Nerve Growth Factor and Insulin

Structural similarities indicate an evolutionary relationship reflected by physiological action.

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Nerve growth factor (NGF) has been shown to enhance the growth of sympathetic ganglia in vitro and in vivo and to elicit neurite outgrowth from explanted embryonic sympathetic and sensory ganglia in vitro (*1*). The biological significance of this protein has been demonstrated by the rapid degeneration of the sympathetic nervous system of animals injected with an antibody to NGF (*2*), by the marked effects of NGF upon the metabolism of nervous tissue in vitro (*3*), and by its wide distribution in tissues and body fluids of all of the mammals that have been examined (*4*). Its biological effects on nervous tissue have been well documented, particularly with regard to metabolic processes. For example, Angeletti and Levi-Montalcini (*4*) and Partlow (*5*) have shown, in vitro, that NGF stimulates many of the anabolic processes of embryonic sympathetic and sensory neurons. In particular, studies have been done of uridine uptake (*6*) and the synthesis of RNA (*4*), protein (*6*), and lipid (*7*) as well as the uptake and metabolism of glucose (*8*). However, little is known about the mechanism by which NGF exerts its many effects on sympathetic nerve tissue.

In order to establish the molecular basis for these observed effects of NGF, structural analyses of mouse NGF were performed. First, experiments determined that NGF with a sedimentation coefficient of 2.5*S*, as prepared from mouse submaxillary glands (*9*), is composed of two identical polypeptide chains associated by noncovalent forces (*10*). Sequence analysis of the subunit further established it as a polypeptide chain of 118 amino acids with a molecular weight of 13,259 and containing three intrachain disulfide bonds (*11*). In some preparations, one of the constituent subunits was shortened by eight residues from the amino terminus, and this suggested that limited proteolysis of the primary subunit can occur during the isolation procedure (*12*). Comparison of the amino acid sequence of the primary subunit with a number of proteins of similar size did not reveal any obvious structural similarities (*11*). However, a comparison of the physiological effects of NGF with those of other trophic factors suggested that many properties were shared with insulin. For example, insulin in high concentrations (0.5 unit per milliliter) can stimulate uridine uptake (*5*, *6*), macromolecule synthesis (*5*, *6*, *13*), and energy metabolism (*8*) of neurons sensitive to NGF. Hence, a detailed examination of the primary structure of both proteins was made from which we concluded that insulin and NGF possess similar structural features that probably reflect a common evolutionary precursor. These similarities may provide an explanation for the many common functional properties of the two proteins. Similar relationships for other enterosecretory proteins have been suggested (*14*).

Structural Similarities

A comparison of the amino acid sequence of the primary subunit of mouse NGF (*11*), human proinsulin (*15*), and guinea pig insulin (*16*), is shown in Fig. 1. Proinsulin consists of a single polypeptide chain containing the A and B chains and the C peptide, all linked covalently (*15*). Human proinsulin was used for this comparison because it contains the longest C peptide (35 residues) and can be compared to mouse NGF with no deletions in the C peptide. The sequences of the bovine (*17*) and porcine (*18*) C peptides, which have also been determined, display greater variation (in length as well as sequence) than the A and B chain (*19*) regions of the proinsulin molecules. However, comparison of NGF to either of these proinsulin proteins produces approximately the same degree of similarity observed with the human protein. In addition, upon examination of the known structures of other insulin molecules, only guinea pig insulin, whose sequence differs the most from those of other mammalian insulins, supplied a significant number of additional amino acid identities and favored amino acid replacements and, hence, was included in the comparison with NGF shown in Fig. 1.

The degree of similarity between NGF and the insulins was calculated in two ways. These are indicated in Fig. 1 by solid-line boxes, which enclose identical residues, and by dashed-line boxes, which indicate residues considered to be favored amino acid replacements. Favored amino acid substitutions were defined from the relatedness-odds matrix (*20*) as interchanged (amino acid *i* with amino acid *j*) pairs with a value of R_{ij} greater than 10, where R_{ij} is ten times the ratio of the probability that the particular amino acid substitution has occurred during the evolution of proteins of common ancestry divided by the probability that the substitution occurs by chance.

Two features of the alignment of these structures can be readily seen. First, the sequence of 86 residues that makes up human proinsulin may be positioned starting at the amino terminal portion of NGF and aligned so that only five deletions are introduced into the NGF structure to produce the maximum similarity with the proinsulin structure. Second, although the NGF subunit contains 32 more residues than human proinsulin, a repetition of the proinsulin sequence, starting only four residues after the termination of the first proinsulin molecule, allows the incorporation of a second complete B chain in the comparison. The residues comprising this second B chain are designated here as B′.

