Ligand Binding to Ferrocycytochrome *c* at High pH

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Ferrocycytochrome *c* has been shown to bind two molecules of CO at pH 14. The second CO is thought to be bound only when the cytochrome *c* molecule is denatured, and once bound appears to be spectrally silent. Insolubilization of native cytochrome *c* prevents the binding of the second CO molecule. A scheme is proposed to explain these observations based on evidence from static titrations and flash-photolysis experiments, use of carboxymethyl cytochrome *c* and insoluble cytochrome *c*, and use of cyanide instead of CO as a ligand.

Ferrocycytochrome *c* binds CO or cyanide in alkaline conditions (Theorell & Åkesson, 1941; Butt & Keilin, 1962), and this reactivity has been used by George & Schejter (1964) to obtain conformational information for ferrocycytochrome *c* by investigating the dissociation kinetics of the CO and cyanide complexes at pH 7.0, after rapidly lowering the pH from alkaline conditions. More recently, CO has been used to investigate the properties of polymerized cytochrome *c* (Dupré *et al.*, 1974) and urea-denatured cytochrome *c* (Kaminsky *et al.*, 1972).

The results presented in the present paper pursue this course further by investigating the CO and cyanide complexes of ferrocycytochrome *c* and chemically modified forms of ferrocycytochrome *c* at very high pH; in particular insoluble cytochrome *c* has been used to investigate the effects of environmental constraints on the cytochrome *c* molecule. Insoluble proteins have been used to investigate conformational integrity (Gabel *et al.*, 1971), and have been developed as models for membrane-bound systems (Brown *et al.*, 1966, 1967), especially since the advent of affinity chromatography (Guilbault, 1966; Kay, 1968). The characterization of such insolubilized enzyme systems has given much insight into those processes which inactivate enzymes or change their properties (Kay, 1968; Goldstein & Katchalski, 1968). The insoluble cytochrome *c* system was particularly convenient for this research since it enabled a chemical and physical study of the protein in the presence of a chemically inert matrix.

Materials and Methods

Horse heart cytochrome *c* was supplied by Boehringer Corp. (London) Ltd., London W.5, U.K. Cytochrome *c* concentrations were determined

spectrophotometrically by using the millimolar extinction coefficient of $27.6 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 550 nm for the reduced material as found by Margoliash & Frohwirt (1959). All experiments with ferrocycytochrome *c* were carried out in the presence of an excess of sodium dithionite.

Agarose powder was obtained from Miles-Seravac (Pty.), Maidenhead, Berks., U.K., and CO from B.O.C., Wembley, Middx., U.K. All other chemicals were AnalaR grade and all solutions were prepared in double-distilled water.

Cytochrome *c* was insolubilized on to agarose gel by the method of Kay & Lilly (1970) by using 2-amino-4,6-dichloro-*s*-triazine.

Soluble and insoluble cytochrome *c* were modified to the carboxymethyl forms by the method of Schejter & George (1965) and Schejter & Aviram (1970).

NaOH solutions were prepared by using volumetric standard solutions from BDH, Poole, Dorset, U.K.

All high-pH solutions were prepared with NaOH and the pH values calculated from the activities of NaOH published in the Handbook of Chemistry and Physics (1972–73); a Radiometer concentric electrode in conjunction with a Radiometer pH-meter, PHM 51, was used to measure pH values lower than 10.

Small additions of titrant solution of known concentration were injected into the cuvette with an Agla syringe fitted into a micrometer screw gauge.

Absorption spectrophotometry was performed on either a SP.700 A Unicam spectrophotometer, or a split-beam spectrophotometer, constructed according to the design of Yang & Legallais (1959) by using a Hilger & Watts Monospek 600 monochromator filter, and an Aminco-Chance dual-wavelength/split-beam recording spectrophotometer.

Flash-photolysis experiments were conducted by the use of the instrumentation and methods described by Gibson & Greenwood (1965). A Bausch and Lomb monochromator (550mm grating, 1200 grooves/mm, $f = 4.4$) was used in conjunction with the photolysis apparatus. The glass cell holding the sample had a 5 cm light-path and was fitted with quartz end windows.

The CO solution was prepared by degassing double-distilled water in a tonometer and equilibrating it with CO by repeatedly introducing CO into the tonometer and shaking the solution well. CO concentrations were measured by titrating a known concentration of myoglobin with CO solution at pH 7.0 by using an Agla syringe as described above.

