John D. Baldeschwieler, professor of chemistry, and a test tube full of mouse, ready for the lab equivalent of a ride on a very slow merry-go-round.

Tiny Bubbles

by Dennis Meredith

The day may soon come when minuscule drug-carrying spheres injected into the body will home in on tumors and diseased organs. When it does, we can thank Perturbed Angular Correlation spectroscopy.

INSIDE the modern Noyes Laboratory of Chemistry at Caltech is the laboratory of Professor of Chemistry John D. Baldeschwieler. Inside this laboratory is a modest-looking machine featuring four cylindrical gamma ray counters, each at 90 degrees to its neighbor and aimed at a slowly rotating translucent tube.

Inside this rotating tube rests a standard, white, laboratory mouse, who looks so relaxed that you might think he is enjoying the ride. Inside this white mouse circulate millions of microscopic hollow spheres, each about one-hundredth the size of a cell and made of phospholipid, a common building block of living membranes.

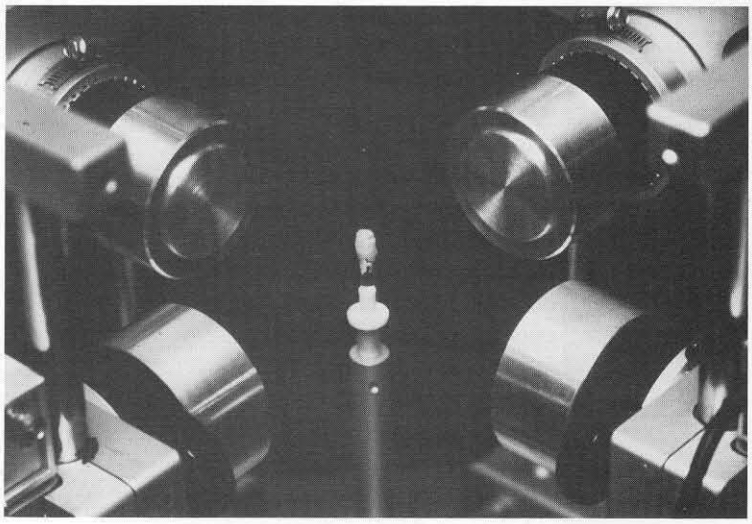
And inside each of these spheres — called vesicles — is a load of radioactive indium, each atom clutched within a molecule known as a chelator and emitting a rapid-fire salvo of two gamma rays as it decays.

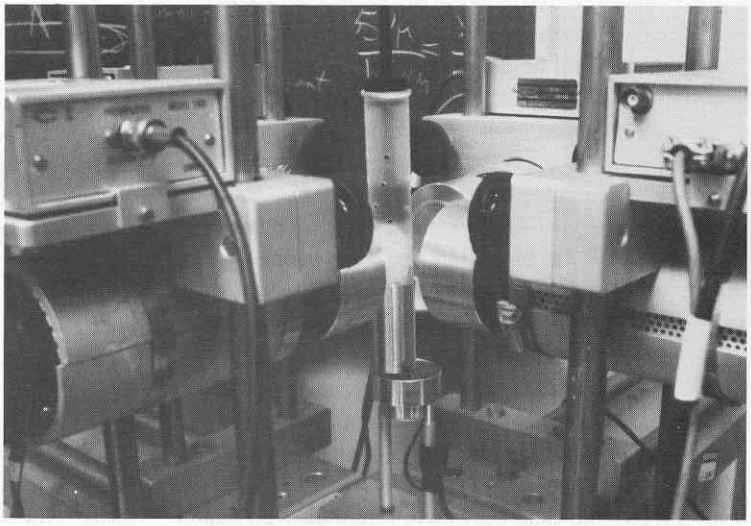
The experiment is an elegant one, say Baldeschwieler's colleagues, and it could significantly advance the day when microscopic man-made vesicles are used as the medical equivalent of guided missiles. Injected into the body and containing a dose of either drugs for treatment or radioactive tracer for diagnostic tests, they would home in on diseased organs. Such vesicles, lodged in a tumor, for example, might be designed to be enveloped by the tumor cells, where they would be attacked by protective enzymes. An opened vesicle would prove to be a lethal surprise package, because its contents would kill the cell with little or no injury to other healthy body tissues. Or, the vesicles might be built with special substances that would allow them to fuse with a cell, like two soap bubbles joining, and then to inject their contents to kill the cell or treat a disorder.

