Thus, the results obtained here demonstrate that NGF has a potent stimulatory effect on the regenerative sprouting and growth of severed central noradrenaline axons.

There have been few studies of the effect of NGF on regenerating neurons. With sensory neurons, Scott and Liu (*8*) found in the kitten that systemic injections of NGF caused an acceleration of the regenerative growth of the central process of the dorsal root ganglion cells. To our knowledge, observations on the effect of NGF on the regeneration of peripheral sympathetic neurons are lacking. Silberstein and co-workers (*9*) reported that NGF stimulated the reestablishment of a sympathetic nerve plexus in rat iris from superior cervical ganglion cells in vitro. From this it seems conceivable that NGF has a similar effect on the regenerative growth of central noradrenergic neurons and on the peripheral sympathetic and sensory neurons.

This is, to our knowledge, the first demonstration of an effect of NGF on central neurons, and it raises a number of interesting possibilities. For instance, our results suggest that NGF could be used to accelerate, increase the magnitude of, or improve the final result of, regeneration of central catecholamine neurons. It seems also possible that NGF is an endogenous, normally occurring physiological factor that is required for the normal development, maturation, and growth of certain central neuron systems, thus playing a role in central neurons similar to that in peripheral, sympathetic neurons.

ANDERS BJÖRKLUND
ULF STENEVI

Department of Histology, University of Lund, S-223 62 Lund, Sweden

References and Notes

1. R. Levi-Montalchini and S. Cohen, *Ann. N.Y Acad. Sci.* **85**, 324 (1960).
2. R. Levi-Montalchini and P. U. Angeletti, *Physiol. Rev.* **48**, 534 (1968).
3. R. Katzman, A. Björklund, Ch. Owman, U. Stenevi, K. A. West, *Brain Res.* **25**, 579 (1971).
4. A. Björklund and U. Stenevi, *ibid.* **31**, 1 (1971).
5. A. Björklund, R. Katzman, U. Stenevi, K. A. West, *ibid.*, p. 21.
6. U. Ungerstedt, *Acta Physiol. Scand.* (Suppl.), 367 (1971); A. Björklund, B. Falck, U. Stenevi, *Brain Res.* **32**, 1 (1971).
7. B. Falck and Ch. Owman, *Acta Univ. Lundensis Sect. 2* **7**, 1 (1965).
8. D. Scott, Jr., and C. N. Liu, in *Mechanisms of Neural Regeneration*, M. Singer and J. P. Schade, Eds. (Elsevier, Amsterdam, 1964), p. 127.
9. S. D. Silberstein, D. G. Johnson, D. M Jacobowitz, I. J. Kopin, *Proc. Nat. Acad. Sci U.S.* **68**, 1121 (1971).
10. Supported by grants from NIH (NS 06701-05) and from the Swedish Medical Research Council (B71-14X-712-06A and B71-14X-56-07A).

19 October 1971

The Hill Plot and the Energy of Interaction in Hemoglobin

Abstract. *The Hill plot does not independently yield the average free energy of interaction per binding site, as has been proposed by Wyman, but rather the difference between the free energies of interaction on binding the first and last ligands. It is shown that additional data (or assumptions) and a model for cooperative behavior are required to obtain the average free energy of interaction.*

The concept of the Hill plot as a means of evaluating the average energy of interaction per binding site in cooperative ligand binding processes was first put forward by Wyman (*1*) and has since been employed by him and others (*2*) to study the nature of cooperative ligand binding in hemoglobin. If y is the fractional saturation of ligand and c_o is the concentration (or activity) of ligand, the equilibrium ligand binding behavior may be represented by a plot of log $[y/(1-y)]$ against log c_o, called the Hill plot. Wyman claims that this plot, independent of any model or additional data, yields the average Gibbs free energy of interaction per binding site (defined below). The purpose of this report is to demonstrate that, in fact, both additional data and a model for cooperative binding are required to obtain a value of the average free energy of interaction and, furthermore, that this value depends upon the model chosen.

The binding of oxygen to hemoglobin may be generally treated as four successive additions of one molecule of oxygen at a time. If we represent the equilibrium constant for the reaction $Hb(O_2)_{i-1} + O_2 \rightleftarrows Hb(O_2)_i$ as K_i, then the oxygen saturation y as a function of the oxygen partial pressure c_o is described by the well-known equation of Adair.

$$y = \frac{K_1 c_o + 2 K_1K_2c_o^2 + 3 K_1K_2K_3c_o^3 + 4 K_1K_2K_3K_1c_o^4}{4 (1 + K_oc_o + K_1K_2c_o^2 + K_1K_2K_3c_o^3 + K_1K_2K_3K_1c_o^4)} \quad (1)$$

The free energy of binding each successive molecule of ligand is given by

$$\Delta G_i = - RT \ln \gamma_i K_i$$

where R is the molar gas constant, T the absolute temperature, and $\gamma_1 \ldots \gamma_4$ are statistical factors equaling 1/4, 2/3, 3/2, and 4, respectively.

