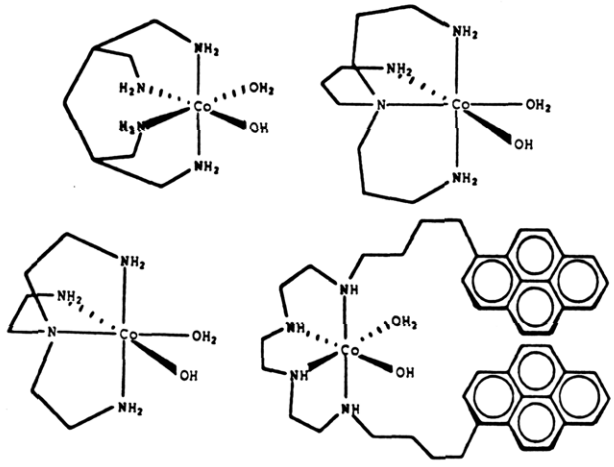
Abstracts, Division of Biological Chemistry, 3rd Chemical Congress of North America and 195th National Meeting of the American Chemical Society, June 5–10, 1988

Monday Morning—Symposium on Enzyme Mechanisms (Cosponsored with the Biotechnology Secretariat)—C. D. Poulter, Presiding

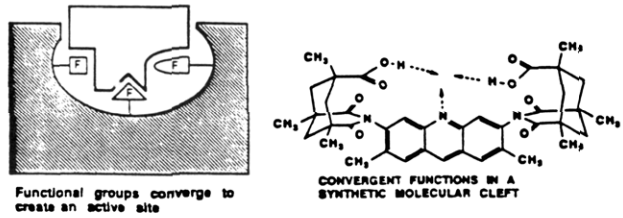
Designing Artificial DNases: Highly Efficient Hydrolytic Cleavage of Phosphate Diesters. *Jik Chin*, Mariusz Banaszczyk, Mark Drouin, Vrej Jubian, Stephen Kawai, Jung Hee Kim, Andrew Moore, Xiang Zou. Department of Chemistry, McGill University, 801 Sherbrooke St. W., Montreal, Quebec, Canada H3A 2K6.

Phosphate diesters bound to cobalt(III) complexes of the type $[(N_4)Co(OH)(OH_2)]^{2+}$ where N_4 represents a tetraammine ligand, are hydrolyzed up to a billion times more rapidly than the corresponding free esters. The reactivity of the cobalt complex is highly sensitive to the N_4 tetraammine ligand structure. This structure–reactivity relationship can be explained in terms of the mechanism of the hydrolytic process. The cobalt complex can be covalently linked to DNA binding molecules. One approach is to link the cobalt complex to various DNA intercalators. Another approach is to link the cobalt complex to sequence specific single strand DNA binding molecules.



Convergent Functional Groups in Recognition and Catalysis. *Julius Rebek, Jr.* Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260.

Recent developments involving model systems have led to a growing sense of confidence that behavior once thought to be characteristic of biochemical systems can be engineered into much smaller molecules. Catalysis, regulation, transport, and recognition are examples of these phenomena, and it has been possible to devise structural features that are responsible for these specific activities. In this paper, the effect of molecular shape on questions of recognition and catalysis will be discussed, and the use of model systems involving convergent functional groups will be described.



Acetolactate Synthase: An Unusual Flavoprotein with a Voracious Appetite for Herbicides. *John V. Schloss.* Central Research & Development Department, Experimental Station E328, E. I. du Pont de Nemours & Co., Wilmington, DE 19898.

Acetolactate synthase (ALS), the first common enzyme in the biosynthesis of branched-chain amino acids, has a rather unusual requirement for FAD in that the reaction it catalyzes involves no net oxidation or reduction. Further, reduction of the endogenous FAD, or replacement of the FAD by flavin analogues with higher or lower redox potentials, has little effect on the rate of the enzymic reaction, suggesting that there is no hidden or internal redox chemistry. As an additional curiosity, ALS has been found to be the site of action of several structurally diverse classes of commercial herbicides, and although flavin reduction has little effect on the rate of the enzymic reaction, it has a dramatic effect on the enzyme's affinity for herbicides. A potential explanation for the presence of FAD in ALS and its predilection for herbicides will be discussed.

Role of Biotin in Enzyme-Catalyzed Carboxylation Reactions. Takeshi Ogita and *Jeremy R. Knowles.* Department of Chemistry, Harvard University, Cambridge, MA 02138.

The mechanism of biotin-dependent enzyme-catalyzed reactions remains obscure, and the role of the biotin cofactor itself is a puzzle. We have earlier shown that, in the ATP-dependent carboxylation of N_1 of biotin by bicarbonate catalyzed by pyruvate carboxylase, the γ-phospho group of ATP suffers inversion at phosphorus. This result eliminates several pathways from further consideration, yet there remain a variety of possibilities for the mechanism of the biotin carboxylation reaction. We shall report the results of several experiments, including positional isotope exchange in ^{18}O-labeled ATP, fast quench trapping experiments to search for the intermediacy of carboxyphosphate, and ^{18}O tracer experiments, that illuminate the pathway followed by the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase.

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MONDAY AFTERNOON—SYMPOSIUM ON ENZYME MECHANISMS (COSPONSORED WITH THE BIOTECHNOLOGY SECRETARIAT)—G. L. KENYON, PRESIDING

Enzymes of the Shikimate Pathway: Mechanistic Inferences from Synthesis of Intermediates and Inhibitors. *Paul A. Bartlett*, Kunio Satake, Alexander Fässler, Robert Nyfeler, David G. Alberg, Paul M. Chouinard, Uday Maitra, Chuck T. Lauhon, Charles R. Johnson, Yumi Nakagawa, Mark E. Hediger, and John H. Bushweller. Department of Chemistry, University of California, Berkeley, CA 94720.

The shikimate pathway contains a number of enzymes which involve unique mechanisms or unusual transformations. The synthesis of reactive intermediates to probe these mechanisms and of enzyme inhibitors based on mechanistic considerations will be described. Among the enzymes being investigated are dehydroquinate synthase, 5-enolpyruvylshikimate-3-phosphate synthase, chorismate synthase, chorismate mutase, and prephenate dehydratase.

Enzymes in the Cholesterol Biosynthetic Pathway. Conversion of Presqualene Diphosphate to Squalene. *C. Dale Poulter* and Todd L. Capson. Department of Chemistry, University of Utah, Salt Lake City, UT 84112.

Squalene synthetase catalyzes the first pathway specific reaction in sterol biosynthesis. The enzyme converts two molecules of farnesyl diphosphate to presqualene diphosphate and catalyzes the NADPH-dependent rearrangement of presqualene diphosphate to squalene. The design, synthesis, and evaluation of mechanism-based inhibitors of the rearrangement step will be discussed.

Fluorinated Carbohydrates as Probes of Mechanism in Phosphoglucomutase and β-Glucosidases. *Stephen G. Withers*, M. David Percival, and Ian P. Street. Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, Canada V6T 1Y6.

An extensive series of fluorinated and deoxygenated substrates and inhibitors for phosphoglucomutase has been synthesized. Kinetic and ^{19}F NMR studies of these compounds bound to the enzyme have provided insight into the hydrogen bonding interactions around the sugar ring and also into the mechanism of substrate reorientation at the active site. Studies with phosphofluoridate analogues have demonstrated the importance of the dianionic phosphate moiety for turnover. A novel class of mechanism-based inhibitors of β-glucosidases is described. These are 2-deoxy-2-fluoro-β-D-glucopyranosides containing an excellent leaving group such as fluoride or dinitrophenol. Reactivation of the purified inactivated enzyme requires addition of glucoside. This requirement for a second sugar molecule has been investigated kinetically and by means of ^{19}F NMR. The stereochemistry of the inactivation of the enzyme by a prochiral fluorinated inhibitor has also been investigated kinetically and by ^{19}F NMR. Such inhibitors permit "active site titration" of the enzyme and can be used to identify essential catalytic residues.

Mechanism-Based Inhibitors of Acyl-Transfer Enzymes. *Allen Krantz*. Syntex Research (Canada), 2100 Syntex Court, Mississauga, Ontario, Canada L5N 3X4.

There is considerable interest, both theoretical and clinical, in the design of mechanism-based inhibitors of enzymes whose reactions are mediated by the formation of acyl enzymes. Within this domain, serine and cysteine proteinases, as well as enzymes engaged in glutamyl transfer, have been the object of inhibition studies by the Syntex Research Group in Canada. This talk will focus on strategies of inhibitor design and the mechanistic thinking that underlies successful inhibition of clinically relevant enzyme targets that form acyl enzyme intermediates.

TUESDAY MORNING—SYMPOSIUM ON ENZYME MECHANISMS (COSPONSORED WITH THE BIOTECHNOLOGY SECRETARIAT)—R. H. KLUGER, PRESIDING

A Quantitative Theory for Enzymic Catalysis. *J. A. Shafer* and N. E. Shafer. Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-0606.

A relationship was established between experimentally determined rate constants for analogous enzymic and nonenzymic reactions for cases where the enzyme appears to be expressing a maximal rate enhancement. The relationship $\log k_e = 5.8(\pm 0.3) + 0.29(\pm 0.02) \log k_{ne}$, where k_e is the measured first-order rate constant for conversion of enzyme-bound reactants and k_{ne} is the measured second-order rate constant for the analogous nonenzymic reaction, is followed for several solvolytic reactions. Absolute rate theory was used to develop a model of enzymic catalysis that not only accounts for the observed relationship but also predicts values for the activation energies of the correlated enzymic and nonenzymic reactions. Moreover, the theory relates values of internal equilibrium constants for interconversion of enzyme-bound reactants and products and external equilibrium constants for interconversion of unbound reactants and products.

Use of Isotope Effects to Deduce Enzyme Mechanisms. *W. W. Cleland*. Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

Adenosine deaminase shows smaller ^{15}N isotope effects in D_2O than in H_2O, while the reaction is faster in D_2O. These data suggest a D_2O equilibrium isotope effect on formation of the tetrahedral intermediate of ~0.4, which is consistent with a sulfhydryl group donating its proton to N-1 to permit addition of water at C-6 to give the intermediate. Tartronate semialdehyde-P (TSP) is an excellent inhibitor of enolase which is thought to become enolized upon binding. 2-Deuteriated TSP has a K_i value 2.2 times that of unlabeled TSP, suggesting that the base which removes the proton from 2-P-glycerate or TSP is a SH group. Secondary ^{18}O isotope effects are being used to determine transition-state structures for phosphoryl-transfer reactions, with dissociative mechanisms giving slightly inverse isotope effects and associative ones expected to give values several percent normal for 3-^{18}O. Alkaline phosphatase shows an isotope effect 0.4% more inverse at pH 6 (where catalysis is more rate limiting) than at pH 8 and thus appears to have a dissociative mechanism.

Structure–Function Relationships in Subtilisin. *David A. Estell*, Thomas P. Graycar, Robin Adams, Scott D. Power, Mark Ulltsch, Richard R. Bott, Brian C. Cunningham, Paul Carter, James A. Wells. Genencor Inc. and Genentech Inc., South San Francisco, CA 94080.

We have chosen to study in detail the relationship of structure to function in the enzyme subtilisin. More than 300 site-directed mutants of subtilisin have been produced. The effects of these mutations on substrate specificity, kinetic parameters, pH profile, and stability have been examined. The structures of several mutant enzymes have been solved, and the structural consequences of the mutations have been de-

termined. From this data base we are constructing a picture of the way in which structural changes are reflected in enzyme function for subtilisin.

Insights into How Fidelity Is Achieved by DNA Polymerase. *S. J. Benkovic* and Robert Kuchta. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

A complete kinetic scheme describing the polymerization of correct and incorrect dNTPs by the Klenow fragment of DNA polymerase I has been developed by using short DNA oligomers of defined sequence. The high fidelity arises from a three-stage mechanism. The first stage of discrimination (10^4–10^6-fold) primarily comes from a dramatically reduced rate of phosphodiester bond formation for incorrect nucleotides but also gains a smaller contribution from selective dNTP binding. After phosphodiester bond formation, a conformational change slows association of the incorrect DNA products from Klenow fragment which in conjunction with the 3′–5′ exonuclease increases fidelity 10–10^2-fold. Finally the enzyme polymerizes the next correct dNTP onto a mismatch, very slowly providing a further 10–10^2-fold increase in fidelity. Surprisingly, the 3′–5′ exonuclease does not differentiate between correctly and incorrectly base-paired nucleotides.

TUESDAY MORNING—SYMPOSIUM ON PEPTIDE HORMONES—D. H. RICH, PRESIDING

1. Design of ANF Analogues of Reduced Molecular Size and Increased Potency. *R. F. Nutt*, T. M. Ciccarone, S. F. Brady, T. M. Williams, R. Winquist, and D. F. Veber. Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

The atrial natriuretic factor Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (ANF), isolated from heart tissue, exhibits potent natriuretic, vasorelaxant, and hypotensive activities. Through structure–activity studies, sidechain and backbone conformational features important for eliciting biological activity have been identified. These studies have led to the successful design of ANF analogues of reduced molecular size, increased metabolic stability, and increased potency. Molecular size was reduced by deletion of the termini and optimization of the ring structure of ANF. The 19-peptide cyclo(Phe-Pro-Phe-Gly-Gly-Arg-Ile-Glu-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly) retains full biological activity but reduced potency. Increased vasorelaxant potency could be attained by incorporating a cationic moiety into the ring structure. Analogues more potent than ANF have been obtained by introducing conformation-stabilizing substitutions for glycine residues. Highly active single point modifications can be combined to give analogues with 5–10 times the potency of ANF. A model of a possible bioactive conformation can be constructed based on the structure–activity results.

2. New Concepts in Peptide Analogue Design. *Peter W. Schiller*. Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7.

A major goal in peptide drug development is the synthesis of peptide analogues with high specificity for a particular receptor class and with altered "efficacy" (superagonists or antagonists). Novel design principles were used for the development of peptide ligands interacting with opioid receptors (μ, δ, κ). Various performed peptide cyclizations resulted in conformationally restricted analogues displaying high μ-receptor selectivity, increased "efficacy", resistance to enzymatic degradation, and long duration of action. To test the recent hypothesis that δ-receptors might be located in a hydrophilic, positively charged environment and that μ-receptors might be contained in an anionic fixed-charge membrane compartment, we synthesized several enkephalin analogues carrying a net electrical charge. As expected, positively charged analogues turned out to be extremely μ-receptor selective, whereas several negatively charged peptides showed high preference for the δ-receptor. Finally, bivalent enkephalin–oxymorphamine ligands were synthesized and, in agreement with the recently proposed μ/δ receptor complex model, one of them showed much higher analgesic activity than expected on the basis of its receptor binding affinities.

3. Influence of Membrane Environments on Peptide and Protein Structure. *Charles M. Deber*. Research Institute, Hospital for Sick Children, Toronto M5G 1X8, and Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Ontario, Canada.

Interactions of proteins and peptides with lipids, particularly those involving membrane binding of extracellular ligands, may exert a broad influence on molecular events at, and within, cellular membranes. Experimental and predictive aspects of these phenomena will be examined: (1) *Lipid-induced secondary structure in proteins and peptides.* We will describe the complexes formed between phospholipids and amphiphilic bioactive peptides such as substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), an undecapeptide neuromodulator involved in the transmission of pain information. Experimental results from NMR, circular dichroism, and other physical techniques demonstrate that membrane binding and penetration induce significant secondary structure in substance P and related tachykinin peptides and identify specific membrane-interactive residues of the peptide. Such membrane-induced reduction in the neuropeptide conformational ensemble is discussed as a possible requirement for a receptor-cognitive conformation. (2) *Membrane penetration of receptor protein segments.* The possibility will be discussed that some residues in receptor aqueous domains may participate in transmembrane signal transduction, conceivably by ligand-activated membrane penetration of hydrophobic "epitopes" which ultimately contact cytoplasmic domains. Such penetration seems plausible in view of the experimental demonstration (in (1) above) of such properties in segments of bioactive peptides. (Supported by MRC and NSERC.)

4. Solid-Phase Reductive Alkylation Is a Powerful Tool for Probing Peptide Backbone and Side-Chain Function. *David H. Coy*. Peptide Research Laboratories, Department of Medicine, Tulane University Medical Center, New Orleans, LA 70112.

Although $NaCNBH_3$-mediated reductive alkylation of α and side-chain amino groups with Boc-amino acid aldehydes or various alkyl aldehydes and ketones has occasionally been used in peptide analogue design, it has been the recent adaptation of the reaction to solid-phase methodology (Sasaki and Coy, *Peptides 8*, 119, 1987; Hocart et al., *J. Med. Chem. 30*, 1910, 1987) which has enabled peptides to be made with sufficient speed for intensive SAR studies. Thus, peptide bond replacement in somatostatin with the CH_2NH group proved to be a useful probe of intramolecular CO–NH hydrogen bonding. Reduced peptide bond replacement in the bombesin tetradecapeptide yielded 2 receptor antagonists, $Ala^9\Psi[CH_2NH]Val^{10}$- and $Leu^{13}\Psi[CH_2NH]Leu^{14}$-BN. The latter

had an unprecedentedly low IC_{50} value of 35 nM against bombesin, and the results might be explained by a conformational shift in a folded receptor-bound peptide due to loss of one hydrogen bond. Turning to another peptide system, N^{α}-alkylation of GRF(1-29)NH_2 was found to have profound effects on GH releasing activity in the rat. Although the N-Me-Tyr^1 analogue was only about 50% as potent, N-Et and iPr analogues were 5 and 41 times more potent, respectively. Reductive alkylation of the α-amino group and both Lys side-chains with $NaCNBH_3$/acetone gave N^{α}-iPr-Tyr^1,N^{ϵ}-iPr-Lys^{12,21}-GRF(1-29)NH_2 which was more than 100 times more potent acutely and many times longer acting than GRF in the rat, perhaps suggesting improved stability to enzymatic degradation. These and other data indicate the potential of this approach in the production of potent and specific antagonists and superpotent agonists. (This work was supported by NIH Grants DK-18370 and DK-30167.)

TUESDAY AFTERNOON—SYMPOSIUM ON ENZYMES INVOLVED IN THE INFLAMMATORY PROCESS (COSPONSORED WITH THE DIVISION OF MEDICINAL CHEMISTRY)—A. KRANTZ, PRESIDING

Pathology of Oxygen-Derived Free Radicals. *Joe M. McCord.* Department of Biochemistry, University of South Alabama, Mobile, AL 36688.

Superoxide free radical ($O_2^{\cdot-}$) contributes to neutrophil bactericidal action and activates a chemoattractant which contributes to the development of the inflammatory response. Superoxide is also produced within postischemic (reoxygenated) tissue by the enzyme xanthine oxidase. The mechanisms of radical production are biochemically distinct in these pathological conditions, but there are logical links whereby an ischemia/reperfusion-induced injury would lead to inflammation, and vice versa. Much evidence now implicates $O_2^{\cdot-}$ and H_2O_2 in reperfusion injury to the heart. In the rat and dog, xanthine oxidase appears to be the primary source of active oxygen species, leading to inactivation of specific enzymes such as creatine phosphokinase, functional impairment of the mitochondria, and conductance abnormalities.

Serine Proteases of Leukocyte, Lymphocyte, and Mast Cell Origin: Substrate Specificity and Inhibition of Elastases, Chymases, and Tryptases. *James C. Powers.* School of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332.

A large number and variety of serine proteases have been isolated from different mammalian tissues and cells types. These enzymes are most active in the physiologic pH range, and several have been implicated in inflammation and diseases which involve tissue destruction. The most commonly studied enzymes are elastase from human polymorphonuclear leukocytes (PMNs), cathepsin G and chymases from mast cells and PMNs, and tryptases from mast cells and lymphocytes. Although the normal function of most of these enzymes is not known, they will attack and cleave a variety of connective tissue proteins including elastin, proteoglycan, and collagen. Inhibitors for elastase have been widely reported and now include both mechanism-based irreversible inhibitors and reversible transition-state analogues. Relatively few inhibitors have been reported yet for chymases and tryptases. Synthetic inhibitors, such as mechanism-based isocoumarins, have great potential for use in treatment of diseases which involve tissue destruction.

Potent New Inhibitors of Cathepsin B. *Roger A. Smith,* Peter Coles, Leslie J. Copp, Heinz W. Pauls, Valerie J. Robinson, Robin W. Spencer, and Allen Krantz. Syntex Research (Canada), 2100 Syntex Court, Mississauga, Ontario, Canada L5N 3X4.

New classes of compounds which act as time-dependent inhibitors of the thiol protease cathepsin B have been developed. In vitro K_2's as low as 10 nM and inactivation rates exceeding 10^6 M^{-1} s^{-1} have been determined, establishing some of these compounds as the most powerful cathepsin B inhibitors yet reported. The inactivation has also been characterized as irreversible, and investigations of enzyme adduct structure have been pursued. Mechanisms of inactivation, structure–activity relationships, and relevant biological activities will also be discussed.

Role of Metalloproteinases in the Physiological and Pathological Breakdown of Collagen. *Jaro Sodek* and Chris M. Overall. MRC Group in Periodontal Physiology, 4384 Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

The biophysical properties of most connective tissues can be attributed largely to collagen fibers which provide the structural framework of the tissue and are responsible for tissue integrity. Although collagen is resistant to almost all proteolytic enzymes, the fibers are continuously and extensively remodeled during the growth and development of connective tissues, in response to functional forces, and in wound healing. Extensive degradation of collagen also occurs in chronic inflammatory diseases such as rheumatoid arthritis and periodontal disease and is also associated with tumor invasion and metastasis. A class of neutral metalloproteinases, synthesized by mesenchymal, epithelial, and inflammatory cells, have been implicated in the degradation of collagen fibers in many of these processes. Collagen fiber degradation appears to be initiated by limited cleavage of the collagen molecule by collagenase and telopeptidase enzymes which render the molecule susceptible to complete degradation by gelatinases. Control of these enzymes may be achieved by specific proteinase inhibitors. To study the activation and regulation of collagenolytic enzymes, highly sensitive assays have been developed. These assays have also proved useful for monitoring connective tissue disease activity.

Enzymes and Receptors in the Arachidonic Acid Cascade as Potential Therapeutic Targets. *A. W. Ford-Hutchinson.* Merck Frosst Canada Inc., P.O. Box 1005, Pointe Claire-Dorval, Québec, Canada H9R 4P8.

Free arachidonic acid may be liberated from membrane phospholipids through the action of phospholipases and further metabolized by either cyclooxygenase or 5′, 12-, or 15-lipoxygenase. Cyclooxygenase is inhibited by non-steroidal antiinflammatory drugs and leads to the production of prostaglandins (PGs) and thromboxane A_2 (TxA_2). Selective therapeutic intervention in the cascade can be achieved through TxA_2 synthetase inhibitors or receptor antagonists (e.g., L-655,240), leading to selective modulation of platelet aggregation and smooth muscle contraction. The leukotriene (LT) pathway is initiated by 5-lipoxygenase which catalyzes both an oxygenase and a dehydrase step to produce the epoxide intermediate LTA_4. LTA_4 hydrolase leads to the production of LTB_4, a potent mediator of leukocyte and lymphocyte function. LTC_4 synthetase produces the LTC_4 which is then converted to LTD_4 and E_4, compounds associated with smooth muscle contraction. Therapeutic manipulation of the leukotriene cascade has been achieved through 5-lipoxygenase in-

hibitors (e.g., L-651-392 and L-656,224) and LTD_4 receptor antagonists (e.g., L-649,923 and L-648,051), and such compounds may have important therapeutic implications in human disease.

TUESDAY AFTERNOON—SYMPOSIUM ON PEPTIDE HORMONES—T. HOFMANN, PRESIDING

5. Novel, Nonpeptidal Gastrin/CCK-B Receptor Antagonists. *Roger M. Freidinger*, Mark B. Bock, Robert M. DiPardo, Ben E. Evans, Kenneth E. Rittle, Daniel F. Veber, Raymond S. L. Chang, and Victor J. Lotti. Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

We previously reported the development of potent, orally active 3-substituted 5-phenyl-1,4-benzodiazepinone antagonists of cholecystokinin (CCK) with high selectivity for peripheral (CCK-A) receptors. [B. E. Evans et al., *Proc. Natl. Acad. Sci. USA 83*, 4918 (1986); R. S. L. Chang and V. J. Lotti, *Proc. Natl. Acad. Sci. USA 83*, 4923 (1986)]. In this presentation, the design and synthesis of potent, nonpeptidal antagonists with selective affinity for gastrin and central CCK (CCK-B) receptors will be described. These compounds show much lower binding to CCK-A receptors. The importance of certain structural features of these molecules for defining their selective biological properties will be emphasized.

6. Synthetic Peptide Vaccines: Potential, Problems, and New Approaches. *B. H. Barber* and G. Carayanniotis. Department of Immunology, University of Toronto, Toronto, Canada M5S 1A8.

Relatively short (10–15 amino acid) peptides can, when coupled to foreign carrier proteins, be used to elicit pathogen-neutralizing antibody responses in experimental animals. However, the generation of effective anti-peptide responses almost always requires the use of a potent, nonspecific immunostimulator such as Freund's complete adjuvant (FCA). Because strong adjuvants such as FCA are forbidden for use in veterinary and human vaccine formulations, there exists an important practical problem in terms of realizing the potential of peptide vaccine agents. We have addressed this concern by developing an adjuvant-independent means of immunization based on the use of monoclonal antibodies (MAbs) as "targeting agents" for the delivery of antigen to specific cells of the immune system. By coupling antigens to MAbs for the class II gene products of the major histocompatibility complex, we have been able to prime mice for significant secondary antibody responses to both protein and synthetic peptide antigens in the absence of any adjuvant, thus offering new opportunities to manipulate the immune system via the use of synthetic peptide determinants.

7. Synthesis and Activity of Photoaffinity Labeling and Conformationally Constrained Analogues of Cyclosporine. *Daniel H. Rich*, Robert D. Tung, J. Aebi, Brian Dunlap, William Mellon, and A. Ruoho. School of Pharmacy, University of Wisconsin—Madison, Madison, WI 53706.

Cyclosporine A (CsA, 1) is an orally active, cyclic undecapeptide which displays potent immunosuppressive and antiparasitic activities and which is used widely to suppress rejection of transplanted human organs. We have developed new, simplified chemical methods for synthesizing the major fragments of the CsA ring (the MeBmt, the 8–11 tetrapeptide, and the 2–7 hexapeptide portions). With these key fragments now readily available to us, we have begun to develop compounds which might be of use in studying the biology of the cyclosporines. We describe in this lecture the chemical synthesis of new, conformationally constrained analogues of CsA, and the synthesis of a new, biologically active photoaffinity labeling analogue of CsA. The synthesis of a carrier-free ^{125}I-labeled AIPPS photoaffinity ligand attached to the ϵ-NH_2 of D-Lys^8-CsA has been accomplished. This analogue, which strongly inhibits Con-A-stimulated thymocyte proliferation, has been found to label isolated, purified cyclophilin when photolyzed in thymocyte cytoplasm.

8. Transforming-Epidermal Growth Factors. *James P. Tam.* The Rockefeller University, 1230 York Ave., New York, NY 10021.

The proliferation of normal cells is a well-controlled and highly conserved process that requires regulation by hormone-like growth factors. However, many tumor cells do not require serum growth factors for their proliferation. It is now believed that transformed cells produce their own factors to sustain their autonomous growth. One of the better characterized growth factors is the family of transforming growth factor type α (TGFα) and epidermal growth factor (EGF), which are small mitogenic proteins and members of a diverse group of proteins that share considerable similarities in sequence homology and structural motif. Whereas EGF is present in normal physiological conditions, TGFα has been known to be overproduced in oncofetal states. Furthermore, the TGFα–EGF family now includes homologous sequences found in domains of blood coagulation factors, extracellular matrix, DNA tumor virus early gene products, and invertebrate embryonic proteins, and none of these putative growth-factor sequences have been biochemically characterized. To understand the relationships between the chemical structures of these growth factors and their biological properties, we used solid-phase peptide synthesis to produce these growth factors, their structural variants, and shortened analogues. Our results showed that full biological activity of these growth factors required a tertiary structure maintained by the disulfide pairs and shortened analogues or extend structure without the disulfide restraint greatly reduced biological activity. However, several shortened analogues showed partial agonist or antagonist activity and may provide useful leads for the design of therapeutic useful agents.