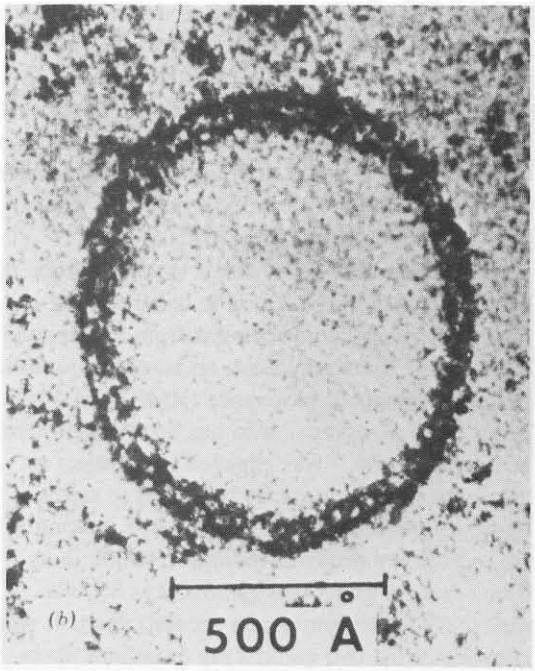
Vesicles designed to rupture only at certain temperatures could be injected into patients to deliver medicine only upon the onset of fever. Similarly, such vesicles carrying growth hormones or insecticides could be introduced into plants, where they would wait until just the right point in the growing season to discharge their cargo. The vesicles also make excellent experimental models of living membranes, since phospholipid — their main constituent — is the same as that of natural membranes. Thus, biologists can build membranes of their own design to test theories of membrane structure and activity.

For over a decade, scientists have known that phospholipid molecules — gangly structures with an ionically charged "head" end and "tails" consisting of two organic carbon chains — tend to form closed spheres in solution when agitated with ultrasound. And they have long believed that these spheres could be used medically to concentrate drugs or other substances at desired places in the body. But despite the promise of the little globes, serious problems prevented their use. Scientists had yet to learn how to construct precisely the little bubbles; to load them with chemicals; to "address" them to the right organs; to track them in the body; and to determine where and when they rupture to deposit their loads.

John Baldeschwieler's attack on these problems began — as do many efforts in basic science — with a discovery quite removed from its eventual application. Baldeschwieler, an inventive pioneer in nuclear magnetic resonance (NMR) and other forms of spectroscopy, who has been called "the Mozart of NMR," was engaged in a project to develop a new spectroscopic method to study biological molecules in 1967. He was looking at the radioactive decay of indium^{111}, which in its decay process emits two gamma rays 85 nanoseconds (billionths of a second) apart. It was known that the two gamma ray emissions show an angular correlation. That is, when the decay of







The instrument for studying the path of vesicles inside a mouse features four cylindrical gamma ray counters focused on a central upright (top), on which is mounted a test tube carrying a mouse (center). Inside the mouse circulate millions of phospholipid vesicles loaded with radioactive indium. A scanning electron micrograph of a vesicle is shown at the right. The 500 Å indicated on the scale is the equivalent of about two-millionths of an inch.

a sample of indium[111] is studied using detectors placed at different angles to one another, it can be shown that the indium atom tends to emit its two gamma rays at certain angles with respect to each other.

He discovered that this angular correlation could be used to reveal how fast an indium-containing biological molecule was tumbling. A molecule free in solution would display one characteristic set of angular correlations, but quite a different set when it became stuck to a surface or to a larger molecule.

The process is called "Perturbed Angular Correlation (PAC) spectroscopy. It was intriguing; it was unique; and, at first, it was useless. The phenomenon was, as Baldeschwieler describes it, "a classic case of a cure looking for a disease." Then, in 1976, he began to investigate the possibilities of using PAC in the promising-but-unfulfilled area of phospholipid vesicles. What was at first merely intriguing became transformed into a powerful research tool.