Computing

An ICL 4-75 computer in the Edinburgh Multi-Access System was used to analyse the results. Experimental points from the rate curves were transferred to the computer storage by using a Ferranti Freescan Digitiser.

The curve-fitting program used to predict the end point of each reaction was derived from the following equation, which is the simplest model which could give biphasic kinetics when $n = 1$ or more, i.e. a curve-fitting program which had some relationship to the system under investigation.

where k_{+1} , k_{-1} , k_{+2} and k_{-2} are rate constants, and Fe and Fe* are different forms of ferrocyanochrome *c* existing at alkaline pH values.

It was assumed that the first equilibrium was set up at a high pH, and n molecules of CO bound in a single step. The latter assumption was made to limit the number of constants to be optimized, and may be justified by the fact that the spectral change is the same whether one or two molecules of CO bind, and that the recombination profiles of carbon monoxyferrocyanochrome were biphasic at all pH values investigated. Practically, the predicted value for the final absorbance change was the same if n was held at a value of 1 or 2. This was important for the results at pH 13.83 and 13.54 where direct titrations could not be performed.

If the concentrations of Fe, Fe*, CO and Fe*(CO)_{*n*} at any time are Y_1 , Y_2 , Y_3 and Y_4 respectively, then their initial concentrations are $\frac{\text{Fe}_0 \cdot k_{-1}}{(k_{+1} + k_{-1})}$, $\frac{\text{Fe}_0 \cdot k_{+1}}{(k_{+1} + k_{-1})}$, L_0 , and 0.0 respectively, where Fe₀ is the total concentration of all forms of ferrocyanochrome *c* and L_0 is the initial concentration of ligand (CO).

Four rate equations were set up from eqn. (1), i.e.

$$\frac{dY_1}{dt} = k_{-1} \cdot Y_2 - k_{+1} \cdot Y_1 \quad (2)$$

$$\frac{dY_2}{dt} = k_{+1} \cdot Y_1 - k_{-1} \cdot Y_2 + k_{-2} \cdot Y_4 - k_{+2} \cdot Y_2 \cdot Y_3^n \quad (3)$$

$$\frac{dY_3}{dt} = n(k_{-2} \cdot Y_4 - k_{+2} \cdot Y_2 \cdot Y_3^n) \quad (4)$$

$$\frac{dY_4}{dt} = k_{+2} \cdot Y_2 \cdot Y_3^n - k_{-2} \cdot Y_4 \quad (5)$$

These equations satisfied the two conservation equations

$$\text{Fe}_0 = Y_1 + Y_2 + Y_4 \quad (6)$$

$$L_0 = Y_3 + nY_4 \quad (7)$$

Eqns. 2–5 were solved by numerical integration.

The computer was programmed to accept estimated or guessed values for the simplex method of Nelder & Mead (1965). Each set of constants tried corresponded to a time-course of absorbance changes which was compared with the observed data by the sum of squared deviations (cost), and when the cost reached an acceptable minimum, the values of the constants were used to predict the final ΔE_{415} . The final absorbance changes were calculated by assuming that the species Fe* and Fe were spectrally identical at 415 nm. The change in extinction between the two spectrally identifiable species was given as that observed when a large excess of CO was bubbled through a ferrocyanochrome *c* solution at the appropriate pH.

The simplex contracted when a reflexion resulted in a cost which was worse than the second-worst cost, and the program accepted this as a minimum when the mean-squared deviation of the cost of each vertex of the simplex, compared with that of the centroid, was less than 1.0×10^{-7} for the first minimum found, and less than 1.0×10^{-9} for minima found after the first re-expansion. The best value for the minimum (i.e. the smallest cost) was stored as the parameter FA and compared with each minimum cost found [$Y(L)$] such that the program was terminated when the criterion

$$\left| \frac{\text{FA} - Y(L)}{Y(L)} \right| < 5.0 \times 10^{-5}$$

was satisfied. After termination, any value for the mean cost (per experimental point) exceeding 1.0×10^{-4} was rejected. Practically, all values accepted were at least one order of magnitude smaller than this limit, and those rejected were at least two orders of magnitude higher than this limit.

