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Protein Fluorescence of Lactate Dehydrogenase

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1. There is a non-linear decrease in the protein fluorescence (F) of lactate dehydrogenase with the increase in the fraction (α) of the coenzyme-binding sites occupied with NADH. 2. By a curve-fitting procedure it is shown that the fluorescence intensity can be represented by the equation $F=[1-\alpha(1-x)]^n$ where n is the number of identical and indistinguishable coenzyme-binding sites per protein molecule and $x=F_s^{1/n}$ (F_s is the protein fluorescence at $\alpha=1$). This equation implies that the relative protein fluorescence of molecules bearing j ligands form the geometric series x^j. 3. Non-linear quenching of protein fluorescence for this enzyme is probably due to radiationless transfer of energy from the protein molecule to the bound NADH and should also be observed when other potential acceptors of protein fluorescence are bound at unique sites. 4. The intercept with F_s of an initial tangent to a curve of protein fluorescence against α will be at a value of α equal to $(K_d+[E_0])\cdot(1-x^n)/n\cdot(1-x)$ and not at a value equal to the sum of the dissociation constant (K_d) and the concentration of identical ligand-binding sites ($[E_0]$). 5. A use of non-linear protein fluorescence quenching to investigate the state of aggregation of a protein is discussed.

Proteins that contain tryptophan residues normally have a fluorescence emission band at about 340nm. This protein fluorescence sometimes changes when the protein forms a complex with a ligand. When lactate dehydrogenase forms a binary complex with NADH (Velick, 1958) or reduced 3-acetylpyridine–adenine dinucleotide (McKay & Kaplan, 1964), the protein fluorescence is quenched by more than 80%. This large change in fluorescence can be used to follow the formation of this enzyme–coenzyme complex. Protein fluorescence is potentially more sensitive than the difference in the fluorescence at 440nm between bound and free NADH when excited at 340nm, but to exploit this sensitivity it is necessary to be able to relate the protein fluorescence (F) to the fraction of the coenzyme-binding sites occupied (α). Velick (1958) implied that there was a linear relationship between F and α, but made no explicit test of the implication. McKay & Kaplan (1964) explicitly state that there was a non-linear relationship. Measurements of the protein fluorescence as NADH was added to pig heart lactate dehydrogenase in a buffer containing oxamate (Fig. 2) clearly confirm a non-linear relationship. Recent measurements on alcohol dehydrogenase also show a non-linear relationship (Theorell & Tatemoto, 1971) and these authors ascribe the non-linearity to radiationless transfer of electronic excitation energy across subunit interfaces.

To exploit non-linear protein fluorescence quenching to measure α, it is necessary to have a function that describes the relation of F to α. The most useful function will be one where any numerical constants can be obtained from the known properties of the system, such as the subunit structure, rather than a series of unrelated constants which define a high-order polynomial function. In the present paper it will be shown that the equation $F^{1/n}=1-\alpha(1-x)$ gives a description of the non-linear relationship to within experimental error. The constant n is the number of ligand-binding sites per molecule (4) and the constant x is obtained as $F_s^{1/n}$, where F_s is the protein fluorescence as α tends to unity. The equation applies to the physical situation where the n sites are indistinguishable, independent and intrinsically identical.

Theory

In this theoretical section protein fluorescence (F) is expressed as a fraction of the fluorescence of the protein in the presence of ligand to that in the absence of ligand (L). It is assumed that the ligand-binding sites are indistinguishable and thus that there is only one form of the protein with j ligands bound ($0\leqslant j\leqslant n$). This allows the fraction of the total fluorescence of the mixture of protein molecules contributed by the jth species to be expressed as the product of the concentration of the jth species ($[PL_j]$) and the fluorescence of that species (Q_j) relative to that of the unliganded species. The relative protein fluorescence of a mixture of partially liganded

protein molecules is obtained by summing the individual contributions:

$$F = \frac{\sum_{j=0}^{j=n} [PL_j] \cdot Q_j}{\sum_{j=0}^{j=n} [PL_j]} \quad (1)$$

This equation is only strictly correct if the polarizations of the various species are constant or zero. Eqn. (1) can only be simplified if assumptions are made about either $[PL_j]$ or Q_j. The fractional saturation of the ligand-binding sites α can be introduced, since:

$$\alpha = \frac{\sum_{j=0}^{j=n} [PL_j] \cdot j}{n \cdot \sum_{j=0}^{j=n} [PL_j]} \quad (2)$$

The first three simplifying conditions make no assumptions about Q_j.

(i) One ligand-binding site per molecule ($n = 1$). For this system $\alpha = [PL_1] / \sum_{j=0}^{j=1} [PL_j]$ and thus:

$$F = 1 - \alpha(1 - Q_1) \quad (3)$$

that is protein fluorescence changes are linearly related to α.

(ii) Complete positive co-operativity between the ligand-binding sites. Only the species PL_0 and PL_n will exist at appreciable concentrations. Thus $\alpha = [PL_n]/([PL_0]+[PL_n])$.

Eqn. (1) reduces to:

$$F = 1 - \alpha(1 - Q_n) \quad (4)$$

that is F is linearly related to α.

(iii) Complete negative co-operativity. Only the species PL_0 and PL_1 exist at appreciable concentrations. Thus $\alpha = [PL_1] / n \cdot \sum_{j=0}^{j=1} [PL_j]$ and eqn. (1) reduces to ($\alpha \leqslant 1/n$):

$$F = 1 - n \cdot \alpha(1 - Q_1) \quad (5)$$

that is F is linearly related to α. For a series of binding sites with widely differing affinities which are saturated sequentially, a plot of F against α will show successive linear sections of decreasing slope.

