# The Microbioassay of Hormones

## The Scientific Basis of Medicine

**Presented by Dr J Chayen, Head of Division of Cellular Biology, Mathilda and Terence Kennedy Institute of Rheumatology.**

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<**Opening titles**>

<**Dr J Chayen to camera**>

A hormone can be regarded as a chemical messenger. It's produced by one cell type and it's carried by the blood through to its target cells where it changes or regulates the metabolic activity of those target cells.

<**Chayen over animated illustration of human body**>

The trophic hormones, such as corticotrophin or ACTH act at a considerable distance from their site of origin, which is the pituitary gland. On the other hand, gastrin is secreted by the cells of the pylorus, even though it's going to act on nearby cells, it too is carried by the blood to the fundus where it stimulates the secretion of hydrogen

ions. The fact that the blood acts as a carrier means that we can use a sample of circulating blood to measure levels of circulating hormones.

<**Chayen over graphs showing hormone levels in the blood**>

In 1ml of blood there may be $10^{17}$ molecules of albumin. There may be only $10^{9}$ molecules of ACTH, that is 100 millionth of the concentration of albumin. In other words, in 1ml of blood you will find 40mg of albumin but only 50pg of ACTH, and perhaps 10pg of gastrin, that is 10 million millionth of a gram. So even though they are very powerful chemicals, there's not much of them in 1ml of blood.

Basically, there are three methods for measuring hormone levels in blood and I will illustrate them by reference to ACTH. In conventional bioassay, we take blood from the patient and inject it into animals to try to measure the biological effect of the hormone in that animal. Such assays require relatively large volumes of blood and they are very insensitive so that they can rarely be used to determine normal blood levels of hormones, that is between 10 and 80pg per ml.

Radioimmunoassays are markedly more sensitive than most bioassays, but in general their sensitivity is not sufficient to detect subnormal levels of hormones. Although they are remarkably sensitive at higher concentrations, they become very insensitive as they approach the lower normal blood levels. Frequently, the only way you can measure normal levels by radioimmunoassay is to concentrate relatively large volumes of blood. In contrast, the cytochemical bioassays that I am going to discuss are about 500 to 1000 times more sensitive than radioimmunoassay. This means that you can measure normal blood levels of a hormone in a sample of 0.1ml of plasma. Or in a large drop contained in, for example, the heel prick from a newborn baby.

<**Chayen over diagram depicting the function of radioimmunoassays**>

The great specificity of radioimmunoassay comes from the fact that you produce a specific antibody to the hormone molecule, but in fact this is also a drawback. The

antibody may recognise only the biologically active part of the molecule, unfortunately it is more likely to recognise the biologically inactive regions so you may measure the biologically inactive or the degraded molecules as well as those which still retain their biological activity. In fact, it is possible to have a sample of a hormone which gives a highly specific radioimmunoassay but which has absolutely no biological activity.

<**Chayen over diagram depicting the function of macrobioassays**>

In contrast, the new macrobioassays depend on the very powerful chemical effect of the hormones. These methods measure the chemical or metabolic change induced by the hormone, acting on its specific target cells. We can do this because we have developed a form of cellular biochemistry, or cytochemistry, which actually measures the biochemical changes produced in each individual cell in the target tissue.

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<**Chayen over detailed filmed demonstration of cytochemical bioassay**>

The general scheme for all of these cytochemical bioassays is very similar and again, I would use the bioassay of ACTH as my typical example. The 2 adrenal glands are removed from a suitable animal, usually a guinea pig. Each of these adrenal glands is cut into 3 pieces, or as we call them, segments, giving us 6 from any one animal. To achieve greatest reproducibility, each assay is a ‘within animal’ assay, that is to say that the response of tissue to a standard hormone preparation and to the plasma, must be done on tissue from the same animal, that is, from one guinea pig.

To do this, we first maintain each segment separately, in vitro, each in its own culture pot. Synthetic culture medium is added, just up to the top of the table in the culture pot so that it soaks the lens tissue on top of the metal grid table. Each segment is then placed on its lens tissue. In this way the tissue is fed with culture medium but it is not drowned in it, the cells can still breathe.

The culture pots are sealed with a seal of lanoline. They are then transferred to a gas slide so that their atmosphere can be replaced by 95% oxygen, 5% carbon dioxide. This concentration of carbon dioxide is used to buffer the bicarbonate in the culture medium. The tissue is then maintained for 5 hours.