Results

CO titration of ferrocyclochrome *c*

Ferrocyclochrome *c* solutions of known concentrations were prepared in NaOH, and titrated with CO solution as described previously. The data were obtained either from a direct spectrophotometric titration or from a computer prediction of the end point for the observed absorbance changes (i.e. a single experiment produced one point on the titration curve). The time-courses all showed biphasic characteristics and equilibrium was reached fast enough in 2.0M- (pH14.13) and 1.5M- (pH14.01) NaOH to make direct titration feasible, but in 1.0M- (pH13.83) and 0.5M- (pH13.54) NaOH the computer prediction from a 15–20min time-course was the only feasible way to obtain experimental points.

Fig. 1 shows the CO titration of ferrocyclochrome *c* at pH14.13 and it can be seen that there is good agreement between the titration values from direct measurement and those from computer prediction. The titration shows that two molecules of CO bind to each cytochrome *c* molecule, and that the ligands are bound very tightly.

Fig. 2 shows the titrations at pH14.01, 13.83 and 13.54 with two, one and one molecules of CO binding per cytochrome *c* molecule respectively, and indicates that binding of the second CO decreases from being tightly bound at pH14.13 to not being bound at pH13.83, whereas the first CO is still bound very tightly at pH13.83 but not at pH13.54. The value for *h* (Hill coefficient) at pH13.54 was calculated from a Hill plot (see Fig. 2) from which the equilibrium constant was calculated to be $1.29 \times 10^6 \text{ M}^{-1}$ corresponding to a standard free energy of formation of $-34.5 (\pm 3.06) \text{ kJ/mol}$ at 25°C; this is approaching the values for those of the CO derivatives of ferrous myoglobin (-42.9 kJ/mol) and haemoglobin (-40.3 kJ/mol) at pH7.0 (Ainsworth & Gibson, 1957), but is considerably lower than that of +97.5 for ferrocyclochrome *c* at pH7.0 (George & Schejter, 1964), suggesting an approach to a condition of full exposure of the iron to the solvent. The rapid change of titration characteristics over this small range of pH, and the change from *h* = 1 to *h* = 2 between pH13.83 (*I* = 1.0) and 14.01 (*I* = 1.5), is indicative of a highly co-operative process such as protein denaturation.

Fig. 2(c) requires some explanation as it may be argued that the experimental points describe a sigmoidal curve indicating the presence of two species of ferrocyclochrome *c* in equilibrium with each other and distinguished by their CO-binding properties. This is difficult to prove, as it is not easy to get meaningful estimations of errors of optimized parameters. However, the standard deviation of the root-squared residuals from the line drawn is of the same order as that of Fig. 2(b) (1.36 compared with

2.07) and thus can be assumed to be within experimental error.

pH titrations of soluble ferrocyclochrome *c*

The titrations were performed by preparing solutions of ferrocyclochrome *c* in NaOH and reading the absorbance at the required wavelength. The pK values were measured from the points of maximal slope.

The change of absorbance of ferrocyclochrome *c* at 415nm showed a single pK of 13.44 in the pH range 12.70–14.20. This was very similar to the value of 13.54 measured by monitoring the absorbance difference between ferrocyclochrome *c* and carbon monoxycyclochrome *c* at 418nm (see Fig. 3), showing that the changes that take place occur as a result of the effect of pH on the protein before CO binding.

Flash photolysis of soluble carbon monoxycyclochrome *c*

The absorbance changes for the recombination of ferrocyclochrome *c* with CO after flash photolysis showed biphasic properties at all pH values investigated, and the variation of the pseudo-first-order rate constants for the fast reaction (*k_f*) and the slow reaction (*k_s*) with pH are shown in Fig. 4 together with the ratio *k_f*/*k_s* which decreases steadily with increasing pH.

The fraction of the total absorbance contributed by the fast reaction (calculated by extrapolation to

Fig. 1. CO titration of ferrocyclochrome *c* at pH14.13

The cytochrome *c* was made up in 2M-NaOH in the presence of sodium dithionite. Wavelength, 415nm; path-length, 1.0cm. ●, Points derived from computer predictions of the equilibrium values for the absorbance changes observed during the first 15–20min of the reaction between cytochrome *c* and CO, for each of the values for $[\text{CO}]_{\text{total}}/[\text{cytochrome } c]_{\text{total}}$ shown. Total cytochrome *c* concentration = $4.926 \mu\text{M}$ (at a $[\text{CO}]_{\text{total}}/[\text{cytochrome } c]_{\text{total}}$ ratio below 1.0), or $1.65 \mu\text{M}$. ○, Points from direct spectrophotometric titration. Total cytochrome *c* concentration = $6.018 \mu\text{M}$. The end point of the titration was that measured from a CO-saturated ferrocyclochrome *c* solution.