WEDNESDAY MORNING—1988 BIOLOGICAL AWARDS SYMPOSIUM—S. J. BENKOVIC, PRESIDING

Eli Lilly Award Address by P. Walter
Pfizer Award Address by J. W. Kozarich
Alfred Bader Award Address by T. C. Bruice

9. Protein Translocation across the Endoplasmic Reticulum (ER) Membrane. *Peter Walter.* Department of Biochemistry and Biophysics, University of California Medical School, San Francisco, CA 94143-0448.

The first step in the secretion pathway of higher eukaryotic cells involves the selective recognition of the signal sequences of secretory proteins by the signal recognition particle (SRP) while these proteins are emerging from the ribosome. Correct targeting is then achieved by a direct interaction of the SRP in the nascent chain/SRP/ribosome complex with the SRP receptor, an ER-specific membrane protein. Thus SRP can be thought of as the adapter between the cytoplasmic translation and the membrane-bound protein translocation machinery. Its structure has been the subject of extensive study, which yielded a detailed molecular picture of the function of the particle in the signal recognition and targeting reactions.

This picture is complemented by studies on protein translocation in yeast, where the translocation step is not obligatorily coupled to protein synthesis, but translocation can occur posttranslationally. A unifying model evolved in which SRP-dependent targeting can be bypassed in yeast and allow the translocation of certain fully synthesized presecretory proteins by interacting directly with a secondary signal sequence receptor (distinct from SRP) in the yeast ER membrane.

10. Problems in Mechanistic Enzymology. *John W. Kozarich.* Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

The role of mechanism and stereochemistry in enzyme catalysis has received increased attention in recent years. Our laboratory has focused on several enzymes which are relevant to a number of problems of interest to bioorganic chemists and enzymologists. Selected topics based on our latest research will be discussed.

11. 30 Years of Bioorganic Research. *T. C. Bruice.* Department of Chemistry, University of California at Santa Barbara, Santa Barbara, CA 93106.

Selections of important advancements in bioorganic mechanisms which have originated from our laboratory over the past 30 years will be discussed. Cameos will be chosen from such research areas as acyl-transfer reactions, pyridoxal catalysis, dihydropyridine oxidations, flavin-catalyzed oxidations, dihydroflavin oxygen chemistry, methoxatin chemistry, $(Fe_4S_4)(SR)^{4n-}$ clusters, and "oxygen" atom transfer to and from metalloporphyrins.

WEDNESDAY AFTERNOON—1988 BIOLOGICAL AWARDS SYMPOSIUM—S. J. BENKOVIC, PRESIDING

Repligen Award Address by R. H. Abeles

12. Inhibition of Serine Proteases by Trifluoro Ketones. *Robert H. Abeles.* Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254.

Peptides containing a trifluoro ketone moiety have been prepared. These are effective inhibitors of chymotrypsin and pancreatic elastase as well as human leukocyte elastase. The structure of the enzyme inhibitor complex has been examined by X-ray crystallography and ^{13}C, ^{19}F, and ^{1}H NMR spectroscopy. X-ray crystallography of chymotrypsin–AcLeuPhCOCF$_3$ complex indicates that Ser-195γ O has added to the carbonyl group of the trifluoro ketone to form a hemiacetal. The oxygen of the hemiacetal is placed well into the anionic hole. The three fluorine atoms are well-defined and in some cases are close to the His residue. The rate of dissociation of the crystalline enzyme–AcLeuPhCOCF$_3$ complex was measured immediately after the complex was dissolved and found to be considerably faster than that of the corresponding complex formed in solution. NMR analysis of the enzyme–AcLeuPhCOCF$_3$ indicates that the inhibitor is bound as an ionized hemiketal. The "catalytic triade" is intact. The pK of the active-site His in the complex is 12.5. The only other pK detected is at 4.9, which we attributed to OH of the inhibitor hemiketal. Extended inhibitors were also prepared in which one of the fluorine atoms was replaced by peptide analogues corresponding to the P_1', P_2', P_3' position of the substrate. The additional interactions decreased $K_i \sim 2 \times 10^3$ fold compared to the parent difluoro ketone. X-ray analysis of the extended inhibitors reveals that the P′ residues can bind in at least three different modes, although the position of the P_1 residue remains constant.

WEDNESDAY AFTERNOON—POSTER SESSION—R. H. KLUGER, C. D. POULTER, E. SANCHEZ, AND J. STUBBE, PRESIDING

General

13. Prostaglandin Accumulation In Vitro by Mouse Reproductive Tissues as a Function of Maturation and Aging. *Simin Saffaripour* and Marjorie A. Jones. Chemistry and Biological Sciences, Illinois State University, Normal, IL 61761.

In our previous study the results suggested a net accumulation of PGE_2 in mouse uterine and oviductal tissues but not in ovary. This report will discuss our recent study of aging mice using the same procedure. Uteri, oviducts, and ovaries from mice of 2, 6, 8, 10, and 12 months of age were collected at autopsy. The tissues were cut into segments, weighted, and then incubated with [^{3}H]PGE_2 in 10 mM sodium phosphate buffer (pH 7.2). [^{3}H]PGE_2 was determined by scintillation spectroscopy after dissolution of tissue in Beckman BTS-450 tissue solubilizer. The tissue to medium ratio (T/M) was calculated as dpm/mg of wet weight divided by dpm/μL of supernatant. The results indicate that there is no significant difference in the T/M ratios for uterus between 2, 6, and 10 month old mice. However, these values are significantly different from the T/M ratio at 8 months, which reaches an apparent maximum. The results suggest that the T/M ratio in ovaries increases with age. For oviduct, no change was observed. We therefore conclude that these tissues all respond differently to PGE_2 as the age of the animal changes. (Supported by grants from Phi Sigma Society and ISU Organized Research Grant Program.)

14. Detection of E-9-Keto Reductase (9K-PGR) in Uteri from Rabbit and Mouse. *K. A. Farley*, M. J. K. Harper, and M. A. Jones. Department of Chemistry, Illinois State University, Normal, IL 61761, and Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, TX 78284.

9K-PGR is an enzyme which converts PGE_2 to PGF_2. Since these two PGs have different physiological actions, this enzyme could have an important regulatory role in reproduction. Therefore, we determined the specific activity of 9K-PGR in uterine tissues from both the mouse and rabbit at different reproductive states. Uteri from estrus or pregnant animals were incubated with [^{3}H]PGE_2. After incubation, the reaction was extracted twice with ethyl acetate; the extract was evaluated by HPLC to separate [^{3}H]PGE_2 from newly produced [^{3}H]PGE_2. Fractions from the HPLC were collected, and radioactivity was monitored by liquid scintillation spectrometry. The specific activities (fmol of product formed per g of wet weight of tissue per h) of 9K-PGR in the estrous rabbit and mouse were determined to be 8.52 and 141, respectively. The specific activity for day-6 pregnant rabbit was 2.68. From these data we conclude that the activity of the 9K-PGR varies from species to species as the reproductive status changes. (Supported by the ISU Organized Research Program.)

15. 2D NMR and Lanthanide-Induced CD Studies of 23-Hydroxychenodeoxycholic Acid. *B. Dayal*, G. Salen, and V. Toome. UMDNJ—New Jersey Medical School, Newark, NJ 07103, V.A. Medical Center, East Orange, NJ 07019, and Hoffmann-La Roche, Nutley, NJ 07110.

Patients suffering from cerebrotendinous xanthomatosis (CTX), an inborn error of metabolism in bile acid synthesis,

excrete excessive amounts of 23-hydroxylated bile alcohol, 5β-cholestane-3α,7α,12α,23*R*,25-pentol into urine. Furthermore, it has been suggested that increased (23*R*)-hydroxylase activity in these subjects results in (23*R*)-hydroxylation of bile acids. In order to investigate 23-hydroxylation/oxidation as a striking feature in (CTX), we have evaluated the structure and stereochemistry of (23*R*)-hydroxylated chenodeoxycholic acid by 2D NMR and lanthanide-induced circular dichroism studies. (This investigation was supported by U.S. Public Health Service Grants HL-17818 and AM-18707.)

16. Diffusion of a Homologous Series of Fluorescently Labeled Isoprenoid Alcohols in Model Membranes. *Bruce J. Balcom* and Nils O. Petersen. Chemistry Department, University of Western Ontario, London, Ontario, Canada N6A 5B7.

Isoprenoid alcohols of a wide range of sizes are known to be major constituents of both plant and animal cell membranes. While the largest homologues (20 isoprene units) are known to have an important role in glycoprotein biosynthesis, the ubiquitous nature of these alcohols also suggests a structural role. The molecules citronellol, geraniol, farnesol, solanesol, and dolichol were labeled with the fluorescent probe *N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-(methylamino)hexanoic acid. Fluorescence photobleaching recovery and related techniques have been used to investigate the diffusion and aggregation behavior of these molecules as a function of temperature and alcohol concentration. These measurements reflect the nature of the interaction between the host lipid and the hydrophobic isoprenoid chains.

17. Substances Which Affect the Bilayer to Hexagonal Phase Transition Temperature and Their Effect on Membrane Fusion. *Richard M. Epand*, James J. Cheetham, and Remo Bottega. Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5.

A variety of hydrophobic and amphiphilic substances have marked effects on the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines. Alkanes and diacylglycerols, for example, are potent hexagonal-phase promoters, while lysolecithin inhibits the formation of the hexagonal phase. Not all hydrophobic and amphiphilic substances, however, strongly modulate the bilayer to hexagonal phase transition. Cholesterol has relatively little effect on this transition, probably because it undergoes lateral phase separation. Epimerization of the 3-OH group to give epicholesterol produces a steroid with less tendency to self-associate. Epicholesterol promotes hexagonal-phase formation. Making the headgroup more polar by sulfation produces cholesterol sulfate, which is a bilayer stabilizer that may have important physiological functions. Many of the bilayer stabilizing agents inhibit membrane fusion and have antiviral activity.

18. Topography of Acetylcholinesterase in Liposomes. *R. F. Barber*, L. Stuhne-Sekalec, P. N. Shek, and N. Z. Stanacev. Department of Clinical Biochemistry, University of Toronto, Toronto M5G 1L5, and Defence and Civil Institute of Environmental Medicine, Downsview, Ontario M3M 3B9, Canada.

Unilamellar liposomes prepared from DMPC in the presence and absence of acetylcholinesterase, as obtained from a commercial source or following purification by column chromatography, were examined by ESR for lipid/protein interactions. Using 5-, 12-, and 16-doxylstearic acid probes incorporated into the phospholipid bilayers, no significant differences in the gel to liquid-crystalline phase transition of DMPC (as determined by various spectral parameters) were observed when the enzyme was present. These results have established that no significant incorporation of acetylcholinesterase into the hydrophobic portion of the phospholipid bilayer is detectable at the protein:lipid ratios used in these experiments. Confirmation of these results was also obtained by differential scanning calorimetry. Interaction with the outermost region of the bilayer was established by trypsin digestion which indicated that as much as 25% of liposome-associated enzyme was removable and must, therefore, have been exposed on the outer surface. The results of this study have established that acetylcholinesterase associated with unilamellar DMPC liposomes was not present in the phospholipid bilayer but was primarily entrapped within the aqueous compartment of the vesicles. (This work was supported by the Department of National Defence, Canada.)

19. Effect of Chemical and Enzymatic Modification of *E. coli* ALP on Immunoreactivity with Serum. Thomas J. Hunter, Pratap Singh, and Susan A. Evans. Dade Division, Baxter Healthcare Corp., P.O. Box 520672, Miami, FL 33152-0672.

A constituent in human serum shows immunoreactivity, when used in enzyme-linked immunoassay, with the conjugate prepared by covalent coupling of hapten with *E. coli* alkaline phosphatase (ALP). Chemical and enzymatic modifications of ALP were carried out to study the effect of the modifications on immunoreactivity. Results of these modifications of ALP on its enzyme activity, kinetic properties, and spectral and HPLC characteristics along with the immunoreactivity to human serum will be presented.

20. Bridging Group Effect in Immunoassay and for *N*-Acetylprocainamide. *Pratap Singh*, Sonia L. Srebro, Mary M. Brotherton, and Williams S. Knight. Dade Division, Baxter, P.O. Box 520672, Miami, FL 33152.

We investigated the effect of bridge length on the recognition of the hapten and hapten–enzyme conjugate by the antibody in an enzyme immunoassay for *N*-acetylprocainamide (NAPA), a metabolite of the antiarrhythmic drug procainamide. Antibodies were produced in rabbits by using an immunogen prepared from 3-*O*-(4-carboxybutyl)-NAPA and albumin. Conjugates were prepared by coupling of alkaline phosphatase with the derivatives of NAPA synthesized for this study. Performance of these derivatives and their conjugates was evaluated on the Stratus fluorometric enzyme immunoassay system. Although four compounds in this series showed better displacement than free NAPA, only one conjugate prepared from 2-*O*-(3-carboxypropenyl)-NAPA showed the most sensitive displacement curve and the lowest nonspecific binding. These results showed that for enzyme labeling a shorter bridge length with a rigid component (double bond) resulted in increased sensitivity in this assay, thus demonstrating the importance of bridging group in influencing the sensitivity of the enzyme immunoassays.

21. Blocked Amino Acids Suitable for Peptide Synthesis. 1. Benzyloxycarbonyl Blocked Amino Acids. *Suresh B. Damle* and James A. Krogh. PPG Industries, Inc., Performance Chemicals, Chicago, IL 60666.

A systematic study was undertaken to develop processes for large-scale synthesis of benzyloxycarbonyl blocked amino acids suitable for peptide synthesis. It soon became apparent that

each amino acid required a specific set of conditions such as pH, catalyst, quantity of reagent, and isolation techniques. Processes for a number of different benzyloxycarbonyl blocked amino acids will be described, and a rationale for the conditions required will be discussed. The use of inexpensive benzyl chloroformate is adequate to produce high-purity benzyloxycarbonyl blocked amino acids contrary to published literature which recommend expensive and/or exotic reagents. A generalized study is under way in our laboratories to produce various blocked amino acids in high purity required for peptide synthesis.

22. Blocked Amino Acids Suitable for Peptide Synthesis. 2. Preparation of Dipeptide-Free *N*-Benzyloxycarbonyl Amino Acids Using Benzyl Chloroformate. *James A. Krogh* and Suresh B. Damle. PPG Industries, Inc., Performance Chemicals, Chicago, IL 60666.

Recent references on the preparation of *N*-benzyloxycarbonyl amino acids promote the use of dibenzyl dicarbonate and other alternative acylation agents as methods of choice for the preparation of essentially dipeptide-free products. All of these acylation agents are prepared from the reaction of benzyl chloroformate with at least one other expensive and/or commercially unavailable reagent which precludes their use in industrial scale syntheses. As a major commercial supplier of benzyl chloroformate, our approach has involved modification of the traditional Schotten–Baumann reaction in which an amino acid is reacted with benzyl chloroformate under pH stat conditions. In an attempt to develop a general methodology of choice for the preparation of *N*-benzyloxycarbonyl amino acids, the optimization of the pH range for a given amino acid is defined from its pK_2 value. The results of several examples of *N*-benzyloxycarbonyl amino acids prepared by our procedure are presented.

23. Selective Pyrophosphorylation of Primary Hydroxyl Groups. *Stephen Bearne*, Khashayar Karimian, and Ronald Kluger. Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A1.

It is often necessary to prepare biologically active pyrophosphates from alcohol precursors. Derivatives of thiamin and nucleosides can be converted to pyrophosphates by reaction with partially dehydrated phosphoric acid. These conditions cause reaction to occur at primary and secondary hydroxyl groups. The use of cresol as a cosolvent promotes selective reaction at primary hydroxyl groups in 2-(1-hydroxyethyl)-thiamin and in pyrimidine nucleosides.

24. Chiral Sequence Analysis of Peptides. *Peter S. Marfey*. Department of Biological Sciences, State University of New York—Albany, Albany, NY 12222.

Ordinary Edman sequence analysis of a peptide involves stepwise removal of an amino acid residue from the N-terminus of a peptide and its identification as a 3-phenylthiohydantoin derivative. The derivative is easily racemized, and this precludes its use for determining optical purity of the removed residue. We have used three different approaches to determine optical purity of an amino acid residue during peptide sequencing. The first approach is based on the use of the FDA reagent (F-DNP-L-Ala-NH_2) to derivatize the N-terminal residue of a peptide, subsequent reduction of nitro groups of the attached reagent, formation of a Cu^{2+} or Co^{3+} chelate, hydrolysis of the adjacent peptide bond, and quantitation of the released diastereomeric complex. The second approach is based on a transformation of the released 2-anilino-5-thiazolinone during ordinary Edman degradation to a diastereomeric derivative and its quantitation by HPLC. The third approach is based on the use of a chiral aromatic isothiocyanate to form a diastereomeric thiohydantoin derivative which can be quantitated under nonracemizing conditions with either TLC or HPLC.

25. Radioiodinated Site-Specific Monoclonal Antibody Conjugates for Tumor Therapy and Imaging. *B. A. Belinka, Jr.*, D. J. Coughlin, and V. L. Alvarez. CYTOGEN Corp., 201 College Road East, Princeton, NJ 08540.

The use of radiolabeled monoclonal antibody carriers for tumor imaging and therapy has been the subject of a great deal of study in recent years. While successful results using metal ion chelates and iodinated antibodies have been reported, MAb conjugates which provide greater in vivo stability while retaining tumor specificity are desired. We now wish to report the results of work in our laboratory which has studied the use of monoclonal antibody conjugates of radioiodinated organic compounds. These organoiodine carriers were designed to provide for site-specific attachment to the oxidized carbohydrate portion of antibodies which allows optimal antibody–antigen recognition. In addition, synthetic planning for maximum metabolic stability of the iodinated conjugates was examined. A discussion of the rational design, synthesis, and radiolabeling techniques of the radioiodine carriers as well as the results of biological studies will be presented.

26. Two-Dimensional Gel Electrophoresis and Computer Analysis of Water/Salt-Soluble Proteins in Corn. *J. N. Neucere*. USDA ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179.

Computer gel matching following isoelectric focusing and SDS–PAGE was established for the water/salt-soluble proteins in fungal-resistant (Yellow Creole) and fungal-susceptible (Huffman) corn. The samples were loaded at 15 micrograms per gel onto a broad range (pH 3–10) ampholine in the first direction and a 12.5% acrylamide second dimension. The contact prints and stained gels were analyzed as a matchset, whereby the Huffman profile was used as the standard in establishing sample spot numbers. The results showed 206 protein in the Huffman profile, whereby 158 spots from Yellow Creol were matched. The data correlated molecular weights and isoelectric points of each protein with intensity of radioactive images and spot staining for each sample gel. Confirmed (resolved) protein spots were obtained through a combination of computer analysis and visual detection of gels and films. The isoelectric point and molecular weight of each protein were approximated within a 10–15% error range. Hence, the data yielded qualitative and semiquantitative information on total dissociated proteins in the two varieties within a specific pH range.

27. New Means for Analysis of Dilute (Micromolar) Compounds Lacking Detection Handles: Microbial Calorimetry. *Rex Lovrien*, Irwin Boe, and Mark Ferrey. Biochemistry Department, University of Minnesota, St. Paul, MN 55108.

Bacteria are effective tools for analysis of many compounds, especially after the bacteria are grown on them. After growth (adaption) to acetate, methanol, sugars, etc., bacteria are reactive effective reagents that can bind such compounds with micromolar K_ms, then "burn" them in 200–400 s if the cells are in excess (ca. 1 mg of cells, 30–200 nmol of compound).

The metabolic heats are large, 30–70 kcal/mol carbon atoms (*Biotech. Bioeng. 22*, 1249–69 (1980)). Microorganisms are easily gotton and grown, yet are quite specific reagents that bind such compounds in a few seconds and metabolize them quickly if adapted. Used in aerobic calorimetry, they enabled us to develop a new means for analysis of compounds that still are inconvenient to detect (unless isolated, worked up, derivatized) in HPLC and related methods. Microbial calorimetry is well suited for use with raw, turbid samples that are difficult for spectrophotometry. There has been considerable development in biosensor devices by numbers of workers, but most of them are confined to millimolar analytes and to narrow classes of compounds. (Support: Bioprocess Institute, University of Minnesota.)

28. Cyclic Voltammetric Studies of Flavins in Dioxane/Water. *R. Marrero* and H. Crespo. Chemistry Department, Humacao University College, University of Puerto Rico, Humacao, PR 00661.

Lumiflavin (7,8,10-trimethylisoalloxazine) undergoes a reversible reduction in dioxane/water solutions as determined by cyclic voltammetry. Preparations of the oxidized form of the flavin were scanned under nitrogen between +1.00 and −1.00 V in varying solvent combinations of purified dioxane/water. Dioxane/water ratios varied from 4:1 to a completely aqueous media. The solutions were prepared with deionized water buffered to pH 7.50 by using HEPES (0.100 M). Sodium chloride was used as the supporting electrolyte. Likewise, the oxidized forms of riboflavin, FMN, and FAD were studied. The results of this study show predicted change in reduction potential of flavins as the dielectric constant of the solvent and hydrogen bonding with the flavin changes. The mechanistic implications of these results on biochemical processes in low dielectric constant environments will be discussed.

29. Regeneration of Orthophthaldehyde Reagent for Amino Acid Analysis by HPLC. *Michael E. May*, Kebreab Gebreselasi, and Laurel L. Brown. Department of Medicine Vanderbilt University, Nashville, TN 37232.

Analysis of leucine by reversed-phase HPLC of the orthophthaldehyde (OPA) derivative is rapid and quantitative. A commercial solution (OPA + HSC_2H_4OH + KBO_3 + Brij-35) has been reported stable for months and is used in our laboratory for isocratic determination of plasma branched-chain amino acids. Deterioration of reagent is noted as low peak heights followed by loss of resolution and occurs if reagent is left at room temperature. We report regeneration of high-resolution chromatograms with addition of thiol (0.4–4 mol/mol OPA) or $NaHSO_3$ (excess) prior to but not simultaneously with addition of amino acid. OPA + $NaHSO_3$ alone does not form fluorescent derivatives of amino acids, and excess thiol inhibits the reaction. We conclude that "reagent deterioration" represents oxidation of the thiol to the disulfide, and regeneration is easily accomplished with minimal thiol or reduction of the disulfide.

30. Functional Coupling between the Yellow Fluorescent Protein of *Vibrio fischeri* Y-1 and the Luciferases from Other Bioluminescent Bacteria. *S. Colette Daubner*, Mary L. Treat, and Thomas O. Baldwin. Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

The bioluminescent marine bacterium *Vibrio fischeri* strain Y-1 (ATCC 33715) emits yellow light (535 nm) rather than the blue-green light (475–490 nm) given off by similar species. This shift in bioluminescent emission is due to the presence of the FMN-containing yellow fluorescent protein (YFP). When added in purified form to a *V. fischeri* Y-1 luciferase assay, YFP caused a bimodal spectrum to occur, with peaks at 484 and 534 nm. A similar bimodal spectrum resulted when the luciferase used in the assay was isolated from other marine strains, namely, *V. fischeri* (ATCC 7744) and *Photobacterium phosphoreum*. No conditions were found which allowed the shape of the emission spectrum from *V. harveyi* to be altered by additions of YFP. An oligonucleotide probe prepared based on the N-terminal amino acid sequence of YFP hybridized in Southern blots to a single band from a *V. fischeri* Y-1 genomic DNA preparation. [This work was supported in part by a grant from the National Science Foundation (NSF DMB 85-10784).]

31. Dietary Butylated Hydroxytoluene Enhances 2-(Acetylamino)fluorene-Mediated Induction of Cytochrome P-450c in Nuclei and Microsomes of Rat Liver. *R. Carubelli*, S. A. Graham, H. Miller, S. S. Park, H. V. Gelboin, and F. K. Friedman. Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, and National Cancer Institute, Bethesda, MD 20892.

Male Sprague-Dawley rats fed semipurified diet (10% w/w fat) containing 0.05% (w/w) 2-(acetylamino)fluorene (AAF) for 3 weeks show marked loss of spectral cytochrome P-450 content in liver nuclear envelopes (NE) but not in microsomes. Western blot analysis of these fractions using monoclonal and polyclonal antibodies against cytochrome P-450c (the major 3-methylcholanthrene-inducible form in liver microsomes) showed that P-450c is induced in rats fed AAF. Since dietary butylated hydroxytoluene (BHT; 0.3% w/w) affords some protection against AAF carcinogenesis in rats fed high-fat diets (e.g., 25.6% w/w beef tallow + corn oil, 9:1) and preserves NE cytochrome P-450 content for 9 weeks, we investigated the effect of BHT and AAF + BHT on nuclear and microsomal P-450's. The inducibility of P-450c of nuclei, NE, and microsomes by AAF was enhanced by AAF + BHT; no P-450c was detectable in controls fed diets with or without BHT. The constitutive P-450d form was similarly induced in rats fed AAF or AAF + BHT. These results suggest that regulation of cytochrome P-450 may play a crucial role in the nutritional modulation of AAF hepatocarcinogenesis. (Supported in part by Am. Inst. Cancer Res. Grant 83B17C84BR86B.)

32. Comparison of Toxicities for Some Analogues of Taurine. *Charles C. Otken.* Department of Chemistry, Pan American University, Edinburg, TX 78539.

Sturman et al. (*Annu. Rev. Nutr. 1*, 401–25, 1981) have shown that taurine (2-aminoethane-1-sulfonic acid) is notable among the free amino acids of milk, brain, and other tissues and that gerbils exhibit higher levels of this compound than do other animals. Taurine appears to be a necessary dietary factor for gerbils (Otken et al., *Nutr. Rep. Int. 31*, 955–62, 1985) in that growth rates were improved when this substance was included in purified amino acid diets fed to this species. Levels of taurine in the gerbil fall rapidly on taurine-free rations. Previously, taurine had been found necessary in the diets of cats and infant monkeys. Homotaurine, the next higher homologue (3-aminopropane-1-sulfonic acid), is quite toxic. Several other analogues of taurine, notably isotaurine, the positional isomer (1-aminoethane-1-sulfonic acid), were synthesized, and relative toxicities were tested by feeding to

gerbils in purified amino acid deits. Some chemical and physical properties of these compounds were compared.

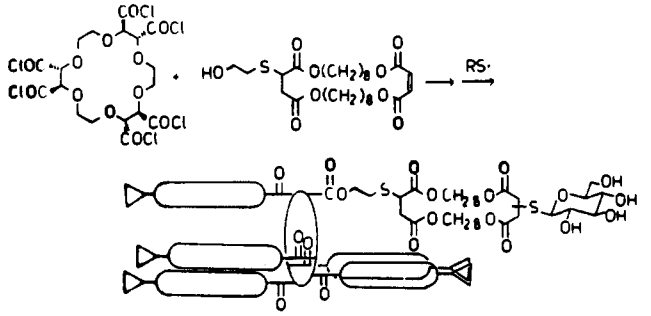
33. Study of the Biochemical Effects in the Cell Composition of Trypanosoma Parasite. Claudia Anaya, Guillermo Gally, Fernando Undapilleta. Culiacán 33-302 Col. Hipódromo, C.P. 06100, México, D.F.

The trypanosoma is a protozoa eventually presented on mammals blood, transmitted to human beings by means of a fly named "tse-tse", and occasions a disease known as "sleeping sickness". This protozoa has the capability to evade the immunological stress of mammals due to specific changes that happen in its cellular structure. The disease typically appears in certain tropical zones. In this paper are presented the results on experimental genetics, considering its biochemical aspects, that originate a change in the parasite structure, according to a new technique developed of change of "genetic information", by means of a recombinant DNA effect. This converts the parasite in a nonpathogenic specia, with obvious favorable results.

