# Effect of Buffer Concentration on the Efficiency of Photosynthetic Energy Conversion*

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ABSTRACT: The efficiency of energy conversion by chloroplasts under conditions of cyclic electron transport but in the absence of ADP was determined as functions of buffer concentration, total ionic strength, and illumination time. Theoretical expressions for the dependence of energy conversion efficiency on buffer concentration were deduced from the proton gradient model, electric potential model, and chemical intermediates model, respectively. A comparison of the experimental and theoretical results rules out the first two of these three energy transduction models for cyclic photophosphorylation.

The molecular mechanism for coupling light-driven electron transport to phosphorylation in photosynthesis is a subject of basic significance and wide interest. The free energy liberated by respiratory or photosynthetic electron transport is often first converted to and stored in various forms generally designated by Z* (Shen and Shen, 1962) or by $X_E$ (Hind and Jagendorf, 1963) before the formation of ATP. The various forms of Z* or $X_E$ suggested in the literature include energy-rich intermediates X~I (Slater, 1953), concentration gradients Mitchell, 1961; Williams, 1961), conformational or configurational changes (Boyer, 1965; Korman *et al.*, 1970), and electric potential energy (Grünhagen and Witt, 1970; Junge, 1970). It is possible that the different phenomenological observations may merely reflect the various features of the same primary molecular process (Wang, 1970).

Let us consider a reacting system in which phosphorylation is coupled to electron transport. If we represent the overall net chemical change including both electron transport and energy storage by

$$\sum_i \nu_i A_i \longrightarrow \sum_j \nu_j A_j$$

where $\nu_1$ moles of molecular species $A_1$, $\nu_2$ moles of molecular species $A_2$, etc., react to form $\nu_n$ moles of $A_n$, $\nu_{n+1}$ moles of $A_{n+1}$ etc. The summation $\Sigma_i$ is over all reactants and the summation of $\Sigma_j$ is over all products. In general at constant temperature $T$ and pressure $P$, the total free energy decrease is given by

$$-\Delta G = \sum_i \nu_i \mu_i - \sum_j \nu_j \mu_j' \geq 0 \qquad (1)$$

where $\mu_i$, $\mu_j'$ represent the electrochemical potentials of $A_i$, $A_j$, respectively, in the mixture, and the equality sign applies to the limiting case in which the system is already at equilibrium.

The electrochemical potential $\mu_i$ of the reactant molecule $A_i$ of charge number $Z_i$ at a location with electric potential $\psi$ in the system may be written as

$$\mu_i = \mu_i^\circ + Z_i \mathfrak{F} \psi + RT \ln m_i + RT \ln \gamma_i \qquad (2)$$

where $m_i$ is the molal concentration, $\gamma_i$ the activity coefficient of $A_i$, $\mathfrak{F}$ the faraday, $R$ the gas constant, and the standard potential $\mu_i^\circ$ is characteristic of $A_i$ but for a given set of arbitrarily chosen standard states is independent of $\psi$ and $m_i$. Likewise the electrochemical potential $\mu_j'$ of the product molecule $A_j$ at another location in the system with electric potential $\psi'$, concentration $m_j'$, and activity coefficient $\gamma_j'$ may be written as

$$\mu_j' = \mu_j^\circ + Z_j \mathfrak{F} \psi' + RT \ln m_j' + RT \ln \gamma_j' \qquad (3)$$

Substitution of eq 2 and 3 into 1 gives

$$-\Delta G = (\Sigma \nu_i \mu_i^\circ - \Sigma \nu_j \mu_j^\circ) + \mathfrak{F}(\psi \Sigma \nu_i Z_i - \psi' \Sigma \nu_j Z_j) + RT(\Sigma \nu_i \ln m_i - \Sigma \nu_j \ln m_j') + RT(\Sigma \gamma_i \ln \gamma_i - \Sigma \nu_j \ln \gamma_j') \geq 0 \qquad (4)$$

In hypothetical coupled reactions with maximum energy storage, the free energy decrease due to oxidation–reduction approaches the free energy increase due to the storage process, consequently $-\Delta G \rightarrow 0$ and the net chemical change can only take place with infinitesimal rate. Conversely for a completely uncoupled system there is no energy storage and the overall free energy decrease, $-\Delta G$, reaches its maximum value $M$. In actual biological energy transducing systems, a compromise between storage efficiency and reaction speed is reached by making $0 < -\Delta G/M < 1$.

The first through the fourth terms on the right-hand side of eq 4 have been used loosely to represent the free energies dissipated in the energy conversion process leading to chemical intermediates, electric potential energy increase, concentration gradients, and additional molecular interactions, respectively. This arbitrary representation is misleading, because in a biological system each of these four effects contributes to two or more terms on the right-hand side of eq 4. For example, since molecules in general react at concentrations $m_i$, $m_j'$ . . . , with activity coefficients $\gamma_i$, $\gamma_j'$, . . . , even in the absence of electric field the formation of chemical intermediates automatically affects the first, third, and fourth terms on the right-hand side of eq 4. For this reason, the experimental detection of concentration gradients generated by energy transducing processes in chloroplasts (Hind and Jagendorf, 1963; Jagendorf and Uribe, 1966; Deamer *et al.*, 1967; Izawa, 1970) and mitochondria (Mitchell and Moyle, 1967, 1968) does not support the proton gradient or chemiosmotic

* From the Kline Chemistry Laboratory, Yale University, New Haven, Connecticut 06520. *Received June 30, 1971.* Supported by research grants from the National Institute of General Medical Sciences, Public Health Service (GM-04483), and the National Science Foundation (GB-7459X).

model in preference to the chemical intermediates hypothesis. For the same reason, the attempted measurements of membrane electric potential and conformation changes, even if experimentally sound, will have little diagnostic value. On the other side, the detection of a phosphorylated intermediate in mitochondrial oxidative phosphorylation (Cross *et al.*, 1970) does not necessarily contradict the chemiosmotic and electric potential models either, because it is thermodynamically possible to drive the generation of chemical intermediates with the free energy stored in concentration gradients or electrically charged membranes.

The successful coupling of phosphorylation to electron-transfer in homogeneous model systems (Brinigar *et al.*, 1967; Tu and Wang, 1970) does demonstrate the feasibility and potential efficiency of the chemical coupling mechanism. Obviously the free energy liberated by electron transfer in these model systems can only be stored in chemical intermediates, since the definition of macroscopic homogeneity precludes electric potential gradient, concentration gradient, and membrane conformation changes. However, results obtained from artificial model systems cannot be used as direct experimental evidence for the coupling mechanism in chloroplasts and mitochondria.

