from the brain and the transformant. Even at the equivalent of 100 gene copies, there was no detectable signal in the normal DNA, but the transformant band hybridized strongly with the probe (Fig. 4A, lane d). Using these conditions, we compared DNA's from primary transformants and the parent 3T3 cells, and tumor X and the brain from the same animal. As we reported earlier (*2*), in the transformants the activated K-*ras* oncogene was amplified three to five times. It showed a strong band of 1.6 kb (Fig. 4B, lanes d and e). The appearance of the band could not be due to the increased number of normal gene copies, because even 100 copies of the normal gene gave no signal under these conditions. More important, we obtained evidence that the mutation in the transformant (Fig. 3) was also present in the original tumor from which it was obtained (Fig. 4B, lane g). As expected from the sequence in Fig. 3, DNA from the brain of the same animal did not hybridize under these conditions. The lower bands appearing in all lanes with genomic DNA's (Fig. 4B, lanes c to g) showed that approximately equal amounts of DNA were loaded in each track.

The presence of the mutation in DNA obtained directly from tumor X rules out the possibility of a mutation during DNA-mediated gene transfer (*15*). Its absence from brain DNA again confirms that the mutation was not genetically inherited but arose de novo in the tumor. Two out of the other three remaining positive tumors had the same mutation G → A in the second base of the 12th codon (data not shown). We have not yet determined the alteration in the fourth positive tumor.

Genes of the *ras* family activated by somatic point mutations have been reported in a variety of human tumors (*6, 9, 10*), and it has been possible to link a genetic alteration in human tumors with a functional assay that transforms cells to the oncogenic phenotype. Ionizing radiation causes the formation of strand breaks in DNA, the release of bases from the DNA backbone, and modification of the bases themselves (*16*). The carcinogenic effect of the radiation is thought to be due to these DNA alterations or to the subsequent DNA repair mechanisms. Radiation has long been implicated in the generation of tumors in humans and experimental animals (*17*). We have now shown that at least three radiation-induced lymphomas have in their DNA's a point mutation that is able to activate normal *ras* genes so that they can transform rodent fibroblasts into tumorigenic cells. This does not demonstrate that *ras* activation is the primary event in radiation-induced lymphomas, but it raises that possibility. That viruses are not the driving force in our system was shown by Mayer and Dorsch-Hasler (*18*).

Similar point mutations are associated with many human tumors and carcinogen-induced malignancies in experimental animals (*2, 3, 19*), and our results suggest a means by which it might be possible to study the association between point mutations and K-*ras* activation in kinetic experiments involving analysis of bone marrow and thymic cells at different times after tumor induction.

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A Novel Nuclear Form of Estradiol Receptor in MCF-7 Human Breast Cancer Cells

Abstract. *Nuclear estrogen receptor from MCF-7 cells undergoes a time-dependent, hormone-inducible transformation to a form that is less extractable from nuclei and less exchangeable with ligand. This receptor-modifying, intranuclear event is independent of receptor loss (processing) and appears associated with hormone responsiveness (progesterone-receptor induction) in these cells. The magnitude of receptor loss, however, is variable and apparently not a prerequisite for hormone action to induce progesterone receptor.*

After treatment of MCF-7 human breast cancer cells with estradiol (E_2), translocation of estradiol-receptor complexes (ER) is followed by processing, a time-dependent irreversible decline in the amount of nuclear ER (*1*). Within 3 to 5 hours, the number of ER's has fallen by about 70 percent, and a new steady-state level is reached. Receptor processing in MCF-7 cells correlates with estrogen induction of progesterone receptor (PR) (*2*). A similar depletion in receptor sites is seen in rat uterine cells (*3*). Processing appears to be a prerequisite for estrogen stimulation since it is either impaired or blocked entirely in the presence of antiestrogens. R_3, an estrogen-independent variant subline of MCF-7, shows no receptor processing, does not respond to estrogens, and has no detectable PR inducible by E_2 (*1, 4*). The molecular mechanism and the precise correlation of this event with hormone-regulated effect in MCF-7 cells remains

obscure. Whether the receptor is degraded or modified and therefore incapable of binding or rebinding hormone is unknown.

