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# Active domains in wild-type and mutant glucocorticoid receptors

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**[$^3$H]Triamcinolone acetonide was used to tag covalently specific glucocorticoid receptors by photoaffinity labelling at $\lambda \geq 320$ nm. Receptors of wild-type mouse lymphoma cells and two glucocorticoid resistant mutants of "nuclear transfer deficient" ($nt^-$) and "increased nuclear transfer" ($nt^i$) phenotypes, respectively, were used. Wild-type and $nt^-$ receptors yielded radiolabelled polypeptide bands of mol. wt. 98 000 as revealed by gel electrophoresis under denaturing conditions and fluorography. In contrast, the $nt^i$ receptor had a mol. wt. of 42 000. Partial proteolysis of the wild-type receptor with α-chymotrypsin resulted in a fragment of mol. wt. 39 000 which still contained the steroid binding site but had increased affinity for DNA indistinguishable from that of the $nt^i$ receptor. Chymotrypsin thus removed a domain from the wild-type receptor polypeptide which is involved in modulating DNA binding. The same domain is missing from the $nt^i$ receptor.**

*Key words:* active domains/glucocorticoid receptors/ mouse lymphoma cells/partial proteolysis/photoaffinity labelling

## Introduction

Cells responsive to steroid hormones contain specific receptors with high affinity for their respective hormone ligands (for reviews, see Gorski and Gannon, 1976; Yamamoto and Alberts, 1976; Higgins and Gehring, 1978; Katzenellenbogen, 1980). The essential role of such receptors in eliciting hormone effects has become particularly obvious through the isolation of unresponsive mutants from glucocorticoid-sensitive mouse lymphoma cells grown in culture (Yamamoto *et al.* 1976; Gehring, 1980b; Bourgeois and Newby, 1980). In addition to those containing negligible glucocorticoid binding activities, two types of mutants have been identified in which roughly normal glucocorticoid binding is seen, but interaction of receptor-steroid complexes with nuclei, chromatin, or DNA is abnormal. Receptors of the "nuclear transfer deficient" ($nt^-$) type are defective in nuclear binding while those of the "increased nuclear transfer" ($nt^i$) type exhibit increased nuclear binding and abnormally high affinity for DNA. The wild-type receptor therefore has to be regarded as a molecular entity which comprises at least two active domains: one for steroid binding and the other for nuclear interaction (Gehring and Tomkins, 1974; Gehring, 1980b).

We have covalently labelled wild-type and mutant receptors by photoactivation of their complexes with a radioactive glucocorticoid of high affinity. This enabled us to analyse crude receptor preparations by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Since the $nt^i$ receptor is of lower mol. wt. than the wild-type, we compared native and chymotrypsin treated wild-type receptors with those of the $nt^i$ type.

## Results

### *Photoaffinity labelling*

Irradiation of crude cytosol receptor complexes with [$^3$H]-triamcinolone acetonide resulted in covalent attachment of the ligand to the extent of ~2% as revealed by precipitation with trichloroacetic acid (TCA). However, when receptor-triamcinolone complexes were chromatographed on DNA- or DEAE-cellulose prior to irradiation, cross-linking was increased to 8 – 12%. Maximum covalent binding was obtained after 45 min of irradiation. SDS gel electrophoresis followed by fluorography revealed a single labelled polypeptide band for wild-type receptors of mouse lymphomas WEHI-7 and S49.1 (Figure 1). The mol. wt. corresponded to 98 000 ± 5000. When excess unlabelled glucocorticoid was added throughout the experimental procedure this band was not detected (data not shown). Minor labelled bands of mol. wts. 62 000, 47 000, 44 000, 30 000, and 23 000 (totalling <30%) were seen in addition to the 98 000-dalton polypeptide in some experiments in which cytosols had been stored overnight in the cold. These are presumably due to partial proteolysis.

### *Activated and non-activated receptor forms*

For steroid-receptor complexes to interact with nuclei, chromatin, or DNA, they need to be activated. Activation can, for example, be achieved by warming receptor complexes to 20°C, by treatment with high salt, or by diluting receptor-containing cytosol preparations (Higgins and Gehring, 1978). Sodium molybdate prevents activation of glucocorticoid-receptor complexes (Leach *et al.*, 1979; Schmidt *et al.*, 1980) and activated and non-activated receptor forms can be separated by chromatography on DEAE-cellulose (Sakaue and Thompson, 1977). We have used these techniques to investigate whether activation involves a change in the basic polypeptide structure of glucocorticoid receptors of lymphoma cells. Within the resolution of the SDS gel electrophoresis we found polypeptides of the same size cross-linked with radiolabelled triamcinolone acetonide independent of whether we subjected the activated or non-activated receptor forms to photolabelling (Figure 1B and C).

