son was made of the covalent binding and the amounts of MBD disemicarbazone produced in pulmonary microsomal incubations in the presence and absence of NADPH and semicarbazide (Fig. 2), there was an inverse relation between the two measures. Semicarbazide inhibited the NADPH-dependent covalent binding of 3-MF, presumably by trapping the reactive aldehyde intermediate (MBD) before it could react and bind irreversibly to the microsomal macromolecules. Enzyme-catalyzed binding was not observed in the absence of NADPH. Although the presence of semicarbazide prevented covalent binding of 3-MF metabolite to microsomal protein, a twofold increase was observed in the amount of 3-MF metabolized.

Finally, we investigated the possibility that the unsaturated aldehyde might require further activation (for example, by oxidation of the olefinic linkage) before it was bound. However, synthetic AA was shown to be bound quickly in microsomal incubations (*19*), and there was no further enhancement by the presence of NADPH (Fig. 4). Thus, it appears that the aldehyde is the principal binding species.

These results provide new insight into the metabolic activation of toxic furans. At least in the case of the alkylfurans, the unsaturated aldehyde intermediates are the products of microsomal metabolism. Therefore, these reactive compounds may be the ultimate toxic metabolites responsible for target tissue alkylation and for toxicity produced by the parent furans in vivo. Epoxides, even if formed transiently, appear not to play a major role in the covalent binding nor in the toxicity of these simple furans.

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17. (A) Acetylacrolein **2** (AA), synthesized by *m*-chloroperbenzoic acid oxidation of 2-MF **1** [N. Clauson-Kaas and J. Fakstorp, *Acta Chem. Scand.* **1**, 415 (1947)], was derivatized to the disemicarbazone **3** with semicarbazide hydrochloride. (B) 3-MF, **4**, on reaction with bromine and methanol, yielded dihydrodimethoxy 3-MF **5** [J. A. Hirsch and A. J. Szur, *J. Heterocycl. Chem.* **9**, 523 (1972)] which was cleaved to methylbutenedial **6** (MBD) in situ with semicarbazide hydrochloride to give the disemicarbazone of MBD **7**. The identities of all the products were confirmed by mass spectral analyses (Fig. 4) and by nuclear magnetic resonance [solvent, D_6-dimethylformamide; internal standard, tetramethylsilane; disemicarbazone of AA, δ (ppm) 10.15 (1H, s), 9.42 (1H, s), 6.52 (3H, m), 2.02 (3H, s); disemicarbazone of MBD, δ (ppm) 10.22 (1H, s), 10.15 (1H, s), 6.34 (1H, s), 6.5 (2H, m), 2.04 (3H, d)].
18. 3-[^{3}H]MF was synthesized as described by R. B. Franklin *et al.* [*J. Labelled Compd. Radiopharm.* **15**, 569 (1978)]. Lung microsomes were prepared from male Sprague-Dawley rats (*10*). Incubations were performed in the presence (+) or absence (−) of 60 m*M* semicarbazide (SC) or 4 m*M* NADPH or both, as indicated, in triplicate at 37°C for 30 minutes with a total volume per incubation of 2 milliliters of phosphate buffer containing 2 mg microsomal protein and 20 m*M* 3-[^{3}H]MF (specific activity, 1 mCi/mmole). Incubations were run in an air atmosphere and terminated by the addition of 2 ml of ice-cold methanol. After centrifugation, the supernatant was analyzed by HPLC for the metabolites and the precipitated proteins were assayed for covalently bound radioactivity (*10*). The HPLC analysis of the supernatants and the synthetic standard MBD disemicarbazone were performed on a Whatman Partisil 5-ODS3 column with 7 percent acetonitrile-water as mobile phase. Similar HPLC conditions were used to separate MBD disemicarbazone from rat hepatic microsomal incubations containing 3-MF and to identify AA-disemicarbazone from rat pulmonary and hepatic microsomal preparations containing only 2-MF in the presence of both SC and NADPH. The HPLC retention volumes for the disemicarbazones of MBD and AA were 14.4 and 9.14 minutes, respectively.
19. [^{14}C]Acetylacrolein was obtained by oxidation of 2-[^{14}C]MF, which was synthesized by reaction of 2-furyl lithium with [^{14}C]methyl iodide and purified by preparative gas chromatography, and the chemical identity was established by mass spectral measurement. Oxidation of 2-[^{14}C]MF (1.2 mmole; specific activity, 0.0017 mCi/mmole) by *m*-chloroperbenzoic acid gave [^{14}C]AA, which was purified by flash chromatography on silica gel (specific activity, 0.0017 mCi/mmole; radiochemical purity > 97.5 percent), and chemical identity was established by mass spectral measurement ($M^+ = 98$) and NMR [solvent, $CDCl_3$; internal standard, tetramethylsilane; δ (ppm) 2.38 (3H, s), 6.15 (1H, dd, J = 12 and 7 Hz), 6.96 (1H, d, J = 12 Hz), 10.2 (1H, d, J = 7 Hz)]. Liver microsomes were prepared from Sprague-Dawley rats (*10*). Microsomal incubations were run in triplicate at 37°C and in a total volume of 5 milliliters of phosphate buffer containing 50 m*M* [^{14}C]AA, 5 mg of microsomal protein, and, where appropriate, 10 m*M* NADPH. Equal portions (0.5 ml) were removed at specified time intervals and transferred into equal volumes of ice-cold methanol, and the microsomal protein was assayed for covalently bound radioactivity (*10*).