We investigated the mechanism of this intranuclear regulatory event by examining time-dependent, hormone-inducible, quantitative, and molecular changes in nuclear ER in MCF-7 cells. We now report that a modification of nuclear ER to a tightly bound, less exchangeable form with distinct molecular alterations occurs after continued exposure of cells to the hormone. Using an antibody to the receptor, we show that the modification occurs in the receptor itself and does not represent ligand transfer to a different molecule. Further, both the receptor-modifying events and estrogen response (as measured by PR induction) appear to be independent of ER processing, which may occur concomitantly.

Previous studies have shown that ER-binding activity in MCF-7 cells is dependent on a variety of factors, including cell density, insulin concentration, and the various growth components in the cell-conditioned medium (*5*). After varying these factors to adjust the initial ER content to either a high or low intracellular concentration, we measured total cellular high-affinity E_2 binding in intact MCF-7 cells previously exposed to [^{3}H]E_2 for 30 minutes or continuously for 5 hours. At low density and in the absence of conditioned medium, the initial total concentration of cellular ER was high, and the apparent loss of receptor from cells continuously stimulated with E_2 for 5 hours was 45 to 72 percent (Table 1), in agreement with previous results (*1, 2*). Medium conditioned by MCF-7 cells was diluted 1:3 with fresh medium and added to cells grown to high density, reducing the initial cellular ER content by about 2.5-fold (Table 1). Under these conditions, E_2-mediated loss of nuclear ER was generally only 19 to 27 percent (Table 1) and was sometimes absent. Thus, both the initial total measurable receptor content of the cell and the time-dependent loss of ER were variable.

We wondered whether the quantitative loss of ER was a prerequisite for hormone action. Estrogen-inducible PR synthesis was examined under conditions designed either to maximize or to minimize E_2-induced quantitative loss of nuclear ER. We found no correlation between the PR induction and the extent of ER loss associated with 5 hours of stimulation with E_2. Amounts of basal cellular PR typically varied by a factor of 2 or 3 and probably reflected differences in growth conditions and low concentra-

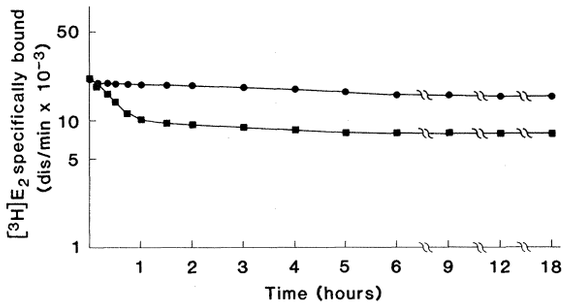

Fig. 1. Dissociation of ^{3}H-labeled estradiol ([^{3}H]E_2) from unprocessed and processed nuclear estradiol-receptor complexes (ER). MCF-7 cells were incubated for 30 minutes (unprocessed ER, ■) or 6 hours (processed ER, ●) at 37°C with [^{3}H]E_2 (5 n*M*) with or without unlabeled E_2 (1 μ*M*). Cells were washed in phosphate-buffered saline (PBS), resuspended in TEM buffer (10 m*M* tris-HCl, 1.5 m*M* EDTA, and 10 m*M* monothioglycerol; *p*H 7.4), and homogenized in a glass Dounce homogenizer. The nuclear pellet was washed, resuspended in TEM buffer (*p*H 8.5) containing 600 m*M* KCl and 10 percent glycerol (by volume), and sonicated (three 5-second bursts at a setting of 2 with a 1-minute cooling interval). The sonicate was centrifuged at 105,000*g* for 30 minutes. The dissociation rate of the [^{3}H]E_2–receptor interaction in the nuclear extract (supernatant) was determined at 15°C after addition of 1000-fold molar excess of unlabeled E_2. Portions were removed and cooled to 0°C, and binding to ER was determined after incubation with HAP (*4*). Each point is corrected for nonspecific binding and receptor stability.

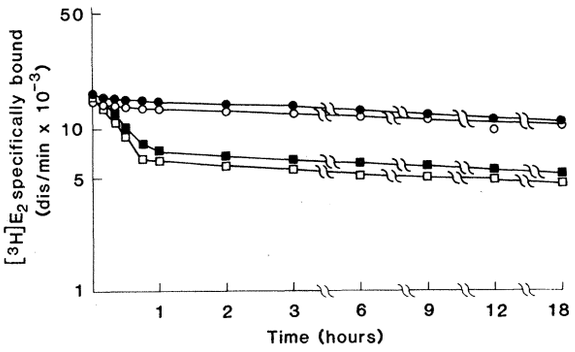

Fig. 2. Dissociation of [^{3}H]E_2 from unprocessed (30-minute treatment) and processed (6-hour treatment) nuclear ER. Details are similar to those described in the legend to Fig. 1 except that specific binding to ER was determined by the protamine sulphate precipitation method (*1*) (unprocessed ER, ■; processed ER, ●) or by adsorption to dextran-coated charcoal (*4*) (unprocessed, □; processed, ○).