In this work, spinach chloroplasts were illuminated in the absence of ADP and $P_i$. The efficiency of photosynthetic energy conversion under conditions of cyclic electron transport, as measured by the yield of Z* or $X_E$, was determined as a function of buffer concentration. A comparison of the experimental data with theoretical expressions for the stored free energy, $\Delta G_s$, deduced from the proton gradient model, electric potential model, and chemical intermediates model, respectively, enabled us to rule out two of these three models for cyclic photophosphorylation.

## Experimental Section

*Materials.* Chloroplasts were generally prepared by grinding fresh spinach leaves in a Waring blender, followed by differential centrifugation as described by Avron (1960). Composition of the medium for grinding spinach leaves: sucrose, 0.2 M–NaCl, 0.01 M–Tricine, 0.2 M at pH 8.0. The slurry was squeezed through four layers of cheesecloth and centrifuged at 2° for 2 min at 500*g*. The supernatant was then taken out and centrifuged again for 7 min at 2000*g*. The pellet from this centrifugation was subsequently resuspended in the homogenizing medium and recentrifuged at 2000*g* for 7 min. The pellet was then suspended in a small amount of homogenizing medium, frozen in liquid nitrogen, and stored at −20°. All the isolation procedures were carried out at 0–2°.

ADP from equine muscle (disodium salt, Grade I) was purchased from Sigma Chemical Co. Radioactive phosphate ($^{32}P_i$) was from New England Nuclear and further purified by treatment with small amounts of weakly basic anion-exchange resin (Amberlite CG-4B) at pH 6.5. This treatment usually removed about 30% of the total radioactivity and reduced the radioactive impurity from 2–3% to 0.02–0.03% as determined by the extraction procedure described below.

All other reagents were of analytical grade and were used without further purification. Deionized water with $19 \times 10^6$ ohm cm resistivity was used to make all the aqueous solutions.

*Reaction Mixtures.* For each experiment, two solutions were required, one for the light stage of the reaction and the other for the subsequent dark stage of the reaction. Basic composition of the light-stage solution: phenazine methosulfate (PMS), $1 \times 10^{-4}$ M–NaCl, $1 \times 10^{-2}$ M–$NaN_3$, $1 \times 10^{-3}$ M–sucrose, 0.2 M–Tricine, $2.0 \times 10^{-4}$ M at pH 6.0. Basic composition of the dark-stage solution: ADP, $2.0 \times 10^{-3}$ M–$P_i$, $1 \times 10^{-2}$ M–$MgCl_2$, $1 \times 10^{-2}$ M–Tricine, 0.1 M at pH 8.0. In each experiment, the chloroplasts were first washed with the light-stage solution and then resuspended in the light-stage solution. The pH of the final mixture was adjusted to 6.2. Radioactive phosphate ($^{32}P_i$) was added to the dark-stage solution. Whenever additional reagents (maleate, succinate, malonate, etc.) were added to either solution, the pH and final concentrations of the other components were maintained constant.

*Illumination.* A 500-W projector lamp with a red filter and a 1.5-in. thick cold water shield was used as the light source. The duration of illumination was controlled by an electric timer.

*Two-Stage Photophosphorylation.* In a typical experiment, 1.5 ml of chloroplast suspension was transferred into a spiral polyethylene tubing by means of a Cornwall continuous pipettor fitted with a B-D MS08 three-way syringe valve. After the suspension was illuminated for a controlled length of time, nitrogen (10 atm) was led into the polyethylene tubing by quickly turning the three-way valve. The chloroplast suspension was forced by nitrogen into a tube in the dark containing 1.5 ml of the dark-stage solution and incubated for 30 sec with constant stirring to utilize the stored free energy, Z* or $X_E$, for ATP synthesis. At the end of the dark incubation period, 0.5 ml of 20% trichloroacetic acid (TCA) was added to the mixture to kill the enzyme-catalyzed transphosphorylation reactions. The whole experiment was performed at 4°. The chlorophyll content of each chloroplast suspension was determined with an aliquot part by the method of Arnon (1949).

*Assay of Phosphorylation.* The assay procedure was essentially the same as that described by Avron (1960). The TCA-denatured reaction mixture was centrifuged at 27,000*g* for 10 min and the supernatant was passed through Whatman No. 2 filter paper to remove a small amount of floating material; 1.01 ml of the filtered aqueous solution was pipetted into a well-cleaned glass tube and mixed with 1.5 ml of water saturated with 1:1 mixture of benzene–isobutyl alcohol and 0.5 ml of 5% solution of ammonium molybdate in 4 N sulfuric acid. The mixture was vigorously stirred by means of a Vortex mixer for 5 min. Then 10 ml of 1:1 benzene–isobutyl alcohol mixture saturated with water was added, and the resulting mixture was again vigorously stirred with the Vortex mixer for 1 min. After the phases separated upon standing, the upper organic layer containing the yellow phosphomolybdate was siphoned out; 0.2 ml of 0.02 M $KH_2PO_4$ solution was then added to react with the excess molybdate and the phosphomolybdate again removed by the above procedure. After this treatment the aqueous layer was extracted once more with 10 ml of benzene–isobutyl alcohol mixture to remove any residual phosphomolybdate; 1.0 ml of the aqueous layer was then mixed with 10 ml of dioxane–base scintillation solution described by Bray (1960), and the radioactivity was measured in a liquid scintillation counter. Two types of control experiments were performed. In the first type, chloroplast suspension was injected into the dark-stage solution without illumination. In the second type, the illuminated suspension was kept in the dark for 2 min, for Z* or $X_E$ to decay, before injection into the dark-stage solution. It was found that these two types of control experiments gave the same small background counting rate. In the photophosphorylation experiments, the amount of ATP produced from Z* or $X_E$ was com-

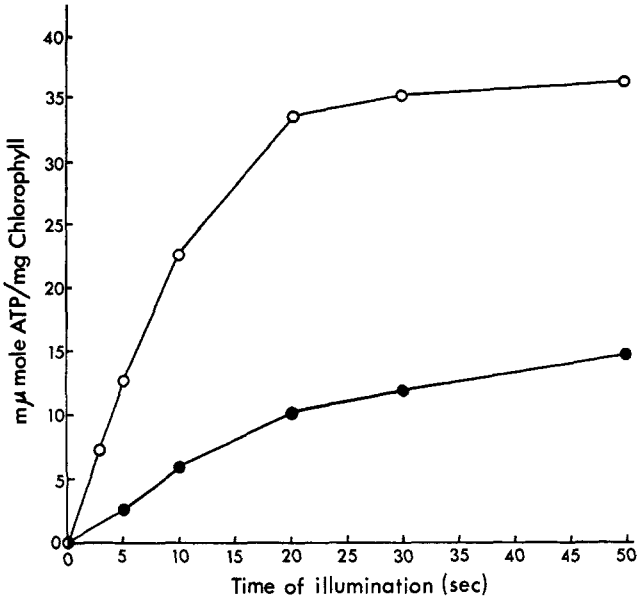



FIGURE 1: Kinetics of $X_E$ formation. Chloroplast suspension at a concentration of 0.50 mg of chlorophyll per ml was illuminated in the presence of 10 mM sodium maleate buffer at pH 6.2 (○—○) and in the absence of sodium maleate at pH 6.2 (●—●). The concentration of NaCl in these suspensions was maintained at 10 mM.

puted from the measured $^{32}P$ counting rate after subtracting out this background counting rate.