(iv) Q_j related by an arithmetic progression. Without any assumptions about $[PL_j]$, eqn. (1) may be simplified if the relative fluorescences of the molecules are given by the arithmetic progression $Q_j = (1 - j \cdot z)$, where z is the arithmetic increment in the fluorescence on increasing j by 1. This, on substitution in eqn. (1), and also by using eqn. (2), gives:

$$F = \frac{\sum_{j=0}^{j=n} [PL_j] \cdot (1 + j \cdot z)}{\sum_{j=0}^{j=n} [PL_j]} = 1 - \frac{z \cdot \sum_{j=0}^{j=n} [PL_j] \cdot j}{\sum_{j=0}^{j=n} [PL_j]} = 1 - n \cdot z \cdot \alpha \quad (6)$$

Thus F is again linearly related to α and this equation cannot describe the non-linear quenching of lactate dehydrogenase fluorescence.

(v) Intrinsically identical binding sites: Q_j related by a geometric progression. A simplification for $n = 4$ will be given. The general simplification for n subunits will be stated. The relative fluorescences of the protein molecules form the geometric progression $Q_j = x^j$. For intrinsically identical, independent and indistinguishable ligand-binding sites it may be shown that:

$$\alpha = \frac{K_a \cdot [L]}{1 + K_a \cdot [L]} \quad (7)$$

(where K_a is the intrinsic association constant for each site, and [L] is the free ligand concentration) and that:

$$[PL_1] = 4 \cdot [PL_0] \cdot K_a \cdot [L],$$
$$[PL_2] = 6 \cdot [PL_0] \cdot K_a^2 \cdot [L]^2,$$
$$[PL_3] = 4 \cdot [PL_0] \cdot K_a^3 \cdot [L]^3$$
and $$[PL_4] = [PL_0] \cdot K_a^4 \cdot [L]^4.$$

[These equations are derived in Tanford (1966).] Substituting for $[PL_j]$ and Q_j in eqn. (1) gives:

$$F = \frac{[PL_0](1 + 4 \cdot K_a \cdot [L] \cdot x + 6 \cdot K_a^2 \cdot [L]^2 \cdot x^2 + 4 \cdot K_a^3 \cdot [L]^3 \cdot x^3 + K_a^4 \cdot [L]^4 \cdot x^4)}{[PL_0](1 + 4 \cdot K_a \cdot [L] + 6 \cdot K_a^2 \cdot [L]^2 + 4 \cdot K_a^3 \cdot [L]^3 + K_a^4 \cdot [L]^4)} = \frac{(1 + K_a \cdot [L] \cdot x)^4}{(1 + K_a \cdot [L])^4}$$

and if $K_a \cdot [L]$ is eliminated by using eqn. (7) this gives:

$$F = [1 - \alpha(1 - x)]^4$$

or for the general case:

$$F = [1 - \alpha(1 - x)]^n \quad (8)$$

Since x will be less than 1, this will be termed geometric quenching. Thus F is non-linearly related to α. This equation can be rearranged to allow a test of the constancy of x for any given value of n to give:

$$x = \frac{F^{1/n} + \alpha - 1}{\alpha} \quad (9)$$

Even for geometric quenching, if $x \geqslant 0.95$ then the approximation $Q_j = x^j \simeq 1-j(1-x)$ may be introduced and eqn. (1) becomes $F = 1+n\cdot(x-1)\cdot\alpha$ as for an arithmetic progression. This leads to the statement that if the relative change in protein fluorescence is small, then protein fluorescence will always be approximately linearly related to α even in the presence of geometric quenching.

(vi) Q_j related by an arithmetico-geometric progression. A more general case would be to relate the relative fluorescence coefficients by partly arithmetic and partly geometric series, that is $Q_j = (1-j\cdot z)\cdot x^j$. With the same assumptions about indistinguishable sites that were made under (v), eqn. (1) simplifies to:

$$F = [1-\alpha(1-x)]^n - n\cdot z\cdot x\cdot\alpha\cdot[1-\alpha(1-x)]^{n-1} \quad (10)$$

This equation also predicts a non-linear relation between F and α, although for a given value of n and x it will be a more linear relation than that given by eqn. (8).

Initial tangent methods for $(K_d+[E_0])$

Assume that the binding of a ligand to all the ligand-binding sites of a protein solution (E_0) may be described by a single dissociation constant (K_d). Application of the law of mass action shows that the total concentration of ligand in the solution ($[L_0]$) is related to the fraction (α) of the binding sites occupied by:

$$K_d/(1-\alpha) = [L_0]/\alpha - [E_0] \quad (11)$$

Rearranging this equation to give $[L_0] = \alpha\cdot[E_0] + K_d\cdot\alpha/(1-\alpha)$, differentiating to obtain $d[L_0]/d\alpha$, and setting $\alpha = 0$ shows that the equation of an initial tangent to a graph of α against $[L_0]$ is $[L_0] = \alpha(K_d+[E_0])$. This tangent will intercept the line $\alpha = 1$ at a value of $[L_0]$ which may be denoted $[L_0]_I = (K_d+[E_0])$. This same intercept will also be obtained if the graph is of a signal change directly proportional to α against $[L_0]$. Such methods are often used to obtain an estimate of $[E_0]$ (when $K_d \ll [E_0]$) or of K_d (when $[E_0] \ll K_d$) (Velick, 1958). However, if there is a non-linear relationship between protein fluorescence and α, which is described by geometric quenching (eqn. 8), then:

$$[L_0]_I = \frac{(1-x^n)}{n(1-x)}\cdot([E_0]+K_d) \quad (12)$$

and the total ligand concentration at the intercept will not be equal to $(K_d+[E_0])$.