Table 1. Total concentrations of cellular estradiol-receptor (ER) and progesterone receptor (PR) in MCF-7 cells grown under different conditions. Cells were replicately plated in plastic dishes (six-well) at a density of 6×10^4 cells per well (low density) or 5×10^5 cells per well (high density) in improved minimal essential medium supplemented with 5 percent dextran-charcoal–stripped serum and 0.02 unit of porcine insulin per milliliter. Medium conditioned by MCF-7 cells for 5 days was diluted 1:3 and added to the high-density cells. Two days after plating, monolayer cells were incubated at 37°C for 30 minutes or 5 hours with different concentrations (0.1 to 5 n*M*) of [2,4,6,7-^{3}H]E_2-17 β (91 to 115 Ci/mmol; New England Nuclear) with or without 1 μ*M* unlabeled E_2. Cells were washed three times with ice-cold PBS (*p*H 7.4) and harvested by adding 2 ml of PBS containing 0.02 percent EDTA. Portions of cell suspensions were taken for cell counting and measurement of the amount of label. In a modified whole-cell binding assay, cells were washed with cold PBS and digested with 1 ml of 1*N* NaOH at 60°C for 1 hour. The NaOH was neutralized and the entire sample counted. The number of cells in each experiment was determined from parallel cultures. Total amount of cellular E_2-specific binding sites was determined by Scatchard analysis with a computer-assisted program (*12*). For the induction of PR, cells were treated 15 to 16 hours after plating with 10 n*M* unlabeled E_2 for 2 to 3 days. The amount of PR was measured by either a single-dose assay with 5 n*M* ^{3}H-labeled R5020 (87 Ci/mmol, New England Nuclear) or Scatchard analysis with 0.1 to 5 n*M* ^{3}H-labeled R5020 in the absence or presence of 1 μ*M* unlabeled R5020. Cortisol (1 μ*M*) was added to all mixtures before incubation.

Basal cellular ER* (site/cell × 10^3)	ER lost during processing (percent)	Basal cellular PR (site per cell × 10^3)	Increase in PR induction
	Cells at high density with conditioned medium		
38	21	47.8	1.6
52	27	36	4.8
86	19.4	17.2	19.1
	Cells at low density without conditioned medium		
98.3	72.1	47.6	2.1
200†	55.5	72	3.4
109	45.7	41.6	10.5

*Cells harvested by PBS and 0.02 percent EDTA. †Cells harvested by digestion with NaOH.

tions of unextracted estrogens. We found almost the same extent of PR induction regardless of the degree of quantitative loss of ER (Table 1). Therefore, PR induction can take place in the absence of extensive ER loss.

We next examined E_2-inducible molecular changes in nuclear ER that might be coupled with hormonal responsiveness. Preliminary experiments showed that, during a 6-hour exposure of MCF-7 cells to [^{3}H]E_2, nuclear estrogen binding became more refractory to extraction into KCl but not if the extraction was performed with sonication (Table 2). Second, we found that receptor released by sonication after a 6-hour exposure to unlabeled E_2 showed a decreased ability to exchange E_2 for [^{3}H]E_2. Both of these changes may have contributed to the decrease in measurable ER binding during processing that was reported earlier (*1, 2*). We compared the dissociation rates of the ER complexes in the unprocessed and processed states. The dissociation of [^{3}H]E_2 from unprocessed receptor was biphasic (Fig. 1), with rate constants (k) of about 8.2×10^{-2} min^{-1} ($t_{1/2}$, ~8.5 minutes) and 3.2×10^{-3} min^{-1} ($t_{1/2}$, ~216 minutes). In contrast, the dissociation of E_2 from the processed receptor was slow and monophasic (k, $\sim 3.4 \times 10^{-3}$ min^{-1}; $t_{1/2}$, ~200 minutes). Similar differences in the binding properties of unprocessed and processed ER were obtained when the degree of dissociation of [^{3}H]E_2 was determined by initially immobilizing ER on hydroxyapatite (HAP) or when nuclear ER was assayed by protamine sulfate precipitation or the dextran-coated charcoal technique (*4*) (Fig. 2).