His research group, sponsored by the National Science Foundation, the National Institutes of Health, Merck, Monsanto, and Caltech Associate Lester Finkelstein, first developed methods of constructing the vesicles from carefully synthesized phospholipids, and of loading them with indium. The loading process, developed by then research fellow Ronald Gamble, represented a major advance, for it allowed the chemists to pump high levels of indium into the vesicles without damaging them.

Basically, loading involves first constructing the vesicles of a purified phospholipid and cholesterol, which is a necessary substance for stable membrane formation. Inside the vesicle at formation is a load of nitrilotriacetic acid (NTA), a chelating molecule that entraps small ions within its structure. Built into the vesicle walls are other specialized molecules called ionophores. These "porthole" molecules create channels in membranes that allow small ions, but not larger molecules, to pass through. The resulting vesicle is the molecular equivalent of a lobster trap, because when such vesicles are immersed in a dilute solution of indium chloride salt, the indium finds its way into the vesicle, but is trapped and held there by the chelator.

In a typical experiment, loaded vesicles are then injected into a mouse, and the animal is placed amid the gamma ray detectors. As long as the vesicles are intact, the indium and its captor chelating molecule tumble about rapidly inside. But once the vesicle breaks, the freed indium, which tends to be more attracted to cellular proteins than to the chelator, escapes the chelator and

binds to a large protein or a cell wall. Then its tumbling slows markedly, producing a sort of alarm signal from the instruments, revealing that the vesicle has broken. In their first experiments, Baldeschwieler and his colleagues quickly found PAC to be both highly sensitive and to work well *in vivo* — an absolute necessity because earlier researchers had discovered that vesicles behave far differently in the test tube than in living animals.

The Caltech chemists confirmed this difference in their first studies. They found, for example, that vesicles made from phospholipids with 16-carbon-atom tails survived intact in test tube solutions until blood serum was added, whereupon they began to leak badly. Such vesicles were similarly fragile when they were injected into mice. On the other hand, vesicles made from 18-carbon-atom phospholipids proved stable even in the animals.

The research group then tested vesicles of different compositions over a wide range of pHs and temperatures. They learned to vary these conditions to produce vesicles with varying lifetimes in the body, and vesicles tailored to destruct only within a narrow range of pH or temperature. But by far their most intriguing finding has been the first discovery of a way to "address" the vesicles to specific tissues in the body.

The key to their success was the finding within the last decade that living cells use various sequences of sugar molecules on their surfaces as recognition markers. Scientists at other research centers had found that there may be as many as 100 different sugar molecule structures found on cell surfaces and recognized by receptors on other cells. Blood type, for example, is determined by such multi-unit sugar molecules arrayed on the surface of blood cells.

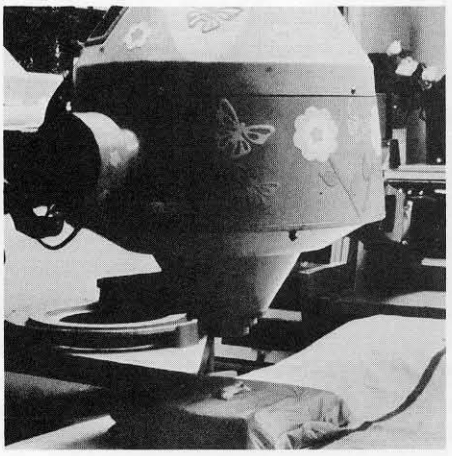
The Caltech scientists began their targeting studies by incorporating into vesicles cholesterol with sugar molecules attached by a sulfur atom "bridge." The sulfur attachment was necessary because the body would readily cleave sugar molecules attached with the usual oxygen linkage. Collaborating with researchers at Merck and Co., who produced the specialized molecules, Baldeschwieler and his colleagues tried modifying the outside of their vesicles with some 15 to 20 different sugars.

