ARV it may be possible to find a means of eliminating the cytopathic effects of the virus or preventing its replication in human cells. This approach may also prove useful in attempts to induce the expression of certain ARV proteins that may be required for vaccine development.

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Mapping Epitopes on a Protein Antigen by the Proteolysis of Antigen-Antibody Complexes

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A monoclonal antibody bound to a protein antigen decreases the rate of proteolytic cleavage of the antigen, having the greatest effect on those regions involved in antibody contact. Thus, an epitope can be identified by the ability of the antibody to protect one region of the antigen more than others from proteolysis. By means of this approach, two distinct epitopes, both conformationally well-ordered, were characterized on horse cytochrome *c*.

THE INTERACTION OF ANTIBODIES to proteins with their respective antigens has been the subject of extensive studies. However, even where the covalent and tertiary structure of a protein antigen is known we rarely know the full identity and conformation of its antigenic sites. The two main approaches to this problem are peptide-binding studies and fine-specificity analyses, but inherent weaknesses limit their usefulness. The concept of using synthetic peptides to probe the antigenicity of a protein was first developed over 10 years ago (*1*). Antibodies that have stringent requirements for native conformation, however, or for surface contacts on the antigen that involve discontiguous regions of the polypeptide backbone, may not be detected by this method. Fine-specificity studies are done with panels of evolutionarily variant proteins. This approach has enabled immunodominant residues in globular proteins to be identified whose epitopes are conformationally dependent, such as in lysozyme, myoglobin, and cytochrome *c* (*2*). However, only one or two evolutionarily variant residues involved in antibody binding can be identified by this method.

These approaches have been used to study the specificities of two monoclonal antibodies, C3 and E8, that were derived from a mouse that had been immunized with cytochrome *c* (*3*). Fine-specificity studies indicated that the determinant for C3 was centered around residue 44. Assignment of the site for E8 was not definitive because the panel of naturally occurring cytochromes *c* was inadequate to distinguish binding at either of the two regions about residues 60 or 89. Further delineation of the antigenic sites with synthetic peptides and large cyanogen bromide–cleaved fragments of the protein was not possible since the antibodies did not bind them (*3*).

We therefore applied a novel approach to detect the surface regions of cytochrome *c* that contact these monoclonal antibodies. Since the regions of antibodies that bind antigen are resistant to proteolysis (*4*) while cytochrome *c* is readily proteolyzed (*5*), we hypothesized that the relative rates of release of peptides from cytochrome *c*–antibody complexes during proteolysis with trypsin, compared to release of peptides from unbound antigen, would indicate which parts of the antigen are in contact with the antibody.

Cytochrome *c* peptides are readily identified in a trypsin-digested protein mixture containing monoclonal antibodies by means of reversed-phase high-performance liquid chromatography (rHPLC). To demonstrate this a monoclonal antibody to dinitrophenol (anti-DNP) that was of the immunoglobulin G1 (IgG1) class and did not bind cytochrome *c* was added to horse cytochrome *c* in a relative amount that would have just saturated binding sites had the two components interacted. The mixture was digested with trypsin for 30 minutes and peptides were separated by rHPLC. One cytochrome *c* peak overlapped with an IgG peptide that had a substantial signal (Fig. 1, A and B), and therefore could not be quantified. This peak lies between peaks 34 and 37 in Fig. 1A and represents the sequence from residues 61 to 72 in the cytochrome *c* molecule. All other cytochrome *c* peptides, however, eluted at positions distinct from the IgG peptides or had significantly larger signals and were quantifiable. Although the IgG had been predigested with trypsin and fractionated on Sephadex G-75, small peptides were still present (Fig. 1B); however, the antigen-binding fragment that was resistant to proteolysis did not elute in the rHPLC gradient.

Essentially the same elution profiles were observed with anti-DNP and anti–cytochrome *c* (Fig. 1B). Fab's prepared from the antibodies also gave a similar profile (Fig. 1C), although the signals at 214 nm were lower. Trypsin (Fig. 1D) contributed little to the peptide signals seen in the total digest in Fig. 1A. Tryptic peptides of horse cytochrome *c* corresponding to the numbered peaks were identified from their amino acid composition and the sequence of horse cytochrome *c* (*5*). In the 30-minute digest of cytochrome *c* there was a peak (peak 39 in Fig. 1A) that was not present in a limit digest (18 hours). This peptide corresponds to residues 56–73 and was an intermediate digestion product in the formation of the peptides containing residues 56–60 and 61–72. Cytochrome *c*, itself, and any peptides larger than those shown did not elute in the gradient.

