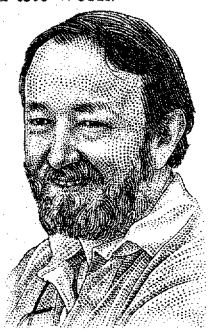
Getting The Message Across

In their 1957 paper, Theodore W. Rall, Earl W. Sutherland, and Jacques Berthet announced the landmark discovery of the biological activity of cyclic AMP (cAMP) and its role as a second messenger. They were studying the activation of liver phosphorylase (the key initial enzyme in the breakdown of glycogen) by epinephrine and glucagon when they found cAMP as a "heat stable factor." The second messenger system began as a "two-staged process" in which the hormone produced the heat stable factor in the membranous fraction, and the membranous fraction activated phosphorylase in the supernatant fraction.

Rall, Sutherland, and Berthet's success in studying hormone action in cell-free homogenates opened a new era in biochemistry. Sutherland, who won the Nobel Prize in 1972 for this research, wrote in the mid-1950s what seems now to be remarkably obvious: "... there is reason to feel that the hormones may act at the molecular level" [Earl W. Sutherland, "Introduction," in *Cyclic AMP*, G.A. Robison, R.W. Butcher, and E.W. Sutherland, Eds. (Academic Press, New York, 1971), p. 2.] Sutherland died in 1974. Although the initial research on cAMP was not funded by NIH, it became the basis for Sutherland's first NIH grant.

In an interview with THE JOURNAL OF NIH RESEARCH, Henry Bourne, chairman of the department of pharmacology at the University of California at San Francisco, spoke of the elegance and value of this paper. Rall, who is now professor of pharmacology at the University of Virginia Medical School in Charlottesville, recalled some of the circumstances that surrounded the work.

Henry Bourne



"I tell my students that this paper is the fountainhead from which all that we presently know about hormone action comes," says Bourne. "It's a genuine landmark paper that's lovely to read and fun to teach." Bourne uses the paper to demonstrate several aspects of science in general and signalling in particular.

"It is the first instance of taking a cell apart to show different components mediating the action of a hormone. Strange as it may seem, at that time there were many researchers who considered the cell to be such a complex and vital object that one would never be able to tease it apart. This paper showed that you could use the fundamental method of biological science, which is to take things apart and put them back together, to understand the cell. That is really the nub of it."

"They knew the beginning of the signalling pathway, that is, glucagon and epinephrine stimulation, and they had an endpoint [the phosphorylase reaction] that was clear and measurable that they could attribute to the action of the hormone. They dissected what was in between.

"Even the footnotes tell us something important about the signalling pathway. In Footnote 3 they gave the composition of cyclic AMP. And they said that there was something in their extracts that degrades it; that, we know now, is the enzyme phosphodiesterase. Footnote 4 tells us about the generality of their findings because homogenates of dog heart membranes behaved similarly. Finally, at the end of the paper they stated that the heat-stable factor [cyclic AMP] did not have a reproducible effect on purified phosphorylase. Now we know that it does not work on purified phosphorylase because there is a cyclic AMP–dependent kinase in between.

"Several elements of luck went into this thing, too. One is that cyclic AMP is itself stable to boiling. If they had taken on some of the other second messengers that we know now, they would have flunked. Secondly, they picked the right incubation time. Under their conditions the phosphodiesterase was chewing up the cyclic AMP almost as fast as it was made. Had they waited 20 or 30 minutes instead of 10, the membranes might have gotten a little soggy and sick and unable to make enough cAMP to let them see their effect in the boiled stuff.

"Finally, there is another point that was not appreciated until the '70s. The ATP they used in the reactions was contaminated with GTP [guanosine triphosphate—required by adenylyl cyclase for the synthesis of cyclic AMP]. If you really wash membranes well and get rid of endogenous GTP, and if you have pure ATP, the cyclase does not work very well. Marty Rodbell, Lutz Birnbaumer, and their colleagues discovered that at the NIH about 15 years later."

Theodore Rall

"I have lost count of how many wrong ideas got us to do the right experiments," Rall says about the experiments in which he and his colleagues identified the biological activity of cAMP and from which the second-messenger concept arose. "There were several incorrect ideas that got us going; in retrospect, some of the notions were downright stupid. But the fact that those mistaken working hypotheses were formulated and acted upon allowed the experiments to be done.