Sensitized fluorescence

The fluorescence at 450nm of the dihydropyridine ring of NADH bound to the enzyme may be excited by radiation that is absorbed by the aromatic amino acids of the protein. In a system where geometric quenching occurs, the electronic excitation energy, which is not emitted as protein fluorescence, is proportional to $(1-x^j)$. If a constant proportion of this non-emitted energy accounts for the excitation of the bound NADH, then the sensitized ligand fluorescence (SLF) (relative to the sensitized fluorescence when $\alpha = 1$) will be given by:

$$SLF = \frac{(1-\alpha)^n}{(1-x^n)}\sum_{j=0}^{j=n}\frac{n!}{j!(n-j)!}\cdot\frac{\alpha^j}{(1-\alpha)^j}\cdot(1-x^j) \quad (13)$$

for a protein with n identical and indistinguishable ligand-binding sites. If $n = 1$ then $SLF = \alpha$ and there is a linear relationship between SLF and α. Differentiation of eqn. (13) at $\alpha = 0$ shows that an initial tangent to a graph of SLF against $[L_0]$ will intercept the value of SLF when $\alpha = 1$ at $[L_0]_I$ where:

$$[L_0]_I = \frac{(1-x^n)}{n(1-x)}\cdot(K_d+[E_0])$$

Thus initial tangents to sensitized-fluorescence curves and non-linear protein fluorescence curves will have the same intercept with the signal at $\alpha = 1$.

Solution of $F = [1-\alpha\cdot(1-x)]^n$ *for n and x*

A curve-fitting procedure was used. An unweighted least-squares deviation (D) was used as the criterion of fit between the protein fluorescence calculated on the basis of eqn. (8) (FC_j) and that measured (F_j) at J values of α_j:

$$D = \left[\frac{1}{J-2}\sum_{j=1}^{j=J}(F_j-FC_j)^2\right]^{1/2}$$

Two methods of solution were used. In the first, no restraint was placed on the value of x at a given value of n. A series of values of n were assumed. For each value of n, x was iterated until D_n was at its minimum. A minimum was always obtained when n was in the range 1–20. These values of D_n were then plotted against n, the best fit being that value of n giving the lowest minimum deviation (Fig. 4).

A restricted solution was obtained by introducing the restraint that $x = (F_s)^{1/n}$, where F_s was the protein fluorescence at saturating concentrations of NADH. This method is very dependent on the value taken for F_s and this might have an error of ± 0.01 for lactate dehydrogenase.

Graphical comparisons. The confidence with which a solution to eqn. (8) can be accepted depends on the error in the original results. The values of FC_j at selected values of n and x were decreased to take into account the fraction of the protein fluorescence absorbed by the NADH in solution by using the same

correction factor that had originally been used to obtain the corrected protein fluorescence. These decreased values were then plotted on tracings of the experimental protein fluorescence curves to allow direct comparison of the fitted points with those measured.

Experimental

To test the relationship between F and α, both these quantities had to be determined independently.

α was determined from the enhanced fluorescence of NADH at 435nm when the coenzyme was bound to the enzyme. The fluorimeter was arranged as shown in Fig. 1(*a*). This arrangement allowed the recording of ΔF, the difference between the fluorescence of NADH added to the enzyme solution and to an equal volume of buffer. The fluorescence was excited with radiation at 320nm, which was focused at the centre of 1cm-square cuvettes. Nucleotide fluorescence was observed at 90° through Kodak–Wratten no. 98 filters (maximum transmission at 435nm). NADH was added at a constant rate ($5\mu l \cdot min^{-1}$) to the 3ml contents of each cuvette, which were stirred at about 10Hz by 25mm^2 stainless-steel paddles. The lowest point of the paddle was 12mm above the base of the cuvette. The measured nucleotide fluorescence (*MNF*) was corrected for the decrease in intensity of the exciting radiation by the added NADH by using a molar extinction coefficient of 3600 litre$\cdot$mol$^{-1}\cdot$cm^{-1} and an effective path length of 0.5cm. The maximum correction was 3%. Before starting a titration the same concentration of free NADH was added to the buffer in both cuvettes and the greater signal was then attenuated until there was no difference signal. This procedure corrected for the imbalance of the optical system.

Protein fluorescence was measured with the fluorimeter rebuilt as shown in Fig. 1(*b*). The contents of cuvette 1 were excited at 305nm and the fluorescence at 90° transmitted by a Kodak–Wratten no. 18A filter (maximum transmission at about 335nm) was amplified and recorded as protein fluorescence. Photomultiplier 2 was arranged to respond to the intensity of radiation at 305nm transmitted by cuvette 2. The anode current of photomultiplier PM2 was converted into a voltage and used to control the cathode voltage applied to both photomultipliers by means of a programmable high-voltage supply unit (ABC 1500M; Kepco, Flushing, N.Y., U.S.A.). The gain and sign of the feedback loop were arranged such that any decrease in the signal from photomultiplier PM2 caused the high voltage to increase. In this way changes in signal caused by variation of the intensity of the exciting radiation were automatically compensated. In particular, decrease in the intensity of the exciting radiation by the added NADH was counteracted. (The path length of cuvette 2 was 1cm, compared with an effective path-length for cuvette 1 of 0.5cm; thus the concentration of NADH added to cuvette 2 was one-half that added to cuvette 1). 305nm was chosen as the exciting wavelength, as it was the highest wavelength which would still excite tryptophan fluorescence and yet allow small, and therefore linear, corrections to be made for the fraction of the incident radiation absorbed by the added NADH by the electrical system just described. Where there are large changes in the protein absorption spectrum on binding a ligand (such as are encountered when pyridoxal phosphate binds to apotransaminase) their effect can be minimized by exciting at 275–295nm. The measured protein fluorescence was corrected for the reabsorption of the protein fluorescence by the added NADH by using a molar extinction coefficient of 5200 litre$\cdot$mol$^{-1}\cdot$cm^{-1} and an effective path-length of 0.5cm. The maximum correction was 5%. In a typical experiment the signal from a solution of 10mM-oxamate in 67mM-H_3PO_4 adjusted to pH7.2 was suppressed. Concentrated protein solution was added to cuvette 1 and the amplification was adjusted to set the signal from this solution to 1000mV. The decrease in this signal as NADH was continuously added was recorded. The temperature was 21.0°C. To minimize absolute errors, the measurements of nucleotide and protein fluorescence were made with the same protein, NADH and buffer solutions within 2h.