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Synthesis of Compounds with Properties of Leukotrienes C_4 and D_4 in Gerbil Brains After Ischemia and Reperfusion

Abstract. *6-Sulfidopeptide–containing leukotriene-like immunoreactivity was synthesized in gerbil forebrains after bilateral common carotid occlusion and reperfusion. At 5, 10, or 15 minutes of ischemia, concentrations increased significantly and became more marked on reperfusion. Immunoreactivity was highest in forebrain gray matter and was below the detection limit of the assay in brain regions remote from the zone of ischemia. In vitro experiments with vascular cells and organ cultures of cerebral arteries indicate that the cerebral blood vessel wall is not a major source of biosynthetic activity in the brain. These experiments demonstrate leukotriene biosynthesis by the brain. Because synthesis occurs during ischemia and reperfusion and because leukotrienes are potent vasoconstrictors and promoters of tissue edema, they may play a role in the pathophysiology of cerebral ischemia.*

The 6-sulfidopeptide–containing leukotrienes [slow reactive substances (SRS's)] are a family of pharmacologically active peptidolipids that are synthesized from arachidonic acid by way of the lipoxygenase pathway (*1*). These molecules are more potent than other derivatives of arachidonic acid (such as the prostaglandins) in their ability to promote plasma leakage and constrict blood vessels (*2*). During cerebral ischemia, deacylation of membrane phospholipids and release of arachidonic acid occurs (*3*). During reperfusion, arachidonic acid is converted enzymatically to products of the cyclooxygenase pathway (prostaglandins) (*4*). It has been suggested that prostaglandin accumulation after stroke may contribute to decreases in blood flow and cerebral edema formation in the affected brain region (*5*). This is partially confirmed by studies showing that blood flow increases in the brain's microcirculation after treatment with a combination of indomethacin, heparin, and prostacyclin (*6*). However, in other experiments brain edema did not abate despite adequate inhibition of prostaglandin synthesis by indomethacin (*4*). Since arachidonic acid is a precursor for SRS's, and since SRS's, in relatively small amounts, produce vasoconstric-

tion and tissue edema, studies were undertaken to determine whether these molecules are synthesized after various periods of cerebral ischemia and reperfusion.

Mongolian gerbils (*Meriones unguiculatus*) were used because they lack the anatomical circle of Willis and well-developed anastomoses between the carotid and vertebrobasilar circulations (*7*). Cerebral ischemia was induced in adult males by occluding the common carotid arteries bilaterally with vascular clips. These occlusions were maintained for 5, 10, or 15 minutes and then removed to allow reperfusion for 5 minutes to 24 hours (*8*).

