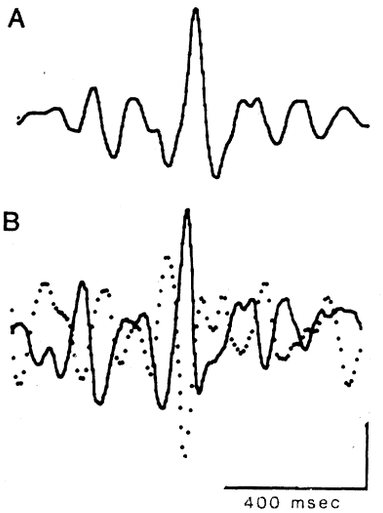
Fig. 3. Fast seizure spiking in animal 1. (A) Averaged rapid seizure spike in the ECoG (negative down). (B) Seizure spikes averaged across three successive seizures recorded in the MEG at each of the bilateral sites (solid and dotted traces) are similar in morphology to the ECoG and are of opposite direction between recording sites. (Emerging and reentering fields are shown by upward and downward traces, respectively.) Vertical calibration is the same as in Fig. 1.



why slow shifts are less difficult to record magnetically; of particular importance is the absence of a tissue-electrode interface and the inherently wide frequency response of superconducting magnetometers (d-c to 10 kHz).

Our data provide measurements of both the fast and slow magnetic phenomena associated with focal electrographic seizures. These phenomena indicate strong electric currents occurring at the cellular level within the epileptic focus. In all animals, the magnetic fields associated with both interictal and ictal activity were consistently reversed in direction between the two MEG recording sites, indicating an organized pattern of emerging and reentering magnetic flux. Although these recordings were performed with a single channel MEG sensor, the repeatability of ictal and interictal magnetic phenomena at a given MEG recording site permitted comparison between separate epileptiform events sequentially recorded at each of the bilateral locations.

This experiment also represents a use of an animal model for the neuromagnetic study of central nervous system events. Although accomplished with a large-coil instrument designed for the study of the human brain, neuromagnetic phenomena produced in much smaller brains like that of the rat may be measured if proper attention is paid to the diameter and separation of the gradiometer coils in relation to the measurement distance (*11*).

The magnetic phenomena observed here in laboratory animals with experimentally induced epileptic foci may also be of practical consequence. The slow field shifts associated with the development of seizures are very strong. For this reason, slow field shifts should be suited for neuromagnetic mapping in patients with focal seizure disorders. Although electrical measurements of slow focal seizure phenomena in humans has been performed during neurosurgery (*12*), noninvasive electrical mapping of these major slow events has not been possible to our knowledge. Neuromagnetic sensing therefore may be a useful method for noninvasively localizing the seizure focus in human beings with focal epileptic disorders.

DANIEL S. BARTH
Departments of Psychology and *Neurology, University of California, Los Angeles 90024*
WILLIAM SUTHERLING
Department of Neurology, University of California, Los Angeles
JACKSON BEATTY
Department of Psychology, University of California, Los Angeles

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Benzodiazepine Receptor Synthesis and Degradation by Neurons in Culture

Abstract. *The benzodiazepine–γ-aminobutyric acid receptor complex was used to study functional receptor synthesis and degradation in primary cultures of neurons. Fifty percent of the receptors turned over with an unusually rapid half-life (4 hours); this was followed by a second, slower phase (32 hours). These results provide the basis for elucidating the mechanism by which neurons derived from the central nervous system control neurotransmitter receptor number, an important problem in cellular neurobiology. The findings may be of significance in the study of neurological and psychiatric disorders.*

Synaptic transmission at chemical synapses involves a well-described sequence of events in which a neurotransmitter is released from the presynaptic terminal and interacts with postsynaptic receptors that transduce ligand binding into a postsynaptic response. One way a neuron can regulate its sensitivity to a ligand is by altering the number of postsynaptic receptors. The mechanism by which receptor numbers are controlled is thus of central importance in neurobiology. Receptor numbers under steady-state conditions are ultimately controlled by the relative rates of receptor synthesis and degradation, and an understanding of such rates should help to elucidate the mechanism of receptor regulation. Except for the nicotinic cholinergic receptor in skeletal muscle (*1*), little is known about the turnover of neurotransmitter receptors (*1*). This is due, in part, to the complexity and relative inaccessibility of the central nervous system (CNS) and to the lack of specific, irreversible probes. Neurotransmitter receptor turnover kinetics have typically been inferred from the rate of cycloheximide-induced receptor loss (*2*) or from agonist-induced decreases in receptor number (down-regulation) (*3*). However, cycloheximide is now known to alter receptor degradation (*4*), and down-regulation may involve mechanisms distinct from

actual receptor degradation, such as receptor internalization (*5*).