Table 1 summarizes a numerical analysis of this comparison with human proinsulin. Although many types of

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analyses have been used to calculate similarities between protein sequences, the relatedness-odds relationship (*20*) seems to be the most appropriate for distantly related proteins. In this analysis, the comparison has been considered in segments that correspond to the polypeptide chains produced by the activation of proinsulin (*17*) as well as the complete alignment. We calculated the degree of similarity, using the relatedness-odds matrix, at each position of the aligned sequences. The average values, R_{ii} and R_{ij} $(i \neq j)$, represent the sum, ΣR_{ii} divided by the total number of identities (the same amino acid appearing in both aligned sequences at the same position) and the sum ΣR_{ij} $(i \neq j)$ divided by the total number of nonidentities. The corresponding values for some pairs of unrelated proteins chosen for comparison are listed in the last line. Clearly, both the B and A chain segments, when compared to the appropriate segment of NGF, show significantly higher values of R_{ii} (and thus more similarity) than would be expected between unrelated proteins. In addition, the B′ segment also yields a value for R_{ii} higher than random. However, it is interesting to note that this calculation depends very much upon the nature of the identical residues; this can be seen from a comparison of the number of identities in the C peptide versus the number in the B′ segment and the resulting R_{ii} values. Whereas the percentage of identical residues in the B′ segment is not appreciably higher than that of the C peptide, the calculated values, R_{ii}, are greater than the random value for the B′ segment but much lower than the random value for the C peptide.

A much different picture emerges from a consideration of the nonidentical residues. We see that the values of R_{ij} $(i \neq j)$ for the segments or groups of segments show essentially random values with the exception of the value of 13.3 for the B chain, which is slightly above the random value of 10. This result is hardly surprising because proteins at an evolutionary distance corresponding to 15 to 20 percent remaining identical residues, such as proinsulin and NGF, have experienced, on the average, four to five amino acid changes at each position with nonidentical residues (*20*). Thus the nonidentical residues cannot be used as indicators of an ancestral relationship. This is apparent in the relatedness odds calculated for the sum of all identical and nonidentical residues, shown in the last column of Table 1. All of the values greater than random are due to the contributions of identical residues which occur within the segments and groups of segments. Therefore, structural relations that reflect evolution from a common precursor can be inferred only in regions of identity. As indicated in Fig. 1, these regions of identity are clustered in the positions of the NGF molecule which correspond to the functionally significant A and B chains of insulin.

Several additional aspects of the comparison strengthen the conclusion that significant structural similarities exist between these molecules. An examination of many other known insulin structures reveals additional identities and favored amino acid substitutions in the comparison with NGF. Table 2 is a summary of these related residues. Significantly, of the four additional identities, three of these are located in the second B chain segment, B′. These additional identical residues plus the identities found in human proinsulin give a total number of seven identical residues in the B′ segment of NGF and the B segment of various insulins. In this manner, the total number of identities in the A, B, and B′ chains is 11 of 21, 9 of 30, and 7 out of 32, respectively. Thus, in addition to supporting the previous conclusions, data from the other known insulin structures provide further support for the alignment of the second B chain segment.

The segments of the NGF sequence which clearly correspond to the A and B chains of insulin are separated by 35 residues, exactly the spacing required to insert the C peptide of human proinsulin (*15*) with no deletions. This observation in itself greatly strengthens the comparison, because proinsulin is in fact the primary gene product. As has been shown (*17*), insulin is formed by proteolytic cleavage of proinsulin removing the C peptide from between the B and A chains, which remain linked by two disulfide bonds. The order of the insulin chains as they occur in the proinsulin molecule is B chain–C peptide–A chain (*15, 17, 18*), and, as seen in Fig. 1, this is in fact the order in which the respective simi-