"The principal stupid hypothesis is my personal claim to fame. Notice that we made sucrose

homogenates of liver cells. As a matter of fact, the experiment will work as well or better if other homogenizing media are used. But at the time, I thought it was crucial, and it convinced me to do the experiment. I had just done my Ph.D. thesis with Albert Lehninger, and I had learned to grind up liver tissue to prepare and study these magic things called mitochondria. The use of isotonic sucrose was very important for getting happy mitochondria. I thought that if sucrose keeps mitochondria happy, we could perhaps keep some other part of the cell—we didn't know what part—happy. So that's what I did.

"You have to put yourselves into the minds of the people who did research in the 1950s, including Earl Sutherland, who had come out of the [Carl and Gerty] Cori lab [at Washington University, St. Louis]. They thought there was something magic about the structure of the intact cell that was necessary for hormones to act. Sutherland seemed reticent to undertake this broken-cell experiment. But I said, 'Give me a couple of months' and talked him into it.

"Then, of course, at the start I used the wrong animal. I reasoned that since so much of mammalian biochemistry had been discovered using rat liver, this tissue would be the best to use initially. We now know that this type of experiment does not work using any kind of rodent liver; in fact, it probably would work only when using the livers of carnivores, such as dogs or cats.

"After a couple of really disappointing months messing around with rat liver, I was getting a little desperate. Sutherland's foot was tapping on the floor, we had some other experiments to do, and I was under the gun. All the work on phosphorylation in Sutherland's lab had used dog liver, and I knew the hormones would produce a large and rapid activation of the enzymes in slices of dog liver. So I decided to incubate slices as if I was to reproduce this observation, but I would try to 'fool' the system by adding the hormones to fortified homogenates instead of to the slices. It was pretty wasteful, because I needed only 10 or 20 grams from a 400-gram dog liver, but I figured it was my last shot.

"I had not seen Sutherland for a day and a half. I do not know how he knew, but for some reason, right at the time the incubation was over, he appeared to watch the outcome. The reaction was so obvious that we just about dropped all the tubes. That first experiment showed nearly a doubling of the rate of phosphorylase activation. Sutherland was not usually a demonstrative person, but you could tell he was absolutely ecstatic. We were scheduled to go away the following weekend (even before we had any confirmation), and we spent the whole weekend fantasizing about what this meant.

"Then there was a Belgian postdoc in the lab, Jacques Berthet, who had just gotten his degree with Christian de Duve. He had spent his six or so years in a cold room making sucrose homogenates of liver and performing very precise fractionations by differential centrifugation. Berthet was very upset with me about the way I did those experiments. Looking over my shoulder, he watched me centrifuge the homogenates in an angle rotor for brief periods timed with a wrist watch, just to get something reasonably smooth that could be pipetted. Then I did the 'Lehninger Hard Pour' where the supernatant material was decanted with a smooth and continuous motion that allowed you to see the pellet string out along the side of the tube. As soon as the hunks and chunks reach the top, you quit. I had done it many times before, and the supernatant material worked.

"Berthet was so offended by this procedure that he wrote me a 'proper' protocol for centrifugation. I must use a horizontal yoke, not an angle rotor, and I must centrifuge a prescribed height of suspension at a certain rpm for a defined time. Moreover, the supernatant must be harvested by careful aspiration, not by pouring. So I did it, but none of the supernatant fractions obtained by this procedure responded to hormone.

"I was furious with him. But as it turned out, that was the way we found out that the supernatant would not respond unless you add back a little bit of the particulate fraction [the hunks and chunks]. Then we did the experiment in two stages, incubating the particulate fraction with the hormones, heating the mixture, and adding the 'cooked stuff' ['Kochsaft' in Figure 4] to the supernatant. Fortunately, for no good reason, we included MgATP in the first stage, and such experiments reproduced the effect of the hormone in the whole homogenate. Voilà, second messenger! You'll notice a much less sexy term was used in the paper, something like "intracellular mediator."

"Things went like fury in the next few months. As I recall, that first experiment was November 5, 1955. By the time the paper was submitted in July 1956, cyclic AMP had been crystallized—it went from a gleam in somebody's eye to crystals in roughly seven months. Some chemistry was done in the next couple months, and a footnote describing the stoichiometric content of adenine, ribose, and phosphate was slipped into the galleys before publication in January 1957.

"Even before the chemistry was done we had a bioassay for the heat stable factor, so we looked in other tissues with other hormones. We found, thanks to our friend and colleague down the hall, Robert Haynes, that ACTH [adrenocorticotropic hormone] stimulated the formation of the 'heat stable factor' in the adrenal cortex. That started the notion that we were doing business with a general phenomenon."

—GAYL LOHSE GALLAGHER