Receptors for γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the vertebrate CNS, are coupled to high-affinity benzodiazepine receptor sites, and the behavioral effects of benzodiazepines are probably mediated through modulation of GABA-related transmission in the CNS (*6*). Binding sites for GABA and benzodiazepines seem to be located on the same macromolecular protein complex (*7*). We previously used primary cultures of cells derived from embryonic chick brain and spinal cord to study the action of benzodiazepines on GABA-related transmission (*8, 9*). In the study reported here we monitored the synthesis and degradation of benzodiazepine receptors in such cultures by photoaffinity labeling. Despite the fundamental importance of the regulation of neurotransmitter receptors, this may be the first time that the synthesis and degradation of a receptor found only in the CNS have been directly measured.

When cultures of intact brain or spinal cord tissue (*10*) are exposed to flunitrazepam and ultraviolet light [photoinactivation (*11*)], reversible binding of 5 n*M* [^{3}H]flunitrazepam decreases approximately 75 percent, as does the ability of chlordiazepoxide to potentiate GABA-induced increases in conductance (Fig. 1A) (*9*). Photoinactivation is specific for benzodiazepine receptors (*11*). In rat brain homogenates, photolabeling irreversibly links [^{3}H]flunitrazepam to 25 percent of the binding sites, while binding of flunitrazepam to the remaining sites is greatly reduced (*12*), apparently by allosteric interactions.

To monitor the appearance of new receptors (recovery), we photoinactivated cultures and incubated them at 37°C. Reversible [^{3}H]flunitrazepam binding and benzodiazepine potentiation of GABA responses recovered to a similar extent (Fig. 1). The similar decrease in binding and potentiation of GABA-induced conductance, the similar recovery of both, and the observed correlation of binding affinities with electrophysiological potencies (*9*) strongly indicate that functional benzodiazepine receptors reappeared in these experiments. Binding returned to a level slightly greater than that in control cultures, suggesting accumulation of benzodiazepine receptors. To minimize accumulation, kinetic analysis was limited to the first 10 hours of recovery. Spinal cord cultures recovered 7 and 9 percent per hour in two experiments and brain cultures at 6 ± 1.5 percent per hour (n = 7). A representative spinal cord experiment gave the following values (specific counts per minute per assay): control, 606 ± 39; inactivated, 114 ± 33; 2 hours of recovery, 217 ± 18; 4 hours, 289 ± 34; 6 hours, 318 ± 28; and 10 hours, 346 ± 4. Although we have extensively investigated the electrophysiology of chick spinal cord neurons in culture (*8, 9*), a detailed analysis of benzodiazepine receptor turnover required a large number of cultures that were difficult to obtain because of the small size of young embryonic spinal cords. Thus most subsequent experiments were performed with brain cultures. When intact brain cultures were photolabeled with [^{3}H]flunitrazepam (*13*) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), cell-associated radioactivity migrated as two peaks consistent with molecular weights of 48K and 51K (Fig. 1B), similar to rat brain (*14*). No radioactive peaks were observed in cultures that were photolabeled immediately after blockade, whereas the peaks reappeared in cultures that were photolabeled after a 24-hour recovery period (Fig. 1B).

Is benzodiazepine receptor synthesis being monitored? The results show that receptor reappearance requires de novo protein synthesis. During a 6-hour period after photoinactivation, receptor recovery was inhibited 76 ± 25 percent (two experiments in triplicate) and 82 ± 19 percent (two experiments in triplicate) by cycloheximide (20 μg/ml) and puromycin (100 μg/ml), respectively. A 5-hour incubation with cycloheximide (20 μg/ml) resulted in a 97 ± 1 percent (two experiments) decrease in the incorporation of [^{35}S]methionine (70 n*M*, ~1000 Ci/mmol, 30 minutes at 37°C) into trichloroacetic acid (TCA; 10 percent)–precipitable material. One hour after washout of the drug, incorporation returned to 92 ± 15 percent of control, indicating that cycloheximide action is reversible and that the drug is not cytotoxic under the conditions used.