### *Mutant receptors*

When receptors of the $nt^-$ type were investigated by photoaffinity labelling a mol. wt. of ~98 000 (Figure 1E), indistinguishable from wild-type, was found. In contrast, the $nt^i$ receptor revealed a major labelled band of mol. wt. 42 000 ± 3000 and a minor band of mol. wt. 39 000 (Figure 1F), but the minor band did not occur in all experiments.

Chromatography of temperature-activated receptor complexes on DNA-cellulose showed profiles similar to those previously published (Yamamoto *et al.*, 1976; Gehring, 1980a) with $nt^-$ receptors eluting at lower, and $nt^i$ receptors at higher, than normal salt concentrations (Table I). Interes-

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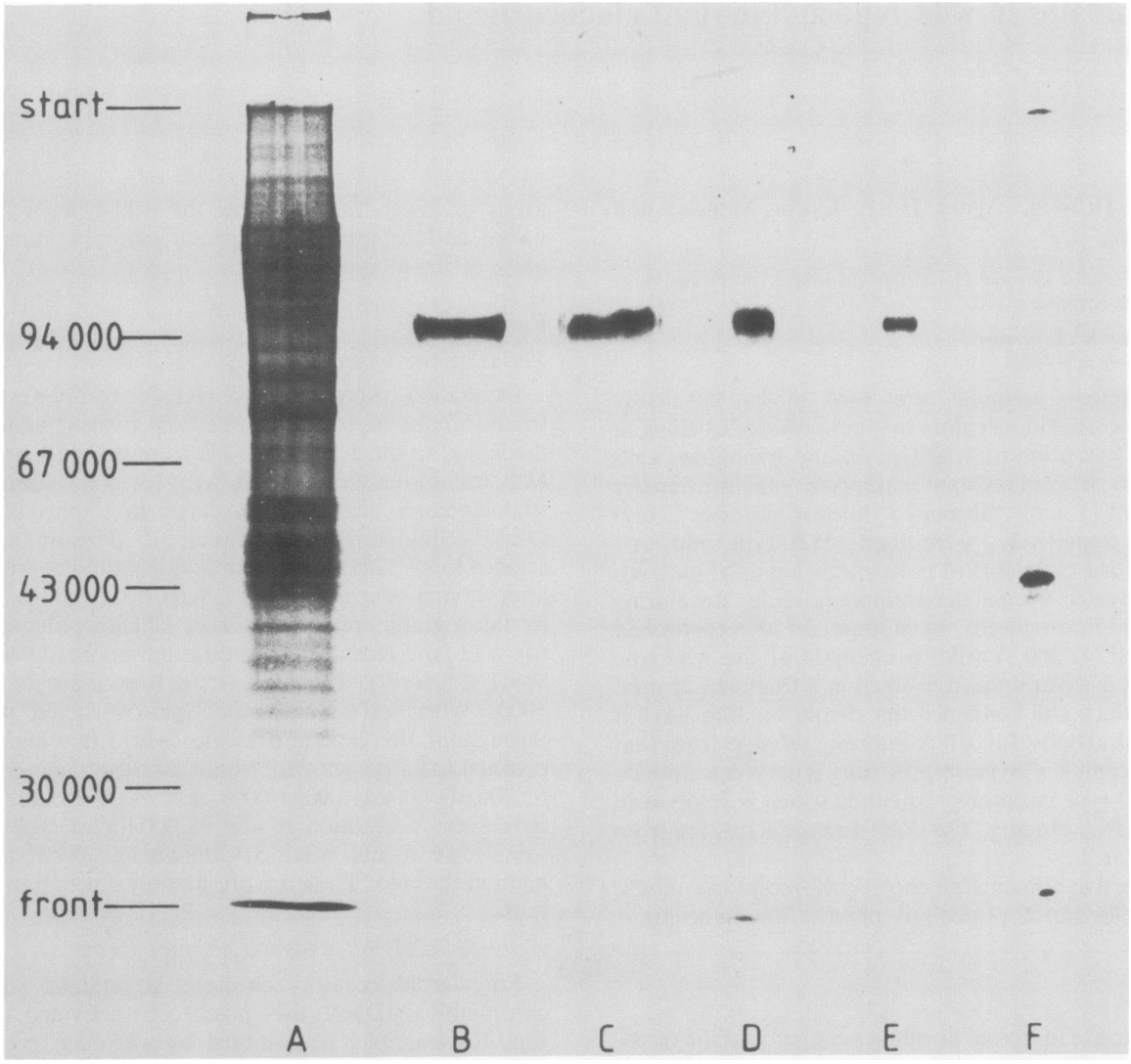


