

## OF SEX HORMONES AND BOWELS

The origins of **phenoxodiol** lie in a request from a friend and colleague, Graeme Cotton. I had worked with Graeme in the same department in the cancer field, although Graeme had left to take up a university administrative post.

In about 1980 Graeme was diagnosed with cancer of the colon. He underwent surgery, but a couple of years later was found to have secondaries in his liver. Chemotherapy was tried, but to little avail. Having spent some 10 years in cancer research, Graeme was as familiar as anyone about the process that was taking place inside his body. He understood that the odds were heavily stacked against him eventually beating the disease, but with a young family, he was determined to give himself every opportunity to fight it. A strict dietary regime, meditation, physical exercise, and a strong sense of spirituality were to be his mantras for his few remaining years. He also wanted to find out everything possible about the disease...I guess he felt that 'knowing thine enemy' was going to be an important basis of his mental battle with the disease. To that end, he approached me in the early 1980s with a view to getting hold of as much relevant information as I could about bowel cancer.

This was pre-internet and no Google to help make literature searching the straightforward task that it is today. The task then involved the methodical and painstaking review of dozens of different scientific and medical journals. It meant scanning through the titles of literally thousands of scientific articles, and then chasing up individual references, most of which would ultimately prove worthless. I guessed that Graeme's energy levels were not up to such a task, and so I was more than happy to help out.

It didn't take too long to work out that there was little useful information that I was going to be able to pass onto Graeme. Neither of us had had any specific experience with cancer of the bowel, but I was surprised at just how little a reasonably exhaustive literature search turned up in the way of any useful or new information about colorectal cancer that was not already generally known. I wasn't even sure what I was looking for, although I assumed that it was information about what started the disease in the first place, what factors within his body may have contributed to its growth, and what new thinkings were around on how to manage the disease. I gave him what I could find as I collected it, but I doubt that it was ever of much practical use.

Then I stumbled by sheer chance across a scientific review paper that caught my attention. It was an article about the relationship between colorectal cancer and estrogens. In the course of conducting this exercise for Graeme, the keyword phrase that was fixed in my brain that I looked for as I scanned through thousands of article titles, was simply *colorectal* (or *large*

*bowel*) cancer. In the vast majority of cases, a look at the full title or the abstract quickly indicated that the study held little relevant information. But the presence of *colorectal cancer* and *estrogen* in the same title immediately caught my attention. If it had been *estrogen* and *breast cancer* or *ovarian cancer* or *uterine cancer* or *prostate cancer*, I doubt that I would have given it a second thought. To my mind, the large bowel was about as removed from a 'reproductive' organ as one might expect, so the linking of *estrogen* and *colorectal* in the same sentence was instantly attention grabbing.

The article was written by an Australian scientist, Dr McMichael. In it he discussed the evidence for the role of estrogen, the female sex hormone, in the development of bowel cancer. He reviewed three bodies of evidence.

- The first was that there was a difference in the nature of cancer of the colon between men and women. The colon anatomically is described as having 3 parts – the first part of the colon where it emerges from the small intestine is known as the *ascending colon* because it heads from the bottom of the abdomen up towards the chest. The second part heads across the abdomen from left to right and is known as the *transverse colon*. The third part heads back down to become the rectum, and this is known as the *descending colon*. Most cases of cancer of the colon (or large *bowel*) in women occur in the ascending colon, whereas most cases in men occur in the descending colon. I am not aware of any other single example of where there is a gender difference involving cancer of a part of the body that both men and women share. It would be like saying that men mainly get skin cancer on the left arm and women on the right arm.

- The second was the finding that in men receiving estrogen therapy, the development of cancer of the colon followed the same pattern as in women – in other words, the cancer predominantly occurred on the ascending loop of the colon. This evidence came from a study of a large number of men with prostate cancer who were treated in the 1960s with the potent estrogen, stilboestrol, as a crude way of slowing down tumor growth.

- The third was the observation made by a San Francisco doctor in the early 1970s that the incidence of colorectal cancer in women fell following the introduction of the contraceptive (estrogen) pill in the early 1960s. In fact, we now know that the pill confers about a 44% lower risk for women for the development of bowel cancer.

Since then, the association between estrogen and colorectal cancer has been confirmed by other observations, including (a) the fact that the incidence of colonic cancer is higher in women under the age of 55, but falls below that of men following menopause, and (b) the fact that hormone replacement therapy lowers the risk of colonic cancer even further in menopausal women.

The link between estrogen and bowel cancer remains largely a mystery to this day. Just why a hormone playing such a key role in our sexuality would have any influence on an organ designed to ferment and to store waste food remains a matter of total speculation. Bowel cancers and the polyps that precede them don't appear to have any particular sensitivity to estrogen, and yet the statistical evidence points pretty clearly towards a role of female sex hormones in predisposing us to colorectal cancers.

However, the fact that this mechanism was (and still is) unknown was not important to me at the time. What did matter, and what immediately fascinated me, was the fact that the five

‘Western’ cancers now had a common link – all five cancers (breast, uterine, ovarian, prostate, colorectal) occurred in parts of the body that were sensitive to sex hormones, either male or female. What had previously seemed to me to be a divergence – the fact that four of the five ‘Western’ cancers occurred in reproductive tissues, while the fifth one didn’t – was no longer a divergence. There now seemed to be a completely justifiable reason for considering all five types of cancer in the same basket, with a strong likelihood of all of them having a common underlying risk factor.

## DIET AND SEX HORMONE FUNCTION

With the benefit of hindsight, a direct link between diet and the five ‘Western’ cancers is far more complex than a simple association with how what we eat affects our sex hormone levels. But back in 1984, that over-simplistic assumption served its purpose, and that was to ignite and focus an interest on just how the human diet might by influencing sex hormone activity to the extent that it might predispose us to certain cancers. For better or for worse, this is the train of thought that led to **phenoxodiol**.

If I had to summarize my thinking back in 1984, it went something like this:

- ❖ that the risk of development of the five ‘Western’ cancers had something to do with sex hormones
- ❖ that the ‘sex hormone’ factor, whatever it was, appeared to be associated with diet.

In other words, what we were eating was capable of changing the activity of sex hormones in our bodies to the extent that it changed our risk of developing cancers of the breast, ovary, uterus, prostate and large bowel.

Before I even got to the point of wondering just how diet could be affecting our sex hormones, the obvious first question was whether there was in fact any detectable difference in sex hormone levels in the bodies of Westerners (where the five cancers were common) and non-Westerners (where the same five cancers were relatively uncommon). I never really expected that if there was a difference that it would be obvious to the extent that it would show up in levels of sex hormones in our bloodstream. I thought that any differences would almost certainly be subtler than that, but it was the only obvious place I could think of starting. So, it was back to the medical library to go trawling through the scientific journals. I could not find any reports of any studies that had done that particular comparison between Western and Asian populations, but there were a number of studies that had compared vegetarian women and non-vegetarian women in Western countries such as the US. These studies mostly had been done because of the significantly lower rate of breast cancer in vegetarian women, and the researchers were looking to see if differences in blood levels of estrogen might be contributing to this. To my surprise, those studies in fact had found a meaningful difference in blood estrogen levels between vegetarian women and non-vegetarian women. The difference was not so much in the overall estrogen levels, but in the level of the main estrogen, estradiol. There are three main types of estrogen in the body, and

estradiol is the most potent. It is the most prevalent type of estrogen prior to menopause, and it is the declining levels of estradiol in middle-age that brings about the menopause. Estradiol, rather than the other two forms of estrogen, is the form of estrogen most implicated in cancers of the female reproductive tract. The studies found that vegetarian women, on the whole, had lower levels of estradiol. Given that both groups of women had the same Caucasian racial background, lived in the same cities, and enjoyed pretty much the same lifestyles with the exception of diet, it was difficult to avoid the implication that it was the vegetarian diet that was producing the lower estradiol levels. This didn't prove that Asian women would have similarly lower levels of estradiol than their Western counterparts, but there were enough similarities between the Asian diet and a vegetarian diet to believe that they well might.

