Ultrafiltration Sampling in the Rodent Workstation to Study the Metabolic Effects of Green Tea on Zucker Diabetic Rats

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Purpose The Zucker Diabetic Rat is an excellent model for Type 2 diabetes. Studying the efficacy of a dietary supplement to ameliorate the diabetes in these animals requires long-term monitoring. Frequent glucose monitoring by blood sampling from the tail is stressful, and glucose can be altered by stress. There is also a limit to blood sampling frequency due to blood volume depletion. The goal of this study was to develop a system to monitor the metabolic status of awake diabetic Zucker rats over long time periods in order to be able to assess the effects of green tea on appetite, weight and glucose. To accomplish this, rats were implanted with ultrafiltration probes to sample interstitial fluid for glucose and were maintained in the Rodent Workstation™.

Methods Male Zucker Diabetic Rats and male Zucker non-diabetic control rats were implanted with subcutaneous ultrafiltration probes at seven weeks of age and placed in a Rodent Workstation™. The ultrafiltration probes consisted of looped microdialysis fibers attached to a non-permeable tube. The fibers were implanted in the tissue and a negative pressure was applied to the tubing. The glucose in the interstitial fluid is an indication of the severity of the diabetes. The diabetic rats were divided into four groups and dosed with four levels TeaGreen® green tea powder. The TeaGreen® was assayed for EGCg using HPLC with electrochemical detection. The doses were calculated to deliver 0, 50, 85 or 125 mg/Kg of EGCg, the main catechin in green tea. The weight, food consumption, and glucose concentration of the interstitial fluid collected over 24-hour periods were monitored for three weeks. Two glucose tolerance tests were then performed at one-week intervals using interstitial fluid samples collected with the ultrafiltrate probe to measure glucose.

Results The Zucker Diabetic control rats consumed more food and had a higher weight gain than the non-diabetic control rats. The diabetic control rats also had a higher average interstitial glucose concentration and than non-diabetic control rats and showed decreased glucose tolerance. There was a great deal of variability among the diabetic rats fed different levels of TeaGreen®. Some rats showed improvement in weight gain, food consumption, average glucose concentration and glucose tolerance while others did not.

Conclusions Studies on diabetic rat models lasting several weeks, can be carried out successfully using ultrafiltration probe sampling with animals housed in a Rodent Workstation™. Under these conditions it is possible to monitor weight gain, food consumption and daily glucose profiles and to perform glucose tolerance tests. Since no blood is removed, glucose can be monitored more frequently and multiple glucose tolerance studies can be done in each animal.

Rodent Workstation is a trademark of Bioanalytical Systems, Inc.

The Effects of Green Tea on Development of Obesity and Diabetes in the Zucker Diabetic Rat

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Green tea has been reported to be beneficial in reducing weight gain, food consumption and glucose levels. We are conducting a dose response study of orally administered decaffeinated green tea extract in the Zucker Diabetic Rat, which is a rodent model of type 2 diabetes.

Male Zucker Diabetic Rats and male Zucker non-diabetic control rats are implanted with subcutaneous ultrafiltration probes at seven weeks of age. Ultrafiltration probes consist of looped microdialysis fibers attached to a non-permeable tube. The fibers are implanted in the tissue and a negative pressure is applied to the tubing. The glucose in the interstitial fluid is an indication of severity of the diabetes. The rats are housed in a Rodent Workstation™. The diabetic rats are divided into four groups and dosed with four doses of green tea extract. The extract is assayed for epigallocatechin gallate (EGCg) using HPLC with electrochemical detection. The doses are calculated to deliver 0, 50, 85 or 125 mg/Kg/day of EGCg, the main catechin in green tea, and rats are dosed by oral gavage twice daily. Weight, food consumption, and glucose concentration of the interstitial fluid collected over 24-hour periods are monitored for three weeks. Two glucose tolerance tests are performed at one-week intervals using interstitial fluid samples collected with the ultrafiltrate probe to measure glucose. Preliminary data shows lower glucose concentrations in some green tea-treated groups.