**Fig. 1.** SDS gel electrophoresis of photoaffinity labelled receptors. Cytosol receptor complexes with [$^3$H]triamcinolone acetonide were subjected to chromatography and photolabelling as described in Materials and methods. **A**: WEHI-7 receptor complex chromatographed on DNA-cellulose, gel stained with Coomassie blue. **B**: Fluorography of **A**. **C**: WEHI-7 receptor complex in the non-activated form chromatographed on DEAE-cellulose, fluorography. **D**: S49.1 wild-type receptor complex chromatographed as in **C**, fluorography. **E**: S49.1 nt$^-$ receptor complex chromatographed as in **C**, fluorography. **F**: S49.1 nt$^i$ receptor complex chromatographed as in **C**, fluorography. Positions of marker proteins are indicated by bars. "Start" denotes the interphase between stacking gel and separation gel, "Front" denotes the position of bromophenol blue. In several samples we detected small amounts of radioactivity at the origin and at the front. Material at the start is probably due to protein not completely dissolved in SDS buffer while radioactivity at the dye front is caused by residual free steroid not completely removed by acetone.

**Table I.** Chromatography of wild-type and mutant receptors

| Receptor type | DNA-cellulose (mM KCl required for elution) | | DEAE-cellulose (mM KCl required for elution) | | | |
|---|---|---|---|---|---|---|
| | | | before chymotrypsin | | after chymotrypsin | |
| | before chymotrypsin | after chymotrypsin | non-activated | activated | non-activated | activated |
| wild-type (WEHI-7) | 186 | 233 | 190 | 55 | 194 | 62 |
| wild-type (S49.1) | 175 | 237 | 188 | 63 | 190 | 59 |
| nt$^-$ (S49.1) | 86 | 87 | 185 | 57 | | |
| nt$^i$ (S49.1) | 225 | 229 | 192 | 56 | | |

Results are reported as means of 2–3 independent experiments.

tingly, chromatography on DEAE-cellulose did not reveal any differences between wild-type, nt$^-$, and nt$^i$ receptor types with respect to salt concentrations required for eluting activated and non-activated complexes (Table I).

*Partial proteolysis with α-chymotrypsin*

Previously we showed that partial proteolysis of receptors with α-chymotrypsin results in altered DNA binding properties (Andreasen and Gehring, 1981). We have now investigated the molecular sizes of these partially ingested receptors.