If diet really was able to change how much estradiol our bodies made, the question then become, how? What evidence was there that what we ate could have any such effect on our bodies? And this is where being an Australian scientist came into play. The most compelling example of diet having a powerful influence on sex hormones activity in the body is almost uniquely an Australian experience..... the so-called 'Clover Disease' of sheep. This is a disease that almost exclusively affects Australian sheep and in the 1940s through to the 1960s was responsible for affecting large numbers of sheep across the country. It is a disease characterised by dramatic disruption of the reproductive capacity of sheep, with affected sheep experiencing all the symptoms of a substantial estrogen-overdose. Ewes show considerable thickening of the uterus, cervix and vagina; pregnant ewes abort and non-pregnant ewes stop ovulating. Even male sheep show significant pathology, to the extent of becoming infertile and even lactating.

In the 1960s, the disease was shown to be due to diet, in particular to a large amount of estrogenic material present in the pasture. The problem was traced back to the presence of a certain type of clover in the pasture known as subterranean clover. Clovers are an important pasture grass in agriculture because they contain high levels of protein, and as Australian scientists were soon to discover, high protein levels in a plant unfortunately equated with



high estrogenic activity of the plant.

**Subterranean clover**

For intensive farming practice such as dairy farming or fat lamb production, increasing the protein content of the diet is a key foundation to ensuring high productivity. Apart from hand-feeding stock a high-protein supplement, the best natural way to increase the protein level in the diet is through sowing grasses such as clovers into the pasture. In the 1950s and 60s, the

wool industry was the main form of livestock agriculture in Australia, and Australia at that time was producing about 90% of the world's wool used to make clothing. Since wool is itself made almost entirely of protein, the idea of boosting the protein level in the diet of the sheep was widely fostered as a way of increasing the national wool output. So, Australian farmers began planting a form of clover known as subterranean clover (or *sub-clover*) on a large-scale basis. There are many different types of clover, although most people would be familiar with red clover that grows in many parks and is a small upright plant with a bright red or pink flower. Sub-clover is so-called because unlike the better-known red and white clovers that grow vertically, it hugs the ground and deposits its seeds directly into the ground. Sub-clover, a Northern European plant, proved to be well-suited to the Australian climate and rapidly became a common component of pastures across Australia.

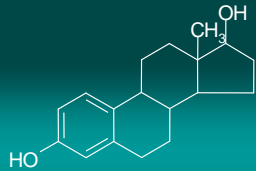
The estrogenic activity of sub-clover was found to be due to very high levels of plant chemicals known as *isoflavones* that just happened to be estrogenic. Isoflavones are a natural component of plants and play a key role in the plant's life. They don't act as estrogens in the plant, simply because plants don't have a need for sex hormones in the way that animals do. But plants, like all living things, need hormones to regulate all sorts of functions including reproductive behaviour such as flowering, and that is what isoflavones do in plants.

Although one of Earth's most primitive life forms, plants still need a range of simple hormones to deal with such basic biological functions as the need to reproduce and to respond to stressors such as drought, predators, and injury. When animal life eventually evolved from plants, these primitive plant hormone systems served as the starting points for the more complex hormone systems that animals, and much later humans, were going to need to support far more intricate biological systems. Rather than reinvent the wheel, animals simply took the pre-existing plant hormone manufacturing systems and adapted them to their more sophisticated needs. The isoflavones just happened to be chosen as the basis of the male and female sex hormone system in animals. In the process of adapting the primitive isoflavone system, animals subtly altered the structure of the isoflavone to make it a more powerful sex hormone, but sufficient structural similarity remained for the isoflavone to have some estrogenic activity.

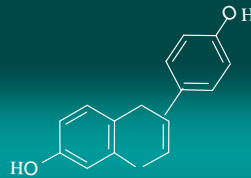
An analogy to this situation is that of beta-carotene and vitamin A. Beta-carotene is the yellow-red pigment that provides such distinctive colour to vegetables such as carrots. One of its functions in the plant is to act as an anti-oxidant, protecting plant tissues from a range of injuries. Animals, once they evolved from plants, found that they required a much higher level of anti-oxidation activity because of their substantially higher metabolic rates compared to plants. The beta-carotene that was being absorbed from the diet simply was insufficient to meet that extra need. So animals evolved a process whereby beta-carotene was absorbed into the body from the diet was converted into vitamin A. The conversion involved a relatively minor structural alteration to the beta-carotene molecule, but that alteration resulted in a molecule with about 1000-times the anti-oxidant capacity of beta-carotene.

The following illustration shows the structural similarities between an estrogenic plant isoflavone and estradiol, the main estrogen made in the body.

## Comparative structure of human estrogen and an estrogenic plant isoflavone

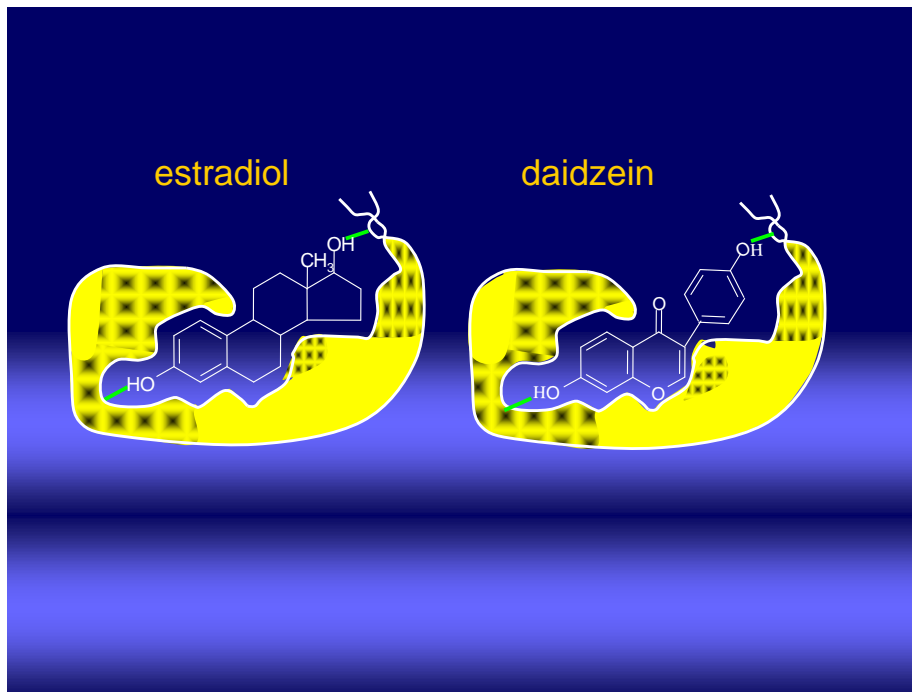


estradiol



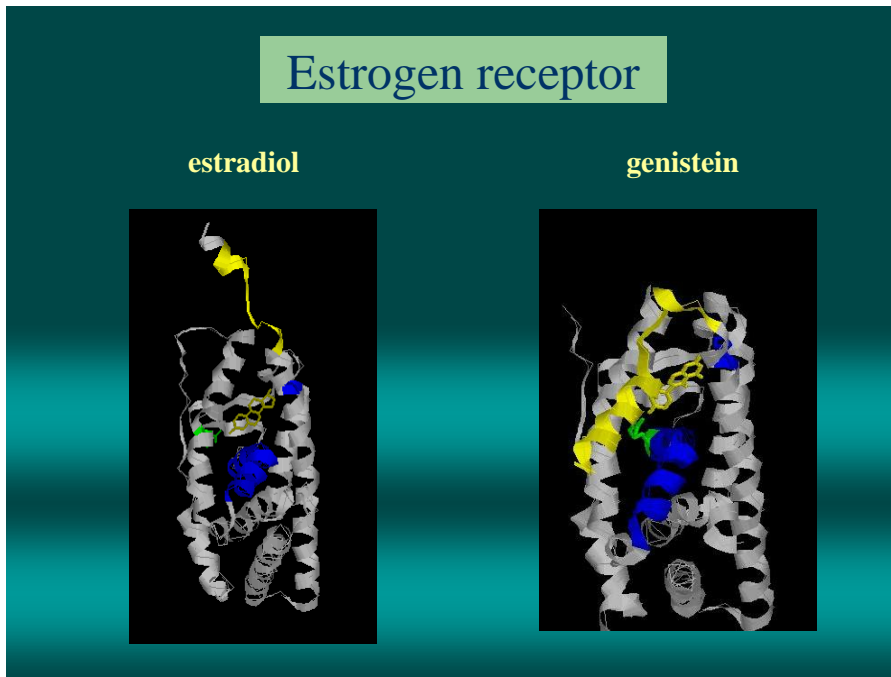
isoflavone

Those structural similarities mean that the plant isoflavone is able to function as an estrogen by interacting with the estrogen receptor, a protein found on all cells in the body. The following illustration shows schematically how that interaction occurs.