Pig heart lactate dehydrogenase (98% H_4 isoenzyme) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. The crystals were centrifuged down and were dialysed against 67mM-H_3PO_4 adjusted to pH7.2 with 5M-NaOH containing charcoal (1g/litre) for 24h at 4°C. For the fluorescence titrations the protein concentration was 0.28mg/ml and the cuvette volume 3ml. The buffer contained 10mM-oxamate, and 2.23mM-NADH was added at $5\mu l \cdot min^{-1}$. There was no change in either nucleotide or protein fluorescence if the addition of NADH was stopped half-way through a titration. The slight acceleration in the rate of change during the addition of the first 0.5μM-NADH was due to the attainment of a steady state between the rate of mixing and the rate of addition. The nucleotide and protein fluorescence titrations were aligned by extrapolating the initial change in signal back to zero change in signal. The uncertainty in the alignment was equivalent to an absolute uncertainty in α of 0.005. Thus, when $0<\alpha<0.05$, there could be considerable relative error in α ($\pm 10\%$) in addition to the overall error of $\pm 1\%$. When duplicate protein fluorescence curves were aligned over the first 40% change, there was a variation of $\pm$10mV over the latter portion of the titration. The fractional saturation of the NADH-binding sites (α) is (corrected nucleotide fluor-

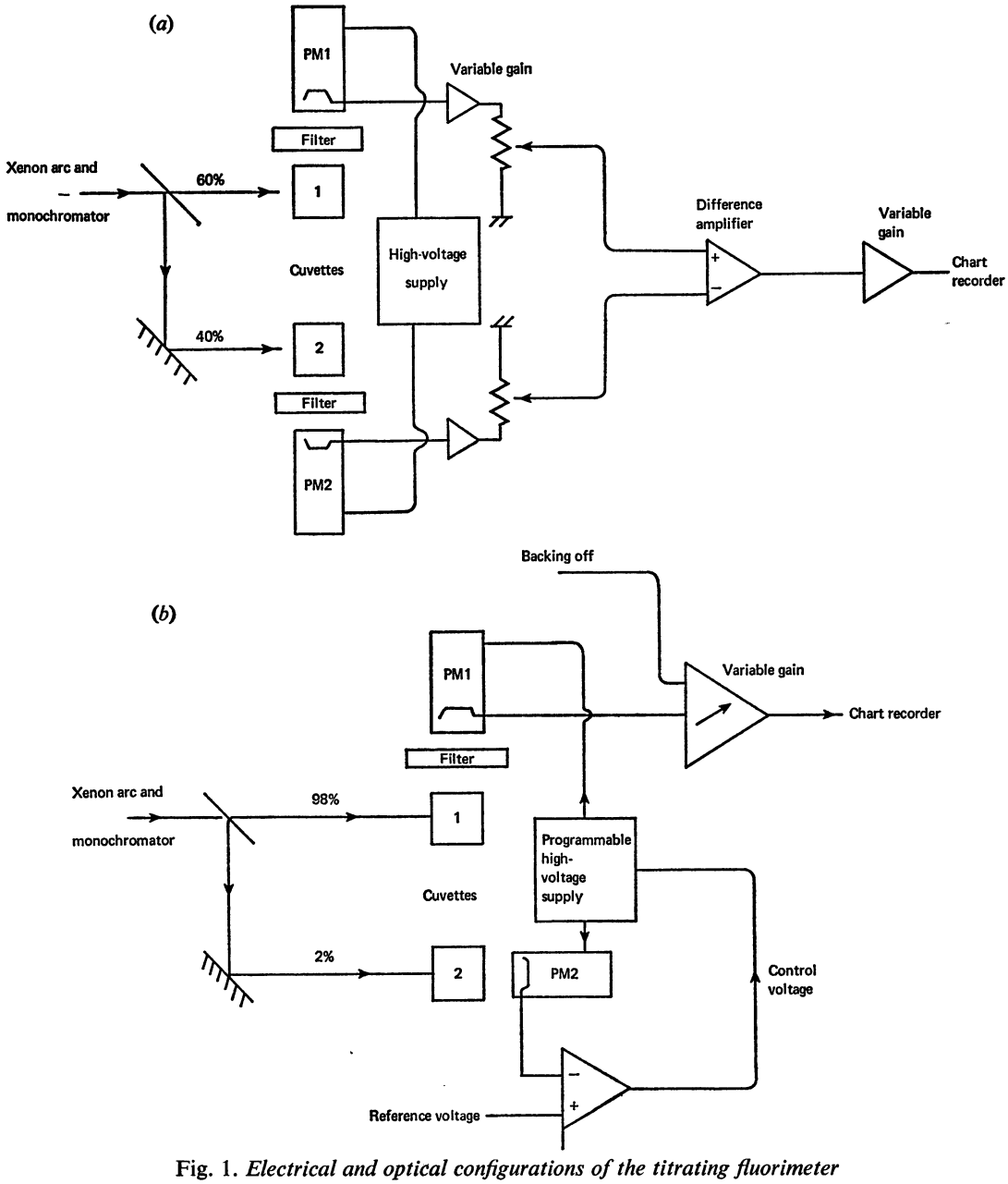

Fig. 1. *Electrical and optical configurations of the titrating fluorimeter*

(*a*) Split-beam differential fluorimeter connected to measure the difference in the fluorescence of NADH added at constant and equal rates to the stirred contents of cuvette 1 (containing enzyme) and cuvette 2 (containing only buffer). Excitation was at 320nm. The emitted radiation was selected with a Kodak–Wratten no. 98 filter (maximum transmission at 435nm). PM, photomultiplier. (*b*) Measurement of protein fluorescence. The enzyme was titrated with NADH in cuvette 1. The excitation was at 305nm. Emitted radiation was selected with a Kodak–Wratten no. 18A filter. NADH was added at half concentration to cuvette 2. The radiation transmitted by this cuvette was measured with photomultiplier PM2. Any decrease in the anode current of photomultiplier PM2 caused the cathode voltage to both photomultipliers to increase, to eliminate the change in current. This compensated for changes in lamp intensity and for the 305nm radiation absorbed by the added NADH.

escence)/(corrected nucleotide fluorescence at saturation). The corrected nucleotide fluorescence at saturation (0.923) was obtained by correcting the measured nucleotide fluorescence at saturation (0.887, obtained from Fig. 2 by inspection) for the exciting radiation being decreased to 0.96 of its initial value by the added NADH.