## Results

The acid sodium salts of phosphoric acid and several dicarboxylic acids which are known to penetrate the chloroplast membrane (Jagendorf and Uribe, 1966; Heldt and Rapley, 1970) were used as buffer systems in the present study. The observed production of Z* or $X_E$ as a function of illumination time is shown in Figure 1. While the observed amount of $X_E$ formed generally increases with the time of illumination, the yield was much higher in the presence of maleate and its plateau value could be reached in a shorter time than in the absence of maleate.

It will be shown in the discussion section that the quantitative treatment of the effect of buffer concentration on the yield of $X_E$ becomes greatly simplified for a very short illumination time during which no significant amount of buffer ions diffuse passively across the chloroplast membrane. On the other hand, if the illumination time is too short, the yield of $X_E$ will be low and consequently the percentage experimental error will be high. As a compromise, the illumination time was set at 5 sec in our studies of the effect of buffer concentration on the efficiency of energy conversion. These data are summarized in Figures 2 and 3.

The data in Figure 2 show that the efficiency of energy conversion increases with the concentration of maleate buffer. The yield of $X_E$ increased almost three times as the concentration of maleate buffer increased from 0 to 10 mM. This pronounced effect cannot be due to a variation in total ionic strength for the following two reasons. First, Figure 2 shows that when the concentration of maleate buffer increased from 0 to 1 mM, the yield of $X_E$ increased 50%, whereas the total ionic strength, due mainly to sodium chloride and endogenous ions, increased by less than 10%. Second, essentially the same yield of $X_E$ was observed when the NaCl concentration was increased from the usual 10 mM to 30 and 50 mM, respectively. Neither can the increase in the yield of $X_E$ with maleate buffer concentration be due to the acid–base transition type of phosphorylation discovered by Jagendorf and Uribe (1966), because both our dark and 2-min decay control experiments show that no significant amount of $X_E$ was formed by this type of phosphorylation under our experimental conditions. Figure 3 summarizes similar results obtained with succinate, malonate, and phosphate buffers, respectively.

## Theory and Discussion

In this section, quantitative treatments of the effect of buffer concentration on the efficiency of energy transduction are developed from the standpoints of proton gradient model, electric potential model, and chemical intermediates model, respectively. The characteristic theoretical results are compared with experimental data and definite conclusions are drawn.

*Effect of Buffer Concentration According to the Proton Gradient Model.* Let us adopt the following notation: $[H^+]_1$, $(H^+)_1$ denote the concentration and activity, respectively, of $H^+$ (or $H_3O^+$) on one side of the chloroplast membrane (side 1); $[H^+]_2$, $(H^+)_2$ denote the similar quantities on the other side of the membrane (side 2); $[X^-]_1$, $[X^-]_2$, $(X^-)_1$, $(X^-)_2$ denote similar quantities for the counterion $X^-$; $(\gamma_\pm)_1$, $(\gamma_\pm)_2$ denote the mean activity coefficients of $H^+$ and $X^-$ on side 1 and side 2 of the membrane, respectively.

The free energy increase due to the transfer of $d\delta$ mole of $H^+$, with accompanying counterions, from side 1 to side 2 of the membrane at constant temperature is equal to

$$dG = RT\left\{\ln \frac{(H^+)_2(X^-)_2}{(H^+)_1(X^-)_1}\right\} d\delta$$

$$= RT\left\{\ln \frac{[H^+]_2[X^-]_2(\gamma_\pm)_2^2}{[H^+]_1[X^-]_1(\gamma_\pm)_1^2}\right\} d\delta \qquad (5)$$

If the ionic strength is maintained approximately constant by an excess of inert salt $M^+X^-$ so that $[X^-]_1 \approx [X^-]_2$ and $(\gamma_\pm)_1 \approx (\gamma_\pm)_2$, then

$$dG_s \approx RT\left\{\ln \left(\frac{[H^+]_2}{[H^+]_1}\right)\right\} d\delta \qquad (6)$$

Let us represent the buffer anion and its conjugate acid by $B^-$ and BH, respectively. Since the chloroplasts were equilibrated with the buffer in the dark for 0.5 hr before the illumination, we have

$$\frac{[BH]_1}{[B^-]_1} = r = \frac{[BH]_2}{[B^-]_2}$$

In the pH range of our experiments, the buffers were so selected that $r$ is not very different from unity. The true dissociation constant $K_a$ and the apparent dissociation constant $K_a'$ of BH are defined by

$$K_a = \frac{(B^-)(H^+)}{(BH)} = \frac{[B^-][H^+]}{[BH]}\frac{(\gamma_{B^-})(\gamma_{H^+})}{(\gamma_{BH})} \qquad (7)$$

and

$$K_a' = \frac{[B^-][H^+]}{[BH]} = K_a \times \frac{(\gamma_{BH})}{(\gamma_{B^-})(\gamma_{H^+})} \qquad (8)$$

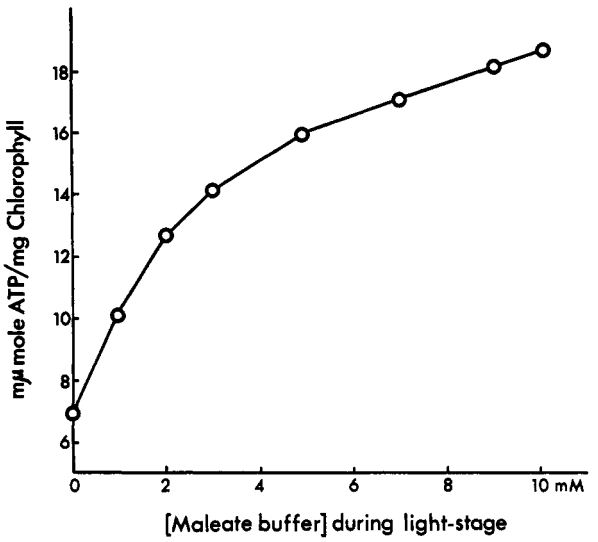



FIGURE 2: The dependence of the yield of $X_E$ on the concentration of sodium maleate buffer. Chloroplast suspensions at a concentration of 0.63 mg of chlorophyll per ml were illuminated for 5 sec in the presence of different amounts of sodium maleate buffer at pH 6.2. Complemental amounts of the maleate were added to the dark-stage solutions so that the dark transphosphorylation reactions were carried out at a constant sodium maleate concentration of 5 mM in all experiments. The concentration of added NaCl was 10 mM in all cases.