The degradation of existing benzodiazepine receptors was next investigated by photocoupling [^{3}H]flunitrazepam to cells (*15*). Cell-associated radioactivity decreased with time and SDS-PAGE showed that the radioactive bands decreased 60 percent in 24 hours (six experiments); 87 ± 2 and 95 ± 4 percent of the specific radioactivity were recovered in the bands at 0 and 24 hours, respectively. Thus the loss of specific cell-associated radioactivity represents degradation of the benzodiazepine receptor. The kinetics of receptor degradation were then monitored by following the release of radioactivity into the medium (Fig. 2). The best fit to the data of four experiments was provided by a two-exponential fit, indicating that receptor degradation has two components with half-lives of 3.8 hours (42 ± 3 percent of total) and 32 hours (58 ± 3 percent of total). The two radioactive peaks observed on SDS-PAGE decreased proportionately and thus do not account for the biphasic kinetics of degradation. The degradation rates for benzodiazepine receptors are about five times higher than

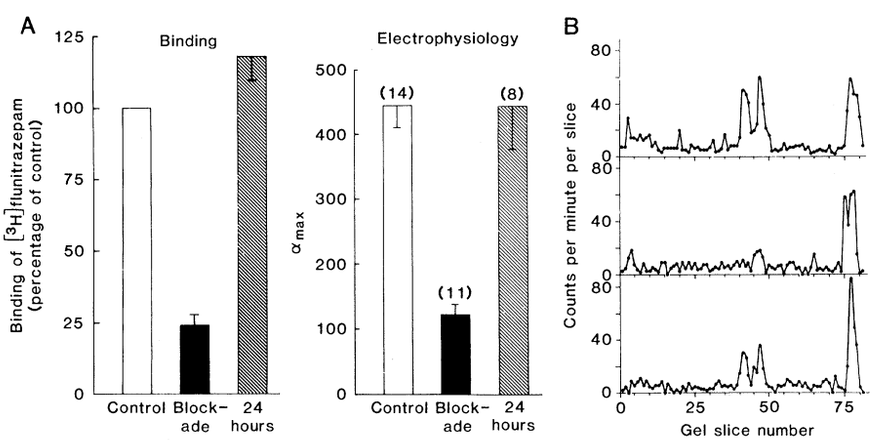

Fig. 1. (A) Reappearance of [^{3}H]flunitrazepam binding and maximum potentiation of the GABA response (α_{max}) after photoinactivation (*11*). Binding data (means ± standard errors) for two experiments are shown, normalized to control values. Values for one experiment are as follows: control, 219 ± 25 count/min per assay tube; inactivated, 76 ± 10; and recovery, 243 ± 24. Electrophysiology was performed as in (*9*). The GABA response, $\alpha_{max} = [(g_{GABA}' - g_{GABA})/g_{GABA}]100$, where g_{GABA} is GABA-induced increase in conductance and g_{GABA}' is GABA-induced increase in conductance in the presence of 300 μ*M* chlordiazepoxide. (B) Analysis by SDS-PAGE. (Top) Brain cultures were mock-inactivated (*11*), washed, photolabeled (*13*), and prepared for SDS-PAGE (*22*). Gels were sliced and counted. (Middle) Cells were photoinactivated, washed, photolabeled, washed again, and processed for SDS-PAGE. (Bottom) Cells were photoinactivated, washed, and, after 24 hours, photolabeled, washed again, and processed for SDS-PAGE.

the rates for general cellular protein (Fig. 2). Photolabeling was specific for neurons (*16*); maximum specific photolabeling was about 60,000 sites per neuron, as compared with a maximum reversible binding of 210,000 sites per neuron, consistent with the presence of four-site complexes (*12*) in living neurons. Photolabeling did not adversely affect cell function, since no affect on protein synthesis, protein degradation, cell number or morphology (*17*), resting potential, input resistance, or GABA response was observed.

We partially characterized the degradation product. Cultures were photolabeled and the radioactivity released after 24 hours at 37°C did not comigrate with [^{3}H]flunitrazepam on thin-layer chromatography (chloroform:methanol, 9:1), and was about 70 percent soluble in TCA. When applied to a Sephadex G-25 column (phosphate-buffered salt solution; 1 by 22 cm; recovery, 85 percent), 80 percent of the radioactivity eluted after one column volume. Radioactivity did not dissociate from photolabeled brain membranes after dialysis in 1 percent Triton X-100 (24 hours at 4°C).