Question and Answer: Notes on the LCEC/IMER determination of ACh

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Determining ACh using LC/EC coupled to IMER technology is a widely accepted method (1). The combination allows for determining basal ACh concentration in dialysate samples without using AChE inhibitors (2,3). The method depends upon the LC separation of ACh and its enzymatic conversion to H_2O_2 in the post-column IMER. EC oxidation of H_2O_2 at a Pt electrode, or its reduction at a wired

glassy carbon electrode (4), is used to quantify the H_2O_2. The amount of H_2O_2 is directly proportional to the mass of ACh injected (1). Losing response to injected ACh can thus be due to a problem with the IMER (less than 100% conversion of ACh to H_2O_2) or to lowered rate of oxidation/reduction at the electrode.

Enzymes are susceptible to denaturation, and thus loss of activity, in both the IMER and the wired electrode. How can one distinguish between the two possibilities? Divide and conquer, a very old strategy! The IMER produces the H_2O_2, and the EC transducer cell detects the H_2O_2. The ACh Kit (5) manual outlines a procedure for testing IMER efficiency. I am going to present an alternative procedure which I think is more practical for day-to-day testing, and which allows easy evaluation of the electrode's condition.

First, establish a benchmark, a fully functional LCEC/IMER* system with an ACh detection limit adequate for the study samples. Inject, **separately**, an amount of ACh and of H_2O_2 that will give a strong response, say 100 pmoles for the standard system and 10 pmoles for the microbore version. It is important that the H_2O_2 be **diluted** to an injectable concentration **with mobile phase**: H_2O_2 comes out at the void volume and any other matrix (solution), which would also come out at the void, interferes with the quantitation of H_2O_2. We now have two benchmark numbers: 1) the response (peak height/area) for a known amount of H_2O_2, and 2) another for a known amount of ACh standard. If we should now lose sensitivity, we can distinguish whether the drop is caused by a change in the IMER or in the electrode. Again, separately inject the known mass of H_2O_2 and ACh. If the IMER efficiency has dropped, the response for H_2O_2 will be similar to the benchmark value and the ACh response will be reduced from the benchmark response. If both the H_2O_2 response and the ACh response are reduced, then the electrode has changed. If a Pt electrode is being used to oxidize the H_2O_2, then it is most likely passivated or the potential has changed. If the wired electrode is in use, prepare a new electrode or replace outdated peroxidase/polymer coating solution (4).

We have found this to be a useful in-house diagnostic procedure. Of course, degraded standards, a faulty injector or recording device, a bad reference electrode and changed chromatography can affect this troubleshooting procedure.

**Any precolumn IMER containing immobilized catalase or peroxidase must be removed prior to initiating the outlined procedure.*

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2. *T. Huang, L. Yang, J. Gitzen, P. T. Kissinger, M. Vreeke and A. Heller, J. Chromatogr. B, 670 (1995) 323.*
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5. *BAS P/N MF-8910 for standard version and MF-8908 for microbore version.*

Guest Column: "Musings on Clinical Trials"

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Subject A recent *Annals of Internal Medicine* had a thoughtful piece about the select patients who make it into clinical trials vs. the diverse patients with the disease who use the product. I've been thinking about this and about the DART group led by Gary Lightfoot, formerly head of the Lilly field clinical trial force, who have been trying to speed enrollment. I've also been thinking about the success of VeritasMedicine and *InVivo* and John Griest's telephone enrollment efforts, and the NIH interest in rapid translation. I put together some re-engineering thoughts about moving from a guru investigator-centered clinical trial paradigm to a patient-centric design. I don't believe the proposals violate any regulations. I think many pieces have been done already. Lilly, for example, captured a lot of data directly from patients in their male sexual dysfunction trial (electronic rulers?). I'd love to see someone take a design along these lines to FDA. I am sure VeritasMedical would love to use their Harvard/MIT resouces to help put up the intake part of the system at least. I think with good planning and contacting all the right patient-advocacy and physician-specialty groups and the web pages in advance, you might have all the patients enrolled and on placebo at the time the FDA finally got around to the end of the 30-day delay.