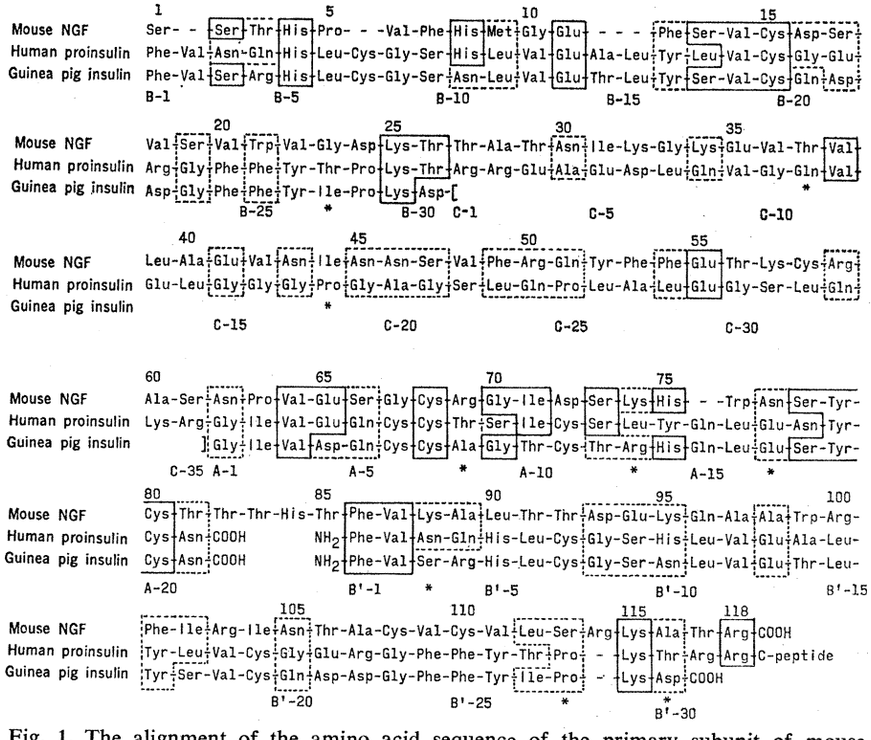

Fig. 1. The alignment of the amino acid sequence of the primary subunit of mouse NGF (*11*) with those of human proinsulin (*15*) and guinea pig insulin (*16*). Numbers above the lines are those of the NGF residue positions and numbers below the lines indicate the positions of the proinsulin or insulin residues. Solid lines enclose sets of identical residues, and dashed lines enclose sets of residues considered to be favored amino acid substitutions, defined as those pairs of residues with an R_{ij} value (relatedness odds) greater than the random value of 10 (*20*). B′ denotes the repeated C chain of insulin (see text). Asterisks indicate positions at which residues from other insulins and proinsulins increase the number of observed similarities (see Table 2).

Table 1. Relatedness-odds calculations for mouse NGF and human proinsulin. Any R divided by 10 gives the ratio of the probability that an amino acid replacement at a particular position has occurred because of common ancestry to the probability that the amino acid pair occurs by chance (*20*).

Corresponding segments		NGF residues (No.)	Identities (No.)	ΣR_{ii}	R_{ii}	Nonidentities (No.)	ΣR_{ij} $(i \neq j)$	R_{ij} $(i \neq j)$	ΣR_{ij} total	R_{ij} total
Proinsulin	NGF									
B-C-A-B′	1–118	118	20	1150	57.5	98	945	9.6	2095	17.8
B	1–26	26	7	368	52.6	19	253	13.3	621	23.9
C	27–61	35	2	42	21.0	33	292	8.8	334	9.6
A	62–81	20	7	549	78.5	13	111	8.5	660	33.0
B′	86–118	33	4	191	47.8	29	289	10.0	480	14.5
B-C-A	1–81	81	16	959	59.9	65	656	10.0	1615	20.0
B + A	1–26 and 62–81	46	14	917	65.5	32	364	11.4	1281	27.9
				Random value						
					40*			10†		15.1‡

* R_{ii}, random $= \Sigma f_i \times R_{ii}$, where f_i is the normalized frequency of occurrence of the amino acid, i (*20*). Based on eight trials with randomly selected actual sequences, the value of 40 given by this formula is probably an upper limit of R_{ii}, random. †R_{ij} $(i \neq j)$, random = 10 for any pair of amino acids which replace each other with equal frequency in related and unrelated sequences (*20*). ‡ R_{ij} (total), random = (identities/total residues) × R_{ii}, random + (nonidentities/total residues) × R_{ij}, random, where R_{ii}, random = 40; and R_{ij}, random = 10.

lar segments occur in residues 1 to 81 of the NGF sequence. This similarity of the primary gene products is an obvious necessity if the observed similarity between these two proteins is the result of the action of evolutionary events on a common ancestral gene.