Fig. 2. CO titrations of ferrocyanochrome *c* at pH 14.01, 13.83 and 13.54

A Hill plot of the data at pH 13.54 is also shown. The cytochrome *c* solutions were made in NaOH of the appropriate strength and in the presence of sodium dithionite. Wavelength, 415 nm; path-length, 1.0 cm. (a) pH 14.01 (1.5 M-NaOH), all points from direct spectrophotometric titration; ●, [cytochrome *c*]_{total} = 6.018 μM; ○, [cytochrome *c*]_{total} = 4.962 μM. (b) pH 13.83 (1.0 M-NaOH), points from computer predictions of end points, [cytochrome *c*]_{total} = 4.96 μM for points where [CO]_{total}/[cytochrome *c*]_{total} is less than 1.0, or 1.650 μM. (c) pH 13.54 (0.5 M-NaOH), points from computer predictions of end points, cytochrome *c* as in (b). (d) Hill plot of data from (c). The end points of all titrations were measured from CO-saturated ferrocyanochrome *c* solutions. The points derived from computer predictions were equilibrium values for the absorbance changes observed during the first 15–20 min of the reaction between cytochrome *c* and CO for each of the values for [CO]_{total}/[cytochrome *c*]_{total} shown.

zero time) was constant at a value of approx. 0.35 over the pH range investigated (see Fig. 4c) indicating two obligatory processes or a pH-independent equilibrium. All absorbance changes were monitored at 550 nm and there was no spectral dependence for k_f and k_s in the region 535–570 nm.

Chemical modification of cytochrome *c*

Insoluble cytochrome *c*, carboxymethylated cytochrome *c* (CM-cytochrome *c*) and carboxymethylated insoluble cytochrome *c* (insoluble CM-cytochrome *c*) were used for further investigations.

At pH 14.13, the spectra of native and chemically modified forms of cytochrome *c* were identical in the region 400–600 nm in all states investigated as were those of soluble and insoluble CM-ferrocyanochrome *c* at pH 7.4.

Table 1 shows the number of molecules of CO bound per molecule of ferrocyanochrome *c* or modified ferrocyanochrome *c* at pH values 14.13 and 7.40.

The titration profiles were all indicative of high affinity binding.

The carbonmonoxy derivatives of insoluble ferrocyanochrome *c* and soluble CM-ferrocyanochrome *c* at pH 14.13 showed biphasic recombination profiles at 550 nm after flash photolysis. The second-order rate constants for the fast and slow reactions (l'_f and l'_s , respectively) together with the ratio l'_f/l'_s are shown in Table 2 with those for soluble CM-ferrocyanochrome *c*. k_s and k_t for insoluble ferrocyanochrome *c* were shown to be independent of wavelength in the region 535–565 nm. The fractional contribution by the fast reaction to the total absorbance was approximately the same for soluble and insoluble ferrocyanochrome *c*, and CM-ferrocyanochrome *c*.

Cyanide titration of soluble and insoluble ferrocyanochrome *c*

The titration of soluble and insoluble ferrocyanochrome *c* by cyanide at pH 14.13 was followed

Fig. 3. Titration of ferrocyanochrome *c*

(a) pH titration of ferrocyanochrome *c*; wavelength, 415 nm, [cytochrome *c*], 4.748 μM ; path-length, 1.0 cm. (b) pH titrations of the absorbance difference between CO-ferrocyanochrome *c* and ferrocyanochrome *c*, wavelength 418 nm, [cytochrome *c*] 4.748 μM ; path-length = 1.0 cm. All solutions were prepared in NaOH and in the presence of sodium dithionite.

spectrophotometrically at 420 nm and indicated a lower affinity than that for CO. The titration curves were analysed by using the Hill equation which gave values for *h* of 1.05 ± 0.10 and 0.95 ± 0.11 for soluble and insoluble ferrocyanochrome *c* respectively, and 4.79×10^2 and $1.66 \times 10^2 \text{ litre} \cdot \text{mol}^{-1}$ for the equilibrium constants ($K_{\text{sol.}}$ and $K_{\text{insol.}}$), corresponding to standard free energy changes of -15.42 ± 0.29 and $-12.60 \pm 0.34 \text{ kJ/mol}$ in comparison with +26.0 at pH 7.0 (George & Schejter, 1964). The ratio $K_{\text{sol.}}/K_{\text{insol.}}$ of 2.88 ± 0.13 shows that insoluble cytochrome *c* binds ligands less easily than soluble cytochrome *c* at this pH, which would be consistent with greater stability towards denaturants and denaturing conditions than soluble cytochrome *c*.

