Sub-cellular Fractionation: Liver Lysosomes And Iron Overload
The Scientific Basis of Medicine

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<Opening titles>

<Peters to camera>

This programme is about the cell: the cell in health and disease. We're going to concentrate on a particular part of the cell, a particular structure, the lysosome; and we're going to talk about its function in health and in a particular disease. And we're going to describe a new technique which we've developed to study the changes in this particular organelle.

<Peter narrates over electron micrograph and diagrams>

This is an electron micrograph of a liver cell, a hepatocyte. In the centre you can see a nucleus. Surrounding this are many mitochondria and other membrane-bound structures. The whole is surrounded by the cell wall or the plasma membrane. Now we're going to concentrate on a particular part of the cell, a particular group of structures known as the Golgi complex and the lysosome. <*Diagram*> In the centre

of the picture is the Golgi complex. This has several functions, one of which is the formation of primary lysosomes. The primary lysosomes bud off from the Golgi complex. Primary lysosomes contain packages of enzymes which between them are capable of degrading all known biological components. They, for example, contain lipases which will break down lipids, carbohydrases, proteases and mucopolysaccharidases.

Primary lysosomes fuse with membranes containing unwanted material to be degraded; for example, mitochondria, which are no longer functioning, enter the primary lysosomes to become secondary lysosomes. In this way, the mitochondria can be broken down to free amino acids and simple sugars; these diffuse out of the cell and act as building blocks for reformation of other cell structures. Material that cannot be degraded remains in the lysosome which form residual bodies or tela lysosomes.

<Peters briefly to camera and then narrates over slide>

Much is known of the functions of these various subcellular organelles. This is the science of cell biology. <*Slide*> Rather less is known, however, about the changes that occur in the various organelles in disease, in other words about cell pathology. In order to look at the changes in the organelles in disease, several techniques are used. First of all, there are the classical biochemical techniques in which the techniques of chemistry are applied to biology. More recently other techniques have been used, including histochemistry, immunochemistry and electron microscopy. Although very powerful, these techniques have disadvantages, in particular they are difficult to quantitate, and we have concentrated on the technique of subcellular fractionation in which the individual organelles are separated on sucrose density gradients with the help of the centrifuge.

<Peters narrates over film>

The tissue is collected in isotonic sucrose in a small glass homogeniser. The art of the technique is to break open the cells without breaking open the subcellular

organelles. The homogenate is then centrifuged at low speed to sediment any unbroken cells, red cells, and nuclei. The cell extracts are subsequently fractionated on sucrose density gradients, conventionally this is done with a swinging bucket rotor. The extract is loaded into a small polythene tube which is placed in the bucket in the rotor. This is a slow process and has numerous disadvantages for our purposes. Although this rotor will take three buckets, the rotor has to be stopped and started during the fractionation procedure. The buckets move through 90 degrees during this procedure, during which there is mixing and turbulence. In addition, there is wobble of the rotor during stopping and starting and there are difficulties with temperature control and with hydrostatic damage to the organelles.

On the other hand, the zonal rotor, shown here, overcomes most of these disadvantages. Here we see the rotor being disassembled. The locknut on the top of the rotor is removed and a brass pulling nut is inserted so that the load may be lifted off the central shaft of the rotor. Here we see the chamber of the rotor.

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<Peters narrates over series of diagrams>

This is a schematic representation of the chamber of the zonal rotor. The sucrose density gradient is in the peripheral part of the rotor, extending from a density of 1.05 to a density of 1.32. In the dark area, near the centre, the cell extract is injected. <*Next diagram*> Schematically, we've shown this as containing two particles: a dense particle and a less dense particle. Under the influence of the centrifugal force, these particles sediment through the sucrose gradient to their equilibrium positions, the denser particle moving further than the less dense particles, as shown here <*next diagram*>.

From the sucrose gradient, while the rotor is still spinning, a series of fractions are collected so that the dense particles may be separated from the less dense particles for subsequent biochemical analysis.

<Peters narrates over film>

Here we see the rotor with the lid removed. The sample and gradient is injected down the central core and along this fine tube to the periphery of the rotor where the annulus is. To empty the rotor at the end of the experiment, the core of the rotor is filled with nitrogen at high pressure. This pumps the sample and the gradient and the separated organelles back along the fine tube into the collecting tubes in the ice bucket.

Here we see the gradient-making device. This consists of a series of accurately made stainless steel syringes, all surrounded by cooling jackets through which water at 0°C is being pumped. The gradient is prepared by arranging two cylinders in parallel. Into this cylinder, dense sucrose of 1.28 density is placed. Special high pressure fittings are necessary on this device because pressures in excess of 2000 pounds per square inch are achieved. Into the second chamber, sucrose of density 1.05 is added, and the small stirring device is placed in the top of the syringe. A locknut is placed and this is clamped home firmly.