The ancient relationship between the plant isoflavone and estradiol is what allows the isoflavone to connect to the estrogen receptor, but there is sufficient difference between the two that the isoflavone does not fit as snugly into the receptor as does estradiol. This means that the isoflavone is not able to trigger the estrogen receptor as powerfully as estradiol. In fact, the isoflavone will only trigger the receptor to about 1% of the amount that estradiol

would trigger. The following illustration is a virtual representation of the protein receptor, showing either human estrogen (estradiol) or the isoflavone, *genistein*, attached. Note the yellow-coloured arm on top of the receptor. This yellow-coloured arm is like a trigger. When estradiol docks perfectly into the estrogen receptor, this arm is released, springing up, and triggering a sequence of events within the cell. When an isoflavone docks, the fit is imperfect and so the yellow-coloured arm is not released to anything like the same degree, with the result that the subsequent estrogenic response by the cell is correspondingly smaller.



Although isoflavones are only weakly estrogenic, plants such as sub-clovers can contain such enormous levels of isoflavones that their total estrogenic power can be so large as to swamp any effect of estradiol. This is what was happening in Australian sheep. It has been estimated that Australian sheep were consuming every day up to 2 g of estrogenic isoflavones, which in estradiol terms is the equivalent of the sheep eating an entire monthly packet of oral contraceptive tablets every day. It was little wonder that they were getting such dramatic changes in their reproductive tracts.

Clover grasses including sub-clovers are a natural part of the diet of grazing animals all over the world, and yet there were no reports of Clover Disease in Europe or North America. What made it a peculiarly Australian problem came down to the make-up of Australian soil. As the oldest and driest continent in the world, Australian soils are fairly fragile and contain significantly lower nitrogen levels from decaying vegetation and animal manure than elsewhere in the world. Clovers planted in Australian soil simply have to work harder to fix nitrogen in the soil and to convert it into plant protein. That nitrogen fixation process is regulated by estrogenic isoflavones, so that the harder the plant works to fix nitrogen, the more estrogenic isoflavones it has to make. Compounding this situation, it just happened that the strains of sub-clover chosen to be planted in Australia happen to have naturally high levels of estrogenic isoflavones to start with. Eventually, once the problem was identified, agricultural scientists bred strains of sub-clover that had much reduced levels of estrogenic isoflavones, thus solving the problem.

The notion that plants might exert some sex hormonal effect on the body was not entirely novel. Traditional folk medicine in Europe and China has used certain plants over the centuries to treat infertility and other reproductive disorders of women. But this effect was very much a medicinal effect, something that was prescribed and used on an occasional basis, and usually came from an unusual herb or plant not normally part of the human diet. What I was searching for was evidence that a change in the pattern of what we ate normally could effect a long-lasting change in our sex hormone profile. The Australian sheep were not being dosed with a rare plant in some sort of traditional medicine concept. They were eating sub-clover grasses that had been deliberately planted as feed. The problem was that the Australian sheep were ingesting hundreds of times more of these compounds in their diet than sheep elsewhere in the world. It is *normal* for sheep to eat clover, and therefore it is *normal* for them to consume small amounts of estrogenic isoflavones. It was just *abnormal* for them to be consuming such a massive amount of isoflavone. This is no different to humans and any essential dietary ingredient. Vitamin A is an essential nutrient – just as too little produces problems with skin and eyesight, so too much produces potentially fatal side-effects in the liver.

Humans don't eat sub-clover, so that was hardly going to be a factor in determining their risk of getting the five 'Western' cancers. However, what the 'Clover Disease' story did was to provide clear evidence that it was possible for an everyday diet to influence the sex hormone activity of the mammalian body to a degree that was biologically significant. That meant that there was a genuine scientific basis to think that any differences between vegetarian and non-vegetarian women in their blood estrogen levels might be explained on the basis of diet.

However, it only took a short while to realise that the sub-clover story was representative of a much larger story that had to do with how humans obtain protein in their diet. At its heart, the **phenoxodiol** story is about dietary protein.

Protein is essential to the structure and function of our bodies. At a macro structural level, protein is the major component of our skin, hair, muscle and blood; if we drill down a bit further, most of the chemical regulators in our bodies including hormones such as growth hormone and insulin are proteins; and drilling down even further into an individual cell, most of the internal workings of the cell are based on thousands of different proteins.

The human body is able to make the tens of thousands of different proteins that it needs to make on a daily basis by having access through the diet to a plentiful supply of amino acids, the fundamental building blocks of proteins. There are 20 different amino acids, and assembling these in different combinations in lengths of hundreds or thousands leads to the almost infinite number of combinations that are the body's complement of proteins. Dietary protein (either plant or animal protein) is disassembled in the gut to the 20 individual amino acids that then are absorbed and reassembled in the body to a whole series of new proteins. To that extent there is no difference between plant protein and animal protein.

Humans could, if they wish, rely solely on either animal protein or plant protein as their sole source of dietary protein. Eskimos for most of the year traditionally relied on seafood as their sole source of protein, while strict vegans (no dairy products or eggs) equally can survive on plant protein. For most of us, however, our diets contain a mixture of animal and plant proteins.

All plants make some protein, which they do by absorbing nitrate salts in the soil and turning them into protein. That method of protein manufacture yields relatively low levels of protein, typically between about 2-8% of the dry weight of the plant. The human diet needs to contain about 20% protein to provide a healthy protein level. A diet based solely on fruits, vegetables and cereals would lead to a life-threatening protein deficiency over time.

If fruit, vegetables and cereals contain such inadequate levels of protein, how then do vegans survive? The answer lies in the same method that farmers use to increase the protein content of the pasture by planting grasses such as clovers. Clovers belong to a family of plants known as *legumes*, distinguished by their high protein content. Vegetarians don't eat clovers, but they do eat other members of the legume family, and that is where much of their dietary protein comes from.

In the French language, the word *legume* is a general term referring to all vegetables. But in strict botanical terms, it refers to a family of plants that are distinguished from all other plants by their high protein levels. Legumes are one of the most important groups of plants in human history, still acting today as an important source of protein for the majority of people in the world, not just vegetarians.

Whereas all plants make a small amount of protein by absorbing nitrate salts from soil, legumes have an additional protein-making ability based on taking nitrogen gas directly from the soil and turning it into protein. This is the ability that sub-clover used to enrich Australian pastures with protein. This ability to turn nitrogen gas into protein is referred to by botanists as 'nitrogen fixation'. Nitrogen makes up about 80% of the atmosphere that we breathe in, so there are relatively high amounts of it in the soil, in which form it is available to the legume.

If you were to pull a legume such as a bean plant from the ground, you would notice that its roots would be covered in small, white nodules. These nodules, called *rhizobiums*, are in fact colonies of soil bacteria that have been induced to grow on the plant's root system. It is these bacteria, and not the plant, that undertake the conversion of nitrogen into amino acids. The plant then absorbs these amino acids from the colonies and turns them into plant proteins. The result is a level of protein several times higher than other plants. Whereas a vegetable such as broccoli, for example, has a typical protein content of about 3%, a legume such as the soya bean has a typical protein content of about 35%. Legumes embrace all members of the bean family (eg. soya beans, chick peas, lentils, navy beans, faba beans etc) and certain nuts (eg. peanuts). Legumes that are eaten by humans are referred to as *pulses*. Crops such as alfalfa (lucerne) and clovers are referred to as *non-pulse* legumes.