The functional nature of the benzodiazepine binding site under investigation is supported by the correlative binding and electrophysiological experiments. Benzodiazepine receptor recovery requires protein synthesis, indicating that recovery reflects de novo receptor synthesis. Rearrangement of receptor subunits in the membrane, resulting in reversion to a high-affinity state, cannot, therefore, account for the recovery data. Furthermore, reappearance of binding is not the result of dissociation of flunitrazepam from these receptors. The observation that recovery is not completely blocked by inhibition of protein synthesis suggests the presence of a pool of receptor precursor.

Benzodiazepine receptor degradation is biphasic, consistent with the presence of two distinct receptor pools of nearly equal size (half-lives, 3.8 and 32 hours). Receptor reappearance agrees closely with the recovery rate predicted from the kinetics of degradation but does not seem to be biphasic, because, at the times examined (up to 10 hours), the rapid phase of degradation dominated. The biphasic degradation may reflect differences in the degradation rates of synaptic and extrasynaptic receptors, by analogy with junctional and extrajunctional acetylcholine receptors (*18*). These results contrast sharply with the monophasic degradation kinetics observed for insulin receptors on hepatocytes (*5*), receptors for epidermal growth factor on fibroblasts and epidermoid carcinoma cells (*19*), and α_1 and acetylcholine receptors on muscle cells (*1, 20*). The rapid rate of benzodiazepine receptor degradation (half-life, 3.8 hours) is similar to the degradation rate of a number of receptors in the "down-regulated" state (*19, 21*).

By using the methods described here, it should be possible to elucidate the mechanisms responsible for the maintenance and control of benzodiazepine receptor number in a defined neuronal preparation. It is important to determine whether the turnover and recycling (if it occurs) of receptors on these cells of neuroepithelial origin occur through mechanisms different from those for receptors on nonneural cells. Such information could provide a basis for understanding, on the cellular level, the factors that regulate receptor number and modulate synaptic activity in the CNS.

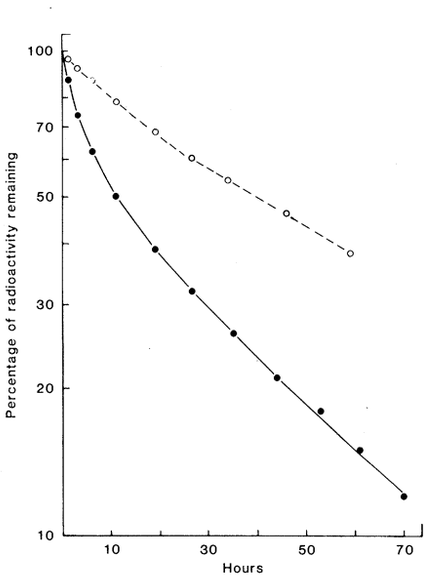

Fig. 2. Degradation of benzodiazepine receptors (●) and general cellular protein (○). To determine receptor degradation, we photolabeled brain cultures (*13*), washed them, and incubated them at 37°C in complete medium. At the indicated times, medium was withdrawn from total and nonspecifically labeled dishes and replaced with fresh medium. Withdrawn medium was centrifuged (1000 rev/min for 5 minutes) and the supernatant was counted for radioactivity. At the end of the experiment, cells were scraped and counted. The appropriate nonspecific value was subtracted at each time point to yield specific photolabeling. The accumulation in the medium of released specific photolabeling is plotted semilogarithmically: percentage release = [1 − (cumulative specific release/total specific incorporation)]100, where total specific incorporation equals cumulative specific release plus specific counts per minute remaining. To determine protein degradation (○), we labeled cells with 6 n*M* [^{35}S]methionine (specific activity, ~1000 Ci/mmol; Amersham) for 24 hours at 37°C, washed them, and added growth medium with 2 m*M* L-methionine. Portions of medium were removed and radioactivity soluble in 10 percent TCA was measured. Data were transformed as described above. Lines (theoretical two-exponential best fits to the data) were obtained by nonlinear regression analysis.