If we assume that the intrinsic free energy of ligand binding to a subunit unperturbed by interactions with neighbors, ΔG_o, is the same for all binding sites, then

$$\Delta G_i = \Delta G_o + \Delta G_i^I$$

where ΔG_i^I is the energy of interaction associated with binding ligand molecule i. The average Gibbs free energy of interaction per binding site, $\overline{\Delta G^I}$, is thus defined

$$\overline{\Delta G^I} \equiv \tfrac{1}{4} \sum_i \Delta G_i^I$$

We designate k_o the binding constant of a hypothetical noninteracting subunit, given by

$$\Delta G_o = - RT \ln k_o$$

The Adair equation may then be rewritten as

$$y = \frac{S_1X + 3S_2X^2 + 3S_3X^3 + S_4X^4}{1 + 4S_1X + 6S_2X^2 + 4S_3X^3 + S_4X^4} \quad (2)$$

where $X = k_oc_o$ and

$$S_j = \exp\left(\sum_{i=1}^{j} \Delta G_i^I / RT\right)$$

Equation 2 may be rearranged to yield

$$\frac{y}{1-y} = \frac{S_1X + 3S_2X^2 + 3S_3X^3 + S_4X^4}{1 + 3S_1X + 3S_2X^2 + S_3X^3} \quad (3)$$

It follows that

$$\lim_{X \to 0} \log\left(\frac{y}{1-y}\right) = \log S_1 + \log k_o + \log c_o \quad (4)$$

$$\lim_{X \to \infty} \log\left(\frac{y}{1-y}\right) = \log S_1 - \log S_3 + \log k_o + \log c_o \quad (5)$$

These two straight lines of slope 1 represent the asymptotic behavior of the Hill plot at limiting low and high ligand concentrations, respectively. The vertical distance D between them will be given by

$$D = \log S_1 - \log S_3 - \log S_1 = \frac{- \Delta G_1^I + \Delta G_1^I}{2.303\ RT} = \sqrt{2}\, N \quad (6)$$

where N is the normal distance between the two straight lines (Fig. 1). Thus, the unsupplemented Hill plot yields only the difference between the free energies of interaction on binding the first and last oxygen molecules. This is insufficient to determine $\overline{\Delta G^I}$.

The free energy of oxygen binding to a hypothetical noninteracting subunit in a tetramer, ΔG_o, cannot be measured directly. Its value may, however, be estimated by extrapolation from experimental data. For the purpose of this discussion we assume that the free en-

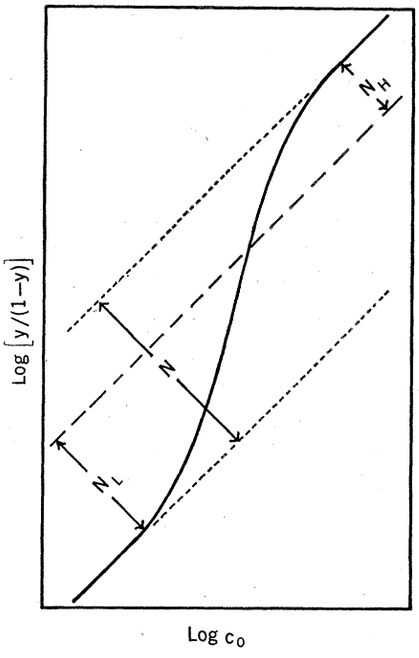

Fig. 1. Parameters of the Hill plot. The dotted lines are extensions of the asymptotes, Eqs. 4 and 5. The dashed line is the reference line, Eq. 8.