The stress of death has released ACTH in the animal, and this ACTH has depleted ascorbate or reducing potency from the adrenals. During the 5-hour culture, the cells recover from this effect of the animal's endogenous hormone levels and they accumulate ascorbate from the culture medium. Consequently, the reducing potency of the critical zone, the zona reticularis, is maximal at the end of this period and it is primed to give the maximum response to ACTH.

At the end of the recovery period, the culture medium from 4 segments is replaced by fresh culture medium containing graded concentrations of the standard preparation of ACTH. This hormone is allowed to act for only 4 minutes. This will give us our calibration graph; it will allow us to measure how much activity is induced by 4 different concentrations of the hormone.

In practice, we use logarithmic doses from 5pg to 5fg per ml. The medium from the other 2 segments is replaced by fresh medium which contains plasma, diluted 1 in 100 and 1 in 1000. They too are treated for 4 minutes only. The tissue must then be chilled rapidly to -70˚C, this avoids the formation of ice. The protoplasm becomes super cooled. There must be no delay in cooling the specimen and this is done by precipitate immersion in the coolant. The super cooled segments are transferred, with cold forceps to avoid thawing, to a dry tube at -70˚C; this is for temporary storage.

To cut the super cooled tissue, it has first to be mounted on a microtome chuck. This is a potentially hazardous procedure and care must be taken to ensure that the tissue will not thaw. The microtome chuck, with its specimen firmly stuck on it, is put into a cryostat. The temperature of the cabinet is kept at about -25, but the knife is

kept at about -70; this is to ensure that the heat generated during cutting is conducted to the knife and so away from the tissue.

Sections are then cut at 10 microns. Although the setting in the microtome reads 10 microns, the actual thickness of the sections will vary with the speed of cutting.

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For our work, the thickness must be rigorously reproducible, so we use an automatic cutting device which allows us to control the speed at which the sections are cut. Now comes the tricky part of the procedure. We have a section of super cooled tissue sitting on the knife at -70. As soon as that tissue warms up, the thermal energy will be used to convert the super cooled water into ice. And if we are to do real cellular biochemistry, we must avoid ice damage which affects chemical activity even more than it affects histological structure. We overcome this by bringing a slide from the temperature of the laboratory up towards the knife. As it approaches the section, we have a temperature gradient of nearly 100˚C between the knife and the slide. This causes the water in the section to boil off onto the knife. This boiling jet propels the section onto the knife, and the water condenses on the knife as free water and, of course, sets as ice.

The section may contain only as much as 5% of water, so we now have a practically dry, that is, flash dry section, and we have avoided ice artefact. We now test the sections for the particular chemical activity which we know is altered by the hormone. For ACTH we measure the change in reducing potency. This is equivalent to the well-established loss of ascorbate induced by ACTH in the cells of the zona reticularis of the adrenal cortex.

Ascorbate and similar reducing substances will reduce ferricyanide to ferrocyanide. This ferrocyanide in the presence of ferric ions produces an intense blue precipitate, or Prussian blue. Now, because ACTH produces a loss of ascorbate, higher concentrations of ACTH will cause a greater loss of ascorbate. This means that there will be less ascorbate in the section to produce ferricyanide, so that less Prussian

blue will be formed. Of course, conversely, lower concentrations of ACTH will have less effect on the ascorbate concentration in the cells, so that there will be more ascorbate left in the cells to produce more Prussian blue in the section.

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We now come to the most powerful part of these new techniques; that is, to microdensitometry. I have just stressed that ACTH causes loss of ascorbate from the adrenal cortex, in particular it removes ascorbate from the thin band of cells that make up the zona reticularis. These reactive cells may constitute perhaps 10% of the adrenal gland, all of which is rich in ascorbate. So, for a sensitive assay we must measure effects just in these cells.

<**Chayen over film demonstrating microdensitometer**>

For this, we use a scanning and integrating microdensitometer. Basically, there's no difference between conventional spectrophotometry and microdensitometry or micro spectrophotometry. In both, you have a monochromator which passes light of the required wave length into your specimen. In spectrophotometry, this specimen is a solution in a glass or quartz cell; in microdensitometry, the specimen is a biological cell coloured by a stoichiometric chemical reaction. The light is then transmitted to a photo cell or in microdensitometry to a multi stage photo multiplier. One major difference is that in microdensitometry the image has to be scanned and transmitted, point by point to the photo multiplier. But we will come to that later.

So basically, the microdensitometer is a conventional spectrophotometer but built around a microscope. We first adjust the monochromator to give the correct wavelength of the light we need for measuring our reaction product. We then position the slide so that we can position the cells that we wish to measure.

