 1967). [b] Values obtained from graphs of Figure 3. [c] Values for variant A from Luzzatto (1967).

velocity at very low $NADP^+$ concentrations were previously described (Luzzatto, 1967).

Calculations. The saturation curves for each enzyme variant with respect to $NADP^+$ were analyzed according to a previously proposed model, according to which the enzyme has more than one $NADP^+$ binding site. It was postulated (Luzzatto, 1967) that the binding of the first molecule of NADP (dissociation constant, K_{s_1}) increases the affinity for $NADP^+$ of another site (dissociation constant, K_{s_2}). The reaction rate, v, as a function of NADP concentration, s, predicted by the model is

$$v = \frac{s^2 V_{max}}{s^2 + s/K_{s_2} + K_{s_1}K_{s_2}} \quad (1)$$

Results

Dependence of Reaction Rate of Different Variants on [NADP⁺]. The sigmoid-shaped dependence of reaction rate on $NADP^+$ concentration, previously described for the A variant, is observed again clearly in two other variants, B and Ita-Bale, but only to a minimal degree in the case of Ijebu-Ode, and not at all in the case of A^- (Figures 1 and 2). The sigmoid kinetic curve of the A variant was previously interpreted as resulting from the $NADP^+$-induced transition of the enzyme from a state I of low affinity for $NADP^+$ to a state II of high affinity for $NADP^+$. It was possible, by the use of the simple quadratic eq 1, to fit the experimental data and to derive the binding constants (K_{s_1}, K_{s_2}) of the substrate for the enzyme in the two postulated states (Luzzatto, 1967). The validity of the treatment used before has now been verified by applying the same equation, with appropriate parameters, to the other four variants. The agreement between expectation and observation is seen by comparing the individual experimental points to the continuous lines in Figures 1 and 2.

The extent to which a saturation curve appears sigmoid on inspection will depend both on the absolute values of the two substrate binding constants and on the ratio between them. Thus, the curve will tend to become hyperbolic when either $K_{s_1} \rightarrow K_{s_2}$, or the value of K_{s_2} is so low that transition from low to high affinity occurs at very low [$NADP^+$]. The actual values of the two constants (Luzzatto, 1967), calculated from eq 1, are listed in Table I. It is seen that the values of K_{s_1} fall within a fairly narrow range for all variants, except Ita-Bale. Among the values of K_{s_2}, that of A^- stands out as being one order of magnitude lower than those of the common variants A and B.

The extent to which binding of one substrate molecule influences the affinity of binding of a second molecule can also be expressed in terms of Hill's interaction coefficient n. It is seen from the first column of Table II that even the variants with saturation kinetics showing little sigmoid character on inspection of the saturation curve have a value of n significantly above unity, consistent with cooperative binding. For all the variants n lies between 1 and 2, confirming the previous suggestion (Luzzatto, 1967) that 2 is the minimum number of molecules of substrate, $NADP^+$, that can be bound by the enzyme.

Inhibition by NADPH. Inhibition of erythrocyte glucose 6-phosphate dehydrogenase by the reaction product, NADPH, has been previously reported (Chung and Langdon, 1963b), and in the variant A the inhibition appears to be competitive, with deviations at low $NADP^+$ concentrations (Luzzatto, 1967). Because of this, the best way to determine the inhibition constant, K_i, was by the method of Dixon (1953). The same procedure was adopted for the other variants in this report (Figure 3a–c: unfortunately Ita-Bale could not be studied due to lack of sufficient material). Qualitatively, the results

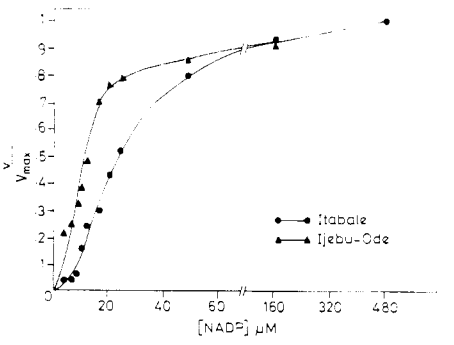

FIGURE 2: Relative reaction velocity of two rare variants of glucose 6-phosphate dehydrogenase as a function of the concentration of the substrate, $NADP^+$. Triangles: Ijebu-Ode. Full circles: Ita-Bale. Other explanations as in Figure 1.