Imagine 100% enrollment in ONE DAY! Also imagine getting valid data ready to lock two days after the last critical visit. Oh well, it's just money, just $32 per second, and $1 billion per year. I would appreciate your thoughts and comments, but I don't need any more Thorazine.

From Montreal to Maryland...

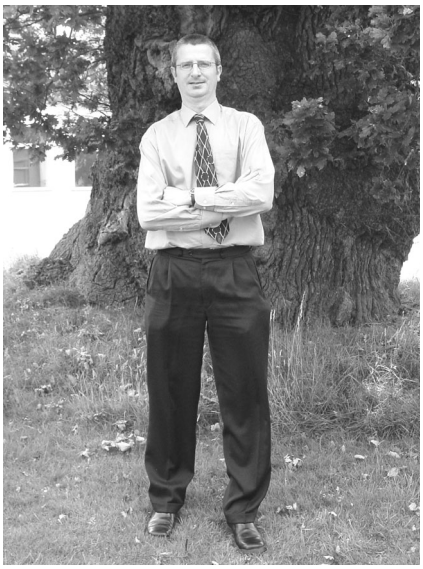
...from accounting to clinical research; from night custodian/clinical tech to Senior Director of Phase I Operations for BAS-Baltimore - that's Christopher Ore!

Chris is obviously a man who has made some huge leaps in his life. Starting out in the banking business, he returned to Concordia University for more education and while there, joined a research laboratory in Montreal at the entry level. He enjoyed the work so much that it was an easy decision to change careers, and he quickly assumed more and more responsibility. Chris has been instrumental in the spin-off and creation of a new clinical/bioanalytical company, and in the founding and start-up of a multi-center clinical trials organization which has grown into a highly successful multi-national CRO. Currently, he oversees in-house early-phase clinical studies at BAS-Baltimore. This includes the Recruiting, Screening and Clinical Operations Department, and organizing and executing everything from first-in-man studies to generic bioequivalency studies.

Chris is one of a vanishing breed - a Montreal Expos baseball fan. He enjoys the thrill of downhill skiing, but "the agony of the knees" has taken its toll on that activity. An admitted political junkie, Chris is also an avid student of history - certainly two very compatible avocations.

Chris enjoys the CRO industry because of the scientific challenges and entrepreneurial opportunities it offers. "In nearly 20 years," says Chris, "the breadth of studies I have conducted covers nearly every therapeutic field and would fill a couple of shelves at your local pharmacy. The best part, though, has been working with some of the most talented, dedicated people in the industry who have shared their knowledge and experience freely."

Out of Sherwood Forest



Robin Hood, Maid Marian, Friar Tuck and...Mike Prow. What do they all have in common? If you said they all work for a drug discovery company, we'll have to suggest that you brush up on your history...or fictional literature, whatever the case may be. The correct answer is that they are all natives of Nottingham, England!

Mike Prow came to BAS early in 2001 as a product specialist in neuropharmacology, and his chief responsibility is building and maintaining BAS business and client relations in the UK and Europe. Although his background is in neuroscience, most notably with Boots/Knoll Pharmaceuticals in the area of CNS disorders, obesity and related diseases, and he specializes in *in vivo* products, Mike enjoys the challenge of working with the entire range of BAS products and services.

Always fascinated by all aspects of biology, it seemed natural that Mike would gravitate to the scientific community, especially with a noted pharmaceutical firm located in his hometown. Since Mike had been using BAS-made LC/EC equipment to analyze brain neurotransmitters for nearly 20 years, it also seemed logical that his next career move was to join the BAS team permanently.