The conservation of three of the six half-cystinyl residues present in both NGF and proinsulin, including one intact disulfide bridge, suggests that at least some regions of the two proteins have similar three-dimensional structures. Figure 2 shows a schematic representation of the alignment of the disulfide bonds of each molecule; the half-cystinyl (Cys) residues are indicated by circles, with closed circles representing those half-cystinyls conserved in the sequence alignment (Fig. 1). Two of the three conserved half-cystinyl residues retain pairing identical to that of the corresponding half-cystinyl residues in insulin. Interestingly, this disulfide bond connects the two regions of the NGF sequence which are most identical with that of insulin. The third conserved half-cystinyl (Cys 68) is not paired to a conserved residue, but rather is linked to a half-cystinyl found in the repeated B segment, B′. The presence of the B′ segment, which contains no conserved half-cystinyl residues, imparts added structure to the NGF molecule, as evidenced by the fact that the two nonconserved disulfide bonds link residues Cys 58 to Cys 108 and Cys 68 to Cys 110. We cannot yet assess the effect of this rearranged disulfide structure on the conformation of the amino terminal portion of the A chain segment of NGF. However, it is likely that the changes in structure necessitated by the incorporation of the B′ segment into NGF are reflected in the properties which distinguish it from insulin.

Finally, the physical aggregation of insulin, proinsulin, and NGF shows a marked parallel. In each of these cases the molecule exists, over a wide range of *p*H, as a specific dimer or higher even-aggregates associated by noncovalent forces (*10, 21*). Of special interest is the observed polymerization of insulin to hexamers following the addition of zinc (*21, 22*). This structure has been shown by crystallographic means to be associated by coordination of two zinc atoms by the histidyl (His) residues at position B-10 (*23*). In view of the fact that this residue is conserved as His 8 in the NGF sequence, it may be possible for NGF to also form hexamers in the presence of zinc. Experiments to test this premise are currently in progress although these may be complicated by the presence of shorter chains of NGF missing the initial eight residues (and, hence, His 8) in some preparations of NGF (*12*).

The data presented clearly provide a more convincing argument for the existence of structural similarity between NGF and insulin in the regions of NGF corresponding to the A and B chains of insulin than in the repeated B chain segment. However, the observed relatedness in the B chain of insulin to the carboxyl terminal region of NGF is clearly suggestive of a gene duplication event. One possible scheme to account for the relation of the carboxyl terminal portion of NGF to the precursor gene of proinsulin by plausible evolutionary events is depicted in Fig. 3. In this scheme, we propose that following a complete duplication of the ancestral gene, a contiguous reduplication occurred resulting in the formation of a structural gene with sufficient genetic information to code for a polypeptide twice as long as proinsulin. An analogous situation has been described for the human haptoglobins (*24*) and for the immunoglobulins (*25*). From this point there are two possible routes, which are at present indistinguishable, for the production of the 118 residue NGF molecule. In the first route, a subsequent deletion event (or sum of events) would have removed the genetic material coding for the carboxyl terminal A chain and C peptide region and left a gene coding for the 118 residues of NGF. The alternative route would involve no genetic events materially effecting the length of the gene, but rather a double length gene product would be formed. A subsequent proteolytic event would be responsible for removing some 50 residues from the carboxyl terminal end, and the NGF molecule that is presently isolated would result.

Although no definitive evidence is available to resolve this question, several observations tend to support the latter alternative. Taylor, Cohen, and Mitchell (*26*) have reported that epidermal growth factor, a protein of about 56 residues, also isolated from mouse sub-

Table 2. Residues from other insulins and proinsulins related to positions of NGF; I, identity; F, favored replacement [see (*58*) for abbreviations].

Mouse NGF position	Residue	Insulin (or proinsulin) position	Residue	Animal source	Relationship	Reference
23	Gly	B-27	Ser	Chicken	F	(*16*)
37	Thr	C-11	Ala	Pig	F	(*18*)
44	Ile	C-18	Leu	Pig	F	(*18*)
69	Arg	A-8	His	Chicken	F	(*16*)
74	Lys	A-13	Lys	Toadfish 2	I	(*16*)
77	Asn	A-17	Gln	Cod	F	(*54*)
88	Lys	B′-3	Lys	Mouse	I	(*55*)
113	Ser	B′-28	Ser	Toadfish	I	(*16*)
116	Ala	B′-30	Ala	Cow	I	(*56, 57*)

maxillary glands, has a carboxyl terminal arginine. Because epidermal growth factor is isolated bound to one of the many arginine esterases of the submaxillary gland, it has been suggested that the epidermal growth factor may be formed through proteolytic cleavage of a larger protein. Furthermore, the carboxyl terminal arginine of NGF corresponds to one of the sites at which proinsulin is cleaved to release the C peptide (*17*), and as such could be located in a region of the molecule which retains the features necessary for the limited proteolysis required in zymogen activation. In fact, NGF exists within the submaxillary gland as a part of a multiprotein complex (*27*) that includes a potent arginine esterase.