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The cytochrome *c*–specific monoclonal antibodies, C3 and E8, both IgG1, were examined for effects on the proteolytic cleavage of cytochrome *c*. Highly purified complexes of cytochrome *c* with monoclonal antibodies were prepared (*6*), digested with trypsin, and analyzed by rHPLC (Table 1). For comparison, the effect of anti-DNP on proteolysis of cytochrome *c* was also examined. A bound monoclonal antibody had a significant effect on the general rate of proteolysis of the antigen. In a 30-minute digestion the peak heights (ratios of free cytochrome *c*:bound cytochrome *c*) for the cytochrome *c* peptides from the antibody-bound antigen were about 25% of those observed for the peptides from free cytochrome *c* mixed with a nonspecific antibody (Table 1). This protection was observed whether the antibody used was the Fab fragment or the whole molecule. Since cytochrome *c* is only about one-fourth the size of the Fab fragment, steric hindrance of the proteolytic cleavage sites may be the major reason for this phenomenon. Within 4 hours of digestion, the peak heights for any given peptide became equal whether cytochrome *c* was originally bound to antibody or not (Table 1).

In addition, the monoclonal antibodies protected some regions of the antigen more than others. Thus, when C3–cytochrome *c* complexes were proteolyzed, peptides that contained residues 39–53 (peak 20), 40–53 (peak 18), and 26–27 (peak 1a) were not present in the digestion mixture at 30 minutes to the same extent as they were when unbound cytochrome *c* was cleaved with the protease (Table 1). In experiments with monoclonal antibody E8 (Table 1), the nonspecific protective effects of the antibody were again observed; however, the regions that were protected by E8 (peaks 37 and 39) were not the same as those that were protected by C3. Peak 37, the peptide corresponding to residues 61–73, was present in the 30-minute digest of E8-bound cytochrome *c* at a level too low to be measured accurately. Thus, only the lower limit of the ratio of the peak height compared to that of free antigen could be estimated; peptide 56–73 (peak 39) was also drastically diminished in quantity. Two peptides, 56–60 (peak 23/24) and 61–73, are breakdown products of 56–73; therefore one would also expect 56–60 to have been present in the antibody-bound cytochrome *c* digest in lower amounts. However, this peptide co-eluted with another (92–97) and could not be quantified. That these specific protective effects were reproducible for both C3 and E8 is also shown in Table 1. Thus, the same peptides are implicated for C3 and E8 in triplicate samples taken from single 30-minute digests within a single assay and also in experiments performed on three different occasions.

Since the specific protective effects on particular peptides were unique to each anti–cytochrome *c* monoclonal antibody tested, they would appear to be due to the binding of the antibody in the immediate vicinity of the protected peptides. Indeed, the data obtained from the proteolysis experiments were consistent with previous results for these antibodies from fine-specificity analyses in which substitutions at position 44 profoundly influenced the binding of C3 (*3*). Of the peptides affected in the present studies (Table 1) two contained the identified immunodominant residue 44. The third peptide protected by C3, residues 26–27, is evolutionarily conserved and spatially situated in the native molecule close to the peptides that contain residue 44 (Fig. 2). In fact, the carbonyl oxygen atom of residue 44 is hydrogen-bonded to the *N*-ε-hydrogen of the side chain of His^{26} (*7*). It is not clear whether the dipeptide 26–27 is part of a discontiguous epitope centered around residue 44 or if the binding of the antibody in that region simply sterically hinders the approach of trypsin to cleavage sites on either side of this dipeptide.