Brain extracts were radioimmunoassayed for SRS's, 12-hydroxyeicosatetraneoic acid (12-HETE), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), and $PGF_{2\alpha}$ (*9*). The assay procedures and serologic specificities for 12-HETE, 6-keto $PGF_{1\alpha}$, and $PGF_{2\alpha}$ have been reported elsewhere (*10*). Analyses for SRS's were performed with an antiserum to leukotriene C_4 (LTC_4) (*11*). Because of its serologic heterogeneity (*12*), some SRS determinations were made by radioimmunoassay after subjecting the brain extract to high-performance liquid chromatography (HPLC). When immunochromatography was not performed, data were calculated from the inhibition of the tritiated LTC_4 anti-LTC_4 binding generated by a standard LTD_4. The antiserum used to measure 12-HETE was also serologically heterogeneous, and data are expressed as immunoreactive 12-HETE (i12-HETE).

Reperfusion of ischemic brain tissue significantly increased iSRS activity in gerbil forebrains (Fig. 1). These increases were observed both at the end of the ischemic period (and before reperfusion) and at every measurement thereafter (5, 10, 15, and 30 minutes and 1, 2, 6, 12, and 24 hours). At 15 minutes of reperfusion, the iSRS concentration reached a maximum regardless of the duration of ischemia. By 2 hours concentrations had dropped to 50 to 75 percent of peak values, and were less than 10 percent of maximum by 24 hours, but nevertheless were still elevated compared to those in animals that underwent sham procedures or in untreated controls. We do not know why brain iSRS levels do not differ between animals rendered ischemic for 5 or 15 minutes, but this could reflect excess levels of substrate or differences in the rate of clearance, degradation, or reacylation of arachidonic acid. Levels of iSRS in control animals were below the sensitivity limit of this assay (0.2 ng of $iLTD_4$; 0.12 ng of $iLTC_4$ gives 10 percent displacement). Values for i12-HETE were measurable at all time points in the brains of control as well as animals subjected to surgery. These values varied between 3 and 30 ng/g by weight but did not bear any obvious relation to the period of ischemia or reperfusion. A relation may exist but may be masked by the heterogeneity of our antiserum. The relation may reflect the synthesis of several serologically active monohydroxyeicosatetraenoic acids by many different cells.

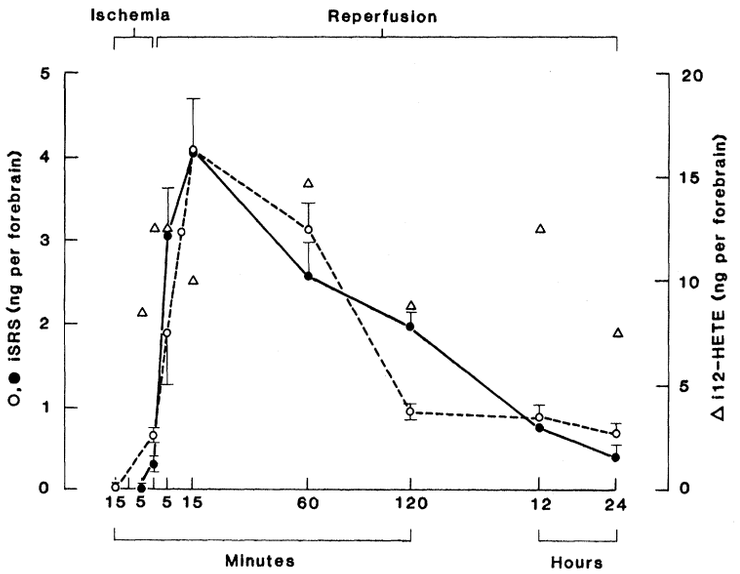

Fig. 1. Concentrations of iSRS and iHETE in forebrain homogenates after bilateral carotid occlusion for 5 minutes (●) or 15 minutes (○) and reperfusion. Values of iSRS are means ± standard errors for six data points. Levels of i12-HETE were determined in duplicate by pooling six samples from each time point. These values agreed with one another to within 20 percent. Results shown are from a representative experiment repeated five times. Increases in iSRS activity after ischemia and after reperfusion were highly reproducible and statistically significant in each of the five experiments ($P < 0.01$).