Results

Winer & Schwert (1959) proposed that a ternary complex was formed between lactate dehydrogenase, NADH and oxamate by compulsory order of addition of ligands (E is the enzyme):

$$E + NADH \rightleftharpoons E\text{–}NADH + oxamate \rightleftharpoons E\text{–}NADH\text{–}oxamate$$

When NADH is added to a solution of the enzyme containing excess of oxamate, the NADH will be bound with an apparently lower dissociation constant ($K_{app.}$) than in the absence of oxamate ($K_{E,NADH}$). If $K_{E-NADH,oxamate}$ is the dissociation constant of oxamate from E–NADH then it is readily shown that:

$$K_{app.} = K_{E,NADH}/(1 + [oxamate]/K_{E.NADH,oxamate})$$

At 21°C and in 67mM-sodium phosphate buffer, pH7.2, it is known that $K_{E,NADH} = 0.5\mu M$ and $K_{E-NADH,oxamate} = 10\mu M$ (J. J. Holbrook & R. A. Stinson, unpublished work). Thus at an oxamate concentration of 10mM, $K_{app.} = 0.5$nM. In experiments such as that shown in Fig. 2, where the concentration of NADH-binding sites (8μM) was 10000 times the dissociation constant, all the NADH added (up to the concentration of sites) will be bound. The linear relationship between the change in the nucleotide fluorescence (ΔNF) and the increase in NADH concentration is evidence that the quantum yield of bound NADH is a constant. Anderson & Weber (1965) also concluded that this was so for reduced 3-acetylpyridine-adenine dinucleotide and this justifies equating α to $\Delta NF/\Delta NF_{max.}$.

The change in protein fluorescence in Fig. 2 is non-linearly related to the increase in concentration of bound NADH. Values of the corrected protein fluorescence as a function of the fraction of the NADH-binding sites occupied are given in Table 1.

The results can be represented mathematically by assuming geometric quenching. For $n = 4$, the plot in Fig. 3, based on eqn. (9), is linear, indicating that x is constant and independent of α.

By using the unrestrained curve-fitting procedure, the 15 data points of Table 1 were fitted to $F^{1/n} = 1-\alpha(1-x)$. The deviations at each value of n are plotted in Fig. 4. The best fit to the results was at $n = 3.8$ and $x = 0.5706$. For $n = 4$ the best fit was at $x = 0.58905$. Experiments were done to determine how sensitive the valnes of n and x were to the likely errors in the results. Alternate pairs of data points from Table 1 ($J = 8$) were used. Uncertainty in the start of the titration was equivalent to an absolute error in α and the effect of adding or subtracting increments in the values of α was determined. Errors in pipetting enzyme, or in estimation of the end-point of the perturbed-nucleotide-fluorescence trace, all lead to a relative error in α. The effect of increasing or decreasing the values of α by 5% of their value was examined. The results are given in Table 2. The correspondence between the fitted points (decreased to simulate trivial reabsorption) and the original experimental results for $n = 1, 2, 4, 6$ and 10 is shown in Fig. 5. On the basis of these comparisons it is estimated that experimental error in the original results limits the precision in n to $4^{+1}_{-0.5}$. The asymmetry arises from the shape of the curve in

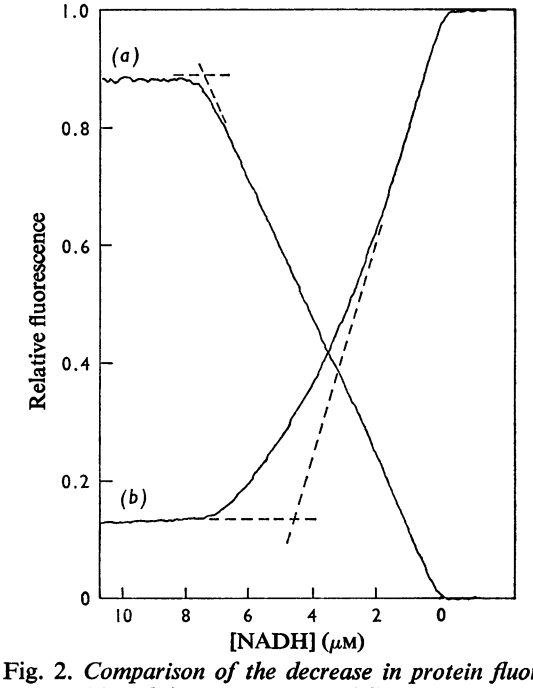

Fig. 2. *Comparison of the decrease in protein fluorescence (b) and the increase in the difference in NADH fluorescence (a) as NADH was added at a constant rate to a solution of pig heart lactate dehydrogenase* The broken lines are tangents to the initial and final portions of each curve. The buffer solution contained 10mM-oxamate and, as the nucleotide fluorescence of the complex enzyme–NADH–oxamate is lower than that of free NADH, the nucleotide fluorescence signal was inverted before recording.

Table 1. *Dependence of protein fluorescence of pig heart lactate dehydrogenase on the fractional saturation of the NADH-binding sites in oxamate*

The table shows the derivation of the corrected protein fluorescence (*F*) and the degree of saturation of the NADH-binding sites (α) from the measured protein fluorescence (*MPF*), the measured difference in nucleotide fluorescence (*MNF*), the total NADH concentration, and the corrected difference in nucleotide fluorescence (*NF*). The uncorrected, assumed, end-point for nucleotide fluorescence was 887 and the corrected value was 923. The method used to obtain the results is given in the Experimental section.