Mike is an avid sports fan, especially of football (which we think of as soccer in the U.S.), and likes nothing more than trekking through the beautiful Nottinghamshire countryside with his fashion-conscious 12-year-old daughter Gemma and dog Taz.

According to Mike, "What I enjoy most about BAS is the team-based approach to building client relationships. I know that if I don't have the answer to a client's question, one of my colleagues, either in the UK or in the US, will be able to provide a swift response."

Move Over, Bill Gates and Norm Abrams!

Vern LeBlanc is surely one of our most versatile and talented BAS staff members. As the Information Systems Engineer at McMinnville, Oregon, Vern handles support for all computers, computer systems, security and building maintenance, phone and communication systems and web site support. At home, he is a skilled carpenter and do-it-yourselfer.

Vern grew up in Massachusetts, studied electronic engineering at Northeast Institute of Industrial Technology and then went to work for Hewlett Packard's electrocardiograph division. For 18 years he managed manufacturing, technical support and information systems for them, and it was through a job transfer with HP that Vern landed in Oregon. Breaking out of the corporate world, Vern first started an internet provider service and then became a consultant helping small businesses find and improve network solutions. He was retained to update the McMinnville systems and BAS, always on the lookout for the best people, convinced Vern to become a permanent part of the staff to maintain and keep updating those systems. It has been a wonderfully productive association!

"The people here really want to have technology work for them," says Vern. "That has given me the opportunity to step way out on the edge, to learn an incredible number of new things. With that kind of attitude and encouragement, the future has limitless possibilities and looks mighty exciting!"

As we said, Vern is a very versatile guy. He built his first house, a chalet on 10 acres in New Hampshire, and this winter plans to restore totally the interior of his 1987 red Corvette. And oh my, how Vern loves to travel! Everything from long rides in that Corvette with his wife, to a month in China, trips to Mexico, Spain, Morocco and other exotic destinations. And then he loves to return home to his wife, three sons, a daughter and their Chihuahua, Pepe´.

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in•scrú•ta•ble (*adjective*): not readily investigated, interpreted, or understood, MYSTERIOUS

One might say that is an apt description of Ralph Wheeler, Vice President of Marketing and Sales for BAS-Evansville. At least he certainly *looks* inscrutable to us in these photos!

Ralph has been an invaluable part of BAS since 1989 when he made what he considers the most important career move of his life by accepting BAS' invitation to make the transition from part-time marketing consultant to full-time employee. Since then, he has been responsible for preparing bids, client visits, planning exhibits and meetings, and preparing and approving research agreements, representing BAS to clients in his always professional and elegant manner.

After completing his education at Trinity University in San Antonio, Texas, Ralph first managed a chemistry department that provided analytical services to a large bioassay program and later became its Principal Investigator. During those years, he was also responsible for marketing and for providing the studies and funds necessary to support the group, and he built a lot of relationships in the toxicology field that have been an invaluable asset at BAS.

Something of a Renaissance man, Ralph enjoys travel, Broadway shows and history, and spends many rewarding hours reading. He is careful to maintain his inscrutable aura by revealing limited information about himself.

What Ralph is not reticent to discuss, however, is the rewarding nature of his association with BAS. "Things are never dull here; the challenges are endless. No two studies are alike, and there is always a new set of problems to solve."

Marathon Sea Turtle Hospital

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Halfway down U. S. 1 in Florida, a highway to nature lovers' heaven, you can take a travel break on Marathon Key, the last big key before you get to Key West, 50 miles farther out. To put this in a modern-day travel perspective, there are no Burger Kings or McDonalds between Marathon and Key West.

Marathon Key is a fisherman's haven. Deep-sea charter yachts line the marinas and fresh fish restaurants and oyster bars skirt the docks. In the middle of the island, surrounded by all of this fishy fun, there remains what used to be Fanny's, which used to be the only topless bar on Marathon. I say used to be, because the bar that once was haven to scantily clad women is now shelter to sick and injured sea turtles.