ergy of oxygen binding to an isolated subunit is a reasonable approximation to ΔG_0. According to this approximation, the isolated subunit will bind ligand in accordance with the expression

$$y = \frac{k_o c_o}{1 + k_o c_o} \quad (7)$$

This is equivalent to

$$\log\left(\frac{y}{1-y}\right) = \log k_o + \log c_o \quad (8)$$

which is a straight line of slope 1 on a Hill plot, which we will call the reference line. When this line is plotted together with the extended asymptotes of the Hill plot for the cooperative system (Fig. 1), the vertical distance between the high-saturation extended asymptote and the reference line is

$$D_H = \log S_4 - \log S_3 = \frac{-\Delta G_4^I}{2.303\ RT} = \sqrt{2}\, N_H \quad (9)$$

and the vertical distance between the low-saturation extended asymptote and the reference line is

$$D_L = \log S_1 = \frac{-\Delta G_1^I}{2.303\ RT} = \sqrt{2}\, N_L \quad (10)$$

where N_H and N_L are the corresponding normal distances between the lines. Thus, the energies of interaction of binding the first and fourth ligand molecules may be evaluated from the Hill plot of the cooperative system together with that of the isolated subunit. But in the absence of a model this is still insufficient to determine $\overline{\Delta G^I}$.

We now demonstrate that (i) a model of some kind is required to obtain a value of $\overline{\Delta G^I}$ from the information supplied by a Hill plot and (ii) the value of $\overline{\Delta G^I}$ so obtained depends on the model employed. Within the context of this discussion the question of whether a given model for cooperative ligand binding is a "good" model, or more appropriate than another model, is irrelevant. We therefore need not consider general models such as those of Koshland *et al.* (*3*) or Monod *et al.* (*4*). For the sake of ease in calculation and reduction of ambiguity, we instead employ three highly simplified models for cooperative behavior. In all three of these models the hemoglobin molecule is represented by a regular tetrahedron of identical subunits, which interact isotropically in pairwise additive fashion.

1) *The Pauling model* (*5*): There exists a stabilizing interaction between two liganded subunits only. The magnitude of the free energy of stabilization in this model is given by $\Delta G_P \equiv -RT\ \ln\alpha_P$, and the binding curve is represented by

$$y = \frac{k_o c_o + 3\ \alpha_P k_o^2 c_o^2 + 3\ \alpha_P^3 k_o^3 c_o^3 + \alpha_P^6 k_o^4 c_o^4}{1 + 4\ k_o c_o + 6\ \alpha_P k_o^2 c_o^2 + 4\ \alpha_P^3 k_o^3 c_o^3 + \alpha_P^6 k_o^4 c_o^4} \quad (11)$$

2) *The Coryell model* (*6*): There exists a stabilizing interaction between two unliganded subunits only, which is eliminated (broken) by liganding either of the pair. The magnitude of the free energy of stabilization in this model is given by $\Delta G_C \equiv -RT\ \ln\alpha_C$, and the binding curve is represented by

$$y = \frac{\alpha_C^3 k_o c_o + 3\ \alpha_C k_o^2 c_o^2 + 3\ k_o^3 c_o^3 + k_o^4 c_o^4}{\alpha_C^6 + 4\ \alpha_C^3 k_o c_o + 6\ \alpha_C k_o^2 c_o^2 + 4\ k_o^3 c_o^3 + k_o^4 c_o^4} \quad (12)$$

3) *The mixed interaction model* (*7*): There exists a destabilizing interaction between a liganded and an unliganded subunit. Two unliganded or two liganded subunits do not interact. The magnitude of the free energy of destabilization in this model, relative to the liganded or unliganded pair, is given by $\Delta G_m \equiv -RT\ \ln\alpha_m$, and the binding curve is represented by

$$y = \frac{\alpha_m^3 k_o c_o + 3\ \alpha_m^4 k_o^2 c_o^2 + 3\ \alpha_m^3 k_o^3 c_o^3 + k_o^4 c_o^4}{1 + 4\ \alpha_m^3 k_o c_o + 6\ \alpha_m^4 k_o^2 c_o^2 + 4\ \alpha_m^3 k_o^3 c_o^3 + k_o^4 c_o^4} \quad (13)$$

Equations 11, 12, and 13 yield binding curves (y plotted against $\log c_o$) of identical shape when $\alpha_P = \alpha_C = 1/(\alpha_m)^{1/2}$ (*7*). Figure 2 illustrates these curves for $k_o = 1$ and $\alpha_P = \alpha_C = 1/(\alpha_m)^{1/2} = 5.0$. The three binding curves may be superimposed by suitably adjusting the values of k_o in the three equations. If they are so superimposed, then the three equations yield a single Hill plot as well.