Legumes have served as an important source of dietary protein for most of human development. With the shift from a hunter-gatherer lifestyle to that of agriculture, legumes proved to be a reliable source of protein and a foodstuff that could be stored for long periods without refrigeration. Suddenly the dependency on meat and dairy products for protein was ameliorated. That historical reliance on pulses as a source of dietary protein persists to this day in most traditional cultures. In Africa, Mediterranean countries, Asia, and Central and South America, pulses provide between about 40-60% of daily protein requirements. In those regions, meat and pulses have been blended together over the centuries to form a complementary source of dietary protein that has changed little over thousands of years.

The outstanding exception is the so-called 'Western' diet. Increasing industrialisation in Europe in the 18<sup>th</sup> and 19<sup>th</sup> centuries saw the growing use of animal products as a mark of

prosperity. Pulses, for so long the staple source of dietary protein in the human diet, now became known as the 'poor man's meat'. The result of this progressive change has been that in the modern Western diet, about 90% of protein is in the form of animal products and 10% in the form of plant protein, almost all coming from vegetables and cereals.

This preponderance of animal products in the modern Western diet has long been a topic of study for nutritionists and epidemiologists for its impact on community health. With there being no essential difference in the nutritional value of plant protein versus animal protein, it was only natural that scientific attention would focus on non-protein factors that come as a result of eating a lot of animal protein. The main factor that has come under scrutiny has been fat type. Animal proteins are associated with a high level of saturated fats, whereas plant protein is associated with high levels of unsaturated fats. A high saturated : unsaturated fat ratio is implicated as a strong risk factor for heart disease, obesity and diabetes. Receiving only slightly less scrutiny is dietary fibre and fermentable sugars, both lacking in animal products, and both implicated in maintaining a healthy bowel.

However, try as they may, scientists have not been able to pin any hard evidence of a causal link between a high saturated : unsaturated fat ratio or low dietary fibre levels to common community cancers, particularly those associated with sex hormone function.

But one thing had been overlooked. That was the fact that plant protein had another important difference compared to animal protein beyond that of the type of fat. And that was the presence of isoflavones. Isoflavones were not present in fruits, nuts, cereal grains and most vegetables because those plants could not fix nitrogen. But they were present in legumes, and legumes (or pulses) were the principal source of plant protein for those diets where meat is not the dominant source of protein. The legume is able to fix nitrogen because of the presence of isoflavones, and eating legumes means consuming relatively high levels of isoflavones. The two factors are inseparable.

There are four main isoflavones (known as *biochanin*, *genistein*, *formononetin* and *daidzein*). **Genistein** is a derivative of **biochanin**, and **daidzein** is a derivative of **formononetin**. Most legumes contain all four isoflavones, although the proportion of each of the four varies from plant to plant. In sub-clovers, **formononetin** and **daidzein** emerged as the dominant isoflavones and the ones responsible for the estrogenic effect in the sheep. Scientists discovered that sheep could not absorb **biochanin** and **genistein**, and so by selecting strains of sub-clovers that were high in those two isoflavones, the nitrogen-fixing capacity of the plant could be retained while its estrogenic effect was greatly diminished.

If, as it appeared, many societies around the world were eating diets containing relatively high levels of isoflavones, the issue then became what biological consequences that might have for humans.

An estrogenic effect was one possibility. Back in the 1980s where this story has its beginnings there was no evidence that dietary isoflavones were having any estrogenic effect in humans, but the experience with Australian sheep pointed very clearly to that possibility. The isoflavone level in the sheep diet was way beyond anything that a human might encounter, but the sheep experience nevertheless proved that plant isoflavones could be absorbed into the body where they could act as estrogens.

The second possible biological effect was a direct anti-cancer action. This is a function that isoflavones share in common with a small number of other chemicals found in certain other vegetables. The best known of these are chemicals known as *isothiocyanates* that are found in members of the cabbage family (Brussel sprouts, cauliflower, kale, kohlrabi, savoy cabbage etc). When added to human cancer cells growing in the test-tube, compounds such as isothiocyanates show modest ability to stop the growth or even to kill a wide range of human cancer cells. In the 1980s, isoflavones such as **genistein** were shown to have the same type of cytotoxic activity against human cancer cells. **Genistein** was also shown to have the ability to block the growth of blood vessels supplying cancer tissues (known as an *anti-angiogenic* effect). The significance of these findings from a biological perspective was that **genistein** levels in the diet of a vegetarian could be thousands of times greater than isothiocyanates.

### *The theory*

Bit by bit a theory was emerging to explain why those of us who had embraced a Western diet based predominantly on animal protein were at increased risk of developing cancers, particularly of those parts of the body most sensitive to sex hormones, the so-called 'Western' cancers.

The basis of that theory was the level of plant isoflavones in the diet.

There was ample evidence pointing to the huge discrepancy in the level of legumes in different diets. Legumes contribute a significant proportion of dietary protein for people in Asia, the Mediterranean region, North Africa, and Central and South America. The diets in these regions provide about 40-60% of dietary protein in the form of legumes. Legumes are far less common in the typical Western diet; plant protein (from vegetables and cereals) might provide up to about 10% of the dietary protein, but very little of this comes from legumes.

Without going to the trouble of analysing diets and blood samples for levels of isoflavones, even a cursory glance at the range of typical diets showed a vast discrepancy in the level of isoflavones in different diets. Without any significant use of legumes in the typical Western diet, isoflavone levels clearly were going to be very low. The occasional Mexican dish (refried beans), Indian dish or hummus dip (chick peas), baked beans (navy beans), or French provincial meal (Puy lentils) would be delivering the occasional burst of estrogenic isoflavones, but it has been estimated that the average level of estrogenic isoflavones in the typical, non-vegetarian, Western diet varies somewhere between the barely detectable, up to a maximum of about 3 mg per day.

At the other end of the scale, a vegan who is relying entirely on plants for protein, and where most of that would by necessity come from legumes, would be consuming about 100-150 mg isoflavones per day.

In between these two extremes are typical Asian and Mediterranean diets where legumes contribute between about 40-60% of dietary protein. The isoflavone content of these diets is between about 30 and 80 mg per day.

So there was the range of daily isoflavone intake ..... roughly about 1-2 mg for the typical Western diet, 50 mg for the typical Asian and Mediterranean diet, and 120 mg for the typical vegan diet. By any measure, that was an enormous range.

So, my theory, in a nutshell, became the following:

- ✚ that legumes have been an integral part of the human diet for thousands of years, meaning that isoflavones also have been an integral part of the human diet;
- ✚ that dietary isoflavones arguably contribute to the overall ability of the body to fight the development of cancer in general;
- ✚ that if plant isoflavones have a biological effect when eaten by humans (and the symptoms of 'Clover Disease' certainly pointed to that possibility), then it seems likely that humans must have adapted to the estrogenic effect of isoflavones to the extent that they are an important contributor to the way we manufacture and use our own sex hormones;
- ✚ that like any key nutrient, it is possible to get too much, with 'Clover Disease' being an example of that;
- ✚ that, again, like any key nutrient, it is possible to get too little, and the very low levels of isoflavones in the typical Western diet represents a deficiency, the consequence of that deficiency being a change in the regulation of our sex hormones to the extent that we have become predisposed to certain sex hormone-associated cancers.

If you were to test your blood several hours after eating a pulse such as chickpeas or lentils or tofu or baked beans or Mexican refried beans or hummus or after drinking soymilk, you would find substantial levels of isoflavones in the blood. And to put those levels into perspective, the isoflavone levels would be several thousand times greater than the levels of the body's own sex hormones. These compounds have been in our bloodstream for thousands of years at levels that clearly are biologically significant. The notion that an evolving human body somehow would have managed to ignore them or to bypass them is too silly to contemplate. Nature would have been far more likely to have adopted and adapted existing systems to its own end in the course of developing more complex systems.

## THE FIRST EXPERIMENT

What was not clear back in 1988 was which of the two known biological functions of isoflavones .....a direct anti-cancer effect or an estrogenic effect .... was the most relevant to cancer prevention in the community. Were the two actions completely independent and supplementary, or were they complementary, with one action depending on the other?