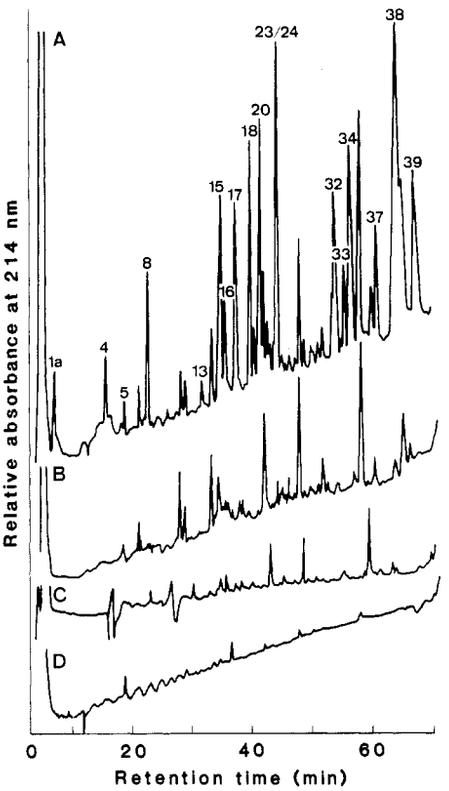

Fig. 1. Identification of cytochrome *c* peptides by rHPLC of a proteolyzed mixture containing IgG1. Cytochrome *c* (Sigma Chemical Co., St. Louis, MO) was further purified on carboxymethylcellulose (*14*). The components in the digests were as follows: (A) horse cytochrome *c* (0.17 mg) and anti-DNP (1 mg), (B) anti-DNP (1 mg), (C) Fab (0.75 mg), and (D) trypsin (0.06 mg). The quantities of IgG1, Fab, and cytochrome *c* employed were equimolar with respect to binding sites. The proteins were incubated with 5% trypsin (w/w of total protein) in 50 m*M* ammonium bicarbonate (*p*H 8.3) for 30 minutes at 37°C with end-over-end mixing. The digested proteins were applied to a 4.6 × 250 mm C-18 (octadecylsilane) column (Spherisorb ODS-I, Custom LC Inc., Houston, TX) and eluted at a flow rate of 1 ml/min in a linear gradient of from 0 to 70% acetonitrile (0.1% TFA) over a time course of 90 minutes. Numbered peaks from a parallel run of 1 mg of digested cytochrome *c* were collected as they eluted and were analyzed for amino acid composition with a Beckman 6300 Amino Acid Analyzer after hydrolysis in 6*N* HCl for 18 hours at 110°. The absorbance scale used in this experiment was 0.2 OD units Full Scale. Fab's were prepared by digestion of the IgG with papain and were purified by chromatography on DEAE-cellulose (*15*).

To define the binding site for E8 could not be uniquely defined by the previous fine-specificity studies, but was known to involve either residue 60 or 89 (*3*). Applying the present methodology, however, the region around residue 60 (Fig. 2) was clearly distinguished as the epitope for monoclonal antibody E8 since both peptides that were observed to be affected by E8 contained residue 60 (*8*).

Having determined the contact regions on cytochrome *c* that bind to these two monoclonal antibodies, we tested their ability to bind peptides that contained them. Peptide 56–73, which contained the epitope for E8 (GITWKEETLMEYLENPKK) (*9*), was isolated by rHPLC from a partial tryptic digest of cytochrome *c*. Peptides that represent the region of cytochrome *c* involved in binding C3, and a shorter peptide (57–63) containing residue 60, were synthesized by the standard solid-phase procedure with *tert*-butyloxycarbonyl–protected amino acids, dicyclohexylcarbodiimide couplings, and trifluoroacetic acid (TFA; 30%) for deprotection (*10*). The peptides were cleaved from the resin by hydrogen fluoride. Four synthetic peptides were prepared:

(I) ITWKEET (residues 57–63);

(II) GQAPGFTYT (residues 41–49);

(III) EKGGKHKT (residues 21–28);

(IV) CH_3CO-EKGGKHKTKGG-NH_2
|
GQAPGFT

Peptide IV is a branched peptide designed to orient the two discontiguous regions 21–28 and 41–47 in the direction in which they are arranged in the native molecule. It is likely that these two branches exist in a number of conformational states; they were covalently linked, however, in such a way that residues 46 and 28, and 44 and 26,