The rotor is now spinning at 5000 rpm and we need to connect the filling device to the rotor. This is done by pulling a switch which allows the clutch to engage with the top of the rotor. Meanwhile, the liver homogenate has been spinning at low speed in the centrifuge to sediment the nuclei and undisrupted cells seen here. The supernatant, or extract, is loaded into a syringe which is connected to the fine tube leading to the centre of the rotor. The extract, or suspension, of subcellular organelles is injected into the rotor, and this fine tube is then immediately connected to the selector switch so that the gradient can be pumped into the rotor after the extract. The stirring motor on the gradient-making device is switched on and the two pistons move up in exact parallel to create a linear sucrose gradient.

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Here the contents of the gradient-making device are being pumped by these accurately monitored stepping motors into the rotor. Two syringes are pumped in

parallel to allow the gradient to be formed. After injecting the gradient, a cushion of 6 mls of sucrose, density 1.32, is injected into the rotor. Having completely filled the rotor, the speed is taken up to 10,000 rpm, and by moving the switch on the filling device the rotor is disengaged from the filling unit. The rotor is then speeded to 35,000 rpm or approximately 100,000 g. Checks are made to ensure that the filling device and rotor are fully disengaged.

When the rotor has reached 35,000 rpm, the clock is started, the centrifugation is carried out for 35 minutes. This is approximately a third or a quarter of the time necessary using more conventional swinging bucket type rotors. Here you see the display of the total integral to which the particles are being subjected during the centrifugation process. After 35 minutes, the rotor is slowed to approximately 8,000 rpm by switching on the breaking device, and the filling unit is re-engaged to the centre of the rotor.

The tube is removed from the filling selector switch and connected to a nitrogen cylinder, and the central part of the rotor filled to a pressure of 300 pounds per square inch. By observing the bubble flow chamber, it can be readily seen that the rotor is full of nitrogen. The nitrogen supply is then removed and fractions are collected from the periphery of the rotor down this fine tube into previously weighed tubes. Fractions of approximately 2 ml are collected and the tube on the right is used as a measure. When emptying the rotor, it is necessary to adjust very finely the speed of the rotor. As the rotor empties, the effective nitrogen pressure within the core of the rotor falls and it is therefore necessary to reduce the speed of the rotor so that emptying continues smooth and steadily.

Throughout the entire procedure, from homogenisation, low speed centrifugation, high speed centrifugation and collection of the fractions, the samples are maintained at 0°C. This gives excellent preservation of the organelles and of their constituent marker enzymes. After collecting the fractions, tubes are reweighed so that the accurate weight of sample collected can be determined. Using a refractometer, the density of the sucrose can be accurately determined so that the volume of the individual fraction can be readily calculated. The fractions are then assayed for

various marker enzymes so that the distribution of the various subcellular organelles, for example, the lysosomes, can be determined in the series of 15 to 16 tubes which have been collected.

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<Peters to camera>

This technique of microanalytical subcellular fractionation is applicable to several different areas of clinical medicine. We are applying this technique to the study of cardiac tissue, intestinal tissue, isolated granulocytes and to liver tissue. To exemplify the technique, I should like to describe our work on subcellular fractionation of liver tissue from patients with iron overload. This work was carried out in collaboration with Dr Carol Anne Seymour at the Hammersmith Hospital.

Haemochromatosis can be divided into two types: primary and secondary.

<Peters narrates over slides, interspersed with talk to camera>

The patients with secondary haemochromatosis we've studied have been those who've had massive transfusional iron overload due to beta thalassaemia. <*Next slide*> In haemochromatosis, there is a massive accumulation of iron. Initially, the damage to the cells, to the liver cells, is reversible, but with prolonged high levels of iron accumulation the damage is irreversible and leads to cirrhosis. <*Next slide*> If it is possible to remove the iron, the damage may be partially reversible and the severity of the lesion may be limited. <*Next slide*> However, the mechanism by which the excess iron damages the cell is unknown.

<*Next slide*> We have investigated the hypothesis that iron accumulates within the lysosomes leading to their disruption which leads to the release of highly active degradative enzymes which can damage the cell.

<*To camera*> Liver biopsies collected from patients with primary and secondary haemochromatosis: <*next slide*> the levels of iron and N-acetyl-beta-glucosaminidase and acid phosphatase were assayed in these liver biopsy specimens. You can see that in both primary and secondary haemochromatosis, there is a massive increase in the concentration of the liver. There is also an increase in the level of N-acetyl-beta-glucosaminidase and an increase in the level of acid phosphatase. These levels appear to be unique to iron overload conditions. We've studied a few patients who've been successfully de-ironed by venesection and the levels of iron, N-acetyl-beta-glucosaminidase and acid phosphatase have returned to normal. These results suggest that in iron overload, there is an accumulation of undegradable material within the lysosomes which has caused them to become activated. In order to demonstrate this, we carried out subcellular fractionation studies on biopsies from iron-overloaded patients. <*To camera*> The biopsies were homogenised and fractionated as described previously […]

<Peters narrates over film>

[…] and a series of 15 fractions collected from the rotor. Using highly sensitive, fluorometric assays the levels of these enzymes were determined in the fractions. The analiquot of the fraction is mixed automatically with the substrate and incubated in a water bath at 37°C. The reaction is stopped by adding a glycine buffer which also develops the fluorescence. The released methylumbelliferone, which is a measure of the enzyme activity – which is a measure of the lysosome activity – is assayed fluorogenically.