The fact that the five ‘Western’ cancers involved tissues that were sensitive to sex hormones (both male and female sex hormones) seemed to point towards the critical role of isoflavones in modifying the behaviour of sex hormones in the body. And yet the ability of a dietary component as common as **genistein** to kill human cancer cells in the test-tube was just far too obvious to ignore. The levels of **genistein** that were killing cancer cells such as human breast cancer cells in the test-tube, were within the range of isoflavones found in the blood of sheep after consuming the same amount of dietary isoflavones that a human vegetarian might consume each day. So the indications were that a direct killing effect on cancer cells was more than possible.

By 1988, I had managed to track down virtually every scientific paper ever written about dietary isoflavones....not that it was a particularly big body of study. The great majority of the information came from agricultural and botanical chemistry journals, with ‘Clover Disease’ featuring heavily. Journals devoted to medicine and nutrition didn’t seem to have heard of the word ‘isoflavone’, and the whole issue of the relevance of isoflavones in the human diet had barely permeated medical consciousness.

The one notable exception was a vague reference to the ‘anti-cancer’ properties of **genistein**, and a literature search led me to a patent that had been awarded to Kikkoman, the huge Japanese manufacturer of soy sauce. It seems that their scientists had decided to search soybeans for natural compounds with anti-cancer properties as a potential explanation of why Japanese, who are the highest consumers of soya in the world, had much lower rates of certain cancers. Whereas my path of realization had been somewhat tortuous and based around the rather nebulous connection with sex hormone activity, the Kikkoman scientists had taken the classic ‘trawling through Nature’ approach, looking for the ability of a particular plant component to kill cancer cells. Once they had established that certain chemical extracts of soybeans did have anti-cancer activity in the test-tube, they eventually narrowed that effect down to **genistein**. The irony of this is that the National Cancer Institute, with its vast resources and an extensive program over decades of searching North American plants, managed only a couple of home-runs over hundreds of thousands of plant

specimens. The Kikkoman scientists hit a home-run from a single plant. The even greater irony is that soybeans, along with corn, are the two most widely grown plants in North America, and yet there is no evidence that they soybeans were ever sampled as part of the National Cancer Institute program. How medical science might have changed if they had focused on the obvious rather than the obscure.

The patent had been awarded to Kikkoman in 1978, so by the time I became aware of it 10 years later in 1988, it was pretty obvious that there was no commercial interest within the pharmaceutical industry in pursuing **genistein** as a potential drug candidate. At the time, that struck me as amazingly odd – how could it be that in the same era that two naturally-occurring plant chemicals, **taxol** and **genistein**, were shown to have potent anti-cancer properties, that **taxol** should have the spotlight turned on it, while **genistein** be totally ignored. I guess that it came down to the fact that a rare chemical from an exotic plant such as a yew tree and which was discovered by such a reputable body as the National Cancer Institute in a highly publicised and expensive program, was always going to be sexier than a chemical that occurs in common soybeans, and which is present in the blood of billions of the world's population, and which was discovered by a company that produces soy sauce.

There were a couple of things about the Kikkoman patent that intrigued me. The first was that **genistein** was killing cancer cells directly, and that this effect seemed to be totally independent of its weak estrogenic effect. When I came to work with **genistein** myself a couple of years later, it soon became clear that its ability to kill cancer cells in the test-tube had nothing to do with its estrogen function. **Genistein** proved just as capable of killing human cancer cells such as brain cancer (glioma) and lung cancer cells where estrogen plays no known role in their growth, as it was with breast cancer cells where estrogen plays a key role in their growth.

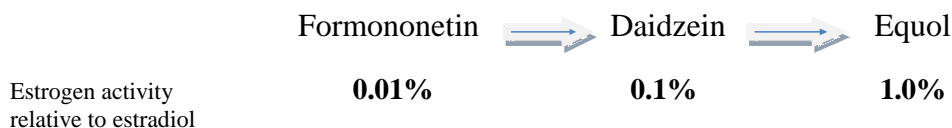
The second aspect of the Kikkoman patent that puzzled me was the fact that the other main isoflavone in soybeans, **daidzein**, had no ability to kill cancer cells. The reason why this was so intriguing was that **genistein** and **daidzein** were almost identical in their chemical structure, and they both shared the common biological functions of being responsible for initiating the nitrogen-fixing activities of plants, and being estrogenic. Yet, one was able to kill cancer cells, and the other could not.

This observation was particularly relevant to the question of whether it was a direct killing effect or an effect on estrogen function that was behind the health benefits of dietary isoflavones. If it was a direct killing effect, then that meant that it must be due to **genistein** (because **daidzein** was inactive), and that is where potential trouble with this theory lay. That trouble was that in sheep, **genistein** was completely degraded in the gut. If the same thing happened in humans, then the theory that **genistein** was providing some direct anti-cancer effect in humans was dead and buried, leaving the estrogenic effect of isoflavones as the only possible cause of anti-cancer benefits.

As part of the research into 'Clover Disease', Australian scientists in the 1960s and 70s had put considerable effort into tracking the fate of the dietary isoflavones once they entered the sheep's gut. What they found was that the plant isoflavones were subjected to extensive fermentation by bacteria within the gut. The rumen (or forestomach) is little more than a huge fermentation tank in ruminants. The rumen is where the plant material is received and the very large and complex carbohydrates such as cellulose that make up the great bulk of the plant material are broken down to the much smaller and simpler types of sugars that we

humans generally eat. The way the sheep does this is by bacterial fermentation. The rumen is a large, fluid-filled sac containing a range of different types of bacteria that are specialists in fermenting plant material. These bacteria attack the tough plant material with enzymes, which along with warm temperatures, bring about changes in the chemical structure of the plant components. In the same way that yeast organisms ferment large complex plant sugars and convert them into alcohol, so the large, complex, indigestible plant fibre is converted by fermentation into smaller sugars that are readily absorbed by the body. While the main purpose of the fermentation process is to break down complex sugars, anything else present in the plant will also inevitably get caught up in that process. That is the case with the isoflavones. They get caught up in the fermentation process that results in changes to their chemical structure. Of the large amount of isoflavones present in the sheep's diet, only a very small proportion managed to escape the fermentation process unchanged and be absorbed and appear in the animal's blood in that original form.

In the case of the sub-clover, the two main isoflavones present were **formononetin** and **biochanin**. The **formononetin** was converted in the sheep's gut into **daidzein**, which in turn was converted into a compound called **equol**. The large amount of **formononetin** being eaten by sheep ended up in their bloodstream almost completely as **equol**. The significance of this conversion for the sheep was that the estrogenic activity of the compounds increased 100-fold going from **formononetin** to **equol**.



The **biochanin** content of the sub-clovers, on the other hand, underwent degradation within the sheep's rumen. It first was converted into **genistein** and that in turn was broken down to an inactive compound. This degradation appeared to be virtually complete, with little or no **biochanin** or **genistein** appearing in the bloodstream.

In the late-1980s I had managed to obtain some **equol** and shown that, like **daidzein**, it had no anti-cancer activity against human cancer cells in the test-tube. So the answer to the question of how dietary isoflavones might be influencing the development of cancers associated with sex hormone-sensitive tissues, lay with understanding whether humans treated dietary isoflavones as did sheep. If **biochanin** and **genistein** were degraded as occurred in sheep, then the likelihood that these compounds were contributing a direct anti-cancer effect could be effectively ruled out.

The one saving grace was that humans don't need such a super-charged fermentation process as sheep do because we don't eat so much roughage. So there was some chance that **genistein** would not be subjected to so much rigorous treatment and at least some might get through the human gut unchanged. Humans carry out a limited amount of fermentation (where essential nutrients such as short-chained fatty acids are produced), but this takes place in the large bowel and is a very gentle process compared to what takes place in a rumen. The hope was that enough **genistein** might be absorbed before it got to the large bowel, or that

the fermentation processes within the large bowel of humans would not be so destructive as to inactivate all the **genistein**.

In 1989 I decided to answer this question by feeding people a diet rich in isoflavones, and then looking to see how the body metabolized those isoflavones. Arms were twisted and 10 guinea pigs were recruited from family, friends and work colleagues. These people agreed to eat 2 large cups of soy flour a day for 10 days, the flour being made into a type of bread. Mutiny threatened once they discovered on Day 1 that a loaf of soy flour is about as appetising and as easy to swallow as Styrofoam, but fortunately all went on to finish the study including collecting their urine every day for later analysis.