In the above experiments, total loss of ER sites after 5 to 6 hours of E_2 treatment and measured by labeling and salt extraction of nuclear ER in vivo in combination with sonication was only 10 to 15 percent. We also determined the dissociation rates of ER from subconfluent MCF-7 cells growing in the absence of conditioned medium (which results in high initial concentrations of intracellular receptor and extensive loss of receptor). The greater degree of ER loss (2.47 pmol per milligram of DNA 30 minutes after addition of E_2 compared to 1.34 pmol/mg 5 hours after addition) did not affect this qualitative change in dissociation kinetics (Fig. 3). The hormone-induced transition to a slowly dissociating state appears to be compatible with the decreased exchangeability or rebinding characteristics of processed nuclear ER and may result in the tighter binding of ER to chromatin. An analogous shift from a rapidly dissociating state to a slowly dissociating state of cytoplasmic ER occurs with the conversion of nonactivated to activated ER (*6*).

Processed nuclear ER was physically distinguishable on a sucrose density gradient. The receptor underwent a conversion from 4.4*S* to 5*S* that was associated with processing (Fig. 4a). Because the

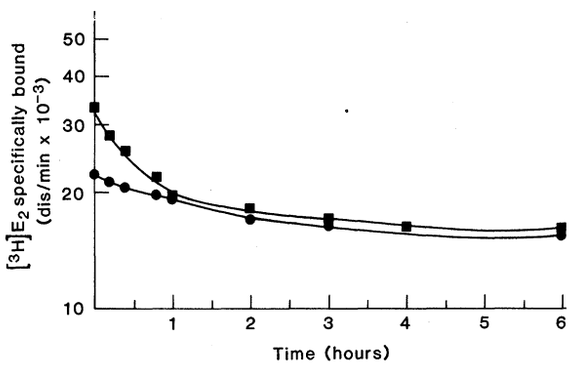

Fig. 3. Dissociation of [^{3}H]E_2 from unprocessed (30-minute treatment, ■) and processed (5-hour treatment, ●) nuclear ER obtained from MCF-7 cells grown at low density without insulin or conditioned medium (which results in maximum loss of measurable ER over time).