Richie Moretti, an ex-Volkswagen mechanic from Orlando, and his partner Tina Brown own the Hidden Harbor Hotel on Marathon Key. Nine years ago they started the Hidden Harbor Marine Environmental Project, Inc. with the intent to help the sick and injured sea turtles in the Florida Keys area. To expand their efforts they needed a facility where the infirmed sea creatures could get state-of-the-art medical care. After seven years of toeing the property line with the topless bar, Richie bought it and created the world's first "sea turtle only hospital." He and Tina invested almost $300,000 of their own money to purchase and remodel the saloon, complete with a human hospital-quality surgical suite. The duo got their first patient in 1985 when a fisherman brought an injured turtle to them. They estimate that since then, they have received over 350 turtles in need of medical care.

They treat a myriad of turtle maladies, but by far the two most common problems are trauma cases, turtles injured from boats, propellers, shrimp nets, lobster traps and sharks, and a horribly disfiguring disease called fibropapillomatosis. Fibropapillomatosis, which has been likened to "elephant man disease," results in the animal being covered with multiple tumors, some as large as a basketball. As many as fifty tumors have been found on a single turtle. The tumors themselves don't kill the turtle; what happens is the tumors grow in delicate areas, such as the cornea of the eye or around the mouth, and the animal goes blind or can't eat and eventually starves.

If caught in time, these tumors can be removed surgically and after a long rehabilitation in Hidden Harbor's private sea turtle convalescent pool, which can last six months to a year, the animals can be released if no new tumors develop. Presently surgical removal is the only treatment available, and fortunately for Richie and Tina, and especially for the sea turtles, Dr. Lisa Bramsen, a veterinarian from Key West, volunteers her surgical skills in her free time.

Unfortunately, these tumors can also grow inside the turtle's body. About 20 percent of the affected turtles develop fibropapillomas in their kidneys, liver, heart, lungs and intestines. There is no way to diagnose these tumors easily, and often they are only found during a necropsy, which is an animal autopsy, after the patient dies.

The problem has grown to near epidemic proportions. Although fibropapillomatosis was first recognized in sea turtles in 1938, its incidence and distribution have increased in recent years. Initially the fibropapillomas were found only on the Green Sea Turtle (*Chelonia mydas*). But recently, the tumors have been diagnosed on Loggerheads (*Caretta caretta*) as well. Studies by the Florida Department of Natural Resources estimate that over 50 percent of the wild Green Sea Turtles are affected by the disease. Green Sea Turtles around the Hawaiian Islands are affected at a similar rate. The fibropapillomas have also been found in the warm waters off Australia, Belize, Puerto Rico, Barbados, Venezuela, Columbia and the Cayman Islands, and the disease is now found in the Caribbean and the Atlantic, Pacific and Indian Oceans.

The scope of this disease was so broad that Richie and Tina enlisted the help of researcher, veterinarian Dr. Elliott Jacobson. Dr. Jacobson is a virologist and teaches veterinary medicine at the University of Florida, School of Veterinary Medicine in Gainesville. He has teamed up with the Hidden Harbor Environmental Project, Inc. in an effort to discover the cause of the disease and, hopefully, find either a cure or vaccine for it. The project has a series of isolated tanks where sea turtles are raised and the mode of transmission of the disease is studied.

Currently, it is suspected that the fibropapillomas are caused by a type of Herpes virus, however this is has not been proven. Other factors seem to play an important part in development of the tumors as well. Most of the diseased turtles are found near the shore where the waters are the most heavily polluted. The pollution may suppress the animals' immune system, making them more susceptible to the virus, or whatever is causing the disease.

According to Richie, this serious problem could potentially cause the demise of an entire species, and possibly other species as well. The disease is being seen primarily in juveniles, but when the turtles become adults at 15 to 20 years of age, they could potentially pass it on to their offspring through the eggs.