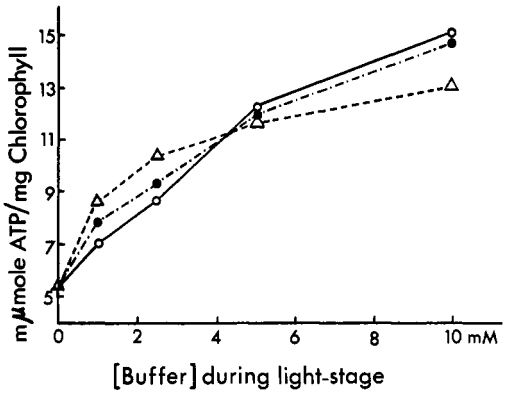



FIGURE 3: The effect of different buffer systems on the yield of $X_E$. Experimental conditions were similar to those of Figure 2, except that chlorophyll concentration was 0.58 mg/ml and that different buffer systems were used: sodium phosphate (○—○); sodium malonate (●·····●); sodium succinate (△- - - - -△). In the experiments with phosphate buffer, complemental amounts of phosphate were added to the dark-stage solutions so that the final concentration of $P_i$ was 5 mM in all tubes. The concentration of added NaCl was 10 mM in all cases.

According to the proton gradient model, the number of moles of $H^+$ translocated is proportional to the number of moles of electron transported. By using intense light for a very short illumination period, it is possible to translocate a sufficiently large number, $\delta$, of moles of $H^+$ from one side of the chloroplast membrane to the other, with accompanying $X^-$ which is present in excess, without appreciable amounts of $B^-$ and BH diffusing across simultaneously. Under such experimental conditions, the concentrations of $H^+$ on the two sides immediately after the light-driven proton translocation for the case in which $\delta < a_2r/(1 + r) < a_1r/(1 + r)$ and $\delta < a_2/(1 + r) < a_1/(1 + r)$ are given by

$$[H^+]_2 = K_a'\left\{\frac{[BH]_2}{[B^-]_2}\right\} = K_a'\left\{\frac{a_2r/(1 + r) + \delta}{a_2/(1 + r) - \delta}\right\} \quad (9)$$

$$[H^+]_1 = K_a'\left\{\frac{[BH]_1}{[B^-]_1}\right\} = K_a'\left\{\frac{a_1r/(1 + r) - \delta}{a_1/(1 + r) + \delta}\right\} \quad (10)$$

where $a_2$ and $a_1$ represent the total number of moles of buffer, $B^-$ plus BH, on side 2 and side 1 of the membrane, respectively.

Substituting eq 9 and 10 into eq 6 and integrating (see Appendix A), we get

$$\Delta G_s \approx \frac{(1 + r)^2}{2r}\left(\frac{1}{a_1} + \frac{1}{a_2}\right)RT\delta^2 \quad (11)$$

Equation 11 shows that for a given number, $\delta$, of moles of proton translocated, the free energy stored should decrease as the buffer concentration increases according to the proton gradient model. Since $a_1$ and $a_2$ represent the total amounts of buffer, rather than its concentrations, eq 11 should remain valid even when the chloroplasts swell or shrink during illumination, provided that the light was sufficiently bright and that the illumination period was sufficiently short so that the number of moles of $B^-$ or BH diffused across was small as compared to the number of moles of proton translocated. For longer illumination periods, eq 11 should include correction terms due to the diffusion of $B^-$ and BH, but the above qualitative conclusion concerning buffer concentration should still hold.

Figures 2 and 3 show that when equivalent samples of chloroplasts suspended in the same supporting electrolyte solution are illuminated in the absence of ADP for 5 sec with the same light source, the free energy stored increases rapidly as the buffer concentration is increased. Inasmuch as each experimental point in Figures 2 and 3 was produced by the absorption of the same number of photons of the same frequency distribution and over the same interval of time, the number of electrons transported along the cyclic electron transport path must be the same in each case. Consequently the number, $\delta$, of moles of proton translocated in each experiment must also be the same according to the proton gradient model which assumes a stoichiometric relationship between electron transport and proton translocation (Mitchell and Moyle, 1966).

Therefore the data in Figures 2 and 3 show that for the same number, $\delta$, of moles of proton translocated the free energy stored increases rapidly as the concentration of the buffer is raised. Since this observation contradicts eq 11, we can only conclude that most of $Z^*$ of $X_E$ in photosynthetic phosphorylation is not of proton gradient nature.

Attempts have been made to improve the proton gradient model by postulating that protons are not translocated from side 1 to side 2, but from the exterior to the interior of the thylakoid or inner mitochondrial membrane. This type of proton translocation would produce a much larger proton gradient for the same number of protons translocated. But the improved model also contradicts the observed rapid increase of $\Delta G_s$ as the buffer concentration is raised.

*Effect of Buffer and Salt Concentration According to the Electric Potential Model.* It is assumed in this model that the free energy of light-driven electron transport in photosynthesis is first used to charge the thylakoid membrane and stored as electric potential energy. This electric energy is later utilized to translocate protons and phosphorylate ADP (Grünhagen and

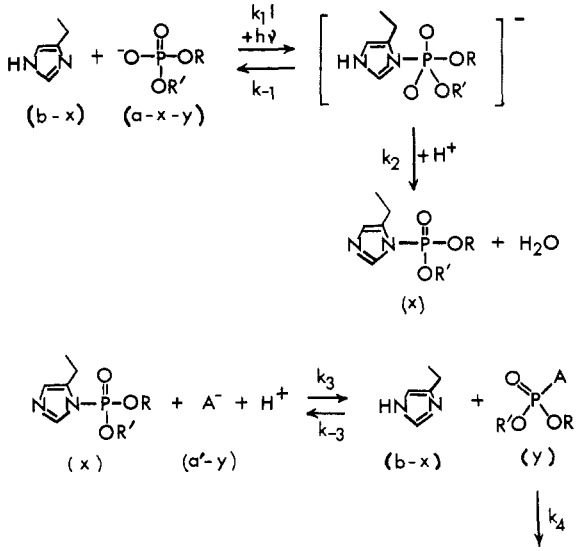


FIGURE 4: A reaction scheme involving chemical intermediates for photosynthetic phosphorylation. The letters in parentheses under each structure denote the molar concentrations of the corresponding molecular species.