Model Studies

34. Construction of a Unimolecular Channel for Bilayer Membrane Ion Transport. Vicky A. Carmichael, *Philip J. Dutton*, Thomas M. Fyles, and Jennifer Swan. Department of Chemistry, University of Victoria, B.C., Canada V8W 2Y2.

A prototype of a unimolecular ion channel capable of spanning a bilayer membrane was designed from elementary considerations of shape, size, and complementary functionality. Structures of suitable size can be efficiently assembled from crown ether polycarboxylic acids and macrocyclic tetraesters as illustrated below. Transport of ions across bilayer membranes was studied by using unilammelar egg phosphatidylcholine vesicles. Ion influx was monitored by fluorescence techniques. Prospects for biomimetic ion channels assembled by this strategy may be assessed from the kinetic behavior of simple structural variants.



35. Mechanism of Amine Oxidation by Methoxatin and 9-Decarboxymethoxatin. *Evelyn J. Rodriguez* and Thomas C. Bruice. Department of Chemistry, University of California—Santa Barbara, Santa Barbara, CA 93106.

The finding that methoxatin is the cofactor for several bacterial as well as mammalian enzymes has created interest in its reactivity. The intact enzyme as well as the cofactor itself catalyzes the oxidation of primary amines and alcohols to aldehyses. We have studied the mechanism of amine oxidation by methoxatin and its analogue 9-decarboxymethoxatin using benzylamine and its α,α-dideuterio derivative as substrates. Our results suggest the formation of a covalent adduct between benzylamine and the cofactor from which two pathways for amine oxidation are available. These mechanistic results will be discussed in detail.

36. Kinetics and Mechanism of Alkene Epoxidation Employing Manganese(III) Porphyrin and Hypochlorite in a Homogeneous Organic System. *Robert W. Lee*, Paul C. Nakagaki, and Thomas C. Bruice. Department of Chemistry, University of California—Santa Barbara, Santa Barbara, CA 93106.

The modeling of the peroxide shunt mechanism of cytochrome P-450 has been the subject of numerous investigations. The use of various oxygen atom donors has contributed greatly to our present understanding of how the enzyme functions. However, due to the rate-limiting oxygen transfer from oxidant to metalloporphyrin, the determination of the second-order rate constant for alkene epoxidation by the hypervalent metalloporphyrin has been difficult. In a homogeneous methylene chloride system, the rate constants for the oxygen transfer from lithium hypochlorite to [5,10,15,20-tetrakis(2,4,6-trimethylphenyl)porphinato]manganese(III) chloride and subsequent oxygen transfer to alkenes has been determined. The mechanistic aspects of this work will be discussed.

37. Model Studies Relating to the Reactions Catalyzed by Enzymes of the Organomercurial Detoxification System in Bacteria. *Enona Gopinath* and Thomas C. Bruice. Department of Chemistry, University of California—Santa Barbara, Santa Barbara, CA 93106.

Bacterial detoxification of organomercurials is believed to result from the cleavage of the carbon–mercury bond by an organomercurial lyase enzyme followed by the reduction of Hg^{2+} to Hg^0 by a mercuric reductase enzyme. The catalytic mechanism of neither enzyme is well understood. Protodemercuration of organomercurials occurs under strongly acidic conditions. However, organomercurial lyase enzymes carry out what is formally a protonolysis with optimal activities at neutral or basic pH. In this investigation we show that bis(thiol) ligation of an organomercurial salt allows cleavage of the C–Hg bond by reversible stepwise ligation with a weakly basic oxyanion followed by specific acid protodemercuration at pH values between 4 and 8. These reactions investigated mark the clearest cases of nucleophilic catalysis of protodemercuration, which is shown to occur through a two-step mechanism. A similar mechanism is proposed as a plausible means for enzyme catalysis.

38. Boronic Acids Catalyze the Hydrolysis of Salicylaldehyde Schiff Bases. Galla Rao, Liliah Anand, and *Manfred Philipp*. Department of Chemistry, Lehman College/City University of New York, and Biochemistry Program, Graduate Center of City University of New York, New York, NY 10468.

Boronic acids are well-known transition-state analogue inhibitors of serine proteases. They are also known to reversibly bind to amino alcohols, diols, and hydroxy acids. Boric and boronic acid catalyses have been a topic of interest for some time. We have found that boronic acids catalyze the hydrolysis of salicylaldehyde Schiff bases. The kinetics of these catalyses show a binding step preceding catalysis. The range of second-order rate constants at pH 6.0 extends from 0.094 M^{-1} s^{-1} for 4-tolueneboronic acid to 2.35 M^{-1} s^{-1} for 2,5-bis(trifluoromethyl)benzeneboronic acid to 75 M^{-1} s^{-1} for diphenylborinic acid. Boronic acid catalyses are inhibited by fructose and require the *o*-hydroxy group on salicylaldehyde. First-order catalytic rate constants are hardly affected by Hammett sigma. Observed dissociation constants decline with higher values of sigma. The pH profile suggests that the neutral boronic acid is the effective catalyst. (We thank the

Research Corporation and ACS Project SEED for support.)

39. Protein-Mediated Activation of Aromatic Substitution Reactions. *Elisa M. Woolridge* and Steven E. Rokita. Department of Chemistry, State University of New York, Stony Brook, NY 11794.

Tryptophanase and tryptophan synthase have been chosen as models for our investigation of protein-mediated aromatic substitution reactions. These PLP-containing enzymes facilitate the reversible C-3 alkylation of indole. While the mechanism of electrophilic catalysis intrinsic to PLP is well documented, activation of indole toward substitution is not as thoroughly understood. The protein-dependent transformation of indole analogues is used to identify the method of aromatic activation. Specifically, 3-haloindoles and 3-halomethylindoles have been developed to probe for general acid–base catalysis.

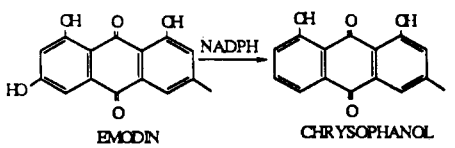
Biosynthesis

40. Biosynthesis of 5-Deazaflavin. *S. S. Lee*, J. M. Beale, Jr., H. G. Floss, and A. Bacher. Department of Chemistry, The Ohio State University, Columbus, OH 43210, and Lehrstuhl für Organische Chemie and Biochemie, Technische Universität München, Garching, West Germany.

Coenzyme F_{420} is one of the cofactors involved in the net reduction of CO_2 to CH_4 in methanogenic bacteria. The redox-active moiety of this molecule is 7,8-didemethyl-8-hydroxy-5-deazaflavin. The structural similarity of riboflavin and 5-deazaflavin raises the question whether the two types of chromophores are produced by similar metabolic pathways. The heterocyclic and ribityl moieties of 5-deazaflavin and riboflavin are biosynthesized from guanosine triphosphate in *Methanobacterium thermoautotrophicum*. However, the carbocyclic ring of the 5-deazaflavin is derived from the shikimate pathway via a symmetrical intermediate. Feeding experiments with labeled precursors were conducted in *Streptomyces aureofaciens* to probe the origin of the carbocyclic ring and to compare the streptomycete pathway with that of *M. thermoautotrophicum*.

41. Mechanism of Emodin Deoxygenation to Chrysophanol. *J. A. Anderson* and B.-K. Lin. Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409. H. J. Williams and A. I. Scott. Department of Chemistry, Texas A&M University, College Station, TX 77840.

A cell-free system from *Pyrenochaeta terrestris* catalyzes the NADPH-dependent reductive removal of oxygen from the 6-position of the hydroxyanthraquinone, emodin, to give chrysophanol. The mechanism of the transformation involves reduction of a ketonic form of the substrate as shown by (a) exchange of the 5- and 7-protons with deuterium when the incubation is performed in D_2O and (b) incorporation of deuterium (up to 40%) from NADPH-4-d_R exclusively at the 6-position of the product, chrysophanol. The implication of this result for the reductive removal of hydroxyl groups during the biosynthesis of natural products, such as aflatoxin, is discussed.



42. Synthesis and Degradation of Biogenic Amines in B_{16} Murine Melanoma Cells and Normal Melanocytes. *S. B. Wilde*, F. A. Lashley, C. Putnam-Evans, and C. S. Brown. Clemson University, Clemson, SC 29631.

Melanocytes are neural crest derivatives committed to the synthesis of melanin from the amino acid tyrosine. Analysis of extracts of murine B_{16} melanoma cells by high-performance liquid chromatography (HPLC) with electrochemical detection revealed that these cells could also synthesize classical neurotransmitters derived from tyrosine (dopamine, norepinephrine, and epinephrine) and from tryptophan (serotonin). Some breakdown products of these amines have also been identified. An HPLC assay for aromatic amino acid decarboxylase revealed the presence of an enzyme which can convert L-dihydroxyphenylalanine to dopamine and 5-hydroxytryptophan to serotonin. A monoamine oxidase activity was demonstrated with tritiated tyramine as substrate. This activity was competitively inhibited by serotonin. HPLC analysis of an extract of normal melanocytes from the C57BL/6 mouse, from which the B_{16} melanoma cell line was derived, also indicated that the normal cells produce these neurotransmitters and their metabolites.

43. Rubber Polymerase. *C. R. Benedict* and S. Madhavan. Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

Washed rubber particles (WRP) were isolated from stem homogenates of *Parthenium argentatum* on a column of LKB Ultrogel Ac 34 with an exclusion limit of 700 000. The incorporation of [^{14}C]IPP into polyisoprene in the WRP required the addition of Mg^{2+} and an allylic-PP but not a chain-elongating enzyme. The polyisoprene was identified by ozonolysis and ^{13}C NMR spectroscopy. The reaction was linear for 60 min at 25 °C and the optimal concentrations of IPP, Mg^{2+} and DMAPP, GPP, or NPP were 0.18, 1.0, 0.20, 0.47, and 0.33 mM, respectively. The enzymatically synthesized polyisoprene was chromatographed on linear columns of 1×10^6 Å to 500 Å of ultrastyragel in a Waters 150 gel permeation chromatograph. The mol wt distribution of the polymers ranged from 10^4 to 10^6 with a peak mol wt of 2×10^5. The WRP contain a rubber polymerase which catalyzes the repeated additions of IPP to an allylic-PP initiator to form high mol wt polyisoprene. The regulation of the polymerization reaction in situ by cold temperature and chemical elicitors will be reported.

44. HPLC Characterization of Isomeric Incomplete Corrinoids. *S. H. Ford*, A. Nichols, M. Shambee, and L. Jackson. Physical Sciences Department, Chicago State University, Chicago, IL 60628.

Cobalamin (vitamin B-12) belongs to a group of complex organocobalt compounds called corrinoids, the biosynthesis of which is as yet not fully understood. Several incomplete corrinoids are currently being prepared and characterized for use as enzyme substrates in the study of cobalamin biosynthesis. Three of the incomplete corrinoids under study are cobinic acid pentaamide isomers which were formed by mild acid hydrolysis of cobalamin followed by cerium hydroxide hydrolysis of the resulting cobamic acid pentaamides. The purpose of this study was to develop a separation procedure for these isomers using high-pressure liquid chromatography (HPLC). Initial work on separating the cobinic acid isomers was conducted by following a published method describing the separation of the structurally similar cobamic acids (Binder et al., 1982 *Anal. Biochem. 125*, 253–258). The cobinic isomers were chromatographed separately and as a mixture. A

gradient system employing pyridine acetate buffers on a Waters NH_2 column was found to effect separation of the 3 isomers, and to differentiate the cyanoaqua forms. Separation on reverse phase (Waters C-18) was also successful, although retention times were quite long. (Supported by NIH-DRR-RR08043-MBRS.)

45. *Heliothis virescens* Aldehyde Oxidases. *María L. Tasayco* and Glenn D. Prestwich. Department of Chemistry, State University of New York at Stony Brook, Stony Brook, NY 11794.

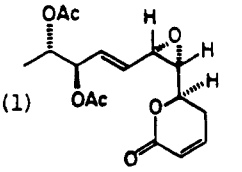
The role of the pheromone-degrading enzyme in the olfaction process of moths is still unknown. The examination of the enzymatic activity in several tissues, particularly in the whole antenna, by means of tritiated pheromone, radio TLC assay, and enzyme staining in gel electrophoresis indicates the presence of a group of antennal soluble proteins, tissue- and sex-unspecific, with similar molecular weights and isoelectric points responsible for aldehyde degradation. The two major aldehyde components of the pheromone, Z9-14:Al and Z11-16:Al, are degraded to the corresponding carboxylic acid with a K_m value in the micromolar range.

46. NMR Spectroscopy of the Excretory Products of *Mesocestoides corti* Exposed to Monensin in Vitro. *Barry J. Blackburn* and Marie M. Novak. Department of Chemistry and Biology, University of Winnipeg, Winnipeg, Manitoba, Canada R3B 2E9.

Excretory products of the larvae (tetrathyridia) of the tapeworm *Mesocestoides corti*, fed D-[$^{13}C_6$]glucose in a solution containing 10 μM of the carboxylic ionophore monensin for 2 h, were studied by using ^{13}C and ^1H nuclear magnetic resonance (NMR) spectroscopy. Signals due to unlabeled and labeled succinate, lactate, acetate, and alanine were identified in the spectra. Several differently labeled species of these metabolites were present; the major labeled lactates were U-^{13}C and 2,3-$^{13}C_2$, and the major labeled succinates were 1,2,2′-$^{13}C_3$ and 2,2′-$^{13}C_2$. Monensin decreased significantly the amounts of unlabeled and total labeled succinate and of total labeled acetate and lactate. The potential significance of these changes on the glucose metabolism of this tapeworm is discussed.

47. Constituents of *Hyptis* Species (Labiatae). *Rogelio Pereda-Miranda* and Isabel Navarro. Departmento de Quimica Organica, Escuela Nacional de Ciencias Biologicas Instituto Politecnico Nacional, Mexico, D.F. 11340.

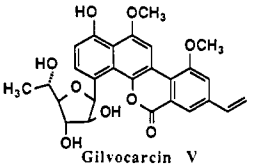
For several years our research group has been interested in the metabolism of some genera of Labiatae. In continuation of our studies on the constituents from *Hyptis*, we have now investigated the chemical composition of *H. albida*, *H. mutabilis*, and *H. oblongifolia*, which are commonly used in the traditional medicine of Mexico. From the aerial parts of *H. albida* eight terpenoids were isolated, two of which are new oleanane derivatives with 13β,28-olide system. Two rare naturally occurring flavonol-3-methyl ethers have also been isolated from this species. *H. mutabilis* does not contain flavonoids but a rich triterpene fraction, from which a new ursene was isolated and identified as the 3-*epi*benthamic acid. A minor poliketide derivative, 4-deacetoxy-10-epiolguine (**1**), was isolated from *H. oblongifolia*. Phytochemical aspects, as well as some quemotaxonomic implications, of these metabolites occurring in *Hyptis* are discussed.



Nucleic Acids

48. DNA Covalent Bonding and Backbone Nicking by Gilvocarcin V. *Lawrence R. McGee*, Yuk Ching Tse-Dinh, Greg M. Cole, and Pat N. Confalone. DuPont CR&DD, E328/310, Wilmington, DE 19898. Renuka Misra. NCI-FCRF Frederick, MD 21701.

Light activation of gilvocarcin V in the presence of double-stranded DNA leads to a covalent adduct with the DNA and, under certain conditions, single-strand nicking of DNA.



Gilvocarcin V

Other antitumor agents which induce DNA breakage generally do so by degradation of the ribosyl groups, while the evidence for gilvocarcin supports phosphate ester cleavage. We will describe the covalent photoadduct and the evidence for phosphodiester bond cleavage by gilvocarcin V, as well as conditions where strand cleavage is and is not observed.

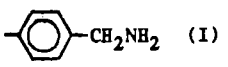
49. Conformation-Specific Oligonucleotide Photochemistry. *Steven Rokita*, Bernard Lau, and Lorraine Romero-Fredes. Department of Chemistry, State University of New York, Stony Brook, NY 11794.

Sequence dependence of DNA secondary structure has become the anticipated norm from many recent crystal and solution studies of oligonucleotide duplex conformation. Our approach to characterizing the heterogeneity of duplex structure is to contrast the photochemical reactivity of a defined series of oligomers individually and in their duplex forms. By this process, the conformation-specific reactivity is identified through competitive photochemical degradation. The contribution of aberrant structures in the formation of hyperreactive premutagenic sites is also surveyed in this manner. The nucleotide sequences currently under investigation derive from a fragment of pBR322 and are photolyzed by using an environmentally relevant model of aerobic solar irradiation. Analytical ion-exchange chromatography is used for separating and quantifying photochemically modified oligonucleotides.

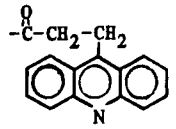
50. Interaction of Acridine-Anchored, Positive-Periphery Porphyrins with DNA. David K. Lavallee, Zhengjiu Xu, Richard Pina, and Alex Scarborough. Department of Chemistry, Hunter College of City University of New York, 695 Park Ave., New York, NY 10021.

Several groups have shown that positive-periphery porphyrins bind strongly with DNA, in some cases by intercalation and in other cases at the "surface" of DNA. The ability of acridine and similar planar aromatic systems to bind strongly with DNA is also known. We are interested in developing molecules combining the intercalating ability of acridines and the high affinity for DNA surface binding of positively charged porphyrins and to use such molecules as DNA alkylators or DNA cleavage reagents. The porphyrins which have been synthesized for this study have 4-(aminomethyl)phenyl sub-

stituents (I) at all four methine positions and include *N*-methyl derivatives. Using dilute conditions, an acridine moiety (II) has been added to one of the four methine substituents. The interactions with sonicated calf thymus DNA evaluated by fluorescence observation of the competition with ethidium bromide and by induced circular dichroism spectroscopy will be presented.



(I)



(II)

Structural Studies of Macromolecules

51. Differential Binding of Peptide-Analogue Inhibitors to Porcine Pancreatic Elastase. *E. F. Meyer, Jr.*, A. Karrer, R. Radhakrishnan. Biochemistry Department, Texas A&M University, College Station, TX 77843-2128. D. A. Trainor. Stuart Pharmaceuticals, ICI Americas, Wilmington, DE 19897. W. Bode. Max-Planck-Institute of Biochemistry, Martinsried (Munich), W. Germany.

High-resolution crystallography has established the binding geometry of naturally occurring peptide (e.g., Kunitz, Bowman-Birk) inhibitors to members of the serine protease family of enzymes. The tight binding of modified analogues (fluoromethyl ketones) raises questions about the extent of geometric homology. Two X-ray crystallographic structure analyses (1.7- and 2.5-Å resolution) reveal the binding geometry of related compounds to porcine pancreatic elastase. The binding of an aldehyde inhibitor, leupeptin, to trypsin to form an hemiacetal complex has been studied to 1.5-Å resolution, revealing subtle geometric differences that provide mechanistic explanations for binding affinity (high) and turnover (low) that characterize many such complexes. These and homologous complexes will be discussed as a means for using binding, catalytic mechanism, and modeling to explore rational inhibitor design. (Research supported by the Welch Foundation, O. N.R., and the Tobacco Council.)

52. X-ray Crystallography Explores Multiple Binding Geometries of Heterocyclic Serine Protease Inhibitors. *R. Radhakrishnan*, E. F. Meyer, Jr. Biochemistry Department, Texas A&M University, College Station, TX 77843-2128. J. C. Powers. Chemical Division, Georgia Institute of Technology, Atlanta, GA 30332.

It is generally the exception that the receptor binding geometry of a new compound is known. Yet, such information can be seminal for compound optimization; it can provide insight into the mechanism of action of an enzyme, and it can serve as a template for molecular modeling. We now have high (1.7 Å) resolution crystallographic data of the structure of porcine pancreatic elastase bound to three heterocyclic inhibitors. All three bind differently: Such a result removes some of the euphoria associated with molecular modeling. It naturally is a function of both the "constant" factor, the receptor, and the chemical and structural variables associated with the heterocyclic ligands. Experimental details and results will be presented, and a method employing quick, low-resolution X-ray diffraction measurements as an entre to molecular modeling will be discussed. (Research supported by the Welch Foundation, O.N.R., and the Tobacco Council.)

53. ^{15}N and ^{1}H NMR Studies of the Reduced and Oxidized States of Cytochrome c_2 from *Rhodospirillum rubrum*. *Liping P. Yu* and Gary M. Smith. Department of Food Science and Technology, University of California, Davis, CA 95616.

Significant ^{15}N and ^{1}H NMR resonances of the ^{15}N-enriched cytochrome c_2 from *Rhodospirillum rubrum* in both oxidized states have been unambiguously assigned. In the reduced state, a conformational change is observed having a pK_a of 6.8, which is assigned to the imidazole group of His-42. The protein in the oxidized state exists in multiple conformations whose populations are pH dependent. These multiple conformations are found to be in slow exchange, and only one of them is able to transfer electrons efficiently. The heme nitrogens are found to have very small contact shifts compared to the heme methyl groups. The structural differences between the oxidized and the reduced protein will be compared in detail in terms of NOE experiments, ionization effects, hydrophobicity, hydrogen bonding and exchange rates, and conformational variations. The redox variations with pH now can be explained by the observed structural differences found at different pH. (Supported by NIH Grant GM-34194.)

54. Characterization of *Coprinus cinereus* Mushroom Peroxidase. *Gudrun S. Lukat*, Mark N. Jabro, Kenton R. Rodgers, and Harold M. Goff. University of Iowa, Iowa City, IA 52242.

Examination of the peroxidase isolated from the *Coprinus cinereus* mushroom shows that the 42 000-dalton enzyme contains a protoheme IX prosthetic group. Reactivity assays and the optical absorption spectra of native *C. cinereus* peroxidase and several of its ligand complexes indicate that this enzyme has some characteristics similar to those reported for horseradish peroxidase. However, EPR and NMR studies reveal differences between the heme sites of the two enzymes. Carbon-13 (ferrous CO complex) and nitrogen-15 (ferric cyanide complex) NMR studies together with the proton NMR studies of the native and cyanide complexed *C. cinereus* peroxidase are consistent with coordination of a proximal histidine ligand. Protein reconstitution with deuteriated hemin facilitated assignment of the methyl resonances in the proton NMR spectrum. (Support: NIH GM-28831.)

55. NMR Structural Studies of the Heparin-Binding Protein, Platelet Factor 4. C. J. Talpas, D. A. Walz, and *L. Lee*. Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4, and Department of Physiology, Wayne State University, Detroit, MI 48201.

The release of the protein platelet factor 4 (PF4) by activated platelets, the binding of PF4 to heparin, and the resultant neutralization of the physiological activity of heparin suggest that PF4 plays a key role in blood coagulation. Although preliminary studies of the crystallization of bovine and human PF4 have been reported, the structure of PF4 in solution and its interaction with heparin have not been elucidated. Bovine and the highly homologous human PF4 both contain 2 histidine residues in homologous positions along the amino acid sequence. Our initial ^{1}H NMR studies on bovine PF4 indicate that the pK_a values of His-38 and His-50 differ significantly. We have begun to assign the resonances to specific residues. An analysis of the NMR spectra of bovine and human PF4 will enable us to probe the role of these amino acids in the structure and function of the protein. (Supported by NSERC, the J. P. Bickell Foundation, and the University of Windsor Research Board.)

56. NMR Studies of Deuteriated Analogues of the Trp Re-

pressor from *Escherichia coli*. *Cheryl H. Arrowsmith*, Laszlo G. Szilagyi, Lynda G. Treat-Clemons, Branimir Klaič, and Oleg Jardetzky. Stanford Magnetic Resonance Lab, Stanford University, Stanford, CA 94305-5055.

We have isolated a series of selectively deuteriated analogues of the Trp repressor from *E. coli* and are analyzing their ^1H NMR spectra with the goal of assigning the majority of resonances in this dimeric protein of 108 amino acids/monomer and subsequently studying its interaction with DNA. In each protein analogue, approximately two-thirds of the amino acid residues are deuteriated with the remaining 40–50 protonated residues consisting of only 5–7 different amino acid types. The 2D ^1H NMR spectra of these analogues are greatly simplified compared to the fully protonated protein and are readily interpretable in terms of the spin systems of the protonated amino acid residues. Consequently, we are able to assign most of the spin systems of the protonated residues from a variety of two-dimensional NMR spectra that are normally too complex or uninformative for a protein of this size.

57. NMR Studies of the Interaction of Polymyxin B with Dipalmitoylphosphatidylcholine Model Membranes. *Bruce J. Forrest*. Department of Chemistry, Brandon University, Brandon, Manitoba, Canada R7A 6A9.

Variable-temperature studies of the interaction of the cyclic peptide antibiotic polymyxin B with dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes have been carried out by using ^2H and ^{31}P NMR. At a molar ratio of lipid:antibiotic of 4:1, ^2H spectra of chain-labeled DPPC-d_{62} and headgroup-labeled DPPC-d_9 as well as ^{31}P spectra of the headgroup are all dominated by narrow singlet resonances between 10 and 42 °C. Above the normal lipid gel–liquid crystalline phase transition temperature, these resonances, indicative of the presence of small lipid particles undergoing rapid rotational reorientation, gradually decrease in intensity with increasing temperature. The amount of water present was found to be an important factor in dictating which types of structures are formed.

58. Study of Fluoride Ion Binding to Cytochrome P-450-CAM. *George B. Crull* and John H. Dawson. University of South Carolina, Columbia, SC 29208.

The interaction of fluoride ion with the ferric high-spin heme iron of cytochrome P-450-CAM has been investigated by using ^{19}F NMR relaxation measurements. An enhanced relaxation rate has been observed for the fluoride ion in the presence of varying amounts of cytochrome P-450-CAM. This change in relaxation rate is consistent with the fluoride ion coming in close proximity (<3.3 Å) of the paramagnetic heme iron. Because the rate of fluoride ion exchange between the binding site near the iron and the bulk solution, determined from the temperature dependence of the relaxation rate, is faster than the rate of paramagnetic relaxation, it is impossible to determine an exact Fe–F distance. The long lifetime of the fluoride ion near the iron of ferric P-450-CAM may be indicative of direct binding to the heme iron as is known to occur with ferric myoglobin. However, the Fe–F distance in the P-450 system does not change upon addition of cyanide, a known ligand to the heme iron. This result suggests that the fluoride ion is entering the substrate binding pocket of P-450-CAM and is binding to an amino acid side chain, most likely via a hydrogen bond, and not to the heme iron. (Support: NSF DMR 86:05876 (J.H.D.) and CHE 84:11172 (Department Instrument Grant).)

59. Characterization of Bacterial Cell Walls Using Solids NMR and GC/MS. *Theresa M. Forrest*, G. E. Wilson, Jr., James Hardy, Terrence Lee, and Jacob Schaefer. Department of Chemistry, University of Akron, Akron, OH 44325, and Department of Chemistry, Washington University, St. Louis, MO 63130.

A combination of solids NMR and GC/MS experiments has been used to quantify bacterial cell wall cross-link density and the metabolic fates of cell wall components in *B. subtilis*. Solids NMR techniques such as DCPMAS and REDOR are used on ^{13}C- and ^{15}N-labeled systems to study particular C–N bonds selectively. In cases where metabolism of precursors leads to label scrambling, GC/MS analysis of the derivatized cell wall hydrolysates allows complete interpretation of the NMR data. This method has been applied to *B. subtilis* where labeling of the cross-linking nitrogen of *meso*-diaminopimelic acid from the precursor L-[^{15}N]aspartic acid also results in labeling of cell wall D-glutamic acid via the transaminase. Evaluation of both GC/MS and NMR data from cells grown in D-[1-^{13}C]alanine and L-[^{15}N]aspartic acid provided a cross-link index of 0.47. Experimental details and extensions of this technique will be discussed.