When the urine samples were tested, levels of **genistein**, **daidzein** and **equol** prior to the start of the study were virtually undetectable in all test subjects, which was consistent with the fact that non-vegetarians in Western societies are consuming little or no dietary isoflavones. However, all three compounds began appearing in the urine on the first day of consumption of the soy flour, reaching a peak by the second day that was maintained for the rest of the study. **Equol** levels were substantially higher than **genistein** and **daidzein** levels, but most importantly, **genistein** was there, albeit not in great amounts.

So that answered three key questions: first, that humans absorb dietary isoflavones and that they circulate throughout the body; second, that bacterial fermentation does occur in humans, as evidenced by the appearance of large amounts of **equol**, which from the sheep studies we concluded represented bacterial conversion of **daidzein**; third, that humans don't destroy **genistein** the way that sheep do, or at least not to the same extent that sheep do. At the very least, this meant that the ability of isoflavones such as **genistein** to attack and kill cancer cells directly as had been seen in the laboratory, potentially could occur in the human body, although the levels were not of the order that I would have thought would be contributing a significant direct anti-cancer effect.

That was all very reassuring and interesting, but it was only the beginning, and the real scientific challenge still lay ahead. For when we calculated how much isoflavone was being eaten and how much we identified in the urine, there was a gap of about 70%. That is, 70% of the isoflavones that were consumed in each lot of soy flour could not be accounted for by what came out in the urine. There was any number of reasons that could have accounted for this. For example, some of the isoflavones may have stayed trapped in the food in the gut and not have been absorbed. Or they may have been absorbed and passed out of the body through means other than urine. Or they may have been broken down to inactive compounds as the sheep did with **genistein**. Or they may have been converted into other isoflavonoid compounds in the same way that **equol** was being produced.

Twenty years later, we know that the 'isoflavone gap' is due to all four such factors – a small proportion (about 15%) of dietary isoflavones is not absorbed, some **genistein** is destroyed by bacterial fermentation, and some of the absorbed isoflavones leave the body via bile and not urine. But the overwhelmingly largest part of the 'isoflavone gap' we now know is due to conversion by bacterial fermentation in the large bowel into a range of other compounds (like **equol**). Twenty years ago, however, that was all unknown. We had been able to detect plant isoflavones and **equol** in the urine because their chemical structures were well known, thanks to the work on sheep affected by Clover Disease. There also had been a couple of small studies conducted with other species such as cattle and horses in relation to clover diets, and they had found some evidence of trace amounts of a couple of compounds apart from **equol**

in the urine, but beyond that we were in the dark as to what to look for, or indeed, if there was anything else to look for.

Identifying whether there was ‘anything else’ was important because it had a big potential effect on the vexed question relating to the way in which dietary isoflavones might be contributing to protection from cancer. We already knew from the sheep studies that the progressive chemical conversion of **formononetin** to **daidzein** to **equol** increased estrogenic activity 1000-times. That raised the prospect that a similar thing might be occurring with a direct cytotoxic effect of isoflavones on cancer cells. What if humans were converting most of the **genistein** in food into ‘something else’? And if that ‘something else’ showed the same 100-times increase in anti-cancer activity that occurred in estrogenic activity when **daidzein** was converted into **equol**? If that was the case, then the relatively small amounts of **genistein** in the body might not be relevant in terms of direct anti-cancer effect.

All highly speculative of course, but nevertheless essential knowledge if we were ever to understand the hypothesised connection between dietary isoflavones and cancer risk. If we had been unable to account for just a small proportion of the isoflavones that the volunteers had eaten in the study, I doubt that this question would have been all that important. But 70% was just too large an amount to dismiss. Buried in that 70% might be some highly active compounds that could give this story an entirely different complexion.

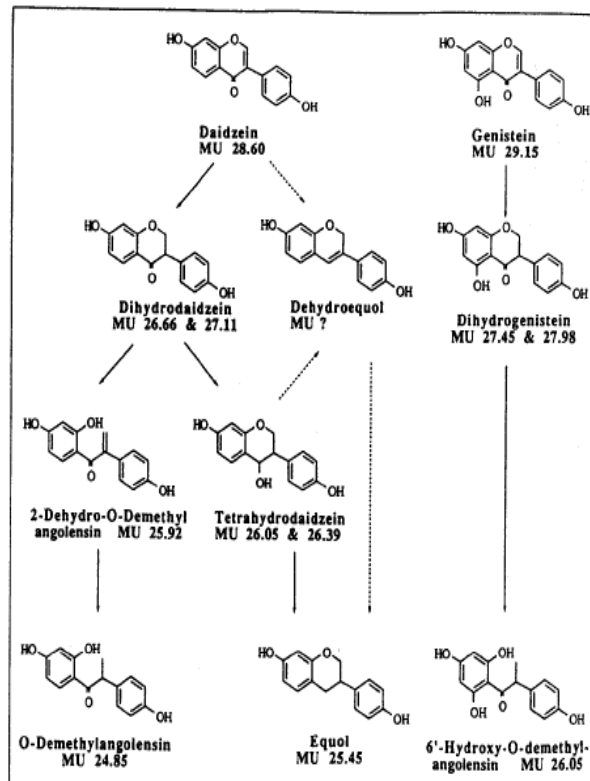
Searching for compounds where you have no idea what they look like or even if they exist is about as difficult as it sounds. It’s like searching for a needle in a haystack without knowing what a needle looks like. That was when the experts were called in, and the expert in this case was a steroid chemist known as Dr George Joannou of the Royal Prince Alfred Hospital in Sydney. Dr Joannou’s main focus was on developing techniques to measure steroid hormone levels in the urine of new-born babies with a view to diagnosing certain diseases associated with malfunction of hormone production. As steroids and isoflavones are very similar in structure, and he was very familiar with searching human urine for previously unidentified steroid hormones and their metabolites, he was an obvious person to have involved in the study. In retrospect, I now realise what an extraordinary coincidence it was that I happened to be working in the same hospital as someone with George’s particular skills, as there would have been just a handful of chemists in the world at that time with those skills.

This part of the project took 4 years, from 1988-1992, and involved a level of chemical skills that cannot be done justice here. Dr Joannou, with the assistance of Mark Waring and Tony Reader, took the urine samples and subjected them to analysis by complex analytical techniques such as nuclear magnetic resonance technology and gas chromatography. The outcome was that they reported that there were 9 additional isoflavone compounds apart from **genistein** and **daidzein** and **equol** in the urine. [These are listed in the diagram below, and I won’t confuse the reader by mentioning them by name here, except to say that 3 compounds – **dihydrodaidzein**, **tetrahydrodaidzein** and **dihydrogenistein** – each existed as two different isomeric forms].

Three of these 9 additional compounds had been described before. They had not previously been found in human urine, but they were not new in the sense that they had been created in a laboratory by synthetic chemistry. Six compounds, however, were new in the sense that they had never been described before either in urine or in any form.

We presumed that, like **equol**, all 9 compounds were the result of bacterial fermentation processes within the large bowel. Some of them were present in trace amounts only, while others were there at very high levels. Collectively, these 9 compounds accounted for almost three-quarters of the 'isoflavone gap', confirming our suspicion that most of the plant isoflavones that humans eat are converted by the bacteria in their large bowel into new isoflavonoid compounds that are peculiar to animals, with some of them possibly even being peculiar to humans.

The following diagram details the different isoflavonoid compounds found in the urine of the volunteers.



The next step, once the new compounds had been identified, was to try and determine which ones came from **genistein** and which from **daidzein**. Organic chemists see chemical compounds in much the same way as an engineer looks at a structure like a bridge and sees the different components and their contributions to the overall structure. Chemists can look at an organic compound and predict reasonably accurately how it was made, in the same way that an engineer could describe exactly the sequence of how a bridge was constructed. Dr Joannou looked at the 9 new compounds and concluded that 6 were derived from **daidzein** and 3 from **genistein**. He then took the matter one step further by predicting the synthetic pathway that the bacteria must have taken in order to convert **daidzein** and **genistein** into their various end-products. In the case of **daidzein**, he concluded that the first step in that conversion process must have been the production of a compound he called **dehydroequol**. This was an entirely hypothetical compound that he speculated must have been made by bacteria as the initial step in the fermentation process that eventually yielded end-compounds such as **equol**. And as **dehydroequol** was not detected in the volunteers' urine, we assumed that it never left the bowel bacteria where it probably had an ultra-short lifespan.