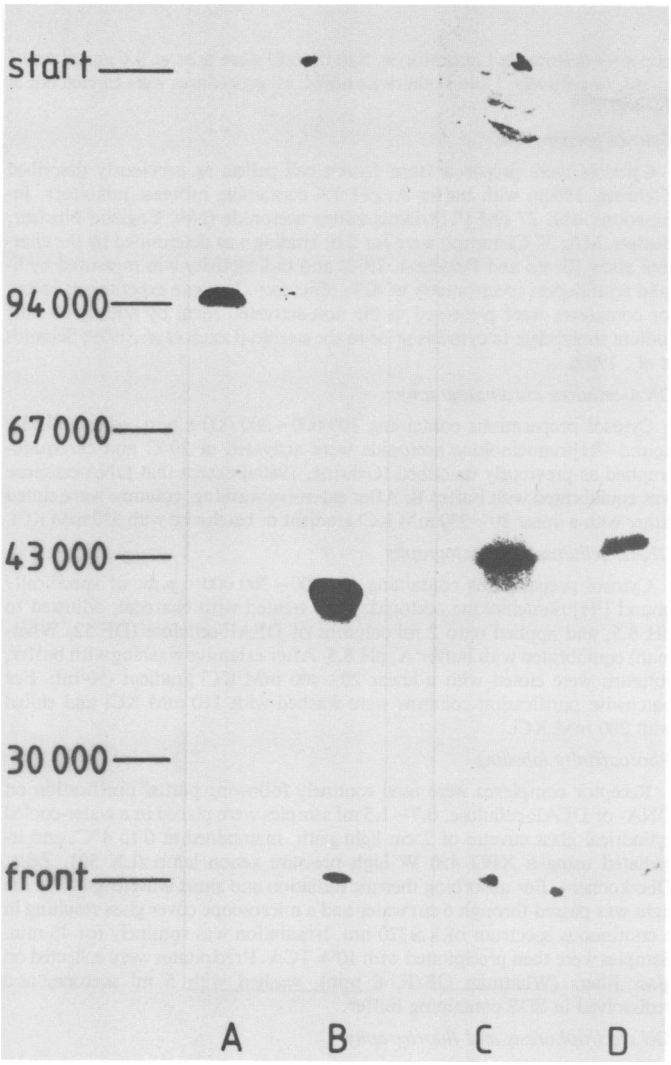


**Fig. 2.** SDS gel electrophoresis and fluorography of native and chymotrypsin-treated receptors. Cytosol receptor complexes with [$^3$H]triamcinolone acetonide in the non-activated form were chromatographed on DEAE-cellulose and photolabelled as described in Materials and methods. Samples were split and one half was treated with α-chymotrypsin (10 μg/ml) for 10 min at 0°C before acid precipitation and electrophoresis. **A**: S49.1 wild-type receptor. **B**: S49.1 wild-type receptor, treated with chymotrypsin. **C**: S49.1 nt$^i$ receptor. **D**: S49.1 nt$^i$ receptor, treated with chymotrypsin.

When the wild-type S49.1 receptor was treated with α-chymotrypsin, a labelled polypeptide of mol. wt. 39 000 ± 3000 was recovered (Figure 2B). This polypeptide band was observed whether protease treatment was performed before DEAE-cellulose chromatography and irradiation or after, as in Figure 2B. A chymotryptic fragment of similar size was also recovered from the nt$^-$ receptor (data not shown). However, chymotrypsin did not lower the mol. wt. of the nt$^i$ receptor (Figure 2D).

Native and chymotrypsin-treated receptors were also compared by chromatographic procedures. Upon treatment with α-chymotrypsin the wild-type receptor exhibited increased DNA binding properties indistinguishable from those of the nt$^i$ receptor (Figure 3, Table I), while nt$^-$ and nt$^i$ receptors retained their affinities for DNA. Chymotryptic digestion of wild-type receptors, however, did not change their chromatographic behaviour on DEAE-cellulose (Table I).

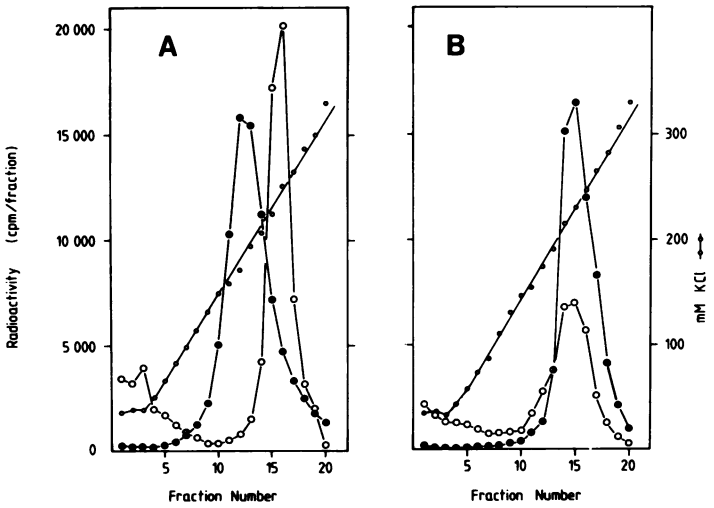


**Fig. 3.** DNA-cellulose chromatography. S49.1 wild-type (**A**) and S49.1 nt$^i$ (**B**) receptor complexes with [$^3$H]triamcinolone acetonide were activated at 20°C and chromatographed on DNA-cellulose either in the native state (●) or following a 10 min treatment with 10 μg/ml α-chymotrypsin at 0°C (○). Fractions of 15 drops (~800 μl) were collected.