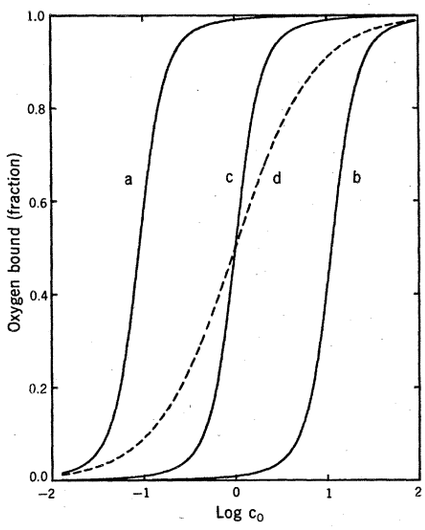

Fig. 2. Oxygen binding curves for $k_o = 1$. (Curve a) Eq. 11, $\alpha_P = 5.0$; (curve b) Eq. 12, $\alpha_C = 5.0$; (curve c) Eq. 13, $\alpha_m = 1/(5.0)^{1/2}$; (curve d) Eqs. 11–13, $\alpha_P = \alpha_C = \alpha_m = 1$ (statistical equation).

For the case of curves of identical shape we may define an energy parameter $U \equiv RT\ \ln\alpha_P = RT\ \ln\alpha_C = -\frac{1}{2}\ RT\ \ln\alpha_m$. Because of the simplicity of the three models developed above, the ΔG_i^I may be readily calculated for each model, and they are shown (in units of U) in Table 1. This table provides a graphic demonstration that different models exhibiting the same Hill plot [and identical values of the apparent Hill n (*8*)] may be characterized by widely varying values of $\overline{\Delta G^I}$.

Tyuma *et al.* (*9*, *10*) have presented Hill plots of oxygen binding for both tetrameric hemoglobin and the isolated α and β chains simultaneously. The tetrameric hemoglobin displays an upper asymptotic behavior apparently identical to that of the β chains. (The α chains are slightly different, but for the purpose of this semiquantitative discussion we ignore the small difference.) Following the treatment given above, one may calculate from the data of Tyuma *et al.* (*9*) for phosphate-stripped hemoglobin at 25°C (their figure 2) that $\Delta G_1^I \approx +2300$ cal/mole and $\Delta G_4^I \approx 0$ cal/mole. Of the three models presented here, only the Coryell model is consistent with the

Table 1. Stepwise free energies of interaction on liganding. See the text for definitions of the models and the energy parameter U.

ΔG_i^I	Model		
	Pauling (Eq. 11)	Coryell (Eq. 12)	Mixed interactions (Eq. 13)
ΔG_1^I	0	$3U$	$6U$
ΔG_2^I	$-U$	$2U$	$2U$
ΔG_3^I	$-2U$	U	$-2U$
ΔG_4^I	$-3U$	0	$-6U$
$\overline{\Delta G^I}$*	$-3/2U$	$3/2U$	0

*$\overline{\Delta G^I} = \frac{1}{4}\Sigma\Delta G_i^I$

observed limiting behavior of hemoglobin and the β chains. We note that in the Coryell model three of the six pairwise interaction energies are broken on binding of the first ligand molecule. Because of symmetry, the sum of these three energies, which is equal to ΔG_1^I, must be equal to one-half the total interaction energy. Thus, $\overline{\Delta G^I} = \frac{1}{2}\Delta G_1^I$, or about 1150 cal/mole. It may be verified that this result is valid even if the subunits do not interact isotropically and the molecule possesses only dihedral symmetry rather than the full tetrahedral symmetry specified earlier. However, if the assumption of pairwise-additive interactions is invalid, then we cannot use the symmetry argument given above to relate ΔG_1^I to the total energy of interaction and hence $\overline{\Delta G^I}$.

In conclusion we note that, following Wyman (*1*), many workers (*2*, *9*, *10*) have identified the quantity 2.303 $\sqrt{2}$ *RTN* obtained from a Hill plot as the average interaction energy per site, or $\overline{\Delta G^I}$ in our terminology. It is shown here that this quantity is not $\overline{\Delta G^I}$, but rather $(-\Delta G_4^I + \Delta G_1^I)$, and that it cannot be related to the average free energy of interaction per site without reference to a specific model for cooperative ligand binding.