We have also examined the extent to which those residues of NGF and insulin that are related reflect the functionally important residues of insulin. Table 3 lists the positions at which residues have been found to be invariant among the insulins of all species examined (*16, 20, 23*). As already noted, the predominant relatedness in this comparison is also clustered around the B-19 to A-20 disulfide bridge. The extent of relatedness between these residues of NGF and their invariant counterparts in the insulins is approximately 25 percent and 30 percent for identities and favored amino acid replacements, respectively. Although these numbers may appear to be somewhat low for comparisons involving functional residues, it is perhaps germane to note that comparisons of the extent of similarity in functional residues of hormones are obscured by the often undefined distinction between residues which maintain intrinsic activity and those which meet the stringent steric requirements for maximum interaction with a receptor. Thus, those comparisons that have classically been used to relate functional residues of enzymes may not apply with equal force to comparisons among this class of residues in hormones and related factors. For example, many synthetic insulin derivatives containing alanyl residues in normally invariant positions display significant activity (*28*). One such active derivative contained an alanyl residue incorporated in place of Cys A-7. In view of the fact that the replaced half-cystinyl residue, which normally forms a disulfide bridge with half-cystinyl B-7, is among the invariant residues in all naturally occurring insulin molecules examined to date, it may be concluded that this feature of the three-dimensional structure of insulin is not a prerequisite for its biological activity. As noted above, this disulfide bridge is not conserved in the NGF structure, and this supports the view that the relation of structure to function in related hormone molecules may be reflected in more subtle ways than it is in other biologically active protein molecules.

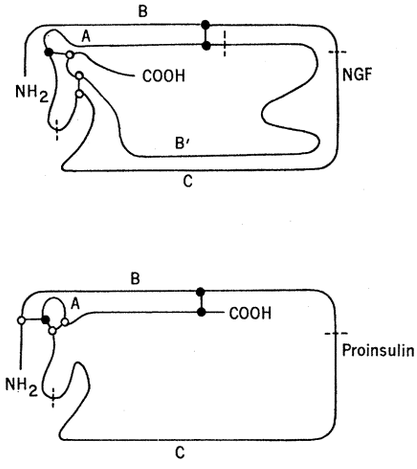

Fig. 2. A schematic representation of the alignment of disulfide bonds in the NGF and proinsulin molecules. Circles represent half-cystinyl residues; those indicated as closed circles have been conserved in the NGF sequence while open circles have not. Dashes divide the schematic peptide chains into the regions of the proinsulin molecule corresponding to the B chain, C peptide, A chain, and repeated B chain, B'.

Functional Similarities

On the basis of the apparent structural relatedness of insulin (or proinsulin) and NGF, it is reasonable to ask whether these molecules display any functional relatedness as well. As mentioned earlier, several authors have noted that NGF and insulin elicit similar metabolic responses in vitro in neural tissue sensitive to NGF (*5, 6, 8, 13*). Although these similarities include many anabolic processes, a comparison of the action of the two proteins is best done by considering the effects of each only on its corresponding target tissue. This approach has the added advantage of eliminating negative responses which might be due solely to differences in the receptors.

It has been known for some time that insulin stimulates many anabolic processes in its target cells (*29*). Recently, the effect of insulin on this set of cellular responses has been restated