60. 2D NMR Conformational Studies of a Cyclic Decapeptide Bradykinin Analogue. *Rickey P. Hicks*, Paul R. Blake, Donald J. Kyle, and W. Janusz Rzeszotarski. Nova Pharmaceutical Corp., Drug Discovery, Baltimore, MD 21224.

NMR study of a conformationally restricted cyclic decapeptide (**1**) analogue of bradykinin has been initiated. Peptide

Cys-Arg-Pro-Pro-Gly-Phe-Cys-Pro-Phe-Arg (**1**)

1 was selected as a candidate for study for two reasons. J. M. Stewart and co-workers reported that the Raman spectrum of **1** in water was similar to that of bradykinin, under similar conditions; this implies a similar solution conformation. Stewart also reported that **1** binds to the bradykinin receptor site with low affinity (Stewart et al., *7th American Peptide Symposium*). The solution conformation of **1** has been determined in DMSO by 2D NMR techniques. Under these conditions **1** exists in at least two distinct conformations. The major conformer of **1** is characterized by a cis peptide bond between the Arg^2 and Pro^3 amino acid residues. The minor conformer is characterized by having all-trans peptide bonds. NOE and coupling constant data from the NMR studies have been used in conjunction with molecular dynamics calculations to correlate the solution conformation of both conformers. The relevance of these data to bradykinin and its possible conformations in solution will also be discussed.

61. Conformational Studies on a Cyclic Peptide Bradykinin Agonist Using Molecular Dynamics. *D. J. Kyle*, R. P. Hicks, P. R. Blake, and W. J. Rzeszotarski. Nova Pharmaceutical Corp., Drug Discovery, Baltimore, MD 21224.

A study of the conformational and dynamic properties of a decapeptide, cyclized through a disulfide bond, has been

Cys-Arg-Pro-Pro-Gly-Phe-Cys-Pro-Phe-Arg

accomplished by using the molecular dynamics algorithms available in CHARMm. This peptide has been reported to be conformationally similar to bradykinin and exhibit activity throughout various bradykinin assays [Fox et al., *Peptides 1*, 193 (1982)]. The initial conformation was fully extended with all peptide bonds trans, and subsequently located local minima were characterized by minimization of coordinates taken at

2-ps intervals along the dynamics trajectory. NOEs obtained from 2D NMR experiments in DMSO indicated that two predominant backbone conformations existed, the most populated of which had a cis peptide bond between Arg^2 and Pro^3. A new starting conformation was generated when a cis peptide bond was inserted into the original structure at this location. Subsequent dynamics analysis resulted in the discovery of a family of conformers that correlated extremely well with the experimentally determined NOEs.

62. Molecular Dynamics Simulation of Cyclic Urea Artificial Enzymes. P. V. Maye and *C. A. Venanzi*. Chemistry Division, New Jersey Institute of Technology, Newark, NJ 07102.

Molecular dynamics (MD) calculations were carried out on a full mimic of chymotrypsin proposed, but not yet synthesized, by Cram and co-workers, in order to extend molecular mechanics (MM) structural studies of this molecule. Previous MM conformational analysis by Venanzi and co-workers focused on determining the orientation of the catalytic functionalities and showed that an imidazole–carboxylate hydrogen bond characterizes the low-energy conformations of the mimic. In the present work, a long (300 ps) MD simulation indicates that the key structural hydrogen bonds and bond torsions of the MM minimum energy structure are conserved at 300 K. Examination of MD-derived RMS atomic and torsional fluctuations of the macrocycle shows a rigid binding site. Energy optimization of selected configurations from the MD trajectory results in a slight but potentially significant modification of the MM-derived global energy minimum structure.

63. Computer Graphics Applied to the Interaction between Cytochrome *c* and Negatively Charged Inorganic Complexes. *Arun Majumdar* and Ann M. English. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve Blvd. West, Montreal, Quebec, Canada H3G 1M8.

The interaction between tuna cytochrome *c* and small negatively charged reagents has been studied by using computer graphics and semiempirical methods of simulating the electrostatic potential surface around the protein. The results are used to assess possible binding modes of negatively charged ruthenium polypyridyl complexes and their distances from the heme edge which is involved in electron transfer. The validity of the cytochrome–polypyridyl binding will be discussed in light of NMR studies with paramagnetic chromium analogues of the ruthenium complexes.

64. Probing the Europium Binding Environments of tRNA Using Laser-Induced Lanthanide Ion Luminescence Spectroscopy. *Emily H. Cornwall* and William DeW. Horrocks, Jr. Department of Chemistry, Pennsylvania State University, University Park, PA 16802.

Several classes of magnesium and lanthanide ion binding sites have been proposed to stabilize the structure of tRNA in solution using a variety of physical techniques including laser-induced lanthanide ion luminescence spectroscopy. Metal titration binding curves reveal the presence of 4–5 strongly bound metal ions. Further information about the ligand environments can be gained from the Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ transition from the analysis of lifetime measurements using a time-resolved technique and spectral deconvolution methods developed in our laboratory. Lifetime measurements in D_2O and spectral deconvolution of europium-bound tRNA suggest the presence of at least two europium environments. The relationship between the number of europium environments, lifetimes, and spectrally resolved peaks will be reported for these europium-bound tRNA species. (Supported by the National Science Foundation.)

65. Investigation of the Interaction of Calmodulin with Target Enzyme Analogues Using Luminescence Spectroscopy. *Barbara C. Thompson, Steven T. Frey*, Joel M. Tingey, and William DeW. Horrocks, Jr. Department of Chemistry, Pennsylvania State University, University Park, PA 16802.

In an effort to understand the interaction of calmodulin and its target enzymes, the binding of several enzyme analogues to lanthanide-bound calmodulin has been investigated. These analogues, which include melittin and fluphenazine, were chosen for their ability to transfer energy to lanthanide ions in accordance with the Forster energy-transfer mechanism. This energy transfer provides a spectroscopic means for determining the distance between the fluophor donor on the analogue and the lanthanide ion acceptor bound to calmodulin. Changes in the affinity of calmodulin toward Eu^{3+} in the presence of a target enzyme analogue are observed by means of binding curves of each Eu^{3+} environment obtained by time resolution of luminescence decay curves. These experiments were carried out by using pulsed dye laser excitation and lifetime spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+}. (Supported by National Institutes of Health Grant GM23599.)

66. Lanthanide(III) Ion Luminescence Studies of Calmodulin and Its Tryptic Fragments. *JoAnne Bruno*, Tritaporn Choosri, Joel M. Tingey, and William DeW. Horrocks, Jr. Department of Chemistry, Pennsylvania State University, University Park, PA 16802.

Calmodulin contains four similar calcium-binding sites which complicate the interpretation of experimental data regarding the effect of metal-ion binding. Tryptic digestion of calcium-bound calmodulin occurs initially at Lys-77 to yield two fragments, each containing two metal ion binding sites. Replacement of calcium by tripositive lanthanide ions provides a spectroscopic probe of the tryptic fragments and intact calmodulin. In an effort to ascertain the validity of these fragments as models of the calcium-binding domains in calmodulin, the laser-induced luminescence excitation of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} has been exploited to compare metal ion binding, metal–metal distance measurements, and changes in the Eu^{3+} excitation spectrum as a function of pH for whole calmodulin and its tryptic fragments. (Supported by National Institutes of Health Grant GM23599.)

67. Studying the Metal-Binding Sites of Parvalbumin through Use of Laser-Induced Europium(III) Ion Luminescence Spectroscopy. *Charles W. McNemar*, Donald T. Cronce, and William DeW. Horrocks, Jr. Department of Chemistry, Pennsylvania State University, University Park, PA 16802.

The laser-induced luminescence excitation of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} demonstrates quantitative binding to the two metal-binding sites of parvalbumins. These two sites are difficult to study separately because of their similar affinities for Eu^{3+}. In some cases, as with the pI = 4.75 codfish isotype, diffusion-enhanced energy transfer to small molecules (e.g., $Co(NH_3)_6^{3+}$) selectively changes the Eu^{3+} excited-state lifetimes, providing the means by which two otherwise indistinguishable Eu^{3+} sites may be studied. Changes in pH have distinct effects on the excitation spectra. The peaks in the spectra above 579.0 nm, corresponding to the two metal sites,

diminish and a peak at 578.0 nm grows with increasing pH, indicating a change in the Eu^{3+} environment. Resolution of the spectrum into two peaks is possible if competing metal ions (e.g., La^{3+} and Lu^{3+}) are present. (This work was funded by NSF and NIH grants.)

68. Structural Studies of Hevein, a Protein from *Hevea brasiliensis* Latex. Adela Rodríguez, Barbarín Arreguín, Andrés Hernández y Manuel Soriano. Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, Coyoacán, 04510 México, D.F.

The conformation of hevein, a small disulfide-rich protein present in the latex of *Hevea brasiliensis* (rubber tree), was investigated by using circular dichroism and the Chou–Fasman method for structure prediction. The unusual circular dichroism spectrum of hevein in the far-ultraviolet region shows a positive band at 221 nm and negative signals below 210 nm. These spectral features suggest the presence of irregular structure and β-turns and the absence of helical regions. The results from the secondary structure prediction indicate a predominance of β-turns (60%) and the presence of a small amount of β-sheet (19%). On the other hand, hevein has been crystallized by the vapor diffusion method using 2-methyl-2,4-pentanediol and $CaCl_2$. X-ray examination of the crystals has shown that the protein crystallizes in the orthorhombic system, space group $P2_12_12_1$, with a = 21.77 Å, b = 31.73 Å, c = 51.17 Å, and one molecule in the asymmetric unit.

69. Isolation and Conformational Study of Multiple Forms of Chymopapain. *Arturo Rojo*, Rafael Zubillaga, Silvia Solís, and Andrés Hernández. Departmento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Apartado Postal 55-534. México, D.F.

Five molecular forms of chymopapain were isolated by ion-exchange high-performance liquid chromatography. Their molecular weights, determined by gel filtration in 6 M guanidine HCl, ranged between 24 100 and 24 900. Circular dichroism (CD) spectra in the far-ultraviolet zone (250–182 nm) indicate that the five forms have similar secondary structure content. Analysis of the spectral data by the Hennessey and Johnson method [*Biochemistry 20*, 1085 (1981)] showed the following average values: 23% helix, 17% β-sheet, 18% turns, and 42% unordered. These figures are only slightly different from those determined for papain. In the near-ultraviolet region (320–250 nm) the CD curves of the chymopapains closely resemble that of papain, suggesting that many of the interactions of the aromatic groups present in the latter also occur in the chymopapain forms. Thus, as far as the molecular weight, secondary structure content, and environment of aromatic groups are concerned, the multiple molecular forms of chymopapain seem to be very similar to papain, supporting Lynn's idea that these enzymes have a common ancestry [*Phytochemistry 22*, 2485 (1983)]. (We thank the financial support of PRONAES-SEP, México.)

70. Thermal Unfolding of Human Orosomucoid [α_1-Glycoprotein]. Rafael Zubillaga, Arturo Rojo, and Andrés Hernández. Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa. Apartado Postal 55-534. México, D.F.

The thermal unfolding of human orosomucoid (α_1-glycoprotein) was studied in the pH range 3.1–5.2 by using circular dichroism and differential spectroscopy. The changes observed in the negative differential bands (277 and 283.5 nm) and in the ellipticity at 220 nm gave nearly coincident transition curves, which were found to be reversible. This suggests that the unfolding of orosomucoid closely follows a one-step mechanism. However, in the differential spectra an intense positive band at 294 nm appeared at a lower temperature than the main transition; it is proposed that this "anomalous" band is due to some local changes occurring around two tryptophanyl residues of the glycoprotein. The enthalpy change of unfolding (ΔH_d) was calculated from the individual transition curves by a van't Hoff analysis. From a plot of ΔH_d versus the melting temperature (T_m) a value of 0.09 cal g^{-1} deg^{-1} was calculated for the heat capacity change (ΔC_p^d). This value lies in the lower limit of the range observed for small globular proteins. (We thank the financial support of PRONAES-SEP, México.)

71. Abstract withdrawn.

72. Detection of Conformational Changes in Cytochrome Oxidase Using Tryptophan Fluorescence. *Leonor Ferreira* and Bruce C. Hill. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve W., Montreal, Quebec, Canada H3G 1M8.

Cytochrome oxidase is an integral part of the respiratory chain in the inner mitochondrial membrane. Its function involves the transfer of electrons from cytochrome *c* to the terminal electron acceptor, oxygen. The transfer of electrons is associated with a large negative free energy change, ca. 96kJ, which is conserved by cytochrome oxidase as an electrochemical proton gradient across the inner mitochondrial membrane (Wikstrom et al., *Cytochrome Oxidase: A Synthesis*, 1981). Cytochrome oxidase's ability to link electron transfer and proton translocation may be dependent on conformational changes. Cytochrome oxidase exhibits measurable tryptophan fluorescence with an emission maximum at 328 nm and a quantum yield of 30–40% in comparison to *N*-acetyltryptophanamide (Hill et al., *Biochemistry*, 1986). We are interested in employing tryptophan fluorescence as a tool in the detection of conformational changes in cytochrome oxidase dependent on the enzyme's environment, in particular, conformational changes arising from the solubilization of the oxidase in various detergents and lipid vesicles. Preparations of cytochrome oxidase specifically depleted of subunit 3, which is thought to be involved in the proton translocation process, will also be investigated.

73. Energy Transfer in the Zinc Cytochrome *c*: Cytochrome *c* Oxidase Couple. *Monique Laberge* and Jack A. Kornblatt. Concordia University, Departments of Biology and Chemistry, 1455 de Maisonneuve W., Montreal, Quebec, Canada H3G 1M8.

The triplet absorption decay of zinc cytochrome *c* (64.5 s^{-1}) is quenched in the presence of cytochrome *c* oxidase. Two components are observed in the decay profile of the complex: a fast process with k = 727 s^{-1} and a slower one with k = 204 s^{-1}. Radiationless dipole–dipole transfer of triplet excitation energy to Cu_A of the oxidase is believed to contribute significantly to the quenching mechanism of the faster process, as shown by calculations of intermolecular distances using the respective lifetimes of both processes. The r value calculated from the fast process (16 Å) is thought to reflect the cytochrome *c* to Cu_A distance. The value obtained from the slow process (21 Å) is in good agreement with singlet quenching experiments evaluating the cytochrome *c*–heme *a* distance.

74. Triplet State Lifetime of Tryptophan in Globular Proteins in Solution and Local Structural Dynamics. William C. Galley. Department of Chemistry, McGill University, 801 Sherbrooke St. W., Montreal, Quebec, Canada H3A 2K6.

Nonradiative decay rates for molecules in electronically excited states depend, in addition to an electronic coupling which for triplet excited states involves spin–orbit coupling, on the Franck–Condon factors which reflect the probability of an accompanying vibrational transition from the lowest vibrational level of the excited state to an isoenergetic high vibrational level of the ground electronic state. Experimentally it is found that even in the absence of oxygen very large variations in the tryptophan phosphorescence lifetimes of globular proteins in solution occur, and this has been attributed to variations in the local viscosity surrounding the chromophore. A model is presented which suggests that both static and dynamic perturbations from the environment on the vibrations of the emitting residues create more favorable F–C factors enhancing radiationless decay. The model is discussed in relation to observations on both the viscosity dependence of triplet decay and the pressure dependence of phosphorescence lifetimes.

75. Characterization of the Ferrous Ion Binding Site in Phthalate Oxygenase. *Christopher J. Batie*, David P. Ballou, William R. Dunham, James E. Penner-Hahn, and Him-Tai Tsang. Departments of Biological Chemistry, Biophysics, and Chemistry, University of Michigan, Ann Arbor, MI 48109.

Phthalate oxygenase (PO) is a dioxygenase isolated from *Pseudomonas cepacia* that converts phthalate to the 4,5-*cis*-dihydrodiol by using one equivalent each of NADH and O_2. PO contains a Rieske-type [2Fe-2S] center and a mononuclear iron (Fe^{2+}), which is probably at the active site. We have investigated the Fe^{2+} site by removing the Fe^{2+} and reconstituting with Fe^{2+} and other metals (Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Cu^{2+}). All these ions caused identical changes in the EPR spectrum of reduced PO (probably due to g-strain), but only Fe^{2+} reconstituted activity. EXAFS spectra of Zn^{2+}-reconstituted PO indicate that the site includes 6 N or O ligands. Reconstitution with $^{57}Fe^{2+}$ allows examination of the site by Mössbauer spectroscopy. CD and MCD spectra have also been used to characterize the changes in PO structure accompanying Fe^{2+} binding. (This work was supported by GM-20877 and GM-38047.)

Enzymes and Proteins, General

76. Characterization of Mutants of the Cloned Gene for Mandelate Racemase That Lead to Enhanced Production of Enzyme. *A. Y. Tsou*, V. M. Powers, S. C. Ransom, G. L. Kenyon, and J. A. Gerlt. Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, and Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143.

Pseudomonas aeruginosa (ATCC 15692) transformed with the cloned and sequenced gene for mandelate racemase from *P. putida* (ATCC 12633) (Ransom et al., *Biochemistry* (in press)) is able to utilize *R*-mandelate as sole carbon source. Growth of this transformed host on *R*-mandelate but not *S*-mandelate is largely prevented by the active site directed irreversible inhibitor α-phenylglycidate. However, colonies that are resistant to the inhibitor eventually appear despite the presence of α-phenylglycidate. The resistance to the inhibitor is the result of an at least 15-fold increase in the amount of mandelate racemase synthesized by the plasmid-encoded gene. The enzyme produced has the same sequence as that produced by *P. putida*. The enhanced synthesis of mandelate racemase is solely the result of a single base change 87 base pairs upstream of the initiation codon in a sequence that is likely to be the promoter for the mandelate operon. The elevated levels of mandelate racemase in this mutant allow the facile isolation of large amounts of homogeneous enzyme.

77. Structural and Functional Analysis of Yeast Hexokinase 2. *Leslie M. Bloom*, Hong Ma, David Botstein, and Chris T. Walsh. Department of Biological Chemistry and Molecular Pharmacology, HMS 45 Shattuck St., Boston, MA 02115, and Biology Department, MIT, 77 Massachusetts Ave., Cambridge, MA 02139.

The relationship between the structure and function of *S. cerevisiae* hexokinase 2, a glycolytic enzyme involved in catabolite repression, has been clarified by studying the effects of *hxk2* mutations in vivo and analyzing the proteins produced by these mutant strains in vitro. A mutant plasmid (initial ATG altered) was generated which encodes HK 2 without the first 15 amino acids. Although the protein encoded by this mutant dimerizes much less efficiently than nonproteolyzed wild-type protein, growth and catabolite repression of strains producing the mutant protein are similar to wild type. The second class of mutants is defective for growth on fructose. These *hxk2* genes have been sequenced, the kinetic parameters of the HK 2 mutant proteins are being measured in vitro, and their structures are being analyzed using monoclonal antibodies in order to identify the defects in these enzymes and relate them to changes in protein structure.

78. Purification of the Insoluble Aggregate Obtained from Expression of Creatine Kinase in *E. coli* Yields Greatly Improved Specific Activity in the Refolded Protein. *P. C. Babbitt*, B. L. West, I. D. Kuntz, and G. L. Kenyon. Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

We have expressed creatine kinase from *Torpedo californica* in *E. coli* using a *trc* promoter. In this system, the creatine kinase constitutes about 1–5% of the total bacterial protein. The expressed protein forms an insoluble aggregate under these conditions and must be refolded from 6 M guanidine-HCl to obtain active soluble enzyme. Purification of the aggregate is crucial to the level of activity that can be obtained in the refolded protein. The crude pellet purified by sonication and centrifugation of the aggregate and then refolded has a specific activity about 2% that of wild type. The same pellet extracted with *n*-octyl glucoside, unfolded in the presence of 1% β-mercaptoethanol in 6 M guanidine-HCl and then refolded has nearly normal specific activity. While it is uncertain exactly how the octyl glucoside extraction improves the activity of refolded creatine kinase, it does remove a major proportion of contaminating proteins from the crude pellet, as shown by polyacrylamide gel electrophoresis.

79. Substrate Kinetics and Specificity of a Cloned *Bacillus* Endoglucanase. M. Hefford, *K. Laderoute*, A. Lo, V. Seligy, and G. Willick. Division of Biology, National Research Council of Canada, Ottawa, Canada K1A 0R6.

We have studied the specificity and kinetics of hydrolysis of cellodextrins with dp = 2–6 of the cloned endoglucanase from *Bacillus subtilis* PAP115. This enzyme was found to be initially expressed as an inactive form with M_r 52 200, which is processed to an active form with M_r 35 800 by removal of

a portion of the COOH-terminus. The enzyme has a pI = 5.75 and showed two pH optima of 5.4 and 6.7 and a temperature optimum of 60 °C with carboxymethylcellulose (CMS) as substrate. The viscosity profile of CMC hydrolysis was typical of an endoglucanase. Cellobiose (<100 mM) stimulated the reaction up to 1.5× and then became inhibitory. The comparative rate of hydrolysis of cellodextrins was cellohexaose = cellopentaose ≫ cellotetraose. Neither cellobiose nor cellotriose was attacked. Cellopentaose hydrolysis gave only the expected cellotriose and cellotetraose, whereas cellotetraose hydrolysis yielded the cellotriose as well as the expected cellobiose. The kinetics of a deletion mutant with M_r 33 800 will also be described.

80. Recombinant Plasminogen Activator with in Vivo Thrombolytic and Pharmacokinetic Superiority to nt-PA. *G. F. Smith*, J. L. Bobbitt, P. J. Burck, B. Grinnell, S. R. Jaskunas, J. F. Quay, and C. V. Jackson. Lilly Research Laboratories, Indianapolis, IN 46285.

We have constructed a genetically engineered novel plasminogen activator (mt-PA 6) comprised of the second kringle domain sequence and the serine protease domain sequence of native tissue plasminogen activator (nt-PA). The highly purified single-chain enzyme (95% active site titration) contained ~200 000 fibrin plate units (FU)/mg and was highly fibrin dependent. In vitro fibrinolytic activity was shown in whole plasma (human and dog) clot systems by using a ^{125}I fibrin microfibrinolysis assay. In vivo evaluation of the enzyme in a well-defined dog model of coronary thrombolysis revealed that mt-PA 6 achieved a more rapid and more persistent reperfusion of the thrombosed coronary artery than did equivalent doses of single chain nt-PA (64 300 FU/kg). Plasma fibrinogen remained between 75 and 100% of base line during all treatments. The in vivo half-life found for the mt-PA 6 molecule ($t_{1/2}$ for mt-PA 6 was 58 ± 7 min) was substantially greater than the 3.3 ± 0.3 min half-life found for nt-PA. These findings suggest that mt-PA 6 would be a superior coronary thrombolytic agent as compared to nt-PA.

81. Anticoagulant and Antithrombotic Activities of Recombinant Human Activated Protein C: Apparent Lack of Species Specificity. *G. F. Smith*, S. B. Yan, B. Grinnell, J. L. Sundboom, and C. V. Jackson. Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285.

We have produced from a human kidney cell line a genetically engineered molecule (r-aPC) identical with plasma human activated protein C. The in vitro anticoagulant properties of r-aPC in human plasma were different from the effects of warfarin and heparin. The APTT assay was prolonged 2× by 0.2 μg/mL r-aPC, whereas the recalcification time and prothrombin time assays were much less affected (the thrombin clotting time was unaffected). The in vitro anticoagulant effects of r-aPC in fresh blood/plasma of the monkey, dog, cat, rabbit, and rat revealed that all species responded to r-aPC (needed to double APTT: 0.3, 0.4, 0.5, 0.6, 1.0 μg/mL r-aPC, respectively). We found r-aPC to behave virtually identical with plasma aPC. In a well-defined rat model of carotid artery thrombosis doses of 2.6 and 5.2 mg/kg, iv, r-aPC caused significant antithrombotic effects and persistent APTT prolongations, while factors Vc and VIIIc were not affected. The half-life of r-aPC was approximately 20 min in the rat. The antithrombotic efficacy shown by r-aPC in the rat (the species least sensitive to r-aPC) supports the hypothesis that r-aPC should prove efficacious in human antithrombotic therapy.

82. Site-Directed Mutagenesis Studies on the Divalent Cation Sites of *E. coli* Glutamine Synthetase. *Bin Li*, Pamela J. Keck, and Joseph J. Villafranca. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

E. coli glutamine synthetase (GS) catalyzes the ATP-dependent biosynthesis of glutamine from glutamate and ammonia. It requires two divalent cations per subunit for catalytic activity. It is known that binding of a divalent cation at the high-affinity site (n_1) induces a conformation change, converting the inactive enzyme to the fully active form. Binding of a metal ion at the lower affinity site (n_2) is also necessary for catalysis. From the X-ray crystallographic structure (Almassy et al. (1986) *Nature 323*, 304) and the sequence of the enzyme, ligands of the two metal ion sites have been proposed. Histidine 210 is at or near the n_1 site and histidine 269 is at the n_2 site. These residues have been changed to asparagines by site-directed mutagenesis. Studies on the mutant enzymes include determination of dissociation constants of Mn^{2+}, steady-state kinetics, and oxidative inactivation of the mutant enzymes. Electron paramagnetic resonance spectroscopy of GS–Mn–MSOX complexes has provided information about the metal ion environments. (The work was supported by NIH Grant GM 23529.)

83. Production and Purification of Human Insulin Chain B Synthesized in *E. coli* by Recombinant DNA Technology. Xôchitl Alvarado, Paulina Balbás, Salvador Antonio, and Francisco Bolívar. Centro de Investigación sobre Ingeniería Genética y Biotecnología. UNAM, Apartado Postal 70-479, México, D.F. 04510, Mexico.

Human insulin chain B has been highly purified from *E. coli* extracts by recombinant DNA technology. The overproduction of a β-galactosidase chain B hybrid polypeptide was chemically induced with IPTG (1). This protein was cleaved with CNBr and then purified by molecular filtration (Sephadex G-50), ion-exchange (DEAE-Sephadex A-25), and high-performance liquid chromatography (RP-C18) (3). The highly purified peptide was characterized by amino acid analysis, and it showed complete identity with the human chain B standard. Results of polyacrylamide gel electrophoresis (18% polyacrylamide) showed the same physicochemical properties as the standard. The association procedure of chain B to chain A (4), both synthesized and purified from *E. coli* extracts, gave 60% of correctly joined active insulin with respect to B chain as detected by radioimmunoassay.

84. Purification and Characterization of 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG CoA) Synthase from *Saccharomyces cerevisiae*. *I. Mukharji*, K. A. Jarman, and R. L. Ausich. Amoco Research Center, Naperville, IL 60566.

HMG CoA synthase, which catalyzes the condensation of acetyl CoA with acetoacetyl CoA to give HMG CoA, has been purified to apparent homogeneity from *S. cerevisiae* and the N-terminal sequence of its first 25 amino acids determined. The purification scheme involves chromatography of yeast cytosol on DE-52 and phosphocellulose P-11, chromatofocusing on PBE-94, and hydroxylapatite as the last step. A single band of MW 50 000 on SDS–polyacrylamide gel electrophoresis established homogeneity of the final preparation. The native enzyme, MW 105 000, is a dimer with identical subunits. At pH 8.2, the K_m for acetyl CoA and acetoacetyl CoA are 40 and 2 μM, respectively. Substrate inhibition by acetoacetyl CoA, which is competitive with respect to acetyl CoA binding, is observed, K_i = 14 μM. Both HMG CoA and free coenzyme

A exhibit product inhibition. The structural gene encoding HMG CoA synthase in *S. cerevisiae* has been isolated and sequenced (Jarman et al., 1987). Seventeen of the 21 amino acids from the conserved active-site region of avian liver (Miziorko et al., 1985) and hamster (Gil et al., 1986) synthase are homologous to the derived amino acid sequence of the yeast enzyme. However, very little homology exists between the first 25 N-terminal residues of the yeast and hamster HMG CoA synthases.

85. Highly Processive Synthesis by DNA Polymerases α and δ from Calf Thymus. *T. W. Myers*, R. D. Sabatino, P. H. Frickey, and R. A. Bambara. Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642.