TABLE II: Influence of NADPH on Cooperativity of $NADP^+$ Binding.

Enzyme Variant	Values of Interaction Coefficient, n,[a] at NADPH (μM)				
	0	20	40	80	160
A[b]	1.7		1.3	1.1	1.0
B	1.6	1.9	2.0	1.9	1.5
A^-	1.8	1.7	1.6	1.4	1.3
Ijebu-Ode	1.4	1.3	1.3	1.2	1.1
Ita-Bale	2.0				

[a] See Monod *et al.* (1965). [b] Values from Luzzatto (1967).

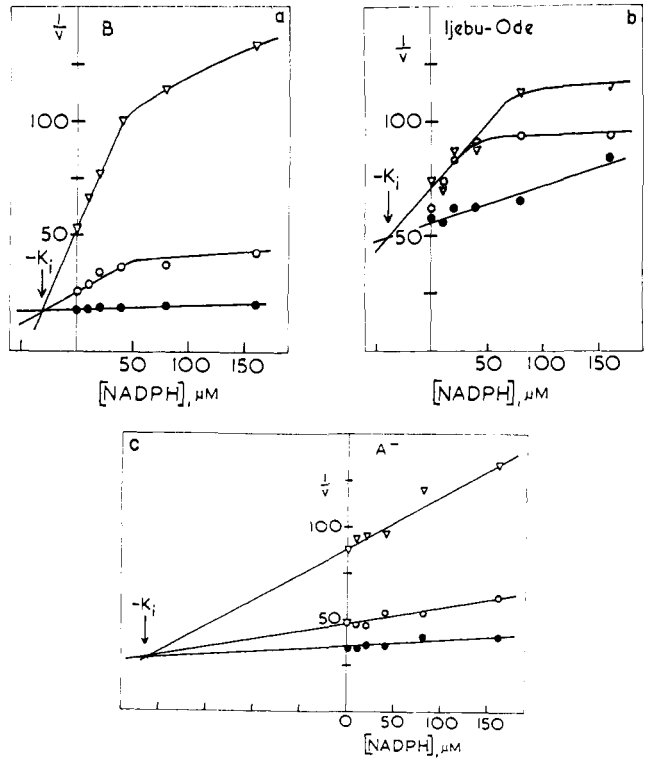

FIGURE 3: Dixon plots of reaction velocity as a function of the concentration of the product, NADPH. Each curve represents an experiment performed at different concentration of the substrate, $NADP^+$, as follows: (▽—▽) $NADP^+$ = 16 μM; (○—○) $NADP^+$ = 48 μM; (●—●) $NADP^+$ = 160 μM. Each panel represents results on a different enzyme variant. (a) Variant B, (b) variant Ijebu-Ode, and (c) variant A^-. The values of K_i derived from these graphs are listed in Table I.

are similar to those obtained with A. Quantitatively, the value of K_i obtained for A^- stands out as being one order of magnitude higher than for the other variants (Figure 3c and Table I). NADPH not only competes with NADP, but also affects the cooperativity of $NADP^+$ binding. In the case of A, A^-, and Ijebu-Ode the values of the coefficient n decrease monotonically with increasing NADPH concentrations; in the case of B, n first increases with NADPH concentration, reaches a maximum at [NADPH] = 40 μM and then decreases again (Table II).