Leukotriene C_4 and LTD_4 are potent vasoconstrictors of human cerebral arteries in vitro (*13*). If one calculates the concentrations of iSRS in the gerbil brain after 5 to 15 minutes of ischemia alone or after ischemia plus reperfusion, the concentrations greatly exceed those reportedly required to constrict human cerebral arteries in vitro (< 2 n*M*). Since the entire gerbil forebrain is not rendered ischemic by bilateral carotid occlusions, levels are probably even more elevated in zones of ischemia than those we report. Because others have shown potent effects of leukotrienes on vascular permeability (*2, 14*), leukotrienes locally generated may be important in the development of cerebral edema after ischemia or ischemia plus reperfusion (*15*). In addition, LTD_4 may be important in the pathogenesis of seizures in this model (or in humans with embolic strokes), as it has been shown to markedly increase the firing rate of cerebellar Purkinje neurons after its iontophoretic application (*16*).

Brain extracts from animals subjected to 15 minutes of ischemia and 60 minutes of reperfusion were separated by reverse-phase HPLC. Radioimmunoassay was performed on the eluates (Fig. 2). LTC_4 and LTD_4 account for most of the immunoreactivity present. Low levels of LTE_4 could escape detection by this assay, since LTE_4 cross-reacts with antiserum to LTC_4 by only 6 percent. However, this may indicate that cells which synthesize SRS-like immunoreactivity in the brain do not contain high cysteinylglycine dipeptidase activity.

We then tried to determine the origin of SRS-like immunoreactivity in the brain. SRS production has been reported in lung tissue, macrophages, peritoneal cells, polymorphonuclear leukocytes, leukemic white cells, and mastocytoma cells (*17*). Organ cultures of cerebral arteries have been found to exhibit iSRS activity when challenged with calcium ionophore (*18*). We separated cerebral gray and white matter from frozen brains that had been subjected to 15 minutes of ischemia and 15 minutes of reperfusion (*19*). Concentrations of iSRS in cortical gray matter (18.23 ± 1.60 ng/g by weight) were more than three times higher than those in whole forebrain (5.06 ± 1.76 ng/g) under identical conditions of ischemia and reperfusion ($P < 0.001$, Student's two-tailed *t*-test). Levels of iSRS in white matter from these same brains were consistently be-

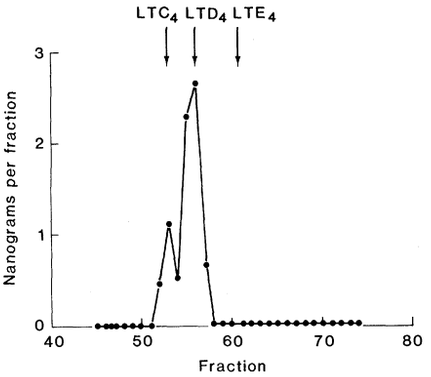

Fig. 2. Results of HPLC and radioimmunoassay of forebrain extracts from animals subjected to ischemia (15 minutes) and reperfusion (60 minutes). Leukotrienes were separated by reverse-phase HPLC with the Waters model 6000A pumps, model 660 solvent programmer, model U6K injector, and a 3.9 by 300 mm Waters μbondapak column. Samples were separated by using a linear gradient program from 100 percent solvent A to 100 percent solvent B over 100 minutes at a flow rate of 1 ml/min, and 1-ml fractions were collected. Solvent A consisted of 93.4 percent 0.01*M* phosphate buffer (*p*H 7.4) and 6 percent methanol (HPLC grade, Fisher) with 0.6 percent *t*-amyl alcohol (Aldrich); solvent B was 99.4 percent methanol with 0.6 percent (by volume) *t*-amyl alcohol. HPLC fractions were dried under nitrogen and resuspended in tris buffer for radioimmunoassay. Recovery after tissue extraction and HPLC ranged from 48 to 70 percent and was 56 percent in this experiment (7.64 ng recovered from a total of 13.73 ng of iSRS injected). This experiment was performed twice and both experiments produced similar results. Data from the first experiment are shown.