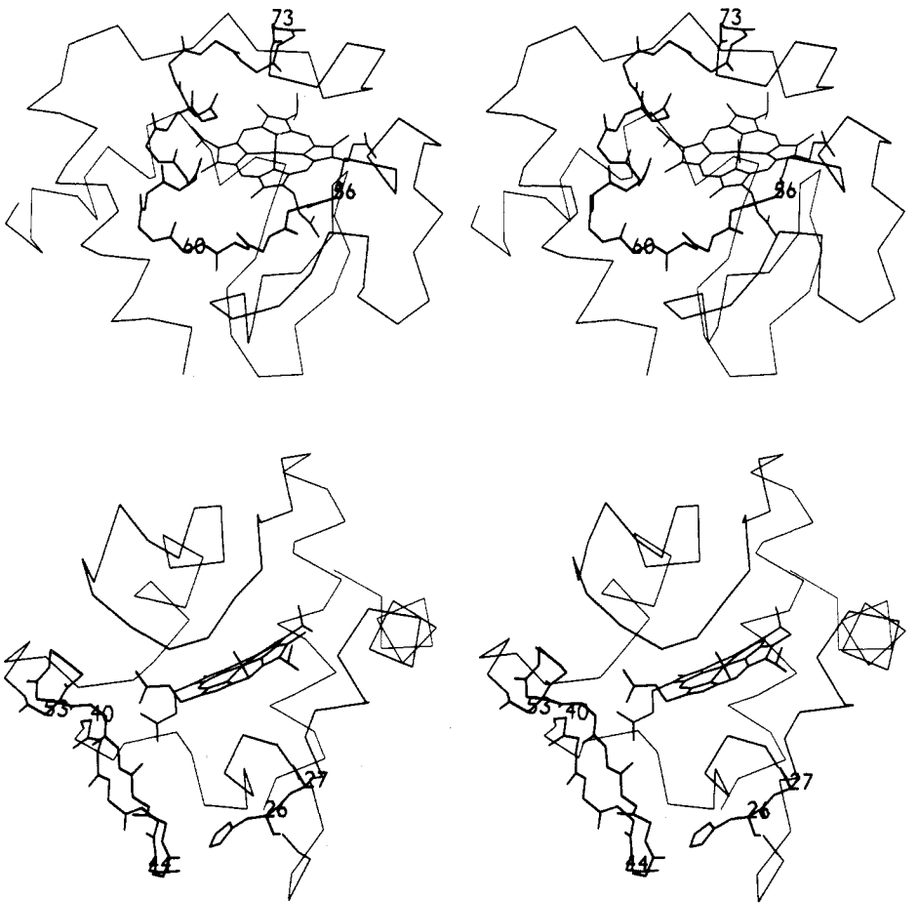

Fig. 2. Stereo computer graphic views by E. Getzoff and J. Tainer (Scripps Clinic) showing the carbon backbone of tuna cytochrome *c* (*7*) with the programs GRAMPS and GRANNY (*16*). The atomic coordinates were derived from the crystal structure of tuna cytochrome *c* at 2.0 Å resolution (Protein Data Bank, Brookhaven National Laboratory, Upton, NY). The three-dimensional structures of horse and tuna cytochrome *c* are similar (*7*). All side chains (except for His^{26} in the bottom view) have been omitted to simplify the representation. Peptides mapped in these experiments, 56–73 in the top view and 26–27 and 40–53 in the bottom view, are indicated by the thickened lines.

Table 1. Peak-height ratios (free cytochrome *c*:monoclonal antibody–bound cytochrome *c*) of rHPLC-fractionated peptides. Mixtures containing affinity-purified monoclonal antibodies and cytochrome *c* were digested with 5% trypsin (w/w of total protein) at 37°C with end-over-end mixing. The total volume for each sample was about 6 ml. At various times, 1 ml aliquots were removed and acetic acid (200 μl, 2.0*N*) was added to stop further tryptic degradation. Samples were lyophilized to dryness, dissolved in 250 μl 0.4*M* acetic acid containing 0.1% TFA, centrifuged to remove any particulate material, and chromatographed by rHPLC (Fig. 1). Peak heights for the cytochrome *c* peptides were determined for both anti–cytochrome *c*–antigen complexes and anti-DNP–cytochrome *c* mixtures; ratios of these peak heights were then calculated. Average values ± standard deviations of peak-height ratios obtained from three different experiments are given for the 30-minute digestion time point for C3 and E8 to indicate the reproducibility of the data between experiments. For C3, data obtained from triplicate samples taken from single 30-minute digests are also shown, to demonstrate the reproducibility within an experiment.

