Questions of differences between versions of recombinant proteins have taken on great commercial importance—well beyond their effects on therapeutic activity within the body. Regulatory agencies and the courts have determined that such differences—between first-generation molecules engineered differently or between recombinant versions of native proteins and their second-generation analogs—do matter. The U.S. Food and Drug Administration (FDA, Bethesda, MD) gave the first versions of human growth hormone (hGH) and erythropoietin (EPO) Orphan Drug status, closing off competition by guaranteeing market exclusivity to their producers. And, in a case involving Genentech (So. San Francisco, CA) and Burroughs Wellcome (London), a U.S. trial court said last spring that second-generation analogs of tissue plasminogen activator (t-PA) are not sufficiently different from the recombinant version of the native protein to avoid patent infringement. At stake here are shares of multi-hundred-million-dollar per year drug markets.

Legislators and industry have hotly debated amendments to the Orphan Drug Act over the past year (*Bio/Technology* 8:182, Mar. '90), but President Bush rejected a compromise bill passed by Congress in November. The t-PA trial result (*B/T* 8:385, May '90) is scheduled for a hearing on appeal this month. And the FDA is currently grappling with the Orphan Drug issue as a competitive version of EPO awaits approval. Against this backdrop, the Institute of Medicine (IOM) of the National Academy of Sciences (NAS, Washington, DC), at the request of the FDA, convened a November symposium on Microheterogeneity of Macromolecules. In the words of chairman James Wyngaarden, associate vice chancellor for health affairs at Duke University (Durham, NC) and foreign secretary for the NAS and IOM, its purpose was to take "a step back before critical decisions are made about what constitutes a new agent to look at the scientific basis for such an issue."

Historically, Wyngaarden noted, drug developers, to avoid patent infringement (and more recently, the provisions of the 1982 Orphan Drug Act), have modified molecules slightly, while retaining their desired effect. Any difference in the chemical structure of a small molecule drug's active moiety, whether or not that difference altered its clinical effect, was sufficient to classify it as a new molecular entity. As applied to larger macromolecules, however, these issues become more complicated: minimal heterogeneities may exist in a portion of a protein that is far removed from its active site; changes in the transcription of the gene may arise from minor changes in process, not from differences in genetic structure; and criteria for determining the significance of differences in post-translational modifications must be established.

In effect, each molecule in a recombinant protein or peptide product exists in a population of closely related but not identical structures. "Is it enough," Wyngaarden asked the audience at the outset, "to have a small structural change in some silent part of the molecule when one can demonstrate no difference in pharmacological properties or in clinical activity?" One possibility would be to treat macromolecules encoded by the same structural gene as identical. Post-translational modifications could still make a difference in activity. Or if post-translational modifications simply vary the number of residues without altering properties, is that a sufficient difference? Glycosylation of recombinant proteins—and the twin challenges of knowing what to look for and how to test for it—thus become paramount considerations here.

For some proteins, noted Charles Gooch of Stanford University (Palo Alto, CA), site variability and site occupancy, as well as variabilities in branch structure and N-terminal processing, have definite effects on activity. EPO, for example, has three N-linked structures and one O-linked structure that compose almost 50 percent (by weight) of the mature glycoprotein. "Removing terminal neuraminic acid," said Gooch, "reduces solubility, increases susceptibility to protease digestion, decreases thermal stability, increases specific activity *in vitro*, and increases clearance rate *in vivo*." During attempts to amplify EPO in CHO cells, he observed, one clone made EPO with a preponderance of bi-antennary structures instead of the usual predominance of tetra-antennary structures. And with t-PA, he contin-

ued, variable site occupancy "can also affect *in vitro* activity, as has been demonstrated in the difference in specific activity between type one and type two.... There are now many glycoproteins for which it has been demonstrated that removal of their oligosaccharide structures or production in the absence of their oligosaccharide structures affects their *in vitro* activity."

Yet such observations do not, by themselves, help regulators establish guidelines. Ken Seamon of the FDA Center for Biologics Evaluation and Research division of biochemistry and biophysics pointed out that "Clinical efficacy is really the most crucial part of the evaluation of the given product because, if we cannot determine what the most heterogeneous aspect is of the product, we will not be able to determine the most important testing to apply to that product." Heterogeneities intrinsic to the expression system exist: glycosylation, the presence of N-formyl methionine, different species due to proteolytic processing, truncated forms due to internal initiation. So do those due to production and purification, including deamidation, aggregation, and incomplete disulfide formation.

"Clinical efficacy will reflect the composite activities of all of the species," Seamon observed, "[including] not only the specific activity of the species at its particular target but also...its bioavaila-

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bility." Reviewing characterization data on the protein (biochemical and physical chemical analyses to determine the extent of heterogeneity), followed by determining the bioactivity of individual isolated forms of the protein (via appropriate *in vivo* or *in vitro* bioassays), accomplishes this. As well, researchers must be aware that clinical development may have changed heterogeneity, which would mandate clinical equivalency studies. The goal, said Seamon, is to define what critical heterogeneity is important to the efficacy of the product, "and to design an appropriate analytical test to insure that the heterogeneity is produced consistently between lots." ■