[NADH] (μM)	*MPF*	*MNF*	*NF*	*F*	α
0.9	823	104	104.38	827.56	0.1130
1.4	735	163	163.90	741.11	0.1774
1.9	658	221	222.62	665.31	0.2410
2.3	588	276	278.54	596.17	0.3015
2.8	518	334	337.69	526.65	0.3655
3.3	458	388	393.00	466.94	0.4254
3.7	404	437	443.45	413.02	0.4800
4.2	351	490	498.14	359.83	0.5391
4.7	312	546	556.08	320.73	0.6019
5.1	272	598	610.16	280.39	0.6604
5.6	232	654	668.52	239.81	0.7235
6.1	200	705	721.97	207.31	0.7814
6.5	171	763	782.80	177.74	0.8472
7.0	148	815	837.68	154.26	0.9066
7.4	139	856	881.44	145.28	0.9540

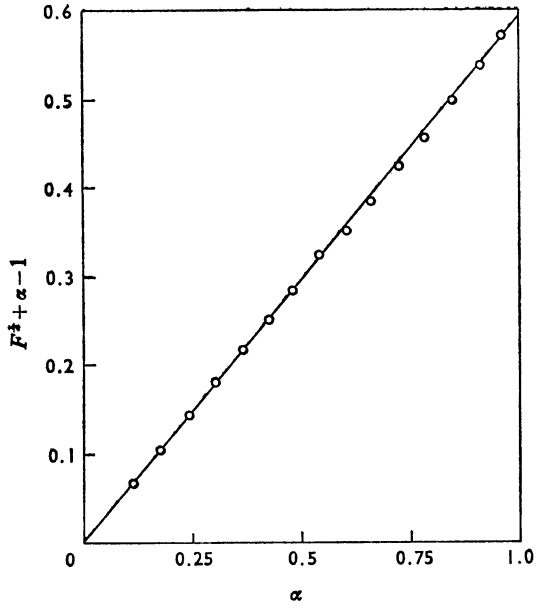

Fig. 3. *Demonstration that the value of x for the equation* $F^{\frac{1}{2}}+\alpha-1=x\cdot\alpha$ *is a constant and is independent of* α

Results are taken from Table 1.

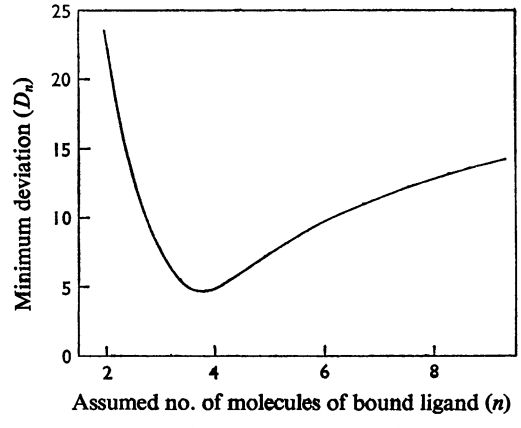

Fig. 4. *Solution of* $F=[1-\alpha(1-x)]^n$ *for x and n by using unrestrained curve-fitting*

For assumed values of *n*, the value of *x* was varied until a minimum least-squares deviation of the calculated values from the measured results was found. The minimum deviations are plotted against the assumed values of *n*. Results are taken from Table 1. The lowest minimum deviation was at $n = 3.8$ and $x = 0.5706$. For $n = 4$, $x = 0.58905$.

Fig. 4, which shows that values of *n* that are too low give far greater deviation than those that are too high.

A curve-fitting solution to eqn. (8), which was restrained by setting $x = (0.13)^{1/n}$, was attempted. The best fit to the results of Table 1 was at $n = 4.9$. This method was not reliable, since even a 2% impurity in

Table 2. *Simulation of the effect of experimental error in the measured dependence of F upon α*

The values of α taken from Table 1 were either multiplied by a constant factor or increased or decreased absolutely by a constant amount to simulate the effect of experimental error. The effects of these errors were then observed in the solution to the equation $F = [1-\alpha(1-x)]^n$ for n and x obtained by the unrestrained-curve-fitting procedure.

Simulated experimental error	Equivalent error in α	n	x
None	0	3.75	0.565
Wrong alignment of the two titration curves	α+0.01	3.2	0 51
	α−0.01	4.5	0.62
Pipetting error, or wrong estimate of the nucleotide fluorescence end-point	α×0.95	3.7	0.58
	α×1.05	3.7	0.54

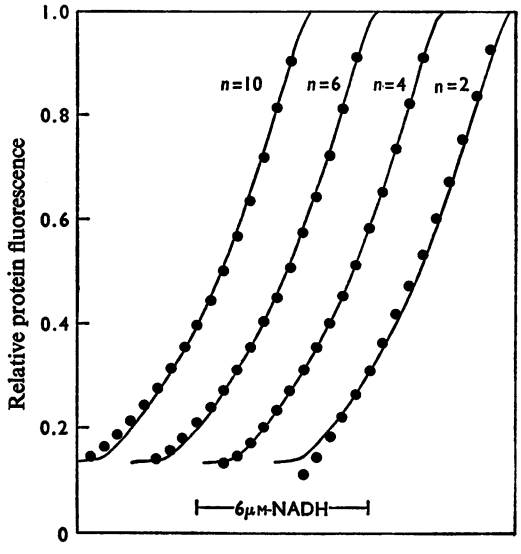

Fig. 5. *Comparison of the protein fluorescence (F) calculated from measured values of α by using the values of n and x obtained from the procedure outlined in the legend to Fig. 4*

The calculated protein fluorescence was decreased to account for absorption of protein fluorescence by the added NADH. The continuous curves are measured. The solutions to $F = [1-\alpha(1-x)]^n$ used were: $n = 2$, $x = 0.2804$; $n = 4$, $x = 0.5891$; $n = 6$, $x = 0.7128$; $n = 10$, $x = 0.8216$.

the enzyme protein [$x = (0.11)^{1/n}$] decreases the value of n for the best fit to 3. Thus, since all proteins will be less than pure, the most that can be concluded is $n \leqslant 5$.