DNA polymerases α and δ have been thought to be only minimally processive—synthesizing from 5 to 20 nucleotides per binding event. We have demonstrated that these polymerases are capable of becoming highly processive upon lowering the pH and magnesium concentration of the reaction from standard synthesis conditions. Processivity values of up to 2000 nucleotides were observed by using poly(dA)·oligo(dT) at pH 6.0 and 1 mM $MgCl_2$. These conditions were found to differ from pH 7.0 and 5 mM $MgCl_2$, which support the maximum rate of synthesis on this template. During highly processive DNA synthesis the K_m value of the reaction was lower with respect to primer terminus concentration. Furthermore, under these same conditions, both polymerases became insensitive to dissociation from the template as a result of encountering the 5′-ends of primers. Overall, these results suggest that the affinity of the polymerases for the primer termini is higher throughout the polymerization reaction when the pH and magnesium concentration are lowered from conditions which favor maximum synthetic rate.

86. Identification, Purification, and Characterization of Human Serum Nucleases. Ph. Tuduri and *P. Englebienne*. Research and Production Department, Sopar-Biochem, 124 Rue J. Besme, B-1080 Brussels, Belgium.

The hydrolysis of calf thymus DNA by human serum at neutral pH in the presence of calcium and magnesium ions is tremendously activated near 55 °C. Preliminary attempts at characterizing that enzymatic activity provided us with elements likely to exclude that activity being exclusively the result of DNase I (EC 3.1.4.5) action. By a combination of two affinity (on ssDNA and heparin) and one gel permeation chromatographic procedure, we succeeded in purifying to homogeneity several nucleases from a pool of normal human serum. The main interesting feature resulting from the partial characterization of these enzymes is that some are both calcium and magnesium dependent, while others are calcium dependent only.

87. Plant Nitrate Reductase Has Interchain Disulfide. *Wilbur H. Campbell*, Ellen R. Campbell, and Gregory Hyde. Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931.

Squash cotyledon NADH:nitrate reductase was purified on blue Sepharose and then a monoclonal antibody column. Reduced nitrate reductase (i.e., treated with 5% 2-mercaptoethanol) had M_r 110000 when electrophoresed in 5% acrylamide denaturing gels, while the unreduced enzyme had M_r 200000. Thus, the dimer structure of native nitrate reductase appears to contain an interchain disulfide. Interestingly, when native and reduced/denatured nitrate reductase were chromatographed on a reverse-phase HPLC C-4 column, they had the same retention time, which suggests the presence of the interchain bond does not alter the hydrophobic contact region of the enzyme's polypeptide. To further locate the interchain disulfide, native enzyme was degraded with V8 protease, resulting in two major peptides of 55 and 45000, which could be separated by the monoclonal antibody column with the smaller one not binding and having the dehydrogenase activity of the original enzyme. The 55000 domain appeared to be a larger M_r species (about 200000) in nonreducing, denaturing gels. This larger domain of the enzyme is believed to be the amino-terminal portion of the polypeptide and to largely account for subunit contact in nitrate reductase. Corn nitrate reductase yielded similar results. (Supported by NSF Grant DMB 85-02672).

88. Ectoenzyme Activity of Human Erythrocyte Adenosine Deaminase. Ken Bielat and *George L. Tritsch*. Roswell Park Memorial Institute, Buffalo, NY 14263.

Adenosine deaminase (ADA) activity was localized at the erythrocyte cell membrane by incubation of intact cells with 6-chloropurine ribonucleoside, which is dechlorinated by ADA to form Cl^-. At loci of ADA activity, Cl^- is precipitated with added Ag^+, and electron-dense Ag^0 is formed upon exposure to light. Electron microscopy revealed a nonuniform distribution of Ag^0 deposits at the periphery of the cells, which indicated appreciable membrane-associated ADA activity. Diazotized sulfanilic acid (DASA) cannot penetrate the red cell membrane and inactivates membrane proteins whose active sites are exposed to the medium. DASA inactivated about two-thirds of the membrane-associated ADA; it is concluded that this fraction of membrane-associated ADA faces the external medium and is thus designated ecto-ADA. From the hydropathic profile of the amino acid sequence of ADA, a model is proposed for the orientation of ecto-ADA with the erythrocyte cell membrane in which hydrophobic domains near the middle of the peptide chain anchor the enzyme to the cell membrane, and the predominantly hydrophobic portions near the amino-terminus and the predominantly hydrophilic portions toward the carboxy-terminus are juxtaposed to form the ectoenzyme.

89. Study of Adenosine Deaminase and Its Conversion Factor in Normal and Pathological Human Serum. Jeffrey T. Starkey, Rebecca K. Smith, Cynthia Ma, and *Pang F. Ma*. Center for Medical Education, Ball State University, Muncie, IN 47306.

A large and small molecular form of adenosine deaminase have been reported in advanced mammals, including man. The molecular weights of these enzyme forms are estimated to be 200000 and 35000, respectively, by gel filtration column chromatography. The small form can be reversibly converted into the large form in the presence of conversion factor, a high molecular weight glycoprotein that is aggregated during this process. Previous studies have shown that the distribution of the two molecular forms of adenosine deaminase is tissue specific (for instance, the large form of the enzyme is predominant in human liver, while the small form is predominant in human heart). It follows that the conversion factor is present in higher amounts within those tissues where the large form predominates. With this in mind, an attempt is made to study the conversion process and determine whether or not the serum level of conversion factor is elevated in certain pathological conditions. (Supported by a grant from Delaware

County Cancer Society.)

90. Substrate Specificity of a tRNA Ribose Methyltransferase. *Guojun Bu* and Thomas O. Sitz. Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

We have detected a 2′-*O*-methyltransferase in nuclei isolated from Ehrlich ascites cells which has high specificity for tRNA isolated from liver in mice treated with ethionine (a methionine analogue that inhibits methylation). This methyl-deficient tRNA was an excellent substrate but represents a heterogeneous population of many different tRNAs with different levels of methylation. This enzyme did not use tRNA isolated from yeast, *E. coli*, and non-ethionine-treated mouse liver as a substrate but did use tRNA from Ehrlich ascites cells at half the efficiency of tRNA isolated from ethionine-treated mouse liver. Synthetic substrates such as poly(A), poly(G), poly(I), and poly(U) were not substrates, but poly(C) was a poor substrate. A cloned phenylalanine tRNA gene transcript (a yeast tRNA with no modifications) also was not a substrate. When the methylated RNA was isolated from a reaction and hydrolyzed, an alkaline stable fragment about five nucleotides long was found. Kinetic data would suggest that the enzyme was acting processively under our assay conditions. We will report on which tRNAs are being methylated and the characterization of the sequences methylated.

91. Characterization of Lugworm Protease C. *Y. M. Lin.* Department of Chemistry, Tennessee State University, Nashville, TN 37203.

Lugworm protease C is one of the four proteases, isolated by us from lugworm (*Arenicola cristata*), that activate cyclic AMP phosphodiesterase. Protease C has been obtained in homogeneous form. It exhibited a typical UV–visible spectrum for protein with absorbance maximum at 280 nm. SDS–PAGE showed a single polypeptide of 23 000 daltons, considerably smaller than 30 000 daltons obtained previously by HPGC. Amino acid analyses indicated high content of aspartate, glutamate, and their amide relative to arginine and lysine residues. Examination of its peptidase specificity using oxidized insulin B and its fragments as substrates revealed that, consistent with trypsin-like proteases, protease C was highly active toward arginyl peptide bond and also hydrolyzed lysyl bond, though at a much slower rate. Also hydrolyzed at slow rates were valyl, alanyl, and leucyl bonds and, at high enzyme-to-substrate ratio and with prolonged incubation, Asn–Gln, His–Leu, Ser–His, and phenylalanyl bonds. K_m and V_{max} were determined to be 0.81 mM and 100 μmol/min/mg for Arg–Gly bond in oxidized insulin B, 0.24 mM and 0.15 μmol/min/mg for Val–Cya in Phe_1–Arg_{22} fragment, and 0.042 mM and 400 μmol/min/mg for TAME, a synthetic trypsin substrate. A broad specificity appears consistent with digestive function of the protease.

92. Spectroelectrochemical and Resonance Raman Investigations of Glutaryl-CoA Dehydrogenase. *Colleen M. Byron*, Mazhar Husain, Bridget Brennan, and Marian T. Stankovich. Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, and Molecular Biology Division, Veterans Administration Medical Center, San Francisco, CA 94121.

Glutaryl-CoA dehydrogenase (GCD) from *Paracoccus denitrificans* is an FAD-containing enzyme that catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA. The *Paracoccus* GCD shares several characteristics with mammalian acyl-CoA dehydrogenases, and it is able to cross-react enzymatically with electron-transferring flavoprotein (ETF) and ETF-Q-oxidoreductase. Characterization of the *Paracoccus* GCD, it is hoped, will aid in the study of mammalian GCD which is present only in small amounts in mitochondria. This presentation will focus on the spectroelectrochemical investigation of *Paracoccus* GCD regulation by substrate/product binding. Further, these results will be supported by resonance Raman data to understand the structure–function relationship in GCD.

93. Hydrophobic Ligand–Protein Interactions: Binding Kinetics and Immunochemical Studies on the 4S Mouse Protein. H. A. Barton and *M. A. Marletta.* College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109-1065.

The 4S protein purified from mouse liver cytosol is a 29-kDa protein that binds polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene (B[*a*]P), with high affinity, specificity, and saturability. Kinetic studies showed that B[*a*]P had a k_{off} = 0.28 ± 0.06 min^{-1} and a $k_{on} = 7.0 \pm 0.2 \times 10^7$ M^{-1} min^{-1}. The K_d calculated from these rates is 4.0 ± 0.9 nM, which agrees with equilibrium measurements. This provides support for the description of this ligand–protein interaction as a simple mass action binding of one ligand per protein molecule. Polyclonal antibodies were raised against this protein and were specific toward this 4S protein by western blot analysis. Western blots from mouse tissues showed highest levels in liver, heart, kidney, and lung with lower levels in intestine, spleen, thymus, testes, and brain. Serum was negative. The protein was also found in eight different strains of mice. High-affinity specific binding for B[*a*]P, assayed by dextran-coated charcoal, was found in rat, rabbit, guinea pig, scup, and chicken liver cytosols. This binding had low nM equilibrium binding constants and was not competed by tetrachlorodibenzofuran distinguishing these sites from the Ah receptor. (Supported by DHHS Grant CA-37770.)

94. Macrophage Synthesis of Nitrite, Nitrate, and *N*-Nitrosamines. R. Iyengar and *M. A. Marletta.* College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109-1065.

Macrophages immunostimulated with *E. coli* lipopolysaccharide (LPS) and or γ-interferon synthesize nitrite, nitrate, and *N*-nitrosamines. The nitrogen for all the products is derived exclusively from one of the two equivalent guanido nitrogens of L-arginine as determined by ^{15}N-labeling experiments. Citrulline was the only other product found. Other precursors included only close structural analogues of arginine such as homoarginine. D-Arginine was not a precursor, and it did not inhibit L-arginine-derived nitrite/nitrate synthesis. Recent experiments have been directed toward the enzymology of this pathway. To induce the enzymatic activity, the cells were treated with LPS and γ-interferon for 18 h and lysed. The activity was present in the 100000*g* supernatant and required NADPH. Experiments to probe the involvement of metal ions showed Mg^{2+} slightly enhanced the activity, while Zn^{2+} was a strong inhibitor. Other divalent metals either had no effect or were weak inhibitors. A scheme, consistent with the evidence to date, will be presented for this novel metabolism of arginine. (Supported by DHHS Grant CA-26731.)

95. Structure and Function of Synthetic Growth-Factor-like Domains in Human Blood Coagulation Factor. *Linda H. T. Huang* and James P. Tam. The Rockefeller University, 1230 York Ave., New York, NY 10021.

The proliferation of normal cells is a well-controlled and highly conserved process that requires regulation by hormone-like growth factors. A well-characterized growth factor family is the transforming growth factor type α (TGFα) and epidermal growth factor (EGF). These are small mitogenic proteins that share the same cell surface receptor and considerable similarities in sequence homology and structural motif. Based on sequence comparisons, the TGFα–EGF homologous sequences are found in domains of blood coagulation factors which include factor IX, X and protein C, S, and Z. None of these putative growth-factor sequences have been biochemically characterized. To study these growth-factor-like domains, we have prepared these domains and their analogues by the solid-phase peptide synthesis method. These peptides are vigorously purified, and their structure is characterized. Their structure and biological activity relationship will be discussed. To understand the relationships between the chemical structures of these growth factors and their biological properties, we used solid-phase peptide synthesis to produce these growth factors, their structural variants, and shortened analogues. Our results showed that full biological activity of these growth factors required a tertiary structure maintained by the disulfide pairs and shortened analogues or extend structure without the disulfide restraint greatly reduced biological activity. However, several shortened analogues showed partial agonist or antagonist activity and may provide useful leads for the design of therapeutic useful agents.

96. Purification of Human Chorionic Renin. *David A. Egan* and Patrick A. Marcotte. Abbott Laboratories, Abbott Park, IL 60064.

Native human renin has been purified on a milligram scale by using a five-step purification scheme. The starting material was the culture media of human chorionic trophoblasts that contained 0.05–0.2 Goldblatt units of prorenin/mL. The prorenin was partially purified by ammonium sulfate fractionation and chromatographies on QAE-Sephadex and cibracon blue agarose. Following conversion of prorenin to renin with porcine trypsin, purification to homogeneity was achieved by affinity chromatography and gel filtration. Western blot analysis indicates that prorenin (MW 43K) in the presence of trypsin is totally converted to active renin (MW 40K). Chorionic renin is a single polypeptide chain by SDS–PAGE. The amino acid composition and amino-terminal analysis (22 residues) is identical with the kidney enzyme. Heterogeneity of chorionic renin is demonstrated by isoelectric focusing, showing three major and four minor bands of enzyme activity (pH 4.8–5.2). Treatment with endoglycosidases is ineffective, whereas neuraminidase decreases heterogeneity and shifts the protein bands to more basic species. Since these species all have identical MN by SDS–PAGE, variances in carbohydrate composition and particularly in sialic acid content is a probable cause of the heterogeneity.

97. Purification and Characterization of Peptides Which Cause Contraction of *Aplysia* Muscles. *Susan L. Knock* and David J. McAdoo. Marine Biomedical Institute and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550.

Gastropod neuropeptides have been intensively studied as models for understanding the transmission of neurochemical information. Peptides which modulate the neural control of *Aplysia* muscles were chemically characterized. Peptides were extracted and chromatographed by using gel filtration chromatography followed by reverse-phase high-performance liquid chromatography (HPLC). Active peptides eluted from a Sephadex G50 column with a molecular weight between 2000 and 5500. Two distinct peptide fractions can be separated by these means, which cause rhythmic and tonic contractions of the arterial and body wall muscles. Peptides eluted from a C_{18} column at 23.5% and 26% CH_3CN in trifluoroacetic acid. Preliminary sequence analysis indicates that the peptide which eluted at 26% has not previously been described. Further sequence data will be discussed. Post column fluorescence derivatization techniques give a fluorescence signal that is enhanced 55-fold over the UV signal at 220 nm. This indicates the peptides have a Lys residue, an unblocked N-terminus, or both.

98. Semisynthetic Fluorohydrolase. *David E. Albert* and Melvin H. Keyes. Anatrace, Inc., 1280 Dussel Drive, Maumee, OH 43537.

Semisynthetic enzymes that have the ability to catalyze the hydrolysis of organophosphates structurally related to Soman have been prepared from bovine ribonuclease. The method (Keyes et al. *Enzyme Microlab. Technol. 61*, 97–100 (1984)) for inducing new enzyme activity in naturally occurring proteins involves three steps: perturbation of the conformation of the starting protein by chromatography on a gel filtration column with 1 mM HCl as the eluent; modification by adding a competitive inhibitor of the desired enzyme to be mimicked such as hexamethylphosphoramide; and cross-linking the protein with a diimidate while the competitive inhibitor is still bound. The semisynthetic fluorohydrolases have activities between 50 and 200 units/gram using either diisopropyl fluorophosphate or phenylmethanesulfonyl fluoride as substrates. In addition, turnover numbers in the range of 50–100 have been obtained. The results of purification of the crude mixtures will be discussed.

99. 2-[4-Bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 5′-Diphosphate: A New Fluorescent Nucleotide Affinity Label of the Active Site of Pyruvate Kinase. *Dianne L. DeCamp* and Roberta F. Colman. Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716.

A new reactive fluorescent ADP analogue has been synthesized: 2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 5′-diphosphate (BDB-TϵADP). Rabbit muscle pyruvate kinase is inactivated by 200 μM BDB-TϵADP at pH 7 and 25 °C. Protection against inactivation is provided by ATP, Mg^{2+}, K^+, and phosphoenol pyruvate (PEP) or by Mg^{2+}, K^+, and PEP, suggesting that reaction occurs in the region of the active site. Incorporation of 1.7 mol of reagent/mol of enzyme subunit accompanies 90% inactivation in 80 min. However, in the presence of the protective ligands Mg^{2+}, K^+, and PEP, 1.0 mol of reagent is incorporated while the enzyme is only 14% inactivated, indicating that reaction of BDB-TϵADP at one of the two sites is primarily responsible for the inactivation. Modified enzyme was digested with trypsin, and the nucleotidyl peptides were isolated by chromatography on phenylboronate-agarose followed by reverse-phase HPLC. Two labeled peptides were isolated and identified as Asn^{162}-Ile-Cys-Lys^{165} and Ile^{141}-Thr-Leu-Asp-Asn-Ala-Tyr-Met-Glu-Lys^{150}. Only the tetrapeptide (162–165) is modified in the presence of Mg^{2+}, K^+, and PEP, when the enzyme retains most of its activity. We conclude that the decapeptide (141–150) is in the active site of pyruvate kinase and that its modification by BDB-TϵADP causes inactivation. (Supported by NSF Grant DMB-87-40243.)

100. Inactivation of *E. coli* 2-Amino-3-ketobutyrate (AKB) CoA Ligase by Arginine-Modifying Reagents. *Jagat J. Mukherjee* and Eugene E. Dekker. Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-0606.

E. coli AKB ligase [2-amino-3-oxobutyrate glycine lyase (CoA-acetylating), EC 2.3.1.29: 2-amino-3-oxobutyrate + CoA ⇌ glycine + acetyl-CoA] catalyzes the second step in the threonine dehydrogenase initiated pathway for threonine utilization in eukaryotes and prokaryotes. It is a pyridoxal 5′-phosphate-dependent, dimeric enzyme with MW 85 000 and 26 arginine residues/subunit. Treatment of this enzyme with arginine-specific reagents, i.e., phenylglyoxal (PGLX), 2,3-butanedione, 4′-(oxyacetyl)phenoxyacetic acid, or 1,2-cyclohexanedione, shows a time- and concentration-dependent loss of ligase activity. PGLX is most effective; incubation with 10 mM PGLX in 50 mM phosphate buffer at pH 7.0 for 20 min causes >90% inactivation. Biphasic inactivation kinetics are seen. Acid hydrolysis of enzyme >95% inactivated by PGLX followed by amino acid analyses shows the loss of ~1.5 arginine residues/subunit with no significant change in other amino acid residues. Ligase incubated with 10 mM PGLX for 20 min in the presence of 1.5–2.0 mM acetyl-CoA retains >50% of the initial activity. The results indicate that an arginine residue (or residues) is (are) at the catalytically active site of *E. coli* AKB ligase. (Supported by USPHS–NIH Grant AM-03718.)

101. Identification of Amino Acid Residues of Glutamine Synthetase Modified by Pyridoxal 5′-Phosphate. *Carolyn L. DiIanni* and Joseph J. Villafranca. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

Chemical modification studies with pyridoxal 5′-phosphate have indicated that lysine(s) appear(s) to be at or near the active site of *E. coli* glutamine synthetase [Colanduoni, J., and Villafranca, J. J. (1985) *JBC 260*, 15042; Whitley, Jr., E. J., and Ginsburg, A. (1978) *JBC 253*, 7017]. Samples containing ~1, ~2, and ~3 pyridoxamine 5′-phosphate/subunit, corresponding to 100, 25, and 14% of the original activity remaining, respectively, were subjected to CNBr cleavage. The peptides containing pyridoxamine 5′-phosphate were identified by their absorbance at 320 nm and isolated by HPLC. These peptides were analyzed and sequenced. The N-terminus of the protein (a serine residue) was modified by pyridoxal 5′-phosphate and resulted in no loss of activity. Lysines 352 and 383 are also modified by pyridoxal 5′-phosphate, and each modification results in partial loss of activity. When methionine sulfoximine phosphate/ADP and ADP/P_i were used to protect the enzyme from inactivation, glutamine synthetase is not modified at Lys-352, indicating that Lys-352 is at the active site. (Work supported by NIH Grant GM 23529.)

102. Site-Directed Modifiers of Proteins: Acyl Phosphate Monoesters and Their Bifunctional Analogues. *Andrew Grant*, Stephen Bearne, and Ronald Kluger. Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A1.

Methyl acetyl phosphate has previously been shown to acetylate nucleophiles adjacent to cationic sites in hemoglobin and D-3-hydroxybutyrate dehydrogenase. Generalized methodology for the preparation of other acyl phosphate monoesters has now been developed through the use of improved acyl bromides, bifunctional reactants, and crown ether catalysts. These methods permit specific analogues to be prepared which are used as probes of enzyme structure and as potential chemotherapeutic agents.

103. Diethyl Pyrocarbonate as a Probe of Histidine Reactivity in Cytochrome *c* Peroxidase. *Ted Fox* and Ann M. English. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve West, Montreal, Quebec, Canada H3G 1M8.

Three surface histidine residues of cytochrome *c* peroxidase (CCP) are modified by the histidine-specific reagent diethyl pyrocarbonate (DEPC) at pH 7. The histidines have different pK_a's which can be correlated with DEPC reactivity since only deprotonated histidines are reactive. The surface histidines can also be modified by $Ru(NH_3)_5H_2O^{2+}$, yielding stable derivatives which are being used to study intramolecular electron transfer in CCP. Using DEPC, we aim, first, to identify $Ru(NH_3)_5$–CCP derivatives containing 1 Ru/CCP and, second, to distinguish between these derivatives according to their DEPC reactivities. DEPC reactivity will be assigned to each histidine by using HPLC analysis of tryptic digests of the stable $Ru(NH_3)_5$–CCP derivatives and, hopefully, also correlated with histidine assignments by NMR.

104. Preparation and Characterization of a Tris(4,4′-dicarboxy-2,2′-bipyridine)ruthenium(II) (Ru(DIB)) Derivative of Horse Heart Cytochrome *c*. *Theodore Tsekos* and Ann M. English. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve West, Montreal, Quebec, Canada H3G 1M8.

A mixture of covalent derivatives between Ru(DIB) and horst heart cytochrome *c* is formed via carbodiimide-catalyzed amide formation. Addition of *N*-hydroxysulfosuccinimide greatly enhances product yield. The reaction involves amide formation between the carboxylates on the ruthenium compound and the lysine ϵ-amino groups of cytochrome *c*. The characterization of the derivatives obtained and their potential usefulness in the study of electron transport phenomena will be discussed.

105. Alternative Mechanism To Account for the Beneficial Effects of 1-Hydroxybenzotriazole on Peptide-Bond-Forming Reactions. *Francis M. F. Chen* and N. Leo Benoiton. Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5.

The reactions of an *N*-alkoxycarbonylamino and an *N*-acetylamino acid with an amino acid ester effected by carbodiimides in the presence and absence of 1-hydroxybenzotriazole (HOBt) and the reactions of 2-alkoxy-4-alkyl-5-(4*H*)-oxazolones, 2,4-dialkyl-5(4*H*)-oxazolones, symmetrical anhydrides, and mixed anhydrides with HOBt and other additives and an amino acid ester have been examined. The rates of formation of intermediates and products have been monitored by 60-MHz ^1H NMR spectroscopy. An alternative mechanism to explain the beneficial effects of HOBt in accelerating aminolysis, eliminating *N*-acylurea formation, and minimizing racemization during the coupling of acyl amino acids and N-protected peptides will be presented.

106. Isolation and Characterization of Proteins Involved in *Callinectes sapidus* Biomineralization. *Sybil K. Burgess* and Robert D. Roer. Department of Chemistry and Institute for Marine Biomedical Research, University of North Carolina at Wilmington, Wilmington, NC 28403-3297.

Cuticles from the blue crab, *Callinectes sapidus*, were removed immediately prior to and after ecdysis. Both types of cuticle were stripped of underlying hypodermal tissue and successively immersed in 0.5 M $CaCl_2$ and 0.5 M $NaHCO_3$. Observations with polarized light microscopy revealed no mineralization in the preecdysis cuticle, whereas in the postecdysis cuticle mineralization did occur. Biochemical analysis of the pre- and postecdysial cuticles revealed a progessive change in protein composition. Three proteins, observed by the technique of polyacrylamide gel electrophoresis, decreased in abundance as the crab progressed through ecdysis. These proteins have been isolated and characterized with respect to molecular weight, isoelectric point, and carbohydrate content as well as by their ability to inhibit mineralization in vitro.

107. Insect Apolipophorin III: Evidence for the Existence of More Than One Molecular Species in the Hemolymph of *Manduca sexta*. *Marta S. Fernández* and Michael A. Wells. Department of Biochemistry, Centro Investigación I.P.N., A.P.14-740, 07000 México City, México, and Department Biochemistry, University of Arizona, Tucson, AZ 85721.

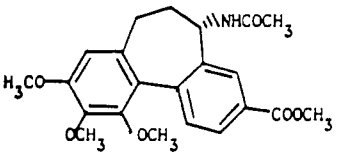
SDS–PAGE and immunoblotting of lipophorin isolated by KBr gradient ultracentrifugation of hemolymph from *Manduca sexta* adults treated with adipokinetic hormone show three distinct bands of very similar MW (ca. 18 000). Apo-III was purified from such lipophorin, the procedure involving exhaustive delipidation followed by aqueous extraction and reversed-phase HPLC. In this way, two peaks were obtained, the ratio between their areas being ~6:1. The major fraction is a protein of ca. 18 000 daltons which, except for a slight tendency to dimerize, is quite stable. Its fluorescence spectrum is characteristic of polypeptides containing tyrosine but not tryptophan. The minor fraction exhibits two close, albeit distinct, bands in SDS–PAGE (MW ca. 18 000); these proteins are very unstable and tend to decompose into low MW species. The fluorescence spectrum is typical of polypeptides containing phenylalanine as the sole aromatic amino acid. Immunoblotting performed subsequently to SDS–PAGE shows that proteins from both HPLC fractions react strongly with IgG obtained against an Apo-III purified by gel filtration, a procedure which has been reported to yield only one molecular form of Apo-III. (This work was performed at the University of Arizona during the sabbatical leave of M.S.F.)

108. Enzymatic Properties of Avidin. *Meir Wilchek*, Gerry Gitlin, Edward A. Bayer, and T. Viswanatha. The Weizmann Institute of Science, Rehovot, 76100 Israel.

During the course of our studies on the biotin-binding site of avidin, we attempted to affinity label avidin using biotinyl *p*-nitrophenyl ester (BNP). When BNP was added to avidin at pH 4 and the pH was raised to 9, a rapid release of *p*-nitrophenol was observed. However, no biotin-containing peptide could be detected. Furthermore, upon denaturation of avidin, free biotin was released, indicating that a hydrolysis reaction occurred and not a covalent labeling of avidin by BNP. Other biotin-containing derivatives, including the amide, *p*-nitroanilide, methyl, and *N*-hydroxysuccinimide esters, were not hydrolyzed. When BNP was combined with bacterial streptavidin, the protein failed to hydrolyze the ester; hydrolysis of BNP was thus *protected* up to pH 12! This indicates a qualitative difference between the binding sites of avidin and streptavidin. In an attempt to identify which amino acid(s) is(are) involved in the observed hydrolysis, we could tentatively exclude His, Tyr, Asp, and Glu using chemical modification techniques. Acetylation of lysines in avidin caused the protection of the hydrolysis reaction. Avidin apparently binds biotin in a way that resembles the transition state of an enzyme, thereby generating the hydrolysis of BNP. This contention is further supported by the fact that *p*-nitrophenyl esters of biotin analogues (homobiotin and norbiotin) were not hydrolyzed.