LAURENCE A. BORDEN
CYNTHIA CZAJKOWSKI
CHRISTOPHER Y. CHAN
DAVID H. FARB

Department of Anatomy and Cell Biology, State University of New York, Downstate Medical Center, New York 11203

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10. Brain or spinal cord cultures prepared from 7-day chick embryos (*9*) were exposed to arabinosylcytosine (Ara-C; 1 μ*M*) on day 2 for 24 hours. Cultures were used after 7 days for biochemical studies and after 21 days for electrophysiological studies.
11. To photoinactivate cultures, we incubated them with flunitrazepam (100 n*M*, 30 minutes at 4°C) in phosphate-buffercd salt solution (*9*) and irradiated them with ultraviolet light (30 minutes at 4°C). Controls were mock-inactivated (flunitrazepam, no ultraviolet). Cells were then washed ten times with phosphate-buffered salt solution, medium was added, and the cultures were returned to the incubator. At the appropriate times, cultures (three 60-mm dishes per time) were washed, scraped, homogenized, and centrifuged (30,000*g* for 20 minutes). The pellets were resuspended in phosphate-buffered salt solution. Reversible binding of 5 n*M* [^{3}H]flunitrazepam (85 Ci/mmol; New England Nuclear) was measured in triplicate by filtration (Whatman GF/B). Nonspecific binding in 1 m*M* flurazepam (10 percent of total) was subtracted to yield specific binding. Photoinactivation is completely blocked by 1 m*M* flurazepam and does not alter binding to opiate, muscarinic, and GABA receptors (determined with 7.5 n*M* tritiated D-Ala, [D-Leu]enkephalin, 1 n*M* tritiated L-quinuclidinyl benzilate, and 10 n*M* tritiated muscimol, respectively), suggesting that inactivation is specific for benzodiazepine receptors.
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16. Cultures with few nonneuronal cells (treated with Ara-C) and cultures with a monolayer of nonneuronal cells (no Ara-C) were photolabeled and the number of neurons per dish was determined (about 7000 neurons were counted in three experiments with six dishes per experiment). Specific photolabeling was 6.2 ± 1.5 × 10^4 and 5.7 ± 0.9 × 10^4 sites per neuron for

control and dense nonneuronal cultures, respectively.

17. To determine protein degradation, we exposed cultures to 2 n*M* [^{35}S]methionine (~1000 Ci/mmol) for 24 hours at 37°C and washed and photoinactivated them. The efflux of 10 percent TCA-soluble material into complete growth medium was then monitored for 48 hours at 37°C. To determine protein synthesis, we photoinactivated cultures, washed them, and measured the incorporation of 30 n*M* [^{35}S]methionine (~1000 Ci/mmol, 37°C) into 10 percent TCA-precipitable material immediately or after 24 hours at 37°C. In addition, no difference in the number or morphology of neurons was observed 24 hours after photoinactivation.
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13 February 1984; accepted 15 August 1984

Homologies Between Signal Transducing G Proteins and *ras* Gene Products

Abstract. *The guanosine triphosphate–binding proteins (G proteins) found in a variety of tissues transduce signals generated by ligand binding to cell surface receptors into changes in intracellular metabolism. Amino acid sequences of peptides prepared by partial proteolysis of the α subunit of a bovine brain G protein and the α subunit of rod outer-segment transducin were determined. The two proteins show regions of sequence identity as well as regions of diversity. A portion of the amino-terminal peptide sequence of each protein is highly homologous with the corresponding region in the* ras *protein (a protooncogene product). These similarities suggest that G proteins and* ras *proteins may have analogous functions.*

Cells respond to their environment by means of cell surface receptors that are capable of binding specific ligands. These "signals" are transduced into changes in cellular function and metabolism. One system that mediates the transduction process involves a ubiquitous family of guanosine triphosphate (GTP)–binding proteins (G proteins). These proteins transduce signals generated by ligand interactions with specific cell surface receptors into changes in intracellular levels of cyclic nucleotides (*1*). Different G proteins are found in different kinds of specialized cells. In some types of cells adenylate cyclase is regulated by both a stimulatory G protein (G_s) and an inhibitory protein (G_i) (*2*). In the visual transducing system the G protein analogue, transducin, regulates cyclic guanosine monophosphate concentrations in the rod outer segment by inactivating an inhibitor of a specific phosphodiesterase (*3*). In brain tissue, two G proteins have been found: G_i, and a relatively abundant protein, G_o, whose function is not yet known. The relative ease with which the G_o protein can be purified, however, makes it useful for studies of protein structure (*4*).