The equation $F = [1-\alpha(1-x)]^n$ was used to describe the quenching in the protein fluorescence of horse liver alcohol dehydrogenase when NADH binds to the enzyme in the presence of isobutyramide. The values of F and α were taken from Fig. 4 of the paper by Theorell & Tatemoto (1971). For this dimer, n was 2 and x was taken as $(0.375)^{\frac{1}{2}} = 0.612$. Table 3 shows the excellent agreement between the measured protein fluorescence and that calculated from $FC = [1-\alpha(1-0.612)]^2$. The values of ($K_d$+[$E_0$]) estimated by using initial tangents to the curves labelled I, II and III of Fig. 4 of Theorell & Tatemoto (1971) were 1.10, 1.13 and 1.33 μM. The ratio of I to III is 0.83 (=1.10/1.33) and is close to the value of 0.81 predicted by eqn. (12) by taking $n = 2$ and $x = 0.612$. These authors did not present sufficient results to allow an attempt to solve eqn. (8) by curve-fitting.

Eqn. (8) was used to describe the non-linear decrease in the fluorescence of ox heart lactate dehydrogenase on binding reduced 3-acetylpyridine–adenine dinucleotide. The results were taken from McKay & Kaplan (1964; Fig. 2). The agreement between $FC = [1-\alpha(1-0.656)]^4$ and the measured protein fluorescence shown in Table 4 was not as good as for the more recent results on alcohol dehydrogenase. However, the agreement was well within the greater experimental error of McKay & Kaplan (1964). For the results of McKay & Kaplan (1964), an initial tangent to the protein-fluorescence curve intersected F_s at 1.98 μM-nucleotide. K_d+[E_0] was estimated by these workers to be 3.38 μM. There is agreement between the ratio of these two values (0.58) and the ratio predicted from eqn. (12) [$(1-0.656^4)/4\cdot(1-0.656) = 0.59$].

Discussion

One of the aims of the present work was to find a mathematical function that would describe the non-linear relationship between the fluorescence of a protein (F) and the fractional saturation of the NADH-binding sites (α). Any constants in the function should be available from inspection of the

Table 3. *Use of the equation* $FC = [1-\alpha(1-0.612)]^2$ *to describe the changed protein fluorescence of alcohol dehydrogenase on forming a complex with NADH and isobutyramide*

Protein fluorescence (F) and fractional saturation with NADH (α) are taken from Fig. 4 of Theorell & Tatemoto (1971).

α	0.15	0.3	0.45	0.60	0.75	0.85
F	0.880	0.780	0.680	0.588	0.502	0.450
$[1-\alpha(1-0.612)]^2$	0.887	0.781	0.681	0.589	0.503	0.449

Table 4. *Use of the equation* $FC = [1-\alpha(1-0.656)]^4$ *to describe the decrease in protein fluorescence* (F) *with the increase in the fractional saturation* (α) *of the reduced 3-acetylpyridine–adenine dinucleotide-binding sites of ox heart lactate dehydrogenase*

Values of F and α are taken from Fig. 2 of McKay & Kaplan (1964). x was taken as $(0.185)^{\frac{1}{4}}$, and n was 4. Allowance was made for the fluorescence of the free nucleotide in calculating α.

α	0.161	0.335	0.536	0.689	0.818	0.927
F	0.767	0.628	0.406	0.346	0.269	0.232
$FC = [1-\alpha(1-0.656)]^4$	0.796	0.613	0.443	0.339	0.267	0.215

results or should reflect the known properties of the enzyme. For proteins where all the ligand-binding sites are identical and do not interact the function $F = [1-\alpha(1-x)]^n$ was suggested. The two constants are easily assigned, since n is the number of NADH-binding sites per molecule and x is the $1/n$th root of the protein fluorescence as α tends to 1.

It was demonstrated that this function describes the non-linear quenching in the protein fluorescence as NADH is added to pig heart lactate dehydrogenase in a buffer containing oxamate, as NADH is bound to alcohol dehydrogenase in a buffer containing isobutyramide and as reduced 3-acetylpyridine–adenine dinucleotide is bound to ox heart lactate dehydrogenase. The function described the results to within experimental error. For pig lactate dehydrogenase it was possible to use a curve-fitting procedure to show that the best fit to the experimental results occurred at $n = 4^{+1}_{-0.5}$. This suggests that it may be possible to use the curve-fitting procedure to determine the number of identical and non-interacting binding sites in unknown systems, thus making the non-linearity an indicator of molecular structure. Very accurate results are required. F and α should preferably be measured by using a dual-wavelength fluorimeter on the same solution, since this eliminates the major cause of error in estimating n.

In the Theory section it was shown that there will be a linear relation between F and α (i) when there is only one ligand-binding site per protein molecule, or when there is either complete (ii) positive or (iii) negative co-operation between the ligand-binding sites. The form of the titration curve will allow a distinction between these three possibilities, since a plot of $\log[(1-\alpha)/\alpha]$ against log(free ligand concentration) will have a slope of 1, >1 or <1 for the three cases respectively.

In the Theory section it was shown that any physical process resulting in an arithmetic progression in the relative fluorescence of protein molecules with j molecules of bound NADH predicts a linear relationship between F and α. Changes in properties that are restricted to the subunit at which the NADH is bound (such as changed environment of tryptophan residues) can only give an arithmetic increment in the fluorescence coefficients. Non-linear quenching must therefore involve changes in properties of the tryptophan and tyrosine residues in the whole protein, not just in the subunit which binds the NADH. Co-operative changes in conformation could cause such changes. Neither pig heart lactate dehydrogenase (Anderson & Weber, 1965; Holbrook *et al.*, 1970) nor alcohol dehydrogenase (Theorell, 1970) show any measurable co-operativity in binding NADH. NAD^+ that has been highly purified to remove traces of impurities (Dolin & Jacobson, 1964) does not change the fluorescence of pig lactate dehydrogenase at concentrations that give 70% saturation of the NAD^+-binding sites (J. J. Holbrook & R. A. Stinson, unpublished work). It has been usual to ascribe the quenching in protein (mainly tryptophan) fluorescence by NADH to resonance transfer of excitation energy (Förster, 1959) to the dihydropyridine ring (Velick, 1958; McKay & Kaplan, 1964; Theorell & Tatemoto, 1971). Calculations indicate that the critical distance, at which the rate of deactivation of the excited state of tryptophan by resonance transfer to NADH equals that by fluorescence emission, is 2.5nm (Karreman *et al.*, 1958).