The relevance of this matter is that **dehydroequol** eventually came to be called, **phenoxodiol**.

Back in 1992 at the conclusion of this small study, **dehydroequol** hardly seemed an exciting prospect, certainly looking a non-event in terms of the anti-cancer story. After all, how could a compound be biologically interesting that most likely only existed for milliseconds in a reaction process in bacteria, and apparently never left those bacteria? The two fermentation products of **genistein** held far more interest from an anti-cancer point of view.

The hypothesis gradually taking shape in my mind in 1992 was

- ✚ that dietary isoflavones somehow were providing an anti-cancer effect, particularly in relation to cancers of the breast, ovary, uterus, prostate and large bowel,
- ✚ that this effect was due in part to **genistein** and some of its fermentation derivatives
- ✚ that in the same way that **daidzein** became a more potent estrogen when converted by bacteria into **equol**, so plant isoflavones such as **genistein** became considerably more potent anti-cancer agents as a result of the same fermentation process.

The next step then was to test all the newly discovered isoflavone compounds for their anti-cancer activity. The amount of **genistein** exiting the body of these volunteers could certainly have been enough to provide a meaningful anti-cancer effect, but it was an intriguing possibility that the bulk of any anti-cancer effect lay with the newly discovered 'humanised' isoflavones and not with the original plant isoflavones such as **genistein**.

## NOVOGEN

The next stage was to get hold of all 9 isoflavone compounds (plus **dehydroequol**) that Dr Joannou had identified in human urine, and then to screen them for anti-cancer activity. Conducting the anti-cancer assays was not an issue, since that was something that my university laboratory was already geared up to do. The challenge was getting hold of the 10 compounds. I already had obtained some **equol** from scientists who had been involved in the original Clover Disease studies in sheep and had extracted it from urine, but the rest of the compounds were unavailable, especially seeing that 6 of them had not been previously described.

The first obvious thought was to isolate them from urine. If the body was already making them, then it seemed a logical step to take advantage of that. The problem with that approach, however, was the amount of urine that would have been needed. The early work with **taxol** used a similar painstaking approach, collecting bark from thousands of yew trees in order to isolate enough drug to conduct early studies. There also was a classic example at the time of a drug derived from urine. **Premarin**, the main hormone replacement therapy drug on the market in the early-1990s was made from horse urine. Urine was collected from thousands of horses in North America and put through separation columns to isolate the estrogen hormones that went into **premarin**. The isoflavone compounds that I was looking for were remarkably close in their chemical structure to the **premarin** hormones, so I figured that it at least was technically possible. That is when the practicalities of that approach hit home. It would have meant having several volunteers collect all their entire urinary output every day for several months just to get enough of each of the 9 compounds to work with. That would have meant working with hundreds if not thousands of litres of urine, not exactly what a university cancer research laboratory was equipped for. Carrying 5 litre bottles of urine into the medical school each day as I had been doing for some months wasn't remarkable, but a truckload of urine arriving at the front door of the medical school might have raised a few eyebrows.

That led me to toy with the fanciful idea of creating a large artificial human gut in the laboratory, where I would fill a tank with human faeces and pour in soy flour and let the little bugs go at it in the hope that they would do in the laboratory what they were doing in the body, and deliver a bunch of isoflavone metabolites that I hopefully could recover in due course. Happily, that was just a fleeting thought, as I quickly realised that a truckload of urine arriving at the front door of the medical school would have been nothing compared to the

problem of explaining the presence (not to mention smell) of an artificial large bowel in a medical school

Commonsense eventually prevailed with the realisation that the only alternative was chemical synthesis.... that is, creating the drug from scratch by chemical manufacture. In this way, I could make as much as I wanted whenever I wanted. The problem was that for a non-chemist such as me, chemical synthesis was as much a mysterious and dark art as its medieval origins, alchemy – the futile belief that it was possible to create gold from another heavy metal such as mercury. But to a trained organic chemist, creating a new organic compound is nothing more than a series of chemical reactions, usually starting with a readily available, simple compound that gradually changes shape until you end up with the desired new compound. These various step-by-step reactions can range from the simple (such as adding an acid and applying heat), through to complex, highly toxic or dangerous reactions (such as conducting reactions in hydrogen under high pressure) that need to take place in highly controlled, secure environments.

If you are lucky, the synthetic process is relatively straight-forward (such as making margarine from sunflower oil). If you are unlucky, the process is highly complex and/or difficult and/or dangerous (such as the manufacture of **taxol**). At the start of this process I had no idea just how difficult the synthetic process was going to be. I assumed that since bacteria could do the job, then humans must be able to find a way as well. Although against that was the fact that it had taken the best part of 10 years of effort by teams of chemists to work out how to make **taxol**, and yet dumb yew trees had been making it for hundreds of thousands of years.

So that was the task....to synthesis all 9 isoflavone metabolites (plus the hypothetical **dehydroequol**) that had been found in human urine, and then when we had enough of each compound, to screen them in the laboratory for anti-cancer activity. I estimated that the first part of that task was going to require 2-3 chemists working full-time for 2-3 years, with a well-equipped chemistry laboratory. The manpower cost alone was going to be in the order of \$1M. And that assumed that I could find a chemistry laboratory with synthetic chemistry expertise that was willing to cooperate.

Not surprisingly, no such cooperation was forthcoming either within the university or outside. The whole concept proved to be just too far-fetched to attract anyone's interest. Having a half-baked idea presented by someone with no track-record in the area of pharmaceutical research was never going to inspire much confidence in the listener.

Nevertheless, the underlying concept was far too fascinating to be allowed to slide into obscurity, so with a big, deep breath, the concept was converted into a private venture that would hopefully tap into venture capital. In 1992, two local businessmen stepped in with cash to bankroll the venture. A scientist was employed, a contract negotiated with the University of Sydney to lease some laboratory space, and so the fledgling enterprise began. Looking back, I am not sure what I imagined was going to happen. There was no business plan, let alone any grand plan...I had no clear idea how much money it was going to take or how long it was going to take. There wasn't even any guarantee of success ...it was entirely possible that we would get to the end of the project and find that none of the 10 compounds that we were chasing would be any more active as anti-cancer agents than **genistein**, or even worse, that they would have any anti-cancer activity at all.

The most significant achievement of the first 18 months or so after setting up the private company was coming to the realisation of the enormity of the task ahead. It quickly became abundantly clear that this was a task well outside of the resources of a tiny private company with very limited financial resources. So by late 1993, we accepted the need of turning the company into a public company that would list on the Australian Stock Exchange and raise sufficient funds by public subscription to enable the project to proceed.

Looking back, I marvel now at the willingness of investors to part with their hard-earned cash for a concept that by all measures was a bit odd, and being presented by a scientist, who while fully convinced of and committed to the concept, was nevertheless a total innocent abroad in the world of big business. We even picked one of the worst times to raise capital – mid-1994, a time of a major slump in the stock market worldwide. Nevertheless, perhaps because of rather than despite our naivety, the listing was successful, and the Company (Norvet Ltd, later to change its name to Novogen Ltd) listed on the Australian Stockmarket on 11th August, 1994. After almost 8 months of daily discussions with lawyers, bankers, underwriters, stockbrokers and investors, repeating the isoflavone story hundreds of times along with what we were planning to do with the money and the projections of how long it all would take etc, I still have vivid memories of sitting in my new office on the 12<sup>th</sup> August, stunned that it had actually happened, suddenly aware of the awful responsibility that I had taken on, and wondering what the hell I was going to do next. That's when I realised that dreams are easy....it's reality that is difficult.

