methods of fixation performed on frozen tissue sections, paraffin-embedded sections, vibratomed fresh sections and intact cultured tumour cells.

Szego's comment regarding the equivalence of paraffin-embedded materials to those discussed in our report is an oversimplification. The immunocytochemical literature contains ample evidence that many antigens easily localized in frozen or fresh tissues are obscured by the more extensive fixation and processing required for the preparation of paraffin blocks[7,8]. We have already shown[3] that extended fixation of oestrogen-receptor-containing tissues results in a progressive loss of nuclear staining for the receptor, with no change in the nonspecific extranuclear staining. With regard to the question of whether oestrogen receptors are located in or on the nucleus, recent electron micrographs (in which the ultrastructure is well preserved) show receptor localized in the chromatin and not on the nuclear (or other) membrane[9]. We cannot rule out the possibility that low levels of membrane-bound or diffuse cytoplasmic receptor have been missed by the immunoperoxidase method used, especially at the light microscopic level.

Finally, it is important to note that our findings are consistent with the hypothesis[1] that interaction of receptor with steroid induces the formation of a complex that binds more tightly to the nucleus and that this 'activated' steroid-receptor complex is responsible for the biological effects of the hormone.

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GORSKI REPLIES—In our original paper[1], we did not comment on membrane localization of steroid receptors, but in our review article[2] we pointed out that the cytoplasts contain intact cell membranes yet do not contain appreciable concentrations of oestrogen receptors. The cytoplasts prepared from GH_3 cells contain normal concentrations of cytosolic marker proteins, exclude dyes and can incorporate labelled amino acids into proteins and specifically prolactin.

These protein synthetic activities require transport of, for example, amino acids and energy sources, which we assume requires a reasonably normal membrane, and the dye exclusion also suggests an intact membrane. Our observations plus those of King and Greene[3] and more recently McClellan *et al.*[4] provide no support for a membrane-localized receptor. I believe that there is no substantive support in the literature for membrane localization of steroid receptors.

Finally, the concern of Szego and Pietras about steroids getting into cells is surprising. They readily get out of the cells where they are synthesized and they readily pass through many layers of cells during tissue incubations. There is nothing in most normal cells that one would imagine to be an effective barrier to diffusion of steroids unless one conceptualizes a cell as being simply a lipid sack surrounding a drop of water.

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Mobility and evolutionary variability factors in protein antigenicity

IN a recent *Nature* article[1], a correlation was made between segmental mobility and the location of continuous antigenic determinants in proteins. Temperature factors obtained from X-ray crystallographic data were used as a measure of local conformational flexibility for three proteins, namely, the tobacco mosaic virus (TMV) protein, myoglobin and lysozyme. The antigenic sites had deen deduced previously from a number of studies that examined the binding of peptides to anti-protein antibodies.

Can generalizations about the total antigenicity of a protein be made from studies that rely heavily on peptide binding to detect antigenic sites in proteins? In assessing the binding of antibodies to a protein, it seems reasonable that regions of local flexibility are more likely to be mimicked by linear peptides than are regions that are more rigid. A peptide adopts an ensemble of conformational states in solution, some of which may be close to the conformation of that sequence as it appears in the native antigen. Thus, some peptide molecules may be induced into a conformation that fits an antibody-combining site. Studies in which peptides are used to identify antigenic sites, are, therefore, biased towards detecting epitopes that are flexible. As Westhof *et al.* pointed out, it is possible that the anti-protein antibodies that bind peptides do not represent all of the antibodies present in the antisera in the studies they cited[1].

Another method that is used extensively to study protein antigenicity involves comparison of evolutionarily related proteins such as lysozyme, myoglobin and cytochrome *c* in fine specificity studies[2]. Generally, the results of this type of analysis have shown a correlation between antigenicity and evolutionary variability[3,4]. The detection of antigenic sites by such fine specificity studies, however, may be biased towards detecting antigenicity in regions of evolutionary variability. Naturally occurring amino-acid substitutions within protein families are likely to occur in regions of polypeptide chain flexibility as natural selection clearly favours those mutations that do not either perturb the overall tertiary structure of a protein or reduce its functional capacity. Surfaces of close contact within the packed secondary structure in the interior of a protein are likely to be both inflexible and structurally conserved evolutionarily. In contrast, regions of the polypeptide chain in which changes in local conformation arising from point mutations can be tolerated are most likely to be on the surface, where they are not involved in the long-range interactions that stabilize the internal folding of the molecule, and, hence, are more likely to be flexible. It is probable that antigenicity, polypeptide chain flexibility and evolutionary variability are related. Therefore, correlations that have been made between atomic mobility in proteins and antigenic sites for anti-protein antibody responses[1,5,6], although significant, may be explained by factors other than an inherent ability of flexible regions to invoke an immune response.

A complication that arises in correlating published data on the antigenicity of proteins with their molecular mobility is that many protein antigens that have been studied have a high degree of homology with the host animal's protein. The TMV protein cited in ref. 1 is an exception. In general, antibodies elicited in a particular host appear to arise in response to regions of the immunogen that differ in amino-acid sequence from the homologous host protein[2-4]. This phenomenon derives from immunological tolerance[7] and is particularly well documented for cytochrome *c*, but it also seems to hold for myoglobin, insulin, and other well-characterized protein antigens in mammalian hosts[2]. Certain sites on a molecule may not elicit an immune response even though they are flexible, if B cells reactive to these regions are not present as a result of either tolerance[7] or possible evolutionary effects on the germline repertoire[8].

Existing methodologies for studying antigenic sites cannot completely deter-