low the sensitivity limit of the assay (< 0.2 ng per ~ 350 mg of tissue by weight). Regions not supplied by the carotid arteries (brainstem and cerebellum) did not contain measurable levels of iSRS activity at any time. Since white matter contains glia, axons, and blood vessels and gray matter contains glia, axons, blood vessels, and neuronal perikarya, these results suggest that iSRS activity in the brain may reflect synthesis by ischemic cortical neurons or by populations of glial cells found in greater abundance in cortical gray matter (such as astrocytes) (*20*). Luminal white cells are unlikely to be the major source of SRS in the ischemic brain since white blood cells are contained in blood vessels of both gray and white matter.

To determine the extent to which blood vessels and its component cells synthesize iSRS-like immunoreactivity, we incubated cerebral blood vessels with Krebs-bicarbonate buffer (*21*). Bovine blood vessels were used because larger amounts were more easily and more inexpensively harvested. Baseline iSRS activity was low but measurable. The amount present (0.21 ng per gram of blood vessel by weight) was significantly lower than that measured in brain homogenates (3 ng/g) after ischemia and reperfusion and did not significantly increase upon the addition of arachidonic acid or the calcium ionophore A23187. Furthermore, the rate of iSRS formation in superfused cerebral arteries (0.15 ng/g per minute) was considerably lower than that in brain tissue which was made ischemic and then reperfused (1.33 ng/g per minute). It is, therefore, unlikely that the major source of iSRS synthesis in brains subjected to ischemia and reperfusion is cerebral blood vessels. This is further supported by the results of cell culture experiments in which conditioned media from bovine aortic endothelium, brain capillary endothelium, and smooth muscle cells (5×10^5) showed no measurable amounts of iSRS activity even after stimulation with arachidonic acid or calcium ionophore (*22*).

In conclusion, we demonstrated biosynthesis of leukotrienes in brain tissue under conditions of ischemia and reperfusion. Brain leukotrienes were not measurable in control animals and in brain tissues remote from the region of ischemia. Since brain damage may be incurred during the period of recirculation after ischemia (*23*), a possible role for leukotrienes should be sought in the pathogenesis of brain edema and changes in brain blood flow that complicate vascular occlusion. Also, it is important to develop drugs that block leukotriene biosynthesis or its receptor interactions. Finally, the possibility that leukotriene biosynthesis occurs under similar conditions in other organs deserves further investigation.