109. Spectroscopic and Kinetic Features of the Association of Allocolchicine with Tubulin. *Susan Bane Hastie*. Department of Chemistry, State University of New York—Binghamton, Binghamton, NY 13901.

Allocolchicine (**1**) is a colchicine analogue in which the tropone C ring of colchicine is replaced by an aromatic ester.



1: allocolchicine

Like colchicine, **1** is weakly fluorescent in aqueous solution, and ligand fluorescence is enhanced some 300-fold upon binding to tubulin. Unlike colchicine, allocolchicine fluorescence may be induced by decreasing solvent dielectric constant. Fluorescence enhancement upon tubulin binding was monitored to analyze the equilibrium and kinetic features of the association reaction. The association constant for **1** with tubulin was found to be 3.5×10^5 M^{-1} at 37 °C. The kinetic features of the association of **1** with tubulin are virtually identical with the colchicine–tubulin association reaction. The kinetics are biphasic, with an activation energy for the fast phase of 21 kcal/mol. These results indicate that although the electronic properties of the two molecules differ, their interactions with tubulin are quite similar.

110. Immobilization of Cytochrome *c* Peroxidase on a Conducting Polymer Matrix. *Pete Bozel* and Ann M. English. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve West, Montreal, Quebec, Canada H3G 1M8.

Physical entrapment of cytochrome *c* peroxidase (CCP) on a polypyrrole matrix has been achieved in an aqueous medium. Codeposition of enzyme and polypyrrole occurs at a platinum cathode in a typical three electrode cell. The entrapped CCP is active and the electrode may detect the presence of hydrogen peroxide via the reactions

$$\text{CCP[Fe(III)]} + H_2O_2 + 2H^+ \rightleftharpoons \text{CCP[Fe(IV)]} + 2H_2O$$

$$\text{CCP[Fe(IV)]} + \text{C[Fe(II)]} \rightleftharpoons \text{CCP[Fe(IV/III)]} + \text{C[Fe(III)]}$$

$$\text{CCP[Fe(IV/III)]} + \text{C[Fe(II)]} \rightleftharpoons \text{CCP[Fe(III)]} + \text{C[Fe(III)]}$$

where C stands for cytochrome *c*. Characterization of the electrode and its possible use as an amperometric biosensor (glucose sensor in specific) will be discussed.

Enzyme Mechanisms and Kinetics

111. Kinetic and Structural Studies of L-Aspartase from *Escherichia coli*. *Ronald E. Viola* and William E. Karsten. Department of Chemistry, The University of Akron, Akron, OH 44325.

The enzyme L-aspartase from *Escherichia coli* has absolute specificity for its amino acid substrate. An extensive survey has not uncovered any alternate substrates, although a number of good competitive inhibitors have been characterized. A divalent metal ion is required for enzyme activity above neutral pH, with one metal ion binding site per subunit. Paramagnetic NMR relaxation studies have shown that the metal ion binds at the recently discovered activator site on L-aspartase (Karsten et al., *Biochemistry 25*, 1299 [1986]) and not at the enzyme active site. This activator site is remote from the active site of the enzyme since the relaxation of inhibitors that bind at the active site is not affected by paramagnetic metal ions bound at the activator site. Since the substrate L-aspartic acid also serves as the activator for the enzyme, the role of the metal ion may be to direct the partitioning of L-aspartate between the active site and the activator site. The kinetic mechanism of L-aspartase has been established by initial velocity, product inhibition, and metal ion and activator binding studies. [This work was supported by an NIH grant (GM34542) to R.E.V.]

112. Mechanistic Studies of the Bifunctional Enzyme Aspartokinase–Homoserine Dehydrogenase I. *Thelma S. Angeles* and Ronald E. Viola. Department of Chemistry, The University of Akron, Akron, OH 44325.

The bifunctional enzyme aspartokinase–homoserine dehydrogenase I has been purified to homogeneity from *Escherichia coli* by using a purification scheme involving dye–ligand chromatography (Karsten et al., *Anal. Biochem. 147*, 336 [1985]). A series of structural analogues of L-aspartic acid have been investigated as potential alternate substrates or competitive inhibitors of this enzyme by using a coupled spectrophotometric assay for ADP production. A wide range of compounds led to the production of ADP, raising the possibility of an induced ATPase activity rather than acyl-phosphate production. This hypothesis has been explored by kinetic and NMR studies of this enzyme system with several structural analogues of aspartic acid. Analogues with substitutions at the α-carboxyl or β-carboxyl groups have been observed to form acyl-phosphate products that are recognized by aspartate-semialdehyde dehydrogenase, the next enzyme in the biosynthetic pathway. The nature of the enzyme groups involved in binding or catalysis are being examined by pH and chemical modification studies. (Supported by a grant to R.E.V. from the Charles E. Culpeper Foundation.)

113. Presteady-State Kinetic Studies of CTP Synthetase. *Deborah A. Lewis*, Kenneth A. Johnson, and Joseph J. Villafranca. Department of Chemistry and Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802.

CTP synthetase from *Escherichia coli* catalyzes the formation of CTP from ATP, UTP, and glutamine (or NH_3) with a turnover number of 0.38 s^{-1}. UTP-dependent ATP hydrolysis is also catalyzed but at a much slower rate, with a turnover number of 0.03 s^{-1}. The time courses for CTP formation and UTP-dependent ATP hydrolysis are both characterized by a burst of acid labile phosphate equivalent to 90% of the enzyme active sites with transient burst rates of 2.07 and 1.78 s^{-1}, respectively. These results along with positional isotope exchange experiments (von der Saal, W., Anderson, P. M., and Villafranca, J. J. (1985) *J. Biol. Chem. 260*, 14993–14997) indicated that the mechanism for CTP formation involves phosphorylation of UTP by ATP followed by attack of NH_3, producing CTP, ADP, P_i, and glutamate. The positional isotope exchange experiments also suggested that the order of addition of substrates is UTP followed by ATP and then NH_3. Isotope partitioning experiments agree with the positional isotope exchange experiments in that trapping of ATP was not observed, indicating that ATP does not bind first. Preliminary isotope partitioning experiments indicate that UTP can bind first to form a competent E-UTP complex. (Work supported by NSF Grant PCM 8409737.)

114. Studies with the B_{12}-Dependent Methionine Synthase from *E. coli*. *Ruma V. Banerjee*, Verna Frasca, Nancy L. Johnston, David P. Ballou, Prasanta Datta, and Rowena G. Matthews. Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109.

The terminal step in the de novo biosynthesis of methionine is the transfer of a methyl group to homocysteine (Hcy) from methyltetrahydrofolate (MeH_4F), in a reaction catalyzed by methionine synthase (EC 2.1.1.13). The B_{12}-dependent enzyme, as isolated, has an optical spectrum corresponding to cob(II)alamin. It can be reductively methylated with adenosylmethionine and a chemical or physiological reducing system to generate methylcobalamin on the enzyme. Stopped-flow studies with enzyme activated in this manner have been performed under anaerobic conditions at 25 °C. The implications of our studies are (1) the reaction catalyzed by methionine synthase involves a ternary complex, (2) the cobalamin shuttles between cob(I)alamin and methylcobalamin during turnover, and (3) demethylation of methylcobalamin by Hcy is largely rate limiting, while remethylation by MeH_4F is rapid. The gene for the B_{12}-dependent methionine synthase (*met*H) has been cloned into a plasmid vector. It was shown to complement methionine auxotrophy in the double mutant RK4536 (*met*H, *met*E) which lacks both the B_{12}-dependent and independent methionine synthase enzymes. Nucleotide sequence determination of the gene using Sanger's dideoxynucleotide method is in progess. (This work was supported in part by NIH Grant GM24908.)

115. Transition States for Rate Limitation of the Specificity Ratio in Chymotrypsin-Catalyzed Hydrolysis of Peptidyl Substrates. *M. S. Matta*, M. E. Andracki, and R. Elghanian. Department of Chemistry, Southern Illinois University at Edwardsville, Edwardsville, IL 62026.

Temperature dependences and proton inventories of the specificity ratio ($k_c/K_m = k_E$) were determined for three peptidyl substrates of α-chymotrypsin. For Z-Ala-Ala-Pro-Phe-pNA and Succ-Ala-Ala-Pro-Phe-pNA, the Eyring plots exhibit curvature, suggestive of a change in rate-limiting step with temperature. Proton inventory curves for both pNA substrates are dome shaped: $k_E > 10^5$ M^{-1} s^{-1} in H_2O (pH 7.03), $^Dk_E \sim 1.6$. Z-Ala-Ala-Pro-Phe-MBTH (MBTH = 3-methyl-2-benzothiazolinone hydrazone) is more slowly hydrolyzed than the pNA substrates: $k_E = 2.61 \times 10^3$ M^{-1} s^{-1} (pH 7.03), $^Dk_E = 1.69 \pm 0.02$. As for the pNA substrates, the proton inventory curve is dome shaped, but the Eyring plot is linear. The temperature dependence and proton inventory data conform to a model in which chemical acylation of the enzyme is the only step contributing to rate limitation of k_E for the MBTH substrate. A second process, possibly enzyme–substrate association, contributes 10–20% to the rate-limiting transition state for the pNA substrates.

116. Kinetics of Fe Oxidation and Reduction in Ferritin Cores: A Comparison of Natural and Engineered Proteins. J. S. Rohrer, S. P. Sreedharan, M.-S. Joo, E. Dartyge, A. Fontaine,

G. Tourillon, D. E. Sayers, and *E. C. Theil.* Departments of Biochemistry and Physics, North Carolina State University, Raleigh, NC 27695, and Laboratoire pour l'Utilisation du Rayonnement Electromagnetique, Universitē Paris-Sud, F-91405 Orsay Cedex, France.

The protein coats of ferritin, hollow spheres of 24 peptides, react with Fe(II) and dioxygen to form Fe(III)–oxo cluster/protein complexes; later, cores of bulk iron form. Using protein coats from the ferritin of horse spleen, kinetic analyses of core formation, using UV–vis for polynuclear Fe formation (amber color) and X-ray absorption spectroscopy (XAS) for oxidation (2-ev shift in edge), indicated vast differences in rates. Study of the reverse reactions, reduction of Fe(III) and conversion to monoatomic Fe, showed a complex pathway with both rapid (ca. 50 s) and slow (24–48 h) reactions. Clearly, the protein coat influences the chemistry of iron core formation and dissolution. The results of comparing core formation and dissolution for protein coats with native sequences and genetically engineered alterations (subunit dimer interface) will be evaluated to determine regions of the protein important for function. [Supported in part by NIH Grants DK20251 and GM34675 (E.C.T.) and DOE DE-AS05-80-ER10742 (D. E.S.); XAS measurements were made at NSLS and LURE.]

117. Flow Kinetics of an Enzyme Immobilized at a Rotating Disk. *Joseph N. Daka* and Rajender Sipehia. Artificial Cells and Organs Research Centre, Faculty of Medicine, 3655 Drummond Street, McGill University, Montreal, Quebec, Canada H3G 1Y6.

A flow kinetics equation for an enzyme immobilized at a rotating disk surface has been developed. Results of lactate dehydrogenase (LDH) covalently bound to nylon show promising behavior. It was found that the covalent linkaging technique involving 3,3′-diaminobenzidine produces an LDH–nylon surface with a very stable activity. The new theory, coupled with this immobilization procedure, leads to the conclusion that the apparent Michaelis constant, K_m(app), decreases with the speed of rotation, f. Extrapolation to infinite speed of rotation gives the reaction rates, v, and the K_m corresponding to the diffusion-free behavior. At $f^{-1/2} = 0$, the affinity of LDH for NADH, its substrate, was much smaller than when the enzyme was unbound. Since according to our equations, diffusion effects are assumed eliminated at infinite f, the observed drop in the affinity for the substrate was interpreted to be largely due to the sterric hindrances imposed on NADH as a result of having a solid-supported enzyme.

118. Nicotinate Phosphoribosyltransferase of Yeast: Analysis over a pH Range. *Donald L. Sloan* and Susan L. Hess. Chemistry Department, City College of City University of New York, New York, NY 10031.

Nicotinate phosphoribosyltransferase (NPRTase) has been purified from yeast and observed to produce nicotinate mononucleotide (NMN) from nicotinate and phosphoribosyl pyrophosphate (PRibPP) by way of an ordered Uni Uni B Ter Ping-Pong kinetic mechanism, during which ATP is hydrolyzed concomitant with PRibPP utilization (L. Hanna, S. Hess, and D. Sloan, *J. Biol. Chem. 258*, 9745, 1983). Incubations of NPRTase with pyridoxal phosphate (PLP), followed by sodium borohydride reduction, led to inactivation of the enzyme. The presence of Mg(II) and either ATP or PRibPP in the incubation solution reduced competitively and noncompetitively the inhibition by PLP alone and protected the enzyme from inactivation by PLP and borohydride. These effects have been quantitated by pH 7.5 with tritiated PLP (a gift from Dr. J. Preiss). Higher rates of inactivation were observed in the alkaline pH range (7.5–9). These results suggested the presence of an essential lysine residue at the ATP binding site. A kinetic analysis of the stability of NPRTase activity was completed over a pH range of 4.1–9.6. The enzyme was observed to remain 100% active for 30 min from pH 5 to 9. Dixon plots, generated from studies of NPRTase activity over a pH range and concentration ranges of PRibPP and ATP, defined pK values of 5.7 ± 0.3 and 8.5 ± 0.5. (This research was supported by NIH Grants DK-20183 and RR-01868.)

119. Expedient Assay for a Pterin-Dependent, Alkyl Ether Lipid Cleavage Enzyme. *D. Dick* and D. S. Lawrence. Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214.

A thorough investigation of the alkyl ether cleavage (AEC) enzyme, a pterin-dependent monooxygenase, has been hampered due to the lack of a conventient and reliable assay. Interest in the AEC enzyme has been warranted since the discovery that a variety of neoplasms are deficient in this monooxygenase. The AEC enzyme assay employed in our laboratory circumvents the necessity of radio-labeled substrate analogues as well as tedious extraction procedures. To better elucidate the substrate specificity of this catalytic entity, several N-substituted, 1-alkylphosphatidylethanolamine analogues have been synthesized and evaluated as substrates. Optimal conditions and requirements for the monooxygenation reaction are more readily ascertained as a consequence of the improved assay technique. The synthesis, structures, and substrate efficiency of the lipid conjugates will be presented.

120. Substrate Specificity of cAMP-Dependent Protein Kinase. *M. Prorok* and D. S. Lawrence. Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214.

The catalytic subunit of cAMP-dependent protein kinase from bovine heart phosphorylates serine and threonine residues in protein and peptide substrates. Phosphoryl group transfer is not restricted to the hydroxyl groups of serine and threonine, as the enzyme's limited capacity for the phosphorylation of water, phosphatidylinositol 4-phosphate, and hydroxyproline has been previously demonstrated. With this information in hand, we embarked upon a study of the substrate specificity of the kinase, employing analogues of the synthetic heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, wherein serine is replaced by other phosphorylatable residues. Our presentation will focus upon the kinetic constants obtained for the kinase reaction with these novel substrates, as well as the spatial and heteroatom requirements for phosphorylation. The design and synthesis of potential inhibitors as it relates to our knowledge of substrate specificity will also be emphasized.

121. 2′-*O*-Anthraniloyl- and 2′-*O*-(*N*-Methylanthraniloyl) Derivatives of cAMP and cGMP as Active Site Probes of Cyclic Nucleotide Phosphodiesterases. N. Karuppiah, C. J. Talpas, and *B. Mutus.* Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4.

Calmodulin-dependent 3′:5′-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17) M_r 63 000 and 60 000 isozymes from the brain as well as M_r 60 000 species from the heart and the placenta, isolated by conformational-specific monoclonal antibody affinity chromatography, have been compared with

respect to their steady-state kinetic parameters (K_m and V_m) for the hydrolysis of the fluorescent 2′-*O*-anthraniloyl- (ANT) and 2′-*O*-(*N*-methylanthraniloyl) (MANT) derivatives of cAMP and cGMP. In general, ANT derivatives resulted in higher K_m and V_m values in comparison to the MANT derivatives. The phosphodiesterase forms that displayed the largest pseudo-substrate specific variation in their kinetic parameters were the low M_r brain and the placenta enzymes. The higher K_ms and V_ms observed with the ANT derivatives of the cNMPs are thought to be related to the formation of an intramolecular H-bond which leads to decreased electrostatic interactions between active-site cationic side chain(s) and the cyclic ribose phosphate of the pseudo-substrate. (Supported by grants from NSERC and the Research Board, University of Windsor.)

122. Intramolecular Interactions in 2′-*O*-Anthraniloyl and 2′-*O*-(*N*-Methylanthraniloyl) Derivatives of cAMP and cGMP. T. Leung, R. Aroca, B. A. Keay, and *B. Mutus*. Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4.

2′-*O*-Anthraniloyl (ANT) and 2′-*O*-(*N*-methylanthraniloyl) (MANT) derivatives of cAMP and cGMP have been used as pseudo-substrates and as active-site probes of the enzymes cyclic nucleotide phosphodiesterase. This communication attempts to reconcile the differences in K_m and V_m displayed by the ANT and MANT derivatives of the cNMPs on the basis of pseudo-substrate specific intramolecular interactions, which have been detected by a combination of spectroscopic techniques including ^{31}P NMR, surface enhanced Raman, and FT-IR. (Supported by grants from NSERC and the Research Board, University of Windsor.)

123. Function of Cys-16 of Spinach Phosphoribulokinase (PRK). *Michael A. Porter* and Fred C. Hartman. Biology Division, Oak Ridge National Laborabory, P.O. Box Y, Oak Ridge, TN 37831.

Regulatory deactivation of PRK entails intrasubunit oxidation of Cys-16 and Cys-55 to a disulfide. Chemical modification studies place Cys-16 at the binding domain for ATP, thereby suggesting that one of the regulatory sulfhydryls may also be involved in catalysis. To explore this possibility, we have determined the consequences of modifying Cys-16 with a sterically unobtrusive methyl group. Treatment of PRK with methyl 4-nitrobenzenesulfonate leads to a 50% loss of activity with a concomitant 2-fold increase in the K_m for ATP, demonstrating that the residual kinase activity is not due to incomplete modification. Methylation of a regulatory sulfhydryl is indicated by the resistance of the modified enzyme, in contrast to the native, to 5,5′-dithiobis(2-nitrobenzoic acid). Peptide mapping of tryptic digests of the modified enzyme, following its [^{14}C]carboxymethylation, documents Cys-16 as the site of methylation. Thus, Cys-16 cannot be essential to catalysis, and regulatory deactivation may reflect conformational or steric effects. (Supported by Martin Marietta Energy Systems, Inc., under Contract DE-AC05-84OR21400 with the USDOE and USDA grant 84-CRCR-1-1520.)

124. Carbocyclic Analogue of Glycinamide Ribonucleotide Is a Substrate for Glycinamide Ribonucleotide Transformylase. *Carol A. Caperelli* and Martyn F. Price. College of Pharmacy, University of Cincinnati, Cincinnati, OH 45267.

The carbocyclic analogue of glycinamide ribonucleotide has been synthesized from the parent racemic trihydroxycyclopentylamine. This analogue was accepted as a substrate by mammalian glycinamide ribonucleotide transformylase (K_m = 18 μM, V_{max} = 0.23 mM/min), the first folate-dependent formyl group transferase of de novo purine biosynthesis, with an efficiency comparable to that of the natural substrate glycinamide ribonucleotide (K_m = 10 μM, V_{max} = 0.27 mM/min). For each molecule of 10-formyl-5,8-dideazafolate cosubstrate consumed, 0.92 molecule of *N*-formyl carbocyclic glycinamide ribonucleotide was produced in the enzymatic reaction, indicating a 1:1 stoichiometry. These studies afford an analogue that should facilitate investigations on the early steps in de novo purine biosynthesis and suggest that the ribose ring oxygen of the natural substrate is not critical for enzyme recognition and binding.

125. Effects of Monovalent Cations of Enolase Activity. *M. J. Kornblatt*. The Enzyme Research Group, Departments of Chemistry and Biology, Concordia University, Montreal, Quebec, Canada H3G 1M8.

The activity of mammalian enolases (EC 4.2.1.11) is stimulated by some monovalent cations (K^+, NH_4^+, Cs^+, Rb^+) and inhibited by others (Li^+, Na^+). Both the stimulation by K^+ and the kinetic isotope effect have been measured as a function of pH. The results suggest that K^+ stimulates a step that is at least partially rate limiting at pH 7.0 and that this step is not proton abstraction. Yeast enolase is also inhibited by Li^+ and Na^+ but is not stimulated by any of the monovalent ions tested. These results, plus the larger kinetic isotope effect for yeast enolase at pH 7.0 (k_{ie} = 1.65 for yeast vs 1.34 for the mammalian enzyme), suggest some differences between yeast and mammalian enolases in the details of the catalytic mechanism.

126. Kinetic and Spectroscopic Investigations of Phosphoglycolate Phosphatase. *David E. Ash*. Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140.

Phosphoglycolate phosphatase catalyzes the hydrolysis of phosphoglycolate in a reaction which requires both divalent cation and monovalent anion activators. We have found that the enzyme from spinach will also catalyze the hydrolysis of thiophosphoglycolate and hydroxylamine phosphate. The hydrolysis reactions are potently inhibited by submicromolar concentrations of vanadate, while molybdate and tungstate are much less effective as inhibitors. The paramagnetic vanadyl ion (VO^{2+}) has been shown to bind to phosphoglycolate phosphatase, presumably at the divalent cation binding site. Although VO^{2+} does not support catalysis, EPR experiments have shown that ternary complexes of enzyme–VO^{2+} with glycolate and vanadate are readily formed. These experiments demonstrate the utility of VO^{2+} as a paramagnetic probe in studies of the metal ion binding site of phosphoglycolate phosphatase. (Supported by a grant from the American Heart Association, Pennsylvania Affiliate.)

127. Substrate Specificity and Kinetics of a Fungal β-Glucosidase. A. C. Lo and *G. E. Willick*. DOMTAR Research Centre, Senneville, Quebec, Canada, and Division of Biological Sciences, National Research Council, Ottawa, Canada.

β-Glucosidases release glucose from cellobiose and many R-β-D-glucosides and are essential in the total breakdown of cellulose to glucose. We have studied the β-glucosidase ex-

pressed by the wood-rotting fungus *Schizophyllum commune*. Two forms of the enzyme, designated β-glucosidase I (βGase-I) and β-glucosidase II (βGase-II), are secreted. The two appear to be very similar in sequence, bu βGase-II is much more highly glycosylated. They differ in their pH optima, being 5.75 and 5.08 for βGase-I and βGase-II, respectively. Little specificity is shown for the R-group, but analysis of Michaelis–Menten kinetics for oligosaccharides of dp 2–6 indicated a binding site for three glucose residues. βGase-I was much more inhibited by glucose than βGase-II, whereas both were equally inhibited by glucono-δ-lactone, a transition-state analogue. Cellobiose was much more sensitive to glucose inhibition than the higher oligosaccharides, resulting in its accumulation during hydrolysis of the higher oligomers.

128. Kinetic Probes of Heme Accessibility of Cytochrome *c* in Complexes with Cytochrome *c* Peroxidase. *Paul Ala* and Ann M. English. Enzymology Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve West, Montreal, Quebec, Canada H3G 1M8.

The accessibility of the cytochrome *c* heme in the complexes formed between cytochrome *c* peroxidase (CCP) and cytochrome *c*'s from yeast, horse, and tuna has been investigated. The kinetics of nitric oxide binding to cytochrome *c*, in the presence and absence of CCP, were used to probe heme accessibility. Nitric oxide was used because its association rates will only be marginally influenced by electrostatic factors and its reacts rapidly with ferricytochrome *c* at neutral pH.

129. Photosensitivity Studies of the Ligand Complexes of Cytochrome Oxidase. *Bruce C. Hill.* Enzyme Research Group, Department of Chemistry, Concordia University, 1455 de Maisonneuve Blvd. West, Montreal, Quebec, Canada H3G 1M8.

Cytochrome oxidase reacts with oxygen at a site that is thought to involve two metal centers, one heme of cytochrome a_3 and one of the copper atoms, Cu_B. The oxidase also binds the inhibitory ligands CO, NO, and CN^- at the O_2-reactive site when cytochrome a_3 is in the ferrous state. The CO and NO adducts have been reported to be photosensitive. In these studies I have measured the photosensitivity of the cyanide complex. The quantum yield and recombination kinetics for these ferrous cytochrome a_3–ligand complexes will be reported. In addition, the dependence of the parameters on temperature and pH shall be presented.

130. Role of Heme Propionate Groups in Myoglobin Oxidation–Reduction Behavior. *Anthony R. Lim*, Bhavini P. Sishta, and A. Grant Mauk. Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

The oxidation–reduction equilibrium and reduction kinetics of dimethyl ester heme-substituted horse heart myoglobin (DME-Mb) have been studied under a variety of solution conditions to evaluate the involvement of the heme propionate groups in myoglobin electron transfer. Under standard conditions (pH 7.0, μ = 0.1 M, 25 °C) E_m for DME-Mb is 100.0(2) mV vs NHE ($\Delta H°$ = 12.9(2) kcal/mol and $\Delta S°$ = −51.0(8) eu), which is 39 mV higher than the E_m of native Mb. The pH dependence of the DME-Mb reduction potential can be fitted with the assumption of a single, redox-linked titratable group with pK_{red} = 7.8 [8.4] and pK_{ox} = 6.4 [7.7] (comparable values for native Mb shown in brackets). Under standard conditions, reduction of DME-Mb by $Fe(EDTA)^{2-}$ proceeds with k_{12} = 1.3(5) × 10^3 M^{-1} s^{-1} ($\Delta H^‡$ = 9.2(3) kcal/mol and $\Delta S^‡$ = −13(1) eu), which is 10^3 times that of native Mb. The pH dependence of k_{12} shows an apparent pK_a = 7.4 [5.9] with k_a = 1.9(7) × 10^3 M^{-1} s^{-1} and k_b = 1.1 M^{-1} s^{-1}. The possible involvement of His-97 (to which one of the heme propionate groups is hydrogen bonded) in these observations is considered. (Supported by MRC of Canada Grant TM-7812.)

131. Mutation-Induced Perturbation of the Cytochrome *c* Alkaline Isomerization. *Linda L. Pearce*, Alfred L. Gartner, Michael Smith, and A. Grant Mauk. Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

During $Fe(EDTA)^{2-}$ reduction of yeast iso-1 cytochrome *c* mutants altered at position 82, two kinetic phases were observed at mildly alkaline pH, conditions under which the wild-type protein reduces as a single exponential. The possible role of the alkaline isomerization in this observation was evaluated by spectrophotometric pH titrations of the variants in which the S→Fe charge transfer band (695 nm) is monitored. The pK_a for the exchange of the Met-80 heme iron ligand was found to be 8.5 for the wild type, 7.7 for Ser-82, 7.7 for Gly-82, 7.2 for Leu-82, and 7.2 for Ile-82. pH-jump experiments (Davis et al., *J. Biol. Chem. 249*, 2624 (1974)) established that substitutions at position 82 affect the alkaline isomerization by lowering the pK_a of the "triggering" group as much as 1.9 p*K* units; for the Ser-82 and Gly-82 variants, there is also a small effect on the K_{eq} for the ligand-exchange equilibrium. One critical role for Phe-82 in the wild-type protein appears to be stabilization of the heme binding environment. (Supported by NIH Grant GM-33804 and MRC Grant TM-1706.)