Discussion

Enzymatic Differences among Variants. Different genetic variants of glucose 6-phosphate dehydrogenase showed distinguishable interactions with the substrate, $NADP^+$, and the reaction product, NADPH. For the three common variants (A, B, and A^-), results obtained on three different preparations of each have been consistent. These findings corroborate the earlier suggestion that the deviation from Michaelis–Menten kinetics previously reported for variant A (Luzzatto, 1967) must be attributed to an inherent property of the enzyme, since they indicate that the specific behavior of the enzyme with respect to the substrate is subject to genetic change. Similar considerations apply to the features of inhibition by NADPH.

The model previously suggested for the dependence of reaction rate on $NADP^+$ concentration has now been tested by asking whether eq 1, which described adequately the experimental data for variant A, would maintain its validity for other genetic variants. It was found that, for suitable different values of the kinetic parameters K_{s_1} and K_{s_2}, all the experimental data fit eq 1 (see Figures 1 and 2).[2]

The structural difference between the two variants A and B has been shown by Yoshida (1967) to consist in a single amino acid substitution. If we assume that the same is true for all other variants, we can conclude that the binding of $NADP^+$ can be extremely sensitive to small differences in the structure of the protein. By contrast, the Michaelis constants of the same five variants for glucose 6-phosphate, the other substrate, are all within the limits of experimental error (Luzzatto and Afolayan, 1968). We suggest that $NADP^+$ binding is more affected by structural changes in the enzyme by virtue of the critical role of this ligand in determining the conformation of the protein molecule, and the transition from state I to state II.

If we compare these data on substrate binding by a set of homologous protein species to those already available in a more extensively investigated system, human hemoglobin, some similarities and some differences are apparent. Also in the case of hemoglobin binding of a ligand (*e.g.*, azide) is influenced by single amino acid substitutions, even when they are relatively distant from the heme group (the active site): but what is affected is mainly the enthalpy change of binding, rather than the free-energy change (Bailey *et al.*, 1970). It is possible that in the case of substrate binding by glucose 6-phosphate dehydrogenase the free-energy change, and therefore the dissociation constant, are affected as well simply because of the larger size of the $NADP^+$ (compared to the azide) molecule, determining a much wider field of interactions with the polypeptide chain of the enzyme.

Mechanism of Inhibition by the Reaction Product NADPH. Because of the very close structural similarity between $NADP^+$ and NADPH, one would expect inhibition by NADPH to be competitive. This has been previously observed (Chung and Langdon, 1963b; Luzzatto, 1967; Soldin and Balinsky, 1968). The Dixon plots in Figure 3 confirm this conclusion for the variants studied here, since the intersection of curves corresponding to different concentrations of $NADP^+$ is found to lie above the zero ordinate line.

[2] Bonsignore *et al.* (1970) have now measured by equilibrium dialysis the association constants for $NADP^+$ of erythrocyte glucose 6-phosphate dehydrogenase type B, and have obtained values of 20 and 12 μM at low and high NADP concentration, respectively, in perfect agreement with our kinetic data (Table I).

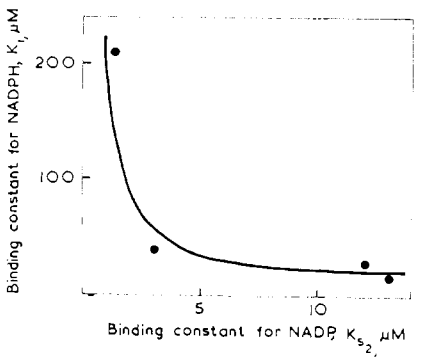

FIGURE 4: Inhibition constants for the reaction product, NADPH, as a function of the "high-affinity" binding constant for the substrate, $NADP^+$, in four different genetic variants of glucose 6-phosphate dehydrogenase. Data from Table II.