rHPLC peak	Residues in cyt *c* sequence	Peak-height ratios at 30 minutes		Peak-height ratios within C3 experiments		
		E8	C3	30 Min.	1 Hr.	4 Hr.
1a	26–27	5.1 ± 2.5	8.4 ± 1.5	6.7 ± 0.6	4.5	1.7
4	89–91	4.7 ± 2.7	3.6 ± 0.3	3.8 ± 0.3	3.8	1.5
5	88–91/98–99	3.1 ± 0.3	1.8 ± 1.5	1.8 ± 0.5	2.0	1.0
8	1–5	4.0 ± 1.7	3.9 ± 0.8	4.8 ± 1.1	3.6	1.5
13	28–33	2.2 ± 0.9	3.1 ± 1.9	3.0 ± 1.2	2.4	1.8
15	74–79	2.3 ± 1.9	4.0 ± 1.1	4.3 ± 0.5	3.0	1.3
16	73–79	2.6 ± 1.5	3.7 ± 1.9	4.6 ± 0.3	2.8	1.6
17	9–13	4.7 ± 2.8	4.2 ± 1.0	4.8 ± 0.4	3.2	1.3
18	40–53	4.5 ± 3.1	5.6 ± 2.0	7.5 ± 1.0	4.8	1.7
20	39–53	5.1 ± 3.3	7.0 ± 2.4	9.1 ± 1.8	4.7	1.4
23/24	56–60/92–97	3.1 ± 1.7	4.3 ± 1.9	4.8 ± 1.8	4.0	1.4
32	80–86	2.6 ± 1.1	2.5 ± 0.7	2.8 ± 0.3	2.1	1.2
33	28–38	1.7 ± 0.7	4.0 ± 2.5	5.4 ± 0.2	5.0	1.3
34	92–99	3.6 ± 1.8	3.5 ± 1.1	3.6 ± 0.2	2.4	2.2
37	61–73	>20	4.1 ± 1.5	4.8 ± 0.5	4.6	2.0
38	14–22	3.8 ± 0.8	2.6 ± 0.5	3.1 ± 0.3	2.8	1.3
39	56–73	15.3 ± 4.2	2.8 ± 1.6	4.6 ± 1.0	2.0	0.6

which are close together in the native conformation (*7*), are also proximal in the peptide (*11*). All four synthetic peptides and the tryptic peptide, 56–73, were purified by rHPLC.

None of the five peptides displayed antigenic specificity when examined (i) for their ability to compete with ^{125}I-labeled horse cytochrome *c* in binding to E8 or C3 in solution-phase Farr assays (*3*), (ii) for binding to the monoclonal antibodies in a standard solid-phase radioimmunoassay in which the peptides were coupled to hemocyanin by means of glutaraldehyde (*12*), and (iii) by immunoblotting where the peptides were absorbed onto nitrocellulose (*13*). This indicates that the epitopes that monoclonal antibodies C3 and E8 bind are conformationally well ordered and cannot be easily mimicked by peptides. Thus, by this approach even highly conformation-dependent epitopes can be mapped without dependence on either peptides or a panel of cross-reacting antigens for their identification. In addition, conserved residues as well as evolutionary variants involved in an epitope can be discerned whereas methods utilizing panels of structurally related antigens are only effective in identifying the variant residues. Due to possible steric hindrance of the antibody on the protease, not all peptides that are retarded in their release from proteolyzed antigen/antibody complexes are necessarily in direct contact with the antibody-combining site. However, this problem in estimating the boundaries of an epitope may be overcome by means of a variety of proteases having different amino acid residue specificities. While trypsin was employed in the present study, preliminary experiments with chymotrypsin indicate that it, too, will be useful in this type of analysis.

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6. The monoclonal antibodies (3 mg), affinity-purified on the respective antigen, were incubated with 5% trypsin (w/w of total protein) overnight at room temperature and then separated from trypsin and any released peptides by chromatography on Sephadex G-75. The cytochrome *c*–specific antibodies were then incubated with a saturating amount of the antigen (1 mg) for 1 hour at 4°C. Unbound cytochrome *c* was separated from the IgG-bound antigen by chromatography on Sephadex G-75 in 50 m*M* ammonium bicarbonate, *p*H 8.3. Between 1.4 and 2.0 moles of cytochrome *c* remained bound to each mole of trypsinized IgG1. An equimolar amount of cytochrome *c* that just saturated the binding sites on the specific antibodies was added to anti-DNP IgG1 in control experiments.
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9. The following abbreviations were used for amino acids: A, alanine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; T, threonine; W, tryptophan; Y, tyrosine.
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11. The α-amino group of 21–28–KGG was acetylated while the peptide was still protected and attached to the benzhydrylamine resin. Then residues 41–47 were synthesized on the side chain *N*-ε-amino group of the lysine following residue 28 (which does not occur in the natural sequence). In the synthesis of 21–28–KGG, the lysine following residue 28 was side chain–protected with the 9-fluoroenylmethyloxycarbonyl group. This allowed deprotection of its ε-amino group under basic conditions without affecting other side chain protecting groups (*10*). The COOH-terminal glycine residues were used to separate the growing end of the peptide chain from the resin to increase the efficiency of subsequent coupling.
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Steroid Hormone Metabolites Are Barbiturate-Like Modulators of the GABA Receptor