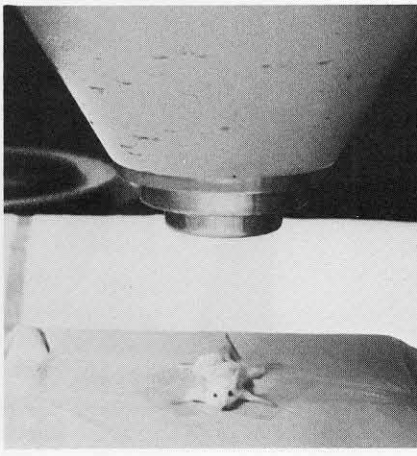
The most dramatic finding in this effort at code-breaking was the discovery by postdoctoral fellow Marcia Mauk that vesicles modifed with aminomannose sugars showed enormously enhanced survival times — lasting some 600 hours in the body versus 20 hours for plain vesicles. Such vesicles also showed considerable tissue specificity, tending to deposit first temporarily in the lungs, and finally accumulating in the liver and spleen. These findings were revealed by histological studies of the mouse tissue done by Raymond Teplitz of the City of Hope Medical Center, Duarte, California, and were confirmed by studies with isolated cells in culture by Caltech research fellow Po-Shun Wu.

This initial discovery of specificity, first reported in 1980, represents only the beginning of a long process of biological code-breaking, but it is already on the verge of being turned to medical use. Intrigued by the Caltech researchers' discovery — as well as by their ability to manufacture precisely and load vesicles with high levels of indium — three other City of Hope investigators — Cary Presant, MD, director of the division medical oncology; Richard Proffitt, assistant research scientist and the 1982 Fred Marik Research Fellow at the City of Hope; and radiological physicist Lawrence Williams — have begun to use the vesicles in diagnostic tests for cancerous tumors.

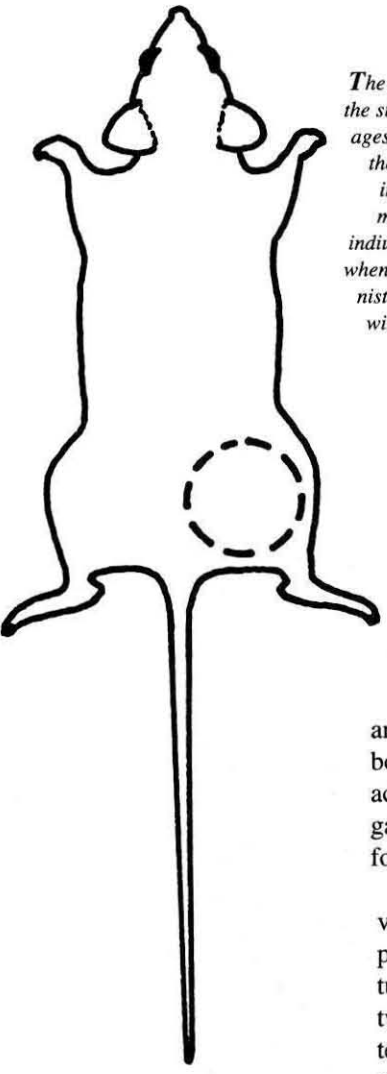
Beginning work in late 1980, the three performed detailed studies of the travels of aminomannose vesicles inside mice. Their basic approach has been to inject the vesicles into mice that have tumors implanted into their thighs. After being anesthetized, the mice are positioned beneath a device called a gamma camera, which can produce a picture revealing the distribution of gamma-emitting isotopes in the body. The camera consists of a 10-inch-diameter sodium iodide crystal, within which a flash of light is produced by each emitted gamma ray. The flashes are detected by an array of photomultiplier tubes, and the resulting signals are interpreted by a computer, which can create an image from the data.

These City of Hope scientists quickly discovered they could obtain excellent images using the vesicles. First of all, indium was already employed as a useful isotope for diagnostic work; its double gamma ray emission yielded good images,