## Discussion

Several recent investigations have used photoactivation of steroids, containing α,β-unsaturated ketones, for covalent linking to steroid metabolizing enzymes, binding proteins, and receptors (Benisek, 1977; Dure *et al.*. 1980; Gronemeyer and Pongs, 1980; Taylor *et al.*, 1980; Westphal *et al.*, 1981; Nordeen *et al.*, 1981). In the present paper we used the high-affinity glucocorticoid triamcinolone acetonide to label wild-type and mutant receptors covalently. In each case only one major polypeptide band was found to be radiolabelled as revealed by SDS gel electrophoresis and fluorography. The mol. wts. of 98 000 and 42 000 for wild-type and nt$^i$ receptors, respectively, compare quite well to those previously determined for the native receptors (Yamamoto *et al.*, 1976). Thus, glucocorticoid receptors are monomeric proteins.

While this work was in progress a paper by Nordeen *et al.* (1981) described photoaffinity labelling of wild-type and nt$^i$ receptors with the progesterone analogue promegestone (R5020). However, photolabelling of glucocorticoids with triamcinolone acetonide may be superior to the use of R5020 for the following reasons: (1) Due to the high affinity of triamcinolone acetonide, specific photoaffinity labelling of receptors can be done after removal of unbound ligand thus avoiding cross-linking to unspecific binding components. By comparison, R5020 binds weakly to glucocorticoid receptors (Raynaud, 1977; Lippman *et al.*, 1977; Nordeen *et al.*, 1981) and thus dissociates rapidly. (2) Yields of cross-linking are ~2-fold higher with the procedure described here with triamcinolone acetonide than with R5020 (Nordeen *et al.*, 1981). (3) R5020 had no detectable glucocorticoid effect on S49.1 lymphoma cells, even at 10 μM, and exhibited only slight anti-glucocorticoid activity at this concentration, in that it merely slowed the action of 10 nM triamcinolone acetonide (unpublished results). Similarly, in other cell types R5020 had at most a weak glucocorticoid activity (Raynaud, 1977; Lippman *et al.*, 1977).

Nordeen *et al.* (1981) report a mol. wt. of 87 000 ± 3000 for the wild-type receptor while our data suggest a mol. wt. of 98 000 ± 5000. This discrepancy is probably due to different

experimental procedures since both investigations are dealing with the same receptor molecule. Similarly, Nordeen *et al.* (1981) report a slightly lower mol. wt. for the $nt^i$ receptor: 39 000 ± 3000, as compared to 42 000 ± 3000 found in the present investigation.

It is of particular interest that the receptor of S49.1 $nt^i$ cells consists of a polypeptide which is much smaller than the wild-type receptor. Also in the P1798 mouse lymphoma system a mutant of the $nt^i$ type has been found (Stevens and Stevens, 1981). A similarly abridged molecule is obtained by partial proteolysis of wild-type receptors with chymotrypsin. Even though it is somewhat different from the $nt^i$ receptor in molecular size it has the same abnormally high affinity for DNA. Interestingly, the $nt^-$ receptor did not gain in affinity for DNA upon chymotrypsin treatment.

The present data suggest a molecular model for the wild-type receptor in which one polypeptide chain comprises three active domains: one for steroid binding; one for nuclear interaction; and a third domain which modulates nuclear interaction. Mild proteolysis with chymotrypsin removes that part of the polypeptide which contains the modulating domain and leaves the steroid binding and nuclear interaction domains together. Treatment with trypsin, however, yields a receptor fragment of even lower mol. wt. containing only the steroid binding domain (Wrange and Gustafsson, 1978; Stevens and Stevens, 1981). The sequential order of the three active domains along the 98 000 polypeptide chain will have to be determined in future experiments with purified receptors.

There are several possible explanations for the origin of the $nt^i$ mutant receptor: (1) a deletion mutation in the receptor gene might lead to synthesis of a molecule from which the modulating domain is missing; (2) a nonsense mutation might cause premature protein chain termination; and (3) substitution of an amino acid in a hinge region of the molecule might render it particularly susceptible to intracellular proteolysis. Unfortunately, we cannot distinguish among these alternatives at present. The possibility of overexpression of an endogenous protease that degrades the receptor can be ruled out because two distinct receptor types have been found in hybrids of $nt^i$ and wild-type or $nt^-$ cells (Yamamoto *et al.*, 1976; Gehring, 1979). Since antibodies raised against the rat liver glucocorticoid receptor react preferentially with that part of the receptor polypeptide which is cleaved off by chymotrypsin from wild-type or missing from $nt^i$ receptors (Okret *et al.*, 1981; Stevens *et al.*, 1981) it may be possible to use these antibodies to find out whether $nt^i$ cells contain such a receptor fragment. A positive reaction would support explanation (3), while a negative result would argue in favour of alternatives (1) or (2).