Fig. 4. Recombination kinetic parameters after flash photolysis of CO-ferrocyanochrome *c* as functions of pH

(a) Change of pseudo-first-order rate constant for the slow reaction (k_s) (○) and fast reaction (k_f) (●) with pH. (b) Change of k_f/k_s with pH. (c) Fraction of the total absorbance contributed by the fast reaction extrapolated to zero time. All cytochrome *c* solutions were prepared in NaOH and in the presence of sodium dithionite. Path-length, 5.0 cm; wavelength, 550 nm; [cytochrome *c*] = 10 μM ; [CO] = 0.5 mM.

Table 1. Static carbon monoxide titrations

The number of CO molecules bound per ferrocyanochrome *c* molecule or modified ferrocyanochrome *c* molecule are given. The pH was maintained at 7.4 by 0.2 M-sodium phosphate buffer, and at 14.13 by 2 M-NaOH. All titrations were carried out as direct spectrophotometric titrations at 415 nm. The reductant was sodium dithionite.

pH	Soluble		Insoluble	
	Ferrocyanochrome <i>c</i>	CM-ferrocyanochrome <i>c</i>	Ferrocyanochrome <i>c</i>	CM-ferrocyanochrome <i>c</i>
7.4	No binding	1.05	No binding	0.95
14.13	1.94	1.95	0.77	1.13

Table 2. *Flash-photolysis results*

The second-order rate constants for the fast and slow reactions for soluble and insoluble carbon monoxy-ferrocyanochrome *c* and soluble carbon monoxy-CM-ferrocyanochrome *c* recombinations after flash photolysis are given. Wavelength = 550 nm, path-length = 5 cm and pH = 14.13.

	Soluble		Insoluble
	Ferrocyanochrome <i>c</i>	CM-ferrocyanochrome <i>c</i>	Ferrocyanochrome <i>c</i>
$10^{-3} \times k_f$ ($M^{-1} \cdot s^{-1}$)	38.01 ± 2.08	19.48 ± 1.21	2.57 ± 0.10
$10^{-3} \times k_s$ ($M^{-1} \cdot s^{-1}$)	6.02 ± 0.22	3.01 ± 0.31	0.41 ± 0.01
k_f/k_s	6.35 ± 0.66	6.50 ± 1.07	6.27 ± 0.42

The absorbance peaks of soluble cyanoferrrocyanochrome *c* at pH 14.13 were identical with those of the insoluble species.

Discussion

Ferrocyanochrome *c* in certain denaturing conditions, ferrocyanochrome *c* polymers, and CM-cyanochrome *c* will bind CO, unlike the native protein (Theorell & Åkesson, 1941; Butt & Keilin, 1962; Kaminsky *et al.*, 1972; Dupré *et al.*, 1974). This property is thus linked with changes in tertiary structure. A certain 'loosening' of structure in response to extremes of pH is reflected in changes of various molecular properties (Theorell & Åkesson, 1941; George & Schejter, 1964; Rupley, 1964; Greenwood & Palmer, 1965; Greenwood & Wilson, 1971; Kaminsky *et al.*, 1972) and is sufficiently above pH 11.0 for CO to bind although no gross changes in structure, as reflected in the specific viscosity, occur until approx. pH 13.0 (Rupley, 1964). The fact that the changes occur at this pH is consistent with the pK, the rapid change in kinetic parameters after flash photolysis, and the change in CO-binding characteristics, all noted previously in this paper. It is therefore consistent with the observation that insoluble ferrocyanochrome *c* binds only one CO molecule at pH 14.13, even when carboxymethylated.