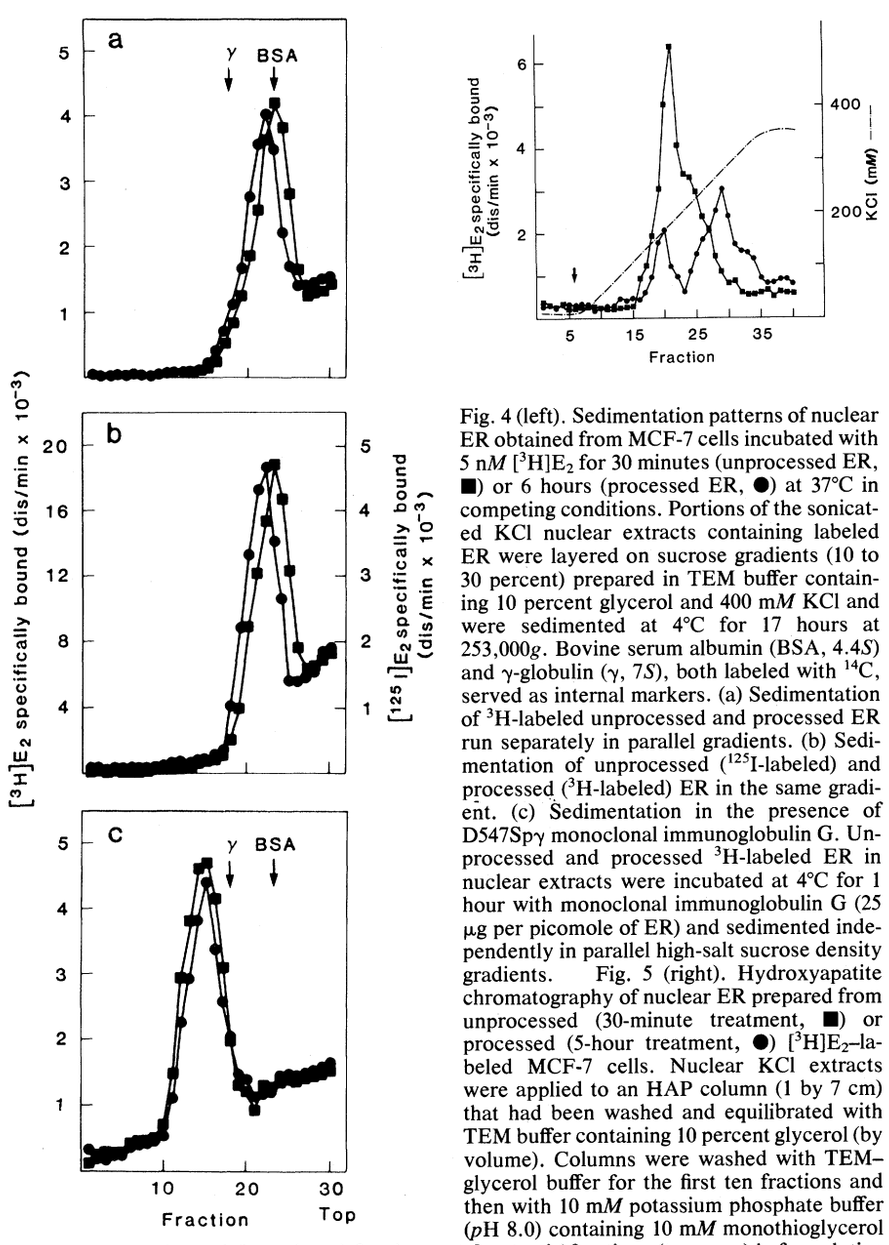

Fig. 4 (left). Sedimentation patterns of nuclear ER obtained from MCF-7 cells incubated with 5 n*M* [^{3}H]E_2 for 30 minutes (unprocessed ER, ■) or 6 hours (processed ER, ●) at 37°C in competing conditions. Portions of the sonicated KCl nuclear extracts containing labeled ER were layered on sucrose gradients (10 to 30 percent) prepared in TEM buffer containing 10 percent glycerol and 400 m*M* KCl and were sedimented at 4°C for 17 hours at 253,000*g*. Bovine serum albumin (BSA, 4.4*S*) and γ-globulin (γ, 7*S*), both labeled with ^{14}C, served as internal markers. (a) Sedimentation of ^{3}H-labeled unprocessed and processed ER run separately in parallel gradients. (b) Sedimentation of unprocessed (^{125}I-labeled) and processed (^{3}H-labeled) ER in the same gradient. (c) Sedimentation in the presence of D547Spγ monoclonal immunoglobulin G. Unprocessed and processed ^{3}H-labeled ER in nuclear extracts were incubated at 4°C for 1 hour with monoclonal immunoglobulin G (25 μg per picomole of ER) and sedimented independently in parallel high-salt sucrose density gradients. Fig. 5 (right). Hydroxyapatite chromatography of nuclear ER prepared from unprocessed (30-minute treatment, ■) or processed (5-hour treatment, ●) [^{3}H]E_2–labeled MCF-7 cells. Nuclear KCl extracts were applied to an HAP column (1 by 7 cm) that had been washed and equilibrated with TEM buffer containing 10 percent glycerol (by volume). Columns were washed with TEM–glycerol buffer for the first ten fractions and then with 10 m*M* potassium phosphate buffer (*p*H 8.0) containing 10 m*M* monothioglycerol and 10 percent glycerol (by volume) for the next five to six fractions (to arrow) before eluting the extracts on a linear gradient of 10 m*M* to 400 m*M* potassium phosphate buffer.

difference in sedimentation was small, we performed a mixing experiment in which nuclear extracts containing unprocessed and processed ER labeled with ^3H or ^{125}I were mixed and sedimented together. Receptor treated for 6 hours with E_2 sedimented more rapidly than that treated for 30 minutes (Fig. 4b). We also reacted nuclear ER with a monoclonal antibody (D547Spγ) to receptor from MCF-7 cells (*7*) and analyzed the receptor-antibody complexes on sucrose density gradients. With both receptor forms, the receptor-antibody complexes were shifted to 8.8*S* (Fig. 4c), suggesting that in both cases E_2 was bound to putative ER. We conclude that the physical changes reported occurred in ER and do not represent transfer of the ligand to some alternative nuclear moiety. Receptor ionic properties during processing were further examined by HAP chromatography. Most of the ER labeled for 30 minutes eluted as a single peak at 170 to 180 m*M* potassium phosphate (Fig. 5). In contrast, processed receptor gave two peaks, one at 180 m*M* and one at 280 m*M* potassium phosphate. Peak fractions from both receptor forms showed appreciable (19 to 26 percent) binding to DNA-cellulose (data not shown).