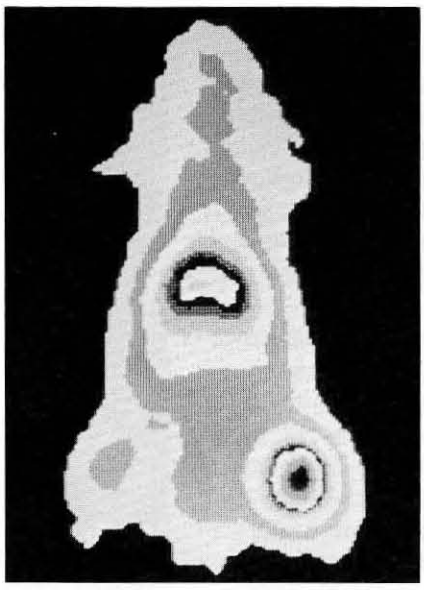


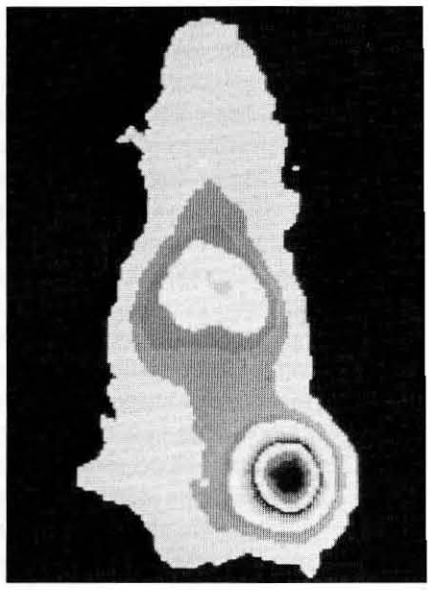


The gamma camera used in diagnostic radiology focuses on a very small patient. The mouse is anesthetized, and feels neither pain nor fright.



The drawing shows a mouse approximately the size of the one in the gamma camera images at the right, the broken line indicating the location of the implanted tumors. The image at the immediate right is that of a mouse injected with vesicles loaded with indium^{111}. At far right is the image obtained when the indium-loaded vesicles were administered after blocking the liver and spleen with vesicles packed with aminomannose.





and it was a substance that tended to linger in body tissues. Second the high loading levels achieved by Baldeschwieler and his colleagues gave the medical researchers the maximum bang for the vesicle.

In early experiments, the gamma camera revealed that the aminomannose vesicles did lodge preferentially in the liver and spleen but not in the tumor. This was an expected result because the two organs are among the sites of the body's protective reticuloendothelial system, which removes such foreign bodies. Most promising, however, was the discovery that a significant fraction of other types of vesicles also lodged in the implanted tumors. Although the scientists have some preliminary theories about why this happens, the phenomenon is not well understood. Dr. Proffitt hypothesizes an altered vascular permeability near tumors — that is, the way materials diffuse through blood vessels may contribute to the tumor's ability to concentrate vesicles.

In their next series of experiments, the City of Hope scientists, working with Caltech research fellows Joseph Uliana and George Tin, tried injecting vesicles of varying sizes and surface charges, in an attempt to enhance the tumor uptake over that of the liver and spleen. The results, while promising, were not outstanding, since the researchers were able to achieve uptake by tumors only about equal to that of liver and spleen.

But then, last fall, came an important new success. Reasoning that they might be able to first saturate the liver and spleen, the City of Hope scientists injected mice with "cold" vesicles modified with aminomannose, that is, vesicles with no indium. After waiting an hour, they then injected other types of vesicles loaded with indium. The results were remarkable; the gamma camera revealed that they had achieved tumor indium levels more than two times higher than liver and spleen levels, measured on a per gram tissue basis. The scans showed the tumor as an intense blotch, while the liver and spleen were only faintly visible.

What's more, the method was able to detect tumors smaller than 100 milligrams. This is potentially better resolution than diagnostic techniques that are now employed. For this reason, the medical researchers believe the technique may eventually aid in the solution of one of the major problems in cancer treatment — the need to detect tumors while they are still extremely small.

Presant, Proffitt, and Williams plan further studies aimed at eventual clinical use of the "blocking and tackling" diagnostic technique, and they suspect that they can improve dosages and timing of vesicle injections to achieve even higher specificities. They are also developing early strategies to use the vesicles to deliver anti-cancer drugs to tumors. For example, says Proffitt, many anti-cancer drugs are soluble in organic solvents, and such substances might be incorporated right into the phospholipid walls of vesicles, where they would be protected from inactivation until the vesicle delivers its contents within the tumor.

Clearly, it may be some time before patients can be treated with guided-missile vesicles to deliver drugs or diagnostic chemicals to body targets. But just as clearly, understanding these tiny man-made bubbles has come a long way since John Baldeschwieler first began wondering how to apply his arcane technique with the jawbreaking name of Perturbed Angular Correlation spectroscopy. □