H. A. Saroff
Allen P. Minton
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Bethesda, Maryland 20014

References and Notes

1. J. Wyman, *Advan. Protein Chem.* **19**, 223 (1964).
2. See, for example, E. Antonini, J. Wyman, M. Brunori, J. F. Taylor, A. Rossi-Fanelli, A. Caputo, *J. Biol. Chem.* **239**, 907 (1964); R. Banerjee and R. Cassoly, *J. Mol. Biol.* **42**, 351 (1969).
3. D. E. Koshland, G. Nemethy, P. Filmer, *Biochemistry* **5**, 365 (1966).
4. J. Monod, J. Wyman, J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
5. L. Pauling, *Proc. Nat. Acad. Sci. U.S.* **21**, 186 (1935).
6. C. D. Coryell, *J. Phys. Chem.* **43**, 841 (1939).
7. H. A. Saroff and W. T. Yap, *Biopolymers*, in press.
8. The apparent Hill n is a parameter appearing in the empirical Hill equation which approximately describes the saturation y as a function of oxygen partial pressure c_o. The equation is $y = Kc_o^n/(1 + Kc_o^n)$.
9. I. Tyuma, S. Shimizu, K. Iwai, *Biochem. Biophys. Res. Commun.* **43**, 423 (1971).
10. I. Tyuma, K. Imai, S. Shimizu, *ibid.* **44**, 682 (1971).

27 September 1971

Rabies Virus in Nasal Mucosa of Naturally Infected Bats

Abstract. *Rabies virus was demonstrated in the olfactory mucosa of naturally infected bats by staining with fluorescent antibody and by isolation of the virus from the nasal tissues. The olfactory mucosa is a potential portal of entry and exit for airborne rabies virus in bat caves.*

During a study of the pathogenesis of airborne infection with rabies virus, we detected the virus in the nasal mucosa of naturally infected bats, both by virus isolation and by fluorescent antibody staining techniques. This finding suggests that nasal tissues may play a role in airborne transmission of rabies —a mode of transmission previously shown to be associated with certain bat habitats.

We collected 55 Mexican free-tailed bats, *Tadarida brasiliensis mexicana* (Saussure), at Frio Cave, Uvalde County, Texas, in July 1970. Of these, 15 bats, all immature, were either ill or dead; and 40 bats, including 30 immature animals, were alive and apparently normal. Each live bat was killed by exsanguination from the heart, and serum was saved. Using separate, sterile instruments for each animal, we dissected out the two ethmoturbinals and the nasal septum and made impression smears by touching the surfaces of one ethmoturbinal and the septum against sterile glass slides. The tissues, sealed in glass ampules, were frozen at −70°C for storage. Each bat carcass was sealed in a separate container and also stored at −70°C. The impression smears from nasal mucosa were tested for rabies virus antigen with the fluorescent rabies antibody (FRA) technique (*1*).

Certain frozen ethmoturbinals (pair members not used to make impression smears) were sectioned at a thickness of 2 or 4 μm, and the sections were similarly tested by the FRA technique.

Additional tissues (brain, submaxillary salivary glands, lungs, and kidneys) were later dissected from certain bat carcasses, again with separate, sterile instruments; and cut surfaces of these organs were used to prepare impression smears for FRA staining. These tissues and corresponding nasal tissues were also tested for virus by inoculation of tissue suspensions into separate litters of suckling albino Swiss mice (Rockefeller Foundation strain), with the use of standard techniques (*2*, *3*).

Smears from nasal mucosa of 5 of the 15 ill bats were strongly positive for rabies virus antigen when examined by the FRA test. Additionally, ethmoturbinals from two of the five bats were completely sectioned, examined by the FRA technique, and found to be strongly positive. Antigen was prominent in the body and rod (dendrite) of some olfactory cells; the fluorescent-staining cells occurred either individually or in groups.

Mouse inoculation tests for virus in nasal tissues (the septum and one or both turbinals) were positive for only two of the five bats. For one bat, the inoculum (prepared from the septum and one turbinal) produced rabies in one of six mice; and for the other bat, the inoculum (prepared from the septum and two turbinals) produced rabies in all of eight mice.

Additional tests were performed on tissues from the five bats that contained rabies viral antigen in cells of the nasal mucosa. Brain, submaxillary salivary gland, lung, and kidney tissues were tested by FRA and mouse inoculation tests. All of these tissues were positive by one or both tests. (Mouse inoculation tests were negative for virus in lungs and kidneys of one of the bats.)

Impressions from nasal mucosa from the remaining 10 ill bats and from the 40 asymptomatic bats were negative to FRA tests for rabies virus antigen. Negative results were also obtained from additional tests performed on nine of these bats (three ill, six asymptomatic). Nasal, brain, salivary gland, lung, and kidney tissues were tested by the FRA and mouse inoculation techniques; ethmoturbinals were completely sectioned and examined by the FRA test.

Several investigators have successfully infected laboratory animals by the intranasal route, either by instilling rabies virus into the nostrils or by