132. Interaction of Cytochrome b_5 and Porphyrin Cytochrome *c*. *Lindsay Eltis* and A. Grant Mauk. Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

Complex formation between cytochrome b_5 and porphyrin cytochrome *c* has been studied by fluorescence quenching titrations. At pH 7 (μ = 2 mM, 25 °C), the apparent binding stoichiometry was found to be 1:1, with K_a = 3.3(4) × 10^6 M^{-1}. Based on Forster energy-transfer theory (FETT), the quenching maximum (Q_{max}) of 29% under these conditions is consistent with a distance of 18 Å between the cytochrome b_5 heme and the cytochrome *c* porphyrin in the complex. The stability of the cytochrome b_5–porphyrin cytochrome *c* complex was highly dependent on ionic strength, and the dependence of Q_{max} on pH suggested that the conformation of the complex varies with pH. Similar experiments using dimethyl ester heme-substituted cytochrome b_5 also indicated 1:1 stoichiometry, with K_a = 1.1(3) × 10^6 M^{-1} and Q_{max} = 11% (FETT distance of 22 Å) (pH 7, μ = 2 mM, 25 °C). We conclude that the availability of the cytochrome b_5 heme propionate groups affects the structure of the complex formed in solution. This result agrees with previous measurements and computer modeling studies on the native complex (Mauk et al. *Biochemistry 25*, 7085 (1986)). (Supported by NIH Grant GM-28834.)

133. 3-Hydroxy-3-methylglutaryl-CoA Synthase: Identification of the Cysteine Involved in Forming the Acetyl-S-Enzyme Intermediate. S. H. Vollmer, L. M. Mende-Mueller, and *H. M. Miziorko*. Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226.

Avian liver mitochondrial HMG-CoA synthase contains an active-site cysteine involved in forming the labile acetyl-S-enzyme reaction intermediate. Identification of this cysteine has been recently accomplished. The experimental strategy relies upon generation and rapid purification of the cysteine-containing peptide under mildly acidic conditions that stabilize the thioester adduct. [^{14}C]Acetyl-CoA is used to form [^{14}C]acetyl-S-enzyme, which is isolated and brought to pH 5.0 prior to chymotryptic digestion. High levels of the protease (0.3:1 w/w) are used to achieve effective digestion at this suboptimal pH. The digest is loaded onto a mono-S FPLC cation-exchange column (20 mM formate, pH 3.8); the weakly bound ^{14}C-labeled fraction elutes prior to gradient elution of the column as a broad peak well resolved from unbound material. Further purification was achieved by reverse-phase HPLC, using a 0–60% gradient of CH_3CN in aqueous TFA. A single radioactive peptide is recovered and sequenced: RESGN TDVEG IDTTN ACY (C is determined by detection of a [^{14}C]PTH-aa). The acetylated cysteine corresponds to Cys-129 in the cytosolic enzyme. This cysteine has previously been shown to be a target of the affinity label chloropropionyl-CoA. (Supported by NIH (DK-21491).)

134. Active-Site Similarities among Aconitase, Isopropylmalate Isomerase, and Homoaconitase. *Mark H. Emptage.* Central Research and Development Department, E. I. du Pont de Nemours & Co., Wilmington, DE 19898.

Aconitase, isopropylmalate (IPM) isomerase, and homoaconitase are the second enzymes in the citric acid cycle, leucine biosynthetic pathway, and lysine biosynthetic pathway of yeasts, respectively. These enzymes catalyze similar dehydration/hydration reactions in which the common structural element of the allylic substrates is a maleate moiety modified at C-2. Aconitase has been shown previously to contain a catalytically active [4Fe-4S] cluster which binds the substrate at a single Fe site (Fe_a). Oxidation of the cluster results in loss of Fe_a and inactivation of the enzyme. Both IPM isomerase and homoaconitase are inactivated by air oxidation, during which the loss of activity correlates with the release of a single Fe. The loss of both iron and activity is greatly retarded by either removal of oxygen or addition of substrate. Reduced IPM isomerase has an EPR signal which is typical of [4Fe-4S]$^{1+}$ clusters and which shifts from a g_{avg} of 1.96 to 1.90 upon addition of substrate. This is analogous to that observed for aconitase and is evidence that substrate coordinates to the Fe-S cluster of IPM isomerase. These results suggest that the three enzymes have structurally similar active sites containing an Fe-S cluster and may have evolved from a common ancestral gene.

135. Inactivation of *E. coli* Pyruvate Formate-Lyase by [^{32}P]- and [^{3}H]Hypophosphite. *K. A. Lipsett*, L. A. Ulissi, E. J. Brush, and J. W. Kozarich. Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

Recently, Knappe and co-workers (*Proc. Natl. Acad. Sci. USA* (1984) *81*, 1332) demonstrated that the catalytically active form of pyruvate formate-lyase (PFL) is associated with a protein-bound organic free radical which is quenched upon enzyme inactivation by oxygen or hypophosphite. Our interest in the chemical mechanism of this unusual enzymatic reaction has led us to investigate several key aspects of PFL inactivation by hypophosphite, in particular whether inactivation ultimately results from quenching of the protein radical, covalent protein modification, or both. Our initial results suggest that phosphorus–hydrogen bond cleavage is at least partially rate-limiting during inactivation and that the inactivated enzyme can be fully reactivated. Furthermore, in agreement with Knappe's findings, stoichiometric radiolabeling of PFL occurs upon inactivation by [^{32}P]hypophosphite. Present studies with [^{32}P]- and [^{3}H]hypophosphite and 3-[^{14}C]pyruvate will reveal the fate of the protein-bound radiolabel upon PFL reactivation, the involvement of the thioester bond of the acetyl enzyme during inactivation, and if one or both hypophosphite hydrogens are labilized in the inactivation mechanism. (Research supported by NIH Grant GM 35066.)

136. Synthesis of 2(*R*)- and 2(*S*)-Glutathiolactaldehydes as Probes for the Stereochemical Course of Glyoxalase I Catalyzed Reactions. *J. A. Landro*, E. J. Brush, and J. W. Kozarich. Department of Chemistry, University of Maryland, College Park, MD 20742.

The stereochemical course of the reactions catalyzed by glyoxalase I (GX-I) has remained elusive as the substrates are mixtures of rapidly interconverting thiohemiacetals. We have reported (Brush, E. J., Kozarich, J. W. *Fed. Proc. 46*, abstract 1814) the preparation of the diastereomeric mixture of glutathiolactaldehyde (GLA) which was processed by GX-I to glutathiohydroxyacetone (GHA) presumably via a nonstereospecific proton abstraction from C-2 of GLA, followed by stereospecific incorporation of solvent deuterium into the hydroxymethyl group of the GHA product. This unusual example of nonstereospecific enzymatic proton abstraction prompted us to synthesize the individual GLA diastereomer so that we could follow the kinetics of their processing by GX-I via UV and NMR. Our present data suggest that these two diastereomers are processed in an equivalent manner, thereby suggesting nonstereospecific processing of the normal thiohemiacetal substrates. (Research supported by NIH Grants GM 35066 and 35067.)

137. Citrate Synthase Catalyzed Enethiolization of Dithioacetyl-CoA. *Ivan Wlassics* and Vernon E. Anderson. Department of Chemistry, Brown University, Providence, RI 02912.

Citrate synthase (CS) catalyzes the synthesis of 1-thiocitrate from dithioacetyl-CoA and oxalacetate with a V_{max} of 0.024 min^{-1}. When excess dithioacetyl-CoA is reacted with 18 μM CS, a rapid exponential decrease in dithioester absorbance (ϵ_{306} = 11 000 cm^{-1}) is observed followed by a slow linear decrease. The spectrum of the rapid decrease has a λ_{max} at 306 nm. The rate constant for the rapid exponential process increases from 2.16 min^{-1} at pH 8.5 to at least 10 min^{-1} at pH 6.5. The amplitude of the exponential process increases over 4-fold between pH 6.5 and 8.5. That the enzyme is rapidly catalyzing the enolization of the dithioacetyl-CoA is demonstrated by the direct observation of CS-catalyzed exchange of the α-protons by ^{1}H NMR. The ^{1}H NMR monitored exchange is over 4-fold faster at pD 6.6 than at pD 7.4. Both the rapid exponential process and the proton exchange required the presence of oxalacetate.

138. Transition-State Structure of the Crotonase Reaction. *Brian J. Bahnson* and Vernon E. Anderson. Department of Chemistry, Brown University, Providence, RI 02912.

The rate-determining transition-state structure of the crotonase reaction has been investigated by measuring the primary deuterium and secondary deuterium kinetic isotope effects at C-2 and C-3 of crotonylpantetheine by perturbation and direct

comparison methods. The kinetics of the reaction were followed spectrophotometrically by measuring changes in absorbance at 263 nm resulting from the reversible hydration of an α-β unsaturated thioester to form the 3-hydroxy thioester. Crotonylpantetheine, which reacts at 1% of the near diffusion controlled rate of crotonyl-CoA, was used to ensure a rate-limiting chemical step. A small but significant primary deuterium kinetic isotope effect of 1.1 was measured. The secondary deuterium kinetic isotope effect was measured at C-2 to be 1.20 and 1.35 and at C-3 to be 0.75 and 0.95 for the hydration and dehydration reactions as written. The primary ^{18}O kinetic isotope effect and transition-state-based inhibitors presently being studied will further define the transition-state structure.

139. Use of Isotope Effects in Elucidation of Enolase Reaction Mechanism. *Louise Penn* and Vernon E. Anderson. Department of Chemistry, Brown University, Providence, RI 02912.

The partitioning of the carbanion intermediate in the enolase-catalyzed dehydration of 2PGA has been examined by a variety of methods. A 10-fold decrease in $V/K_{2PGA}K_{mg}$ between pH 9.0 and 10 indicates a solvent proton is required to form the rate-determining transition state. The inverse solvent isotope effect on dehydration of 0.6 ± 0.1 at pH 9.0 indicates that this proton has fully equilibrated prior to the rate-determining transition state. The location of this rate-determining transition step relative to the two bond cleavages is being determined by measuring of 2PGA when [2-^{2}H]2PGA is incubated with enolase in $H_2{}^{18}O$. Unreacted 2PGA will be analyzed by mass spectroscopy (NICI) of the 1-pentafluorobenzyl-2,3-diacetyl derivative of glyceric acid obtained after treating 2PGA with bacterial alkaline phosphatase. The M^+, M + 1, M + 2, and M + 3 peak intensities reflect the amount of the three possible proton and ^{18}O exchanges.

140. Isotope Effect and Stereospecificity Studies of the Interactions of NAD^+ with Lactate Dehydrogenase. *Richard D. LaReau* and Vernon E. Anderson. Department of Chemistry, Brown University, Providence, RI 02912.

An isotope ratio method based on the parent ion of nicotinamide has been developed to study the interaction of the nicotinamide ring of NAD^+ with lactate dehydrogenase. A mixture of [4-^{2}H]- and [4-^{1}H]NAD^+ (1:1) was allowed to bind to lactate dehydrogenase in a dialysis cell. Bound and unbound NAD^+ were isolated and hydrolyzed, and the nicotinamide was isolated. Careful measurement of the parent ion ratios (M/M + 1) with the mass spectrometer permits detection of isotope effects on binding to within ±0.5%. The stereochemical interaction of the nicotinamide ring was studied with a recycling assay with ^{3}H originally present in achiral [4-^{3}H]NAD^+. The results demonstrate that the enzyme adds and removes the same hydrogen with better than 99.99998% specificity, a discrimination which requires a difference of over 15 kcal/mol between the two transition states.

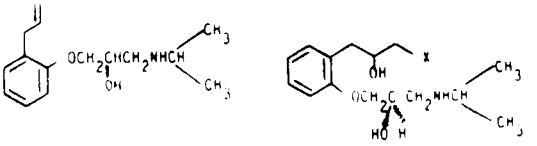
141. Computer-Enhanced Probes of Site-Specific Mutant Enzymes by Equilibrium Isotope-Exchange Kinetics: Aspartate Transcarbamylase. *F. C. Wedler*, Y. Hsuanyu, E. R. Kantrowitz, and S. A. Middletown. MCB, Althouse Lab, Pennsylvania State University, University park, PA 16802, and Chemistry Department, Boston College, Chestnut Hill, MA 02167.

Isotopic exchange kinetics at chemical equilibrium (EIEK) are highly insightful, allowing simultaneous observation of both rate-limiting and faster steps. New PC-based programs have dramatically extended the theoretical and quantitative base of this method by allowing simulation of EIEK data under a wide variety experimental conditions. ISOBI calculates exchange rates as a function of any [substrate pairs], with options for cooperative binding and dead-end complex formation. ISOMOD simulates variation of [modifier] at fixed [S], with any kinetic constants designated as targets for activation or inhibition. Data fitting can provide reliable quantitative values for both "outer" and "inner" kinetic constants. Analysis of EIEK data for *E. coli* transcarbamylase mutated at Tyr-240 → Phe revealed changes in kinetic mechanism, the T–R transition, and responses to allosteric modifiers ATP and CTP. (Supported by NIH Grant GM-33359.)

Inhibitors

142. β-Adrenergic Receptor Blocking Agents Related to Alprenolol. Alprenolol Halohydrins and Related Molecules. *Leah Gotlib*, Tikam Jain, Robert Shorr, and Stanley T. Crooke. Research and Development, Smith Kline & French Laboratories, Swedeland, PA 19406.

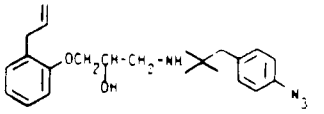
Previously reported chemical and photolabile covalent probes for β-adrenergic receptors (βAR) position their alkylating moieties on the propanolamine-containing side-chain of the known βAR ligands, such as alprenolol (**1**) and others. We



1

2 x = -Br

3 x = -I

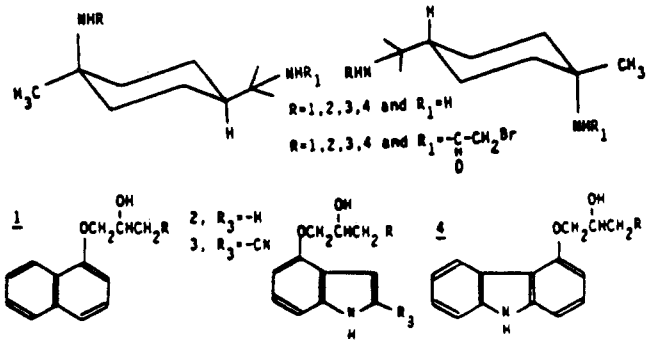


4

have prepared the halohydrins (**2** and **3**) wherein the alkylating moiety is in the ortho position to the phenoxypropanololamine chain. These molecules bind to βARs with high affinity (K_d < 10^{-8} M). The bromohydrin analogue (**2**) irreversibly binds to βARs but only at high concentrations and elevated pH. Our studies suggest that the alkylation by bromohydride (**2**) occurs at a poorly accessible, weak nucleophilic binding site on the receptor. Additionally, biological data on some analogues of alprenolol such as **4** and others will be presented.

143. Active-Site Directed Irreversible β-Adrenergic Receptor Inhibitors Containing Isomeric Bromoacetyl-1,4-diaminomenthanes. *Israil Pendrak*, Leah Gotlib, Drake Eggleston, Tikam Jain, Robert G. L. Shorr, and Stanley T. Crooke, Research and Development, Smith Kline & French Laboratories, Swedeland, PA 19406.

We have synthesized stable and active ligands **1**–**4** which react reversibly at the receptor site (e.g., K_d 1.0×10^{-10} M for **1**, 1.3×10^{-10} M for **4** with R_1 = H). The introduction of a bromoacetyl function in these molecules leads to an irreversible alkylation of the receptors (e.g., K_d 3.8×10^{-10} M for Z-1 isomer of **3** with R_1 = $COCH_2Br$). Despite similar



binding affinities of β_1AR and β_2AR, dramatic selectivity in alkylation of β_2AR was observed. The X-ray crystal structure of **4** (R_1 = bromoacetyl) reveals conformational features of the molecule which may have some significance in interactions with βAR.

144. Design of Alternate Substrate Inhibitors of Human Leukocyte Elastase. *Leslie J. Copp*, Robin W. Spencer, Tim F. Tam, Everton M. Thomas, Heidi LeBlanc, Stephen Heard, and Allen Krantz. Syntex Research (Canada), 2100 Syntex Court, Mississauga, Ontario, Canada L5N 3X4.

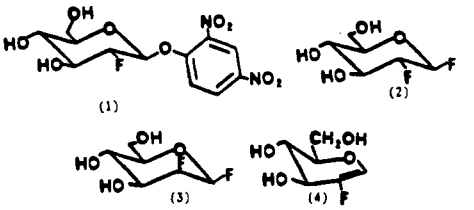
Benzoxazinones are alternate substrate inhibitors of human leukocyte elastase and other serine proteases. Through simple substitution on the benzoxazinone, inhibitors have been designed with deacylation rates as low as 10^{-5}–10^{-6} s^{-1}, while the compounds themselves have enhanced stability. These particular substituent effects will be discussed.

145. Investigation of Enzyme–Substrate Complexes of Phosphoglucomutase by ^{19}F NMR. *M. David Percival* and Stephen G. Withers. Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Y6.

The complexes of phosphoglucomutase (PGM) and various fluorinated substrate analogues have been studied by ^{19}F NMR. Large changes in chemical shift (up to 14 ppm) occur on binding of substrate analogue to the enzyme, and these shifts are modulated by addition of metal ion. In the case of the complex of 6-fluoro-α-D-glucose 1-phosphate (an inhibitor), addition of lithium (an inhibitory metal ion) increases the magnitude of the chemical shift, whereas magnesium (an activating metal ion) causes a broadening of the signal, which is construed as a rapid conformational change of the enzyme. The ternary complexes of PGM, Cd^{2+}, and either 3- or 4-fluoro-α-D-glucose 1-phosphate (3FGIP, 4FGIP), which are both substrates, both give rise to a single bound species. However, the spectrum of an equivalent ternary complex with 2FGIP shows two bound species. These two species may be 2F-glucose bisphosphate bound in different orientations to the enzyme, thus providing evidence for the proposed "exchange mechanism".

146. Mechanism-Based Glycosidase Inhibitors. *Ian P. Street* and Stephen G. Withers. Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Y6.

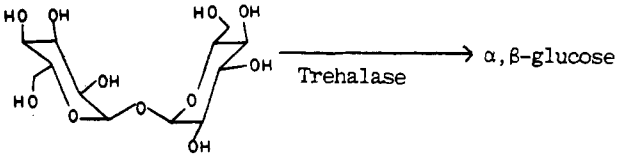
The 2-deoxyfluoroglycosides **1**–**4** are excellent covalent inhibitors of β-glucosidase from *A. faecalis*. Inhibition by **1** and **2** proceeds by formation of a stable 2-fluoroglucosyl intermediate. This can be purified by gel permeation chromatography and stored for weeks at 4 °C. However, the 2-fluoromannosyl enzyme generated from **3** is unstable and hydrolyzes slowly, producing active enzyme. Full reactivation of the 2-fluoroglucosylated enzyme can only be accomplished



by incubation of the intermediate with a second ligand such as β-D-glucosylbenzene. The compound 2-fluoro-D-glucal (**4**) also inhibits this β-glucosidase, again by formation of a stable 2-fluoroglucosyl intermediate. The stereochemical course of the protonation step in the inhibition reaction has been established by using a variety of different techniques.

147. Trehalase from *S. cerevisiae*: Purification and Active-Site Studies. Hamzah Mohd. Salleh and John F. Honek. Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

Due to the increase in fungal infections observed in leukemic and immunosuppressed patients, enzymes unique to fungi should be excellent targets for the development of chemotherapeutic agents. Trehalase, α,α-glucoside 1-glucohydrolase (EC 3.2.1.28), specifically hydrolyzes the nonreducing disaccharide trehalose and is essential for growth and sporulation events in fungi. We have purified and characterized the cytosolic trehalase from *S. cerevisiae* by combination of ion-exchange and affinity methods. Active-site studies were performed by utilizing chemical modifying reagents and substrate analogues. Knowledge of the active site of the enzyme will be presented especially in relation to inhibitor design.



148. Putrescine Aminopropyltransferase in *E. coli* HT527: Product Inhibition Studies. *John E. Gavagan* and David L. Anton. Central Research and Development Department, E. I. du Pont de Nemours Co., Wilmington, DE 19898.

Putrescine aminopropyltransferase (spermidine synthase) catalyzes the transfer of the aminopropyl moiety from decarboxylated *S*-adenosylmethionine to putrescine, forming spermidine and 5-methylthioadenosine. A ping-pong mechanism has been proposed for the *E. coli* enzyme [Zappia, V., et al., *J. Biol. Chem.* (1980) *255*, 7276–7220]. We have previously shown through computer modeling that the enzyme kinetics data fit best to the equation of a sequential mechanism. Additionally, we have been unable to isolate or show evidence for the existence of a covalent reaction intermediate, a requirement for a ping-pong mechanism. To help resolve the reaction mechanism, product inhibition experiments were performed. The results demonstrated that spermidine had 5-methylthioadenosine appear to act as mixed-type inhibitors of the enzyme. When modeled to equations for ping-pong and sequential mechanisms, the data best fit the latter type. The fit was improved when the data were fit to equations for bisubstrate reactions involving the formation of abortive ternary enzyme complexes. Based on these and earlier results we conclude that the putrescine aminopropyltransferase reaction mechanism is sequential rather than ping-pong.

149. Inhibition of Enzyme-Catalyzed Claisen Condensations by Tetravalent Phosphorus Compounds. J. Chen, G. Mrachko,

and *K. Calvo*. Department of Chemistry, The University of Akron, Akron, OH 44325.

The use of phosphoramidates and phosphinates as transition-state analogue inhibitors of serine proteases is well established. In many ways, the enzymes catalyzing Claisen condensations have mechanistic requirements similar to the serine proteases. We are investigating whether phosphinic acids and phosphine oxides can mimic the tetrahedral intermediates that occur during enzyme-catalyzed Claisen condensations of acetyl-CoA. Our initial studies focus on the cytoplasmic thiolase enzyme. In this reaction, two molecules of acetyl-CoA are condensed to produce the thermodynamically less stable acetoacetyl-CoA (AcAc-CoA) and CoASH as products. The cytoplasmic enzyme is the first step in the conversion of acetyl-CoA to HMG-CoA. Initially, a series of compounds of general structure $CH_3P(O)(R)CH_2C(O)R'$ were synthesized and tested as inhibitors. We report on the characterization of the inhibition by these compounds of the thiolytic cleavage of AcAc-CoA catalyzed by thiolase and compare rates of inactivation and inhibition constants for these compounds with other inhibitors of thiolase.

150. Inactivation of Yeast Alcohol Dehydrogenase by Nitrilopropionamides. *Grace C. Shiao* and Ronald E. Viola. Department of Chemistry, The University of Akron, Akron, OH 44325.

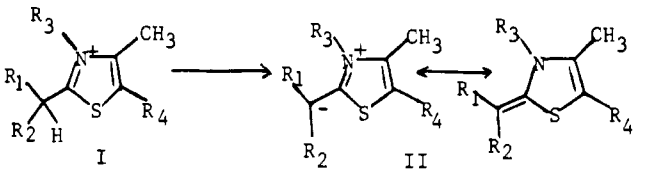
Yeast alcohol dehydrogenase is inhibited by a series of nitrilopropionamides with K_i values ranging from several millimolar to submicromolar depending on the nature of substituents. Both the nitrile and the acetamide groups are required, as well as a good leaving group on the α-carbon. A geminal electronegative atom is necessary for maximal activity. The more potent inhibitors have been observed to cause a slow, time-dependent inactivation of the enzyme with half-times ranging from several seconds to several hours depending on the structure and concentration of the compound. The inactivation appears to be irreversible, since no activity was recovered upon dilution or removal of the inactivator. Substrate protection studies have implicated the nucleotide binding site as being involved in the inactivation process. The results of kinetic, binding, and spectroscopic studies have suggested several possible mechanisms of inactivation of yeast alcohol dehydrogenase by nitrilopropionamides that are currently being explained. (This project is supported by a grant to R.E.V. from the Dow Chemical Co.)

151. *p*-Halomethylbenzylidenepyruvic Acids: Novel Mechanism-Based Pyruvate Decarboxylase Inactivators. Nikoi Annan, George Dikdan, and *Frank Jordan*. Department of Chemistry, Rutgers University, Newark, NJ 07102.

p-$BrCH_2C_6H_4CH{=}CHCOCO_2H$, *p*-$ClCH_2C_6H_4CH{=}CHCOCO_2H$, and *p*-$CH_3C_6H_4CH{=}CHCOCO_2H$ were synthesized and tested vis-â-vis brewer's yeast pyruvate decarboxylase (PDC). The *p*-$BrCH_2^-$ and *p*-$ClCH_2^-$ (but not the *p*-CH_3) derivatives inactivated PDC in a time-dependent manner. Similarly to the report on benzoylformate decarboxylase (Dirmaier, Garcia, Kozarich, & Kenyon, *J. Am. Chem. Soc.* 1986, *108*, 3149), the halomethyl derivatives eliminated halide ion in a PDC-catalyzed reaction. Similarly to earlier reports from this laboratory (Kuo & Jordan, *Biochemistry*, 1983, *22*, 3735), the halomethyl compounds were mechanism-based inactivators, presumably resulting from a partitioning of the enamine intermediate between halide elimination and perhaps Michael addition of an enzymic nucleophile. Current understanding of this complex behavior will be presented. (Supported by NSF-DMB 87-09758, the Rutgers University Busch Fund, and the Research Council.)

152. Further Characterization of Enamines Related to the Key Intermediate on Thiamin Diphosphate Dependent Enzymes. Carlos B. Rios, Alex Chung, and *Frank Jordan*. Department of Chemistry, Rutgers University, Newark, NJ 07102.

Recently the generation and ^1H NMR spectroscopic properties of enamines related to the central intermediate in thiamin diphosphate dependent enzymatic reactions were reported (Jordan, Kudzin, Rios, *J. Am. Chem. Soc.* 1987, *109*, 4415) according to the scheme:



Further support for the assignment and physical properties will be presented employing a variety of R_1 and R_2 groups (including conjugated systems) and R_3 (groups resembling the pyrimidine of thiamin). Variation in pK_a of I with substituent and the double-bond character in II with substituent and solvent polarity will be presented. (Supported by NSF CHE 86-17087, PRF 18846-AC4, NIH MBRS 2506 RR08223-04.)

153. Modification of Bacterial Luciferase by 2-Bromo-2′,4′-dimethoxyacetophenone. Howon Choe and *James Becvar*. Chemistry Department, University of Texas at El Paso, El Paso, TX 79968.

Enzyme active sites can be altered by modification of specific amino acid residues. We report progress in the attempt to convert a catalytically essential sulfhydryl (cysteine) residue to a hydroxyl (serine) residue in bacterial luciferase, which catalyzes the light reaction in luminous bacteria. The thiol-specific reagent 2-bromo-2′,4′-dimethoxyacetophenone (BrDMAP) inactivates luciferase with a second-order rate constant of 5.8×10^3 M^{-1} min^{-1} at 25 °C. Phosphate ion partially protects against inactivation by the thiol reagent iodoacetamide but not against BrDMAP. Flavin mononucleotide protects well against both reagents but protects more effectively against BrDMAP. Photolysis of the BrDMAP-derivatized luciferase at 310 nm is used for the attempted conversion through an α-hydroxy thiol intermediate and yields partially restored enzyme activity. (Supported by NIGMS R15 GM38451.)

