Late news. As this is being posted, I am pleased to report a successful low speed field cycle run in a 500 MHz spectrometer. The sample is about 10 mg/ml by weight lysozyme direct from the bottle, into D$_2$O unbuffered, no salt added, about 150 µl in a 4 mm standard NMR tube. The spectrometer is our homemade microbore 500, recently converted back from Dmitri Ivanov's field cycling PQR experiment. I did not attempt to optimize the spectrometer at all, once I could see a half-way decent proton spectrum in ~100 Fids. It has a lock freeze (what is now called track and hold).

The experiment is simply a field cycle at low air pressure (±0.05 Atm.) with a round trip time of ~150 msec to about 5 T, followed by a hard 90 and FID. As a control, the air system was left off but the timing was the same. The main results are:

1. I did not break the 4 mm thin-wall Wilmad NMR tube, or probe, cycling into a 5 mm probe (built by Craig Bradley, many years ago). The hole in this probe is about 5.4 mm I. D. This is non-trivial since the end of the NMR tube is 13.5 cm below the holder, which is 6 cm long, and the radial clearance in the probe is 0.07 cm.

2. Virtually no time is needed after the tube lands, before the 90° pulse, i.e. 10 ms is probably enough time.

Spectra look identical with and without shuttling except that the spectrum is a couple of percent higher with no shuttling. Note that the sample run in this way gives an overall average T$_1$, not the selective cross-relaxation T$_1$ of ~100 msec that is relevant for NOESY; and that the sample spends very little time (~30 msec) out of the high field, during the cycle.

1. Introduction. This is an update to the previous report. It is not supposed to be readable by itself, and updates the earlier report section by section, pretty much. As before, anyone can use the designs and ideas herein, but I request that publications and other literature acknowledge this contribution if it is useful. This request includes commercial use, if any.

Support. My student Dmitri finished his thesis on a field-cycling study of PQR of $^{11}$B in boronic acid inhibitors bound to proteases. Therefore I applied some of the grant USPHS GM20168 that supported him, to the present project. He is now happily employed by Gerhart Wagner. I have received a generous grant from the Petroleum Research Fund of the ACS for the future development of the shuttler.

Publication. The following is a poster that I presented at the 2001 Chianti conference on Relaxation, near Pisa, May 27-31, 2001.
WHY SHUTTLE A SAMPLE FROM A COMMERCIAL 500?

ABSTRACT
Field-Cycling in a Commercial High Resolution Instrument: Unmasking Dipolar Relaxation with Special Application to $^{31}$P and Spin Labeled Samples.

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I am building a sample shuttler (1) that will move a standard 4 mm NMR tube upward about 27 cm from the center of a commercial 500 MHz NMR to the fringe field, and back after a programmed delay, with minimum round trip time of 0.10 sec with no bounce (preliminary version, not tested in an instrument as of 12/00; Vibration recovery time unknown). The full pulse repertoire of the instrument will be usable. The aqueous solution is confined at the bottom of the tube and is nearly bubble-free. I expect this device to be useful for many kinds of T1, and some NOE, observation in biopolymers. Obvious applications include: testing and improving the current dynamics methodology for labeled proteins; study of dynamics of small ligands bound weakly to enzymes as proposed by Bob Bryant; and especially applications to revive the neglected nucleus $^{31}$P. Cycling to lower field would nearly eliminate CSA relaxation and unmask dipolar $R_1$ and NOE effects, and be especially useful for smaller nucleic acids duplexes, which are likely to have long enough $T_1$'s and high S/N. The obvious application is a dynamic study using $T_1$ measurement from zero to 23.4(?) T. The obvious proton to $^{31}$P NOESY experiment—proton frequency label, then cross relax at low field, and then $^{31}$P observe at high field—is probably feasible in D20. It would yield distances, and be useful for resolution and identification as well. Combined with dipolar splitting seen for aligned samples, H-to-P vector orientations might also be determined. In general the main problem will be to make the proton $T_1$ long enough, or to measure specific relaxation rates, using $^2$H labeling in some cases. For studies of spin labeled proteins (2), the spin-label-enhanced $R_1$ would be increased compared to all other relaxation mechanisms (including fast-relaxing electron spins) except for heteronuclear dipolar relaxation. Field cycling $R_1$ measurement could provide an alternative to deuteration in these experiments.