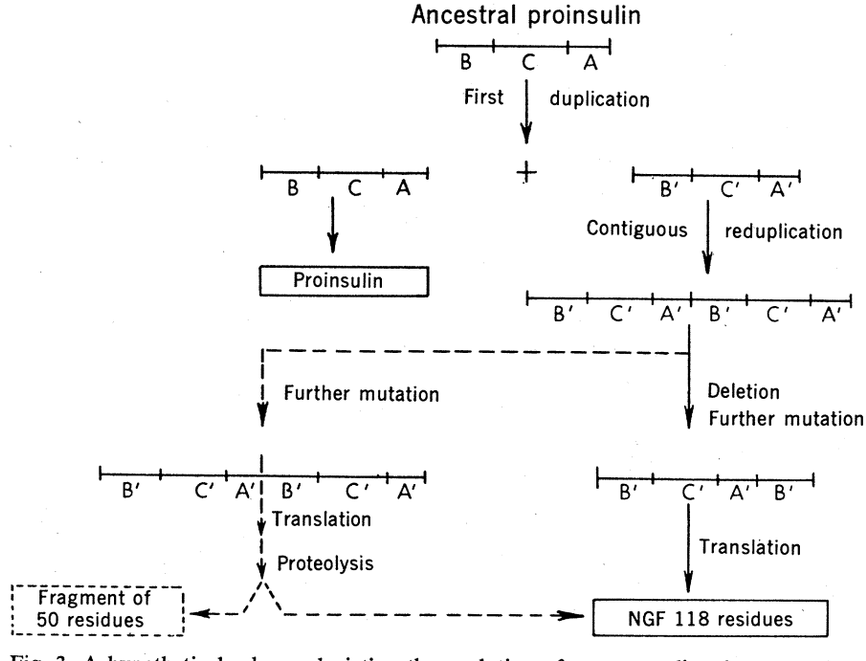

Fig. 3. A hypothetical scheme depicting the evolution of a gene coding for NGF from an ancestral proinsulin gene by way of accepted genetic mechanisms. Genes are shown as lines with bars indicating segments corresponding to those of the gene products. Gene products (proteins) are enclosed in boxes; the dashed-line box indicates a hypothetical protein.

Table 3. The relation of insulin and NGF at the invariant positions of insulin; I, identity; F, favored replacement; X, not related or related only by unfavored single base change (*58*).

Invariant positions of insulin*		Corresponding positions of mouse NFG		
Position	Residue	Position	Residue	Relation to insulin positions
B-6	Leu	5	Pro	X
B-7	Cys	†	†	X
B-8	Gly	6	Val	X
B-11	Leu	9	Met	F
B-12	Val	10	Gly	X
B-15	Leu	†	†	X
B-16	Tyr	12	Phe	F
B-18	Val	14	Val	I
B-19	Cys	15	Cys	I
B-23	Gly	19	Ser	F
B-24	Phe	20	Val	X
A-1	Gly	62	Asn	F
A-2	Ile	63	Pro	X
A-5	Gln	66	Ser	F
A-6	Cys	67	Gly	X
A-7	Cys	68	Cys	I
A-11	Cys	72	Asp	X
A-16	Leu	76	Trp	X
A-19	Tyr	79	Tyr	I
A-20	Cys	80	Cys	I
A-21	Asn	81	Thr	F

* Taken from (*16, 20, 23.*) † Deletion.

in a more unified concept designated as the "pleiotypic" response (*30*). The processes which have thus far been found to be under pleiotypic control are uridine uptake (*31*), RNA synthesis (*32*), polysome formation (*33*), protein synthesis (*34*), protein degradation (*35*), and glucose utilization (*36*). In the positive pleiotypic response, initiated in serum-starved 3T3 cells by serum or insulin, all of these processes are stimulated with the exception of protein degradation, which is inhibited (*30*).

This response closely parallels that of sensitive neurons to NGF in vitro. As demonstrated with explanted sensory ganglia, from 9-day chick embryos, which consist of responsive and nonresponsive cells (*4*), in serum-free Eagles medium, NGF increases uridine uptake (*6*), the synthesis of all classes of RNA (*4*), protein synthesis (*6*), and glucose utilization (*8*). Also, NGF increases lipid synthesis (*3, 7*), an aspect of cellular metabolism which might reasonably be expected to be a part of a pleiotypic growth response. Evidence that NGF causes an increase in polysome formation comes from electron microscopic studies of sensory ganglia cultured with NGF in vitro in which it was found that NGF increases rough endoplasmic reticulum and polysomal aggregates (*37*). In fact, one of the earliest detectable effects after injection of an antibody to NGF is the breakdown of polysomes (*38*). Also, NGF maintains and promotes the growth of cultures of sympathetic nerve cells in serum-free Eagles medium (*1*). At present, no data on the effect of NGF on protein degradation is available.

It is well established that hormones selectively amplify preexisting cellular processes, often by modifying the amounts of tissue-specific proteins. The most obvious specialized proteins of sympathetic neurons, the normal target tissue of NGF, include those for metabolism of the neurotransmitter (noradrenaline) and those involved in maintenance of the axon and its terminal specializations. Nerve growth factor can affect both these classes of proteins. For example, NGF increases the activity of tyrosine hydroxylase and dopamine β-hydroxylase four- to fivefold in the superior cervical ganglia of the newborn rat by an as yet undefined mechanism (*39*). Moreover, the stimulation by NGF of nerve fiber outgrowth probably requires the synthesis and assembly of components normally present in neuronal processes. Particularly conspicuous, after treatment with NGF, is the accumulation of large masses of neurofilaments (*37*), which are found in developing axons (*40*).