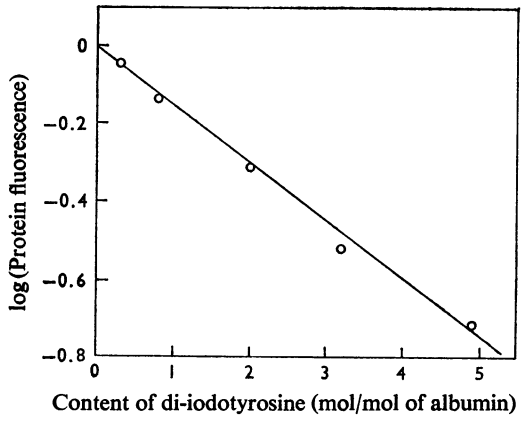

Fig. 6. *Demonstration of the exponential decrease in the relative fluorescence of human albumin with increasing content of di-iodotyrosine*

The results were taken from Fig. 1 at pH6 in the paper by Perlman *et al.* (1968).

Energy transfer over such distances provides a mechanism to change the fluorescence of aromatic amino acids in subunits other than that at which the NADH was bound.

Geometric quenching, however, requires that the fluorescence of the protein is decreased by the same fraction (x) irrespective of the number of equivalent and indistinguishable sites on the molecule that are already occupied. Several geometrical arrangements could give rise to this situation. In the unlikely event of all the NADH-binding sites being at the same place then the aromatic amino acids would always be at exactly the same distance from the NADH site, irrespective of the number of NADH molecules bound, and geometric quenching would occur. A completely symmetrical arrangement of identical subunits would also give rise to geometric quenching, since each NADH molecule would always be bound at the same distance from, and with the same orientation towards, any given aromatic amino acid. In proteins such as lactate dehydrogenase, incomplete symmetry could also give rise to geometric quenching if the large number of aromatic residues (McKay & Kaplan, 1964) are randomly distributed throughout the molecular volume. Many dehydrogenases rich in aromatic amino acids are composed of identical subunits and it will be interesting to see if their protein fluorescence is quenched geometrically.

Experimental support for geometric quenching has been obtained. For example Perlman *et al.* (1968) remarked that the protein fluorescence of human serum albumin decreases approximately exponentially with increasing di-iodotyrosine content. The results of these workers obtained at pH6 are plotted in Fig. 6. The adherence to a logarithmic plot indicates that $Q_J = x^J$, where $x = 0.7$. These workers interpreted their results in terms of resonance transfer from tryptophan to iodotyrosine, because there was no evidence for any conformational change on iodination.

Failure to detect non-linear quenching, where it occurs, can lead to erroneous interpretations of equilibrium and kinetic experiments. Concentrations of binding sites estimated from initial-tangent methods will be too low. Dissociation constants estimated from the whole of a titration curve will be too high. For example, inspection of the protein-fluorescence titration given in Fig. 2 suggests that the binding is described by a dissociation constant of about 0.1–1 μM, whereas the true value is 1000 times smaller.

Estimates of the kinetic order of a reaction from non-linear protein-fluorescence-quenching curves will be too high. A first-order reaction should show constant half-times. Non-linear protein fluorescence would lead to shorter, but successively increasing, half-lives and the reaction might be interpreted as having second or higher order.

The conditions which are thought to give rise to geometric quenching would be fulfilled by the introduction of an aromatic molecule, whose absorption spectrum overlaps the protein emission spectrum, at unique and indistinguishable sites in a protein composed of identical protomers. Such aromatic molecules could be specific cofactors like pyridoxal phosphate, the various haems, the flavins and tetrahydrofolate derivatives. They might equally be molecules which specifically modify a unique amino acid in each protomer, such as 5,5′-dithiobis-(2-nitrobenzoate) (Ellman, 1959), 2-hydroxy-5-nitrobenzyl bromide (Koshland *et al.*, 1965) or chromophoric mercurials (McMurray & Trentham, 1969). Such molecules could then be probes for the state of aggregation of a protein, for dissociation of a fully liganded protein with n sites occupied would cause a change in relative protein fluorescence from x^n to x.

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References

Anderson, S. R. & Weber, G. (1965) *Biochemistry* **4**, 1948
Dolin, M. I. & Jacobson, K. B. (1964) *J. Biol. Chem.* **239**, 3007
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70
Förster, Th. (1959) *Discuss. Faraday Soc.* **27**, 7
Holbrook, J. J., Roberts, P. A., Robson, B. & Stinson, R. A. (1970) *Abstr. Int. Congr. Biochem. 8th.* p. 83

Karreman, G., Steele, R. H. & Szent-Györgyi, A. (1958) *Proc. Nat. Acad. Sci. U.S.* **44**, 140
Koshland, D. E., Jr., Karkhanis, Y. D. & Latham, H. G. (1965) *J. Amer. Chem. Soc.* **86**, 1445
McKay, R. H. & Kaplan, N. O. (1964) *Biochim. Biophys. Acta* **79**, 273
McMurray, C. H. & Trentham, D. R. (1969) *Biochem. J.* **115**, 913
Perlman, R. L., van Zyl, A. & Edelhoch, H. (1968) *J. Amer. Chem. Soc.* **90**, 2168
Tanford, C. (1966) *Physical Chemistry of Macromolecules*, p. 526, Wiley, New York
Theorell, H. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), p. 121, Springer-Verlag, Berlin, Heidelberg and New York
Theorell, H. & Tatemoto, K. (1971) *Arch. Biochem. Biophys.* **142**, 69
Velick, S. F. (1958) *J. Biol. Chem.* **233**, 1455
Winer, A. D. & Schwert, G. W. (1959) *J. Biol. Chem.* **234**, 1153