(1) Wagner et al., JMR 140, p. 172 ('99); Wu and Johnson, JMR 116, p. 270 ('95); Kerwood and Bolton, JMR 75, p. 142 ('87). (2) Gaponenko et al., Protein Science 9, p. 302 ('00).

WHAT WE HAVE DONE SO FAR:

Our pneumatic shuttler replaces the “upper stack” (sample-raising tube, and spinner) of a 500 MHz Varian instrument that is otherwise unmodified. It also requires a moderate amount of other items such as solenoid valves. As I will describe in Torino, it can be expected to raise a sample about 25 cm to the fringe field of the magnet, and back, with a round trip time of about 0.1 sec; and must remain a minimum time at the low field of about 20 millisec. Therefore, we can operate with nuclei having $R_1$’s in the range smaller than around 10 sec$^{-1}$. This includes many species in macromolecules including possibly even protons. This hardware has been developed over the last 9 months, and is about to be tested in a spectrometer. I will use it with an
APPLICATIONS.

In general these applications use the fact that dipolar relaxation is enhanced, and CSA relaxation reduced, at low field, so that CSA can be made negligible, and dipolar relaxation, and in some cases NOE’s, can be favorably enhanced. I hope to apply it to a range of problems that will demonstrate its fantastic utility.

1. **Dynamics of labeled proteins.** We can test the usual model-free predictions for nuclear spin relaxation in proteins at fields well below 11.7 T (limited only by the requirement that $T_1$ be longer than $\sim 0.1$ sec) by measuring $R_1$ and, perhaps, NOE, of $^{15}$N or $^{13}$C interacting with protons. We will use standard sequences with only the variable delay time replaced by a field cycle to the fringe field, and back, with various delays. The measurements can be combined with now-standard high field measurements to get better values of apparent correlation times, for example. The magnitude of the dipolar contribution to relaxation can be accurately measured at lower field where CSA is negligible, and then extrapolated to high field to get an improved determination of CSA relaxation and, possibly, high frequency motion. NOE’s from protons to $^{15}$N or $^{13}$C might also be enhanced, provided that proton $R_1$ can be made long enough, for example by deuteration, for proton frequency-labeling (done at 11.7 T) to survive the journey to low field.

2. **Dynamics of ligands bound in fast exchange.** Some of these applications will be outlined by R. Bryant at this meeting or at the field cycling meeting. It is of great interest to determine the dynamics of enzyme active site ligands, since variations in such dynamics are often invoked, with little experimental justification, in mechanistic discussions.

3. **Enhancement of spin-label-induced relaxation for structural studies.** Shortening of $R_1$’s by engineered single spin labels can be used to determine folds and protein docking, as recently demonstrated beautifully by Rosevear’s lab (see ref. 2 of the abstract). Such relaxation should be enhanced at lower field, compared to cross-relaxation to other protons, and compared to relaxation by rapidly relaxing paramagnetic centers such as iron. Thus, use of field cycling could somewhat extend the reach of this important method, which is in the 25-30 $\Delta$ range.

4. **Unmasking of $^{31}$P dipolar relaxation and NOE from protons.** This is probably the most predictably useful and exciting application. NMR of $^{31}$P in macromolecules has been shockingly neglected in recent years, probably because at 500 MHz and above $^{31}$P relaxation is virtually entirely due to CSA. At low fields (in the range 1 to 5 T, probably) dipolar relaxation will dominate and can be used for distance estimates in conjunction with specific deuteration. In
many interesting cases (e.g. small duplexes and loops) hetero-NOESY is likely to be easy, either proton to $^{31}$P, or the reverse. The methodologies implied in the first two applications above could also be used with $^{31}$P. Phosphorylated proteins, or those binding nucleotide phosphates provide a tremendous range of applications. The main problem is to find good questions.

2. Overall. I have repeatedly achieved a round trip time of 100 msec with several hours of tests. The only failure occurred when I tried to achieve 65 msec, and the NMR tube broke. Therefore, I declared that 100 msec was fast enough for now, though eventually it could be faster.