Witt, 1970; Junge, 1970). Accordingly, Z* or $X_E$ is equal to the work required to charge a membrane condenser by redistributing the electric charges in the chloroplast system.

To simplify the problem, let us assume that a certain salt, say $Na^+$ and $Cl^-$, is present in the system at a much higher concentration than that of the buffer, $B^-$ and BH, *i.e.*

$$C_s \equiv [Na^+] \approx [Cl^-] \gg [B^-] + [BH] \qquad (12)$$

Since practically all charges in a chloroplast system reside on ions and since the capacity of a membrane condenser also depends on the distribution of ions, we expect the free energy stored by charging such a membrane condenser to vary with the concentration of the principal ionic species, $Na^+$ and $Cl^-$, but to be practically independent of the concentration of the buffer $[B^-] + [BH]$, which does not contribute significantly to the total ionic strength.

As a convenient example, let us consider the free energy $\Delta G_s$ stored through charging an infinite planar membrane by bringing sodium ions to it from the sodium chloride solution of molar concentration $C_s$ on one side of the membrane until the membrane potential becomes $\psi_0$. For $C_s < 0.05$ M and $\psi_0 < 1$ V, approximate theoretical considerations (see Appendix B) show that the free energy stored per unit area of the membrane is equal to

$$\Delta G_s = \sqrt{\frac{2DC_sRT}{1000\pi}}\left\{\psi_0 \sinh\left(\frac{Ze\,\psi_0}{2kT}\right) - \frac{2kT}{Ze}\left[\cosh\left(\frac{Ze\,\psi_0}{2kT}\right) - 1\right]\right\} \qquad (13)$$

where $D$ is the dielectric constant of the solvent, $k$ the Boltzmann constant, and $Z = 1$ for sodium chloride solution. Therefore, in the range of experimental data for which eq 12 and 13 are both applicable, $\Delta G_s$ at a given salt concentration should be independent of buffer concentration according to the electric potential model.

For a perfect planar condenser with no leakage, we expect the potential $\psi_0$ of the membrane under illumination for a long time to reach its maximum or saturation value as determined by the photo-emf of the system. For a condenser with a small leakage, we expect the steady-state value of $\psi_0$ to be lower than the maximum value. But in either case, $\Delta G_s$ should be proportional to $C_s^{1/2}$ for $< C_s$ 0.05 M according to eq 13, whereas for a given $C_s$ it should be independent of the concentration of the buffer which does not contribute significantly to the total ionic strength. To obtain the free energy stored by both interfaces of the planar membrane, we need only replace $\Delta G_s$ by $2\Delta G_s - \Delta G'$, where $\Delta G'$ is the interaction free energy between the oppositely charged interfaces. For a membrane 65 Å thick, $\Delta G'$ is small and independent of $C_s$.

Our experimental results show that the above theoretical result based on the charged membrane model is not right, because the observed $\Delta G_s$ increases rapidly as the buffer concentration is raised and that at a given buffer concentration $\Delta G_s$ does not increase as $C_s$ changes from 10 to 50 mM.

Therefore we can only conclude that most of the Z* or $X_E$ produced by photosynthetic energy coversion under the conditions of cyclic electron transport is not in the form of electrically charged thylakoid membrane. The experimental results also preclude a combination of proton gradient and electric field, *viz.*, the electrophysical potential gradient, as the principal driving force for cyclic photophosphorylation. But they do not contradict coupling mechanisms which involve all four terms on the right-hand side of eq 4, *viz.*, chemical mechanisms.

*Effect of Buffer Concentration According to the Chemical Intermediates Model.* In order to treat the effect quantitatively, it is convenient to adopt as working hypothesis an explicit chemical mechanism. Let us tentatively adopt the coupling mechanism discovered in model systems (Brinigar *et al.*, 1967; Tu and Wang, 1970) for photophosphorylation in chloroplasts, and assume that under illumination an imidazole group of a cytochrome molecule is first photooxidized to a substituted imidazolyl radical. If the substituted imidazolyl radical is adjacent to a suitable phospholipid or phosphoprotein, $(RO)(R'O)PO_2^-$, of the chloroplast membrane, it can rapidly react with the latter to form a substituted phosphoimidazolyl radical which can subsequently be reduced to substituted orthophosphoimidazole. The unstable substituted orthophosphoimidazole can either decompose to the original reactants or capture a proton to form water and substituted 1-phosphoimidazole. The latter can then transfer its substituted phosphoryl group to an acceptor group $A^-$ or AH on the membrane to form the intermediate (RO)(R'O)POA. Both the substituted 1-phosphoimidazole and (RO)(R'O)POA are "nonphosphorylated" energy-rich intermediates and either of them can subsequently react with inorganic phosphate $P_i$ and ADP to form ATP and regenerate the original reactants.

This hypothetical reaction scheme is summarized in Figure 4. With the notation of Figure 4 and the assumption of steady state for the substituted phosphoimidazolyl radical, the rate equations may be written as follows

$$\frac{dx}{dt} = k_1 I(b - x)(a - x - y)\left\{\frac{k_2[H^+]}{k_2[H^+] + k_{-1}}\right\} + k_{-3}(b - x)y - k_3(x)(a' - y)[H^+] \qquad (14)$$

$$\frac{dy}{dt} = k_3(x)(a' - y)[H^+] - k_{-3}(b - x)(y) - k_4(y) \qquad (15)$$

where $I$ represents the intensity of light. For illumination per-

iods much shorter than the spontaneous decay time of $X_E$, the term $-k_4(y)$ in eq 15 may be neglected.

Since experimental data show that the number of moles of Z* or $X_E$ formed is much larger than the number of moles of each electron carrier, $y \gg b > x$, we may use the steady-state approximation for $x$, *i.e.*, $dx/dt = 0$. Therefore

$$\frac{d(x+y)}{dt} = k_1 I(b-x)(a-x-y) \times \left\{\frac{k_2[H^+]}{k_2[H^+] + k_{-1}}\right\} - k_4(y)$$

$$\approx k_1 I(b-x)(a-y) \times \left\{\frac{k_2[H^+]}{k_2[H^+] + k_{-1}}\right\} \approx \frac{dy}{dt} \quad (16)$$

With the same notation and approximation used in the above treatment of the effect of buffer concentration according to the proton gradient model, we may write

$$[H^+] = \left\{\frac{rc/(1+r) - 2y}{c/(1+r) + 2y}\right\} K_a' \quad (17)$$

where $r = [BH]/[B^-]$ before the illumination, and

$$c = [BH] + [B^-] \quad (18)$$

Substituting eq 17 in eq 16 and integrating, we obtain for $y/a \ll 1$ and short $t$ the approximate expression (see Appendix C)

$$\frac{y}{a} \approx \frac{1}{2}st - \frac{1}{8}\left[1 + \frac{2m(1+r)^2a}{(1+m)rc}\right]s^2t^2 + \ldots, \quad (19)$$

where $m = k_{-1}/(k_2K_a'r)$ and $s = 2k_1I(b-x)/(1+m)$.