## Materials and methods

*Cell cultures*

The S49.1 mouse lymphoma sublines S49.1G.3 (wild-type), S49.1G.3.83R ($nt^-$ type), and S49.1TB.4.55R ($nt^i$ type) were those previously used (Gehring, 1979). The mouse lymphoma line WEHI-7 (Harris *et al.*, 1973) was kindly provided by A.W. Harris. Cells were grown and harvested as previously described (Gehring, 1980a) and stored at −90°C.

*Buffers and reagents*

Buffer A contained 20 mM potassium phosphate (pH 7.4 or 8.5, at 20°C), 20 mM KCl, 2 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol. Buffer B contained 10 mM Tricine (pH 7.8, at 20°C), 30 mM KCl, 2 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol. α-Chymotrypsin was obtained from Serva (Heidelberg, FRG), mol. wt. marker proteins were from Pharmacia (Uppsala). Protease inhibitors Trasylol (Bayer, Leverkusen), and leupeptin (Peninsula Laboratories, San Carlos) were used at 0.6 μg/ml and 1 μg/ml, respectively. Unless otherwise noted, all procedures were carried out at 0–4°C.

*Cytosol preparations*

Cytosols were prepared from frozen cell pellets as previously described (Gehring, 1980a) with buffer A, pH 7.4 containing protease inhibitors. Incubations with 27 nM [$^3$H]triamcinolone acetonide (New England Nuclear, Boston, MA; 37 Ci/mmol) were for 2 h; binding was determined by the charcoal assay (Beato and Feigelson, 1972) and radioactivity was measured by liquid scintillation spectrometry at 42% efficiency. In some experiments receptor complexes were preserved in the non-activated form by adding 10 mM sodium molybdate to cytosols prior to the steroid (Leach *et al.*, 1979; Schmidt *et al.*, 1980).

*DNA-cellulose chromatography*

Cytosol preparations containing 100 000–300 000 c.p.m. of specifically bound [$^3$H]triamcinolone acetonide were activated at 20°C and chromatographed as previously described (Gehring, 1980a) except that DNA-cellulose was equilibrated with buffer B. After extensive washing, columns were eluted either with a linear 30–330 mM KCl gradient or batchwise with 330 mM KCl.

*DEAE-cellulose chromatography*

Cytosol preparations containing 100 000–300 000 c.p.m. of specifically bound [$^3$H]triamcinolone acetonide were treated with charcoal, adjusted to pH 8.5, and applied onto 2 ml columns of DEAE-cellulose (DE 52, Whatman) equilibrated with buffer A, pH 8.5. After extensive washing with buffer, columns were eluted with a linear 20–400 mM KCl gradient (50 ml). For batchwise purification columns were washed with 110 mM KCl and eluted with 200 mM KCl.

*Photoaffinity labelling*

Receptor complexes were used routinely following partial purification on DNA- or DEAE-cellulose. 0.7–1.5 ml samples were placed in a water-cooled cylindrical glass cuvette of 2 cm light path, maintained at 0 to 4°C, and irradiated using a XBO 450 W high pressure xenon lamp (LX 501, Zeiss, Oberkochen). For absorbing thermic radiation and short wavelength u.v. the light was passed through 6 cm water and a microscope cover glass resulting in a continuous spectrum of $\lambda \geq 320$ nm. Irradiation was routinely for 45 min. Samples were then precipitated with 10% TCA. Precipitates were collected on glass filters (Whatman GF/F, 8 mm), washed with 5 ml acetone, and redissolved in SDS containing buffer.

*Gel electrophoresis and fluorography*

Electrophoresis in SDS containing polyacrylamide gels (9% acrylamide, 0.24% bisacrylamide) was performed as described (Laemmli, 1970) using phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), and carbonic anhydratase (30 000) as mol. wt. markers. At least 20 000 c.p.m. of protein bound radioactivity were applied per slot. Gels were stained with 0.25% Coomassie blue R250, photographed, destained with TCA/acetic acid/methanol/water (1:1:3:5), treated with EN$^3$HANCE (New England Nuclear), dried, and then fluorographed using Kodak XR5 film.

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