Varian delivered a 500 MHz 5 mm indirect H-X probe. I was overjoyed to find that the sample hole of this probe is actually about 5.7 mm inside diameter. Therefore, I will certainly try, and expect to succeed, to cycle a standard 5 mm thin-wall tube (4.97 mm diameter) into this probe. Direct observation of the alignment of my 4 mm tube leads me to be optimistic (see below).

3. Sample tube. The general approach described before works but has to be modified. The "Redfield boat" (previous report) sank when I spun in the clinical centrifuge with epoxy (liquid) above it, because it became a cartesian diver. The next two attempts were bottle floats, made by drilling an axial hole in the top of a plug, then plugging the top of the hole with a little plug, then re-machining to remove excess epoxy. The floats are rather long, about 2 cm, and the first one, which fit tightly, still had a tiny bubble after spinning (about 20 G's ) for ½ hour, even though the bottom end was slightly tapered for 6mm. The next version fit less tightly (outside diameter 0.122 inch in a 4 mm tube whose maximum inside diameter is .1275 inch. It seems to be bubble-free but there is some crud (denatured protein). This crud does not seem to swirl around when I shuttle, indicating that imaging of the sample may be possible, to map $R_1$ vs height and thereby get more information in a single-position run. (It came to my attention that typical sample lengths might be 1.5 cm in our 500, rather than 1 cm as I previously assumed. At a center field of 1.5 T my field map indicates that the field will vary from 1.2 to 1.8 T between the top and bottom of the sample, and if $R_1$ is proportional to $1/B^2$, $R_1$ could then vary by a factor of 2.25. Despite all this, I'm not convinced that imaging is worth the loss in S/N).

Because the little bottles are laborious to make, I'm now trying upper plugs of polyethylene, which floats in water, of various diameters. A looser fit will make it easier to spin out bubbles, but perhaps harder to seal in the plug with epoxy on top because the epoxy is slightly more dense than water, and wants to slide down during the brief spin that I use to de-bubble it. A first test of a polyethylene float plug works well! Probably it can be sealed with only one dose of epo-tek 301. Diameter of plug .122 inch, height ~½ inch, sealed above with ~½ inch of epoxy.

I also tried nearly filling a 4 mm tube with epoxy and a smaller glass tube, as a preliminary test to make a break-resistant tube. This appeared possible, but I did not test it shuttling.
4. **Sample holder** (formerly called "piston"). In connection with the geometry of my home built 500 MHz instrument, which I will use for early tests, I had to either make the holder shorter, or get an NMR tube longer than the standard 8 inches. Also, tests of the NMR tube's alignment (see below) suggested to me that the tube and holder were too flexible; a 4 mm tube is very flexible! The tube may have been bent by the bottom disc of the holder that screwed on without proper alignment. Therefore, the new holder was only about 5 cm long, and was hardly turned down in the space between the two end precisely turned bearing surface. The sleeve that is epoxied onto the NMR tube is now near the top of the tube (see Fig. II-1), and the removable disc of the holder that traps the tube is correspondingly at the top of the holder (Fig. II-2). This disc has a lip that fits into the main holder body to radially align it, and is held on with two fillister-head screws that should not perturb the radial alignment. Finally, the lower part of the hole that goes through the center is now a snug fit over a long distance (/1.5 inch) at the bottom of the holder. Fig. II-2 includes a picture of this holder; it is from a talk I gave at the 2nd Field Cycling in Torino, Italy, June 2001. At this talk, and my earlier poster at Pisa, I demonstrated a short version of the earlier 25.4 mm inside diameter shuttler with de-bouncers, described in my first report, using my lungs to generate the vacuum and pressure.

This holder with tube is about twice as heavy (~30 gm) at the previous one, and the round-trip time is 25% longer. So we will start turning it, or the next one, down.