For approximate considerations, let us neglect the contributions to $\Delta G_s$ due to changes in the concentrations and activity coefficients of the reactants and products in the system, and assume that the free energy stored per unit volume is proportional to $y/a$. Although the washed chloroplasts must still contain a small amount of endogenous buffer, we expect from eq 19 that the additional free energy stored due to the added buffer will increase with the buffer concentration $c$ at low $c$ when

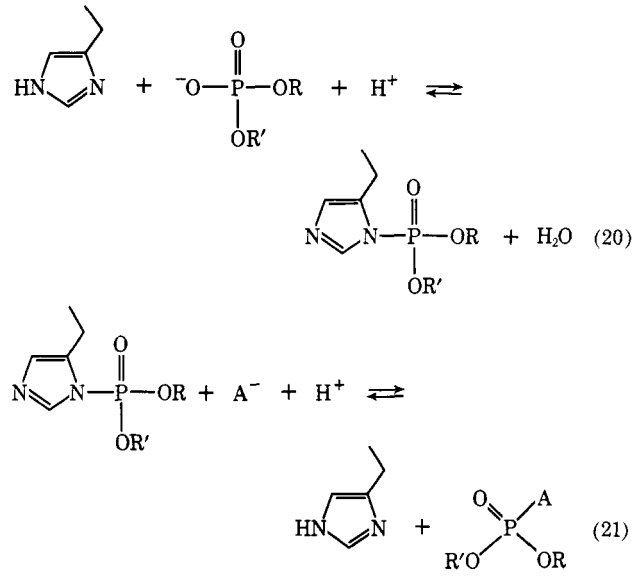
$$\frac{2m(1+r)^2a}{(1+m)rc} \geq 1, \; c \neq 0$$

But at very high $c$, we expect this additional $\Delta G_s$ to become independent of $c$ when

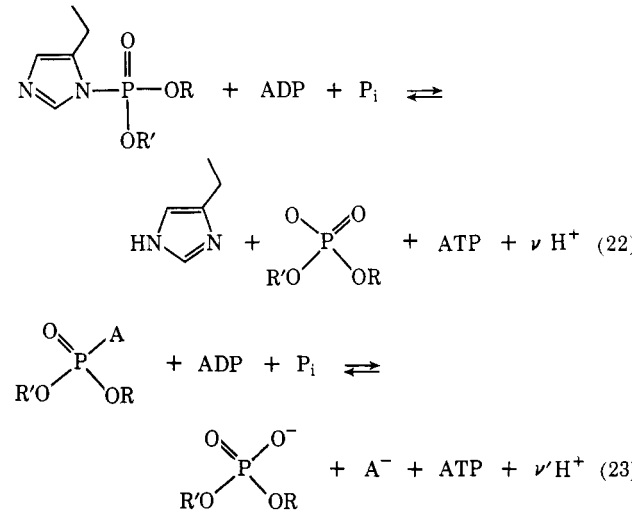
$$\frac{2m(1+r)^2a}{(1+m)rc} \ll 1$$

The experimental data in Figures 2 and 3 are consistent with this inference at low $c$. However, the yield of $X_E$ did not reach a saturation value. We do not know whether this failure to reach a saturation value indicates that the term $2m(1+r)^2a/[(1+m)rc]$ in eq 19 is still appreciable as compared to unity at $c = 10$ mM, or that the rate factor $st$ increases slightly with $c$. The latter case is particularly likely for longer illumination periods in which the yield of $X_E$ may be augmented by supplying a part of the protons required for the production of $X_E$ according to the scheme in Figure 4 from the outside at a rate which increases with the concentration of the added buffer. An appreciable error may also have been introdnced in eq 19 by neglecting the term $-k_4y$ in its derivation, since $k_4$ is known to increase rapidly with pH.

The net reactions leading to Z* or $X_E$ in the present chemical coupling mechanism are




The net reactions for the formation of ATP from $X_E$ in the present scheme are




Since reactions 20 and 21 involve the uptake of protons, whereas reactions 22 and 23 involve the release of protons, we can drive the first two reactions to the right by lowering the pH and drive the last two reactions to the right by raising the pH. Consequently according to the present chemical mechanism it should be possible to make ATP from ADP and $P_i$ by incubating the mixture with chloroplasts in the dark at low pH and then suddenly raising the pH to a high value, but it should not be possible to do the same by incubating with chloroplasts in the dark at high pH and then suddenly decreasing the pH to a low value. These inferences are consistent with the well-known experimental work of Jagendorf and Uribe (1966).

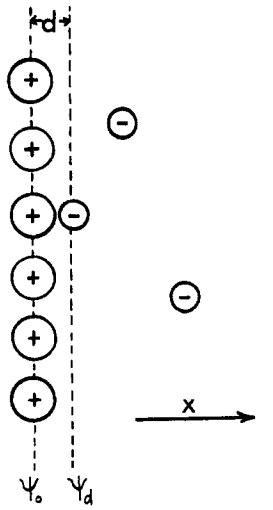



FIGURE 5: The diffuse double layer of hydrated ions with finite radii. $d$ is the distance of closest approach of a negative ion to a positively charged interface.

Similarly when a chloroplast suspension is illuminated under the conditions of cyclic electron transport in the absence of ADP and $P_i$, the external pH is expected to rise initially due to reactions 20 and 21 and gradually to reach a steady-state value when the rate of production of Z* or $X_E$ equals the rate of its hydrolysis ($k_4$ step in Figure 4). After the light is turned off, a drop in external pH should follow the hydrolysis of Z* or $X_E$. Under optimum conditions at low pH (~6), the molar ratio of Z* or $X_E$ photosynthesized to $H^+$ taken up is expected to approach 0.5 according to reactions 20 and 21.