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Two metabolites of the steroid hormones progesterone and deoxycorticosterone, 3α-hydroxy-5α-dihydroprogesterone and 3α,5α-tetrahydrodeoxycorticosterone, are potent barbiturate-like ligands of the γ-aminobutyric acid (GABA) receptor–chloride ion channel complex. At concentrations between 10^{-7} and 10^{-5} *M* both steroids inhibited binding of the convulsant *t*-butylbicyclophosphorothionate to the GABA-receptor complex and increased the binding of the benzodiazepine flunitrazepam; they also stimulated chloride uptake (as measured by uptake of $^{36}Cl^-$) into isolated brain vesicles, and potentiated the inhibitory actions of GABA in cultured rat hippocampal and spinal cord neurons. These data may explain the ability of certain steroid hormones to rapidly alter neuronal excitability and may provide a mechanism for the anesthetic and hypnotic actions of naturally occurring and synthetic anesthetic steroids.

STEROID HORMONES ACT ON THE central nervous system (CNS) to produce diverse neuroendocrine and behavioral effects (*1*). Both adrenal and gonadal steroids interact with intracellular receptors in the CNS and trigger genomically directed alterations in protein synthesis, which occur in minutes to hours (*2*). In addition, many steroids produce more rapid alterations in CNS excitability (*1*). Over 40 years ago Selye (*3*) described the rapid and reversible CNS depressant actions of various steroids in the rat. The gonadal steroid progesterone, and the mineralocorticoid deoxycorticosterone, as well as several of their metabolites, were the most potent among a series of steroids in inducing sedation and anesthesia (*4*). On the basis of these observations a class of steroidal anesthetics was developed and has been used clinically (*5*).

The mechanisms responsible for the rapid effects of steroids on neuronal excitability are poorly understood, although the short latency (seconds to minutes) of the effects makes it unlikely that they are mediated by "classical" intracellular receptors. The anesthetic and hypnotic actions of certain drugs, including the benzodiazepines (*6*), barbiturates (*7*), and the anesthetic steroid 3α-hydroxy-5α-pregnane-11,20-dione (alphaxalone) (*8*) may be due in part to their enhancement of the inhibitory action of the neurotransmitter γ-aminobutyric acid (GABA). The potentiation of GABA-mediated synaptic inhibition by these drugs occurs by a direct interaction with $GABA_A$ receptors, which are coupled to chloride (Cl^-) ion channels (*6–9*). The $GABA_A$ receptor is an oligomeric receptor complex consisting of several subunits with independent but interacting binding sites for GABA, benzodiazepines, and barbiturates (*9*). Because the $GABA_A$-receptor complex is an important site of anesthetic and hypnotic drug action, we examined a series of naturally occurring steroids for their ability to interact with one or more sites on this receptor complex. We now report that the ring A reduced metabolites of progesterone and deoxycorticosterone—namely, 3α-hydroxy-5α-dihydroprogesterone (3α-OH-DHP) and 3α,5α-tetrahydrodeoxycorticosterone (3α-THDOC), respectively—are potent modulators of the GABA-receptor complex and interact at a site close to or identical with that for barbiturates.

A series of steroids was tested for their ability to inhibit the specific binding of the ^{35}S-labeled convulsant *t*-butylbicyclophosphorothionate ([^{35}S]TBPS), a ligand that labels a site close to or on the GABA-operated Cl^- channel (*10*). The specific binding of [^{35}S]TBPS to brain membranes is inhibited both by barbiturates and by GABA antagonists such as picrotoxin, and there is a good correlation between the pharmacological potencies of these compounds and their ability to displace [^{35}S]TBPS binding (*10*). Both 3α-OH-DHP and 3α-THDOC were relatively potent inhibitors of [^{35}S]TBPS binding to the $GABA_A$ receptor–Cl^- channel complex in crude synaptosomal membranes from rat

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