5. **Shuttle tube and its holder alignment.** Since the last report most tests have been with a smaller glass shuttle tube, from Wilmad, 36" long and 0.8" (~20 mm) precision inside diameter. The outside diameter is not "precision" but is slightly less than 1", quite uniform. Fig. II-2 shows a sketch of the holder, tube, and mounting brass tube. The latter is now a 1.5 O. D. brass tube, and is still a preliminary version. The drawing omits several uninteresting details. The smaller diameter glass tube (20% smaller than described in the first report) increases the maximum expected velocity of the shuttle which is the speed in cm³/sec of gas entry or exit through the control valves, divided by the shuttle tube area (around 10 M/sec now). It also leaves more room for an optical sensor and for a heater for temperature control of the glass at the upper sample position.

An important detail omitted in the drawing is the presence of slots in the metal piece at the bottom of the metal tube, to allow gas flow from the bottom of the shuttler around the outside of the glass tube to the top of the system. A smaller shuttle tube makes it easier to provide this space. We will show more details of all this after the system is tested in a commercial magnet and probe.

The disadvantage of a smaller shuttle tube is: Less space for the antibounce discs and the center holes for gas and sample that pass through them. Currently I use a ¾ inch Greenlee hole punch to make the debounce discs, after they have been drilled with a $\frac{3}{8}$" center hole. A smaller tube also gives smaller initial acceleration for a given shuttle mass and pressure, but this is offset by the smaller shuttle mass, and the smaller latency for evacuating the tube.

Generally the smaller tube works in a similar way as the large tube.
Turning to alignment, please focus on the drawing of the lower end of the tube, lower left of Fig. II-2. An open cylinder, the "alignment cup", projects from the bottom support of the glass tube, into a cylindrical hole in the top of the probe. This arrangement is the key to having the NMR tube enter the probe without breaking its fragile insert, by having a close fit between the cylinder at the bottom of the shuttler, and the hole in the top of the probe. At the same time, the fit will not be too tight because I do not want the momentum of the shuttle, when it hits the bottom end, to be transmitted to the probe. Instead, the momentum will be transmitted to the top of the magnet, to which the shuttler support is attached. The probe will be supported from the bottom of the magnet. [This may be similar to a Bruker instrument. In contrast, in Varians, the probe and upper stack are jammed together.]

Varian kindly offered to remove the pins that connect to the gradient coil, and run its gradient wires to the bottom where they already are in Bruker and Nalorac. The probe is now in California for this modification. Currently, the alignment cup's outside diameter is slightly less than 1¾", to slide into the Oxford shim coil assembly of our home-made 500 system's Oxford magnet (a "microbore", 1¾ room temperature bore). Our probes fit snugly into this diameter, and are not recessed at the top like the Varian ones. I contemplate trying a similar arrangement for the Varian (using the shim I. D. for radial alignment); then the shuttler will not directly touch the probe.

All this has not yet been tested by assembling and trying and seeing if the probe breaks! Instead we study alignment by simulating the probe with a small disc of aluminum that fits into the bottom of the shuttler's alignment cup, with a projecting shoulder to keep it at the bottom. This disc has a 5 mm tube at its center, close to where the probe's sample hole will be. Then the shuttle, with a 4 mm NMR tube, is inserted into the glass tube which is in turn in its metal housing with the alignment cup, and placed horizontally on the bench with the disc in place. Then we can see, with bare eyes, how well centered the NMR tube is in the hole; and we can feel how much force is required to make the tube touch the side of the hole. This test reassures us that a 4 mm tube will hit a 5 mm probe hole, and that a 5 mm tube will, in the future, hit the 5.7 mm hole of the Varian probe.

By pushing the shuttler a few cm up into the hole, by rotating the shuttler, and by repeating the test after rotating the glass tube relative to its holder by 180°, we can test for curvature or misalignment of the glass tube and NMR tube.

This alignment test could be applied routinely to all NMR tubes, if necessary, to check for alignment; and it can be quantitated by applying measured transverse force, to see how much force, at some fixed distance, needs to be applied to have the NMR tube touch the side of the hole.

In machining the tube assembly (Fig. II-2) it is desirable to have the bottom end (the alignment tube) be coaxial with the glass tube, because the probe hole is at least 5 cm below the end of the glass tube. Therefore finishing machining was done with the metal parts assembled, and chucked into the lathe so that the upper end (36" away) did not wobble more than a millimeter as the lathe turns.
6. **Debouncers.** Because of the smaller diameter shuttle tube, the debouncers have significantly smaller area than before, but still work well. Sometimes I detected a small, short bounce (probably <3 mm, a few millisec). This poses no serious problem; see "testing" below.