Metabolic inhibitor studies by Partlow (*5*) and Levi-Montalcini (*3*) indicate that the neurite outgrowth response of sympathetic ganglia from 12- to 17-day chick embryos is not inhibited by actinomycin D (*5*) and would thus appear not to be linked to new RNA synthesis (*5*). However, fiber outgrowth does appear to be inhibited by protein synthesis inhibitors such as puromycin (*3, 5*), *p*-fluorophenylalanine (*3*), and cycloheximide (*5*). These observations suggest that NGF may promote fiber outgrowth (and perhaps the synthesis of tyrosine hydroxylase and dopamine β-hydroxylase) by acting in a selective fashion on protein synthesis at a stage after transcription. This type of control is exerted by insulin in the case of tyrosine aminotransferase induction (*41*).

Further similarities between insulin and NGF are demonstrated by the effects of their respective antibodies on certain cell populations. In 1960, Levi-Montalcini and Booker described massive and rapid degeneration of the sympathetic ganglia of animals injected with an antibody to NGF (*42*). This striking phenomenon, designated "immunosympathectomy" (*2*) is characterized by gross ultrastructural changes (*38*), the breakdown of electrical function (*2*), and ultimately cell death (*42*). Recently, the term microangiopathy has been applied to the manifestations of the degeneration of many classes of supportive cells—glial and Schwann cells, the mural cells of the retina, pericytes of bone, and the mesenchymal cells of the kidney—which occur in diabetics (*43*). It has been suggested that the degeneration of these cells is due to the accumulation of insulin antibodies as a result of prolonged administration of animal insulin (*44*). Although the exact mechanism of the cytotoxic effect of the antibody is unknown for either insulin or NGF, the death of a limited class of cells in each case suggests that the hormones may have an active role in cell stabilization.

Similarities of Biological Origin

Adelson (*14*) has recently described the functionally diverse glandular system of vertebrates in terms of a "vertebrate enterosectory system." As support for this hypothesis some general similarities in evolution, development, and secretory products of several types of endocrine and exocrine glands were noted. Because insulin and NGF share many structural and functional properties, we examined their possible relationship as "enterosecretory" proteins

by comparing their organs of origin.

It is well known that insulin is synthesized and stored in the β cells of the pancreas, which lie in the endocrine portion of the gland (*45*). The synthesis of NGF has been demonstrated in the region of the submaxillary gland referred to as the convoluted tubules (*46*), where, at least in mice, NGF is also stored (*47*). A comparison of the embryological development, morphology, and function of the pancreas and submaxillary glands reveals them to be quite similar. Both glands develop from the entoderm of the embryonic gut through interactions with the underlying mesenchyme (*48*). Morphologically, both glands have an exocrine (acinar) portion that secretes digestive enzymes via an opening into the digestive tract (*49*). In addition, each gland has a richly vascularized region (*45, 48*) which, in the pancreas, is unequivocally associated with endocrine function (*45*). Although an endocrine role for mouse submaxillary glands has not been established, the NGF activity found in blood and peripheral organs has been shown to be immunologically identical to NGF isolated from the submaxillary glands (*50*).

The evolution of the salivary glands in vertebrates is of interest, for their appearance parallels or slightly precedes that of NGF. Nerve growth factor activity has been demonstrated in many vertebrates, including reptiles, with the neurite outgrowth biological assay and with immunologic techniques. Salivary glands of the type present in vertebrates are absent in fish and first appear in amphibians (*51*). The pancreas and its product, insulin, developed earlier, being present even in primitive fish. Insulin can be divided into two main classes on the basis of amino acid sequence—fish and mammalian. Examination of Fig. 1 and Table 2 reveals that mouse submaxillary NGF is more closely related to the general class of mammalian insulins than to fish insulins; this indicates that the first gene duplication event (Fig. 3) probably occurred after the divergence of fish and higher vertebrates. With the sequence of only one NGF available (*11*), it is impossible to define more closely the timing of later events leading to the production of a functional NGF molecule. The elucidation of the sequence of snake venom NGF should be of great help in establishing the temporal relationships in the evolution of NGF.