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8. Mongolian gerbils (50 to 70 g) were purchased from Tumblebrook Farms, allowed free access to food and water, and kept under diurnal lighting conditions for up to 3 weeks before experimentation. For bilateral carotid occlusions, the animals were lightly anesthetized with diethyl ether and the carotid arteries were exposed by a midline incision. Small vascular clips were placed on both common carotid arteries and removed after reperfusion. Before being decapitated, the animals were submerged in liquid nitrogen for 45 seconds. Frozen brains were removed on dry ice, weighed, placed in 2.5 ml of cold ethanol, and homogenized on ice with the Polytron C (Brinkmann) at a setting of 5 for 3 minutes. Supernatants were stored at −70°C until being assayed.
9. Cyclooxygenation products used as standards were obtained from Upjohn Co.; HETE standards from W. Hubbard; leukotrienes and SRS from R. E. Lewis; the μbondapak column from Waters Associates; SRS antisera from E. C. Hayes; and the radiolabeled products of cyclooxygenation and lipoxygenation from New England Nuclear.
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12. LTC_4, LTD_4, 11-*trans*-LTD_4, and LTE_4 react 100, 43, 48, and 6 percent, respectively, with this antiserum. Serologic analyses for the cyclooxygenase products, $PGF_{2\alpha}$ and PGI_2 (measured as its stable hydrolytic product, 6-keto $PGF_{1\alpha}$) were made with serologically homogeneous antisera. Radioimmunoassays for all arachidonic acid metabolites were performed in 3.5-ml polypropylene test tubes. The diluent for all reagents was 0.01*M* tris-HCl (*p*H 7.4) containing 0.014*M* NaCl and 0.1 percent gelatin.
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19. To separate gray and white matter we divided brains sagittally at the midline. Gray matter shavings were removed with a scalpel and pooled from five brains. White matter was removed from frontal lobes just under cortical gray matter. Both tissues (100 to 300 mg each) were kept frozen throughout the procedure. In all other respects the tissues were treated as described in text for forebrains.
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21. M. A. Moskowitz, M. Brody, L.-Y. Liu-Chen, *Neuroscience* **9**, 809 (1983). Pial arteries (1.5 g) were removed from bovine ventral and convex surfaces, washed with Krebs-bicarbonate buffer, and placed in the superfusion at 37°C. At various intervals Krebs-bicarbonate buffer containing arachidonic acid (0.1, 1.0, or 10.0 μ*M*) or A23187 was used to superfuse the arteries. Conditioned buffer was collected, extracted, dried, and radioimmunoassayed. This experiment was repeated twice.
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tion. Arachidonic acid (1 or 10 μ*M*) and A23187 (0.1 μ*M*) were each added to three wells. The conditioned medium was then concentrated and measured for iSRS activity (*11*). These experiments were repeated twice.
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24. We thank E. C. Hayes for SRS antiserum and C. Zervas for technical assistance. Supported by grant NS19038 from the National Institute of Neurological and Communicative Disorders and Stroke. M.A.M. is an established investigator of the American Heart Association. L.L. is an American Cancer Society research professor (award PRP-21).

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Synthetic Competitive Antagonists of Corticotropin-Releasing Factor: Effect on ACTH Secretion in the Rat

Abstract. *Polypeptide analogs of the known members of the corticotropin-releasing factor (CRF) family were synthesized and tested in vitro and in vivo for enhanced potency or competitive antagonism. Predictive methods and physicochemical measurements had suggested an internal secondary α-helical conformation spanning about 25 residues for at least three members of the CRF family. Maximization of α-helix–forming potential by amino acid substitutions from the native known sequences (rat/human and ovine CRF, sauvagine, and carp and sucker urotensin 1) led to the synthesis of an analog that was found to be more than twice as potent as either of the parent peptides in vitro. In contrast, certain amino-terminally shortened fragments, such as α-helical CRF or ovine CRF residues 8 to 41, 9 to 41, and 10 to 41, were found to be competitive inhibitors in vitro. Selected antagonists were examined and also found to be active in vivo.*

Corticotropin-releasing factor (CRF), a 41-residue peptide first characterized in ovine hypothalamic extracts (oCRF) (*1*), is the principal neuroregulator of the secretion of adenocorticotropic hormone (ACTH), β-endorphin, and other pro-opiomelanocortin products of the anterior pituitary gland (*2, 3*). Rat hypothalamic CRF (rCRF) has been isolated and sequenced (*4*), and an identical structure has been proposed for human CRF on the basis of the DNA sequence of the human CRF genome (*5*). Mammalian CRF's have approximately 50 percent homology with the frog skin peptide sauvagine (*6*) and the fish urophysial peptide urotensin 1 (U_1) (*7*); all of these peptides are equipotent stimulators of ACTH secretion in vivo and in vitro (*3*). The broad distribution in the central nervous system (*8*) of CRF and several demonstrated autonomic (*9*) and behavioral (*10*) actions of CRF suggest that this peptide may play important roles within the brain, especially during stress.

Peptide analogs are generally designed to fulfill certain needs that are either not satisfied or only partially satisfied by the parent compound. Analogs with higher affinity for their receptor and higher resistance to biodegradation may be more potent and longer acting. Some other substitutions may result in better chemical stability (for example, substitution of a methionine by norleucine or norvaline). Similarly, competitive antagonists of several regulatory peptides have been developed and shown to be useful for studying the physiologic roles of the corresponding endogenous peptides (*11, 12*) or for therapeutic applications (*13*).