7. **Sample changing.** The upper stop assembly (right side of Fig. II-2) is arranged to be easily removable, exposing the 0.8 inch inside diameter of the glass shuttle tube (and a short length of a plastic retaining ring and a flange that retains it), not shown. The clamp that holds the central pressure/vacuum feed line (top right, Fig. II-2) is soldered to another flange, held in by two knurled set screws. (All screws may be revised to prevent loosening during long runs!) I broke one glass shuttle tube by carelessness in removing it; I did not hold it properly aligned at the top. Therefore, we decreased the diameter of the stop assembly, just above the stack of debounce rings.

Once the above assembly is removed, the sample is loaded (already mounted in its piston-holder) by dropping it in, and the stop assembly is re-inserted.

To remove the sample, the upper assembly is again removed, and suction is applied to the top via a short round piece of plexiglass. A small valve is attached to the side of this plug, and it is connected to the main vacuum reservoir by a rubber tube (Fig. II-1, suction assembly not shown). The lower end of the plexiglass tube has a roughly ½” hole connected to the valve, and a small recess about 0.85” diameter and 0.1” deep, into which the sample holder-piston slides loosely after it is sucked up. The flange (that retains the plastic that holds the upper end of the long glass shuttle tube, see above) has a shallow depression (0.05” deep) that aligns the round plexiglass to the top of the shuttle tube, while the user holds the plexiglass cylinder and opens the valve.

8. **External gas flow controls.** The previously described Skinner valves were assembled as two pairs into a manifold using two standard ½" NTP crosses and a Tee with standard ½" to ¼ NTP adapters to the valves. A smaller single Brukert valve to atmosphere was added. It connects to the brass pipe that goes down to the shuttle with a ~40cm heavy rubber tube, including a short right angle turn for flexibility. This and many other connections are made with large diameter metal quick disconnect valves [McMaster Carr "high flow quick disconnect"] (that do not have check valves!); these also serve as swivels. This manifold is quite heavy!

The manifold's top has to be at the same height as the top of the tube to the shuttle, more than 8 feet. Therefore it is mounted to a ¾” solid aluminum rod (tapped ½ NTP to fit the plumbing) that fits vertically downward into holes in a wheeled tower (see tower in Fig. II-1, top drawing). Using primitive lab clamps the manifold can be lowered to fit through the lab doors, then raised when the tower is next to the magnet. The tower also carries two reservoirs, about 2 meters by 8 cm PVC sewer pipe, connected with two large diameter heavy tygon tubes to the manifold valves. The tower is a lightweight design based on a delicate aluminum angle-iron A-frame. It is much admired by passing students, as we wheel it down the hall. A white plastic lab cart (Rubbermaid!) carries a beautiful aluminum rack (Budd, from Newark but you have to get the 12-20 screws from McMaster!). Two rigid (1¼” PVC) plastic pipes, connected with quick-disconnects, carry the pressure/vacuum to the reservoirs from regulators on the cart. These
regulators get pressure from the same source as the NMR (but dry air will not be needed) and vacuum from a Jun-air 600 pump down the hall.