We have thus described a hormone-inducible, intranuclear receptor-processing event (or events) that results in the transformation of nuclear ER to a less exchangeable, slowly dissociating form of receptor. This receptor form is separable from remaining nuclear ER by sucrose gradients and HAP chromatography. While most models of estrogen action are based on obligatory transformation of receptor from 4*S* to 5*S* for mediation of nuclear entry, our results in conjunction with those of other studies (*8*) show that, at least in MCF-7 cells, this transformation is a nuclear process. Further, we have observed a contemporaneous nuclear event associated with receptor processing. After 5 to 6 hours of perfusion with E_2 a rapidly dissociating population of ER is generated, with a subsequent true loss of binding associated exclusively with these sites (*9*). The question now arises as to which of these processing events takes part in initiating hormone action. We believe that true ER loss and receptor modifications may occur simultaneously during processing, since the two events are temporally related. While the quantitative loss of ER is variable and dependent on a variety of growth factors, estrogen responsiveness (as manifested by the induction of PR) is equivalent under all conditions. Therefore we suggest, as have others (*10*), that there is no direct correlation between the extent of ER processing and PR induction in MCF-7 cells. Further, in rat uterus the total concentration of cellular ER is quantitatively conserved after continuous availability of E_2 to rats for 6 hours; within the same period, hormone-induced biochemical and immunological changes occur in both cytoplasmic and nuclear receptor (*11*). Thus, quantitative changes in receptor content may not be directly related to the onset of hormone action. The changes in molecular characteristics of nuclear ER that we report are invariant and independent of receptor loss. Elucidation of the functional significance of these receptor modifications in eliciting specific changes in gene expression will require further experimentation.

Table 2. Effect of assay conditions on concentrations of nuclear estradiol-receptor complexes (ER). MCF-7 cells were treated with 3 n*M* [^{3}H]E_2 (direct assay) or 3 n*M* unlabeled E_2 (exchange assay) for 1 or 6 hours under competing conditions at 37°C. Cells were harvested and washed, and nuclear extracts were prepared by either sonication or Dounce homogenization. Extracted receptor was bound to HAP and measured in two ways. In the direct-binding assay, the amount of specific ER was determined by subtracting nonspecific binding from the total binding. In the exchange assay, ER bound to HAP was incubated at 30°C for 3 hours with 3 n*M* [^{3}H]E_2 plus 300 n*M* diethylstilbestrol to estimate the degree of nonspecific binding.

Time of hormone exposure (hours)	Assay technique	Receptor concentration (picomoles per milligram of DNA)
	Dounce homogenization	
1	Direct	1.61
6	Direct	1.16
	Sonication	
1	Direct	1.98
6	Direct	2.02
1	Direct	4.9
6	Direct	4.6
1	Exchange	3.9
6	Exchange	2.1

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Bimolane: Structure Determination Indicates Anticancer Activity Is Attributable to ICRF-154

Abstract. *X-ray diffraction studies of crystals from samples of bimolane synthesized in China and in the United States showed that the crystals consist of the related compound ICRF-154. Analysis of the results of biological tests did not show any significant differences between the anticancer activity of bimolane and ICRF-154. It appears that the anticancer activity of bimolane is due to ICRF-154.*

The synthetic diketopiperazine derivative bimolane (Fig. 1a) has been reported to have a high chemotherapeutic index against various malignant tumors in lengthy clinical trials in China (*1*). These results have generated considerable interest (*2*), culminating in contractual syntheses, in vitro testing, and wide distribution of bimolane for biological tests by the National Cancer Institute (NSC 351358). We have been interested in correlating stereochemistry and biological efficacy in diketopiperazines (*3*) and have investigated the structure of crystals obtained from samples of bimolane. The structure determination showed the