Unfortunately, in addition to this terrible disease, the sea turtles around the Keys and other warm water areas are also victims of man's encroachment on the environment. Entanglements with fishing lines, shrimp nets, trap ropes and more can all result in severe trauma to these gentle creatures. When a turtle gets caught in a net or a line, the fibers wrap around the flippers and cut off circulation to the limbs. In a short time gangrene sets in and, even if the animal is rescued in time, loss of an extremity is often the result. These trapped turtles are also easy prey for passing sharks. Since turtles do have lungs and must breathe air to live, if entangled in a net and unable to surface for air, they will drown.

Dr. Jacobson is a colleague of mine, and he suggested I visit the Sea Turtle Hospital on Marathon the next time I visited the Keys. So, on a diving trip to

Key West, my wife and I stopped by the Turtle Hospital. No one appeared to be there, so we continued on our way.

In the next few days, on one of our dives off Key West we came across a young Green Sea Turtle secreted under a ledge, and we watched it from a distance. Soon, the creature pushed away from the reef and gracefully swam away, its large wing-like flippers effortlessly propelling its streamlined body into the dark blue. Back on the dive boat we all marveled at the beauty and grace of the turtle and vowed that we would make another attempt to visit the Marathon Turtle Hospital.

The next morning I placed a call to the Hidden Harbor Hotel. Richie Moretti took my call, but was harried and didn't have time to talk. The hospital had just received an injured Loggerhead sea turtle. Some scuba divers had found it caught in a rope net, and it looked as though it had been attacked by a shark. Richie had been frantically trying to get in touch with Dr. Bramson, the Turtle Hospital's veterinarian. Apparently she was out of town, and now he was trying to get in touch with someone else who could help. I told Richie I was a veterinarian and had experience with reptiles, and that I was more than happy to help him if he was interested. He declined my offer politely, but about fifteen minutes later the phone rang and it was the Turtle Hospital, asking me to come and help out. (Apparently they had checked my credentials and I passed.) We quickly headed up to Marathon. In the meantime, Richie, Tina Brown and their crew prepared the patient, a 200 pound Loggerhead, for emergency surgery.

When we arrived, we were awestruck by how modern and well equipped the facility was. Technicians and volunteers were preparing for the surgery. We were escorted to a holding room where an anesthesia machine had already been hooked up to the patient, using an empty plastic bottle as a giant face mask. An adult male Loggerhead missing two flippers was secured in the bottom of an empty wading pool. One of the first images to strike me was that of long, intersecting scars cutting through his brown shell, remnants of past encounters with power boat propellers. His scars looked much like those I had seen in the coral reefs while we were driving the length of the Keys. One of the rear flippers had been severed, most likely by a shark bite. This was an old wound, and had long since healed. The opposite front flipper was partially torn off, and what remained was a thickened, greenish-black chunk of dead meat. After a careful examination, Dr. Ritchie and I decided it would be best to amputate the limb at the shoulder joint. Since the turtle still had one front flipper, and the opposite flipper on the back, Richie and Tina felt it might still be able to swim, should it recover from its serious wounds.

By this time Eagle, as he had been named, was asleep from the effects of the anesthesia. We lifted him onto a customized turtle gurney and wheeled him off to the surgery suite. Once inside, we propped Eagle's mouth open with a large plastic gag, then carefully inserted a sterile silicone tube into his windpipe. Once a reptile is anesthetized it will stop breathing. Unlike mammals, reptiles do not have a diaphragm, and sea turtles are no different. After the endotracheal tube was in place, the anesthesia machine took over breathing for him, and continued doing so for the entire length of the surgery.