G proteins share the following characteristics: They are a complex of three polypeptide subunits termed α, β, and γ. Activated receptors stimulate the α subunit to bind GTP. The α subunit with bound GTP interacts with the effector, for example, adenylate cyclase or the phosphodiesterase inhibitor, and the interaction is terminated when the bound GTP is hydrolyzed to guanosine diphosphate by the α subunit. Finally, the guanosine triphosphatase (GTPase) activity and the receptor coupling properties of the α subunit can be modified by adenosine diphosphate (ADP) ribosylation catalyzed by cholera toxin or pertussis toxin (*5*). The α subunits of the different G proteins appear to be characteristic of each G protein and may be encoded by a family of genes, whereas the β subunit is highly conserved and may be encoded by only one or two genes. For example, the α subunits of G_s, G_i, and transducin have different amino acid compositions, and their peptide maps differ (*6*). However, there is little variation in amino acid composition and peptide maps of the β subunits of these proteins (*6*).

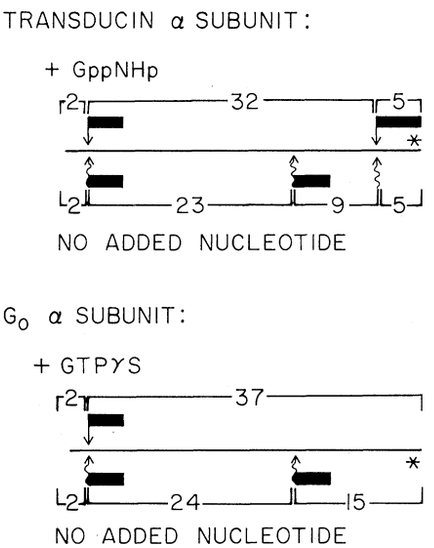

Fig. 1. The distribution of partial proteolytic fragments from the α subunits of transducin and G_o. The black bars indicate the locations on the fragment corresponding to the amino acid sequences that were determined. The wavy arrows represent the cleavage sites in the absence of added nonhydrolyzable guanine nucleotide analog. The G_o was bound to GTPγS (guanosine 5′-[3-*O*-thio]triphosphate) (*4*) and the transducin was bound to GppNHp (guanosine 5′[β,γ-imido]triphosphate) (*18*). The asterisks mark the fragments that were found to be ADP-ribosylated by pertussis toxin. The numbers indicate the size of the fragments in kilodaltons. The amino terminal of the protein is at the left side of the figure.

In order to understand the nature of the genes that encode G proteins, we have determined amino acid sequences from certain G protein subunits. The complete amino acid sequence of the bovine transducin γ subunit and the nucleotide sequence of the corresponding complementary DNA (cDNA) were determined (*7*). The α and β subunits, however, were refractory to Edman degradation suggesting that the NH_2-terminus was "blocked." In this report we describe partial sequences of proteolytic fragments derived from the α subunits of two G proteins, transducin and G_o.

Tryptic proteolysis of the α subunit of transducin results in the formation of a discrete pattern of peptides, which depends on the time of proteolysis and on the nature of the bound guanine nucleotide (*8*). Similar digestion patterns are obtained with the α subunit of the bovine brain G_o protein (*4*) (Fig. 1). These fragments were purified either by high-performance liquid chromatography (HPLC) gel filtration, by electroelution from polyacrylamide gels, or by reversed phase HPLC. The amino acid sequences of the purified peptides were determined by Edman degradation on an automated gas-liquid-solid phase sequenator.

The order of the tryptic fragments for which partial sequences have been determined is outlined in Fig. 1. The order of the proteolytic fragments shown in Fig. 1 is based upon the following arguments.

1) The initial event in proteolysis of both $G_{o\alpha}$ and T_α is a decrease in size from 39 to 37 kD. The 39-kD subunits are blocked, whereas the 37-kD fragments have free amino termini susceptible to Edman degradation. The initial event in proteolysis of the α subunits is thus the removal of about 10 to 20 amino acids from the amino terminus.

2) The 37-kD fragment of T_α can be digested further to 32-kD and 5-kD fragments. The 32-kD fragment has the same amino terminal sequence as the 37-kD fragment, which indicates that it is derived from the amino terminal portion of the 37-kD fragment.