<Peter narrates over series of slides>

This diagram shows a histogram of the distribution of N-acetyl-beta-glucosaminidase or the distribution of lysosomes from a liver cell extract of a control subject. Horizontal axis indicates the density increasing from left to right, the vertical axis is the level of enzyme or the concentration of lysosomes. The area on the left corresponds to the original starting position – this is the position on the gradient to which the liver extract is injected. The darkened area shows the distribution of the

lysosomal enzyme – the distribution of the lysosomes. There is a broad distribution with a modal density at 1.20.

Biopsies were then collected from patients with iron overload and these were fractionated in exactly the same manner and the distribution of the lysosomal enzymes determined in exactly the same manner <*next slide*>. Here there is a striking difference. The modal density of 1.20 is lost and is replaced by a striking peak at a density of 1.30. This indicates that the lysosomes are extremely dense and this is due to the accumulation of iron and iron-containing compounds.

<*Next slide*> If patients with treated haemochromatosis are studied, there is a loss of this high dense lysosome peak. We have thus shown that the lysosomes are activated and that they are accumulating large amounts of iron-containing compounds. Ultrastructure studies with electron microscope have also confirmed this accumulation of iron-containing compounds within the lysosomes.

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<*To camera*> What is the evidence though that this accumulated iron damages the lysosome? <*Next slide*> In order to study this, we've measured the latent and sedimentable N-acetyl-beta-glucosaminidase. The latency and sedimentability are measures of lysosomal integrity. The higher the sedimentability, the greater the stability of the lysosomes. You can see that there is no difference in the fragility of lysosomes isolated from liver biopsies of control subjects or patients with cirrhosis. However, in both primary and secondary haemochromatosis, there is a striking reduction in this lysosomal integrity. We studied liver biopsies from patients with a wide variety of liver diseases and these changes appear to be unique to haemochromatosis. Again, patients with primary haemochromatosis who have been successfully de-ironed by venesection show a return to normal in the lysosomal fragility.

<*Next diagram*> In summary then, we've shown in iron overload the lysosomes accumulate iron, they are activated and are fragile. And we believe this fragility is implicated in the pathogenesis of the iron mediated damage.

<*To camera*> I've asked my colleague Dr Graham Neale to come and view this programme and to bring out any points that he feels need further discussion.

<Neale, seated beside Peters>

Well, Timothy, you've shown us a powerful new weapon for looking at human tissues. I wonder if you would like to say a few words about how it was developed.

<Peters>

Yes, the original rotor was designed and constructed by Dr Henri Beaufay in the University of Leuven in Belgium. He constructed this rotor essentially to bring about separations of organelles from large amounts of animal tissues and we have modified and adapted the technique so that we can apply it to the milligram quantities of tissue as can be obtained by biopsies from human tissue.

<Neale>

Alright, so you've got then a highly sophisticated tool for looking at the biochemical structure of the cell. I wonder if you can see a way ahead for further development of this technique.

<Peters>

We're using this technique to explore diseases about which we know very little. There are many diseases where we're completely at a loss as to determine whereabouts in the cell there is damage. Using this technique as a biochemical screen, we can look for defects in particular organelles; for example, we've been studying biopsies from patients with congestive cardiomyopathy. To date, there is no

clear evidence as to which part of the cell is abnormal. Using this technique we've shown that there are abnormalities in the mitochondria. This will enable us to cone down and concentrate on the possible mitochondrial defect in this disease. Using this technique, we can isolate and fractionate out the mitochondria and then study them by more conventional techniques.

<Neale>

Now, of course, you've done this best with iron overload where you've shown very clearly that the lysosomes are damaged in this condition. What do you think can be done about this in therapeutic terms.

<Peters>

I think in iron overload, the real therapeutic problem is in the removal of iron from patients with secondary iron overload. In primary iron overload, venesection is a perfectly satisfactory, effective form of treatment, but in secondary iron overload, for example due to beta thalassaemia associated with multiple transfusion, removal of the iron is extremely difficult. I think, as we've shown, as we've postulated that the lysosome is the mediator of the damage, and if we can selectively remove the iron from the lysosome, we should have a better form of treatment. Conventional chelating agents as used at present, by nature of their biochemical properties and the acid environment within the lysosome, would be expected to be ineffective. And knowing that there is a lysosome mediated damage, I think we should set out to develop lysosomal tropic or lysosome-direct drugs which will selectively remove the iron from these lysosomes.

<Neale>

Well thank you very much, Timothy, for letting us have a look at your very fascinating work. Thank you for coming along.

<End credits>