Eagle's damaged limb was scrubbed with antibacterial soap, just as in human surgery. After it was prepped, Dr. Ritchie and I placed sterile towels all around the area where we were going to operate. (Veterinary patients receive all the same sterile techniques that human patients receive.) Since Eagle was so badly injured and had probably been in pain for a long time, it was very important that we paid utmost attention to every detail to prevent infection in his stressed body. As planned, we amputated his leg at the shoulder joint. Fortunately the skin immediately around the shoulder was not damaged and using pieces of plastic tubing as little braces, we were able to reinforce the skin at the surgery site so it didn't tear when Eagle got back into the water. Almost three hours later we had Eagle back in his empty wading pool. We took him off the anesthesia machine, and after just a few minutes he was breathing on his own. That was no simple task, because it is not uncommon for sea turtles to hold their breath for over an hour at a time. For a patient coming out of anesthesia, this could prove fatal. But, Eagle was a trooper, and we knew he was in the best possible place in the world to be if you were a sick sea turtle. I prescribed some antibiotics for my turtle patient, and Richie and Tina spent the next twenty-four hours with Eagle, rotating shifts through the night. Their competent care carried Eagle through to the next sunrise, the dawn of his new life.

Four days after the surgery, Tina and Richie got Eagle back into a shallow therapy pool where Tina could administer his daily antibiotics. Eagle started eating right away, a wonderful sign in turtle medicine, and slowly began swimming around his enclosure. It wasn't long before he was moved into the larger rehabilitation pond where he could really develop his swimming skills without his two fins. As Richie had predicted, Eagle had no trouble adapting, easily chasing and catching his own feeder fish.

In the most dramatic recovery ever at the Turtle Hospital, Eagle made the big splash back to freedom just four months after his brush with death in the trap rope when Richie and Tina released him back into the waters where he was rescued.

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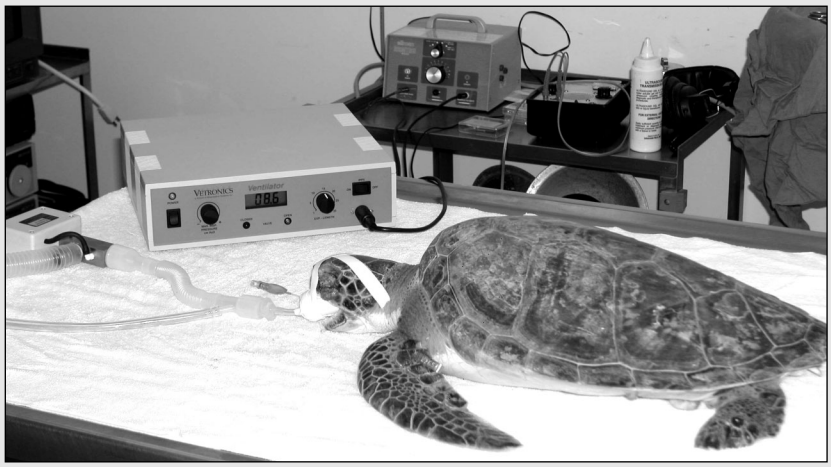
Sidebar

Once hunted for turtle steaks, turtle soup and their shells, sea turtles have had a long battle against their number one enemy, man. All sea turtles are protected as an endangered species, and hunting them, or any commercial trade of turtle products, is forbidden. Even with this political protection, sea turtles are still in a constant battle against man and his dangers, loose and unclaimed fishing nets, trap lines and fishing line. Now, in addition to this, the fibropapilloma disease threatens entire species. The Hidden Harbor Environmental Project is dedicated to

rehabilitating sick and injured sea turtles, and also to efforts to find a cure, or at least a vaccine, for the deadly tumor disease. Research funds for this type of work are scarce. Continued support for the project relies heavily on donations. For more information about the sea turtle rehabilitation project, or to send a donation to the Hidden Harbor Environmental Project, Inc., write to them at 2396 Overseas Highway, Marathon, FL 33050.



Juvenile Green Sea Turtle (*Chelonia Mydas*) with Fibropappilloma. Anesthetized with Isoflurane. Vetronics Ventilator set at 10 cm H_2O, with six breaths per minute. Vetronics is a division of BAS.