However, the same plots show that, like in the case of A (Luzzatto, 1967), there are deviations from standard competitive kinetics for B and Ijebu-Ode. This becomes apparent at low concentrations of $NADP^+$, when the inhibition is less than expected. Qualitatively, these deviations can be explained if, at low $NADP^+$ concentrations, NADPH helps the transition of the enzyme from state I (low affinity for NADP) to state II (high affinity for $NADP^+$). Similar situations have been described for the action of valine on threonine deaminase (Freundlich and Umbarger, 1963; Changeux, 1963); for the action of maleate on aspartate transcarbamylase (Gerhart and Pardee, 1963); and for the action of dAMP on deoxycytidylate aminohydrolase (Rossi *et al.*, 1967). The extent of inhibition by NADPH for each variant and for each $NADP^+$ concentration depends then on the combination of two different effects: (i) competition for binding between substrate and product, which always causes inhibition; (ii) transition of enzyme molecules from state I to state II, which entails activation.

When we attempt to describe these effects in quantitative terms, a major difficulty arises from the fact that, since our determinations are based only on reaction rates, we are not yet able to measure the two binding constants of NADPH (K_{i_1}, K_{i_2}) for state I and state II of the enzyme (*i.e.*, we can measure $NADP^+$ binding in the absence of NADPH, but not *vice versa*). In order to determine K_{i_1} and K_{i_2}, it will be necessary to measure the binding of NADPH directly, by methods not dependent on the enzyme reaction. Here we assume that each value of K_i listed in Table I is a good approximation of the corresponding K_{i_2}, since the experimental data from which it was derived (Figure 3) included some at very high NADP concentration, when most of the enzyme molecules are expected to be in state II. Since NADPH can in some cases help the transition of the enzyme from state I to state II, we infer that $K_{i_2} < K_{i_1}$. This explains why in some cases (A, A^-, and Ijebu-Ode in Table II) the cooperativity of $NADP^+$ binding may appear reduced (see Monod *et al.*, 1965). However, this may not occur if the ratio K_{s_1}/K_{s_2} differs from the ratio K_{i_1}/K_{i_2}. In such a case, apparent cooperativity of substrate binding will first increase with NADPH concentration, reach a maximum at approximately the value of K_{i_1}, and then decrease again when both $NADP^+$ and NADPH favor state II of the enzyme (see Rubin and Changeux, 1966). This situation seems to be verified with enzyme B (Table II).

When we compare the parameters for the binding of $NADP^+$ and NADPH of different variants (Table I) an unambiguous pattern emerges. There is a reciprocal relationship between affinity for the substrate and affinity for the product (Figure 4). These data indicate that, although in each variant the binding site for $NADP^+$ and NADPH is the same (on the basis of competitive inhibition kinetics), its conformation is different for each variant; and the closer the fit for $NADP^+$, the looser the fit for NADPH. No similar correlation holds between the binding constants K_i and K_{s_1}. Thus, the highly selective discrimination between $NADP^+$ and NADPH seems to depend strictly on the conformation of the binding site in state II. One cannot, of course, infer that the structural differences among variants need always be near the binding site, but rather that, wherever they are in the molecule, they affect dramatically the ratio between the binding affinities for $NADP^+$ and NADPH.

Possible Significance of the Kinetic Characteristics of the A^- Variant. It has previously been shown that the variant A^-, in spite of its electrophoretic identity with A, is structurally different from it (Luzzatto and Allan, 1965; Luzzatto and Okoye, 1967; Yoshida *et al.*, 1967). Several enzymic properties, such as K_m for glucose 6-phosphate, pH dependence of activity, etc., of the variant A^- have so far been found to be undistinguishable from those of the variants A and B (Marks *et al.*, 1961; World Health Organization, 1967). Here we have shown that kinetic differences do nevertheless exist between A^- and the normal variants A and B. A^- stands out from other variants in at least two ways. (i) It has the highest affinity for $NADP^+$ in state II (the lowest value of K_{s_2}). (ii) It shows by far the lowest affinity for NADPH (highest value of K_i).