## Appendices

*A. Derivation of Equation 11.* Let us define

$$\xi_1 = (1 + r)\delta/a_1;\ \xi_2 = (1 + r)\delta/a_2 \qquad (1')$$

$$d\delta = \left(\frac{a_1}{1 + r}\right)d\xi_1 = \left(\frac{a_2}{1 + r}\right)d\xi_2 \qquad (2')$$

Substitution of eq 1′ into 9 and 10 gives

$$[H^+]_2 = K_a'\left(\frac{r + \xi_2}{1 - \xi_2}\right);\ [H^+]_1 = K_a'\left(\frac{r - \xi_1}{1 + \xi_1}\right) \qquad (3')$$

Substituting eq 2′ and 3′ into eq 6 and integrating, we get

$$\Delta G_s \approx \frac{RTa_2}{1 + r}\int_0^{\xi_2}\left\{\ln\left(1 + \frac{\xi_2}{r}\right) - \ln(1 - \xi_2)\right\}d\xi_2 + \frac{RTa_1}{1 + r}\int_0^{\xi_1}\left\{\ln(1 + \xi_1) - \ln\left(1 - \frac{\xi_1}{r}\right)\right\}d\xi_1 \qquad (4')$$

For $\xi_2/r \ll 1$, the first integral on the right-hand side of eq 4′ becomes

$$I_2 = \left\{\left(1 + \frac{\xi_2}{r}\right)\ln\left(1 + \frac{\xi_2}{r}\right) - \left(1 + \frac{\xi_2}{r}\right)\right\}r + r + (1 - \xi_2)\ln(1 - \xi_2) - (1 - \xi_2) + 1$$

$$= (r + \xi_2)\left\{\frac{\xi_2}{r} - \frac{1}{2}\frac{\xi_2^2}{r^2} + \ldots\right\} + (1 - \xi_2)\left\{-\xi_2 - \frac{\xi_2^2}{2} - \ldots\right\}$$

$$= \frac{1}{2r}\xi_2^2 + \ldots + \frac{1}{2}\xi_2^2 - \ldots \approx \frac{1}{2}\left(\frac{1}{r} + 1\right)\xi_2^2 \qquad (5')$$

Similarly for $\xi_1/r \ll 1$, the second integral becomes

$$I_1 \approx \frac{1}{2}\left(\frac{1}{r} + 1\right)\xi_1^2 \qquad (6')$$

Substitution of 1′, 5′, and 6′ into 4′ gives eq 11.

*B. Derivation of Equation 13.* Let us consider an infinite planar membrane as a diffuse double layer of finite ions illustrated in Figure 5. The potential $\psi$ at any point obeys the Poisson equation

$$\Delta\psi = -\frac{4\pi\rho}{D} \qquad (7')$$

where the charge density $\rho$ is given by

$$\rho = \sum_i Z_i e n_i \qquad (8')$$

and the number $n_i$ of ionic species $i$ per unit volume is related to the average number $n_{io}$ of that ionic species by the Boltzmann equation

$$n_i = n_{io}e^{-Z_i e\psi/(kT)} \qquad (9')$$

Combination of eq 7′, 8′, and 9′ gives

$$\Delta\psi = -\frac{4\pi}{D}\sum_i Z_i e n_{io}e^{-Z_i e\psi/(kT)} \qquad (10')$$

For the infinite plane in Figure 5, $\partial\psi/\partial y = 0$, $\partial\psi/\partial z = 0$, and $\Delta$ reduces to $d^2/dx^2$. Multiplying both sides of eq 10′ by $2d\psi/dx$ gives

$$2\frac{d\psi}{dx}\times\frac{d^2\psi}{dx^2} = -\frac{8\pi}{D}\sum_i Z_i e n_{io}e^{-Z_i e\psi/(kT)}\frac{d\psi}{dx} \qquad (11')$$

Since $\psi \to 0$ and $d\psi/dx \to 0$ as $x \to \infty$, integration of eq 11′ yields

$$\left(\frac{d\psi}{dx}\right)^2 = \frac{8\pi kT}{D}\Sigma n_{io}\left\{e^{-Z_i e\psi/(kT)} - 1\right\} \qquad (12')$$

For a simple, symmetrical binary electrolyte, eq 12′ simplifies to

$$\frac{d\psi}{dx} = -\sqrt{\frac{8\pi nkT}{D}}\left\{e^{Ze\psi/(2kT)} - e^{-Ze\psi/(2kT)}\right. \qquad (13')$$

Since the total surface charge $\sigma$ should be equal to the total space charge per unit cross-sectional area, we have

$$\sigma = -\int_d^\infty \rho dx = \frac{D}{4\pi}\int_d^\infty \frac{d^2\psi}{dx^2}dx = -\frac{D}{4\pi}\left(\frac{d\psi}{dx}\right)_{x=d} \qquad (14')$$

Substitution of 13′ into 14′ yields

$$\sigma = \sqrt{\frac{DnkT}{2\pi}}\left\{e^{Ze\psi_d/(2kT)} - e^{-Ze\psi_d/(2kT)}\right\} \qquad (15')$$

For a dilute solution containing $C_s$ moles of salt per liter and a moderate surface potential $\psi_0$, *e.g.*, $C_s < 0.05$ M and $\psi_0 > 1$ V, we may use the approximation $\psi_d \approx \psi_0$. Consequently the free energy stored by charging the planar membrane to a surface potential $\psi_0$ is equal to

$$\Delta G_s = \int_0^\sigma \psi_0 \, d\sigma$$

$$\approx \sqrt{\frac{DnZ^2e^2}{8\pi kT}} \int_0^{\psi_0} \{\psi_0 e^{Ze\psi_0/(2kT)} + \psi_0 e^{-Ze\psi_0/(2kT)}\} \, d\psi_0$$

$$= \sqrt{\frac{DnZ^2e^2}{8\pi kT}}\left(\frac{2kT}{Ze}\right)^2\left[e^{Ze\psi_0/(2kT)}\left(\frac{Ze\psi_0}{2kT} - 1\right) - e^{-Ze\psi_0/(2kT)}\left(\frac{Ze\psi_0}{2kT} + 1\right)\right]_0^{\psi_0}$$

$$= \sqrt{\frac{2DnkT}{\pi}}\left\{\psi_0 \sinh\left(\frac{Ze\psi_0}{2kT}\right) - \frac{2kT}{Ze}\left[\cosh\left(\frac{Ze\psi_0}{2kT}\right) - 1\right]\right\}$$

$$= \sqrt{\frac{2DC_sRT}{1000\pi}}\left\{\psi_0 \sinh\left(\frac{Ze\psi_0}{2kT}\right) - \frac{2kT}{Ze}\left[\cosh\left(\frac{Ze\psi_0}{2kT}\right) - 1\right]\right\} \qquad (13)$$