The next step is to instruct the machine to measure only what is seen inside this white mask. The light path is switched away from the eye pieces so that light can pass through the multi stage photomultiplier – I say it can pass, but if we projected

the image of this cell straight onto the photomultiplier we would get a very erroneous reading. This is because the dye is not present as a homogeneous solution as it is in normal spectrophotometry. It is present as an optically inhomogeneous precipitate. To measure this accurately, we scan the cell with a flying spot. Each point on the scan is then optically homogeneous and each point is sent separately to the photomultiplier; the machine then integrates all the spot readings.

If we intercept the results as they are produced by the photomultiplier and show up the actual trace we can see how the intensity varies as the spot traces across the specimen. All these intensities or absorption values are integrated, and in this particular case we can see the integration of all the absorption values in this single linear trace, as it happens.

In practice, the spot makes many such traces across the selected field and all the absorptions are integrated and are presented on a digital meter. These readings can then be converted into absolute extinction, as you measure in conventional spectrophotometry.

<**Chayen over table showing readings from microdensitometer, then readings are plotted in graphs**>

We measure a number of cells in each of two serial sections, and you can see that we get good reproducibility in a number of sections taken from each segment. In each sample, that is from segments treated with different concentrations of a standard preparation of ACTH, or treated with plasma, the variation between results from serial sections is not greater than + or -5%.

We begin by taking the microdensitometer results from the 4 segments treated with graded concentrations of the standard preparation of ACTH, and we plot them on a semi logarithmic scale. This gives us our calibration graph. Then, knowing the integrated extinction of our plasma sample, diluted 1 in 1000, we can read off the concentration of ACTH that must have been present in our diluted plasma. We can do the same for the same results obtained with the same plasma diluted 1 in 100.

After correcting for dilution, these readings give us the concentration of ACTH in the plasma. The results should agree to within + or -15%.

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Now one question we have to answer is how is it possible to measure such low concentrations of hormones, that is 1000 times less than is needed in the human or the animal body, to produce physiological effects. The answer is that we are measuring the initial rate of a chemical response, which, if maintained for sufficient time will produce a physiological effect. Now, how does this work? It means that 1000th of the physiological level of ACTH will produce a loss of ascorbate, but it will be very quick and the cells will recover very quickly. The loss of ascorbate will be very transitory. Higher concentrations of the hormone will produce a faster and a greater change, so if we measure the amount of change at the time of maximal response we can measure a simple dose-related loss of ascorbate.

In contrast, the amount of cortisol produced by the adrenal in response to ACTH depends not only on the loss of ascorbate but also on the time during which there is a diminished concentration of ascorbate in these cells. That is to say, it is proportional to the area under the curve. The area under the curve, produced by 1000th of the normal blood level of ACTH, produces very little cortisol. The area produced by higher concentrations is appreciably greater. What we find is that the maximum rate of ascorbate depletion is achieved by concentrations of ACTH equivalent to low normal blood levels. Higher concentrations of ACTH produce more cortisol because their effect is maintained for longer periods of time.

So, to summarise, very low concentrations of hormone do have a very transitory effect on their target cells. We can measure this effect by measuring the quick change in chemical activity, but to produce appreciable amounts of the product of the hormone action we need the hormonal effect to be maintained for longer time as it is in the body, in vivo, in response to higher concentrations of the hormone.

<**Chayen over series of tables showing cytochemical bioassays for various hormones**>

Basically, these are the techniques which are now being used to assay hormones, not just ACTH, at very low concentrations. They all go down to about 1000th of normal blood level. Obviously each particular hormone has to be tested against its particular target cell. We then measure the specific chemical change produced by the particular hormone in its specific target cell.

The first of these cytochemical bioassays was the ACTH assay. Then, at St Bartholomew's, Dr Lesley Rees and her colleagues in Professor Landon and Professor Chard's groups developed a very similar method for assaying luteinising hormone, LH. This depends on the fact that this hormone, acting on the luteinising cells in the ovarian follicles causes a loss of ascorbate from these cells. So, they measure reducing potency as we do in the ACTH assay. Thyroid stimulating hormone, that is TSH, and the somewhat similar long-acting thyroid stimulator, LATS, both affect the cells lining the thyroid follicles. Although TSH produces a quick response and LATS produces a more retarded response, both act by increasing endocytosis of thyroid colloid. This is necessarily accompanied by an increased permeability of the lysosomes in the endocytosing cells. Dr Lucille Bitensky in our own laboratories has used this increased lysosomal permeability to assay these hormones. In the case of gastrin, we make use of the fact that this hormone increases gastric acid secretion by stimulating the carbonic anhydrase activity of the parietal cells of the stomach. This is the basis of Nigel Loveridge's cytochemical bioassay of gastrin. We also want to assay releasing hormones. We have begun by looking at TRH, the hypothalamic regulating hormone that releases TSH from the pituitary. This work is being done by Diane Gilbert.