Although antibodies to CRF proved useful in early studies of the role of endogenous CRF (*2*), they are of limited value because of their size, species specificity, antigenicity, and poor distribution in the brain (even when administered in the cerebral ventricles). Competitive antagonists of CRF were therefore developed to facilitate studies of the physiologic and pathophysiologic significance of endogenous CRF in experimental animals and, possibly, in human beings.

We have used pituitary cells in vitro (*14*) to assay the potency of our synthetic analogs relative to synthetic oCRF and to discover partial agonists and antagonists (*11*). In vivo experiments were performed as described (*2, 15*) (see Figs. 1 and 2). Peptides were synthesized by the solid phase method (*16*); purification of the crude synthetic peptides generated after treatment with HF and cleavage from the resin was achieved by prepara-

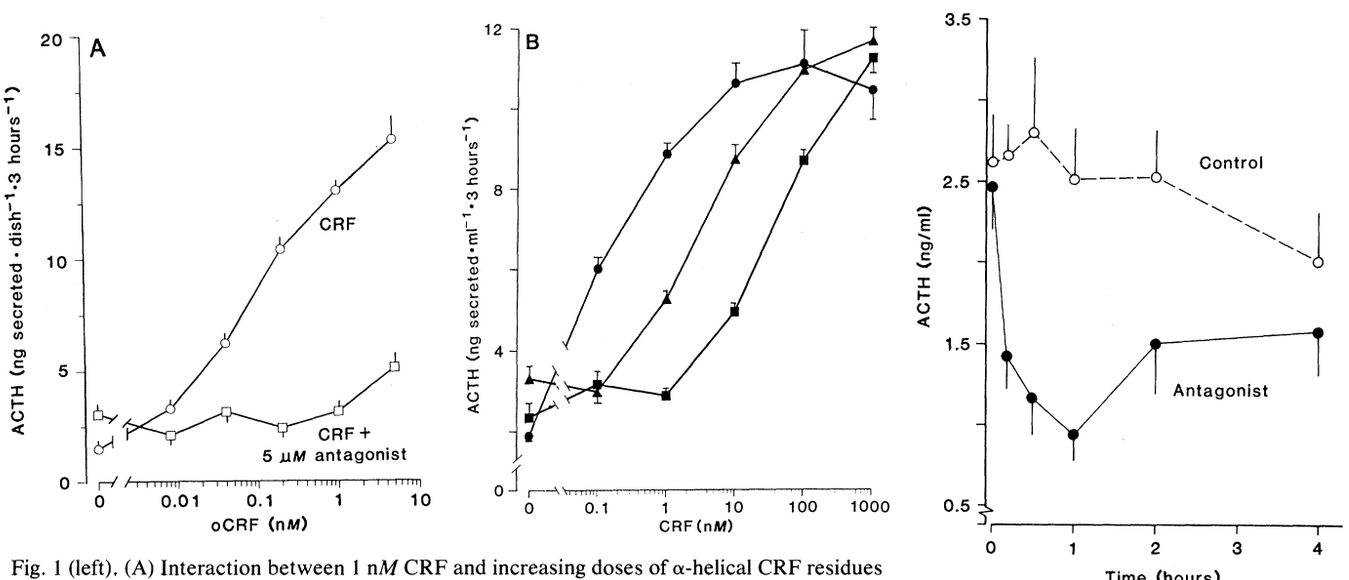

Fig. 1 (left). (A) Interaction between 1 n*M* CRF and increasing doses of α-helical CRF residues 9 to 41 on ACTH secretion by rat anterior pituitary cells in monolayer culture. Results are expressed as nanograms of ACTH secreted per tissue culture dish in 3 hours. (B) Effect of increasing doses of CRF on ACTH release in the presence of 500 n*M* or 5 μ*M* α-helical CRF (9 to 41). ●, Control; ▲, 500 n*M* α-helical CRF (9 to 41); ■, 5 μ*M* α-helical CRF (9 to 41). Fig. 2 (right). Effect of α-helical CRF residues 9 to 41 (antagonist) on ACTH release in nonanesthetized, adrenalectomized rats. Data represent mean ± S.E.M. (N = 6).