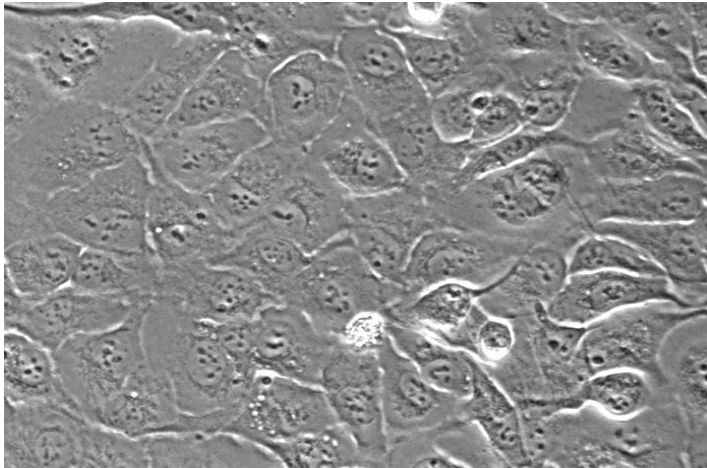
Once I had caught my breath, we settled down and got under way. Offices and laboratories were leased, staff recruited, and strategic plans drawn up. In due course we recruited our first chemist, Dr Andrew Heaton, who took control of the task of synthesising the isoflavone metabolites.

While we didn't anticipate any particular difficulties in synthesising the isoflavonoid compounds, it nevertheless came as a relief when Dr Heaton successfully ticked the first couple of compounds off the to-do list. At least it seemed that we were not going to be faced with the same lengthy process that it took to synthesise **taxol**. Each of the 10 compounds presented its own individual challenges, but the task was successfully completed within 18 months. About 10-20 mg of each compound was produced which was more than enough to conduct initial tests on cancer cells in the test-tube. They even produced some compounds chemically related to the 10 isoflavone metabolites but not found in human urine. The final package for testing purposes comprised the four plant isoflavones (**genistein**, **biochanin**, **formononetin** and **daidzein**), the 10 isoflavones identified in human urine (including the hypothetical **dehydroequol**), and 3 additional new isoflavonoid compounds.

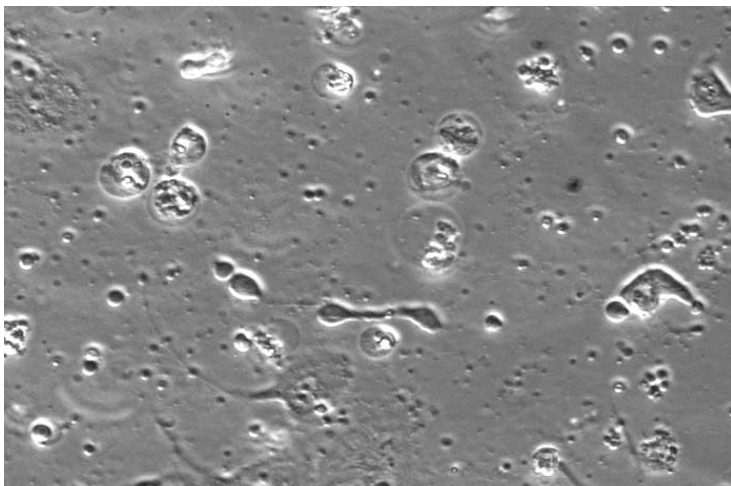
The next step was to establish a laboratory that would conduct the assays for anti-cancer activity. Dr. Michael Thurn, a biologist, was appointed to head this laboratory. He quickly established the assay known as an *in vitro* cytotoxicity assay (*'in vitro'* meaning *in the laboratory*, and cytotoxicity meaning *to kill cells*). This is a very standard initial screen for anti-cancer activity that involves growing cells in a test-tube to which a test drug is added and its effect on the viability of the cells determined. It really is as simple as that. Most cancer cells readily grow in tissue culture, which entails putting about 10,000 cells into a small well in a plastic tray, adding sterile fluid containing all essential nutrients for the cells, and placing the tray in an incubator set at about body temperature (37C degrees) and containing the appropriate mixture of oxygen and carbon dioxide. The cancer cells quickly attach themselves to the plastic and begin to divide, doubling their numbers every day or so. Once

they are established, the test drug is added to the well, left there for 24 – 48 hours, and the cells then re-examined for viability.

The following figure shows ovarian cancer cells growing on the bottom of the plastic wells some 3 days after being sown there. It is the nature of most cancer cells that they are sticky and want to adhere to a solid surface. Note that they form a confluent layer, much like pavement stones.



The following figure is the same cell type, this time grown in the presence of an anti-cancer drug. Note that there are very few cells binding to the plastic surface.



Almost all the cells have died, creating small pieces of debris seen floating in the tissue culture medium. This outcome would be scored as 100% mortality of the cancer cells.

Each drug is tested at a range of different concentrations. Quite a few drugs will kill cancer cells when used at high concentrations, but the real test is to see if they will still kill at the very low levels that are likely to be achievable in blood. The way that drugs are compared for this anti-cancer activity is to determine the lowest concentration at which 50% of the cancer cells die. It is purely an arbitrary number for the purposes of comparison. Fifty-percent provides confidence that a discernible and measurable killing effect has taken place. This

end-point is known as the Inhibitory Concentration where 50% killing has occurred (or IC<sub>50</sub>). The IC<sub>50</sub> normally is expressed as the amount of drug per mL of culture medium. Thus, a drug with an IC<sub>50</sub> of 1 micrograms per millilitre (mL), would be 100-times less active than another drug with an IC<sub>50</sub> of 0.01 micrograms per mL.

When conducting an initial screen for anti-cancer activity, it is usual to include a range of different cancer cell types, just to reduce the risk that a particular test drug has a limited range of activity. Large pharmaceutical companies normally would use about 60-70 cell lines, representing almost all forms of human cancer. We were certainly not equipped to go to that extent, so our initial screening was done with 2 different breast cancer cell lines, 2 different prostate cancer cell lines, and 2 different leukaemia cell lines. Given the fact that the initial theory revolved around cancers arising in sex hormone-sensitive tissues, it made sense to use breast and prostate cancer cell lines, and to add leukaemia cell lines out of interest as a representative of a cancer from a part of the body not associated with sex hormone activity.

The outcome from this initial screening assay was critical to the future of the Company. This was a key foundation of the establishment of the public company, so to say that the result was looked forward to with some apprehension would be an understatement. The assays were conducted on a rolling basis as the different compounds successfully emerged from the synthetic chemistry laboratory. Of the four estrogenic plant isoflavones, **genistein** proved to be the only compound with anti-cancer activity. Just as the Kikkoman scientists had found so many years before, **genistein** showed modest activity against all 6 cancer cell lines. The anti-cancer activities of **daidzein**, **formononetin** and **biochanin** were so weak as to be virtually negligible. As the different isoflavonoid metabolites were progressively tested, they all repeated the same pattern as these latter 3 plant isoflavones .... no anti-cancer activity of any significance. At this stage, it was looking as though any direct anti-cancer effect of dietary isoflavones could be attributable only to **genistein**, but its level in the body of the subjects who took the soya challenge was sufficiently low as to bring into question its clinical significance.

But then the surprise. **Dehydroequol**, the phantom metabolite that Dr Joannou had speculated must exist but which had never actually been found in humans, came up trumps. It showed itself to be up to 1000 times more potent than **genistein** in killing cancer cells in the test-tube....and not just active against the 6 original cancer cell lines used in the initial screen. It eventually was tested against a broad range of human cancer cell lines representing almost all forms of cancer, and it proved to be active against all of these forms including cells such as melanoma and mesothelioma cells that are notoriously resistant to most forms of chemotherapy.

The fact that a compound that might have existed for milliseconds inside of bacteria was shown to have such potent and fascinating anti-cancer activity didn't help my understanding of how diet was helping reduce the risk of certain cancers, but it did offer a marvellous opportunity as an exciting new drug prospect for a pharmaceutical company.

One of the early tasks was to give the new drug a name. **Dehydroequol** was its common chemical name, but the Company needed a name that could be trademarked. So the name **phenoxodiol** was born. Some years later, the international body that governs what new drugs can be called generically (as opposed to a brand name), required that it be called **idrinoxil** for reasons that are too complicated to go into here. So its formal name became **idrinoxil**, and its working name, **phenoxodiol**.

We had our drug candidate and our opportunity. Suddenly all the theory and speculation were justified. The sense of relief and excitement in the Company were palpable. We now had the basis of a drug development program that we dared hope would put us at the forefront of medical science.