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<**Chayen over graph comparing different types of bioassay**>

We now need to consider what are the advantages of being able to measure such low concentrations of hormones. The first is that we can define the lower limits of normality, even though the radioimmunoassay for ACTH is one of the best radioimmunoassay, it is still too insensitive to give a precise figure for the lower levels in normal people. Other radioimmunoassay, like that for TSH, are certainly too insensitive to define the lower levels of normality. So, if we cannot define the limits of normality, we're certainly not in a position to decide whether certain clinical conditions are caused by slightly subnormal levels.

The second advantage is that we require very little blood so we can measure changes that occur quite rapidly in the blood. For example, we can demonstrate that the blood level of ACTH in a normal human volunteer fluctuates very rapidly. In this study, Dr Daly and his colleagues in the Charing Cross Hospital took 11 samples over a period of half an hour. For cytochemical bioassay, this involved removing 11 half ml samples, that is, a total of 5 ½ ml of blood. For radioimmunoassay this would have needed 11 samples but each of 16 ml, that is, a total of 176 ml. But, of course, radioimmunoassay is too insensitive at these relatively low levels to have detected some of these fluctuations. This then is the second advantage: because we require so little blood, we can study the physiology of ACTH without exsanguinating our unfortunate volunteer.

<**Chayen over animated illustration of human body**>

And this leads us to examine some of the physiological studies that now can be made. We have just seen that the ACTH level in the blood can fluctuate minute by minute. It is well known that the secretion of ACTH by the pituitary is greatly influenced by the concentration of cortisol in the blood. This is the well-known feedback mechanism.

When the cortisol level drops significantly, the pituitary is induced to secrete ACTH which then instructs the adrenals to produce cortisol. When the cortisol level has risen sufficiently, it switches off the secretion of ACTH from the pituitary. The sensing centre and the pituitary stimulating centre is the hypothalamus. Now, although this

story is probably correct in general outline, we still know remarkably little about the details of this feedback mechanism. It is true that we can suppress the secretion of ACTH if we inject relatively large doses of cortisol.

<**Chayen over series of animated graphs charting levels of cortisol and ACTH**>

We can show this if we take a volunteer and measure his endogenous ACTH before and after injecting cortisone. You can see that provided he is kept very quite and recumbent, the ACTH concentration in his blood goes down to 35 fg per ml, and we can still measure it. The half life of this endogenous ACTH in the plasma is 8 ½ minutes. From another experiment where we have measured both the ACTH and the cortisol, you can see how quickly this feedback mechanism works. As the cortisol level begins to rise, so the ACTH level begins to drop. This means that the cortisol has cut off secretions of ACTH; what was present disappears with a half life of 8 ½ minutes.

In order to study what happens when you inject ACTH, we need to suppress the secretion of endogenous ACTH in the volunteer. We can do this by giving him a glucocorticoid. We usually find it preferable to give him the synthetic glucocorticoid, dexamethasone, which does not interfere with our measurements of the cortisol which he produces in response to the injected ACTH. You can see that the concentration of ACTH in his blood rises rapidly over the first 5 minutes, and is followed by a rapid rise in cortisol.

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This gives us the tied relationship between ACTH stimulation and cortisol production by the adrenal in the human. You can also see that the level of ACTH drops away fairly rapidly. It is of interest that the half life of injected ACTH is that of the endogenous ACTH in the previous studies, namely 8 ½ minutes. This study also gives support to the accuracy of the cytochemical bioassay; we inject a known amount of ACTH and assuming that the blood volume of the volunteer is 5 litres, we do recover all of the ACTH by the cytochemical bioassay.

<**Chayen to camera**>

Well, in conclusion, I have tried to describe to you a totally new way of bioassaying hormones. I have naturally used, for most of my examples, I've used the ACTH work which we have used originally. But, of course, this has already been applied and the same principles do apply to the hormones TSH, LATS, LH and gastrin. In principle, there's no reason why the same systems can't be used for pretty well any hormone. In fact, you don't have to restrict it to hormones, it can be used for measuring the effect of many biologically active substances including toxic substances and drugs. So, I hope you will agree that it is quite a powerful new tool to be added to the armamentarium of medical research.

<**End credits**