Concluding Remarks

The observations that have been summarized regarding the apparent similarities in structure, function, and origin of NGF and insulin (or proinsulin) suggest an evolutionary and functional relationship for these molecules. This evolution of NGF from an ancestral proinsulin is a clear example of the formation of new function from a preexisting protein and may be compared to the evolution of α-lactalbumin from lysozyme (*52*) and the development of several pancreatic serine proteases with varying specificity from a primitive precursor (*53*). The related structural features of NGF and insulin, indicated by the similarities in their primary sequences, may be responsible for the metabolic stimulation that insulin produces in neurons sensitive to NGF. Definitive evidence establishing this relationship is lacking, but it may still be taken as an indication that NGF acts primarily as a hormone, presumably through a factor-receptor complex.

Although the similarities between insulin and NGF have been stressed here, they are in fact different proteins that serve different functions within the organism. Several features of NGF and the NGF-mediated response are not in line with classical definitions of endocrinology. In particular, the fact that NGF produces maximum stimulation of sensitive cells only during certain periods in embryonic development has led to the suggestion that NGF might best be considered among growth factors as occupying a position intermediate between protein hormones and inducers (*4*). This distinction among hormones, growth factors, and inducers may be an artificial one. Perhaps all of these agents can be described as hormonal, having their actions modified by the temporal changes in susceptibility of their target tissues or by the action of similar agents of opposite effect during the complex course of development. Whatever the ultimate classification assigned to NGF, the primary importance of these observations is the direction which they provide for elucidation of the mechanism of action of NGF. At present, this action may be viewed as that of a protein hormone whose effect is temporally limited and which exerts positive pleiotypic stimulation on developing sympathetic nerve tissue and which is required in small amounts to maintain the mature sympathetic nervous system.

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58. Abbreviations: Ala, alanine; Asn, asparagine; Asp, aspartic acid; Arg, arginine; Cys, half-cystine; Gly, glycine; Gln, glutamine; His, histidine; Ile, isoleucine; Lys, lysine; Leu, leucine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Trp, tryptophan; Val, valine.
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The Overmedicated Society: Forces in the Marketplace for Medical Care

Prescribing levels are determined by converging interests of manufacturers, doctors, and others.

Charlotte Muller

Overmedication is one source of reduced human welfare. This reduction is expressed directly in economic terms insofar as avoidable expenditure on drugs occurs. Avoidable expenditure is a class of outlay that includes ineffective drugs and drugs or dosages that do harm to health. Loss of welfare through death, disability, and fetal damage traceable to inappropriate drug use is translatable into economic terms to the extent that productive years are taken away, although accurate measurement of intangible costs is lacking.

The use of a variety of drugs and the use of drugs in frequent doses open the door to confusion and mishaps in the dispensing and distribution of drugs, in adherence by patients to a dosage schedule, and even in the prescribing process. The probability of side effects when many drugs are used is not simply additive, but multiple, because of interactions. Besides wasting money on a course of medication, the patient may waste time that could have been invested in a superior method of improving his health. He is also getting less value from the doctor's input because the memory and judgment of the doctor are to some extent devoted to sorting out the properties of the profession's cluttered armamentarium.

The evidence that overprescribing occurs is varied. It includes the sale of specific drugs and classes of drugs in volumes far out of proportion to the known incidence of diseases in which such drugs are of known value, as well as practitioners' own statements of what drugs they select for given diagnoses and purposes. Fixed combinations belong in the group of drugs whose rational basis has been sharply questioned. The evidence for overmedication also includes the proportion of total prescribing made up of drugs for which the practitioner has only a probable, possible, or placebo expectation of success. The indirect evidence of the content of pharmaceutical advertising is also pertinent. (If the doctor is using the drug for the reasons and symptoms suggested by the advertising, overmedication *must* exist.) Finally, the uneven quality of the experimental and statistical demonstrations of efficacy used to support marketing approval and to justify prescribing decisions is also indicative of a use of drugs beyond rational limits.

Some of these issues are brought out in connection with psychoactive drugs (part of the class of pharmaceuticals acting on the central nervous system), which accounted for 28.3 percent of total manufacturers' domestic sales of drugs in 1969. An example of the type of promotional efforts conducive to questionable prescribing is found in an advertisement carried in the *New England Journal of Medicine* for an amphetamine-like substance widely promoted "for children with an entity that is virtually without definite limits." Four physicians from Duke University Medical Center wrote to the journal to express their disturbance over this promotion, stating their belief that the manufacturer "seems to blur intentionally the distinctions between hyperkinesis and minimal brain dysfunction" (*1*).

The problem of validity of clinical research underlying drug use is illustrated with respect to another class of psychoactive drugs, the antidepressants.

Analysis of the research done to

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