The solenoid valves are controlled by solid-state 24V. DC relays driven in turn by standard TTL monostables ("one shots") and logic. The sequence will be: (starting with the sample at the bottom, and the pressure open to the atmosphere) wait $\tau_o$, one to ten seconds for magnetization recovery; start preparative pulses; close atmosphere valve and open vacuum valve; finish preparative pulses if they are of fixed time, while the values open and before the sample starts moving; wait an interactive time $\tau_f$, around 40 ms. for shuttle to rise, hit the stop at the top, and stay there $\sim20$ ms to "forget" the upward trip; wait a programmed relaxation delay $\tau_r$, close the vacuum valve; wait about $\tau_x \equiv 10$ msec for the vacuum valve to close; open the pressure valve; wait $\tau'_f$, possibly interactive for the sample to have dropped and stopped bouncing sufficiently; perform read-out pulses and record the FID, while waiting a further time $\tau_f''$, around 100 msec; close the pressure valve and open the atmosphere valve (at this point nothing interesting happens, it is better to start the next sequence with no excess pressure in the shuttle tube). Setup of $\tau_f$ will be discussed later. The delay $\tau_r$ will generally be one of several arrayed times from zero to a long time, in a $T_1$ series, or be a single mixing time for NOE. The small delay $\tau_x$ was used because valves close more slowly than they open, and it seemed undesirable to have both pressure and vacuum valves open simultaneously. The time $\tau'_f$ will be determined with experience, to be long enough to give a good spectrum, probably set by looking at a signal from the bottom optical sensor or, if possible, eventually triggered by this sensor.

All this is pretty simple from the shuttle controller's point of view. It sits most of the time during the relaxation-recovery time $\tau_o$. It receives a positive level during $\tau_x+\tau_f$ which goes directly to open the vacuum valve; logic closes the valve to the atmosphere at the start of this time. At the end of this time a "one shot" (monostable multivibrator) fires, length controlled by a knob on a panel, for the short time $\tau_x$. This time is determined by looking at the pressure vs time from the solid state pressure sensor (see first report), to not overlap the pressure and vacuum intervals. It is not critical. Finally at the end of $\tau_x$ we fire a second one-shot for the time $\tau'_f+\tau_f''$ which will be around 0.5 sec. The pressure valves are on for this time. The final FID is supposed to occur while the pressure stays on. At the end of this one-shot time the pressure is turned off and atmosphere turned on.

More details on how this will be set up are given later. For tests outside the NMR lab we also have a circuit that provides the $\tau_f+\tau_r$ stobe, and than a long $\sim3$ sec delay, to simulate the future Varian output.

9. Testing. A pair of large square-ish holes were drilled and filed into the 1½" brass tube that supports the glass tube, at a height and size to allow me to tape the lower optical sensor pair into them just above the top of the shuttle when it is in its normal position (with the NMR sample, eventually, in the center of the 11.7 T magnet). The LED/sensor pair is an Omron E3T-FT13 (the second "T" stands for "thin"), as found with some difficulty (Newark Electronics). These are turned off by the passage of the plastic holder but not the glass NMR tube. About 25 cm above this we drilled two slightly elongated holes to use a Pasco optical sensor pair as described in the last report, with the upper stop just above this typical position. Tests so far were done with these, and pressure/vacuum of ±0.15 Atm, using the test strobe (above). The strobe
was varied in length; below a certain length (30-40 msec) the shuttle did not pass the upper sensor; then greater than this strobe length, it stayed above for a minimum time ~10 to 20 millisecond, as mentioned briefly, possibly bouncing as mentioned (bounce would be an artifact due to the flimsy mounting that we now have).

Do we need the upper sensor? It is inconvenient! I hope not; the criterion for determining the length of the minimum strobe $\tau_f$ will be: If the time $\tau_f + \tau_r$ is increased beyond it, the time between the end of the input (vacuum) strobe and the arrival of the shuttle back at the bottom (sensed by the Omron sensor) must remain constant! So I will leave out the upper sensor, and test this assumption.

Incidentally, as expected, there are long delays between the input strobe, and the sample hitting the top; for the shortest delays, the strobe is off well before the sample reaches the top.

The fact that there is a minimum time of 10-20 msec for the sample to stay at the top must be due to the further evacuation of the feed tube when the sample reaches the top, which must then be re-pressurized before it starts down. I believe that this delay can be mostly eliminated, and will describe how later, if I am successful.

FIGURES

II-1. Top: Cartoon of the equipment wheeled up to the magnet. Bottom: Left schematic of the solenoid valve manifold. Right, method of confining the liquid sample in the NMR tube, and its upper end.

II-2. Top left, sample tube in its holder. Bottom left, and right, sections of the shuttle tube. The alignment cup shown may not be used as indicated (see text). The lower optical sensor is not close to the bottom as shown, but is ~8 cm higher, somewhat above the top of the sample holder when the holder rests on the debounce assembly.