The similarity at pH 14.13 between the spectra of carbon monoxy derivatives of soluble ferrocyanochrome *c* and of its chemically modified derivatives, together with the biphasic kinetics after flash photolysis under all conditions investigated, indicate that the second CO bound is spectrally 'silent'. It is most likely that both molecules of CO bind to the iron and, as k_s and k_f values rise rapidly above pH 13.5, it is probable that both are photosensitive although one alone is able to produce both phases seen after flash photolysis. The fast and slow rates are dependent on CO concentration so there must be two ways of binding; one can therefore postulate

that the binding site exists in two forms (a and b), the relative proportions of each being independent of pH, carboxymethylation of the methionines, and of overall protein conformation, i.e.

The binding of the second CO will be dependent on general protein conformation at the appropriate pH. The scheme above allows both pseudo-first-order rate constants to be pH-dependent and for k_f/k_s of soluble ferrocyanochrome to be identical with that for the chemically modified derivatives at pH 14.13, without explaining why this is so. 'a' and 'b' may represent species with the same kind of binding-site relationships postulated by Gibson (1959), so that 'a' decays to 'b' with a half-life such that only 35% of the CO has an opportunity to bind to 'a'.

Cyanide binds far less strongly to ferrocyanochrome *c* than CO and, in agreement with previous discussion, even less strongly with insoluble ferrocyanochrome *c*; the Hill coefficient for both soluble and insoluble ferrocyanochrome *c* is equal to 1.

The CO binding might indicate that the binding of the second ligating molecule is a displacement reaction (probably replacing histidine-18) and/or that the binding of one CO (but not cyanide) enhances the binding of a second molecule, possibly by weakening the histidine-18-iron linkage. If CO does displace histidine-18 as the fifth ligand, then one strong field ligand replaces another and so it is reasonable to suppose there is little or no change in the spectrum.

Hence it can be seen that even at very high pH, the structure of cytochrome *c* still partially maintains the haem in an environment which prevents it from reacting like free haem and which is of importance to its function in the electron-transfer chain.

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References

- Ainsworth, S. & Gibson, G. M. (1957) *Nature (London)* **180**, 1416–1417
- Brown, H. D., Chattopadhyay, S. K. & Patel, S. K. (1966) *Biochem. Biophys. Res. Commun.* **25**, 304–306
- Brown, H. D., Chattopadhyay, S. K. & Patel, S. K. (1967) *Enzymologia* **32**, 205–212
- Butt, W. D. & Keilin, D. (1962) *Proc. Roy. Soc. Ser. B* **156**, 429–458
- Dupré, S., Brunori, M., Wilson, M. T. & Greenwood, C. (1974) *Biochem. J.* **141**, 299–304
- Gabel, G., Steinberg, I. Z. & Katchalski, E. (1971) *Biochemistry* **10**, 4661–4669
- George, P. & Schejter, A. (1964) *J. Biol. Chem.* **239**, 1504–1508
- Gibson, Q. H. (1959) *Biochem. J.* **71**, 293–303
- Gibson, Q. H. & Greenwood, C. (1965) *J. Biol. Chem.* **240**, 2694–2698
- Goldstein, L. & Katchalski, E. (1968) *Z. Anal. Chem.* **243**, 375–396
- Greenwood, C. & Palmer, G. (1965) *J. Biol. Chem.* **240**, 3660–3663
- Greenwood, C. & Wilson, M. T. (1971) *Eur. J. Biochem.* **22**, 5–10
- Guilbault, G. G. (1966) *Anal. Chem.* **38**, 527R–536R
- Handbook of Chemistry and Physics* (1972–73) 53rd edn.
- Kaminsky, L. S., Burger, P. E., Davidson, A. J. & Helfet, D. (1972) *Biochemistry* **11**, 3702–3706
- Kay, G. (1968) *Process Biochem.* **3**, 36–39
- Kay, G. & Lilly, M. D. (1970) *Biochim. Biophys. Acta* **198**, 276–285
- Margoliash, E. & Frohwirt, N. (1959) *Biochem. J.* 570–572
- Nelder, J. A. & Mead, R. (1965) *Comput. J.* **7**, 308–313
- Rupley, J. A. (1964) *Biochemistry* **3**, 1648–1650
- Schejter, A. & Aviram, I. (1970) *J. Biol. Chem.* **245**, 1552–1557
- Schejter, A. & George, P. (1965) *Nature (London)* **206**, 1150–1157
- Theorell, H. & Åkesson, Å. (1941) *J. Amer. Chem. Soc.* **63**, 1812–1818
- Yang, C. C. & Legallais, V. (1959) *Rev. Sci. Instrum.* **25**, 801–807