The odd kinetic properties of A^- may be of considerable physiological significance, in that they suggest an explanation of the paradox that the metabolism of A^- red cells is nearly normal, though they are usually termed "deficient" in glucose 6-phosphate dehydrogenase. With normal enzyme, an increase of $NADP^+$ will promote transition of the enzyme from the state of low to high affinity; the enzyme then becomes more active, which removes the excess $NADP^+$. Because an increase in NADPH inhibits the enzyme, further production of NADPH is limited. Thus, the activity of glucose 6-phosphate dehydrogenase in intact red cells might be regulated by the concentration of $NADP^+$ and NADPH (Luzzatto, 1967). The affinity for $NADP^+$ of A^- in state II is so high, that the transition from state I to state II presumably occurs, or is already almost complete at physiological concentrations of $NADP^+$. Furthermore, inhibition by NADPH is so weak, that it is unlikely to be at all significant at physiological concentrations of NADPH. Both of these peculiarities, will cause the enzyme to be always predominantly in state II and its activity not to be limited by product inhibition. Thus, it is likely that the A^- enzyme, although present in the cell at a much lower concentration than A or B, will utilize the substrate in a relatively more efficient way, perhaps to the extent of almost completely offsetting the deficiency in actual number of molecules per cell. The accompanying paper (Luzzatto and Afolayan, 1971) presents strong evidence in support of this counterbalancing effect in intact erythrocytes.

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Added in proof

Purification of variants A, B, and A^- to a state of electrophoretic homogeneity has been recently obtained in collaboration with R. Cancedda and O. Babalola. On these pure preparations the saturation curves for $NADP^+$ were very similar to those shown here. The K_i values for NADPH were 17 μM and 30 μM for variants A and B, respectively, in excellent agreement with the values given in Table I. The K_i value for variant A^- was confirmed to be higher, but not as high as had been found with the less pure preparation (approximately 80 μM instead of 210 μM). These results will be published in detail elsewhere.

References

Bailey, J. E., Beetlestone, J. G., Irvine, D. H., and Ogunmola, G. B. (1970), *J. Chem. Soc., A*, 749.

Bonsignore, A., Lorenzoni, I., Cancedda, R., and De Flora, A. (1970), *Biochem. Biophys. Res. Commun. 39*, 142.

Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Chung, A. E., and Langdon, R. G. (1963a), *J. Biol. Chem. 238*, 2309.

Chung, A. E., and Langdon, R. G. (1963b), *J. Biol. Chem. 238*, 2317.

Cohen, P., and Rosemeyer, M. A. (1969), *Eur. J. Biochem. 8*, 1.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Freundlich, M., and Umbarger, H. E. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 505.

Gerhart, J. C., and Pardee, A. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 491.

Luzzatto, L. (1967), *Biochim. Biophys. Acta 146*, 18.

Luzzatto, L., and Afolayan, A. (1968), *J. Clin. Invest. 47*, 1833.

Luzzatto, L., and Afolayan, A. (1971), *Biochemistry 10*, 420.

Luzzatto, L., and Allan, N. C. (1965), *Biochem. Biophys. Res. Commun. 21*, 547.

Luzzatto, L., Allan, N. C., and DeFlora, A. (1965), *Biochem. J. 97* 19P.

Luzzatto, L., and Okoye, V. C. N. (1967), *Biochem. Biophys. Res. Commun. 29*, 705.

Marks, P. A., Szeinberg, A., and Banks, J. (1961), *J. Biol. Chem. 236*, 10.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Rossi, M., Geraci, G., and Scarano, E. (1967), *Biochemistry 6*, 3640.

Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21*, 265.

Soldin, S. J., and Balinsky, D. (1968), *Biochemistry 7*, 1077.

World Health Organization (1967), Technical Report Series, No. 366, Geneva.

Yoshida, A. (1966), *J. Biol. Chem. 241*, 4966.

Yoshida, A. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 835.

Yoshida, A., Stamatoyannopoulos, G., and Motulsky, A. G. (1967), *Science 155*, 97.