*C. Derivation of Equation 19.* For simplicity let

$$f = \frac{c}{2(1+r)} \text{ or } c = 2(1+r)f \qquad (16')$$

Substituting 16′ and 17 into 16 and integrating, we get

$$k_1I(b-x)t = \int_0^y \left\{1 + \frac{k_{-1}}{k_2K_a'}\left(\frac{f+y}{rf-y}\right)\right\}\frac{dy}{a-y} = \ln\left(\frac{a}{a-y}\right) + \frac{k_{-1}}{k_2K_a'}\left\{\int_0^y \frac{f\,dy}{(rf-y)(a-y)} + \int_0^y \frac{y\,dy}{(rf-y)(a-y)}\right\} \qquad (17')$$

The first integral on the right-hand side of 17′ is equal to

$$I_3 = \frac{f}{a-rf}\ln\frac{(a-y)rf}{(rf-y)a} = \frac{f}{a-rf}\left\{\ln\left(1-\frac{y}{a}\right) - \ln\left(1-\frac{y}{rf}\right)\right\} \qquad (18')$$

For $y/a \ll 1$ and $y/(rf) \ll 1$, 18′ may be written as

$$I_3 = \frac{f}{a-rf}\left\{-\frac{y}{a} - \frac{1}{2}\left(\frac{y}{a}\right)^2 - \ldots + \frac{y}{rf} + \frac{1}{2}\left(\frac{y}{rf}\right)^2 + \ldots\right\}$$

$$= \frac{1}{r}\left(\frac{y}{a}\right) + \frac{1}{2}\left(\frac{a+rf}{r^2f}\right)\left(\frac{y}{a}\right)^2 + \ldots \qquad (19')$$

Similarly for $y/a \ll 1$ and $y/(rf) \ll 1$, the second integral is equal to

$$I_4 = \frac{1}{a-rf}\left\{a\ln\left(1-\frac{y}{a}\right) - rf\ln\left(1-\frac{y}{rf}\right)\right\} = \frac{y^2}{2arf} + \ldots \qquad (20')$$

Let

$$m = \frac{k_{-1}}{k_2K_a'r} \text{ and } s = \frac{2k_1I(b-x)}{1+m}$$

Substitution of 19′ and 20′ into 17′ gives

$$k_1I(b-x)t = -\left\{-\left(\frac{y}{a}\right) - \frac{1}{2}\left(\frac{y}{a}\right)^2 - \ldots\right\} + m\left\{\left(\frac{y}{a} + \left(\frac{a+rf}{2rf}\right)\left(\frac{y}{a}\right)^2 + \ldots + \frac{a}{2f}\left(\frac{y}{a}\right)^2 + \ldots\right\}$$

$$= (1+m)\left(\frac{y}{a}\right) + \frac{1}{2}\left[\frac{(1+m)rc + 2m(1+r)^2a}{rc}\right]\left(\frac{y}{a}\right)^2 + \ldots$$

or

$$\frac{1}{2}st = \left(\frac{y}{a}\right) + \frac{1}{2}\left[\frac{(1+m)rc + 2m(1+r)^2a}{(1+m)rc}\right]\left(\frac{y}{a}\right)^2 + \ldots \qquad (21')$$

Except when $c \to 0$, the third and higher terms on the right-hand side of 21′ may be neglected. Then by solving the resulting approximate quadratic equation, we obtain

$$\frac{y}{a} \approx \frac{(1+m)rc}{(1+m)rc + 2m(1+r)^2a} \times \left\{-1 + \left[1 + \frac{(1+m)rc + 2m(1+r)^2a}{(1+m)rc} \times st\right]^{1/2}\right\}$$

For short $t$, binomial expansion gives

$$\frac{y}{a} \approx \frac{1}{2}st - \frac{1}{8}\left[1 + \frac{2m(1+r)^2a}{(1+m)rc}\right]s^2t^2 + \ldots \qquad (19)$$

The exclusion of $c \rightarrow 0$ is not a serious limitation, since in order for the experiments to be meaningful, the concentration of added buffer must be substantially higher than that of the endogenous buffer groups.

Acknowledgment

We thank Mrs. Josephine Lee for her able assistance in preparing chloroplasts. We are also grateful to Professor A. Ian Scott for making his scintillation counter available to our work and to Mr. James J. Keirns for correcting several errors in the original manuscript.

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# Primary Structure of C-1-$a_1$—a Cyanogen Bromide Fragment of Heavy Chain from Inbred Guinea Pig Immunoglobulin G(2), Which Contains a Markedly Variable Segment*

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ABSTRACT: The primary structure of a 49-residue CNBr fragment from the N-terminal quarter of strain 13 guinea pig immunoglobulin G(2) heavy chain has been determined. This fragment, which spans residues N-35 to N-83 of the $\gamma_2$ chain, has two regions of almost constant sequence joined by a markedly variable region spanning 12 residues. Automated sequential degradation of C-1-$a_1$ from strain 13 and strain 2 animals and from anti-dinitrophenyl antibody purified from strain 13 animals confirmed the presence of a constant sequence for residues N-36 to N-47 of $\gamma_2$ chain. There was greatly restricted variability in purified antibody within the region shown to be markedly variable in normal C-1-$a_1$ of both strains. Positions N-49, N-51, and N-58 within this variable region seem constant in all these sources of C-1-$a_1$.

We have presented the procedures for the isolation of CNBr fragments accounting for 303 residues from the C-terminal three-quarters of the $\gamma_2$[1] chain from strain 13 inbred guinea pig IgG(2)[2] (Birshtein *et al.*, 1971a). Sequence data of two of these fragments and of a tryptic peptide containing methionine have shown a constant sequence for that segment of the molecule extending from position ~N-120 to the hinge region in the middle of the chain (Turner and Cebra, 1971; Birshtein *et al.*, 1971b). Position N-120 is very close to that residue position which, in myeloma proteins, is termed the "switch point," that point N terminal to which these proteins have differing sequences.

The isolation of two additional fragments (C-1-$a_1$ and C-1-$a_2$) was reported (Birshtein *et al.*, 1971a). These fragments as well as a third, C-1-n, have been shown to account for the

* From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland. *Received July 9, 1971.* Supported by National Science Foundation Grant GB 7852.

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[1] The nomenclature is in accord with that suggested in *Bull. W. H. O.* (1964), *30*, 447, and with that proposed by the Conference on Nomenclature for Animal Immunoglobulins, Prague, June 1969.

[2] Abbreviations used are: IgG(2), immunoglobulin G(2); DNP and TNP, di- and trinitrophenyl; HSA, human serum albumin; PTH, phenylthiohydantoin; PCA, pyrrolidonecarboxylic acid.