154. Mechanism-Based Inactivators for Hog Liver 4-Hydroxyphenylpyruvate Dioxygenase. *Brian J. R. Forbes* and Gordon A. Hamilton. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

A number of substrate analogues of 4-hydroxyphenylpyruvic acid were synthesized, and the reactions of these compounds with 4-hydroxyphenylpyruvate dioxygenase from hog liver were studied. One of the model substrates, pentafluorophenylpyruvic acid (F_5PP), is a competitive inhibitor of the enzyme with a K_i of 14 μM. Three of the substrate analogues, 4-hydroxytetrafluorophenylpyruvic acid (HF_4PP), 2-thienylpyruvic acid (2TP), and 3-thienylpyruvic acid (3TP), have proven to be mechanism-based inactivators for the enzyme. The first compound, HF_4PP, has the following kinetic values: partition ratio = 4.2, K_m = 49 μM, V_{max} = 1.9 mkat/kg, k_{inact}

= 1.5 min^{-1}, and K_i = 72 μM. 2TP has the following values: partition ratio = 41, K_m = 490 μM, V_{max} = 6.8 mkat/kg, k_{inact} = 0.62 min^{-1}, and K_i = 410 μM. The following values were found for the final mechanism-based inactivator, 3TP: partition ratio = 22, K_m = 250 μM, V_{max} = 2.9 mkat/kg, k_{inact} = 0.62 min^{-1}, and K_i = 360 μM. The compound thiophenol oxalate and *p*-thiocresol oxalate were synthesized but exhibited no catalytic activity. The implications of these results with regard to the mechanism of action of 4-hydroxyphenylpyruvate dioxygenase are discussed.

155. Sterically Restricted Mechanism-Based Inhibitors of Dopamine β-Hydroxylase. *G. K. Farrington*, Alok Kumar, and J. J. Villafranca. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

p-HO-styrene and *trans*-β-methyl-*p*-HO-styrene have been shown to be mechanism-based inhibitors of dopamine β-hydroxylase (DβH). *trans*-β-Methyl-*p*-HO-styrene has a high K_i (>20 mM), whereas *p*-HO-styrene has a K_i = 0.7 mM. Cyclic analogues of *trans*-β-methyl-*p*-HO-styrene (5-HO indole and 5-, 6-, or 7-HO-benzofurans) have also been shown to be mechanism-based inhibitors of DβH. The cyclic analogues have K_i values of 3.2–0.04 mM, indicating that cyclization relieves the steric hindrance caused by the *trans*-β-methyl group. The cyclic analogues exhibit at least a 10-fold decrease in the partition ratio. A radical cation has been proposed as the inactivating species. The ability of the 5-, 6-, and 7-HO-benzofurans and 5-HO-indole structures to better stabilize the inactivating radical cation intermediate through additional resonance forms may be responsible in part for the decrease in the partition ratios. Indole is not a mechanism-based inhibitor but is a competitive inhibitor with a K_i of 1.5 mM versus tyramine. (Work supported by NIH Grant GM 29139.)

156. Peptide Sequences of Bovine Dopamine β-Hydroxylase: Study of Chemically Modified and Disulfide-Containing Peptides. *James Robertson*, Donald Flory, Alok Kumar, Parimal Desai, and Joseph J. Villafranca. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

In our continuing effort to study the active site of dopamine β-hydroxylase (DβH), we have employed various peptide mapping strategies to identify possible active-site amino acid residues. We report here the results of trypsin-generated peptides modified by two known irreversible inhibitors, namely, [^{14}C]phenylhydrazine (Fitzpatrick and Villafranca, *J. Biol. Chem. 261*, 4510–4518, 1986) and 1,1-[^{3}H]-1-Br-2-(*p*-HO-phenyl)ethane (Flory et al., *Fed. Proc. 45*, 1538, 1986). We have also isolated and analyzed several disulfide-containing peptides obtained under various conditions in order to identify the amino acid environment of the cysteines. Lastly, we report the sequence data for several trypsin-generated peptides which are being used in the construction of oligonucleotide probes for cDNA screening. (Supported by NIH Grant 29139.)

157. Fluorinated Substrates as Probes of Anthranilate Hydroxylase (Deaminating) Mechanism. Justin Powlowski, *David Ballou*, and Vincent Massey. Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606.

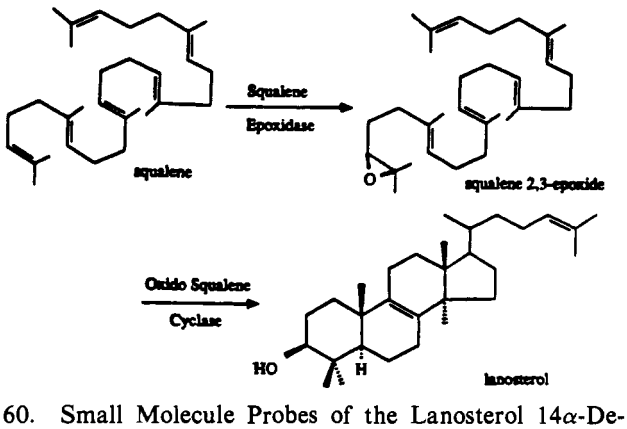
Anthranilate hydroxylase from *Trichosporon cutaneum* is a simple flavoprotein which catalyzes the conversion of 2-aminobenzoate to 2,3-dihydroxybenzoate. One atom from O_2 is incorporated at the 3-position, while oxygen from H_2O is inserted at the 2-position with the elimination of ammonia. Fluorinated anthranilates are reasonably good substrates for the enzyme. When fluorine is present at the 4- or 5-position of the ring, most of the resulting product is the fluorinated 2,3-dihydrobenzoate. At pH 7.4, a small proportion of the substrate is hydroxylated but fails to eliminate ammonia. The proportion of product formed with retention of the amino group increases dramatically with increasing pH; at the same time, uncoupling to form H_2O_2 also increases. The kinetics of the oxidative half-reaction with these fluorinated substrates is examined, and the results are interpreted in terms of the previously proposed mechanism for flavoprotein hydroxylases.

158. Amphipathic Amine Inhibitors of Human Lysosomal Cholesteryl Ester Hydrolase and Phospholipase A Activity. *P. S. Schlom*, K. Lindsley, Y. P. Wei, G. P Ma, and G. N. Sando. The Cardiovascular Center, University of Iowa, Iowa City, IA 52242.

Lysosomotropic amines such as chloroquine and ammonium chloride are believed to inhibit lysosomal hydrolysis in intact cells by increasing the pH of lysosomes above that required for optimal acid hydrolase activity. We report here that a number of amphipathic amines, including zwitterionic detergents, long-chain alkylamines, and physiological phospholipid and sphingolipid derivatives, have a direct effect on the in vitro activity of two lysosomal fatty acyl ester hydrolases: Acid cholesteryl ester hydrolase activity in crucial for the cellular metabolism of lipoprotein cholesteryl esters and triglycerides; acid phospholipase A may be important for membrane remodeling and in providing precursors for bioactive eicosanoids and acylglycerides. By exploiting differential effects of the inhibitors on the two enzymes, we have developed a method for the simultaneous analysis of both activities in cell extracts, with the use of a sensitive fluorimetric rate assay system. Our results suggest that specific physiological lipid amines may have a critical role in the regulation of cellular cholesterol and phospholipid metabolism.

159. Synthesis and Inhibition Studies of Novel Mechanism-Based Inhibitors of Squalene Epoxidase and Oxido Squalene Cyclase. *Stephanie E. Sen* and Glenn D. Prestwich. Department of Chemistry, State University of New York, Stony Brook, New York 11794-3400.

Squalene epoxidase and oxido squalene cyclase are essential in the production of sterols and bile acids in eukaryotic cells. Several novel acetylene-, allene-, cyclopropane-, and silicon-containing analogues of squalene and squalene epoxide have been synthesized. The synthesis and inhibitory effects of these analogues will be presented.

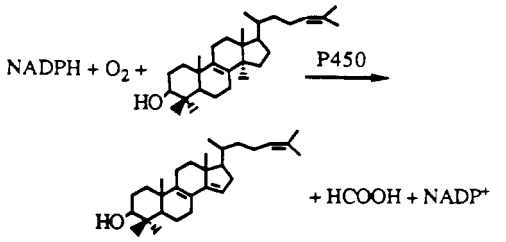


160. Small Molecule Probes of the Lanosterol 14α-De-

methylase from *Saccharomyces cerevisiae*. G. D. Wright and J. F. Honek. Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

Interest in the development of chemotherapeutic agents which interact with uniquely fungal enzymes has increased in view of the susceptibility of immunocompromised individuals to opportunistic fungal pathogens. One such target enzyme is the lanosterol 14α-demethylase, cytochrome $P450_{DM}$, which is essential in the biosynthesis of the major fungal sterol, ergosterol (Scheme I). We have investigated $P450_{DM}$ from *S. cerevisiae*, from a commercial source and an overproducing strain (Kalb, V. F., Loper, J. C., Dey, C. R., Woods, C. W., and Sutter, T. R. (1986) *Gene 45*, 237) with lanosterol analogues altered in positions 3 and 7 and the side chain to determine the substrate promiscuity of the enzyme. Furthermore, the enzyme has been found to be unreactive toward traditional substrates of mammalian P450's and shows narrow substrate specificity. These results are being considered in the development of specific inhibitors of this enzyme.

Scheme I:



161. Rationally Designed Inhibitors of Phospholipase A_2. *Wei Yuan* and Michael H. Gelb. Department of Chemistry, University of Washington, Seattle, WA 98195.

Phospholipid analogues that contain fluoro ketone, ketone, and alcohol replacements for the ester at the 2-position of the glycerol backbone have been prepared and tested as inhibitors of phospholipase A_2 fro *Naja naja naja* venom. Analogues were studied that contain two alkyl chains as well as single-chain compounds that lack carbon-1 of the glycerol backbone and the attached acyl unit. Both long- and medium-length alkyl chain compounds were studied. All inhibitors were examined in a well-defined mixed micelle system which was formed by Triton X-100. Surprisingly, the best inhibitors studied were the single-chain fluoro ketones, despite the fact that the enzyme has a strong preference for two-chain lipids. The most potent compound was found to have a dissociation constant some 600–3000-fold lower than the Michael's constant for dipalmitoylphosphatidylcholine substrate. ^{19}F NMR studies of the fluorinated phospholipid analogues in the micelle show that the single-chain compounds are partially in the hydrated ketone form, and the two-chain compounds are less than 0.1% hydrated. In every case studied, potent inhibitors are those that are significantly hydrated in the micelle, and it is suggested that the hydrated form is the species responsible for the inhibition. In addition, the single- and double-chain alcohol and nonfluoronated ketone analogues were worse inhibitors. Evidence is also presented for the binding of the hydrated fluoro ketone inhibitors selectively to the activated enzyme.

162. Artificially High Inhibition Constants for Vanadate Due to Reversible Interactions with Buffers. *Debbie C. Crans*, Robin L. Bunch, and Paul K. Shin. Department of Chemistry, Colorado State University, Fort Collins, CO 80523.

The effects of buffer interactions on biological enzyme studies with vanadate have been examined by using ^{51}V NMR spectroscopy and enzyme kinetics. Interactions of vanadate with buffers occur in aqueous solutions in the physiological pH range in the presence of enzymes and other biological materials. When vanadate reacts with Tricine, triethanolamine, malic acid, and citric acid, a distinct vanadium species is formed, and this reaction is observed by ^{51}V NMR. How these interactions of buffers with vanadate interfere when measuring inhibition constants in enzyme reactions is examined. Inhibition constants of vanadate, a potent inhibitor for both acid phosphatase and alkaline phosphatase, have been determined in the absence or presence of Tricine, triethanolamine, malic acid, and citric acid. The vanadate–buffer complex formation has been correlated with changes in the inhibition constants. The numerical changes in the inhibition constants range from a factor of two to several orders of magnitude. In addition the effects on inhibition constants of biomolecules such as EDTA, EGTA, and weak complexing buffers such as Tris were determined.

163. Abstract withdrawn.

164. Preparation of Mercury Ethenoadenosine Nucleotides and Their Effect upon the Activity of Selected Enzymes. *Daniel J. McLoughlin*. Department of Chemistry, Xavier University, Cincinnati, OH 45207.

The etheno-bridged nucleotide compounds ϵ-AMP, ϵ-ADP, and ϵ-ATP were reacted with mercuric acetate at low pH and the resulting mercury derivatives isolated by crystallization. Excess mercury was removed by chromatographic procedures. The Hg-ϵ-ATP UV spectrum was examined as a function of pH, and the spectra were found to be significantly shifted from those of the pure etheno compound. The fluorescence of the ϵ-ATP was found to be totally quenched upon binding mercury. Initial fluorescence titration studies demonstrated that the binding of mercury is extremely tight. The Hg-ϵ-ATP derivative was examined as to its effect upon the catalytic activity of selected enzymes. Glutamate dehydrogenase was inhibited by Hg-ϵ-ATP, and the extent of this inhibition was dependent upon pH. Malate, glycerol-3-phosphate, and 3-hydroxyacyl coenzyme A dehydrogenases were partially inhibited by Hg-ϵ-ATP. Hg-ϵ-ATP did not function as a substrate for firefly luciferase but did inhibit the reaction. Hg-ϵ-ATP serves as a substrate for hexose kinase, and Hg-ϵ-ADP serves as a substrate for pyruvate kinase. (Supportd by ACS-PRF Grant 16627-B3.)

THURSDAY MORNING—SYMPOSIUM ON THE DESIGN OF ENZYME INHIBITORS—J. J. VILLAFRANCA, PRESIDING

165. Interplay between Enzyme Mechanism, Protein Structure, and Inhibitor Design. *Paul A. Bartlett*, John E. Hanson, Siegfried H. Reich, and Stephen J. Telfer. Department of Chemistry, University of California, Berkeley, CA 94720.

The rational(ized) design of enzyme inhibitors has traditionally been based on considerations of enzyme mechanism. Strategies based on transition-state analogy and suicide inhibition, for example, have led to potent reversible and irreversible inhibitors even without specific knowledge of the three-dimensional structure of the enzyme active site. With the increasing availability of X-ray structural information, design approaches based on mimicry of naturally occurring proteinaceous ligands and on specific knowledge of the geometry of the enzyme active site become possible. Examples of both mechanism-based and structure-based approaches to inhibitor design will be presented.

166. Mechanism-Based Inactivation of Monoamine Oxidase. *Richard B. Silverman.* Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208.

The flavoenzyme monoamine oxidase (MAO) is one of the enzymes responsible for the degradation of biogenic amines. Inactivators of this enzyme exhibit antidepressant activity. This talk will focus on recent examples of mechanism-based inactivators of MAO that we have designed and/or investigated. Chemical model studies and enzymological studies on the mechanisms of inactivation of MAO by these inactivators will be discussed. Reaction specificity for the two isozymes, MAO A and MAO B, by several inactivators will be described. Mechanistic information from the inactivation studies will be related to the catalytic mechanism of MAO.

167. Effects of Lovastatin and Its Analogues on HMG-CoA Reductase. *Alfred W. Alberts.* Biochemical Regulation Department, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.

The association of elevated levels of plasma cholesterol with heart disease coupled with the knowledge that a major portion of the total body pool of cholesterol is derived from de novo synthesis has resulted in numerous attempts to discover potent specific inhibitors of the enzymes involved in this pathway. The focus of these attempts in recent years has been on a key rate-limiting enzyme in the pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR). This has resulted in the discovery and development of lovastatin (I), a compound isolated from the soil microorganism *Aspergillus terreus*. I is a lactone prodrug which can be converted to its active open dihydroxy acid form L-154,819 (II) by either base hydrolysis or enzymatically. II is a potent competitive inhibitor ($K_i < 10^{-9}$ M) of HMG-CoA reductase from all sources studied. This talk will describe studies of II and its analogues with respect to structure–activity relationships, kinetics, and binding to rat liver HMG-CoA.

168. Inactivation of *L. leichmannii* Ribonucleoside Triphosphate Reductase (RTPR): Active-Site Labeling and Chromophore Identification. G. M. Ashley and *J. Stubbe.* Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

Incubation of 1 equiv of mechanism-based inhibitor 5′- or 2′-[^{3}H]ClUTP with RTPR results in loss of 83% of its catalytic activity and incorporation of 0.9 equiv of radiolabel. To stabilize the radiolabel bound to RTPR so that the modified peptide(s) could be isolated subsequent to alkylation with iodoacetamide and hydrolysis with trypsin, the inactivation was carried out in the presence of $NaBH_4$. HPLC analysis of tryptic digested protein gave 3 closely eluting peptides. Sequencing of the first of these peptides demonstrates that the 2-methylene-3(2H)-furanone generated by RTPR-mediated breakdown of ClUTP is covalently bound to one of the active-site thiols of RTPR: -C*-E-G-G-A-C-P-I-K. The inactivation of RTPR by ClUTP is accompanied by formation of a new protein chromophore at 320 nm, which is not produced in the presence of $NaBH_4$. Model studies suggest that the new chromophore is due to the addition of an amino group to the 5-position of the furanone followed by ring opening and tautomerization to give a β-aminoenone structure. The lysine in the "active-site" peptide might be the group responsible for the new chromophore production.

THURSDAY AFTERNOON—SYMPOSIUM ON THE DESIGN OF ENZYME INHIBITORS—J. J. VILLAFRANCA, PRESIDING

169. Use of Mechanism-Based Inhibitors to Elucidate the Mechanism of Dopamine β-Hydroxylase. *Joseph J. Villafranca*, Paul Fitzpatrick, Alok Kumar, Donald Flory, and G. King Farrington. Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

Dopamine β-hydroxylase is a copper-containing monooxygenase that catalyzes the hydroxylation of dopamine to norepinephrine. Multiple experimental approaches have been used in this laboratory to elucidate the chemical mechanism of this enzyme which involves formation of a radical intermediate. In addition many different classes of mechanism-based inhibitors have been used as probes of the catalytic mechanism (P. F. Fitzpatrick and J. J. Villafranca (1987) *Arch. Biochem. Biophys. 257*, 231). A summary of studies on the use of these inhibitors to characterize the enzymatic mechanism and amino acid residues at the active site will be presented.

170. Design of Transglutaminase Inactivators. *Diana Pliura*, Arlindo Castelhano, Bonnie Bonaventura, Roland Billedeau, and Allen Krantz. Syntex Research, Mississauga, Ontario, Canada L5N 3X4.

A novel series of inhibitors containing a 3-halo-4,5-dihydroisoxazole moiety as a latent reactive group have been designed and evaluated as inactivators of transglutaminases. Time-dependent, irreversible inactivation of this class of calcium-dependent acyl-transfer enzymes was observed, and second-order rate constants as high as 220000 M^{-1} min^{-1} have been obtained for the inactivation of epidermal transglutaminase. The identity of substituents at the 5- and 3-positions and the stereochemistry at C-5 play a crucial role in defining inactivator potency and specificity. A plausible mechanism for the inactivation is proposed based on the normal mode of action of the enzyme, specifically a mechanism wherein the active-site thiolate displaces halide from the halodihydroisoxazole to form a stable thioimine adduct. Chemical and biochemical studies in support of this mechanism will be presented.

171. Inhibition of Enzymes in Bacterial D-Alanine Metabolism. *C. T. Walsh.* Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

The D-alanine branch of the biosynthesis of the bacterial peptidolgycan layer in cell wall assembly involves three enzymes: (a) alanine racemase, interconverting L- and D-alanine; (b) D-Ala-D-Ala ligase, an ATP-utilizing D,D-dipeptide forming catalyst; (c) the D-Ala-D-Ala adding enzyme adds preformed D-Ala-D-Ala to UDP-muramyl tripeptide to produce the UDP-muramyl pentapeptide, the end of the cytoplasmic stage of peptidoglycan assembly. Studies from these laboratories on the molecular biology, behavior of time-dependent inhibitors will be summarized.

172. Substrate Variability in Enzyme Inhibitor Design: α- and γ-Substituted Phosphinothricins as Inhibitors of Glutamine Synthetase. *E. W. Logusch*, D. M. Walker, and J. F. McDonald. Monsanto Agricultural Company, 700 Chesterfield Village Parkway, St. Louis, MO 63198.

Considerations of substrate structural variability for the enzyme glutamine synthetase (GS) have led to the design of α- and γ-substituted analogues of the naturally occurring GS transition-state analogue inhibitor phosphinothricin (PPT). Among potent new inhibitors prepared were γ-hydroxyphosphinothricin (GHPPT) and cyclohexanephosphinothricin (CHPPT). We have found that compounds of this type, including PPT itself, are potent competitive inhibitors of both prokaryotic and eukaryotic GS and also display a unique sequence of pseudo-first-order enzyme inactivation, followed by kinetically complex reactivation. A mechanistic rationale has been developed to account for this behavior and provide a better understanding of the interaction of GS with transition-state analogue inhibitors.

173. Phospholipid Analogues as Mechanistic Probes of Phospholipase A_2 Catalysis. *Michael H. Gelb*, Wei Yuan, and Karen Fearon. Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA 98195.

A number of phospholipid analogues have been prepared as inhibitors of snake venom phospholipase A_2 in an effort to understand the mechanism of enzymatic catalysis. Compounds were synthesized in which the ester at the 2-position of the glycerol backbone was replaced by a difluoromethylene ketone unit. These compounds were designed to be tight-binding inhibitors since the hydrated fluoro ketone is a structural mimic of the putative tetrhedral intermediate that forms in phospholipase A_2 mediated hydrolysis. Inhibitors were tested in a mixed-micelle system. It was shown that only those fluoro ketones that were hydrated in the micelle were good phospholipase A_2 inhibitors. The most potent compounds were found to bind some 1000–3000-fold tighter to the enzyme than dipalmitoylphosphatidylcholine substrate. The snake venom enzyme is known to be activated by choline-containing lipids. It was shown that the hydrated fluoro ketone inhibitors bind only to the activated form of the enzyme. Additional phospholipase A_2 inhibitors were designed to probe the role of the calcium ion in catalysis. The results of these studies will also be discussed.

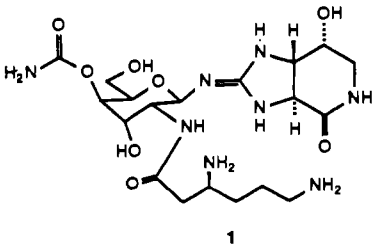
FRIDAY MORNING—SYMPOSIUM ON BIOSYNTHESIS—J. C. VEDERAS, PRESIDING

174. Studies in Natural Products Biosynthesis. *Heinz G. Floss*. Department of Chemistry, University of Washington, Seattle, WA 98195.

The biosynthesis of microbial metabolites is investigated by feeding precursors labeled with stable isotopes followed by analysis of the products by NMR spectroscopy, by analysis of blocked mutants, and by various other techniques. Examples will be presented from ongoing work in the author's laboratory.

175. Metabolism at the β-Position of Amino Acids: Relevance to the Biosynthesis of Antibiotics. *Steven J. Gould*. Department of Chemistry, Oregon State University, Covallis, OR 97331.

Numerous antibiotics are derived from common amino acids where key steps in the biosynthetic pathways involve removal of hydrogen from C-3 and introduction of oxygen, nitrogen, or a double bond at this center. This chemistry will be discussed as it relates to antibiotics such as streptothricin F (**1**), blasticidin S (**2**), and acivicin (**3**).



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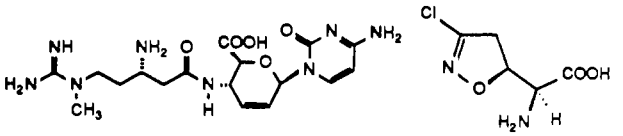

2 **3**

176. Biosynthetic and Biomimetic Studies of Natural Products. *Craig A. Townsend*. Department of Chemistry, The Johns Hopkins University, Baltimore, MD 21218.

Our research program has sought to apply synthetic and spectroscopic methods to understanding natural product biosynthesis in intact organisms and in cell-free systems. Occasionally, the opportunity presents to explore elements of fundamental reaction chemistry revealed in these studies in physical organic or in biomimetic terms. Examples from current work with aflatoxin and β-lactam antibiotics will be presented.

FRIDAY AFTERNOON—SYMPOSIUM ON BIOSYNTHESIS—C. R. HUTCHINSON, PRESIDING

177. Genetic Analysis of the Enzymes Involved in β-Lactam Antibiotic Biosynthesis by *Streptomyces clavuligerus*. *Susan E. Jensen* and Donald W. S. Westlake. Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9.

Streptomyces clavuligerus is a filamentous prokaryotic microbe which produces a variety of β-lactam compounds. Isopenicillin N synthetase (IPNS) is the enzyme which forms the β-lactam ring system of penicillins and cephamycins. This enzyme has been purified to homogeneity from *S. clavuligerus*, and its amino-terminal amino acid sequence has been determined. An oligodeoxynucleotide probe which corresponds to the amino-terminal sequence of IPNS was used to isolate the gene for IPNS from a library of *S. clavuligerus* DNA fragments in the *Escherichia coli* vector pUC119. The gene was subcloned and the nucleotide sequence determined from the entire open reading frame. The IPNS sequecne showed 56% similarity to the corresponding IPNS genes from *Cephalosporium acremonium* and *Penicillium chrysogenum* at the nucleotide level and 62% similarity at the amino acid level. All three IPNS proteins show a stretch of 11 identical amino acid residues in the vicinity of the cysteine-106 residue which has been implicated to be involved in the active site of the enzyme. The IPNS gene was transferred to *Streptomyces lividans* by using the plasmid vector pIJ702, and IPNS activity was detected by enzyme assay and immunological methods. No corresponding activity could be detected when the gene was transferred to an *E. coli* host.

178. Chromopeptide Lactone Biosynthesis in Streptomycetes. *U. Keller*. Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, D-1000 Berlin 10, West Germany.

Chromopeptide lactones such as the actinomycins, virgi-

niamycins, or the quinoxaline antibiotics are produced by a number of *Streptomyces* strains. Previous work on the biosynthesis of actinomycin has revealed that the pentapeptide chains of this compound are synthesized by the action of two multifunctional enzymes. They activate the amino acid residues as thioesters and also harbor the catalytic functions for epimerization and N-methylation of thioester-bound amino acids. Chromophore activation is catalyzed by one separate enzyme which is much smaller than the two pentapeptide synthetases (46.5 kDa vs 225 and 280 kDa). Besides the more detailed description of this enzyme system, data will be presented that a similar assembly of catalytic functions is involved in the biosynthesis of the quinoxaline antibiotics triostin and quinomycin. The general features of peptide lactone synthesis in *Streptomyces* are discussed.

179. Cryptic Genes for Antibiotic Synthesis in Streptomycetes. *G. H. Jones.* Department of Biology, University of Michigan, Ann Arbor, MI 48109.

Phenoxazinone synthase (PHS) is an enzyme involved in actinomycin biosynthesis in *Streptomyces antibioticus*. Two apparently unlinked, cryptic homologues of the PHS gene have been identified in *S. lividans* and cloned in *Escherichia coli*. Inserts of 3.98 and 2.21 kb were identified as PHS homologues in plasmid constructs. The 3.98-kb insert appears to code for a protein with the properties of the PHS subunit in a streptomycete coupled transcription–translation system. Available data indicate that transcription of the PHS gene in the plasmid construct can be initiated at both plasmid- and insert-borne promoters. With regard to the genomic organization of the two PHS homologues in *S. lividans*, it appears that, even though they are not contiguous, they can be isolated together on a 10–12-kb genomic DNA fragment. Homologues of the PHS gene have also been identified in *S. chrysomallus* and *S. parvulus*, two other actinomycin producers, and in *Saccharopolyspora erythrea* and *S. glaucescens*, which do not make that antibiotic.

180. Genetics and Biochemistry of Macrolide Antibiotic Biosynthesis. *R. Nagaraja Rao.* Eli Lilly and Company, Indianapolis, IN 46285.

A variety of DNA cloning methods have been developed for the isolation of antibiotic biosynthetic genes from streptomycetes. Two methods have proven to be particularly useful. The first is the use of specific DNA probes prepared either from the amino acid sequence of an enzyme involved in the biosynthesis of tylosin or from *actI* and *actIII* genes involved in the biosynthesis of actinorhodin. The second is the use of linkage between the resistance genes and the biosynthetic genes. Understanding the physiological significance of the cloned genes requires an ability to modify the cloned genes and to reintroduce them into the chromosome by gene replacement or by site-specific insertion. The development of these methods and their application to the analysis of macrolide biosynthetic genes in streptomycetes will be discussed.